EVALUATION OF GENES ISOLATED BY A FUNCTIONAL GENETIC SCREEN FOR SUPPRESSION OF BACTERIAL GROWTH OR SYMPTOMS IN PIERCE'S DISEASE

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ABSTRACT
Our overall objective is to identify genes from cDNA libraries of either grape or heterologous plants that, when up regulated in grape, will disrupt infection, spread or symptom development by the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). Hence, we are interested in the effect of the genetic disruption of Pierce's disease (PD) symptoms on the movement or establishment of the bacterium in the xylem of susceptible grape plants. Recent published information from our laboratory established that specific transgenes from homologous or heterologous hosts that block programmed cell death (PCD) during plant disease development, can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions. A functional screen was used to evaluate cDNA libraries of grape and tomato for genes that, when overexpressed in tissues stimulated to undergo PCD, would block the death and therefore represent potential anti-PCD (anti-disease symptom) genes. Collectively, more than 500,000 cDNAs were screened and 12 genes were cloned that when overexpressed as transgenes in tomato or grape blocked PCD. Three of these genes when overexpressed as transgenes blocked PCD triggered by a verified ceramide-derived inducer of plant PCD. One of these gene, designated as PR1A in grape, was chosen for further direct characterization. This gene has high sequence homology to a gene family from humans, nematodes, hookworms and several plant species, wherein its expression is correlated with situations in which PCD is blocked in both animal and plant diseases. When overexpressed as a transgene in grape, the PCD sensitive grape tissues is now insensitive to microbial inducers of PCD. We believe that examination of the molecular basis of cell death in pre-symptomatic and symptomatic tissues, along with the immediate assessment of the effect of expressing anti-apoptotic transgenes in PD infected tissues on the development of death-related symptoms in grape, will be very informative in the short run in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic strategies for protection of grape against infection by *Xf* in years not decades.

INTRODUCTION
Published information from our laboratory confirms that specific transgenes from homologous or heterologous plants, that block PCD during plant disease development (4), as well as chemical inhibitors of apoptotic proteases (3), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). The conserved genetically determined PCD process can be studied by biochemical, cytological and genetic techniques and can be transgenically manipulated by techniques developed in our laboratory (3, 4).). PCD is now well established as a key pathway involving many gene products in numerous diseases of animals and plants. We further established that expression of the anti-apoptotic p35 gene in transgenic grape tissue blocked cell death (PD) symptoms in *Xf* infected tissue. This demonstrates that the anti-apototic genes to be recovered from the cDNA library screens have excellent potential to provide protection in grape against PD. Based on previous results we tested the effect of the p35 transgene from baculovirus on viability of roots, produced on *Xf* infected chardonnay and observed protection of the roots against death in the presence of *Xf*. This indicates a role for PCD in PD and provides optimism that novel genetic determinants of resistance can be identified using this screen. Given the strategies used it is likely the genes will function in grape by altering the effect of *Xf* infection in grape through suppression of symptoms either directly on cell death or indirectly by modifying the behavior of the bacterial in the xylem. It should be emphasized that the effect of anti-apoptotic transgenes on plants is not to induce so-called systemic acquired resistance (SAR) as no markers of SAR are induced in the presence of anti-apoptotic genes such as the p35 gene (4). We believe that the effect of expressing anti-apoptotic transgenes in PD infected tissues on the development of death-related symptoms in grape will contribute significant information in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical-based signaling strategies for protection of grape against infection by *Xf* in years not decades, perhaps similar to the effects we reported previously in tomato (4).

OBJECTIVES
1. Create grape transgenic plants over-expressing candidate anti-apoptotic plant genes obtained through cDNA library screens.
2. Evaluate these specific anti-apoptotic plant genes in grape for effect on *Xf* and PD symptoms.
3. Apply signal molecule discovery tactics to elucidation of the molecular basis of susceptibility, focusing first on grape PR1A.

RESULTS

Creation of grape (Thompson seedless) transgenic plants over-expressing genes of interest

Although the construction of a grape cDNA libraries initially proved much more difficult than we had experienced in making libraries from 4 other plant species, we have isolated a number of genes from screens of Chardonnay cDNA libraries as well as tomato cDNA libraries that potentially regulate programmed cell death in plants (Table 1). The inserts for all libraries are cloned into the binary vector B5 for direct transformation into the *A. tumefaciens* for generation of transgenic grape plants by the UCD plant transformation facility. It is important to emphasize that the screens were not dependent on the presence or role of PCD in PD but will detect any gene that affects the integrity of the bacterium in the infected tissue or the ability of the bacterium to elicit symptoms of PD, regardless of whether the step being affected is strictly dependent on the induction of PCD.

Table 1. "Short list" of plant anti-apoptotic genes, derived from functional screen of cDNA libraries, for transformation into grape

Construct	Gene	Originating organism
CBWG3	secretory leader of chitinase but not ORF	Chardonnay
CBWG8	glutathione-S-transferase	Chardonnay
CBWG23	EST of grape, Arabidopsis, rice	Chardonnay
CBWG29	Expressed ORF without significant match	Chardonnay
CBWG33	Expressed ORF without significant match	Chardonnay
CBWG71	cytokine-like	Chardonnay
CBWG75	germin-like	Chardonnay
CBPRIA	PR1A	Chardonnay
CBI35	intron p35 (anti-PCD control gene)	baculovirus
CBP14LD	P14 leader (wild type)	tomato
CB376	mycorrhizal induced	tomato
CB456	nematode induced	tomato
CBMT	metallothionine	tomato

Our goal is to rapidly identify resistance genes in grape genotypes that block any one of several required steps in the infection and spread of *Xf* in the xylem, steps which logically will include genetic factors regulating PCD induced by disease stress in grape. We have begun to evaluate the effect of experimental transgenes both from tomato and from grape on grape tissue bearing GFP-*Xf* in xylem elements with various cell death markers and GFP-marked bacteria. By using the GFP-tagged *Xf*, this also is a direct functional assay for genes that block bacterial movement or accumulation in the xylem of newly differentiated grape tissue (6).

Evaluate transgenic grape (cv. Freedom) plants over-expressing specific anti-apoptotic plant genes for effect on *Xf* and PD symptoms

Last year, over-expressing transgenics of grape (Freedom) were created for several of these cDNAs. Although both Chardonnay and Freedom transgenics were initiated only Freedom transgenics survived. Northern analysis confirmed the over-expression of transgene mRNA in these Freedom lines (Figure1). Pathogenicity tests with any isolated disease-disrupting cDNA will first involve a system using micro-propagated (MP) plants that are vegetative clones of sterile grape plants in small plastic boxes that can be infected with *Xf* under sterile conditions. This ensures that these plants will have uniform physiology without confounding by stress inductions as would likely occur in the field or greenhouse grown plants. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions.

Resistance of grape transgenics to PCD induction

Collectively, more than 500,000 cDNAs were screened and 12 genes were cloned that when overexpressed as transgenes in tomato or grape blocked PCD. Three of these genes when overexpressed as transgenes blocked PCD triggered by a verified ceramide-derived inducer of plant PCD, FB1. One of these gene, designated as PR1A in grape, was chosen for further direct characterization. This gene has high sequence homology to a gene family from humans, nematodes, hookworms and several plant species, wherein its expression is correlated with situations in which PCD is blocked in both animal and plant diseases (Table 2). When overexpressed as a transgene in grape, the PCD sensitive grape tissues is now insensitive to microbial inducers of PCD. The use of PCD inducers other than Xf may allow a rapid analysis for anti-PCD activity of an over-expressed gene in grape. The fungal mycotoxin FB1 has previously been shown by our lab to trigger PCD in tomato and can be protected against by anti-PCD genes (4). We investigated the possibility that grape transgenics can also be assayed by FB1 insensitivity. Both Freedom and Thompson seedless showed high sensitivity to FB1. The symptoms (Figure 2) included necrosis at the leaf margins (at 250nM FB1)and leaf drop (at 1000nM FB1). Interestingly expression of the tomato P14 gene in transgenic grape protects the grape from PCD induced by FB1. (Figure 3)

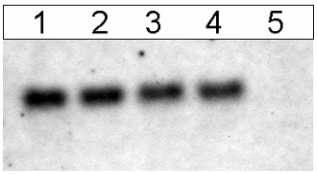
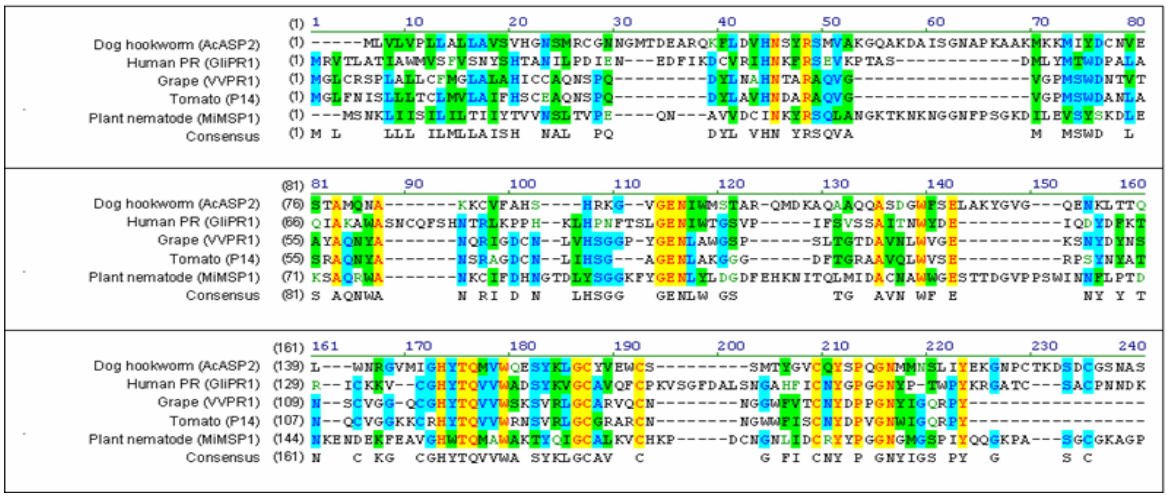


Figure 1. Northern analysis of transgenic grape. RNA isolated from transgenic grape plants (Freedom) were hybridized to a labeled P14 probe. Lanes 1 thru 4 are P14 transgenics; lane 5 is a GFP transgenic.

Table 2. PR1 family amino acid lineup displaying domains of high conservation



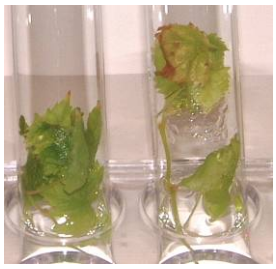


Figure 2. Induction of PCD in grape by sphinganine analog mycotoxin FB1, a widely used inducer of PCD in plants and animals. Left = 0, Right =250nm.

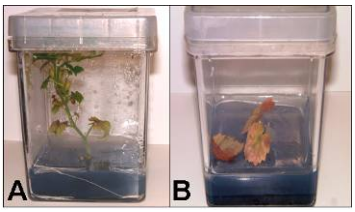


Figure 3. FB1 sensitivity assay. The terminal four nodes of a P14 transgenic (A) or a GFP transgenic (B) grape shoot was pushed into growth media containing 250nM of the programmed cell death inducing FB1. Photo was taken after 2 months. Non transgenic plant is killed while the plant transgenic for the P14 gene is protected and survives

CONCLUSIONS

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 disrupt infection, spread or symptom development by *Xf*. From a functional screen of more than 500,000 cDNAs, a total of 12 genes were scored as capable of blocking PCD in both yeast surrogate system and a plant disease-based system. Significantly we demonstrated that expression of the p35 gene and the PR1A gene, when up-regulated in transgenic grape tissue blocked programmed cell death. Additional potential anti-PCD genes from the functional screen are currently being transformed into whole grape plants (Thompson seedless) for further characterization. We believe that examination of the molecular basis of cell death in symptomatic tissues will be very informative in the short run in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical strategies for protection of grape against infection by *Xf* in years not decades.

REFERENCES

1. Gilchrist, D.G. 1997. Mycotoxins reveal connections between plants and animals in PCD and ceramide signaling. Cell Death and Differentiation 4:1312-1317.
2. Gilchrist, D.G. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. Ann. Rev. Phytopathology 36:393-414
3. 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: 213-221.

4. 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.
5. Gilchrist, D., J. E. Lincoln, and C. Richael, 2001. Inhibiting apoptosis in plants using a baculovirus p35 protease inhibitor gene. Patent # 6310273, United States Patent Office.
6. Gilchrist, D., J. E. Lincoln, A. Walker, B. Kirkpatick. 2002. Application of *Agrobacterium rhizogenes*-mediated transformation strategies for a rapid high throughput screen for genetic resistance to Pierce's Disease in grape that maintains the clonal integrity of the recipient host. Symposium Proceedings, p 11-12, Pierce's Disease Research Symposium. San Diego, CA December 15-18.

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