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

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

James Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

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ABSTRACT

Several relatives of grape and other asymptomatic host plants can harbor high titers of *Xylella fastidiosa (Xf)* without exhibiting symptoms of Pierce's disease (PD). The basis of what is a genetic difference is unknown. We have established that leaf scorch PD symptoms in grape result from apoptosis or programmed cell death (PCD). Clearly, *Xf* does not have to kill in order colonize the vascular system leaving this endophytic association asymptomatic. We have identified from a cDNA library screen several grape genes that block PCD when over-expressed in grape tissue. Preliminary experiments indicate that one of these genes, VVPR1A, is expressed or up-regulated in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. This gene also is upregulated in the presence of *Xf*. We are testing the hypothesis that over expression of one or more of the 12 genes recovered in the anti-apoptotic screen, with an initial focus on VVPR1A, can block both PCD induced by *Xf* and disease symptoms associated with *Xf*. Preliminary results reported here indicate that grape plants over expressing VVPR1A, metallothionein, or a *Meloidogyne incognita* upregulated gene can block symptoms in a cut branch assay. Experiments with whole transgenic plants inoculated with *Xf* are in progress to assess the movement of bacteria, the induction of *Xf* responsive grape genes and if symptoms of PD are affected by the anti-apoptotic transgenes.

INTRODUCTION

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/Glassy-winged Sharpshooter (PD/GWSS) Research Program and the National Academies report. Disease is defined as plants expressing several symptoms resulting from cell death (leaf scorch) or changes in tissue differentiation (green islands). The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when over expressed in grape, will prevent infection, spread or symptom development due to the presence of *X. fastidiosa* (*Xf*) in the xylem (Gilchrist and Lincoln 2004). Currently, several laboratories including our own have begun to carry out systematic studies of the molecular basis of susceptibility of plants to a range of pathogens including bacteria and fungi. The objective of these studies is to identify genetic or chemical approaches that have the potential to block susceptibility in grape to PD, thereby effectively creating cells that are refractory or insensitive to the signals expressed by pathogens that lead to susceptibility. 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 genetically determined pathways leading to apoptosis or programmed cell death (PCD) (Gilchrist 1998, Harvey et al. 2006, Lincoln et al. 2002, Richael et al. 2001). These discoveries parallel investigations now widely reported and accepted in human medicine whereby genes, signaling pathways and chemical signals expressed by animal pathogens initiate or block infection by activating or blocking apoptosis through constitutive genes or signaling pathways present in all cells. These studies are the basis for extensive searches for apoptosis-based therapeutic approaches and agents in plants as well as animals (Greenberg and Yao 2004, Nicholoson 2000).

Dr. Tom Rost reported, both in his 2005 PD Symposium address and his annual report (Rost et al. 2005) that *Xf* moves effectively and quickly through the plant following inoculation or uptake. However, the GFP-tagged bacteria have limited dispersal in the leaf lamina expressing the marginal leaf scorch symptoms of PD. These data, obtained in part using the confocal system in our laboratory, are consistent with our own observations using GFP-tagged *Xf* to visualize the bacteria in vascular elements connected to tissue showing the marginal scorch symptoms. These data suggest two key things. First, the cell death symptom is the result of mobile signals moving from the bacteria to cells distal to the bacteria and that strategies effective in blocking the death pathways will most likely consign the bacteria to an endophytic existence in the vascular system. Consistent with this hypothesis, Dr. Steven Lindow pointed out in his 2005 PD Symposium address that *Xf* is an effective endophyte in many asymptomatic plants and can be said to be "an endophyte gone bad in susceptible grape plants". It is a fact that several *Vitis* species, including wild grape, tolerate extremely high titers of *Xf* but remain asymptomatic, while many genotypes of cultivated grapes express PD symptoms at the same or lower titers. Clearly the presence of *Xf* in the xylem is not the single determining factor in disease. In PD and many other bacterial diseases, bacteria live predominantly as endophytes or epiphytes and only occasionally as pathogens. Susceptibility of the host tissues is determined by sensitivity to the presence of the bacterium and the signals expressed by the bacteria leading to PCD. Using genetic or chemical approaches to block PCD is an viable approach in both animal and plant disease prevention (Greenberg and Yao 2004, Harvey et al. 2006).

OBJECTIVES

1. Produce transgenic grape plants over-expressing candidate anti-apoptotic plant genes obtained from functional cDNA library screens as identified in an earlier project.
2. Evaluate these 12 putative anti-apoptotic plant genes in grape for effect on bacterial population dynamics, movement in the xylem, changes in gene expression and on PD symptoms when the candidate genes are expressed constitutively at high levels.. These assays will use confocal microscopy, GFP-tagged bacteria and RT-PCR.
3. Evaluate the stem and leaf uptake procedures developed recently in our laboratory to enable rapid assessment of grape gene expression in the presence of the PD bacterium. The experiments also will use coincidental transcriptional profiling as a measure of similarity of changes in gene expression between infected whole plants and bacterial uptake assays.

RESULTS

Produce transgenic grape plants over-expressing candidate anti-apoptotic plant genes obtained through cDNA library screens

Stable full plant grape transformations of susceptible Thompson Seedless and cv. Freedom are done by the Ralph M. Parsons Foundation Plant Transformation Facility. We anticipate 10-20 transgenic plants of each construct to be evaluated. The cDNA inserts from the library screens are cloned into the binary vector B5 for direct transformation into the *A. tumefaciens.* These plants will be grown for 2 months, ramets made from cuttings, and leaves assayed for the transgene expression after cut leaf and branch uptake with bacteria (see next section for methods relating to the cut branch and leaf assays). Both Chardonnay and cv. Freedom transformations were initiated in the grant proposal but only Freedom transgenics survived in the first round. The cv. Freedom, a common rootstock, is highly susceptible to PD with the same symptoms as Chardonnay and Thompson Seedless. The Transformation Facility has successfully transformed Thompson Seedless recently and is now confident that most of the transgenics of this cultivar can be delivered by November 2006. Currently, we have begun testing lines transgenic for genes CBPR1A, CB390 and CB456. Northern analysis confirmed over-expression of the metallothionein, the *Meloidogyne incognita* upregulated gene, and the VVPR1A transgenes in the transgenic Freedom lines (Table 1). Prior to initiating full plant transformations, all of these genes in Table 1 were confirmed to block programmed cell death in transgenic roots exposed to the apoptotic inducer Fumonisin B1.

Table 1. Plant anti-apoptotic genes, derived from functional screen of cDNA libraries, for transformation into grape plants

Construct	Gene	Source
CBWG8	glutathione-S-transferase	Chardonnay
CB390*#	metallothionein	Chardonnay
CB456*#	Nematode induced gene	Chardonnay
CBWG23#	unknown function	Chardonnay
CBWG29	unknown function	Chardonnay
CBWG33	unknown function	Chardonnay
CBWG71	cytokine-like gene	Chardonnay
CBWG75#	germin-like gene	Chardonnay
CBPR1A*#	VVPR1A	Chardonnay
CBI35	Intron p35 (anti-PCD control gene)	baculovirus
CBP14LD*#	P14 (homolog of PR1A)	tomato
CB376#	Mycorrhizal induced gene	tomato

* Northern positive transgenic plants available at this time in cv. Freedom

Scheduled to be delivered on November, 2006 as Thompson Seedless transgenic plants

Evaluate effect of specific anti-apoptotic plant genes in grape on *Xf* and PD symptoms *in planta*.

The initial experiments began with CBPR1A, CB390 and CB456 using transgenic whole plants in the greenhouse, as well as branch and leaf uptake assays; the latter conducted in the laboratory. In the whole plant assays for the first three transgenes, *Xf*-GFP movement and relative concentration were assessed by PCR and confocal microscopy in individual stems, petioles and leaves, beginning with the detached branch and leaf assays (Figures 3 and 4). Initial experiments of limited numbers of stem inoculated primary transgenics in the greenhouse, however did not provide useful data. After waiting 2-3 months it was clear that the *Xf* inoculated control plants had not developed symptoms as expected nor did the transgenic plants. Both sets of plants remained asymptomatic even though the GFP-tagged bacteria were confirmed to be present throughout the plant (Figure 1). In a second inoculation, the control and transgenic plants lost most of their leaves within 3 months after inoculation, in part due to inadvertent boron toxicity in the

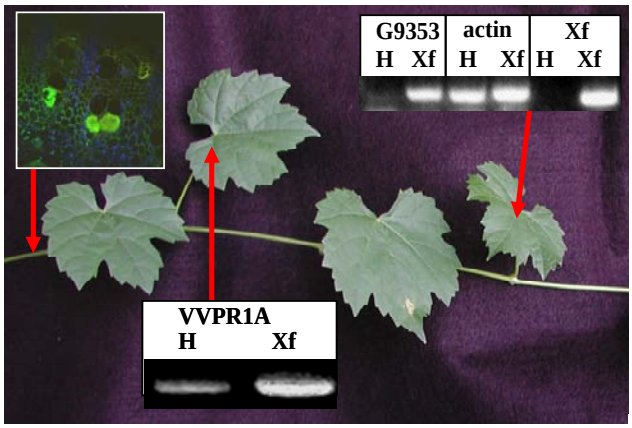

Figure 1. Analysis of transformed and untransformed plants following inoculation with GFP-tagged *X. fastidiosa*.for presence of the bacteria in relation to expression of marker genes and PD symptoms. Plants were analyzed by confocal microscopy and RT-PCR. Both infected and non-infected plants were asymptomatic at the time the assays were done. H=healthy; *Xf* = inoculated.

first month following inoculation. All current transgenic plants in pots are under a newly installed, controlled drip, irrigation with balanced nutrients and deionized water, ramets of each transgenic are ready for the experiment to be repeated with replications.

Measure the effect of blocking leaf scorch symptoms with anti-apoptotic transgenes on bacterial population and movement *in planta*

Bacterial movement and relative concentration will be monitored by RT-PCR and confocal microscopy using *Xf*-GFP. 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. Utilization of these genes in agricultural situations requires that the impact on the pathogen and the host be quantified. This requires extensive sampling of stem, petiole and leaf tissue of all the transgenic plants. We currently sample individual plants with at least 3 stems per plant using the cut branch uptake method with 50 sites per leaf, 20 per petiole and 20 internode sites and each node on the stem. Preliminary experiments have shown that bacteria can first be detected about 1 week at the base of the leaf in the stem uptake experiments but extensive preliminary time course sampling will be required to determine the range of times needed to establish the most revealing time points in the presence and absence of the anti-apoptotic transgenes in both branch and leaf uptake studies (Figure 1).

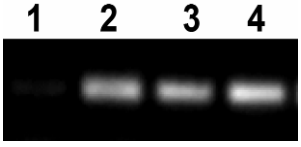

Figure 2. Transcript analysis of branch uptake of *X. fastidiosa*. RT-PCR was performed using G8946 specific primers. Lanes: 1=healthy; 2=PD symptoms; 3 and 4=PR1 transgenic grape.

Determine grape gene expression changes in transgenic compared with non-transgenic plants infected with *Xf*.

Doug Cook previously reported extensive changes in gene expression in *Xf* infected Cabernet Sauvignon on Freedom rootstock by Real-Time PCR followed by expression profiling (Cook 2005). In collaboration with Dr. Cook, we will initially assess the effect of the expression of the anti-PCD genes on a 24 gene subset of the 448 genes he reported to be upregulated only in infected tissue by Real-Time PCR. We also will assess which genetic pathway is affected by the transgenes by difference in expression profiling of inoculated transgenic and non transgenic cohorts on the full set of genes identified by Dr. Cook as differentially expressed in the presence of *Xf*. One of these genes, G8946, was up regulated only in infected stems and expressed only in the phloem and immature xylem cells adjacent to *Xf* in the mature xylem (Gilchrist and Lincoln 2005) and was up-regulated in our cut branch uptake technique described in the following section (Figure 2).

Evaluate the branch and leaf uptake procedures developed recently in our laboratory as surrogate approaches to long term greenhouse or field experiments

The goal is to use rapid response methods to induce and characterize determinants of PD compared with stem inoculation. Demonstration of changes in gene expression in the presence of the bacterial consistent with those recorded under greenhouse or field conditions would at least validate the method for preliminary characterization of plant response to bacteria at the genetic level. The experiments will use coincidental transcriptional profiling as a measure of similarity in transcriptional response of the host tissues relative to the location and appearance of scorch symptoms. In searching for a method to shorten the time from exposure of grape tissue to *Xf* and a measurable plant response, we have explored several *in planta* approaches to introducing the bacteria into the vascular system in a manner that results in changes in host gene expression and the appearance of leaf scorch symptoms. Uptake of *Xf* suspensions into cut grape branches and into cut grape leaves of susceptible grape under our experimental conditions induces typical marginal leaf scorch symptoms of Pierce's disease within 2-4 weeks compared with 12-16 weeks with whole plant stem inoculations in cv Freedom (Figures 3 and 4). Hence, experiments can be replicated many fold by repeatedly treating detached leaves as individual experimental units with clonal genetic identity compared with committing a whole plant to a single assay as one experimental unit. All of the experiments measuring bacterial dynamics and changes in gene expression in infected tissue are tedious requiring extensive serial and time course sampling and analysis. Two methods evaluated in the past few months have proven very useful in this regard are:

Figure 3. Branch uptake. Branches of transgenic grape were allowed to uptake *Xf*. After 3 weeks PR1 (A) and metallothionine (B) transgenics show reduced symptoms compared to GFP transgenic (C).

Branch uptake method. A terminal shoot approximately 60cm long was cut from greenhouse or growth chamber grown grape plants. The shoot is re-cut under water removing an additional 5cm and placed in a beaker of distilled water containing $2x10^7$ *Xf* per ml with mixing in a hood with air flow to increase transpiration. Bacteria were taken up for 2 to 48hrs depending on the experiment, then transferred to individual 50 ml glass culture tubes containing distilled water under low intensity fluorescent lights for symptom development within 2-4 weeks (Figure 3). Initial experiments confirmed that the bacterial taken up through the cut surface move into the vascular system resulting in cell death characteristic of PD leaf scorch. The plants transgenic for VVPR1A and

metallothionein exhibited little or no cell death over the duration of the experiment compared with the control branches. Analysis of the coincident changes in gene expression by transcriptional profiling between control and transformed plants is in progress.

Leaf uptake method. Young, full-sized, mature leaves were cut from greenhouse or growth chamber grown grape, the petioles were re-cut under water and the leaves placed individually in 2ml plastic tubes containing $2x10^7$ *Xf* per mL. The remainder of the uptake and incubation was similar to the branch method. Each petiole provides 20 sections for analysis by RT-PCR and confocal microscopy. Macroscopic leaf scorch symptoms appear on cv. Freedom within 2-4 weeks (Figure 4). Initial transcript analysis indicates that *Xylella*-induced genes are up-regulated in these uptake methods.

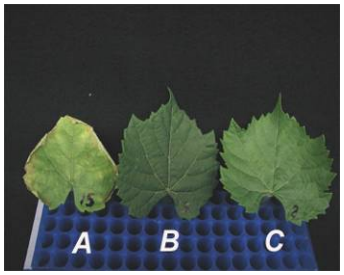

Figure 4 Leaf uptake. Leaves of untransformed (A) develop symptoms 2 weeks after uptake of *X.f.* while VVPR1A (B) and CB390 (C) transgenics are symptom free for 1 month.

CONCLUSIONS

Several relatives of grape and other asymptomatic plants can harbor high titers of *Xf* without exhibiting PD symptoms. We have established that leaf scorch PD symptoms in grape result from apoptosis or programmed cell death (PCD). Clearly, *Xf* does not have to kill in order colonize the vascular system. So, a key question addressed by this research is; are there genes in the plant that respond by triggering programmed cell death in certain grape genotypes, can this response be blocked genetically, and, if so, does this then allow the bacteria to return to the endophytic state, leaving the plant otherwise unaltered and disease symptom free? We have identified from a functional cDNA library screen several grape genes that block PCD when over-expressed. Preliminary experiments indicate that one of these genes, VVPR1A, is expressed in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. We are testing the hypothesis that over expression of genes like VVPR1A can block both PCD induced by *Xf* and disease symptoms associated with PD in both detached branch or leaf uptake assays and in inoculated whole plants.

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THE EFFECT OF DORMANT SEASON SURVIVAL OF *XYLELLA FASTIDIOSA* IN GRAPEVINES ON PIERCE'S DISEASE EPIDEMICS IN CALIFORNIA

Project Leader:
Barry L. Hill
CDFA, PDCP
Sacramento, CA 95832
bhill@cdfa.ca.gov

Cooperators:
Jennifer Hashim
UC Cooperative Extention
Kern County, CA

William Peacock
UC Cooperative Extention
Kern County, CA

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ABSTRACT
The two California Pierce's disease (PD) epidemics associated with population outbreaks of the glassy-winged Sharpshooter (GWSS), at Temecula in the mid-1990s and in Kern County, peaking in 2002, differed in the number of vineyards lost and the grapevine varieties affected. In Temecula, almost half of all vineyards of all varieties were lost to PD, whereas in Kern County only the vineyards of two varieties, Redglobe and Crimson Seedless, suffered losses; all the vineyards of the other four varieties were unaffected. A hypothetical explanation of this epidemiological pattern is that in those parts of California where the winters are more severe, dormant-season die-out of *Xylella fastidiosa (Xf)* is more likely, and only the earlier-season inoculations and infections survive the winter. The likelihood of *Xf* die-out is a function of both winter climate and varietal susceptibility. In Kern County, only the most susceptible varieties were affected by secondary (vine to vine) transmission and early season primary transmission (where insect vectors acquire *Xf* from plant sources outside the vineyard) was of little consequence. Through field experiments, this project expands our knowledge of secondary transmission in the southern San Joaquin valley. The benefit to grape producers in this area will be twofold: 1) more accurate assessment of risk of economic loss from PD, and 2) suggestion of new integrated disease-management practices to control PD.

INTRODUCTION
The glassy-winged sharpshooter (GWSS)-associated Pierce's disease (PD) epidemics in Temecula and in Kern County were the first instances of epidemic secondary transmission of PD in California since the Anaheim epidemic of 1885 – 1895. During the intervening 100+ years, losses from PD in California have resulted from primary transmission, and those losses have been economically manageable in most areas. In the General Beale epidemic in Kern county (which has a colder winter climate and longer dormant season than Temecula), only a small percentage of the vineyards were lost, and all of the lost vineyards were planted in only two of the six varieties in the area, Redglobe and Crimson Seedless.

The losses to vineyards of the other four varieties were very small—in most cases less than 1 in 10,000 vines. By contrast, all 12 of the Redglobe vineyards monitored in the General Beale area were significantly damaged, with a range of 2% to over 50% of the vines lost (Hashim, *et al*, 2003). Most of these vineyards were ultimately removed.

Grapevines acquire new *Xylella fastidiosa (Xf)* infections either by primary or secondary transmission. Primary transmission occurs when vector insects acquire the bacterium from source plants outside the vineyard, then fly into the vineyard to infect vines. Secondary transmission occurs when vector insects acquire *Xf* from an infected vine within the vineyard and then transmit the infection to other vines, known as vine-to-vine transmission.

The risk associated with these two kinds of transmission differs. The disease and vine loss pattern associated with primary transmission is linear; that is, a relatively constant number of vines per year become infected, so the yearly accumulation of PD vines increases additively and predictably. By contrast, the pattern of yearly accumulation of PD vines associated with secondary transmission is typically logarithmic, increasing as a multiple of the infected source vines that are present, so entire vineyards can be lost within just a few years.

Secondary transmission cannot begin to occur until that time in the growing season when the bacterial cells in diseased vines have multiplied and moved within the vine; the cells travel from the refuge site, where they survived the dormant season, up into the new growth where vector insects can feed and acquire them. Secondary transmission of infection can then continue until the end of the growing season. However, infection does not equal disease. The phenomenon of over-winter curing of *Xf* infections is well-documented in most viticulture areas of California (Fiel *et al*, 2003). Early-season inoculations can result in infections which survive the dormant season and progress to chronic disease and vine death. Conversely, later-season infections do not become sufficiently established to survive the dormant season, and the vines are free of infection the following year (Fiel *et al*, 2003).

In most viticulture areas of California (Napa and Sonoma Valleys, for example), secondary transmission of infection regularly occurs, but it cannot begin early enough in the season for the infection to survive vine dormancy and progress to chronic PD. In these areas, secondary transmission occurs but does not result in disease.