Global Gene Expression Profiling During *Medicago truncatula*–*Phymatotrichopsis omnivora* Interaction Reveals a Role for Jasmonic Acid, Ethylene, and the Flavonoid Pathway in Disease Development

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Phymatotrichopsis omnivora (Duggar) Hennebert causes a destructive root rot in cotton, alfalfa (*Medicago sativa*), and many other dicot species. No consistently effective control measures or resistant host germplasm for *Phymatotrichum* root rot (PRR) are known. The relative genetic intractability of cotton and alfalfa precludes their use as model pathosystem hosts for *P. omnivora*. Therefore, we used the model legume *M. truncatula* and its available genetic and genomic resources to investigate PRR. Confocal imaging of *P. omnivora* interactions with *M. truncatula* roots revealed that the mycelia do not form any specialized structures for penetration and mainly colonize cortical cells and, eventually, form a mycelial mantle covering the root's surfaces. Expression profiling of *M. truncatula* roots infected by *P. omnivora* identified several upregulated genes, including the pathogenesis-related class I and class IV chitinases and genes involved in reactive oxygen species generation and phytohormone (jasmonic acid and ethylene) signaling. Genes involved in flavonoid biosynthesis were induced (2.5- to 10-fold over mock-inoculated controls) at 3 days postinoculation (dpi) in response to fungal penetration. However, the expression levels of flavonoid biosynthesis genes returned to the basal levels with the progress of the disease at 5 dpi. These transcriptome results, confirmed by real-time quantitative polymerase chain reaction analyses, showed that *P. omnivora* apparently evades induced host defenses and may downregulate phytochemical defenses at later stages of infection to favor pathogenesis.

Additional keywords: cotton root rot, necrotrophic, Pezizomycetes.

Phymatotrichum root rot (PRR) or cotton root rot is one of most destructive diseases of cotton (*Gossypium* spp.). PRR is

caused by a soilborne fungus, *Phymatotrichopsis omnivora* (Duggar), resulting in significant economic losses every year in the United States. Hennebert (1973) named the fungus as *P. omnivora* (Duggar) Hennebert to emphasize its morphological affinity to *Botrytis*-like ascomycetes. However, phylogenetic trees constructed based on the nuclear ribosomal DNA and RNA polymerase II subunit 2 (RPB2) indicate that *P. omnivora* should be placed in the class Pezizomycetes (operculate discomycetes) within the Ascomycota (Marek et al. in press).

P. omnivora has a very broad host range and infects almost 2,000 dicotyledonous species; however, interestingly, it does not cause disease on monocotyledonous plant species, including maize and sorghum. Furthermore, the disease is more prevalent on plants grown in alkaline and calcareous soils that rarely freeze, restricting the geographic distribution of the fungus to the southwestern United States and northern Mexico (Percy 1983). Since the first report of this pathogen by Pammel (1888), various researchers have studied the biology, epidemiology, and control (chemical, biological, and genetic) of this disease. Although several management practices are used to help reduce the occurrence and severity of this disease, none are highly cost-effective and PRR remains one of the most destructive diseases of cotton, alfalfa, bean, peanut, sweet potato, ornamental shrubs, and several tree species (Lyda 1978; Lyda and Kenerly 1992). Alfalfa (*Medicago sativa* L.) is the most important forage legume in the world, known as “queen of forages” because of its high protein, vitamins, energy, and digestibility. Alfalfa is produced on approximately 162 million hectares in Oklahoma and generates more than \$100 million annually. However, producers in southern Oklahoma and Texas are reluctant to grow alfalfa due, in part, to the persistence of *P. omnivora* in soils.

Despite the extensive research performed on this fungus over the past 100 years, several aspects of the physiological and molecular biology of the disease remains poorly understood. To date, little is known about the cytology of the infection process and almost nothing is known about the molecular processes that occur during *P. omnivora*–host interactions. Also, we know very little about the molecular mechanisms that contribute to the broad host range of *P. omnivora*. More importantly for disease management, no genetic resistance to PRR has been reported. Knowledge about the mechanisms of pathogenesis, including host (alfalfa or cotton) molecular responses to PRR infection, is important to engineer plants to confer

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PRR resistance. The relative genetic intractability of cotton and alfalfa (*M. sativa*) as pathosystem hosts for *P. omnivora* precludes most genomic approaches. The model host plant *M. truncatula* is closely related to alfalfa and, unlike alfalfa, which is a tetraploid and obligate outcrossing species, *M. truncatula* has a simple diploid genome (two sets of eight chromosomes) and can be self-pollinated. *M. truncatula* is fast emerging as a model legume because of its small genome, which is almost completely sequenced (Young et al. 2005); fast generation time; amenability to transformation; availability of Affymetrix gene chips; availability of the Medicago Gene Atlas (Benedito et al. 2008); presence of numerous ecotypes; and availability of ethyl methanesulfonate, fast-neutron, and insertional mutants (Tadege et al. 2005, 2008).

In the present study, we describe the pathology of PRR in alfalfa fields. Furthermore, the model legume *M. truncatula* which is closely related to alfalfa was used to develop an *M. truncatula*–*P. omnivora* pathosystem to investigate PRR disease development. Confocal imaging of *P. omnivora* infection of *M. truncatula* roots revealed characteristic interactions similar to those seen in cultivated alfalfa or cotton. Expression profiling of PRR-infected *M. truncatula* roots using Affymetrix chips identified several differentially expressed genes belonging to different metabolic pathways and suggested a necrotrophic life style for the PRR fungus. To our knowledge, this study presents the first molecular characterization of PRR–host interactions. The PRR-regulated genes identified in this study will help us better understand the infection process and assist efforts to develop PRR-resistant alfalfa.

RESULTS AND DISCUSSION

Field and laboratory observations of PRR in alfalfa.

P. omnivora has been shown to infect alfalfa in artificial inoculations (Streets 1937). Here, we describe the PRR disease

in alfalfa fields (Fig. 1A). PRR disease symptoms in alfalfa fields are most conspicuous during summer when the infected plants suddenly wilt and die in circular infection loci (Fig. 1A). We isolated a virulent strain of *P. omnivora*, confirmed the identity using polymerase chain reaction (PCR) with specific internal transcribed spacer (ITS) primers (data not shown), and designated it as “OKAlf8.”

During its life cycle, *P. omnivora* forms several types of differentiated hyphae. Initially, germ tubes erupt from sclerotia (Supplementary Fig. S1E), the soilborne resting structures that act as the primary inoculum in affected fields. Individual hyphae will either make contact with a host root, leading to infection, or eventually form multihyphal (“mycelial”) structures with a differentiated rind (Rogers and Watkins 1938). Upon contact with the roots, *P. omnivora* forms a mycelial mantle on the root’s surface (Fig. 1B). The epidermis and underlying cortical tissue is killed, resulting in lesions. As the disease progresses, the roots are covered by the characteristic cinnamon-colored mycelial strands covered with acircular sterile hyphae (Fig. 1C), a diagnostic sign of PRR. The roots at later stages of infection show extensive vascular discoloration distal to root necroses. The mycelial strand and symptom development in field-infected roots are generally more conspicuous on cotton. The strands formed on the root surfaces or in the soil form sclerotia, thus completing the life cycle (Fig. 1B and C).

Development

of *M. truncatula*–*P. omnivora* model pathosystem.

Inoculation of 4-week-old axenic seedlings of *M. truncatula* with *P. omnivora* resulted in reproducible disease symptoms among different biological replicates, and symptom development was similar to that seen in field-infected alfalfa or cotton (Fig. 2; Supplementary Fig. S2). With the progress of the disease, aerial parts, including leaves, showed characteristic chlorotic streaks (Fig. 2A and B). The development of leaf chlorosis and vascular discoloration suggest that *P. omnivora* may produce a translocated phytotoxin. The fungus did not infect the aerial parts though, occasionally, necrotic symptoms were seen on the cotyledonary leaves (Fig. 2B). As the disease progressed, necrotic lesions were seen on the root (≈ 7 days postinoculation [dpi]) (Fig. 2H). Infected roots showed browning and typical necrotic lesions at the site of hyphal penetration (Fig. 2H). Furthermore, roots of 4-week-old seedlings of *M. truncatula* and alfalfa infected in Houston black soil also showed necrotic symptoms similar to the agar-grown plants (data not shown). These results suggest that *P. omnivora* is pathogenic on *M. truncatula* (Fig. 2) as well as alfalfa seedlings.

The susceptibility of host plant seedlings to PRR has been debated with respect to Cotton. Henderson (1937) examined infection on cotton seedling roots by *P. omnivora* in aseptic culture and concluded that cotton seedlings were as susceptible as mature cotton plants. However, Blank (1940) observed that cotton seedlings escaped infection by *P. omnivora* in a field study. Consistent with these observations, disease foci in cotton fields tend to occur late in the cotton growing season, which has been suggested to indicate that roots of young seedlings are immune to *P. omnivora*.

Cell biology of infection process in seedlings.

Before designing the expression-profiling experiments aimed at identification of transcript signatures associated with a particular stage of pathogenesis, the successful invasion of host roots by *P. omnivora* were followed using light microscopy and confocal laser-scanning microscopy (CLSM) at different time intervals postinoculation. To capture initial interactions in a noninvasive fashion, 4-week-old *M. truncatula* seedlings

Fig. 1. A, Circular disease foci of *Phymatotrichum root rot* in alfalfa field; **B**, mature mycelial strands (arrows) of *Phymatotrichopsis omnivora* on the root of wilted alfalfa plant; and **C**, acircular and cruciform hyphae (arrows) on mycelial strand.

grown axenically on agar were inoculated with *P. omnivora*-colonized wheat seed (one per seedling) in the near proximity of the tap root (Fig. 2A and B; Supplementary Fig. S6B). The observations using an inverted microscope equipped with Nomarski interference optics showed that the fungal hyphae were observed to initially contact the root surface between 20 and 24 h postinoculation (hpi). Upon contact, hyphae grew

Fig. 2. Stages of *Medicago truncatula* root colonization by *Phymatotrichopsis omnivora* and symptom development. Aerial view of **A**, *M. truncatula* seedlings grown in tissue culture containers and mock inoculated with a wheat seed (arrow) or **B**, with wheat seed colonized by *P. omnivora*. *P. omnivora*-inoculated seedling (aerial) parts including leaves showed characteristic chlorotic streaks (arrow, boxed panel) and, occasionally, the fungus colonized cotyledonary leaves (arrow head). **C**, Fungal hyphae in contact with the root surface and branching along the epidermal cells upon initial contact, 2 days postinoculation (dpi) (arrows). In some cases, epidermal nucleus was located close to the growing hyphal tip (arrow head). **D**, Confocal fluorescence images of WGA-Alexa Flour 488-stained fungal hyphae showing colonization and entry of hyphae between the junctions of epidermal cells, 3 dpi. **E**, Root sections showing WGA-Alexa Flour 488-stained fungal mycelia growing in the intercellular spaces of the cortical cells, 5 dpi. Plant cell walls were stained with calcofluor white. **F**, A z-series projection of infected root. A series of optical sections (z series) were acquired by scanning multiple sections of the image shown in **E** and stacked to obtain a three-dimensional view. **G**, Mycelial mantle covering the root's surfaces (arrows) and hyphae growing in the root epidermis, 7 dpi. **H**, *Phymatotrichum* root rot symptom and necrotic lesion development, 7 dpi. Scale bar = 50 µm.

parallel along the longitudinal groove between the root's epidermal cells and then grew perpendicular and centripetally into the junctions between the epidermal cells (Fig. 2C). Furthermore, we observed that the plant epidermal cell nucleus was located adjacent to the cell wall proximal to hyphal tips (Fig. 2C, arrow head).

Genre et al. (2005), using real-time in vivo imaging, demonstrated that, during the symbiotic interaction of arbuscular mycorrhizal fungi that trigger movement of the *M. truncatula* root epidermal nucleus and during the progressive movement of the nucleus, a penetration apparatus (PPA) is assembled within the epidermal cell which determines the future path taken by the infection hyphae. It is not clear whether nuclear reposition leads to PPA formation and provides any clues for hyphal positioning along the epidermis or penetration in the pathogenic *P. omnivora*-*M. truncatula* interactions (Fig. 2C).

Confocal imaging of infected roots, fluorescently stained to reveal fungal cell walls, showed that *P. omnivora* hyphae directly penetrate epidermal cells (Fig. 2D through G). Within 3 dpi, mycelia started enveloping the roots and infection hyphae penetrating the epidermal cells were noticed (Fig. 2D). Although no macroscopic necrosis was observed at 3 dpi, when examined closely, tiny black lesions were observed on some of the roots (data not shown). Furthermore, no specialized penetration structures such as appressoria were observed. It appears that the fungus penetrates roots directly with simple infection hyphae that branch perpendicularly from the longitudinal hyphae covering the root's epidermis (Fig. 2D). Scanning electron micrographs of *M. sativa* (alfalfa) roots colonized by *P. omnivora* suggested that infection hyphae swell slightly prior to or during penetration, indicating that an increase in hyphal turgor pressure may be involved. The mycelial mantle began to develop multi-hyphal strands and single hyphae branching into the root penetrated the epidermis and invaded the cortex (Fig. 2E and F). Intercellular hyphae infected both epidermal and cortical cells at 5 dpi in *M. truncatula* (Fig. 2E and F) and at 7 dpi in *M. sativa*. By 5 to 7 dpi, hyphae completely covered the root's surfaces and infected epidermal cells collapsed (Fig. 2G). Macroscopically, infected roots displayed necrotic lesions (Fig. 2H).

Expression profiling of *P. omnivora*-inoculated *M. truncatula* roots.

To identify host signaling pathways triggered by *P. omnivora* infection, we used microarrays to monitor the expression profiles and the molecular process associated with initial entry (3 dpi) (Fig. 2C through E) and colonization (5 dpi) (Fig. 2G). The Affymetrix arrays contained 50,900 probe sets from *Medicago* spp., of which 32,167 are *M. truncatula* express sequence tagged- or mitochondrial RNA-based probe sets. When a two-fold cutoff relative to the gene expression in mock-inoculated tissues was used, 915 (196 of which passed stringent statistical criteria) and 692 (509 of which passed stringent statistical criteria) genes on the array belonging to different functional groups were identified as induced or repressed, respectively, at 3 dpi when compared with the mock-controls (Supplementary Tables S1 through S3). However, at 5 dpi, 3,026 (1,402 of which passed stringent statistical criteria) and 7,280 (6,487 of which passed stringent statistical criteria) genes on the array belonging to different functional groups were identified as induced or repressed, respectively. *P. omnivora*-affected *M. truncatula* genes were classified into different functional groups using the new BIN structure recently developed by Goffard and Weiller (2006, 2007) which is based on the hierarchical functional classification modeled of KEGG ontology. Furthermore, we used K-means clustering to visualize the regulatory patterns (10 clusters) of 10,435 genes in all that were differentially regulated during *P. omnivora* infection.

MAPMAN software was used to obtain an overview of *P. omnivora*-affected genes belonging to various metabolic pathways in *M. truncatula* (Fig. 3; Supplementary Figs. S3 and S4). Using automated and manual annotation, we classified genes represented in the Medicago microarray into different functional groups and assigned BIN numbers. The resulting mapping file was used to map the *P. omnivora*-affected genes onto various pathways using ImageAnnotator (as described below). Recently, MAPMAN ontology was adapted to visualize the genes belonging to the pathogen or biotic stress response in solanaceous species (Rotter et al. 2007; Uppalapati et al. 2008). In this study, the MAPMAN ontology is adapted to the *M. truncatula* genes represented on the Affymetrix array and used to visualize biotic stress response genes, thereby identifying several genes correlated with the pathogen lifestyle and suggesting pathogenesis mechanisms used by *P. omnivora* (Fig. 3).

***P. omnivora* infection induces activation of chitinase genes in *M. truncatula* roots.**

Microarray analysis revealed that, during the compatible interaction of *P. omnivora* with *M. truncatula*, several genes encoding chitinase Ib, II (Affy IDs: Mtr.22903.1.S1_at, Mtr.38714.1.S1_at, and Mtr.10332.1.S1_at), endochitinase (Mtr.44506.1.S1_x_at and Mtr.34454.1.S1_at), exo-1,3- β -glucanase (Mtr.45398.1.S1_at), and β 1-4-glucanase (Mtr.1496.1.S1_at) were induced in *M. truncatula* roots. To further confirm these results, we designed primers specific for different *M. truncatula* chitinase gene family members (Salzer et al. 2000) and confirmed the expression of chitinase genes in an independent biological sample by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). Consistent with the microarray results, expression of genes encoding chitinase genes (*Chi-I* and *Chi-IV*) was induced as early as 2 dpi (Fig. 4).

Chitinases catalyze the hydrolysis of chitin oligomers and are generally induced in response to fungal pathogens or abiotic stress, or during plant development (Collinge et al. 1993). Chitinase enzymes are grouped into five different classes (I to IV) based on their amino acid sequences and protein primary structures (Beintema 1994; Collinge et al. 1993). Plant chitinases have been implicated contradictorily as either important or immaterial to plants' resistance to fungi. It is shown that overexpression of a combination of chitinases and β 1-4-glucanase results in fungal resistance in tobacco, potato, and wheat (Bieri et al. 2003; Jach et al. 1995; Lorito et al. 1998). On the other hand, several studies have also demonstrated differential expression of chitinase family members during compatible plant–fungal interactions (Collinge et al. 1993; Salzer et al. 2000). Several of the chitinases are classified as pathogenesis-related (PR) proteins (van Loon et al. 2006). Salicylic acid (SA)-dependent induction of PR proteins PR-1, PR-2, and PR-5 is well demonstrated in *Arabidopsis* and other plant species; however, in general, several chitinases are known to be jasmonate (JA) or ethylene (ET)-inducible in *Arabidopsis* (Thomma et al. 2001; van Loon et al. 2006). The specific induction of different PR proteins, including chitinases to SA or JA/ET in *M. truncatula*, is not well studied; therefore, the possible involvement of SA in induction of PRR-

inducible chitinases cannot be ruled out (Fig. 4). Chitinase gene expression may be induced in *M. truncatula* roots due to recognition of elicitors from *P. omnivora* such as chitoooligosaccharides, or pathogenesis-induced ET (discussed below).

Differential expression of JA, ET, and reactive oxygen species-related genes during infection.

Several genes involved in jasmonate (JA) biosynthesis and JA- or wound-responsive genes were induced in response to initial entry of the fungal hyphae as early as 3 dpi. These genes included lipoxygenases, allene oxide cyclase (*AOC2*), oxophytodienoate reductase (*OPR3*, *OPR-5*, and *OPR-12*), and wound-inducible serine proteinase inhibitors (*PII*) (Figs. 3 and 5). To validate the expression results obtained using microarrays and confirm our results for a subset of genes, we designed gene-specific primers from the available full-length cDNAs and performed real-time quantitative RT-PCR (RT-qPCR). In general, RT-qPCR analyses corroborated the microarray expression data. *P. omnivora* infection strongly induced the expression of *AOS2* and *OPR3*, the key enzymes in the JA biosynthetic pathway (Fig. 5). JA-regulated genes (*PI-II*, *AP2/ERF*, *NAC*, and *MYB* gene family members) were also strongly induced early in the interaction. *P. omnivora* also induced genes involved in ET biosynthesis (Fig. 6) and ET-responsive transcription factors (*AP2/ERF* family members) (Supplementary Fig. S5). The

Fig. 4. Relative changes in chitinase genes (*Chi-I* and *Chi-IV*) upon *Phymatotrichopsis omnivora* infection in *Medicago truncatula* roots at 2 and 3 days postinoculation (dpi) over the mock controls. Transcriptional changes were analyzed using semiquantitative reverse-transcription polymerase chain reaction. The *Actin* gene was used as an internal control.

Fig. 3. MAPMAN illustration of *Medicago truncatula* Affymetrix data showing the genes involved in biotic stress responses during *Phymatotrichopsis omnivora*–*M. truncatula* interactions at **A**, 3 and **B**, 5 days postinoculation (dpi). Genes that are numbered include 1, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (Affy ID: Mtr.25950.1.S1_at); 2, ACC synthase (Affy ID: Mtr.42480.1.S1_at); 3, ethylene response factor-like AP2 domain transcription factor (Affy ID: Mtr.11269.1.S1_at); 4, AP2/EREBP (Affy ID: Mtr.20232.1.S1); 5, lipoxygenase (Affy ID: Mtr.8439.1.S1_s_at); 6, 12-oxo-PDA-reductase (Affy ID: Mtr.318.1.S1_at); 7, cystatin (Affy ID: Mtr.35607.1.S1_at); 8, auxin binding protein-1 (Affy ID: Mtr.6144.1.S1_at); 9, UDP-glucuronosyl/UDP-glucosyltransferase (Affy ID: Mtr.21701.1.S1_s_at); and 10, thiohydroximate S-glucosyltransferase (Affy ID: Mtr.12921.1.S1_at). Red indicates upregulation and blue indicates downregulation.

expression of 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*) and ethylene response factor (*ERF*) were further confirmed by RT-qPCR using gene-specific primers (Fig. 6). Furthermore, several genes involved in generation or homeostasis of reactive oxygen species (ROS), including thioredoxin, glutathione synthetase (*GSHS1*), anionic peroxidase, cationic peroxidase II and III, haem peroxidase, and several glutathione S-transferases, were induced during infection (Fig. 3).

In *Arabidopsis*, JA- and ET-mediated defense responses are implicated in defense against necrotrophic fungal pathogens, including *Fusarium oxysporum* (Glazebrook 2001; McDowell and Dangl 2000). Our results show that JA- or ET-dependent pathways are activated in response to *P. omnivora* infection

and suggest the hemibiotrophic or necrotrophic nature of *P. omnivora*. Cell death triggered by ROS at the interface of necrotrophic fungus–plant interactions has been shown to be required for disease susceptibility (Govrin and Levine 2000; Hano et al. 2008; Okubara and Paulitz 2005). Therefore, we propose that ROS, in conjunction with *P. omnivora*-induced ET, play a role in development of visible necrotrophic symptoms (Fig. 2H). Our results also suggest that *P. omnivora* may produce a toxin-like virulence factor similar to necrosis and ET-inducing peptides characterized in other fungal pathogens such as *F. oxysporum* (Bae et al. 2006), *Botrytis* spp. (Staats et al. 2007), *Pythium aphanidermatum*, and *Phytophthora* spp. (Qutob et al. 2006) during compatible-necrotrophic interactions.

Fig. 5. Jasmonic acid (JA) signaling is altered early during *Phymatotrichopsis omnivora*–*Medicago truncatula* interactions. **A**, Overview of the JA biosynthetic pathway. **B**, Relative changes in JA-pathway genes upon *P. omnivora* infection in *M. truncatula* roots at 3 and 5 days postinoculation (dpi) over the mock controls (MC). Transcriptional changes were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). The expression data are presented for allene oxide synthase (*AOS2*), oxophytodienoate reductase (*OPR12*), and JA-inducible proteinase inhibitor II (*PI-II*). The values represent the average of three independent biological replicates. The *Ubiquitin* gene was used as an internal control.

Phymatotrichopsis omnivora reduces induction of phytochemical defenses at late stages of infection.

Several genes encoding proteins associated with secondary metabolism and cell-wall modification were differentially expressed during infection (Figs. 3 and 7). Interestingly, genes involved in early steps of phenylpropanoid metabolism were induced during both early and later stages of infection. RT-qPCR analyses of a set of genes including *PAL*, *4CL*, *CHS*, *CHR*, and *CHI* further confirmed these results (Fig. 7). In contrast, genes involved in isoflavonoid and dihydroflavonol biosynthesis (*IFS*, *IFR*, *FS*, isoflavonoid glucosyltransferase, and *F3H*) were induced only during the early stage of infection and then declined to the levels of mock-inoculated plants at the later stage of infection (Fig. 7). These transcriptome results, confirmed by RT-qPCR analyses, suggest that *P. omnivora* apparently evades induced host defenses and may prevent induction of phytochemical defenses at later stages of infection.

Flavonoids and isoflavonoids are known to play a significant role in plant defense responses to fungal and bacterial pathogens (Blount et al. 1992; Dixon and Steele 1999; Harborne 1977; Tahara 2007) and as inhibitors of fungal growth in vitro (Bhattacharyya and Ward 1985; Lozovaya et al. 2004). Some fungi, including soilborne *Nectria haematococca* and *F. solani*, have the ability to detoxify or modify the isoflavonoid compounds to a less toxic derivative (Pedras and Ahiaonu 2005; VanEtten et al. 1989). Our transcriptome analyses clearly suggest that *P. omnivora* evades detection by or prevents upregulation of (iso)flavonoid metabolism to favor pathogenesis.

Although detoxification or modification of phytoalexins is linked to virulence or pathogenicity in many pathosystems (Pedras and Ahiaonu 2005; VanEtten et al. 1989), suppression of phytoalexin defense by a virulence factor has been shown to be one of the mechanisms adapted by *Mycosphaerella pinodes* to successfully infect pea plants (Uppalapati et al. 2004). Therefore, it is possible that *P. omnivora* produces effectors that negatively regulate phytoalexin biosynthesis in alfalfa. The ongoing genome sequencing project of *P. omnivora* might shed new light on genes possibly involved in detoxification, modification, or suppression of isoflavonoid biosynthesis. Availability of alfalfa or cotton plants with increased isoflavonoid contents should help us to better understand the role of these compounds in defense against *P. omnivora*.

Conclusions.

In the present study, we describe the pathology of PRR in alfalfa fields and in the model legume plant *Medicago truncatula*, which is closely related to alfalfa. We developed a model pathosystem to investigate PRR disease development. Confocal imaging of *P. omnivora* infection of *M. truncatula* roots revealed characteristic interactions similar to those seen in cultivated alfalfa or cotton. Expression profiling of PRR-infected roots using Affymetrix chips identified several genes belonging to different metabolic pathways and suggested a necrotrophic life style for the PRR fungus and provided clues to the mechanisms of pathogenesis. To our knowledge, this study presents a first molecular characterization of PRR–host interactions. The PRR-regulated genes identified in this study should help us to better understand the infection process and assist in efforts to develop PRR-resistant alfalfa cultivars.

Interpretation of the changes in transcript profiles during compatible interaction is complicated due to the fact that the transcript changes are associated with both general plant responses to the pathogen and changes triggered by the effectors of *P. omnivora*. One outcome of the present study is the identification of gene sets that were suppressed during infection; supporting the notion that *P. omnivora* evades host defense responses during infection (Figs. 3 and 7). Experiments are underway to further analyze *P. omnivora*- and plant-responsive genes and the role of isoflavonoids in pathogenesis. The integration of recent “omic” approaches with traditional physiology and biochemistry should provide new insights into this century-old disease and help with the development of improved disease management strategies and resistant germplasm.

MATERIALS AND METHODS

Isolation of *P. omnivora*

from field-infected alfalfa roots and inoculum preparation.

Strains of *P. omnivora* were isolated from PRR-affected alfalfa fields (near Belleville, OK) in 2003. *P. omnivora*-infected alfalfa roots were washed with running tap water and small sections of root tissues were dissected from the lesions, submerged in 0.525% sodium hypochlorite for 1 min, and rinsed twice with sterile distilled water. The surface-sterilized sections of root tissues were placed on 1.8% water agar containing chloramphenicol (100 mg/liter; Amresco, Inc., Solon,

Fig. 6. Relative changes in ethylene-pathway genes upon *Phymatotrichopsis omnivora* infection in *Medicago truncatula* roots at 3 and 5 days postinoculation (dpi) over the mock controls (MC). Transcriptional changes were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). The expression data are presented for 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*) and ethylene response factor (*ERF*). The values represent the average of three independent biological replicates. The *Ubiquitin* gene was used as an internal control.

OH, U.S.A.) and penicillin G (500 mg/liter; Sigma-Aldrich, St. Louis). Characteristic hyphae of *P. omnivora* growing from root segments were transferred to potato dextrose agar (PDA) (Becton Dickinson Co., Sparks, MD, U.S.A.) and cultures were maintained on PDA or modified ATCC medium 1078 (M1078, containing 1 g of NH_4NO_3 , 0.75 g of MgSO_4 , 0.4 g of KH_2PO_4 , 0.9 g of K_2HPO_4 , 0.1 g of CaCl_2 , 40 g of glucose, 1 g of yeast extract, 1 g of peptone, 100 μl of Vogel's trace elements [Vogel 1964], and 18 g of agar per 1,000 ml of distilled water) and incubated at 28°C. One isolated strain was designated OKAlf8 and was used for all experiments.

Inoculum of *P. omnivora* OKAlf8 for agar-based assays was developed in wheat seed (*Triticum aestivum*). Wheat seed (50 g; Red River Grain Co., Kingston, OK, U.S.A.) was soaked overnight in 50 ml of distilled water and autoclaved in a cotton-plugged wide-mouth glass conical flask. The autoclaved seed were incubated for another 12 h at room temperature, autoclaved for a second cycle (30 min at 121°C), and allowed to cool at room temperature. Two agar plugs (1 cm in diameter) from actively growing cultures of *P. omnivora* were used to inoculate sterile wheat seed and incubated for 10 to 12 days at 28°C in dark to allow the mycelia to completely colonize the seed.

Fig. 7. Overview of phenylpropanoid pathway leading to flavonoid biosynthesis (boxed panel) and expression profiles of selected genes involved in these pathways during *Phymatotrichopsis omnivora*–*Medicago truncatula* interactions. The selected intermediates, end products, and enzymes shown in the pathway include PAL, phenyl alanine lyase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; IFS, isoflavone synthase; IFR, isoflavone reductase; FS, flavanone synthase; and FLS, flavonol synthase. Real-time quantitative polymerase chain reaction (RT-qPCR) expression data are presented for PAL, CHS, CHR, CHI, IFS, IFR, and FS. Relative changes in gene expression upon *P. omnivora* infection in *M. truncatula* roots at 3 and 5 days postinoculation (dpi) over the mock controls (MC) were analyzed using microarrays or RT-qPCR. The *Ubiquitin* gene was used as an internal control. The values represent the average of three independent biological replicates.

Plant material and pathogen infection assays.

Seed of *M. truncatula* cv. *Jemalong* A17 were scarified for 8 min using concentrated sulfuric acid, washed three times with distilled water, and surface sterilized for 15 min in 20% (vol/vol) commercial bleach containing 6% sodium hypochlorite (Clorox Co., Oakland, CA, U.S.A.). Surface-sterilized seed were washed three times with sterile distilled water and germinated on half-strength Murashige and Skoog (MS) medium (0.2% phytagel) (Sigma-Aldrich). Two days after germination in darkness at 24°C, seedlings (usually with 2- to 3-cm-long hypocotyls) were transferred to plant tissue culture containers (58 cm in diameter by 100 cm in height; Greiner Bio-One N.A. Inc., Monroe, NC, U.S.A.) containing half-strength MS medium (0.2% phytagel) with Gamborg vitamins (PhytoTechnologies Laboratories, Shawnee Mission, KS, U.S.A.) and 1% sucrose, pH 5.6 to 5.8, and were maintained in growth chambers (24°C, 40 to 70% relative humidity [RH], 12-h photoperiod, photon flux density 150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Inoculation assays were conducted on 4-week-old plants. To inoculate *M. truncatula* seedlings, a single wheat seed colonized by *P. omnivora* was placed near the main root, at the interface of the root and shoot. One sterile wheat seed was used to mock inoculate a control seedling. The inoculated seedlings were transferred to growth chambers (26°C, 40% RH, 12-h photoperiod, photon flux density 150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and symptoms were monitored at different time intervals postinoculation.

Light and confocal microscopy.

Initial interactions of *P. omnivora* with *M. truncatula* roots were recorded by direct observations of inoculated seedlings grown on agar in petri dishes with cover glass bottom for direct observations using an inverted Nikon TE 3000 microscope equipped with Nomarski interference optics capable of time-lapse video recordings. For fluorescence microscopy, fungal mycelia were stained with wheat germ agglutinin (WGA), coupled to green fluorescent dye Alexa Fluor 488 (WGA-Alexa Fluor 488; Invitrogen Corp., Carlsbad, CA, U.S.A.), whereas the roots cell membranes were visualized by counterstaining the epidermal plasma membranes of roots with the vital lipophilic fluorescent dye FM 4-64 (Invitrogen Corp.) or by staining the root cell walls with fluorescence brightener 28 (Sigma-Aldrich). Inoculated and control 4-week-old *M. truncatula* seedlings at different time intervals postinoculation were carefully removed from agar, and the whole roots were stained with 8.5 μM FM 4-64 and WGA-Alexa Fluor 488 at 10 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS) for 20 min at room temperature. For microscopic observations, after washing with PBS buffer, whole roots or sections of the roots were placed on a glass slide and mounted in PBS using a cover glass with high-vacuum grease (Dow Corning, Midland, MI, U.S.A.) for microscopy. A chamber was created to hold the whole roots on the slide glass using Parafilm.

For microscopic observations of fungal colonization of the root cortical or vascular tissues, 4- to 5-mm sections of infected roots were flash frozen in liquid nitrogen, and 20- μm transverse sections were cut using a Leica CM 1850 cryomicrotome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Root sections were transferred onto a slide glass and were immediately stained with 0.01% Fluorescence Brightener 28 and 10 WGA-Alexa Fluor 488 at 10 $\mu\text{g}/\text{ml}$ in PBS for 10 min at room temperature, washed with PBS, and mounted using a cover glass with Dow Corning high-vacuum grease for microscopy.

Fluorescence microscopy to document the infection process was done using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with $\times 20$ (numerical aperture,

0.70) and $\times 63$ (numerical aperture, 1.2) objectives using appropriate laser and excitation filter settings (WGA-Alexa Fluor, 488-488 nm; FM 4-64-543 nm; Fluorescence Brightener 28-405 nm; blue diode laser). Series of optical sections (z series) were acquired by scanning multiple sections, and the z -series projections were generated with the software provided with the Leica TCS SP2 AOBS CLSM.

Scanning electron microscopy.

For scanning electron microscopy examination, infected roots of alfalfa seedlings were pretreated 20 min in a 2.3% thiocarbonylhydrazide aqueous solution, washed three times for 20 min each with sterile water, fixed in 1% aqueous osmium tetroxide for 30 min, and washed three times for 20 min each with sterile water (Postek and Tucker 1977). Specimens were dehydrated through a graded series of 20 to 100% ethanol; in some cases, freeze fractured in liquid nitrogen; and then critical point dried in a Denton DCP1 dryer for 4 h. Dried specimens were then coated with gold-palladium in a Balzer MED 010 sputter coater and examined in a JEOL JXM 6400 scanning electron microscope.

RNA extraction and microarray experimental design and analyses.

Total RNA was purified from mock-inoculated control roots and roots inoculated with *P. omnivora* using TRIzol Reagent (Invitrogen Corp.) according to the manufacturer's instructions. Total RNA was extracted from three independent seedlings per treatment and, following isolation, the total RNAs were pooled to represent one biological replicate. Three independent experiments were carried out on different days to represent three biological replicates. Prior to microarray analyses, the integrity of the RNA was checked on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, U.S.A.). For each sample, 10 μg of total RNA was used as template for amplification. Probe labeling, chip hybridization, and scanning were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, U.S.A.). Three biological replicates per treatment were hybridized independently to the Affymetrix GeneChip Medicago Genome Array representing 50,900 *M. truncatula* genes. The .CEL data file for each sample was exported from Genechip Operating System (GCOS) program (Affymetrix). All the raw data files were then imported into Robust Multi-chip Average software (RMA) and normalized as described by Irizarry and associates (2003). The presence or absence call for each probe set was obtained from dCHIP (Li and Wong, 2001). Differentially expressed genes between sample pairs were selected using Associative Analyses as described by Dozmorov and Centola (2003). Type I family-wise error rate was reduced by using a Bonferroni-corrected P value threshold of $0.05/N$, where N represents the number of genes present on the chip. The false discovery rate was monitored and controlled by calculating the Q value (false discovery rate) using extraction of differential gene expression (EDGE) (Leek et al. 2006; Storey and Tibshirani 2003). Genes that showed significant differences in transcript levels (twofold or greater and P value $< 9.82318\text{E-}07$) between sample pairs were selected for pathway construction using MAPMAN. Several genes that failed to pass through these stringent criteria but were truly up- or downregulated based on the presence calls were also considered. However, information about the standard deviation, P values, and stringent statistical cutoff values are presented for each gene. We have utilized Spotfire software to visualize the regulatory patterns (10 clusters) of a total 10402 elements that were differentially regulated during *P. omnivora* infection using K-means Clustering. All the data will be submitted to ArrayExpress.

Semiquantitative RT-PCR and RT-qPCR analyses.

Total RNA was purified from *M. truncatula* roots inoculated with autoclaved wheat seed (control) or inoculated with wheat seed infested with *P. omnivora* as described above. Semiquantitative RT-PCR was conducted with cDNA generated using methods described previously (Uppalapati et al. 2008). For semiquantitative RT-PCR, 1 µl of first-strand cDNA (diluted 1:50 in reaction buffer) was amplified using *Taq* DNA polymerase (Promega Corp., Madison, WI, U.S.A.). To check for equal amounts of cDNA in each reaction, PCR was performed with primers specific for the gene encoding Actin. The gene-specific primers used for semiquantitative RT-PCR for chitinases (ChiIV and ChiI) were previously described by Salzer and associates (2000) and are presented in Supplementary Table S4.

RT-qPCR was performed using the gene-specific primers designed based on the target sequence or those described previously (Gao et al. 2007). Total RNA was treated with Turbo DNase (Ambion, Austin, TX, U.S.A.) to eliminate genomic DNA, and 5 µg of DNase-treated RNA was reverse transcribed using Superscript III⁺ reverse transcriptase (Invitrogen Corp.) with oligo d(T)₂₀ primers. The cDNA (1:10) was then used for RT-qPCR. RT-qPCR was performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.) in an optical 384-well plate with an ABI Prism 7900 HT sequence detection system (Applied Biosystems). Melt-curve analysis was performed to monitor primer-dimer formation and to check amplification of gene-specific products. The average threshold cycle (C_T) values calculated from triplicate biological samples were used to determine the fold expression relative to the controls. Primers specific for ubiquitin were used to normalize small differences in template amounts. Data analysis, including normalization and standard deviations, were calculated using Gene Expression Macro (version 1.1; Bio-Rad, Hercules, CA, U.S.A.) with the algorithms described by Vandesompele and associates (2002). Data quantification and analysis was performed using iCycler software (version 3.06.6070) and Gene Expression Macro (version 1.1) (Bio-Rad). The average C_T values calculated from triplicate samples were used to determine the fold expression relative to the controls. Primers specific for ubiquitin were used to normalize small differences in template amounts.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Medicago truncatula Sequencing Resources website:
www.medicago.org/genome/
 University of Oklahoma Advanced Center for Genome Technology
P. omnivora and *E. festucae* Genomic Sequencing website:
www.genome.ou.edu/fungi.html
 University of Washington Department of Biostatistics EDGE software:
www.biostat.washington.edu/software/jstorey/edge
 ArrayExpress database: www.ebi.ac.uk/microarray-as/ae