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

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ABSTRACT

Our overall objective 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 of the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). Furthermore, we are specifically characterizing the effect of the genetic disruption of Pierce's disease (PD) symptoms on the movement and 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 two with PD resistance; b) develop a functional *A. rhizogenes*-based cDNA screen in grape; c) examine the morphological and cytological features of cell death in symptomatic tissues; and d) investigate the potential of blocking PD symptom expression and disease impact with anti-apoptotic transgenes. To date we have developed a *Agrobacterium. rhizogenes*-based transformation procedure that 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. We then used this technique to develop a functional cDNA library screen (each root contains a different cDNA library member) for genes that block either bacterial multiplication, movement, or symptom expression. The only genes that will be identified will be those that directly affect the ability of the pathogen to cause disease and is not dependent on DNA sequence relationships. We have made excellent progress in creating an extensive library of full-length cDNAs from several resistant sources as well as susceptible Chardonnay and conducting initial screens of the libraries.

INTRODUCTION

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 of the xylem-limited bacteria, *Xylella fastidiosa* (*X.f.*). Recent published information from our laboratory confirms that specific transgenes from homologous or heterologous hosts, as well as chemical inhibitors of apoptotic proteases (3) 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 well established as a key pathway involving many gene products in numerous diseases of animals and plants. Since our previous work (4, 5) established that the p35 gene (a gene encoded by an animal virus to block PCD in infected host cells) could block growth of several bacterial and fungal pathogens in infected tissue of several plant species, we transformed grape tissue with the p35 transgene and observed that the infected tissue remained asymptomatic. We are now assessing the effect of the p35 transgene on *X.f.* movement or growth in transformed grape tissues using the GFP-transformed *X.f.*, provided by Dr. Lindow. A corollary to this observation is that the *X.f* -triggered death observed in PD occurs in the absence of apparent water stress and can be observed in young tissues before pronounced symptoms develop. This 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). 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 *X.f.* in years not decades. The immediate goal is to rapidly identify novel resistance genes in grape that block any one of several required steps in the infection and spread of *Xf* in the xylem.

OBJECTIVES

1. Construct cDNA libraries from several different grape backgrounds from infected and uninfected grape tissue, including libraries from lines with PD resistance;
2. Develop a functional *A. rhizogenes*-based cDNA screen in grape;
3. Examine the morphological and cytological features of cell death in symptomatic tissues using Dr. Lindow's GFP transformed *X.f*;

4. Investigate the potential of blocking PD symptom expression with anti-apoptotic transgenes.

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. However, 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 libraries have an average insert size of 1000bp and sequence analysis of random inserts reveals that the cDNAs appear full-length. The goal is to create libraries each containing a minimum of 500,000 cDNAs from PD susceptible *Vitis vinifera* (Chardonnay), and resistant sources, *Muscadinia rotundifolia* (Cowart), and *Vitis shuttleworthii* (Hanes City). These libraries will be immediately available to all grape researchers in this program and will represent the largest available source of full-length cDNAs. We have to date constructed approximately 150,000 independent grape cDNAs derived from healthy and *Xf* infected Chardonnay and then ligated into a plant transformation binary vector, CB5, which is a derivative of pBIN19 and uses the CaMV 35S promoter for high level, constitutive expression. We have generated approximately 100,000 independent grape cDNAs each from *M. rotundifolia* (Cowart) and *V. shuttleworthii* (Hanes City). The susceptible Chardonnay will be used as a recipient host to screen cDNA libraries of Chardonnay, Cowart and Hanes City that express differing levels or forms of resistance to PD. Cowart and Hanes City are two primary sources of resistance being used in Dr. Walker's genetics program. As potential cloned resistance genes become available they also will be used in attempts to identify homologues from the Chardonnay cDNA library that may provide resistance by simple alteration in expression level within the homologous host.

Screening of cDNA libraries

The cDNA library has been moved into *A. rhizogenes* in preparation of transformation of infected grape explants for the purpose of finding cDNAs that will block the death of infected tissues. The library will be screened in sets of 50,000 cDNAs to improve the efficiency in terms of handling numbers of symptom blocking cDNAs. However, 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 *X f*–infected tissue for transformation, we developed a micro-propagation technique for producing clones of sterile grape plants in small plastic boxes that can be inoculated with *Xf* under sterile conditions. The micro-propagated plants 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 perfecting 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 PCD induction by treatment with the mycotoxin FB1. 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 from PCD. These positive grape genes will be tested individually in Chardonnay. Lastly, this analysis of the Chardonnay cDNA library in tomato demonstrates that the cDNA library is functional in transformation and expression. It is important to emphasize that this screen is not dependent on the presence or role of PCD in PD but will, in fact, 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.

Evaluation of the effect of blocking PD symptoms on bacterial growth and movement

We have begun to evaluate the effect of experimental transgenes on grape tissue bearing *X.f* in xylem elements with various cell death markers and GFP-marked bacteria. By using the GFP-tagged *Xf*, this is a direct functional assay for genes that block bacterial movement or accumulation in the xylem of newly differentiated grape tissue (6). Previously we developed a yeast-based surrogate screen for endogenous anti-PCD plant genes from a tomato cDNA library. We obtained 12 genes from tomato from more than 500,000 cDNAs screened in yeast that also block PCD in the *A. rhizogenes*-tomato root functional disease assay. In order to jump-start the functional assay in grape we will immediately test these genes in the *A. rhizogenes*-transformed PD-infected grape system. Homologues of the tomato genes can be cloned from grape to provide authentic grape genes to use in the very near future; the focus being on any grape homologues of tomato genes that block PD in grape. Of particular interest is the possibility that PD blocking signals initiated with transgenes will 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.

Plant transformation with putative resistance genes and whole plant regeneration

Two approaches are being taken. In the first approach, transformed test plants will be obtained by standard *A. tumefaciens* methods by the UC Davis Plant Transformation Facility which now performs grape transformations. To this end, we have initiated transformations of Chardonnay with the baculovirus p35 gene as well as a tomato gene that protects tomato roots from PCD induced by the fungal mycotoxin FB1. In the second approach, we also will pursue regeneration of whole plants from transformed grape roots as a means of direct introgression of cloned resistance genes into a susceptible host plant while maintaining the clonal integrity of the recipient plant following transformation. While derived from basic molecular genetic research, our immediate goal is to rapidly identify resistance genes in grape genotypes that block any one of several required steps in the infection and spread of *X.f* in the xylem and get these into the hands of breeders and viticulturists for immediate

evaluation. As indicated earlier, regeneration in this manner will permit the direct establishment of a propagative plant that will maintain the clonal integrity of the untransformed parental material. More than 120 plants have been transformed by *A. rhizogenes*. Regeneration from transformed roots has been reported from 31 with no indication of unusual difficulties. We do not expect that regeneration will be trivial but are optimistic that, with systematic examination of growth conditions and hormonal regulation of development, regeneration will be possible. However, the first approach will ensure that we have adequate transformed test plants to evaluate under controlled glasshouse conditions in the shortest time possible.

CONCLUSIONS:

We are currently adding monthly an additional 50,000 cDNA inserts each of Cowart, Hanes City and Resistant tester line 8909-15 cloned into the binary vector CB5 (for direct transformation into the *A. rhizogenes* functional screen in Chardonnay). The tissue source for these library sets is from field grown plants. The average insert size is 1000 bases. We anticipate the development of libraries of each of the resistant source with 500,000 members within 12 months. Additional library subsets are being constructed from *X. fastidiosa*-infected tissue that we have developed from micro-propagated sterile cultured plants grown in individual plastic boxes and infected under sterile conditions. This to ensure that the only biotic stress these plants will have experienced is from *X.f* and would, therefore, contain *Xf* specifically induced genes, without confounding by other biotic stress induction as would likely occur in the field or greenhouse grown plants. Plants produced under these same conditions also are the source of *Xf* infected stem sections used for transformation in the *A. rhizogenes* functional screen.

The first grape library has been pre-tested by screening the grape cDNAs in tomato to determine if the library contains anti-PCD genes. Additional considerations that are part of the critical requirements for a comprehensive library from grape lines are that these or other genes will block the development of the bacterium or the symptoms associated with the disease in grape. Hence, we screened 30,000 members of a grape cDNA library by *A. rhizogenes* transformation of tomato cotyledons. Toxin-induced PCD resistant tomato roots were isolated; the protecting grape cDNA insert was recovered by PCR, and sequenced. These genes have now been re-cloned and are being tested in grape against PD-infected stem sections. Based on the pre-test in tomato, a limited number of grape genes appear to effectively protect against PCD as was observed earlier with the screen of tomato libraries in tomato for anti-PCD (disease protecting) genes. Two strongly protecting open reading frames (ORFs) share sequence homology to respectively, glutathione-S-transferase (a protein that has been reported to be involved in disease resistance) and an unidentified expressed plant protein. The fact that a small percentage of the cDNAs appear to protect is encouraging in that we expect that the genome of grape will contain only a few genes that can effectively protect plant cells against pathogen-secreted signaling molecules that lead to disease development. We fully expect to have several novel genes identified within the first year of funding and will proceed to study their mode of action as proposed.

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