Cell Host & Microbe
Article

The Human Fungal Pathogen *Cryptococcus* Can Complete Its Sexual Cycle during a Pathogenic Association with Plants

Chaoyang Xue,[1] Yasuomi Tada,[2] Xinnian Dong,[2] and Joseph Heitman[1,*]
[1] Department of Molecular Genetics and Microbiology
[2] Department of Biology
Duke University, Durham, NC 27710, USA
*Correspondence: heitm001@duke.edu
DOI 10.1016/j.chom.2007.05.005

SUMMARY

Cryptococcus **is a globally distributed human fungal pathogen that primarily afflicts immunocompromised individuals. How and why this human fungal pathogen associates with plants and how this environmental niche influences its life cycle remains a mystery. We established *Cryptococcus-Arabidopsis* and *Cryptococcus-Eucalyptus* systems and discovered that *Cryptococcus* proliferates and mates on plant surfaces. Mating efficiency of *C. gattii* was markedly enhanced on plants and myo-inositol and indole acetic acid were specific plant products that stimulated mating. On *Arabidopsis*, dwarfing and chlorosis were observed following infection with a fungal mixture of two opposite mating-type strains, but not with either mating-type alone. This infection process is countered by the plant jasmonate-mediated defense mechanism. These findings reveal that *Cryptococcus* can parasitically interact with plants to complete its sexual cycle, which may impact an understanding of the origin and evolution of both plant and animal fungal pathogens in nature.**

INTRODUCTION

A wide array of interactions occurs between plants, animals, and microorganisms in nature. Some of these relationships are characterized by close physical associations among species that persist for a significant period of the life cycle. These complex symbiotic interactions can be further distinguished into several categories: mutualism (both members benefit), commensalism (one species benefits, the other one does not), neutralism (no apparent benefit or harm to either), and parasitism (one survives at the expense of the other). Understanding the complex interactions between microbes and natural hosts, especially parasitic interactions, is a primary focus of microbiological studies.

Cryptococcus neoformans and *C. gattii* are closely related dimorphic human fungal pathogens that cause cryptococcosis, a severe life-threatening meningoencephalitis. The medical importance of *C. neoformans* has increased dramatically as a consequence of the acquired immune deficiency syndrome (AIDS) epidemic since the early 1980s (Casadevall and Perfect, 1998). The rarity of human-to-human transmission, and the prevalence of *Cryptococcus* species in the environment indicate that human infection is likely acquired from environmental sources (Casadevall and Perfect, 1998).

Cryptococcus infection is acquired by inhalation of basidiospores or dessicated yeast cells as the suspected infectious propagule. Mating between isolates of the same mating type has been observed in this fungus and may explain why the α mating type predominates in the environment (Fraser et al., 2005; Lin et al., 2005). Nevertheless, both α and **a** mating-type strains can be isolated from nature, and, in some areas, the ratio of the two mating types is close to 1:1 (Halliday et al., 1999), indicating that heterosexual mating likely also occurs in the environment. Mating in association with environmental niches may serve as a selective force to maintain both mating types in nature. However, mating of this pathogen has not been observed in nature and thus, how infectious spores might be produced has been unresolved.

Cryptococcus is distributed worldwide in association with a range of plant species (Chen et al., 2000; Pfeiffer and Ellis, 1992; Sorrell et al., 1996a). The major environmental sources of this fungus are soil contaminated with pigeon guano (*C. neoformans* var. *neoformans* and var. *grubii*) or *Eucalyptus* trees and decaying wood (*C. gattii*). *C. gattii* is endemic in tropical and subtropical regions and primarily infects immunocompetent hosts, mostly in Australia and Papua New Guinea (Casadevall and Perfect, 1998), and has emerged recently in Canada following the Vancouver Island outbreak (Fraser et al., 2005; Fraser et al., 2003; Kidd et al., 2004). Environmental isolations have established that *C. gattii* has a specific ecological association with *Eucalyptus camaldulensis* and *E. tereticornis* (Campisi et al., 2003; Ellis and Pfeiffer, 1990; Granados and Castaneda, 2005; Sorrell et al., 1996a). The

global distribution of these trees appears to correspond to the epidemiological distribution of cryptococcosis caused by *C. gattii* (Casadevall and Perfect, 1998). *Eucalyptus* is not the only environmental niche as *C. gattii* has also been isolated from other tree species (Fortes et al., 2001; Huerfano et al., 2001; Lazera et al., 1998) and from geographic regions where *Eucalyptus* trees are not present (Campbell et al., 2005a). *C. neoformans* var. *grubii* and var. *neoformans* have also been isolated from a variety of plant species (Lazera et al., 1996; Randhawa et al., 2005). These associations between *C. neoformans* and plants suggest that this fungal species may also have an arboreal niche (Sorrell et al., 1996a). Molecular studies on this pathogen are well established following the completion of the *C. neoformans* genome sequence (Loftus et al., 2005), and this organism serves as a model to understand related pathogens of plants and animals (Idnurm et al., 2005). Yet it remains a mystery how and why this human pathogen associates with plants, how this environmental niche is involved with the fungal life cycle in nature, and how this impacts disease epidemiology worldwide.

Many plant fungal pathogens can sense plant hormones, and indole-3-acetic acid (IAA) induces invasive growth of the model yeast *Sacchromyces cerevisiae* (Prusty et al., 2004). Plant hormones could be one environmental signal that triggers morphological changes during the *Cryptococcus* life cycle in nature. Mating and recombination of *C. neoformans* have long been suspected to occur in nature (Campbell et al., 2005a), but mating or sexual spores have not been as yet observed in the environment. Because of the close association between *Cryptococcus* and plants, it will be of considerable interest to establish whether and how plant hormones or other signaling components control development of this human pathogen. One report has shown that *C. gattii* can survive on almond trees (*Terminalia catappa*) for at least 100 days (Huerfano et al., 2001). This fungus can be isolated from fermenting fruit, and sexual reproduction is supported on V8, tomato, or carrot juice media. The ability of a fungus to perceive a plant hormone or product and differentiate into an invasive form has important implications for plant-pathogen interactions.

During pathogen-plant interactions, plants activate distinct defense responses to combat microbial invasion. Induced resistance is regulated by a signaling network in which salicylic acid (SA) and jasmonic acid (JA) function as key signaling molecules (Reymond and Farmer, 1998; Spoel et al., 2003; Thomma et al., 2001). SA and JA accumulate in response to pathogen infection, resulting in the activation of distinct sets of defense-related genes, such as the pathogen-related (*PR*) gene *PR-1* or the plant defensin gene *PDF1.2* (Penninckx et al., 1996). SA- and JA-dependent pathways crosscommunicate (Felton and Korth, 2000; Spoel et al., 2003; Ton et al., 2001), providing potential to fine-tune regulatory defense reactions specific to the attacker.

Here, we established a laboratory-defined *Cryptococcus*-plant interaction system, and discovered that a potential parasitic relationship can exist between *Cryptococcus*, a human fungal pathogen, and plants. Plant-derived signals induce mating and the resulting dikaryotic filamentous form of the fungus infects plants to cause key hallmarks of infection, including chlorosis and wilting as well as induction of a plant defense response. This study provides clues as to the origin and evolution of human fungal pathogens.

RESULTS

Mating and Yeast Colony Formation on Plant Surfaces

We sought to establish a model system in which to explore the ability of *Cryptococcus* to interact with plants. To study potential interactions between *C. neoformans* and plants, both *Arabidopsis thaliana* and *Eucalyptus camaldulensis* were grown on Murashige and Skoog (MS) medium and congenic *C. neoformans* strains of opposite mating type (H99α and KN99**a**; see Table S1 in the Supplemental Data available with this article online) were inoculated as mixtures on the nodes and leaves of plant seedlings under sterile conditions. After 4 weeks of incubation, morphological development of the fungus was examined by microscopy. Mating filaments and basidial fruiting bodies were observed on both *Arabidopsis* and *Eucalyptus* leaves inoculated with a mixture of strains of opposite mating type, even under constant light conditions (Figure 1A), and cell-cell fusion of genetically marked strains was found to occur and produced a filamentous dikaryon that sporulated to produce haploid recombinant meiotic progeny (see Experimental Procedures). We also inoculated strain H99α alone on plant surfaces, and no monokaryotic fruiting was observed (data not shown). The growth of yeast colonies on plant surfaces was also observed (Figure 1A). The number of yeast cells surviving on the plant surface, based on recovered colony forming units (cfu), demonstrated that proliferation occurred (Figure S1). These results indicate that both mating and colonization can occur on plants, which may reflect potential conditions supporting completion of the fungal sexual cycle in nature.

Under previously described laboratory conditions, light inhibits mating of *C. neoformans* (Idnurm and Heitman, 2005). But on MS medium in the presence of growing plants (*Arabidopsis* or *Eucalyptus*), mating between isogenic strains of opposite mating type (H99α and KN99**a**) occurred even under constant light conditions (Figure 1B). In the absence of the plant, mating on MS medium occurred only in the dark, similar to mating on V8 medium. These observations indicate that plant derived materials, which are present in the natural environment of the fungus, can overcome the inhibition of mating by light.

Plant Materials Stimulate Mating of *C. gattii* Strains

C. gattii is capable of infecting immunocompetent hosts and is most commonly found associated with *Eucalyptus* species. Compared with other *Cryptococcus* species, such as *C. neoformans* var. *neoformans* or var. *grubii* strains, mating of *C. gattii* under standard laboratory

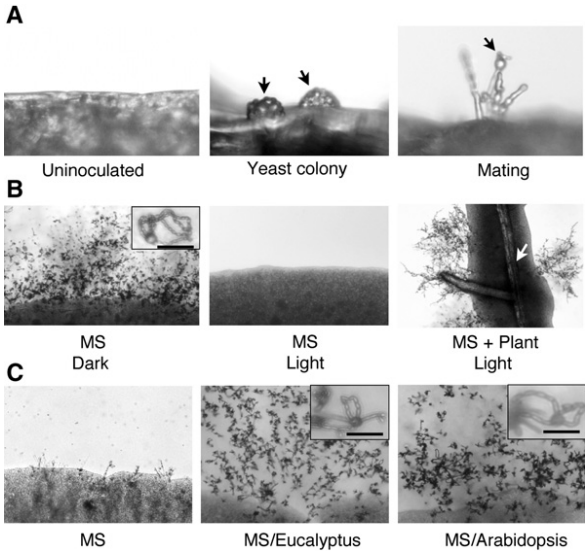

Figure 1. Plant Materials Stimulate Mating of *C. gattii* and *C. neoformans*

(A) A mixture of H99α and KN99**a** cells (2 × 10^7 cfu each) was inoculated on the nodes of *Arabidopsis thaliana* and incubated at room temperature under light for 4 weeks. Formation of yeast colonies (middle panel, indicated by arrows) and mating (right panel, arrow indicates a basidium and basidiospores) were monitored by microscopy.

(B) Mixtures of strains H99α and KN99**a** were inoculated on MS medium (contains 100 mg/l myo-inositol) or MS medium in the presence of *Arabidopsis* plants (+ plant). Mating results were observed after 14 days incubation at room temperature in the dark (left panel) or under light without plant (middle panel) or with plant (right panel). The white arrow indicates roots of *Arabidopsis*. The insert highlights the basidiospore chains. Scale bar = 20 μm.

(C) A mixture of *C. gattii* strains R265α and B4546**a** was inoculated on MS medium, alone (left panel) or in the presence of *E. camaldulensis* (middle panel) or *Arabidopsis* (right panel), and incubated for 3 weeks. The inserts highlight the basidiospore chains. Scale bars = 20 μm.

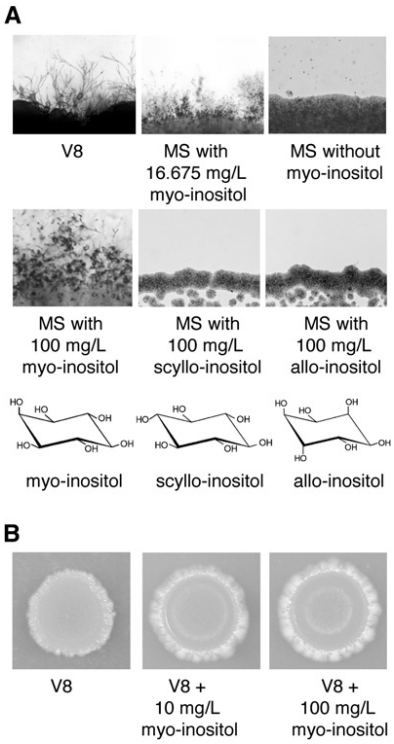

Figure 2. Myo-Inositol Contributes to Stimulate Mating in V8 Mating Medium and MS Medium

(A) A mating assay between H99α and KN99**a** was performed on V8 medium, MS medium containing 16.675 mg/l myo-inositol, MS without myo-inositol, MS containing 100 mg/l myo-inositol, MS containing 100 mg/l scyllo-inositol, and MS containing 100 mg/l allo-inositol, and incubated in the dark at room temperature for 14 days. The structures of inositol isoforms are also presented. Only myo-inositol stimulates mating.

(B) A mating assay between H99α and KN99**a** was performed on V8 mating medium, V8 with an additional 10 mg/l myo-inositol, and V8 with an additional 100 mg/l inositol, and incubated in the dark at room temperature for 14 days. Increasing myo-inositol enhanced mating efficiency.

conditions is much less efficient (Fraser et al., 2003). We discovered that while mating of *C. neoformans* var. *neoformans* and var. *grubii* strains was robust on MS medium, only limited filamentation and sporulation was observed during mating of *C. gattii* strains. However, long mating filaments and abundant basidiospores were observed when *C. gattii* mating pairs (molecular types VGII or VGIII) were cocultured on MS medium on which *Arabidopsis* or *Eucalyptus* seedlings were also growing under light (Figure 1C). Similar results were observed with several mating pairs (R265α × B4546**a**, NIH312α × B4546**a**), but not with any individual strain (data not shown). This enhancement occurred on MS medium even when yeast cells were not attached to plants, indicating that plants secrete compounds that stimulate mating.

Plant-Derived Compounds Induce *Cryptococcus* Mating

Cryptococcus can be isolated from fermenting fruits, and V8, tomato, or carrot juice media support sexual reproduction, indicating the potential importance of plant materials for this fungus. The mating-stimulating compound in MS media was identified by omitting one component at a time. Myo-inositol, whose derivatives play important roles in signaling and in plant development, was found to be necessary to promote *Cryptococcus* mating on this medium (Figures 2A and 3A). No mating occurred on MS medium lacking myo-inositol, whereas readdition of inositol restored mating. MS medium also induced monokaryotic fruiting of *C. neoformans* var. *neoformans* strains such as JEC21, but not var. *grubii* strains such as H99α (data not shown). The completed *Cryptococcus* genome sequence revealed that it contains 7 putative myo-inositol transporters compared to only 2 in other yeasts such as *S. cerevisiae* and *S. pombe* (Figure S2), consistent with the finding that myo-inositol plays an important role in the *Cryptococcus* life cycle.

The concentrations of myo-inositol on leaf surfaces and in total extracts of *Arabidopsis* and *Eucalyptus* were measured (Table 1) and found to be abundant. Interestingly, *Eucalyptus* has almost a 5-fold higher level of myo-inositol

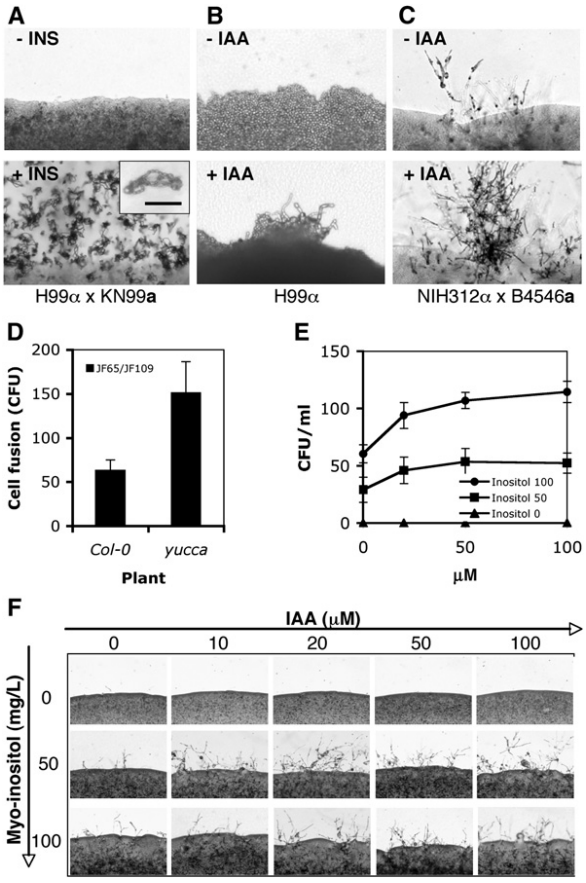

Figure 3. Induction of Mating by Plant-Derived Compounds Myo-Inositol and IAA

(A) A mating assay between H99α and KN99**a** was performed on MS medium (+INS) or MS without 100 mg/l myo-inositol (−INS) and incubated for 14 days in the dark at room temperature. Scale bar = 20 μm.

(B) The wild-type strain H99α was inoculated on filamentation agar (FA) medium without (−IAA) or with 2 mM IAA (+IAA) and incubated at room temperature in the dark. Filamentation on the edge of colonies was observed by microscopy after 3 weeks incubation.

(C) A mating assay between *C. gattii* strains NIH312α and B4546**a** was performed on MS medium (−IAA) or MS containing 50 μM IAA (+IAA) and incubated for 14 days in the dark at room temperature.

(D) Quantitative cell-cell fusion assays between *C. gattii* strains JF65 and JF109 were performed on plant surfaces. Two *Arabidopsis* lines were used for the cell fusion assay: wild-type Col-0 and an IAA overproduction mutant *yucca*. Mating mixtures of JF65 and JF109 were spotted on the leaf surfaces. The cell-cell fusion efficiency was determined by counting the CFU on YPD plates containing nourseothricin (NAT) and G418. This experiment was performed in triplicate. Error bars represent standard deviations.

(E) Quantitative cell-cell fusion assays between JF65 and JF109 were conducted on MS medium containing different concentrations of myo-inositol and IAA (see Experimental Procedures). The stimulation index (SI) was determined as described (see Experimental Procedures). This cell fusion assay was repeated twice with similar results.

(F) Mating assay between *C. gattii* strains JF65 and JF109 was performed on MS medium used in (E). Mating filaments from each condition were photographed after 10 days of incubation at room temperature in the dark.

Table 1. Myo-Inositol Concentrations on Plant Surfaces and in Extracts

Plant Species	Materials Used for Assay	Average Myo-Inositol Concentration
Arabidopsis thaliana	leaf surface extract	1.8 μg/cm^2
A. thaliana	total extract	19.1 μg/g fw*
Eucalyptus camaldulensis	leaf surface extract	6.48 μg/cm^2
E. camaldulensis	total extract	27.38 μg/g fw

* fw denotes fresh weight of the plant tissue.

on leaf surfaces compared to *Arabidopsis*. V8 mating medium contains 16.675 mg/l myo-inositol based on a previous study (Clements and Darnell, 1980), and this level of myo-inositol was sufficient to induce mating on MS medium (Figure 2A). Furthermore, addition of myo-inositol to V8 medium enhanced mating filament production (Figure 2B). Other inositol isoforms (scyllo- or allo-inositol) did not promote fungal mating (Figure 2A). Phytic acid, an inositol metabolite abundant on decaying plant surfaces, has also been tested for mating effect on *C. neoformans* and *C. gattii* strains. MS media (without myo-inositol) containing a range of phytic acid concentrations (from 1 μM to 1 mM) did not stimulate mating of H99α × KN99**a**, JEC21α × JEC20**a**, or NIH312α × B4546**a**.

In addition to myo-inositol, a panel of plant-derived compounds reported to affect microbe activity were tested, but none clearly stimulated *C. neformans* filamentation or mating except the plant growth hormone indole acetic acid (IAA) (Table S2). When the *C. neoformans* var. *grubii* strain H99α was inoculated on filamentation agar (FA) medium containing IAA and incubated at room temperature in the dark, short filaments were observed at the edge of colonies following incubation for 3 weeks (Figure 3B). The filaments observed are not as extensive as those observed during mating, but are morphologically similar to those produced during monokaryotic fruiting, an alternative developmental cascade during which one mating type can sexually reproduce (Lin et al., 2005). This is significant because monokaryotic fruiting has not been reproducibly observed in *C. neoformans* var. *grubii* wild-type strains. IAA has been reported to induce invasive growth of the model yeast *S. cerevisiae* (Prusty et al., 2004). These observations provide evidence that plant derived small molecules may induce *C. neoformans* to undergo fruiting in nature to produce basidiospores, the suspected infectious propagules.

We also tested the effect of IAA on mating of both *Cryptococcus* species by adding IAA to MS medium at a range of final concentrations (from 10 μM to 1 mM). Low concentrations of IAA (such as 50 μM) moderately enhanced mating of *C. gattii* in the presence of myo-inositol (Figure 3C), but had no obvious impact on mating of *C. neoformans*, which may indicate a difference in the association of *C. neoformans* and *C. gattii* with plants. At concentrations

higher than 100 μM, IAA inhibited mating filament production of both *C. neoformans* and *C. gattii* (data not shown). To further support that IAA represents a plant derived molecule that stimulates fungal mating, cell-cell fusion efficiency was compared for two *C. gattii* strains (JF65α and JF109**a**) on *Arabidopsis* wild-type line Col-0 and the IAA-overproduction mutant *yucca*, which contains 50% more free IAA than wild-type plants (Zhao et al., 2001). Cell-cell fusion on *yucca* plants was increased ~2-fold compared to that on Col-0, providing further evidence that IAA enhances fungal mating (Figure 3D).

Since both myo-inositol and IAA were found to promote *C. neoformans* mating, a mating test was performed with both compounds present. While no cell-cell fusion was detected and no mating filaments were produced when only IAA was present in the medium, increased cell-cell fusion efficiency (stimulation index [SI] > 1) and enhanced filament production were observed with a range of myo-inositol and IAA combinations, indicating that myo-inositol and IAA function in a synergistic fashion within defined concentration ranges (Figure 3E and 3F).

Cryptococcus Infects *Arabidopsis* Seedlings to Produce Dwarfing and Chlorosis

We then examined whether *C. neoformans* mating affects its pathogenicity on the plant host. It was recently reported that *Ustilago maydis* can infect *Arabidopsis* seedlings only in the filamentous heterokaryon state (Méndez-Morán et al., 2005). To test whether this is also the case for *Cryptococcus*, *Arabidopsis* seedlings grown on MS medium were inoculated with dH_2O, *C. neoformans* H99α alone, KN99**a** alone, or a mating mixture of strains H99α and KN99**a**. The mating mixture of *C. neoformans,* which results in a filamentous dikaryon, was found to infect *Arabidopsis* seedlings. Interestingly, dwarfing and chlorosis symptoms reproducibly appeared only on plants infected with the mixture of H99α and KN99**a**, but not with H99α or KN99**a** alone or the dH_2O control (Figure 4A). Mating filaments were observed by environmental scanning electron microscopy (ESEM) on surfaces inoculated with mating mixtures, but not with either strain inoculated alone (Figure 4B), and trypan blue staining confirmed that the fungus proliferated inside microlesions of plants infected by the mating mixture (Figure S3A).

To test whether spores produced by mating on plants are viable, we inoculated *Arabidopsis* seedlings with a mating mixture of two mating partners YSB119 and YSB121, which carry dominant markers, *NAT* and *NEO,* respectively. Twenty-five progeny were isolated and confirmed by exchange of the marker genes between mating types. Two progeny (CDX127 and CDX130) with opposite mating type were mixed and used for reinoculation on both sterile *Arabidopsis* Col-0 seedlings on MS medium and soil grown Col-0 plants. After two weeks of incubation, mating filaments were observed on plant surfaces and chlorosis symptoms developed on sterile plants. Inoculation of soil grown plants through infiltration also showed symptom development on locally infected leaves, similar to the original strains (data not shown). These

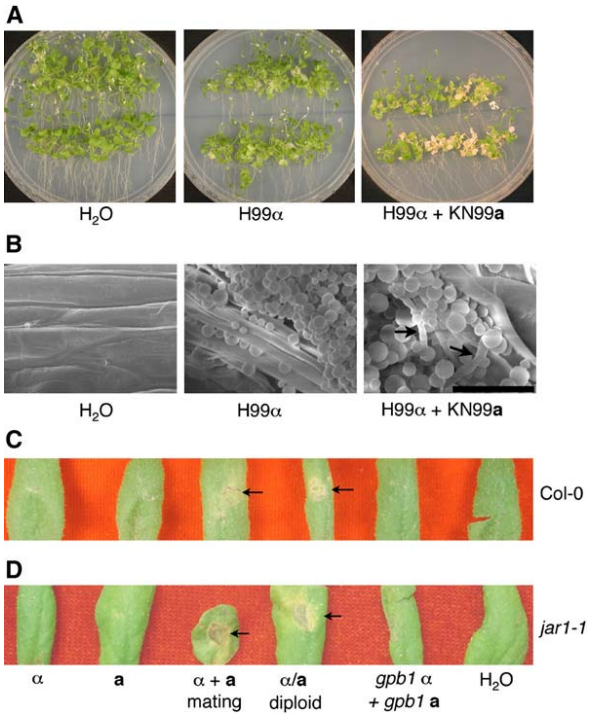

Figure 4. Infection of *Arabidopsis* by *C. neoformans*

(A) A mixture of strains H99α and KN99**a**, or H99α and KN99**a** cells alone were inoculated on the surfaces of *Arabidopsis* seedlings under sterile conditions. A dH_2O control was included. Infected plants were incubated at room temperature with a 16 hr light/8 hr dark cycle. Results were photographed 3 weeks postinoculation.

(B) *Arabidopsis* inoculated with dH_2O, H99α, and a mixture of H99α and KN99**a** were isolated after 3 weeks of incubation and fixed with osmium tetroxide solution. Fixed samples were viewed by ESEM. Arrows indicate mating filaments. Scale bar = 20 μm.

(C and D) Infiltration of a high concentration fungal mixture caused lesion development on *Arabidopsis* leaves. Leaves from 2-week-old *Arabidopsis* plants [Col-0 (C) and *jar1-1* (D)] were inoculated with 100 μl of yeast cells (OD_{600} at 0.1) via infiltration with a 1 ml sterile syringe. H99α, KN99**a**, a mixture of H99α and KN99**a**, α/**a** diploid strain KN191, a mixture of mating-defective *gpb1* α and *gpb1* **a** mutants, or dH_2O were used for the infection. Results were photographed 1 week post-infection. Arrows indicate lesions.

results indicate that viable spores are produced and can reinfect plants, documenting that the sexual cycle can be fully completed in association with plants.

We also attempted to infect mature *Arabidopsis* plants grown on soil with a number of isolates of *C. neoformans* without success (W. Fan and J.H, unpublished data). These efforts included attempts to employ *Arabidopsis* mutants with defects in plant defense responses, such as NahG plants, which cannot accumulate the defense inducing signal salicylic acid (SA) (Spoel et al., 2003). We hypothesized that this might be due to the fact that *C. neoformans* encodes fewer predicted cell wall-degrading enzymes compared to established plant fungal pathogens or *Aspergillus fumigatus* (Kamper et al., 2006) (Table S3). Plant cell wall-degrading enzymes are important for some fungal pathogens to successfully infect host plants

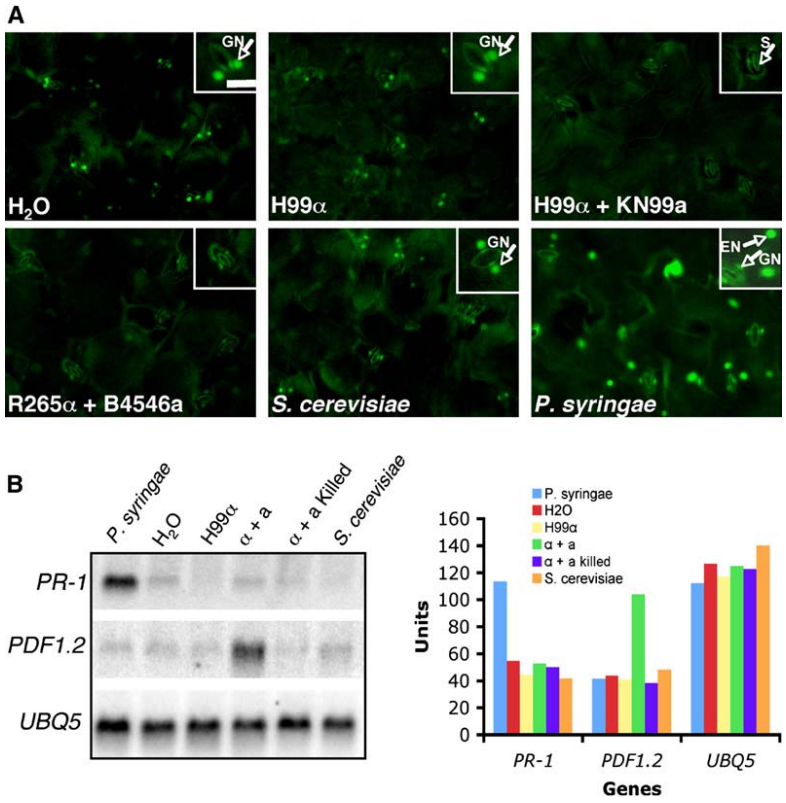

Figure 5. Plant Defense Response after Fungal Infection

(A) Fungal infection triggered the nuclear localization of the NPR1 protein. An NPR1::GFP *Arabidopsis* strain was used to detect the plant response to fungal infection. Two-week-old seedlings were infected with H99α, a mixture of H99α and KN99**a**, a mixture of R265α and B4546**a**, a mixture of *S. cerevisiae* strains ∑1278B and 3962C, or dH_2O. Seedlings infected with *P. syringae* served as a control. The GFP signal was monitored at 96 hr postinfection by direct fluorescence microscopy. Inserts highlight the GFP fluorescence of nuclei of guard cells and epidermal cells. Highlighted structures, such as guard cell nuclei (GN), epidermal cell nuclei (EN), and stomata (S) are indicated by arrows in the inserts. Scale bars = 2 μm.

(B) Plant defense-related genes were induced by fungal infection. Ten micrograms of total RNA from *Arabidopsis* infected with *P. syringae*, dH_2O, H99α, a mixture of H99α and KN99**a**, and a mixture of *S. cerevisiae* strains ∑1278B and 3962C were used for RNA hybridizations, and probed with *PR-1*, *PDF1.2*, or *UBQ5* gene probes (left panel). The hybridization signals were quantified using the ImageJ program (right panel). All of the fungal inocula were at a concentration of 0.1 OD_{600}. This experiment has been performed twice with similar findings.

and also for saprotrophic fungi, such as *A. fumigatus,* to cohabitate on plants (Tekaia and Latge, 2005). To test this hypothesis, soil-grown *Arabidopsis* was infected by infiltration to overcome the plant surface barrier. By 6 days postinfection, small lesions developed on plants inoculated with the mixture of strains H99α and KN99**a** or a *C. neoformans* var. *grubii* **a**/α diploid strain (KN191). Leaves infiltrated with strains H99α or KN99**a** alone, or a mixture of two sterile strains, *gpb1* α and *gpb1* **a,** which lack the G protein β subunit required for pheromone response during mating, showed no symptoms (Figure 4C). Because *C. gattii* strains are more frequently isolated from plants in nature, the infiltration infection assay was also performed for a mating mixture of *C. gattii* strains (JF65 and JF109), and similar lesions were observed (Figure S3B). Thus, *Cryptococcus* has difficulty overcoming plant barriers to cause disease, but can successfully colonize plant tissues once it gains entry.

Plant Defense Response Is Activated during *Cryptococcus* Infection

During a pathogen-plant interaction, plants activate distinct defense responses to combat microbial invasion. Induced resistance is regulated by signaling networks in which salicylic acid (SA) and jasmonic acid (JA) function as key signaling molecules (Spoel et al., 2003; Thomma et al., 2001). In response to biotrophic pathogens, the SA signaling pathway is normally activated, while in response to necrotrophic pathogens, the JA signaling pathway is induced (McDowell and Dangl, 2000). These two pathways are often antagonistic (Spoel et al., 2003). To determine whether either of these pathways is activated in response to *Cryptococcus* infection, we first examined the localization of NPR1, which is a master regulator of different defense signaling pathways.

In an *Arabidopsis* line expressing an NPR1::GFP fusion, infection by *Pseudomonas syringae* (and accumulation of SA) caused NPR1::GFP to translocate to the nucleus in both guard cells and epidermal cells (Figure 5A) (Mou et al., 2003). In contrast, the NPR1::GFP signal was observed only in the nuclei of guard cells in plants surface inoculated with *Cryptococcus* strains H99α or R265α, dH_2O, or a mixture of *S. cerevisiae* strains (∑1278B and 3962C), indicating that NPR1 localization is not affected by these conditions. Moreover, the NPR1::GFP signal was significantly reduced in the guard cell nuclei of plants infected with mating mixtures of strains H99α and KN99**a,** or R265α and B4546**a**, indicating that the filamentous dikaryon or unknown secreted factors produced by mating of *Cryptococcus* may even suppress NPR1 activity. This effect on NPR1::GFP is reminiscent of that observed when plants are exposed to JA (S.H. Spoel and X.D., unpublished data), suggesting that infection by the *Cryptococcus* dikaryon may modulate JA production or signaling.

The expression of several plant defense-related genes was monitored by RT-PCR (data not shown) and northern analysis (Figure 5B) to confirm that the *Cryptococcus* dikaryon modulates JA production or signaling. We observed that transcription of the major SA inducible

gene *PR-1* was induced by *P. syringae* but not by *C. neoformans* infection, whereas expression of the JA-inducible defensin gene *PDF1.2* (Penninckx et al., 1996) was elevated over 2-fold by infection with the *C. neoformans* H99α and KN99**a** mating mixture compared to dH_2O treatment (Figure 5B), which is consistent with a previous report on the systemic induction of *PDF1.2* expression after inoculation with a necrotrophic pathogen (Schenk et al., 2003). There was no significant induction of *PDF1.2* in plants infected individually with strains H99α or KN99**a**, or with a heat killed H99α and KN99**a** mixture. These results provide evidence that infection by the *Cryptococcus* dikaryon may induce JA-mediated defenses in *Arabidopsis* while suppressing the SA-mediated signaling pathway.

To determine whether these defense mechanisms affect the infectivity of *Cryptococcus,* we examined disease symptoms on *npr1-1* mutant plants (SA-insensitive) and *jar1-1* mutant plants (JA-pathway defective). Interestingly, more severe symptoms were observed on *jar1-1* plants compared to wild-type *Arabidopsis* plants after inoculation with a mixture of strains of opposite mating type of both *C. neoformans* (Figure 4D) and *C. gattii* (Figure S3B), while no difference in symptom development was detected on *npr1-1* plants (data not shown). *JAR1* encodes an enzyme that is structurally related to adenylate-forming enzymes of the firefly luciferase family, and it catalyzes formation of the more active JA-amido conjugate, JA-Ile (Staswick and Tiryaki, 2004). The *jar1-1* mutant has reduced sensitivity to JA and is susceptible to necrotrophic pathogens such as the opportunistic soil fungus *Pythium irregulare* (Staswick et al., 1998). Our data showed that *jar1-1* mutant plants are more susceptible to the *C. neoformans* filamentous dikaryon produced by mating, but not to the yeast form. The cell-cell fusion efficiency of *C. gattii* JF65α and JF109**a** on *jar1-1* plants is similar to that on Col-0 (data not shown), which indicates the enhanced susceptibility of *jar1-1* may be attributable to a lack of defense response rather than any enhancement of mating.

DISCUSSION

Cryptococcus is a dimorphic human fungal pathogen that infects the central nervous system and often causes meningitis, which is fatal if untreated. Considerable effort has been expended to address these questions since this fungus was first reported to be associated with *Eucalyptus* species (Ellis and Pfeiffer, 1990). There are reports suggesting that sexual recombination occurs in the environment based on phylogenetic analysis, but it has been unclear if this environmental niche plays a role in completion of the life cycle of *Cryptococcus* in nature (Campbell et al., 2005a; Halliday et al., 1999). Earlier work revealed that **a** and α isolates are both present in *Eucalyptus* trees but with no evidence for mating (Halliday et al., 1999). More recently, isolates have been identified that constitute a recombining population, yet they occur in a region without *Eucalyptus* trees (Campbell et al., 2005a; Campbell et al., 2005b). No direct evidence has been presented to show that mating occurs in nature or in association with *Eucalyptus*.

The *Cryptococcus*-plant interaction systems established here enable analysis of the effects of plant materials on the fungus, as well as plant responses to fungal infection. Fungal mating was stimulated on live plant surfaces, as well as in light by the presence of plants. This provides tools to understand how this human pathogen associates with its environmental niches, with potential benefits in disease control. The observations that yeast proliferate and mate on plant surfaces is the first direct evidence to show that yeast mating can occur on plants, supporting models that plants may provide a unique environmental niche for this fungus to complete its sexual cycle in nature. This is most significant in *C. gattii* strains, in which mating is often inefficient. Given that *C. gattii* strains are frequently isolated from plants, stimulation of mating by plant materials could be critical in nature.

Establishing how the plant stimulates *Cryptococcus* mating will have a significant impact on our understanding of this fungal-plant interaction. Our previous studies revealed that the activity in V8 juice responsible for mating stimulation is water soluble, heat stable, and of molecular weight less than 30 kDa (J.A. Alspaugh and J.H., unpublished data). Here, we report that myo-inositol promotes mating in MS medium and V8 medium is known to contain myo-inositol (Clements and Darnell, 1980). Inositol is essential for growth, promotes mating and sporulation in *S. pombe* (Niederberger et al., 1998), and plays important roles in development both in plants and humans (Clark et al., 2001; Stevenson et al., 2000; York et al., 2001). Whether other inositol metabolites also have a similar effect as myo-inositol is of considerable interest. Because phytic acid is relatively stable in decaying leaves and in soil, we also tested the effect of phytic acid on mating, but no impact was observed. Other inositol metabolites may therefore contribute to mating stimulation. Also, the plant hormone IAA was found to stimulate filamentation of *C. neoformans* and mating in *C. gattii*. IAA stimulates mating in *C. gattii* but not in *C. neoformans,* potentially indicating why *C. gattii* strains are more often isolated from plants. These plant-derived materials could be involved in the induction of mating and filamentation in nature.

In response to *Cryptococcus* mating, the JA responsive gene *PDF1.2* was induced. This result indicates that either mating cells secrete factors, such as pheromones, that induce the JA pathway, or that it is the filamentous dikaryon that does so. By contrast, no *PR-1* gene induction was observed, indicating that the SA-dependent defense pathway is not induced. The reduction of the NPR1::GFP signal in the nuclei of guard cells during infection was unexpected and might be triggered by either factors secreted during mating, the mating filament dikaryon, or even physical penetration by the filaments. A possible explanation for our results could be that induction of the JA signal inhibits the SA signal via cross-communication and thereby suppresses monomerization and nuclear localization of NPR1, thereby preventing *PR-1* gene activation. The inhibition of NPR1 expression following the induction of the

JA pathway has been observed in *Arabidopsis* (S.H. Spoel and X.D., unpublished data). Further studies will be necessary to elucidate this process in detail. We also conducted coinfection using *P. syringae* and a *Cryptococcus* mating mixture (H99α x KN99**a**), and in this situation, NPR1::GFP nuclear translocation was similar to that observed with *P. syringae* inoculation alone. Possible explanations could be that the activation of the SA signal by *P. syringae* infection is predominant compared to the JA signal activated by *Cryptococcus*, in accord with previous studies (Mur et al., 2006).

Although the inoculation of fungal mating mixtures caused dwarfing and chlorosis symptoms under sterile conditions, no significant symptoms developed in plants grown in soil. Compared with other plant fungal pathogens and saprotrophic *Aspergilli* (Tekaia and Latge, 2005), *C. neoformans* has fewer cell wall-degrading enzymes critical for many fungal pathogens to overcome the plant cell wall barrier and infect host plants successfully. The roles of such enzymes have been documented for necrotrophic or hemibiotrophic pathogens, even though their roles in many biotrophic pathogens have not been fully established. Thus, an absence of such enzymes could be one possible factor that causes this fungus to be less aggressive during interactions with plants. In some cases, the filaments produced during mating may also contribute to surmount this barrier by infecting through stomata or wounding sites to cause infection. When plants were grown under sterile conditions, the humidity in plates is high and stomata are consistently open, which may afford fungi the opportunity to gain entry through stomatal openings. Another possibility is that *Cryptococcus* secretes factors during mating that are toxic to plants. Based on the evidence gathered, such as the induction of JA signaling and microlesion development during the fungal-plant interaction, it is more similar to a necrotrophic interaction. But, further study is required to clarify whether it is a true necrotroph, which is beyond the scope of this study.

Our results on the interaction between *C. neoformans* and *C. gattii* with plants provide evidence that this pathogen can complete its sexual cycle in nature in association with plants. This represents a key stage in the replication and propagation of this pathogenic fungus in nature that likely directly impacts its virulence cycle (Figure 6). Our studies are the first to document that plants secrete materials that stimulate mating and, furthermore, define the identity of at least two plant-derived factors that stimulate mating as inositol and IAA. These data strongly support the importance of an association of this human microbial pathogen with plants. Investigation of the interaction between *Cryptococcus* and plants reveals a potential parasitic relationship involving JA-dependent, but SA-independent, signaling. While it has been appreciated for several decades that *Cryptococcus* growth or mating can be supported by plant materials, the biological significance of this phenomenon was not known. Our results showing that association with live plants stimulates *Cryptococcus* mating and causes disease-like symptoms, that

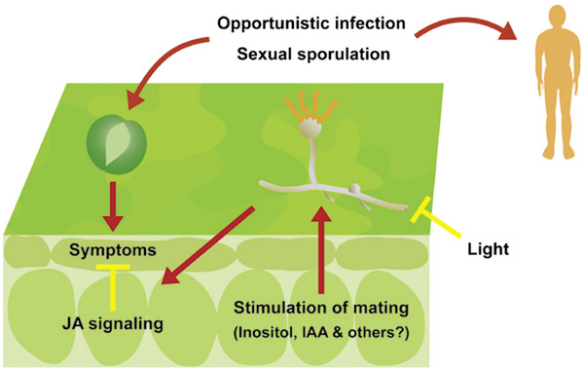

Figure 6. Proposed Model for the Interaction between *C. neoformans* and Plants

Light inhibits mating of *C. neoformans*. Plant materials stimulate mating and can overcome the light inhibition on mating. The mating of *C. neoformans* on the plant surface induces the JA-dependent signal pathway, leading to *PDF1.2* gene expression. The mating dikaryon filaments may gain entry through stomata to cause symptoms on plants. Sexual spores may also disperse to infect susceptible human hosts.

plants secrete molecules that enhance mating, and that fungal mating triggers plant defense response signals provide insights into how the fungal life cycle may be completed in nature.

Questions still remain as to the evolutionary origin of *Cryptococcus* and how this fungus developed into a ubiquitous and successful systemic human pathogen. Because the progenitor of this fungus existed before human or other warm blooded mammals populated the world, environmental niches involving plants could well represent the original hosts for the ancestral progenitor of *C. neoformans* and *C. gattii*. Our results indicate that this microorganism may continue to associate with an original environmental niche to complete its sexual cycle. This type of association should not be limited to pathogens. Therefore, our findings can also provide general insight to understand how microorganisms associate with and benefit from environmental niches.

EXPERIMENTAL PROCEDURES

Fungal and Plant Strains, Media, and Growth Conditions

C. neoformans and *S. cerevisiae* strains used in this study are listed in Table S1. Strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) agar medium and synthetic (SD) medium, which were prepared as described previously (Alspaugh et al., 1997). Filamentation agar (FA) medium was prepared as previously described (Wickes et al., 1996) and used to assay filament production. V8 medium and Murashige and Skoog (MS) medium minus sucrose (Sigma-Aldrich, Steinhelm, Germany) were used for mating assays.

Arabidopsis thaliana wild-type line Columbia Col-0, transgenic NahG plants harboring the bacterial salicylic acid hydroxylase (NahG) gene (Friedrich et al., 1995), mutant *npr1-1* plants (Cao et al., 1994), Jasmonic acid production defect mutant *jar1-1* plants (Staswick et al., 1998), NPR1::GFP expression plants (Kinkema et al., 2000), Indole acetic acid (IAA) overproduction mutant *yucca* plants, and *Eucalyptus* species *E. camaldulensis* and *E. tereticornis* (http://www.jlhudsonseeds.net/) were grown in soil in a growth room with a 16 hr

day (200 μE.m^{-2}.s^{-1} at 24°C)/8 hr night (20°C) cycle at 70% relative humidity. *A. thaliana* Col-0 and NahG, as well as *E. camaldulensis* seeds, were surface sterilized by soaking in 95% ethanol for 5 min, transferred to a sodium hypochlorite solution (1.2% free chlorine) for 10 min, and washed at least three times with sterilized distilled water (dH_2O). The seeds were placed over plates of sterile solid MS medium without sucrose and incubated at room temperature with a photoperiod of 16 hr light. Two-week-old seedlings were transplanted onto new MS plates, incubated at room temperature, and illuminated with a 16 hr light photoperiod.

Recovery of Fungal Cells from Infected Plants

Inoculated plant leaves were collected after 0, 5, and 10 days postinfection. Plant tissue was ground with a sterile mortar and pestle, diluted with dH_2O, and aliquots were inoculated on YPD medium containing 50 mg/l ampicillin and 38 mg/l chloramphenicol. Plates were inoculated at 30°C for 2 days and colony forming units (cfu) were determined.

Mating Assay on Medium and Plant Surfaces

Fungal overnight growth cultures were collected and washed twice with dH_2O, and resuspended in dH_2O at a final concentration of 5 × 10^7 cfu per ml. Equal amounts of opposite mating type cells were mixed, and 5 μl of these mixtures were inoculated on MS medium or on the leaves and nodes of plant seedlings. For MS medium containing growing plants, mixtures were plated on the medium next to the plant roots. Morphological development (filamentation and mating) was monitored by light microscopy. Any alteration of the plant surfaces, such as lesions or cell death, was monitored.

Cell Fusion Assay on the Plant Surface

Cell fusion was performed by mixing 2 × 10^6 cells of two strains, and a 5 μl of the culture suspension washed twice with dH_2O was inoculated onto the plant surface and allowed to incubate for 72 hr at 25°C. The inoculated plant tissues were ground and then resuspended in 1 ml dH_2O and 200 μl of the suspension (~10^5 total cells) was plated onto YPD (NAT and G418) medium and colony forming units (cfu) were determined after 3 days. Meiotic recombination was also tested in a mating event. Dikaryons produced during cell fusion were streaked on MS medium, spores were isolated, and their mating type and drug resistance were determined by crosses with mating type testing strains (H99α and KN99**a**) and on YPD medium containing NAT or G418.

Synergistic Analysis between Myo-Inositol and IAA on Fungal Mating

MS medium was prepared in 6-well tissue culture plates with 8 ml medium per well. Myo-inositol was added horizontally to individual wells at final concentrations of 0, 50 mg/l, and 100 mg/l; IAA was also added vertically at final concentrations of 0, 10 μM, 20 μM, 50 μM, and 100 μM. Five microliter mixtures of *C. gattii* strains at a concentration of 2 × 10^6 cfu per ml were used to inoculate each MS well. Cells were collected from medium after 24 hr incubation, and resuspended into 1 ml dH_2O. 100 μl of resuspensions were spread on YPD plates containing NAT and G418. Cell-cell fusion efficiency was determined by CFU on each plate. The stimulation index was used to determine the effect of chemical combinations. Stimulation index (SI) = CFU (myo-inositol + IAA)/(CFU(myo-inositol) + CFU(IAA)). A synergistic effect was defined by SI > 1, an additive effect was defined by SI = 1, and an antagonistic effect was defined by SI < 1.

Plant RNA Extraction, RT-PCR, and Northern Blot

Infected *Arabidopsis* leaves were collected and stored at −80°C after quick freezing in liquid nitrogen. Total RNA from plant tissues was extracted as described (Cao et al., 1994). First strand cDNA was synthesized following the manufacturer's instructions for the Superscript III kit (Invitrogen, Carlsbad, CA). Ten micrograms total RNA for each sample was transferred to a Nylon membrane and hybridized with *PR-1*, *PDF1.2*, and *UBQ5* ubiquitin gene probes, which were amplified from *Arabidopsis* cDNA with primers JH16590 (5′-ATGAATGAAATGT CGTTCTTTGG-3′)/JH16591 (5′-CTAATATGGACGTTGACCGATG-3′), JH16598 (5′-GGCTAAGTTTGCTTCCAT CATC-3′)/JH16599 (5′-CA TGGGACGTAACAGATA CAC-3′), UBQF (5′-GACGC TTCATCTCGT CC-3′)/UBQR (5′-GTAAACGTAGGTGAGTCCA-3′), respectively. The same pairs of primers were also used to perform RT-PCR to detect differential amplification of the *PR-1*, *PDF1.2*, and *UBQ5* transcripts.

Environmental Scanning Electron Microscopy

Arabidopsis plants were inoculated with a mating mixture at the concentrations described in the results. Four-week-old infected plant materials were isolated and dried in a sterile Petri dish. For fixation, 50 μl of 2.5% osmium tetroxide solution (Sigma-Aldrich, Steinhelm, Germany) was added next to the plant material and the Petri dish was sealed and left in a chemical hood overnight. Fixed samples were viewed by ESEM.

Analysis of Myo-Inositol Concentration on the Plant

For surface extraction of myo-inositol, *Arabidopsis thaliana* or *Eucalyptus camaldulensis* leaves were cut and their areas were drawn on a clean paper and later measured using the ImageJ program. The leaves were dipped into 20 ml boiling dH_2O for 30 s, being careful not to dip the cut end. Leaves were then frozen at −80°C and later used for total tissue analysis. Surface extracts were concentrated in a speed vacuum. The leaf tissues were ground with a mortar and resuspended in 1 ml dH_2O. Resuspensions were spun down at 14,000 rpm for 10 min and supernatants were filtered through a Slide-A-Lyzer Dialysis Cassette (35000 MW, Pierce, Rockford, IL). The filtrates and the concentrated surface extracts were sent out for measurement of the myo-inositol concentrations (HT laboratory, San Diego, CA).

Trypan Blue Staining to Detect Dead Plant Cells and Fungal Cells

Infected plant tissues were submerged in a trypan blue solution (2.5 mg/ml trypan blue, 25% (wt/vol) lactic acid, 23% water-saturated phenol, 25% glycerol), infiltrated for 10 min, and stained for several hours or overnight. The tissues were destained in chloral hydrate solution (2.5 g/ml in dH_2O) for 3 days. Samples were equilibrated with 10% glycerol for microscopic analysis.

Fungal Infection Assay on Plant Surfaces

Yeast cells were cultured in 5 ml YPD medium and incubated at 30°C overnight. Cells were collected by centrifugation and washed with dH_2O three times and diluted into dH_2O to a final concentration OD_{600} = 1.0 or 0.1. Ten microliters of diluted cell suspensions were inoculated on the nodes, leaves, or roots of 2-week-old *Arabidopsis* or *Eucalyptus* seedlings. The infiltration assay was as previously described (Pieterse et al., 1998). A 500 μl cell suspension was added in a 1 ml sterile syringe and injected into the abaxial side of leaves with pressure. Infected plants were incubated under standard growth conditions as described above.

Supplemental Data

The Supplemental Data include three supplemental figures and three supplemental tables and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/1/4/263/DC1/.

ACKNOWLEDGMENTS

We thank Weihua Fan for valuable discussions and preliminary studies and assistance with plant tissue culture, Leslie Eibest for ESEM assistance, James Fraser, Xiaorong Lin, and Lisa Anderson for reagents and strains, Kohki Akiyama for providing 5-deoxy-strigol, Fred Ausubel, Jin-Rong Xu, Kirsten Nielsen, and Alex Idnurm for critical reading and comments on the manuscript, and Zareen Kapadia, Anna Floyd, and Lydia Chen for technical assistance. This study was supported

in part by NIAID R01 grants AI50113 and AI39115 to J.H. and NIH R01 grant GM69594 to X.D.

Received: February 27, 2007
Revised: April 19, 2007
Accepted: May 21, 2007
Published: June 13, 2007

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