Gene Expression Profile Changes in Cotton Root and Hypocotyl Tissues in Response to Infection with *Fusarium oxysporum* f. sp. *vasinfectum*

Caitriona Dowd, Iain W. Wilson, and Helen McFadden

CSIRO Plant Industry, Black Mountain Laboratories, Cnr Clunies Ross Street and Barry Drive, Black Mountain, GPO Box 1600, Canberra, ACT 2601, Australia

Submitted 2 October 2003. Accepted 26 January 2004.

Microarray analysis of large-scale temporal and tissue-specific plant gene expression changes occurring during a susceptible plant–pathogen interaction revealed different gene expression profile changes in cotton root and hypocotyl tissues. In hypocotyl tissues infected with *Fusarium oxysporum* f. sp. *vasinfectum*, increased expression of defense-related genes was observed, whereas few changes in the expression levels of defense-related genes were found in infected root tissues. In infected roots, more plant genes were repressed than were induced, especially at the earlier stages of infection. Although many known cotton defense responses were identified, including induction of pathogenesis-related genes and gossypol biosynthesis genes, potential new defense responses also were identified, such as the biosynthesis of lignans. Many of the stress-related gene responses were common to both tissues. The repression of drought-responsive proteins such as aquaporins in both roots and hypocotyls represents a previously unreported response of a host to pathogen attack that may be specific to vascular wilt diseases. Gene expression results implicated the phytohormones ethylene and auxin in the disease process. Biochemical analysis of hormone level changes supported this observation.

Plants respond to pathogen attack via a complex and integrated set of defenses encompassing both constitutive and induced responses. Interactions between plants and pathogens initiate a series of events in plants, including production of signaling compounds such as ethylene, salicylic acid, jasmonic acid, or reactive oxygen species. These compounds activate downstream responses, including the production of phenolics, phytoalexins, and antimicrobial peptides and the deposition of physical barriers. Host transcriptional activity is modulated substantially over the course of pathogen attack. Over the years, molecular plant pathology has focused on providing a detailed picture of plant–pathogen systems where the interaction is controlled by the operation of a gene-for-gene relationship. Most interest has been focused on host and nonhost resistance to avirulent pathogens during incompatible interactions.

Corresponding author: Helen McFadden: Telephone: +61 (0)2 6246 5377; Fax: +61(0)2 6246 5000; E-mail: Helen.McFadden@csiro.au

Gene sequence data is available in the GenBank database under accession numbers CD485516 through CD486718 and CK738219.

*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains two supplemental tables not included in the print edition.

In contrast, our understanding of compatible interactions and interactions that are controlled by several genes inherited in a complex manner remains relatively limited (Kazan et al. 2001). In addition, there has been a greater focus on foliar pathogens than on soilborne diseases, which are nevertheless of economic importance. This study attempts to focus on some previously neglected areas with an investigation of plant host responses in a system where resistance is believed to be under complex genetic control. We have studied the plant responses during a compatible interaction with a soilborne pathogen.

Fusarium oxysporum f. sp. *vasinfectum* causes Fusarium wilt, an important disease of many crops, including cotton. This pathogen infects through the roots, moves through the cortex, and enters the vascular tissue. It spreads rapidly through the plant in the xylem, where it exists as mycelia and conidia (Beckman and Roberts 1995). Symptoms include wilting due to the disruption of water transport within the vascular system, vascular browning, and eventual death of the plant. For cultivated cotton, adequate resistance to Fusarium wilt disease has yet to be identified. Little currently is known about Fusarium wilt disease in cotton and most research on vascular wilts in this crop has been undertaken using *Verticillium dahliae*. In studies of cotton species showing resistance and susceptibility to *V. dahliae*, similar responses were found to occur in both species, but the difference in susceptibility was associated with the timing and intensity of certain gene expression changes (Bell 1994).

The advent of microarray technology has enabled large-scale surveys of gene profiles leading to a more integrated view of gene expression responses. Despite the suitability of microarray approaches to the study of plant–pathogen interactions, there are surprisingly few published studies of this subject (Schenk et al. 2000), particularly studies dealing with susceptible interactions (Eckardt 2002), with nonmodel plant systems or with soilborne pathogens. There also appear to be few studies examining the plant gene expression responses over the time course of infection as well as the responses within different plant tissues. Such studies are crucial to the development of an integrated understanding of complex plant–pathogen interactions (Diehne and Relman 2001; Schieble et al. 2001; Wan et al. 2002). In this study, we investigated gene expression changes in a crop plant, namely cotton, at different time points and in different tissues during a susceptible interaction with a soilborne, vascular wilt pathogen, *F. oxysporum* f. sp. *vasinfectum*. As far as we are aware, this work represents the first published microarray study of cotton gene expression. Therefore, we provide new information on a neglected type of plant–pathogen interaction. This information is of general

interest and also may aid the development of rational strategies for managing Fusarium wilt disease in cotton.

RESULTS

Experimental system.

We developed a system for *F. oxysporum* f. sp. *vasinfectum* infection of cotton where we grew root-dip-inoculated seedlings in sterile culture pots. This system gave coordinated and reproducible infection of cotton seedlings under controlled environmental conditions. The symptoms characteristic of Fusarium wilt disease in the field were obtained over a 4- to 5-day period in our model system (Fig. 1). Initially, the pathogen attaches to and grows on the root surface, then penetrates the roots at approximately 1 day postinfection (dpi) and enters the vascular system. It moves up through the plant to the hypocotyl vascular tissue (2 to 3 dpi), where infection is associated with the development of vascular browning. Ultimately, the disease results in wilting of cotyledons and death of the plants. The progress of the pathogen through the plant was monitored by reisolation of *F. oxysporum* f. sp. *vasinfectum* from surface sterilized sections of root and hypocotyl tissues (Figs. 1B and 2).

Microarray preparation and analysis.

In order to study cotton-*F. oxysporum* f. sp. *vasinfectum* interactions, we prepared microarrays using clones from two cDNA libraries (discussed below). We prepared one library from infected tissues from several time points after infection and a second library from uninfected cotton tissues. We pre-

pared microarray slides containing 4,000 cDNA clones from the infected cotton library and 2,000 clones from the uninfected library. Gel analysis of polymerase chain reaction (PCR) amplified inserts showed up to 95% of clones contained inserts and the average insert length was 0.7 to 1.5 kb. The redundancy of each library was estimated to be 1% by sequence analysis of 100 randomly selected clones from each library. Labeled cDNA probes did not hybridize to any negative controls on the microarrays (such as the genes for green fluorescent protein [AF078810], luciferase [X65316], NPTII [V00618], or insulin-like growth factor [X07868]), but did bind to many of the positive controls (such as genes for cotton PR10 and cadinene synthase), as expected.

We analyzed gene expression changes in roots and hypocotyls of *F. oxysporum* f. sp. *vasinfectum*-infected and uninfected cotton seedlings at various time points during disease development. At least two sample replicates were harvested for each time point (biological replicates) and at least one of these samples was analyzed with a dye swap (technical replicate) (discussed below). For all samples (except for roots at 14 h postinfection [hpi] and 4 dpi and hypocotyls at 3 and 4 dpi), three or more biological replicates were used. For each spot, the ratio of the fluorescence intensity in the red and the green channels (R/G ratio) was measured. This fluorescence data set then was analyzed using the tools for R Microarray Analysis (tRMA) software suite. Analysis consisted of a normalization protocol (Wilson et al. 2003) based on the log₂ values of the R/G ratio. The normalization procedure of tRMA corrects for spatial and intensity effects across the microarray slide (Dudoit et al.

Fig. 1. Infection of cotton seedlings with *Fusarium oxysporum* f. sp. *vasinfectum*. **A**, Cotton seedling, grown aseptically in a tissue culture pot, 3 days after root-dip inoculation (dpi) with a conidial suspension of *F. oxysporum* f. sp. *vasinfectum*. Surface mycelium was removed as much as practicable prior to RNA extraction. **B**, Mycelial growth of *F. oxysporum* f. sp. *vasinfectum* in the vascular tissue of a surface-sterilized hypocotyl section prepared from a cotton seedling 3 dpi and incubated for 2 days on 25% potato dextrose agar. **C**, Vascular and adjacent tissue of an uninfected hypocotyl section. **D**, Severe browning of vascular and adjacent tissue in a longitudinal section of infected hypocotyl tissue, photographed at 7 dpi.

2002; Jan Klok et al. 2002) and also corrects for possible biases in fluorescence that occur due to differences in the efficiency of incorporation of the two dyes and unequal loading of RNA samples. After normalization, the data were rescaled (Wilson et al. 2003). Differentially expressed genes were identified by comparison of the distribution of the normalized, rescaled $\log_2$ (R/G) data with a normal distribution. Progressive removal of potential outliers was performed until the distribu-

tion of the remaining log (R/G) data represented a normal distribution (using the residual sum of squares of fit of the data distribution to the standard normal distribution in a quantile-quantile plot as the optimization criteria) (Wilson et al. 2003). The outliers were deemed to represent highly differentially expressed genes. Differentially expressed genes were sequenced and analyzed for sequence homology to known genes (BlastN and BlastX). It was found that BlastX search results were more informative, whereas BlastN searches frequently yielded EST sequences without annotation. The complete set of differentially expressed genes was examined for groups of genes with similar expression profiles using K means clustering analysis. Several values of K (from 2 to 8) were used and the clustering that gave the smallest value of K with the maximum value of the tRMA silhouette width (Rousseeuw 1987) was selected. The reliability of the assignment of genes to different profiles also was verified by the presence of multiple copies of the same gene within the same profile. Of the differentially expressed plant and unidentified genes, 12% showed redundancy. As a further manual quality control, when redundant genes were found in different profiles, both genes were removed from the data sets. Genes with sequence homology to known genes were predicted to be of fungal or plant origin on the basis of this homology. In this study, we have concentrated on the analysis of plant gene responses based on putative plant genes with homology to known plant genes. Detailed analysis of putative fungal gene expression will be the subject of future investigations.

Gene expression responses in infected roots.

In all, 484 clones were identified, using the process outlined above, as differentially expressed in roots, with 317 of these clones showing significant matches in databases and 167 clones showing no significant matches with any sequences in the databases. These 317 clones represented 261 unique genes, of which 135 were predicted to be plant genes and 126 were considered putative fungal genes.

The number of nonredundant differentially expressed genes identified at each time point during the infection process in cotton roots is shown in Figure 2A. At 14 hpi, little differential expression was observed with repression of four plant genes in infected tissue observed and no putative fungal clones identified. The pathogen had entered the roots by 1 dpi, as judged by fungal reisolation, and putative fungal genes were first identified at this stage. The number of putative fungal genes identified increased rapidly between 1 and 3 dpi (from 14 to 113) before leveling off at 4 dpi (115). At 1 dpi, repression of plant genes was observed; whereas, at 2 dpi, little differential plant gene repression was observed. At 3 and 4 dpi, there was significant induction and repression of plant genes in infected cotton roots. Most of the plant genes differentially expressed at 3 dpi also were identified at 4 dpi; therefore, few additional genes were differentially expressed at this later stage of infection.

The mean expression profiles of groups of differentially expressed genes sharing similar expression profiles over time in infected roots as determined by K-means clustering are illustrated in Figure 3. Selected genes from these profiles are listed in Table 1. Profiles R1, R2, and R3 (Fig. 3A, B, and C) contain genes that were identified as having increased expression levels in infected tissue from 1, 2, and 3 dpi, respectively. The main differences between the expression patterns of genes in profiles R1, R2, and R3 are quantitative, with gene expression levels increasing in infected roots in all three profiles up to 3 dpi and then showing a slight decline at 4 dpi. Genes in profile R1 were first identified as induced in infected roots at 1 dpi, when the fungus was first reisolated from roots. These genes maintained high expression levels

Fig. 2. Differentially expressed genes in *Fusarium oxysporum* f. sp. *vasinfectum*-infected roots and hypocotyls at various time points after infection. Differentially expressed genes in **A**, *F. oxysporum* f. sp. *vasinfectum*-infected roots and **B**, *F. oxysporum* f. sp. *vasinfectum*-infected hypocotyls. The numbers of nonredundant induced and repressed putative plant, fungal, and unidentified genes are shown. Results of reisolation of *F. oxysporum* f. sp. *vasinfectum* from surface-sterilized sections and disease development stages are indicated for each time point, where + denotes fungal colonies obtained and 0 denotes no fungal colonies observed.

throughout the time course of infection. The majority of genes in this profile are putative fungal genes. Profile R2 contained genes that exhibit increased expression in infected roots from 2 or 3 dpi. Putative stress response genes shared this profile, such as homologues of genes for nonsymbiotic hemoglobin 1 (hypoxia stress), Rab18 (drought stress), and alcohol dehydrogenase 2d (anaerobic stress). Profile R3 included genes identified as showing increased expression in cotton roots at the later stages of infection, at 3 and 4 dpi. A gene for a cotton bacterially induced lipoxygenase was one of the few potential defense-related genes induced in infected roots. This profile also contained putative stress-related genes encoding proteins such as glutathione S-transferase (GST) 18 and nitropropane dioxygenase. Putative sulfur metabolism genes also were present in this profile. Signal transduction-related genes present in profile R3 included those similar to an ethylene inducible gene from *Hevea brasiliensis* and auxin regulated gene homologues.

Profile R4 (Fig. 3D) contained genes that exhibit decreased gene expression in infected root tissue from 1 to 4 dpi, with greater decreases at 3 to 4 dpi. Secondary metabolism gene homologues associated with tannin, anthocyanin, and lignin biosynthesis were repressed in infected roots. Drought-responsive genes, such as those coding for late embryogenesis abundant proteins (LEAs), cysteine proteases, and aquaporins, all shared this profile. Other stress-related gene homologues present in this profile included genes for heat shock proteins, peroxidase, and an abundant Zn class alcohol dehydrogenase (8 cDNA copies). Other genes that were repressed by infection included putative protein synthesis genes, nitrogen assimilation, and sugar me-

tabolism related genes. Signal transduction genes in this profile included those with sequence similarity to genes for a 14-3-3 protein, receptor protein kinases, auxin-repressed genes, and a putative auxin influx protein (aux-1 permease).

Gene expression responses in infected hypocotyls.

In all, 445 clones were identified as differentially expressed in infected hypocotyls, with 361 of these clones showing sequence matches in databases and 84 clones showing no significant matches with any sequences in the databases. These 361 clones represented 214 unique genes, of which 208 were putative plant genes and 6 were putative fungal genes.

The number of differentially expressed genes identified at separate time points during the infection process in cotton hypocotyls is shown in Figure 2B. Hypocotyl tissues were first examined at 2 dpi when relatively small changes in gene expression occurred. At 2 dpi, the fungus was reisolated from a few hypocotyl sections; however, only two putative fungal genes were identified at this stage. At 3 dpi, plant gene induction and repression in response to infection increased; also, vascular browning could be observed at this stage. At 4 dpi, the largest gene expression changes were observed, with the number of induced and repressed plant genes more than doubling.

The mean expression profiles of groups of differentially expressed genes detected in hypocotyls with similar expression profiles over time are shown in Figure 4. Profiles H1, H2, H3, and H4 are shown in Figure 4A, B, C, and D, respectively. Selected genes from these profiles are listed in Table 2. Profiles H1 and H2 showed similar qualitative trends, with gene ex-

Fig. 3. Expression profiling of genes differentially expressed in infected root tissues. The expression ratios were calculated from normalized, rescaled ratios of the fluorescence intensity in the red and the green channels. Error bars show the standard deviation of expression levels at each time point. **A**, Profile R1, **B**, profile R2, and **C**, profile R3 represent genes induced over time whereas **D**, profile R4, represents genes repressed over time. Normalized, rescaled log ratio values greater than 3.0 or less than -3.0 were estimated (tools for R Microarray Analysis) to indicate differentially expressed genes.

Table 1. Selected genes that showed differential expression in *Fusarium oxysporum* f. sp. *vasinfectum*-infected cotton seedling roots^a

Profile, functional category, GenBank ID	Putative protein name ^b	Putative function
Profile R1		
Protein synthesis		
CD485549	40S Ribosomal protein S5	Translation
Profile R2		
Stress related		
CD486338	<i>Nonsymbiotic hemoglobin 1*</i>	Low nitrogen stress
CD486441	<i>Alcohol dehydrogenase 2d*</i>	Anaerobic stress
Metabolism		
CD486220	ATP synthase γ chain 1	Energy generation
Signal transduction		
CD486157	AP2 domain-containing transcription factor	Ethylene signaling
Profile R3		
Defense related		
CD486015	Tumor-related protein	Trypsin inhibitor
Stress related		
CD485630	<i>2-Nitropropane dioxygenase</i>	Reactive oxygen species (ROS) generator
CD486292	<i>Bacterial-induced lipoxygenase*</i>	ROS generator
CD485867	<i>Glutathione S-transferase GST 18</i>	Detoxification
Metabolism		
CD485995	ATP sulfurylase precursor	Sulfur metabolism
CD486555	Cyclopropane fatty acid synthase	Fatty acid metabolism
Signal transduction		
CD486177	<i>Ethylene-inducible protein</i>	Ethylene target gene
CD486334	Similarity to GPI-anchored protein	Auxin regulated gene
CD486500	Nt-iaa deduced protein	Auxin inducible gene
Profile R4		
Defense related		
CD486396	<i>Putative endochitinase</i>	Pathogenesis related
Stress related		
CD486406	<i>Water channel protein MipH</i>	Drought stress
CD486675	<i>Tonoplast intrinsic protein DiP</i>	Drought stress
CD486301	<i>Δ-tonoplast intrinsic protein*</i>	Drought stress
CD486465	<i>Plasma membrane intrinsic protein 3 (SIMIP)</i>	Drought stress
CD486697	<i>γ-TIP</i>	Drought stress
CD486305	<i>MipC</i>	Drought stress
CD485819	<i>Late embryogenesis abundant protein Lea5-A*</i>	Drought stress
CD486032	<i>Late embryogenesis abundant protein Lea5-A*</i>	Drought stress
CD486166	<i>Cysteine protease precursor</i>	Drought stress
CD485902	<i>Putative alcohol dehydrogenase</i>	Anaerobic stress
CD486558	Peroxidase precursor*	ROS scavenger
CD485675	Heat-shock protein 80	Heat stress
CD486459	<i>Major latex-like protein</i>	Unknown
Protein synthesis		
CD486315	Elongation factor 2 (EF-2)	Translation
CD486037	Cyclophilin	Chaperone
Cell wall structure		
CD486432	Tubulin α -3 chain	Cytoskeleton formation
CD486065	Reversibly glycosylated polypeptide	Glucan biosynthesis
Secondary metabolism		
CD486307	Leucoanthocyanidin dioxygenase	Anthocyanin biosynthesis
CD486387	Leucoanthocyanidin reductase	Tannin/anthocyanin biosynthesis
CD486034	4-Coumarate-CoA ligase-like protein	Lignin biosynthesis
CD485924	Peroxidase	Lignin biosynthesis
Metabolism		
CD486351	<i>Asparagine synthetase</i>	Nitrogen metabolism
CD486502	<i>Glutamine synthetase cytosolic</i>	Nitrogen metabolism
CD486252	Putative sucrose-proton symporter	Sugar metabolism
CD486175	Sucrose synthase*	Sugar metabolism
CD485922	Δ -9 stearoyl-acyl desaturase	Fatty acid metabolism
Signal transduction		
CD485611	14-3-3-Like protein	Unknown
CD486173	Auxin-repressed protein	Auxin responsive
CD485647	Auxin-repressed 12.5-kDa protein	Auxin responsive
CD486543	Putative AUX-1-like permease	Auxin transport
CD486061	Annexin (fragment)*	Calcium signaling

^a **Profile R1:** 1 putative plant gene, 12 putative fungal genes, and 10 unidentified genes induced. **Profile R2:** 9 putative plant genes, 51 putative fungal genes, and 33 unidentified genes induced. **Profile R3:** 27 putative plant genes, 63 putative fungal genes, and 76 unidentified genes induced. **Profile R4:** 98 putative plant genes, 0 putative fungal genes, and 48 unidentified genes repressed.

^b An asterisk (*) indicates that the gene sequence matched a cotton sequence. Genes coding for putative proteins with names shown in italics were found to be differentially expressed in both root and hypocotyl tissues.

pression levels increasing over the time measured, but they differed in the quantitative nature of this trend. Profile H1 included genes that showed increased expression from 2 to 4 dpi. *F. oxysporum* f. sp. *vasinfectum* was first reisolated from hypocotyl tissues at 2 dpi; and, at 3 dpi, the hypocotyl was visibly responding to infection as seen by vascular browning. Genes encoding cotton pathogen defense-related proteins (PR10, PR5 [thaumatin]) and putative stress-related proteins (GSTs and dioxygenase) were present in this profile. Secondary metabolism gene homologues also were evident in this profile, including those associated with lignan biosynthesis and terpenoid indole alkaloid biosynthesis. Genes for proteins involved in signal transduction with similarity to membrane associated extracellular glycoprotein (SIEP 1) and a Na/H antiporter (Nhx1, involved in salt stress signaling) also were present in profile H1. Genes in profile H2 were identified as showing increased expression in infected hypocotyls later, at 4 dpi. Genes for different classes of pathogen defense-related cotton proteins, such as ribonuclease-like PR10, PR3 (chitinase, class I and IV), and PR2 (glucanase), were induced at this later stage. Cotton genes of the mevalonate/isoprenoid and the sesquiterpenoid pathways were abundant in this profile. The enzymes in these pathways are responsible for the biosynthesis of gossypol phytoalexins. Several putative secondary metabolism genes associated with lignin and flavanoid biosynthesis were identified as induced due to infection (profile H2; Fig. 4B; Table 2). Homologues of stress-related genes observed in profile H2 included reactive oxygen species (ROS) scavengers and ROS generators. Several putative metabolism genes appeared to be induced; specifically, those involved in sugar, amino acid, and fatty acid metabolism. Signal transduction genes sharing sequence similarity to ethylene synthesis genes and ethylene signaling pathway genes were induced at this later stage of infection.

Profile H3 contained genes with decreased gene expression in infected tissues from the earlier time points, 2 and 3 dpi. Profile H4 contained genes with decreased gene expression later, at 4 dpi. The only putative pathogenesis-related gene in this profile was a gene for a chitinase-like protein (CLT1). This profile consisted primarily of stress-related gene homologues encoding proteins such as metallothionein, peroxidase, thioredoxin, and alcohol dehydrogenase. Genes associated with water transport (aquaporins) and drought responses were abundant in profiles H3 and H4. Over 15 different photosynthesis-related gene homologues were observed in profile H4. Several genes for cell wall synthesis and cytoskeleton proteins were repressed at the later stages of infection (profile H4; Fig. 4D). Genes associated with signal transduction present in profile H4 included putative auxin-inducible proteins and a MAP-3 kinase protein (similar to CTR1, a negative regulator of ethylene signaling).

Verification of gene expression changes.

For verification of the differential expression of genes, quantitative real-time PCR (Q-PCR) was performed for selected genes. A representative gene was chosen from each of

Fig. 4. Expression profiling of genes differentially expressed in infected hypocotyl tissues. The expression ratios were calculated from normalized, rescaled ratios of the fluorescence intensity in the red and the green channels. Error bars show the standard deviation of expression levels at each time point. **A**, Profile H1 and **B**, profile H2 represent genes induced over time whereas **C**, profile H3 and **D**, profile H4 represent genes repressed over time. Normalized, rescaled log ratio values greater than 3.0 or less than -3.0 were estimated (tools for R Microarray Analysis) to indicate differentially expressed genes.

the major functional categories identified in this study and expression levels examined at a representative time point. The changes in expression levels observed using Q-PCR were similar to or greater than the levels obtained by microarray analysis (Table 3). It appears that the levels of gene expression change determined by microarray analysis underestimated those determined by Q-PCR. This has been observed previously for comparison of microarray and PCR-based estimates of gene expression changes (Iyer et al. 1999). Therefore, our PCR data serves to verify the identification of the selected genes as differentially expressed.

Several gene homologues associated with the ethylene signaling pathway were differentially expressed in infected tissues. This suggests the involvement of ethylene in cotton's response to *F. oxysporum* f. sp. *vasinfectum* infection. To determine whether this was the case, ethylene production was measured directly. Ethylene concentration measurements

were performed using *F. oxysporum* f. sp. *vasinfectum*-inoculated and mock-inoculated cotton plants generated using the experimental system. Ethylene production was detected in infected plants from 3 dpi and levels increased up to 5 dpi, whereas no ethylene production was detected in uninfected cotton plants from 1 to 5 days after treatment (Fig. 5). *F. oxysporum* f. sp. *vasinfectum* alone first produces detectable ethylene after 3 days of growth. The amount of ethylene produced by the fungus increases up to 4 dpi but then remains constant from 4 to 5 days after inoculation of the medium. *F. oxysporum* f. sp. *vasinfectum* alone produced lower final levels of ethylene than *F. oxysporum* f. sp. *vasinfectum*-infected plants. Although the exact source of the ethylene in the infection is unresolved from these measurements, the significant increase in ethylene concentration detected after 3 dpi does verify the identification of plant ethylene-responsive genes as differentially expressed.

Table 2. Selected genes from profiles H1, H2, H3, and H4 that showed differential expression in *Fusarium oxysporum* f. sp. *vasinfectum*-infected cotton seedling hypocotyls^a

Profile, functional category, GenBank ID	Putative protein name ^b	Putative function
Profile H1		
Defense related		
CD485528	PR protein class 10*	Pathogenesis related
CD486078	PR protein class 10*	Pathogenesis related
CD486279	Thaumatin-like protein PR-5b	Pathogenesis related
Stress related		
CD485630	<i>2-Nitropropane dioxygenase</i>	Reactive oxygen species (ROS) generator
CD485882	In2-1 protein	Detoxification
CD485867	<i>Glutathione-S-transferase GST 18</i>	Detoxification
CD486401	<i>Glutathione-S-transferase GST 13</i>	Detoxification
Secondary metabolism		
CD486690	Secoisolariciresinol dehydrogenase	Lignan biosynthesis
CD486561	Strictosidine synthase-like protein	TIA biosynthesis
CD486597	Cytochrome P450 71D10	Phenylpropanoid biosynthesis
CD485674	Cytochrome P450 82A3	Phenylpropanoid biosynthesis
Metabolism		
CD486309	Mitochondrial formate dehydrogenase	ATP biosynthesis
Signal transduction		
CD486177	Ethylene-inducible protein	Ethylene target gene
CD486389	SIEPIL protein precursor	Membrane signaling
CD486155	Na/H antiporter Nhx1	Salt stress signaling
Profile H2		
Defense related		
CD485829	Endochitinase 1 precursor*	Pathogenesis related
CD485880	Class I chitinase*	Pathogenesis related
CD485989	Class IV chitinase	Pathogenesis related
CD486342	β -1,3-endoglucanase	Pathogenesis related
CD486053	Ribonuclease-like PR 10*	Pathogenesis related
CD485561	Polygalacturonase inhibiting protein	Pathogen responsive
Gossypol biosynthesis		
CD486030	ATP citrate lyase b-subunit	Mevalonate biosynthesis
CD486505	Acetyl CoA C-acyltransferase	Mevalonate biosynthesis
CD486522	HMG CoA reductase*	Mevalonate biosynthesis
CD485579	Mevalonate diphosphate decarboxylate	Mevalonate biosynthesis
CD485919	(+)- Δ -cadinene synthase*	Sesquiterpenoid biosynthesis
CD485887	CYP706B1, (+)- Δ -cadinene-8-hydroxylase*	Sesquiterpenoid biosynthesis
Stress related		
CD486441	<i>Alcohol dehydrogenase 2d*</i>	Anaerobic stress
CD486292	<i>Bacterial-induced lipooxygenase*</i>	ROS generator
CD485672	Peroxidase precursor	ROS scavenger
CD485729	Putative oxidoreductase	ROS scavenger
CD485581	2-Oxoglutarate-dependent dioxygenase	Unknown
Secondary metabolism		
CD486150	Flavonoid 3'-hydrogenase	Flavonoid biosynthesis
CD486034	4-Coumarate-CoA ligase-like protein	Lignin biosynthesis
CD486304	Caffeic acid ortho-methyltransferase	Lignin biosynthesis

(continued on next page)

^a **Profile H1:** 35 putative plant genes, 2 putative fungal genes, and 18 unidentified genes induced. **Profile H2:** 67 putative plant genes, 4 putative fungal genes, and 37 unidentified genes induced. **Profile H3:** 22 putative plant genes, 0 putative fungal genes, and 5 unidentified genes repressed. **Profile H4:** 84 putative plant genes, 0 putative fungal genes, and 24 unidentified genes repressed.

^b An asterisk (*) indicates that the gene sequence matched a cotton sequence. Genes coding for putative proteins with names shown in italics were found to be differentially expressed in both root and hypocotyl tissues.

Several auxin-responsive genes were detected as differentially induced (profile R3; Fig. 3C) and repressed (profile R4; Fig. 3D) in roots. Several auxin-inducible gene homologues appeared to be repressed in infected hypocotyls (profile H4; Fig. 4D). In order to determine if auxin was involved in responses to infection and if different responses occurred in the separate tissues, we measured auxin directly in infected and uninfected tissues at 4 dpi (discussed below). A fivefold increase in auxin content from 4 ng/μg (uninfected roots) to 20 ng/μg (infected roots) was observed in roots, whereas similar levels, approximately 16 and 19 ng/μg, were observed in both uninfected and infected hypocotyls, respectively.

DISCUSSION

In the absence of strong resistance in cotton to Fusarium wilt disease, we sought to gain information about this suscepti-

ble plant-pathogen interaction using microarray analysis. An experimental system was employed that allowed tight control of environmental conditions to reduce changes in gene expression due to factors other than infection. Symptoms observed in this system mimicked those observed for Fusarium wilt disease (Fig. 1) in the field and glasshouse; this serves to validate our experimental system.

Although microarray analysis is an excellent tool for the study of multiple gene expression profiles during plant-pathogen interactions (Kazan et al. 2001), there are few examples of studies involving plants other than *Arabidopsis*, where well-characterized arrays are available. In order to construct the microarray required for this study, we sought to enrich for likely pathogen response genes that may be expressed in different tissues and at different times during infection. Therefore, we constructed a library from infected cotton seedling roots and hypocotyls from several time points after inocula-

Table 2. (continued from preceding page)

Profile, functional category, GenBank ID	Putative protein name	Putative function
Profile H3, Secondary metabolism (continued)		
CD485702	Cytochrome P450	Lignin biosynthesis
CD486014	NADPH:isoflavone reductase	Flavonoid biosynthesis
Metabolism		
CD486446	Sucrose synthase*	Sugar metabolism
CD485614	Mycolic acid methyl transferase-like protein	Fatty acid metabolism
Signal transduction		
CD486312	1-Aminocyclopropane-1-carboxylate oxidase	Ethylene biosynthesis
CD485820	Ckc2 AP2 domain transcription factor	Ethylene signaling
CD486512	GTP-binding protein RAN2 (ras-like)	Ethylene signaling
CD486506	Auxin-repressed protein like-protein	Auxin regulated gene
Profile H3		
Stress related		
CD485873	<i>Late embryogenesis abundant protein Lea5-A*</i>	Drought stress
CD486367	<i>Late embryogenesis abundant protein Lea5-D*</i>	Drought stress
Metabolism		
CD486005	<i>Asparagine synthetase</i>	Nitrogen metabolism
CD486680	Vacuolar ATP synthase 1	Energy generation
Signal transduction		
CD485577	Transfactor, putative CDPK	Calcium signaling
CD486417	Nt-iaa28 deduced protein	Auxin signaling
Profile H4		
Defense related		
CD486396	<i>Putative endochitinase</i>	Pathogenesis related
Stress related		
CD486684	<i>MipC</i>	Drought stress
CD486301	<i>Δ-tonoplast intrinsic protein*</i>	Drought stress
CD486426	<i>Putative aquaporin PIP2-2</i>	Drought stress
CD486348	<i>Tonoplast intrinsic protein, γ-TIP</i>	Drought stress
CD486260	<i>Putative alcohol dehydrogenase</i>	Anaerobic stress
CD486702	<i>Cysteine protease</i>	Drought stress
CD485826	Metallothionein-like protein	Detoxification
CD486551	<i>Major latex-like protein</i>	Unknown
Cell wall synthesis		
CD486691	Endo-xyloglucan transferase*	Cell wall synthesis
CD485620	Expansin	Cell wall synthesis
CD486470	Cellulose synthase*	Cell wall synthesis
CD485678	Actin	Cell wall synthesis
CD486699	Proline-rich protein	Cytoskeleton formation
CD486526	Tubulin α-1 chain	Cytoskeleton formation
Photosynthesis		
CD485569	Plastocyanin A, chloroplast precursor	Photosynthesis
CD485661	Chlorophyll a/b binding protein precursor*	Photosynthesis
CD486450	Rubisco small subunit	Photosynthesis
CD486206	Oxygen evolving enhancer proteins 1	Photosynthesis
Secondary metabolism		
CD486387	Leucoanthocyanidin reductase	Tannin/anthocyanin biosynthesis
CD486476	Cytochrome P450 71A1 (ARP-2)	Tannin/anthocyanin biosynthesis
Signal transduction		
CD485605	Indoleacetic acid-induced protein 8 (iaa8)	Auxin signaling
CD485926	Iaa7-like protein	Auxin signaling
CD485595	G protein β subunit 2	Unknown
CD486173	Auxin repressed protein-like protein	Auxin signaling
CD486140	MAP3K Δ-1 protein kinase	Ethylene signaling

tion. This strategy was similar to that used in previous studies of gene responses to abiotic stress (Jan Klok et al. 2002, Ozturk et al. 2002) that utilized stressed material from different tissues at a range of time points.

In our array design, we also sought to incorporate a proportion of “nonbiased” genes on the microarray by including clones from a library prepared from uninfected tissue. This was to allow the identification of genes that may be suppressed due to infection. Indeed, over 50% of repressed genes identified in this study were from the uninfected library, whereas many of the defense-related genes derived from the infected library. In addition, because the procedure used to identify outliers in the distribution, and hence differentially expressed genes, fails when a high proportion of differentially expressed genes are identified during hybridization of the microarray (Wilson et al. 2003), we wished to include genes that were less likely to be differentially expressed to ensure effective data analysis. During the tRMA analysis, gene expression changes were estimated relative to the expression levels of the

Fig. 5. Ethylene measurements of infected cotton tissues. Measurement of ethylene production in uninfected cotton seedlings (Control seedlings), infected cotton seedlings (Inoculated seedlings), and *Fusarium oxysporum* f. sp. *vasinfectum* alone (Fusarium alone). Inoculated seedlings were dipped in a conidial suspension of *F. oxysporum* f. sp. *vasinfectum*, control seedlings were dipped in water. For *F. oxysporum* f. sp. *vasinfectum* alone, a few drops of conidial suspension of *F. oxysporum* f. sp. *vasinfectum* were placed on the agar surface. Ethylene measurements were made at various days after inoculation. Each ethylene measurement is the average value obtained from six plants. Error bars show the standard deviation of ethylene levels at each time point.

bulk of genes whose expression levels did not change. Ensuring that these genes were of plant origin allowed us to measure gene expression level changes relative to plant genes, removing any bias from different levels of fungal RNA in infected tissue. Therefore, the array was constructed from anonymous clones from a library prepared from infected tissue (4,000 clones) and a library prepared from uninfected tissue (2,000 clones). We examined tissue-specific gene expression changes occurring over time to develop gene expression profiles for cotton during the susceptible interaction with the soilborne vascular wilt, *F. oxysporum* f. sp. *vasinfectum*, by comparison of gene expression levels in infected and uninfected (mock-inoculated) tissue. Microarray results were validated either on a gene-by-gene basis by Q-PCR measurements for selected genes from representative response classes, or by confirming alterations in major plant pathways through measuring hormone concentrations.

Responses of cotton tissues to *Fusarium* wilt infection.

The most marked difference between induced genes identified in the infected root and hypocotyl tissues was the high proportion of putative fungal genes observed in the root tissues (approximately 30%) compared with the hypocotyl tissues (approximately 2%). Increased numbers of fungal clones identified over time in roots may be a result of the fungal biomass that accumulates in and on the surface of the root tissues rather than because of differential expression of fungal genes. Significantly more unidentified induced genes were observed in infected roots (119; profiles R1, R2, and R3) compared with infected hypocotyls (55; profiles H1 and H2). However, the unidentified induced genes in infected roots are likely to be due to additional putative fungal genes because induced genes could represent either fungal or plant genes. Further analysis of the origin of unidentified genes was not considered for this study, but could yield useful information in the future.

Substantially more plant genes were induced in infected hypocotyl tissues (102; profiles H1 and H2) compared with infected root tissues (37; profiles R1, R2 and R3). This suggests that, although the hypocotyls appear to mount a significant induced response to infection, the fungus may be suppressing plant defense responses in the root tissue. There also was little similarity (approximately 20%; Tables 1 and 2, italics) in the identity of the differentially expressed genes detected in infected root and hypocotyl tissues, indicating that, overall, the tissues responded quite differently to infection.

Table 3. Expression ratios for selected genes determined by microarray analysis and quantitative real-time polymerase chain reaction (Q-PCR)

Functional category, GenBank ID	Protein name	Expression ratios ^a			
		Root (3 dpi)		Hypocotyl (4 dpi)	
		Microarray	Q-PCR	Microarray	Q-PCR
Housekeeping CK738219	Polyubiquitin	1.0	1.0	1.0	1.0
Defense CD486053	Ribonuclease PR10	1.0	1.0	4.0	8.7
CD486522	HMG-CoA reductase 1	ND	ND	3.3	7.0
Stress CD486413	Aquaporin (SIMIP)	0.3	0.6	0.5	0.2
CD485819	Late embryogenesis abundant protein Lea5-A	0.2	0.6	ND	ND
Hormone response CD486177	Ethylene inducible protein	2.5	2.1	3.1	13.0
Growth, photosynthesis CD485661	CAB	NA	NA	0.5	0.2

^a Expression ratios determined from the ratio of the expression level of a particular gene in inoculated tissue to the expression level of that gene in control (mock-inoculated) tissue. In the microarray data analysis, genes were identified as differentially expressed using tools for R Microarray Analysis software from expression ratios of the fluorescence intensity in the red and the green channels that were normalized and rescaled. The expression ratios given here were derived from data that had been normalized, but not rescaled. Results presented for microarray analysis and Q-PCR estimations are the means of four replicate determinations; dpi = days postinfection, NA = not applicable, and ND = not done.

There appear to be two possible stages of differential expression in the roots (Fig. 2A). The first early stage in roots, at 14 hpi to 1 dpi, involved mainly repression of genes. The second later stage of gene expression, at 3 to 4 dpi, involved a large increase in the number of differentially expressed genes. At 2 dpi, there appeared to be a shift in gene expression changes, with few plant genes identified as differentially expressed in infected tissue at this stage. It is interesting to speculate that this shift may be related to the change of the pathogen from an endophytic early phase to a more aggressive pathogenic phase once it reaches the vascular tissue in roots (Rodriguez-Galvez and Mendgen 1995).

Although microarray technology has the advantage of allowing the examination of the gene expression changes of hundreds of genes simultaneously, the limitation lies in trying to interpret and communicate such large amounts of data. Therefore, the gene expression changes in cotton will be discussed with reference to four major functional categories that have been observed to alter in the infection process: defense, stress, hormone, and growth responses.

Defense responses: pathogenesis-related genes.

The differences between the responses of the roots and hypocotyls probably are most significant for defense-related responses. We observed no induction of defense-related genes in infected root tissue. Defense-related genes appeared to be constitutively expressed in roots as judged from microarray and reverse transcription PCR data (not shown) and Q-PCR (Table 3; ribonuclease PR10). Therefore, the pathogen may invade the plant successfully by suppressing expression of defense-related genes and thus evading detection (Kuc 1984). In infected hypocotyl tissues, defense responses were mounted coincident with the fungus first being reisolated from the hypocotyl and the onset of vascular browning (profiles H1 and H2; Fig. 4A and B). Several pathogenesis-related (PR) genes were induced in infected hypocotyls from 2 dpi (profile H1), including two classes of PR10 genes (cotton PR10 and ribonuclease-like PR10) and PR5 (thaumatin). PR2 (glucanase) and PR3 (chitinase, class I and IV) were induced later from 4 dpi in infected hypocotyls (profile H2). PR2 and PR3 exhibit antifungal activity in vivo (Leah et al. 1991). The function of PR10 genes is still unknown but they evidently are involved in the response of cotton to *F. oxysporum* f. sp. *vasinfectum* infection because they represent the most abundant of all the genes induced (20 cDNA copies). PR10, PR2, and PR3 all have been found to be induced by vascular wilt disease (*V. dahliae*) in cotton stems (Hill et al. 1999; McFadden et al. 2001).

Defense responses: phytoalexin biosynthesis.

Cotton phytoalexin (gossypol) biosynthesis genes showed similar expression profiles and seven genes encoding enzymes from the biosynthesis pathway were induced in hypocotyl tissues at 4 dpi (profile H2; Figure 4B; Table 2). The majority of these genes are known to be induced by infection with *V. dahliae* (Tan et al. 2000) and gossypol production is thought to contribute to reduced Fusarium wilt disease severity in cotton (Zhang et al. 1993). In this study, the induction of phytoalexin biosynthesis genes occurred once the infection was well established and, therefore, it was too late to provide an effective resistance mechanism. In *V. dahliae* infection of cotton, phytoalexins are induced later in a susceptible rather than in a resistant cotton species (Bell 1994).

Defense responses: secondary metabolism.

Homologues of genes associated with tannin, anthocyanin, and lignin biosynthesis were repressed in infected root tissues

(profile R4; Fig. 3D; Table 1). Lignin deposition is a known response to pathogen attack; therefore, repression of genes for lignin biosynthesis may represent suppression of defense responses by the pathogen in roots. In contrast, in the hypocotyl tissues, homologues associated with phenylpropanoid, flavanoid, and terpenoid indole alkaloid biosynthesis were induced in response to infection (profile H2; Fig. 4B; Table 2). A lignan and several lignin biosynthesis genes were induced in hypocotyls. We identified a cytochrome P450 gene that showed close similarity to a gene for ferulate-5-hydroxylase (Cyp 84A1). Ferulate-5-hydroxylase catalyses the production of syringyl monomers. High syringyl content (compared with guaiacyl content) in lignin polymers previously has been found to be associated with pathogen defense responses in wheat (Ride 1975). Lignin content in infected and uninfected hypocotyl tissues was measured in this study, but there was no apparent difference in the quantity of lignin (data not shown). However, there may be differences in lignin composition (syringyl:guaiacyl ratio) rather than in overall lignin content. Lignan biosynthesis represents a previously unreported response to vascular wilts. Lignans are dimers of monolignols, which are known to possess antifungal activity (Davin and Lewis 1992).

Defense responses: other defense-related genes.

A polygalacturonase inhibitor gene (PGI) homologue was induced in infected hypocotyl tissue. Plant PGIs may contribute to resistance to fungal attack (De Lorenzo et al. 2001). One of the few defense-related genes induced in roots was a homologue of a tobacco tumor-related protein, similar to kunitz-type trypsin inhibitors. Recently, a trypsin inhibitor from corn was shown to have antifungal properties (Chen et al. 1999). A bacterially induced lipoxygenase (Loxy) gene was induced in both tissues and also has been found to be associated with a hypersensitive response and the incompatible interaction between cotton and the bacterial pathogen *Xanthomonas campestris* (Jalloul et al. 2002). All of the above gene responses are reported as being expressed in both a susceptible and resistant interaction, but the salient difference determining the outcome of the plant-pathogen interaction is in the timing and intensity of gene induction.

Stress responses: drought-responsive genes.

LEAs, cysteine protease, and aquaporins (water channels) usually are induced in response to dehydration stress (Ramanjulu and Bartels 2002). Interestingly, homologues of genes for these proteins were repressed in both infected roots and hypocotyls (profile R4; Fig. 3D; Table 1; and profile H3 and H4; Fig. 4C and D; Table 2). Repression of over 10 different members of the aquaporin family in infected cotton may be a specific response of cotton in this particular plant-pathogen interaction because aquaporins are induced in other plant-pathogen interactions (Tyerman et al. 2002). One of the few examples of repression of aquaporin genes is found in the ice plant (*Mesembryanthemum crystallinum*), where aquaporin genes are repressed in response to salt stress and the symptoms observed include immediate wilting. Upon rehydration, the aquaporins are induced and wilting is reversed (Yamada et al. 1995).

Stress responses: other stress-related genes.

Cotton alcohol dehydrogenase (anaerobic stress) was induced in both tissues but another highly redundant Zn-class alcohol dehydrogenase was repressed, possibly indicating different roles for these dehydrogenases. ROS scavengers (peroxidase and oxidoreductase) were induced in hypocotyls only and were repressed in roots. The induction of antioxidant

enzymes at the later stages of infection likely is a response to ROS generated as a secondary response to injury and the oxidative stress effects of infection. A homologue of soybean GST 18 was induced in roots and hypocotyls whereas a homologue of a GST-like gene, *In2-1*, was induced in hypocotyls and repressed in roots. Although generally thought to play a role in xenobiotic detoxification, the exact function of many of the GSTs identified in this study remains unknown; however, they may be involved in plant defense responses or detoxification of phytoalexins. A major latex protein (MLP) gene was the most abundant potential stress-related gene in both tissues (13 cDNA copies identified). The function of this gene is unknown but it has been shown to be repressed in response to ethylene treatment (Schenk et al. 2000) and induced in response to drought stress (Seki et al. 2001).

Hormone responses: ethylene signaling.

Homologues of ethylene synthesis genes were induced in infected hypocotyls whereas putative ethylene-signaling genes, such as a gene for a ras-like GTP-binding protein (similar to RAN1) (Wang et al. 2002), were induced and a gene for a MAP-3-kinase protein (similar to CTR1) was identified as repressed in hypocotyl tissue (Wang et al. 2002). In *Arabidopsis*, direct inactivation of CTR1 by ethylene is required for ethylene signaling to proceed. Genes for AP2 domain transcription factors (homologues of genes for RAP2.3 and 2.8) were identified as induced in infected hypocotyls and roots: these transcription factors belong to the EREBP-like RAP2 proteins (transcription factors possibly involved in ethylene signaling) (Okamuro et al. 1997). Direct measurements of ethylene concentration confirmed that ethylene production was occurring during the infection process (Fig. 5). Ethylene production was detected in infected plants from 3 to 5 dpi, whereas no ethylene production was detected in uninfected cotton plants from 1 to 5 dpi (Fig. 5). Another interesting observation was that the fungus alone produced ethylene in our experimental system, although final levels were not as high as those in the infected plant. It is possible that production of ethylene by the pathogen may be a strategy for infection. Ethylene is thought to aggravate disease symptoms and is associated with susceptibility in tomato plants infected with *Fusarium* wilt (Lund et al. 1998).

Hormone responses: auxin responses.

In infected root tissues, auxin-regulated gene homologues were induced (profile R3; Fig. 3C; Table 1) and genes for auxin-repressed protein homologues were repressed (profile R4; Fig. 3D; Table 1). These results imply that there are changes in auxin levels in infected root tissues. A less clear response was observed in hypocotyls where putative genes for auxin-inducible protein homologues were repressed (profile H4; Fig. 4D; Table 2) while a putative gene for an auxin-repressed protein was induced. Analysis of auxin at 4 dpi revealed a fivefold increase in auxin content in infected root tissues compared with uninfected root tissue, whereas no significant difference in auxin levels was observed between infected and uninfected hypocotyl tissues. These results implicate auxin signaling in the responses of cotton to *F. oxysporum* f. sp. *vasinfectum* infection; however, different responses appear to occur in the two different tissues examined.

Growth responses.

At the later stages of infection (4 dpi), coincident with the induction of defense responses in the hypocotyls, a large number of genes were repressed in infected hypocotyls. Many of these genes are involved in growth responses; specifically cell wall synthesis and cytoskeleton formation (actin and tubulin).

Photosynthesis-related genes were repressed and highly abundant in hypocotyls at 4 dpi (profile H4; Fig. 4D; Table 2). It would appear that there is an overall cessation of cell expansion and growth. Genes for proteins such as CAB and rubisco are known to be repressed by a variety of factors, including water stress (Bartholomew et al. 1991). Cessation of growth and photosynthesis likely is a global response to stress rather than a specific response to pathogen invasion. Another possibility is that switching off growth or photosynthesis may allow the plant to redirect energy resources to the concurrent transcription of defense-related genes. Interestingly, net photosynthesis also is known to be inhibited by ethylene (Pallas and Stanley, 1982).

Conclusion.

In conclusion, our research has revealed some of the complex plant defense responses that occur in cotton in response to *F. oxysporum* f. sp. *vasinfectum* infection. Different gene responses occurred in infected root and hypocotyl tissues, with the induction of defense-related genes in hypocotyls that appeared to be constitutive in infected root tissues. More plant genes were repressed in roots than were induced, especially at the earlier stages of infection. This may reflect suppression of host responses by the pathogen as a strategy for infection. Many known cotton defense responses were identified in this study, such as the induction of PR and gossypol phytoalexin biosynthesis genes. However, previously unreported responses also were identified, including the possible biosynthesis of secondary metabolites such as lignan, syringyl lignin polymers, and terpenoid indole alkaloids. Ethylene appears to be a key player in the cotton-*F. oxysporum* f. sp. *vasinfectum* interaction and auxin was implicated in responses of cotton to *F. oxysporum* f. sp. *vasinfectum* infection in root tissues. The involvement of these hormones was confirmed by direct measurements of hormone levels. Many of the stress-related gene responses were common to both tissues and may represent a general stress response to a common secondary signal of infection, such as oxidative stress, wounding, or dehydration. In addition, the repression of genes for aquaporins and drought-responsive proteins represents a previously unknown response of a host to pathogen attack and may be specific to vascular wilt diseases and the development of wilt symptoms. Cell expansion and photosynthesis-related genes were repressed in hypocotyls and ethylene also may be involved in the generation of this response. This research has provided information on a susceptible plant-pathogen interaction and on a soilborne root pathogen; both aspects that rarely have been examined in plant-pathogen research. It is anticipated that the information obtained will be a valuable first step toward understanding and managing *Fusarium* infection in cotton.

MATERIALS AND METHODS

Plant growth and inoculation system.

Field isolates of *F. oxysporum* f. sp. *vasinfectum* (purified by monoconidial culture) were provided by N. Y. Moore (Queensland Department of Primary Industries). Isolate 24595 (vcg 01111) was used for the inoculation of seedlings in experiments to generate RNA for gene expression analysis. This isolate and isolates 24596, 24500 (vcg 01111), 24597, 24598, and 24599 (vcg 01112) (Davis et al. 1996) were used to inoculate seedlings for the preparation of the cDNA libraries. Cultures were stored at -80°C as conidial suspensions in 15% glycerol and were revived on 25% potato dextrose agar amended with 40 ppm streptomycin and incubated at 26°C . For root-dip inoculation of seedlings, a conidial suspension of 10^6 conidia ml^{-1} was prepared.

Cotton seed (DeltaEMERALD; Deltapine Australia, Narrabri, NSW, Australia) was surface sterilized (70% aqueous ethanol for 5 min, 5% hydrogen peroxide solution for 2 h), then rinsed in sterile water). The seed then was incubated in petri dishes between sheets of moist filter paper for 48 h at 28°C. Germinated seedlings were transferred to tissue culture pots (MS medium [Murashige and Skoog 1962], pH 5.8, with sucrose at 10 g liter⁻¹ and phytagel at 2 g liter⁻¹) and incubated for a further 5 days at 28°C. Seedlings were removed from the pots and inoculated by root dip into either sterile water (for mock inoculation) or a conidial suspension of *F. oxysporum* f. sp. *vasinfectum* for approximately 30 s and transferred to a fresh pot containing the same MS medium. The seedlings were incubated at 26°C in a growth room with mock-inoculated and *F. oxysporum* f. sp. *vasinfectum*-inoculated pots placed in pairs, adjacent to each other, to minimize the effects of different microenvironments. Root and hypocotyl tissues were harvested at various times after inoculation, frozen, and stored at -80°C. Mycelium was removed from the root tissue as much as practical.

Hand sections (approximately 0.5 mm) were cut from surface-sterilized tap roots and hypocotyls from three cotton seedlings per time point. Sections were transferred to plates containing 25% potato dextrose agar and incubated at 24°C for 2 days. Tissue sections then were examined for the presence of mycelial growth from vascular tissue and the percentage of sections showing mycelial growth was recorded.

RNA isolation and analysis.

Total RNA was isolated (Wan and Wilkins 1994) for preparation of the cDNA libraries and for probe synthesis. Poly A+ mRNA was isolated from total RNA using a Qiagen Oligotex mRNA kit (Clifton Hill, Victoria, Australia) following the manufacturer's protocol. The integrity of the mRNA of total RNA samples was checked prior to pooling by in vitro translation using wheat germ extract (Promega Corp., Madison, WI, U.S.A.) and determination of (³⁵S) methionine incorporation (manufacturer's protocol).

cDNA library synthesis.

Two directionally cloned cDNA libraries were synthesized from pooled root and hypocotyl tissues from infected and uninfected plants, respectively. For the infected library, plant tissues were harvested at a range of time points from 5 hpi to 7 dpi. Poly A+ mRNA (5 µg) was used for cDNA synthesis and cDNA library construction using Invitrogen (San Diego, CA, U.S.A.) Superscript lambda system for cDNA synthesis and cloning following the manufacturer's instructions. The cDNA was recovered in autonomously replicating pZL1 using an in vivo excision protocol provided by Invitrogen. Plasmid DNA was excised in *Escherichia coli* strain DH10B. Clones were picked randomly (4,000 clones from the infected library and 2,000 clones from the uninfected library).

Preparation of microarray slides.

PCR products were precipitated (95% ethanol) and resuspended in 10 µl of 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and transferred to 384-well plates. As a quality-control step, PCR products were checked by gel analysis before and after ethanol precipitation. PCR fragments were printed onto CMT-GAPS coated slides (Corning Microarray Technology, Corning, NY, U.S.A.) using a Bio-Rad (Hercules, CA, U.S.A.) VersArray ChipWriter Pro with SMP 3 stealth microspotting quill pins (TeleChem International Inc., Sunnyvale, CA, U.S.A.). In all, 100 control genes consisting of negative controls (nonplant DNA and intron DNA), positive controls (known PR genes), and potential housekeeping genes

were printed twice on the slides. Slides were processed by baking for 3 to 4 h at 80°C.

Hybridization experiments.

For analysis of gene expression, root and hypocotyl tissues were harvested separately from *F. oxysporum* f. sp. *vasinfectum*-infected and mock-inoculated cotton seedlings. Samples were harvested from selected time points (14 hpi to 4 dpi) in order to cover the infection process. At least two samples (biological replicates) were collected per time point, with six to seven plants per sample. Total RNA (100 µg) was reverse transcribed and labeled cDNA probes were generated using a two-step labeling method (Schenk et al. 2000). Prehybridization of slides, application of the probe to the microarray slides, hybridization, and subsequent washing steps were performed according to manufacturer's instructions (Corning Microarray Technology). Slides were scanned and analyzed with a GenePix 4000A microarray scanner and GenePix Pro3 software, respectively (Axon Instruments, Union City, CA, U.S.A.) to give a fluorescence intensity ratio (R/G ratio) for each spot.

Microarray data analysis.

Analysis of the fluorescence intensity (R/G ratio) data was performed using the tRMA analysis suite (Wilson et al. 2003). The analysis included a spatial normalization protocol, corrected for intensity-dependent effects across the microarray slide (Dudoit et al. 2002, Jan Klok et al. 2002), and corrected for possible biases in fluorescence that occur due to differences in the efficiency of incorporation of the two dyes and unequal loading of RNA samples. Using the tRMA suite as previously described (Jan Klok et al. 2002) to compare the normalized and rescaled log₂ R/G ratio data for all replicates with a normal distribution, differentially expressed genes were identified. In the normalized and rescaled distribution of data (mean 0), those genes identified as outliers had a log₂ R/G ratio greater than 3 or less than -3.

Differentially expressed genes were grouped into profiles based on similar expression profiles using a K-means clustering function of tRMA. The redundancy of genes in each profile was used to assess the quality of clustering whereby redundant genes that would be expected to have similar expression profiles were found in the same profiles.

Differentially expressed genes were sequenced in a single pass from the 5' ends of cDNAs using M13/pUC reverse primer with Big Dye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were analyzed using the program package Biomanager (The Australian National Genomic Information Service [ANGIS], Eveleigh, Australia). Codoncode base caller (based on phred) and vector masking (Claverie and States 1993) were used for reading and quality screening of trace files and vector clipping, respectively. Sequences then were analyzed using Blast N, Blast X, and tBlast X searches (Altschul et al. 1990) through NCBI (GenBank main, SwissProt, and SpTrEMBL databases). Generally, Blast X results were used and accession numbers from the databases SwissProt and SpTrEMBL were used. Matches with an E value higher than 10⁻¹⁰ were deemed insignificant. Redundancy was determined by observation of identical accession numbers.

Q-PCR.

Differential gene expression of selected genes was verified by Q-PCR. Polyubiquitin was used (selected using tRMA software) as a housekeeping gene and a control for RNA loading. For Q-PCR, infected and uninfected cDNA samples from hypocotyl tissues (at 4 dpi) and from root tissues (at 3 dpi) were used as templates. Ratio values were determined by com-

paring gene expression in an infected sample with an uninfected sample. Q-PCR was performed using SYBER green following the method described previously (Jan Klok et al. 2002), with a cycling temperature of 60°C. Four replicates of each sample were tested.

Ethylene detection.

Aliquots (2 ml) of headspace gases from the pots (in which plants were growing) were sampled through a rubber septum in the pot lids using a syringe. Aliquots were injected immediately into a flame-ionization detector gas chromatograph (Series 15, Dohrmann Envirotech, Mountain View, CA, U.S.A.). A standard solution of ethylene (1 ppm/liter) was used as a reference.

Auxin measurements.

Plant material (approximately 0.3 to 0.6 g; two to three plants) was extracted and purified by solid-phase extraction using C18 as described by Prinsen and associates (1991). The samples were derivatized using diazomethane and heptafluorobutyrimidazole (HFB) and incubated (Pilet and Saugy 1985). Indole 3-acetic acid (IAA) was quantified using selected-ion-monitoring gas chromatography mass spectrometry with a Hewlett-Packard HP5890 Series 11 gas chromatograph coupled with a 5971 Series mass selective detector with an SGE brand BPX-5 column (25 m long, 0.22 mm in diameter, 0.25- μ m film thickness). Samples (1 μ l) were injected and quantitation calculated using the base and secondary peaks for derivatized IAA-methyl-HFB (m/z 326, 385) and (13 C6)-IAA-methyl-HFB (m/z 332, 391) (Pilet and Saugy 1985).

ACKNOWLEDGMENTS

This work was funded by the Cotton Research and Development Corporation, Australia. We thank J. Jacobsen, A. Ivanova, and A. Poole for help in the analysis of hormones; G. Kennedy for Bioinformatics assistance; and R. Chapple and K. Vinall for technical assistance.

LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipmann, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Bartholomew, D. M., Bartley, G. E., and Scolnik, P. A. 1991. Absciscic acid control of rbc and cab transcription in tomato leaves. *Plant Physiol.* 96:291-296.
- Beckman, C. H., and Roberts, E. M. 1995. On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. *Adv. Bot. Res.* 21:35-77.
- Bell, A. A. 1994. Mechanisms of Disease resistance in *Gossypium* species and variation in *Verticillium dahliae*. Pages 225-235 in: *Proc. World Cotton Res. Conf.* Brisbane, Australia. G. A. Constable and N. W. Forrester, eds. CSIRO, Melbourne, Australia.
- Chen, Z. Y., Brown, R. L., Lax, A. R., Cleveland, T. E., and Russin, J. S. 1999. Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli*. *Appl. Environ. Microbiol.* 65:1320-1324.
- Claverie, J. M., and States, D. J. 1993. Information enhancement methods for large scale sequence analysis. *Comput. Chem.* 17:191-201.
- Davin, L. B., and Lewis, N. G. 1992. Phenylpropanoid metabolism: Biosynthesis of monolignols, lignans, neolignans and suberins. Pages 326-360 in: *Phenolic Metabolism in Plants*. H. A. Stafford and R. K. Ibrahim, eds. Plenum Press, New York.
- Davis, R. D., Moore, N. Y., and Kochman, J. K. 1996. Characterisation of a population of *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt of cotton in Australia. *Aust. J. Agric. Res.* 47:1143-1156.
- De Lorenzo, G., D'Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* 39:313-335.
- Diehne, M., and Relman, D. A. 2001. Comparing functional genomic datasets: lessons from DNA microarray analyses of host-pathogen interactions. *Curr. Opin. Microbiol.* 4:95-101.
- Dudoit, S., Yang, Y. H., Callow, M. J., and Speed, T. P. 2002. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. *Stat. Sin.* 12:111-139.
- Eckardt, N. A. 2002. Plant disease susceptibility genes? *Plant Cell* 14:1983-1986.
- Hill, M. K., Lyon, K. J., and Lyon, B. R. 1999. Identification of disease response genes expressed in *Gossypium hirsutum* upon infection with the wilt pathogen *Verticillium dahliae*. *Plant Mol. Biol.* 40:289-296.
- Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C., Trent, J. M., Staudt, L. M., Hudson, J., Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. 1999. The transcriptional program in the response of human fibroblasts to serum. *Science* 283 (5398):83-87.
- Jalloul, A. A., Montillet, J. L. B., Assigbetsé, K. C., Agnel, J. P. B., Delannoy, E. C., Triantaphylidès, C. B., Daniel, J. F. C., Marmey, P. C., Geiger, J. P. C., and Nicole, M. 2002. Lipid peroxidation in cotton: *Xanthomonas* interactions and the role of lipoxygenases during the hypersensitive reaction. *Plant J.* 32:1-12.
- Jan Klok, E., Wilson, I. W., Wilson, D., Chapman, S. C., Ewing, R. M., Somerville, S. C., Peacock, W. J., Dolferus, R., and Dennis, E. 2002. Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. *Plant Cell* 14:2481-2494.
- Kazan, K., Schenk, P. M., Wilson, I., and Manners, J. M. 2001. DNA microarrays: New tools in the analysis of plant defence responses. *Mol. Plant Pathol.* 2:177-185.
- Kuc, J. 1984. Phytoalexins and disease resistance mechanisms from a perspective of evolution and adaptation. *Ciba Found. Symp.* 102:100-118.
- Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* 266:1564-1573.
- Lund, S. T., Stall, R. E., and Klee, H. J. 1998. Ethylene regulates the susceptible response to pathogen infection in tomato. *Plant Cell* 10:371-382.
- McFadden, H. G., Chapple, R., de Feyter, R., and Dennis, E. 2001. Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 58:119-131.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant* 15:473-497.
- Okamuro, J. K., Caster, B., Villarreal, R., Van Montagu, M., and Jofuku, K. D. 1997. The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 94:7076-7081.
- Ozturk, Z. N., Talamé, V., Deyholos, M., Michalowski, C. B., Galbraith, D. W., Gozukirmi, N., Tuberosa, R., and Bohnert, H. J. 2002. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol. Biol.* 48:551-573.
- Pallas, J. E., and Stanley, J. K. 1982. Inhibition of Photosynthesis by ethylene—A stomatal effect. *Plant Physiol.* 70:598-601.
- Pilet, P. E., and Saugy, M. 1985. Effect of applied and endogenous indol-3-yl-acetic acid on maize root growth. *Planta* 164:254-258.
- Prinsen, E., Rudelsheim, P., and Van Onckelen, H. 1991. Extraction, purification and analysis of endogenous indole-3-acetic acid and abscisic acid. Pages 198-209 in: *A Laboratory Guide for Cellular and Molecular Plant Biology*. I. Negrutiu and G. B. Gharti-Chetri, eds. Birkhauser Verlag, Basel, Switzerland.
- Ramanjulu, S., and Bartels, D. 2002. Drought- and desiccation-induced modulation of gene expression in plants. *Plant Cell Environ.* 25:141-151.
- Ride, J. P. 1975. Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. *Physiol. Plant Pathol.* 5:125-134.
- Rodriguez-Galvez, E., and Mendgen, K. 1995. The Infection Process of *Fusarium-oxysporum* in Cotton Root Tips. *Protoplasma* 189:61-72.
- Rousseeuw, P. J. 1987. Silhouettes: A graphical aid to the interpretation and validation of cluster analysis. *J. Comp. Appl. Math.* 20:53-65.
- Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C., and Manners, J. M. 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc. Natl. Acad. Sci. U.S.A.* 97:11655-11660.
- Schieble, W.-R., Richmond, T. A., Wilson, I. W., and Somerville, C. R. 2001. *Arabidopsis* genetics and functional genomics in the post-genome era. Pages 563-585 in: *Advances in Photosynthesis*. E. M. Aro and B. Andersson, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* 13:61-72.
- Tan, X. P., Liang, W. Q., Liu, C. J., Luo, P., Heinsteins, P., and Chen, X. Y. 2000. Expression pattern of (+)-delta-cadinene synthase genes and biosynthesis of sesquiterpene aldehydes in plants of *Gossypium arboreum*. *Planta* 210:644-651.
- Tyerman, S. D., Niemietz, C. M., and Bramley, H. 2002. Plant aquaporins:

- multifunctional water and solute channels with expanding roles. *Plant Cell Environ.* 25:173-194.
- Wan, C. H., and Wilkins, T. A. 1994. A modified hot borate method significantly enhances the yield of high quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* 223:7-12.
- Wan, J., Dunning, F. M., and Bent, A. F. 2002. Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. *Funct. Integr. Genom.* 2:259-273.
- Wang, K. L. C., Li, H., and Ecker, J. R. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* 14:S131-S151.
- Wilson, D. L., Buckley, M. J., Helliwell, C. A., and Wilson, I. W. 2003. New normalisation methods for cDNA microarray data. *Bioinformatics* 19:1325-1332.
- Yamada, S., Katsuhara, M., Kelly, W. B., Michalowski, C. B., and Bohnert, H. J. 1995. A family of transcripts encoding water channel proteins—tissue-specific expression in the common ice plant. *Plant Cell* 7:1129-1142.
- Zhang, J. X., Mace, M. E., Stipanovic, R. D., and Bell, A. A. 1993. Production and fungitoxicity of the terpenoid phytoalexins in cotton inoculated with *Fusarium oxysporum* f. sp. *vasinfectum*. *J. Phytopathol.* 139:247-252.

AUTHOR-RECOMMENDED INTERNET RESOURCES

- The ANGIS database: www.angis.org.au
- CSIRO tools for R microarray analysis (tRMA) software: www.cmis.csiro.au/iap/tRMA
- CSIRO Plant Industry GENA genomics array database: www.pi.csiro.au/gena