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Pine genes regulated by the necrotrophic pathogen *Fusarium circinatum*

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Abstract A targeted genomics approach was used to construct a cDNA array of potential pathogen-regulated genes for investigating host–pathogen interactions in pine trees (*Pinus* species). This array, containing a nonredundant set of 311 cDNAs, was assembled by combining smaller sets of cDNAs generated by differential display or suppression subtraction hybridization using a variety of pathogen treatments and elicitors. The array was probed to identify host genes regulated by *Fusarium circinatum*, a necrotrophic fungus that incites pitch canker disease on pine stems. A set of 29 cDNAs were induced during the disease state. Notably, cDNAs on the array that were derived from experiments with fusiform rust, incited by *Cronartium quercuum* f. sp. *fusiforme* (a biotrophic fungus) were unregulated by *Fusarium*. The results imply distinct genetic responses in pine to diseases incited by necrotrophs and biotrophs. This cDNA collection expands the genomics toolkit for understanding interactions between conifers and their microbial associates in forest ecosystems.

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Introduction

Plants express a diverse suite of genes in response to pathogen infection. Defense responses include the rapid production of antimicrobial compounds and proteins, physical barriers, and localized host cell death (Thomma et al. 2001). The effectiveness of these defense responses varies, depending in part upon the type of pathogen infecting the plant. For example, localized host cell death is very effective against biotrophs because these pathogens depend upon living cells for nutrient absorption and survival (Thomma et al. 2001), but this response is postulated to benefit necrotrophic pathogens by increasing nutrient availability through accelerated host tissue destruction (Govrin and Levine 2000; Mayer et al. 2001). When defenses fail to protect plants and disease does occur, the genes encoding defense responses often are expressed, but their rate of induction may be slower in susceptible plants than in resistant plants (Lamb et al. 1992). Also, growing evidence suggests that successful biotrophs and plant symbionts are able to suppress host defense responses when they invade a susceptible plant (Lambais and Mehdy 1993; Kapulnik et al. 1996; Lange et al. 1999; Sikorski et al. 1999; Chou et al. 2000; Ditt et al. 2001; Mellersh and Heath 2001). Responses to necrotrophs appear to be mediated largely by the signaling molecules jasmonic acid (JA) and ethylene, while responses to biotrophs are mediated by salicylic acid (SA); however, there are many genes co-ordinately regulated by these molecules and there is significant genetic evidence for cross talk between these signal transduction pathways (Schenk et al. 2000; Glazebrook 2001; Glazebrook et al. 2003).

While resin-based defenses are the most commonly studied defense mechanisms in *Pinus* species (Phillips and Croteau 1999), it is also apparent that pathogenesis-related (PR) proteins can have a role as well. The synthesis of PR proteins is one component of the suite of plant responses to pathogen challenge in a variety of plant species (Dixon and Lamb 1990; Enyedi et al. 1992; Yang et al. 1997). Gymnosperm PR genes include stilbene synthase (Muller

et al. 1999), chalcone synthase (Richard et al. 2000), phenylalanine ammonia lyase (Butland et al. 1998), peroxidase (Fossdal et al. 2001), and chitinase (Davis et al. 2002).

Pitch canker is an episodic disease of *Pinus* species that is incited by the necrotrophic fungus *Fusarium circinatum* Nirenberg and O'Donnell (Nirenberg and O'Donnell 1998). The disease was initially recognized in 1945 in North Carolina (Hepting and Roth 1945) and has since been identified in other areas of the southeastern United States as well as in California, Mexico, Japan, South Africa, and Chile (Viljoen et al. 1997; Wingfield et al. 2002). Visible symptoms include spreading pigmented lesions (cankers) on stems and branches that generate profuse amounts of resin (pitch). Later stages of the disease lead to wilting of succulent tissues and tissue desiccation.

We describe here the assembly and use of a cDNA array designed to investigate host-pathogen interactions in pine trees. Differential display-reverse transcriptase PCR (DDRT-PCR) and suppression subtraction hybridization (SSH) were used to identify potential pathogen-regulated genes as well as genes induced by chemical and wounding treatments known to induce defense responses in other plants. The resulting clone collection was arrayed on nylon membranes and probed with cDNA from healthy and *F. circinatum*-infected pines. We focused these initial experiments on healthy versus diseased tissues because we anticipated a dramatic re-programming of the host transcriptome in association with pitch canker disease and because this comparison would yield a better understanding of the genes that condition disease phenotypes. Transcripts encoding PR proteins and proteins expected in desiccated tissues were elevated in the pitch canker disease state. Notably, a subset of cDNAs on the arrays unregulated by pitch canker came from experiments with fusiform rust, a disease incited by the biotrophic fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*. These results imply a fundamental difference between host responses to necrotrophic and biotrophic pathogens.

Materials and methods

Plant and pathogen material

Pinus elliottii Engelm. var. *elliottii* (slash pine) seed from two open-pollinated seedlots characterized as resistant and susceptible to pitch canker disease, respectively, were inoculated with *Fusarium circinatum* isolate S45 (Forest Pathology laboratory collection, University of Florida)—initiated and grown as described by Davis et al. (2002)—at a concentration of 400–500 spores/μl. An open-pollinated slash pine seedlot characterized as susceptible to fusiform rust disease was germinated and grown as described by Davis et al. (2002). Basidiospores from *Cronartium quercuum* isolate G2 were obtained as infected oak leaves from the USDA Forest Service Resistance Screening Center near Asheville, North Carolina. The basidiospores were collected into water and adjusted to 400–500 spores/μl. Seedlings from susceptible families were inoculated by hand using methods previously described for *F. circinatum* (Davis et al. 2002). *C. q. fusiforme*-infected and healthy *Pinus taeda* L. (loblolly pine) seedlings were provided by the USDA Forest Service Resistance Screening Center as described by Warren and Covert (2004).

cDNA discovery and array construction

The arrays were assembled using cDNAs isolated from a variety of sources, including pitch canker-diseased tissue (this paper); *F. circinatum*-challenged resistant tissue (Davis et al. 2002); *C. q. fusiforme*-challenged susceptible tissue (Warren and Covert 2004 for DDRT-PCR on *P. taeda*; this paper for SSH on *P. taeda*; this paper for *P. elliottii*); exogenous treatment with sodium salicylate (Davis et al. 2002); mechanical wounding (Davis et al. 2002); chitosan-elicited pine cell culture (Mason and Davis 1997).

Differential display was performed as previously described (Cooke et al. 2003). Products were sampled from the gels and cloned if they were reproducibly displayed in a second, independent amplification reaction. DDRT-PCR and array interrogation were performed using RNA isolated from pitch canker-susceptible *P. elliottii* hedges in which one-half of the shoots on a hedge were challenged with *F. circinatum* spores and the other half with water as a control. Stem tissue, including the inoculation site and approximately 1cm of adjacent non-symptomatic tissue, was harvested 8 days after challenge, when disease symptoms were first evident (purple pigmentation of the stem and slight wilting of the shoot apex). Resistant seedlings showed no symptoms at this same point in time (i.e., they were not distinguishable from the water-inoculated controls). *F. circinatum* RNA isolated from mycelia grown in vitro was included in the DDRT-PCR procedure to aid in discriminating between fungal genes and pine genes induced in the disease state. Since the fungal transcriptome in cultured mycelia is not expected to express genes for host colonization as when in planta, the inclusion of this RNA sample was predicted to minimize the number of constitutively expressed fungal genes cloned during the procedure. DDRT-PCR was also performed using RNA isolated from control (water) and *C. q. fusiforme*-challenged slash pine seedlings. When the initial signs of gall development were detectable (8 weeks after inoculation), stem tissues were harvested. For the pathogen-challenged seedlings, symptomatic tissue was collected (stem swelling and pigmentation). For control-challenged seedlings, stem sections of a similar size surrounding the wound site were harvested.

For SSH, total RNA was isolated from healthy or *C. q. fusiforme*-infected loblolly pine as previously described (Warren and Covert 2004) and converted to cDNA with the SMART PCR cDNA Synthesis kit (Clontech/BD Biosciences, Palto Alto, Calif.). Two cDNA libraries were

constructed from these samples using the PCR-Select cDNA Subtraction kit (Clontech/BD Biosciences) and the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturers' protocols. One library was enriched for sequences expressed only in healthy pine stems (i.e., sequences common in both healthy and galled tissues were subtracted from healthy cDNA), while the other was enriched for sequences expressed only in fusiform rust galls (i.e., sequences common in both healthy and galled tissues were subtracted from galled cDNA). Clones from each library were stored in microtiter dishes, and individual colonies were spotted onto nylon membranes in duplicate with a BioGrid Robot (Biorobotics). The cells were lysed and DNA fixed to the membranes with standard colony-blotting procedures. To identify differentially expressed sequences, we used the PCR-Select Differential Screening kit (Clontech/BD Biosciences) to synthesize subtracted probes from the cDNA populations generated during the subtraction procedure. These subtracted probes were hybridized to clone arrays. Clones that hybridized to the appropriate subtracted probe at least twice were isolated with the QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.) and sequenced with the Big Dye Sequencing kit (ABI, Foster City, Calif.). Unincorporated dye terminators were removed with the DyeEx 96 kit (Qiagen) and samples run on an ABI 3700 sequencer. The genome of origin of the selected clones was determined using PCR as previously described (Warren and Covert 2004).

Cloned cDNAs were amplified using vector primers and purified using Qiaquick columns in plate format (Qiagen). DNA loading dye containing 0.05% Tween 20 was added to the PCR products. The appropriate product size and quantity were verified on agarose gels. Membrane arrays were created as described (Desprez et al. 1998). Base-denatured pine genomic DNA (0.1, 1.0, and 10 ng) was hand-spotted in triplicate on each membrane for scaling purposes. Membranes were hybridized, washed, and analyzed by phosphorimagery as reported by Cooke et al. (2003) except that the probes were prepared from 25 μg of total RNA from *P. elliottii*. var. *elliottii* pitch canker-susceptible hedges. Four membranes were probed for each treatment. Northern blot analysis was performed as described by Wu et al. (1997). Membranes were exposed to X-ray film at −80°C and to phosphorimagery screens for quantitation.

Data acquisition and analysis

Array images were visualized and digitized with a phosphorimager and associated ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech, Ithaca, N.Y.). Defective spots, including those with a high local background, were removed from further analysis. All other spots were adjusted using local background except those with adjusted spot volumes less than 128, which were removed. Low and negative adjusted spot volumes are unreliable, as they do not consistently reflect low or near-zero signals (Cooke et al. 2003). Spot volumes on each filter were scaled to the 10 ng genomic controls by dividing the spot volume by the filter mean genomic control volume. Genes exhibiting low treatment means (less than 256) for both treatments were dropped from further analysis.

Global standardization

After scaling to the genomic control spots, cDNA signal intensities were $\log_2$-transformed and globally standardized to remove variation between arrays due to the magnitude of the overall signal intensities and to normalize skewed distributions (Dudoit et al. 2002) using the filter mean and standard deviation $[x'_{ij} = (x_{ij} - \bar{x}_j)/s_j]$. Mean spot intensities were computed from standardized replicates of each treatment. To visualize the data, a modified version of the MA plots used in single-slide microarray data (Dudoit et al. 2002) was applied to the standardized data by plotting the log ratio of spot intensities, M [calculated as M=$\log_2$(pathogen challenged) − $\log_2$(mock challenged)], against the overall spot intensity, A [calculated as A=$\log_2$(pathogen challenged) + $\log_2$(mock challenged)]. Standardized data from the four replicate membranes for each treatment were averaged and the M and A values calculated and used to generate the MA plot.

Internal reference standardization

Scaled $\log_2$-transformed signal intensities were used to identify 42 genes with minimal response to the *F. circinatum* treatment based on a $\log_2$ intensity ratio of between −0.75 and 1.00 after calculating mean values for both treatments. These 42 genes were designated the internal reference set. Standardized volumes were computed using the mean and standard deviation of the signal intensities for the internal reference set of genes in a manner analogous to that used for global standardization. As above, a modified version of the MA plot was used to visualize the internally standardized data.

Treatment means and standard deviations were computed for each gene and used to derive t-tests to test each gene for a response to *F. circinatum* challenge. Bonferroni step-down adjusted P values were computed for the t values to account for multiple testing (in order to reduce the likelihood of identifying false positives). Adjusted P values of less 0.10 were considered to be significant. A quantile-quantile plot (Dudoit et al. 2002) was also used to visually inspect the significance of the gene responses to *F. circinatum*.

Results

Assembly of a disease-relevant array for pines

DDRT-PCR was performed using RNA isolated from pine tissues subjected to various treatments in order to identify pathogen-regulated genes, including genes likely to be associated with defense. All reactions were performed and analyzed in duplicate to minimize false positives, and in each case samples from pathogen or chemical treatments were compared to samples from untreated pines. Tissues screened for up-regulated genes included susceptible and resistant stems challenged with *F. circinatum* and susceptible stems challenged with *C. q. fusiforme* (Table 1). Our complete cDNA collection also includes differentially expressed genes identified in pine seedlings treated with the elicitor SA (Davis et al. 2002), in chitosan-elicited pine cell cultures (Mason and Davis 1997) and in mechanically wounded pine stems (Table 1, data not shown).

To expand our collection of genes potentially regulated by the biotrophic fungus *C. q. fusiforme*, SSH was used to make two cDNA libraries, one enriched for sequences expressed in healthy pine stems and the other enriched for sequences expressed in fusiform rust galls. Differentially expressed clones were identified from each library by arraying on nylon membranes and probing with either healthy or gall-enriched cDNA samples.

Based on BLASTN sequence comparisons with a probability threshold of 10^{-5}, the cDNA collection contains a total of 318 non-redundant clones. Seven of the non-redundant sequences encode host or pathogen rRNAs that were excluded from further analysis, decreasing the final nonredundant number of sequences to 311. Only two sequences, both with no similarity to sequences in GenBank, were in common between the cDNAs identified by DDRT-PCR and SSH.

Assigning functions and genome of origin

To assign putative functions to the nonredundant cDNAs, the sequences were compared to the non-redundant (via BLASTX) and DBEST (via BLASTN) GenBank databases using a maximum probability threshold of 10^{-5} for a sequence match. For cDNA sequences with significant BLASTX matches to genes with known functions, the highest scoring sequence was used for annotation. Although 37% of the cDNAs (115) had sequence similarities limited to expressed sequence tags (ESTs) in the database, predicted functions could be assigned to only 44 of these sequences by using matching ESTs to extend the coding sequence and thus identify putative functional homologs in the nonredundant database (Mould et al. 2003). Where appropriate, function was assigned based on the annotation generated for *Arabidopsis thaliana* by the Munich Information Center for Protein Sequences. Of the nonredundant cDNAs, 29% were classified into functional groups based on sequence similarity to plant genes with known functions (Fig. 1). Three of these groups—defense/stress, regulatory, and secondary metabolism—include genes encoding putative defense-associated proteins such as PR proteins, transcription factors, putative receptor kinases, and enzymes involved in cell-wall biosynthesis. Thus, up to 20% of the cDNAs may have a direct connection to host defense mechanisms.

Although the remaining BLAST searches did not provide functional information on the cDNAs, they did provide clues about the source genome for many of the sequences. Nineteen percent of the non-redundant sequences have sequence similarity to pine ESTs with an additional 3% having similarity to ESTs from other plant species (Fig. 1). Nonredundant sequences with BLAST similarities to plant sequences with unknown function were placed in the 'plant unknown' category (10%). Members of this group

Table 1 Summary of gene discovery projects that generated cDNAs for the array used in this study

Treatment	Plant material[a]	Host species	Cloning method[b]	Number of clones	Accession numbers[c]
F. circinatum	P.C. susceptible	*P. elliottii*	DDRT-PCR	58	CN852203-59 (this paper); AF457097 (Davis et al. 2002)
F. circinatum	P.C. resistant	*P. elliottii*	DDRT-PCR	48	CN852156-02 (this paper); AF457093, AF457095 (Davis et al. 2002)
C.q. fusiforme	F.R. susceptible	*P. elliottii*	DDRT-PCR	42	CN852115-46 (this paper)
C. q. fusiforme	F.R. susceptible	*P. taeda*	DDRT-PCR	19	BE656687, BE656689-91, BE656693-07 (Warren and Covert 2004)
Salicylate	Mixed seed lot	*P. elliottii*	DDRT-PCR	68	CN85228-43; CN949798 (this paper); AF457094, AF457096 (Davis et al. 2002)
Wounding	Mixed seed lot	*P. elliottii*	DDRT-PCR	26	CN852260-82; CN949799 (this paper); AF457092 (Davis et al. 2002)
Chitosan	Cell culture	*P. elliottii*	DDRT-PCR	19	CN852147-55 (this paper); UU55005-14 (Mason and Davis 1997)
C. q. fusiforme	F.R. susceptible	*P. taeda*	SSH	106	CN852344–CN852449 (this paper)

[a]P.C., Pitch canker; F.R., fusiform rust
[b]DDRT-PCR, Differential display-reverse transcriptase polymerase chain reaction; SSH, suppression subtraction hybridization
[c]SSH GenBank accessions represent consensus sequences derived from contigs of overlapping SSH clones

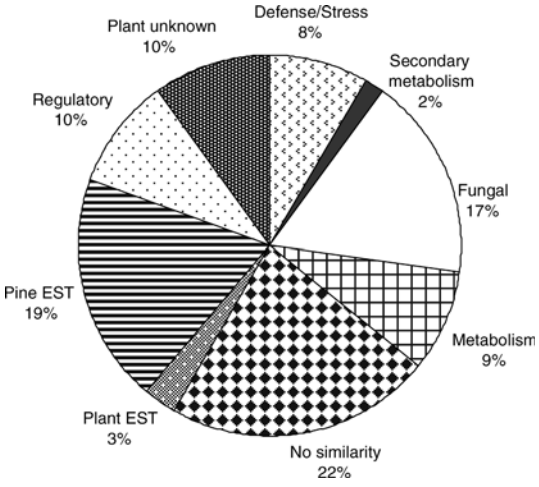

Fig. 1 Classification of nonredundant cDNAs into categories based on predicted function or genome of origin. The 311 non-redundant sequences were sorted according to the best BLAST score for each sequence (*E*-value<10^{-5}) or by gene-specific PCR experiments. The percentage of matches in each category is shown

include sequences with a similarity to expressed proteins or to hypothetical proteins predicted from genomic sequence information. Together, all of the above classes of sequences are likely to represent differentially regulated *Pinus* genes. cDNA sequences with homology to fungal genes or ESTs derived from fungal cDNA sequencing projects were assumed to represent *F. circinatum* or *C. q. fusiforme* genes and as such were placed in the fungal class. In addition, PCR experiments with gene-specific primers indicated that 16 of the clones in the gall-specific subtracted library were *C. q. fusiforme* sequences (data not shown). The inclusion of these sequences in the 'fungal' class brought its representation to 17%. Finally, 22% of the cDNAs contained sequences with *E*-values greater than 10^{-5} that were classified as having no similarity to sequences in the public database. Approximately, two-thirds of the cDNAs in this final group were derived from DDRT-PCR as opposed to SSH; it may be difficult to detect homologs using 3′-untranslated region sequences, which are typical of clones isolated by DDRT-PCR.

Array analysis to identify genes regulated in the pitch canker disease state

We examined the transcript abundance of 276 of the 311 nonredundant cDNAs using nylon membrane arrays because 89% of the nonredundant set of cDNAs were successfully amplified; these were distributed evenly across the two different cloning techniques. The non-redundant set of 276 cDNAs on the arrays was represented by a total of 316 individual clones that were derived from independent cloning of the same transcripts. Total RNA was isolated from the stems of control and treated plants 8 days after *F. circinatum* challenge, when pitch canker disease symptoms were manifest in the susceptible genotypes. Control plants were mock-challenged with water to minimize the detection of transcript abundance changes due to a wound response. Radiolabeled first-strand cDNA synthesized from the RNA was used to probe four replicate membranes for each treatment. Variations in signal intensities associated with differences such as probe synthesis were accounted for by scaling to genomic DNA spotted on the membranes and by standardizing each membrane using both a global approach and an internal reference approach.

For the global standardization procedure, the mean and standard deviation of a given array were used to standardize the signal intensities on that array. As seen in Fig. 2a, a modified MA plot (Dudoit et al. 2002) was used to graphically examine the data after global standardization. MA plots are essentially scatterplots that have been rotated 45° by plotting the log ratio of the spot intensity, *M*, against the overall spot intensity, *A*. We chose to use MA plots over scatterplots of the signal intensities because MA plots increase the ability to see potential artifacts and differentially expressed genes (Smyth et al. 2003).

For standardization using expression data from an internal reference set of genes, we identified a set of genes minimally responsive in the pitch canker disease state. Forty-two clones that fell into this category were all

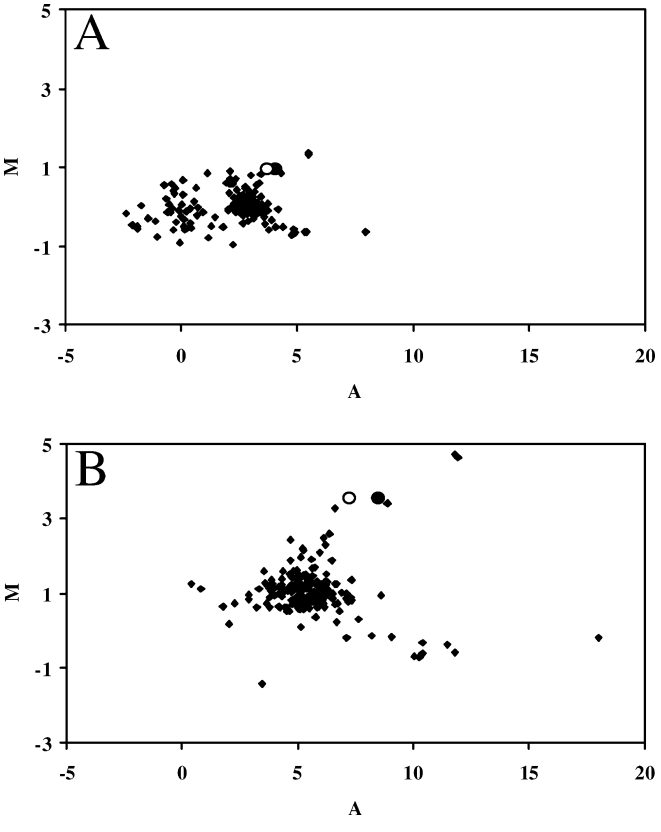

Fig. 2 MA plots of gene expression data generated from 276 nonredundant cDNAs using global (**a**) and internal reference (**b**) standardization procedures. *Pinus* class IV chitinase (pechi270) is indicated by an *open circle* and class I chitinase (pechi1) by a *closed circle*. $M=\log_2(\text{pathogen challenged})-\log_2(\text{mock challenged})$, and $A=\log_2(\text{pathogen challenged})+\log_2(\text{mock challenged})$

derived from the *P. taeda*:*C. q. fusiforme* interaction. The signal intensities of the remaining genes were standardized using the mean and standard deviations of these 42 reference genes and an MA plot (Fig. 2b) generated as described above.

A comparison of the MA plots in Fig. 2 reveals that the two different standardization procedures resulted in visually distinct data sets. In general, the global standardization approach resulted in data that were more 'compact' in both orientations than was found with the internal reference approach, with the majority of transcripts centering on *M* equal to zero. In contrast, when the data were standardized using the internal reference genes, the plot was less compact, with the majority of transcripts greater than *M* equal to zero. Thus, a higher number of genes with increased $\log_2$ ratios were detected with the internal reference approach than with the global approach.

We hypothesized that the apparent lack of differentially expressed genes in Fig. 2a was due to the global standardization procedure removing biological variation. If true, then genes known to be up-regulated in pitch canker-diseased tissue would have lower $\log_2$ intensity ratios after global standardization than after internal reference standardization. Previous work in our laboratory has shown that the transcript abundance of class I and class IV *P. elliottii* chitinase genes is highly induced in pitch canker-diseased tissue (Davis et al. 2002). The locations of the class I chitinase and class IV chitinase genes are indicated in Fig. 2 as closed and open circles, respectively. Global standardization of the two classes of chitinase genes spotted on the arrays gave a twofold induction in the pitch canker disease state, while the internal reference standardization gave a 12-fold induction for each of the classes, which is much closer to levels found in published reports (Davis et al. 2002).

To evaluate the transcript induction values in this experimental system, we performed Northern blot analysis on RNA isolated from stem tissues from two biological replicates of pitch canker-susceptible pines prior to any treatments (day 0) and at 3 days and 8 days after challenge with *F. circinatum*. Challenged plants showed typical disease symptoms at 8 days. Fungal RNA isolated from mycelia grown in vitro was also included to aid in distinguishing host and pathogen genes. To confirm loading, we probed RNA gel blots with a pine 18S ribosomal RNA gene. The results shown in Fig. 3a confirm that expression of the class IV chitinase was induced approximately 25-fold in pitch canker-diseased tissue. Because the internal reference standardization gave a better approximation of chitinase gene induction in response to pathogen challenge and because global standardization procedures are expected to skew small biased arrays, we concluded that using the internal reference set of genes was a more appropriate standardization procedure for these arrays and treatments.

Using the data set standardized with the internal reference genes, the cDNAs on the array were tested for a statistically significant response to *F. circinatum* by calculating test statistics. The normal quantile-quantile (Q-Q) plot (Dudoit et al. 2002) in Fig. 4 reveals the presence of genes with larger *t* statistics than expected for the associated changes in rank. To determine whether these *t* statistics reflect significant changes in gene expression between control and diseased tissues, adjusted *P* values were computed for each gene using Bonferroni step-down to account for multiple testing (family-wise type-I error rate). Adjusted *P* values of less than 0.10 were considered to be significant. Table 2 shows the 29 non-redundant cDNA clones that were differentially expressed between the two conditions and statistically significant. Since a redundant number of cDNAs were spotted on the arrays, our finding that multiple cDNAs encoding the same gene or EST were significantly up-regulated is evidence of the robustness of our array analyses.

To further validate the array analysis, we followed the expression of the PR10 (pi64) and class III peroxidase

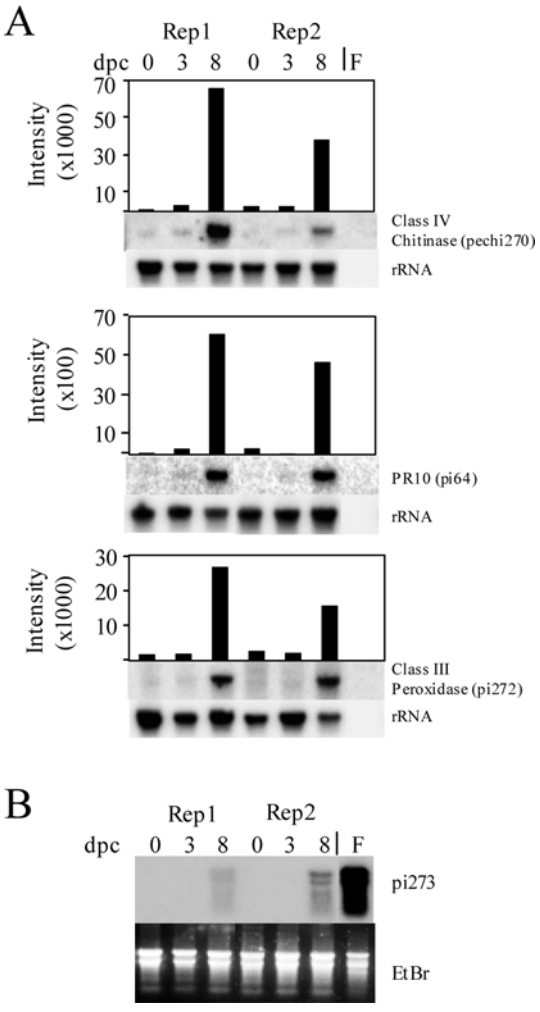

Fig. 3a, b Expression time course for selected cDNAs in susceptible *Pinus elliottii* after challenge with *Fusarium circinatum*. **a** Following the hybridization of selected cDNA clones, each Northern was probed with a pine 18S rRNA probe to confirm loading. Signal intensities of each cDNA are depicted in the graphs *over* each blot after scaling to the rRNA signal. **b** Northern blot showing pi273 expression in samples containing *F. circinatum* RNA. *dpc* Days post-challenge, *F F. circinatum* RNA isolated from mycelium, *Rep 1*, *2* replications of the experiment

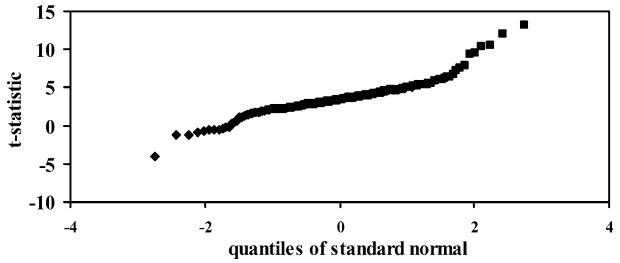

Fig. 4 Quantile-quantile plot using the data standardized with the internal reference procedure reveals genes with extreme *t* statistics. The *X-axis* represents the division of a normal distribution into equal, ranked subgroups (quantile of standard normal). *t* statistics are plotted along the *Y-axis*

(pi279) genes, both identified as significantly up-regulated in the pitch canker disease state (Table 2), during condensed time course experiments. Figure 3a shows the results from these Northern blot experiments. An approximately 32-fold induction for the PR10 gene was found 8 days post-challenge when signal intensities were averaged over both biological replicates. For the class III peroxidase, an approximately 15-fold induction was observed. The magnitude of the response varied depending on the gene being examined, with much higher levels of induction found for the class IV chitinase and PR10 than for the class III peroxidase (Fig. 3a). Thus, the array expression patterns were confirmed using Northern blots.

Genes up-regulated in the pitch canker disease state

Table 3 shows that, of the 29 non-redundant genes identified as significantly induced in the pitch canker disease state, more than 85% came from DDRT-PCR reactions using RNA isolated from either susceptible plants showing symptoms of pitch canker disease or from plants exogenously treated with SA. While the cDNAs derived from pitch canker-susceptible tissue represent only 14.5% of the cDNAs on the array, they constitute more than one-half of the cDNAs up-regulated by *F. circinatum* infection (Table 3). In contrast, cDNAs derived from pitch canker resistant plants and fusiform rust susceptible plants represented, respectively, approximately 10% and 55% of the cDNAs on the arrays (Table 3), yet only two of the significantly induced transcripts came from these subsets (Table 2).

Among those genes with known functions that were identified as being significantly up-regulated in the disease state in our array analyses, some of the most significant encoded PR proteins. These include cDNAs representing the two classes of chitinase proteins (pechi270 and pechi1), four cDNAs encoding a class III peroxidase (pi272, pi279, pi303 and pi304), and a gene encoding a PR10 protein (pi64). This study also implicates an antimicrobial protein (pi117) and a lipid transfer protein (pi130) in pine defense responses. An additional sequence (pi307a) has a similarity to a boiling-stable protein, types of which have been implicated in drought responses in poplar trees (Pelah et al. 1995; Wang et al. 2002). Of the cDNAs identified as significantly up-regulated, 21% were categorized as having no similarities to sequences in the public database. These sequences may represent novel pine or pathogen genes with roles in defense, disease progression, or pathogenesis.

The BLAST comparison of pi273, cloned from pitch canker-diseased tissue and the most significantly up-regulated transcript, revealed sequence similarity to an unknown protein from *Arabidopsis* (Table 2). As seen in Fig. 3b, RNA gel blot analysis of pi273 revealed hybridization only in lanes containing RNA isolated from tissues showing symptoms of pitch canker disease or in those containing RNA from *F. circinatum*. The high degree of hybridization in the lane with *F. circinatum* RNA suggests that pi273 encodes a fungal transcript and not a host-derived transcript. Of note, there was no visible cross-hybridization between host-derived cDNA probes and RNA isolated from fungal mycelia nor was cross-hybridization detected between the pine 18S probe and fungal mycelial rRNA (Fig. 3a).

Discussion

In the study reported here we used transcript profiling techniques to identify genes with the potential for being defense-associated and subsequently found that a subset of these genes showed increased transcript abundance in pitch canker-diseased tissues. These cDNAs expand the number of conifer genes with direct relevance to host-pathogen interactions.

Techniques to enhance gene discovery

While there are some limitations associated with cDNAs identified through DDRT-PCR, such as potential difficulty in functional annotation due to the limited coding sequence present in many clones, there are positive features as well. We believe that the 3′ bias of the DDRT-PCR clones minimized the potential for cross-hybridization between fungal transcripts and the arrayed host cDNAs, and vice versa, that would have existed had the spotted cDNAs been derived from coding regions. The use of 3′-untranslated regions as gene-specific determinants is a common technique in RNA gel blot and high-throughput array analyses. Therefore, in the absence of functional annotation, the DDRT-PCR cDNAs significantly induced in the pitch canker disease state that were originally cloned from host tissues in the absence of pathogen (i.e., in elicitor-treated plants) must be pine in origin and as such reveal changes in host transcript abundance in the disease state. For those cDNAs cloned from pathogen-challenged tissues and showing significant differential expression on the arrays, it is less straightforward to determine in which organism the clone originated. Comparisons to sequences in the database can give clues about the genome of origin (e.g., pine genes are expected to be most similar to other plant genes and less similar to

Table 2 Summary of *Fusarium*-regulated cDNAs at 8 days post-challenge

Clone	Treatment[a]	Annotation[b]	*E*-value[c]	Species[d]	Fold induction[e]	Bonferonni adjusted *P* values
pi273	P.C.-S	Unknown, AAM62667.1 (X)	2.00E-08	*Arabidopsis thaliana*	26.4	0.000
pechi270	P.C.-S	Class IV chitinase, AAL58880.1 (X)	4.00E-45	*Pinus elliottii*	12.0	0.000
pi279, pi303, pi272, pi304	P.C.-S	Class III peroxidase, AAG02215.1 (X)	1.00E-25	*P. sylvestris*	9.6	0.000
pi277, pi278, pi275	P.C.-S	cDNA clone, AI919956 (E)	1.00E-35	*P. taeda*	2.6	0.000
pi293	P.C.-S	No similarity	$>10^{-5}$	–	2.8	0.001
pi276, pi292	P.C.-S	Tar1p, NP_690845.1 (X)	6.00E-18,	*Saccharomyces cerevisiae*	2.1	0.002
pi64	P.C.-R	PR10-related protein, AF197342.1 (X)	2.00E-27	*Picea glauca*	10.6	0.006
pi284	P.C.-S	Ribosomal protein L1, T51935 (X)	1.00E-38	*Spinacia oleracea*	24.8	0.009
pi117	Salicylate	Antimicrobial peptide 2, AL05053.1 (bestEST)	1.00E-102, 9.00E-59	*P. sylvestris*	2.2	0.014
pi274	P.C.-S	no similarity	$>10^{-5}$	–	1.6	0.024
pi263	P.C.-S	Xylem cDNA, BQ700839.1 (E)	6.00E-31	*P. taeda*	2.3	0.026
pechi1	Wounded	Putative class I chitinase, AAL58875.1 (X)	2.00E-32	*P. elliottii*	11.8	0.027
pi280	P.C.-S	no similarity	$>10^{-5}$	-	1.7	0.032
pi76, pi128	Salicylate	cDNA clone, AA739574.1 (E)	1.00E-147	*P. taeda*	2.2	0.032
pi287	P.C.-S	ABC transporter-like protein, NP_191829.1 (bestEST)	5.00E-47	*A. thaliana*	2.4	0.037
pi297	P.C.-S	Pollen cone cDNA, AW754815 (E)	2.00E-35	*P. taeda*	3.7	0.037
pi250	P.C.-S	No similarity	$>10^{-5}$	–	4.2	0.047
pi289	P.C.-S	Hydrophobin 3, AAO16869.1 (bestEST)	1.00E-102, 3.00E-15	*Gibberella moniliformis*	3.0	0.055
pi156-2	Salicylate	Xylem cDNA, BQ107663.1 (E)	2.00E-69	*P. taeda*	2.4	0.066
pi228a	F.R.-S	No similarity	$>10^{-5}$	–	2.4	0.066
pi302	P.C.-S	Xylem cDNA, BF609752.1 (E)	1.00E-140	*P. taeda*	2.3	0.076
pi301	P.C.-S	Expressed protein, NP_564667.1 (X)	1.00E-20	*A. thaliana*	2.6	0.084
pi307a	P.C.-S	Boiling stable protein A, CAC34953.1 (X)	3.00E-09	*Populus tremula*	2.2	0.085
pi139	Salicylate	Xylem cDNA, BI202925.1 (E)	1.00E-10	*P. taeda*	2.3	0.090
pi130	Salicylate	cDNA library, BI416804.1 (E)	3.00E-62	*Heterobasidion annosum* or *P. sylvestris*	2.3	0.093
pidd6-2	Wound	Xylem cDNA, BQ197534.1 (E)	3.00E-71	*P. taeda*	2.5	0.094
pi152-5	Salicylate	Actin-depolymerizing factor, Q8SAG3 (bestEST)	1.00E-101, 9.00E-52	*P. taeda*	2.5	0.096
pi281	P.C.-S	No similarity	$>10^{-5}$	–	1.7	0.098
pi307b	P.C.-S	Photosystem II subunit, P16059 (X)	3.00E-29	*Pisum sativum*	2.3	0.099

[a]Pitch canker-resistant (P.C.-R) and -susceptible (P.C.-S) plants were challenged with *F. circinatum*; fusiform rust-susceptible (F.R.-S) plants were challenged with *C. q. fusiforme*

[b]Annotation based on: X ,BLASTX; E, BLASTN against dbEST; bestEST annotated using EST sequence with the best sequence match as a BLASTX query

[c]*E*-values $>10^{-5}$ were considered not similar

[d]Species of origin for the top BLAST hit

[e]For genes represented by multiple cDNAs, the expression ratios (diseased/control) are given as averages

Table 3 Significant clones categorized by treatment

Treatment[a]	Number of non-redundant clones on array	Percentage of non-redundant clones on array	Number of up-regulated clones on array (percentage of those significantly regulated)[b]
Wounding	13	4.7	2 (6.3)
Chitosan	3	1.1	0
P.C.-resistant	29	10.5	3 (9.4)
P.C.-susceptible	40	14.5	19 (59.4)
F.R.-susceptible	50	18.1	1 (3.1)
F.R. SSH libraries	99	35.9	0
Salicylate	42	15.2	77 (21.9)

[a]Pitch canker (P.C.)-resistant and -susceptible plants were challenged with *F. circinatum*; fusiform rust (F.R.) plants were challenged with *C. q. fusiforme*

[b]Numbers and percentages add up to greater than 29 and 100%, respectively, due to independent cloning of some cDNAs in more than one treatment

fungal ones), but as evidenced by clone pi273, which is a fungal gene (Fig. 3b) with a great sequence similarity to a plant sequence (Table 2), this approach is not fool-proof. Alternatively, gene-specific PCR (Warren and Covert 2004) or hybridization experiments (Fig. 3b) can be used to distinguish between host and pathogen genes, but these approaches are relatively time-consuming when many genes require classification.

In this study, two procedures were used to standardize the array data—an internal reference approach using the expression of an internal standard set of genes that is typical for normalizing gene expression data such as RNA gel blot analysis or real-time PCR and a global approach that assumes that differences in the intensities of the treatment membranes are due solely to assay variation and not biological variation. Global approaches are typically used when the array has many thousands of elements in which only a small subset of genes are regulated (Dudoit et al. 2002; Finkelstein et al. 2002). While global approaches have been used with success on relatively small datasets to account for variation between membranes such that biological treatments can be measured (Cooke et al. 2003), these datasets are most often composed of cDNAs, whose expression is regulated in both directions by the treatment of interest, such that the overall mean of the arrays are the same. For experiments in which the transcript abundance of many genes is biased in the same direction, the overall intensities among treatment membranes are not comparable, since the intensity differences between arrays include biological variation as well as assay variation. Global standardization of these types of biased data sets is therefore predicted to skew the relative transcript amounts because of the removal of the biological variation. The comparison of the $\log_2$ of the intensity ratios for two chitinase genes known to be highly induced in the disease state, following both internal and global standardizations, allowed us to determine that the internally standardized method was more suitable for our experimental design. The method presented here is likely to be applicable to a variety of biased gene sets in which the spotted cDNAs are predicted to show a similar directionality in their regulation.

Genes of known and unknown function

Of the 29 genes significantly up-regulated in the pitch canker disease state, seven have sequence similarities to genes encoding proteins with potential roles in defense or stress response pathways. Thus, a pathogen-induced host signaling mechanism exists in the *P. elliottii-F. circinatum* interaction; this conclusion is in accordance with the results of studies in other plant species in which activation of defense genes in disease states was reported (Glazebrook et al. 1996; Rogers and Ausubel 1997). The PR genes identified as up-regulated include a class III peroxidase, which is a member of the PR9 class (Van Loon et al. 1994) of defense proteins implicated in a number of cellular functions including defense, cell-wall biosynthesis, and hormonal signaling (Halliwell 1978; Kerby and Somerville 1989; Lagrimini et al. 1997). The list also includes an intracellular PR protein of the PR10 class found in a wide variety of plants (Walter et al. 1990) with unknown biochemical and physiological functions as well as two classes of PR3 chitinase enzymes that hydrolyze chitin, a component of fungal cell walls. The expression of PR genes concomitant with the onset of visible disease symptoms (Fig. 3) suggests that the signals that activate PR gene expression are produced in susceptible pines during disease development. Increasing our knowledge of the signaling pathways that activate *Pinus* defense responses may provide a basis for understanding the defense mechanisms of woody plants in general. How defense mechanisms are regulated and maintained over lengthy time periods and large physical distances in trees as compared to short-lived annuals and crops plants is poorly understood but of potential ecological and biotechnological significance.

We predict that the induction of pi307a, which encodes a boiling-stable protein, in the disease state is an example of a molecular symptom of pitch canker disease. Just as

lesion formation and resin production are disease symptoms, changes in gene expression can also be a sign of disease. One of the characteristic symptoms of pitch canker disease in succulent tissues is wilting of the shoot above the lesion. This phenotype is likely caused by the destruction or obstruction of the vascular system by the fungus, resulting in a loss of solute transport, which is turn is followed by a subsequent drought response in the upper parts of the shoot that includes the induction of drought-responsive genes such as pi307a. Other significantly induced genes, whose functions are currently unknown, may also be molecular symptoms of the disease state.

Of the non-redundant genes identified in this study, 22% were classified as having no similarities to sequences in GenBank. Approximately, two-thirds of these cDNAs were generated by DDRT-PCR. This potentially unique class of cDNAs could be a consequence of the fact that many disease-relevant *Pinus* genes have not been discovered in the large EST sequencing efforts directed at understanding wood development. Trees may contain novel genes with roles in pathogen responses that are perhaps associated with the large spatial and temporal scales at which defenses presumably must act in woody perennials. An alternative explanation is that homologs may exist in the public databases that were not revealed due to DDRT-PCR 3′ cloning bias. Specifically, DDRT-PCR preferentially identifies the 3′ ends of expressed genes, while ESTs are often sequenced from the 5′ end. Our success in using ESTs to synthetically extend the length of the DDRT-PCR cDNAs into coding regions and then finding that they increased our ability to assign functions is consistent with this explanation. Finally, we must consider the likelihood that a number of genes in this class could be fungal in origin. These questions will likely be resolved as new gene discovery tools and experimental conditions are brought to bear on defining all of the genes in *Pinus* (Lorenz and Dean 2002).

Pine host responses to necrotrophs and biotrophs are distinct

Most (approximately 60%) of the cDNAs that were significantly induced on the arrays were originally identified by DDRT-PCR from pitch canker-diseased tissues (Table 3). This suggests that DDRT-PCR was a reliable discovery tool because the technique clearly enriched the array for cDNAs that are regulated during pitch canker disease. Studies in progress will directly compare the responses of resistant and susceptible loblolly pine clones in time course studies in order to identify the differences in expression response programs between resistant and susceptible hosts.

Our data suggest that the genetic architecture of disease progression in pitch canker (necrotrophic pathogen) and fusiform rust (biotrophic pathogen) is distinct. Given the dramatic symptoms of pitch canker disease, we surmised that a substantial reprogramming of host gene expression was likely to be associated with the disease state. At the project outset, we predicted that successful *F. circinatum* infection would induce the expression of pine genes involved in defense and disease development. In support of this idea, several cDNAs with known relationships to defense responses, including two classes of chitinase (I and IV), class III peroxidase, PR10, an antimicrobial peptide, and lipid transfer protein were all induced in the pitch canker disease state. A boiling-stable protein associated with drought reponses may reflect shoot desiccation during the disease state.

In contrast to pitch canker disease, fusiform rust is characterized by the development of woody galls on infected stems and branches. These galls result from the proliferation, swelling, and altered differentiation of the cells that make up wood (Jackson and Parker 1958; Jewell et al. 1962). The long-term suppression of host defenses is proposed to be one of the features of compatible biotrophic interactions (Mendgen and Hahn 2002). Consequently, pitch canker and fusiform rust may exhibit fundamentally different gene expression programs in their common pine host. Alternatively, the majority of pathogen-responsive genes may be intrinsic to a state of disease, independent of the inciting pathogen. Our data tend to support the former model, but a direct test will require comparison of disease expression programs on gene expression arrays. These experiments are in progress and should yield greater insight into host processes that are regulated by pathogens during the development of disease.

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