SCREENING OF GRAPE CDNA LIBRARIES AND FUNCTIONAL TESTING OF GENES CONFERRING RESISTANCE TO PIERCE'S DISEASE

Project Leaders:
David Gilchrist
Dept. of Plant Pathology
University of California
Davis, CA 95616

James E. Lincoln,
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
Andrew Walker
Dept. of Viticulture and Enology
University of California
Davis, CA 95616

Steven Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted from October 1, 2003 to October 1, 2004.

ABSTRACT
Our overall objective is to identify genes from cDNA libraries of either grape or heterologous plants that, when induced in grape, will disrupt infection, spread or symptom development by the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). We are interested in the effect of the genetic disruption of PD symptoms on the movement or establishment of the bacterium in the xylem of susceptible grape plants. Specific objectives are to: a) create cDNA libraries from several different grape backgrounds, including three with PD resistance; b) develop a functional *A. rhizogenes*-based cDNA screen in grape; and c) investigate the potential of blocking PD symptom expression and disease impact with anti-PCD (anti-apoptotic) transgenes. To these ends we have created full-length cDNA libraries from resistant and susceptible grape and developed an *Agrobacterium rhizogenes*-based transformation procedure that provides a functional screen for genes that alter the disease phenotype. Transformation of grape explants with *A. rhizogenes* results in the emergence of a transformed root containing a single new DNA insert, from which the transgene can be re-isolated for characterization. The identified genes will be those that directly affect the ability of the pathogen to cause disease and is not dependent on DNA sequence relationships. Pathogenicity tests with any isolated disease-disrupting cDNA will first involve a transient expression 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. Transient assays with test genes involve infiltration of *A. tumefaciens* containing the gene of interest into MP leaf tissue. The bacteria transfer the test gene into leaf cells that are presymptomatic will determine if the expression of the transgene in the leaf can block PD symptoms.

INTRODUCTION:
Published information from our laboratories 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). 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*. We believe that the effect of specifically 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. Construct cDNA libraries from several different grape backgrounds including from lines with PD resistance and from infected and uninfected grape tissue.
2. Conduct functional *A. rhizogenes*-based cDNA screen and clone genes that give altered phenotype in grape.
3. Evaluate specific anti-apoptotic plant genes in grape for effect on *Xf* and PD symptoms.
4. Determine the potential of blocking PD symptom expression with anti-apoptotic transgenes through chemical induction of such genes in transgenic grape tissue or by tissue-specific expression in roots or vegetative tissue of *Xf* infected grapes.
5. Use a combination of genetic and signal molecule discovery tactics to elucidation of the molecular basis of susceptibility

RESULTS

Construction of cDNA Libraries

The construction of a grape cDNA library initially proved much more difficult than we had experienced in making libraries from 4 other plant species. Isolation of mRNA was not difficult but the grape tissue contains high levels of phenolic compounds in an oxidative environment that contaminate the RNA, rendering it difficult to reverse transcribe. We now have an efficient protocol for generating full-length cDNA libraries from grape using an antioxidant cocktail during homogenization and CsCl gradient purification of RNA. The Hanes City (*V. shuttworthii*) and Chardonnay libraries are completed with 300,000 members each with an average insert size of 1000 bases . The tissue source was field grown plants provided by Dr. Walker. The susceptible Chardonnay is used as a recipient host to screen cDNA libraries. We have begun screening these libraries while continuing to develop libraries from Cowart (*M. rotundifolia*) and Dr. Walker's resistant tester line 8909-15. The inserts for all libraries are cloned into the binary vector B5 for direct transformation into the *A. rhizogenes* functional screen in Chardonnay and a transient assay. The transient assay is based on a leaf infiltration approach that we have used successfully for tomato and tobacco disease assays of putative resistance genes. For transient assays, selected cDNA inserts in the B5 vector are used to transform *Agrobacterium tumefaciens* strain GV2260. The resulting GV2260 transformed bacteria are then pressure infiltrated into attached pre-symptomatic leaves of *Xf* infected MP plants. The ability of the expressed gene to inhibit symptoms is then evaluated. As potential cloned resistance genes become available they also will be used to identify homologues from the Chardonnay cDNA library that may provide resistance by simple alteration in expression level within the homologous host in a time and tissue specific manner. These full-length cDNA libraries are available to all grape researchers in this program.

Screening of cDNA Libraries

The *Agrobacterium rhizogenes*-based transformation procedure results in the induction of transformed roots from infected (or healthy) vegetative tissue sections following co-cultivation with the transforming bacteria. Each emerging root is an independent transformation event, contains a single new DNA insert from which the transgene can be re-isolated by PCR for characterization. Figure 1 **(**below) illustrates the successful transformation of all emerging roots from a grape stem explant with the green fluorescent protein (GFP). This technique is a functional cDNA library screen (each root contains a different cDNA library member) for genes from grape libraries that block either bacterial multiplication, movement, or symptom expression. We previously determined that viable roots do not form on host tissue explants that are infected with *Xf* unless protected by transgenes. The genes that will be identified will be those that directly affect the ability of the pathogen to cause disease and are not dependent on DNA sequence relationships. The library is being screened in sets of 50,000 cDNAs to improve the efficiency in terms of handling numbers of symptom blocking cDNAs. Based on previous experience with tomato, we expect that less than 0.01% of the cDNAs will effectively protect against PCD and/or the disease development. This underscores the need for a highly effective functional screen. In order to provide sufficient *Xf*–infected tissue of similar physiological state for transformation, we developed a micro-propagation (MP) technique for producing clones of sterile grape plants in small plastic boxes that can be inoculated with *Xf* under sterile conditions illustrated in Figure 2 at the right. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions (See leaf in foreground). Plants produced under these same conditions also are the source of *Xf* infected stem sections used for transformation in the *A. rhizogenes* functional screen.


Figure 1

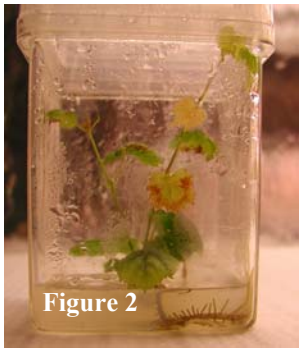
Figure 2

The major advantage of the MP plants is that they are much more efficiently transformed than the greenhouse-derived tissue, which tends to be more highly lignified and produces fewer transformed roots. As a means of fast tracking the cDNA screen while optimizing the grape transformation procedure, we have screened approximately 30,000 members of the Chardonnay cDNA library by *A. rhizogenes* transformation of tomato cotyledons. The resulting roots were subject to disease-dependent PCD induction by treatment with the pathogenic toxin FB1 (1, 2). PCR was used to amplify the Chardonnay cDNA insertion from the surviving tomato roots. The cDNA inserts were then cloned and sequenced. Using this analysis of the Chardonnay cDNA library, we so far have found several grape full-length cDNAs (encoding open reading frames) that protect tomato roots from disease-linked programmed cell death (PCD), a death process that is functionally equivalent to the death of cells in *Xf* infected grape. These grape genes are now being re-evaluated in the *A. rhizogenes*-grape system for protection of Chardonnay grape tissue against symptoms due to the presence of *Xf* in the xylem. Several potentially protecting cDNAs that protect roots are now in the queue to produce whole plant transgenics by the UCD Plant Transformation Facility (Table 1). The expression of these genes in the protected roots was confirmed by northern analysis (unpublished). Most of these genes share sequences homologous with animal genes known to block disease-linked PCD.

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

Name	ID (putative)
404	empty vector
P35	baculovirus p35
G8	glutathione-S-transferase
G71	cytokine-like protein
P14LD	pathogenesis related gene secretory form
P14	pathogenesis related gene non-secretory form
MT	metallothionine
Y376	mycorrhiza up regulated gene
Y456	nematode up regulated gene

It is important to emphasize that this screen is 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.

Two of the genes (P14LD and Y456) were constitutively expressed in grape by *A. rhizogenes* transformation. The transformed roots were protected against *Xf* -induced death, as were those *Xf*-infected grape explants from which the emerging grape roots transformed with the p35 gene. 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) nor were they observed in the case of the P14LD and Y456.

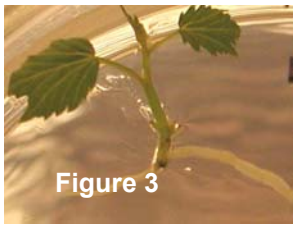
Figure 3

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). Of particular interest is the possibility that PD blocking signals initiated with transgenes may move systemically through the vascular system from transformed rootstocks to upper regions of grafted cultivated grape tissue affording protection against systemic movement or activity of *Xf* without genetically engineering the cultivated grape. To this end, the MP plants provide an excellent experimental system by which transformed roots can be initiated on untransformed shoots. The fact that these transformed roots can be formed within 4-6 weeks means that any gene that protects roots can quickly be evaluated for systemic protection; protection from a transformed root stock (in the real world) to an untransformed susceptible fruit producing scion illustrated in Figure 3 above.

RNA Induced Gene Silencing (RNAi)

This same system will enable us to explore the potential for using RNA induced gene silencing (RNAi) (7,9), the expressed silencing small RNA molecules, comprised of small (21 bp) sequences derived from the gene to be silenced, are known to move systemically throughout the plant (8) and silence transgenes from roots to scions. The RNAi from RNAs expressed in the roots have the potential to silence any gene from our project or from other labs that is induced in either susceptible or resistant responses, and deemed to have a definitive role in disease. The small mobile silencing RNAs further have the potential to move systemically in the plant (8) to silence genetic determinants of susceptibility. If either signals from the transgenic roots (from cDNA library screen) or roots expressing RNAi were to provide protection against PD, the best case scenario would be to simply graft a transformed shoot onto an existing infected plant and block the disease without transforming either the roots or the scion. To this end we have developed a plant transformation vector capable of expressing a hairpin RNA. As proof of concept we have used this vector to construct a GFP RNAi expression vector and have shown it is capable of knocking out GFP expression in transient assays. We are currently using *A. rhizogenes* to produce GFP RNAi roots on GFP-expressing transgenic grape shoots to explore the ability of transgenic roots to knock out expression in the shoot.

The research discussed herein has been reported at the Pierce's Disease Symposium in San Diego and in annual reports to the CDFA Pierce's Disease/GWSS Research Program. Manuscripts are being prepared on the various screens developed for the cDNA libraries and the construction of the libraries.

CONCLUSIONS

Genetic resistance and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program. 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*. Published information from our laboratory established that specific transgenes from homologous or heterologous hosts that block programmed cell death (PCD) (1) during plant disease development (4), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). PCD is now considered as a key pathway involving many gene products in numerous diseases of animals and plants. Blockage of PCD can be achieved by expression of anti-apoptotic transgenes, RNAi suppression of endogenous genes, and by chemical inhibitors of PCD. Significantly we demonstrated that expression of the anti-apoptotic p35 gene in transgenic grape tissue blocked cell death and PD symptoms in *Xf* infected tissue. 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 Differen. 4:1312-1317.
2. Gilchrist, D. G. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. Ann. Rev. Phytopath. 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. Mol. Plant Pathol. 59(4) 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., Lincoln, J.E. and Richael, C. 2001. Inhibiting apoptosis in plants using a baculovirus p35 protease inhibitor gene. Patent # 6310273, United States Patent Office.
6. Gilchrist, D, Lincoln, J., Walker, A., and Kirkpatick, B. 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.
7. Lavorgana, G., et. al. 2004. In search of antisense. Trends in Biochemical Sciences. 29:88-94.
8. Palauqui, J-C, et. al. 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. The EMBO J. 16:4738-4745.
9. Baulcombe, D. and English, J. 1996. Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. Curr. Opinion Biotech. 7:173-180.

FUNDING AGENCIES

Funding for this project was provided by the American Vineyard Foundation and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.