Plant Defense Mechanisms Are Activated during Biotrophic and Necrotrophic Development of *Colletotricum graminicola* in Maize[1][W][OA]

Walter A. Vargas, José M. Sanz Martín, Gabriel E. Rech, Lina P. Rivera, Ernesto P. Benito, José M. Díaz-Mínguez, Michael R. Thon, and Serenella A. Sukno*

Centro Hispanoluso de Investigaciones Agrarias, Department of Microbiology and Genetics, University of Salamanca, 37185 Villamayor, Spain

Hemibiotrophic plant pathogens first establish a biotrophic interaction with the host plant and later switch to a destructive necrotrophic lifestyle. Studies of biotrophic pathogens have shown that they actively suppress plant defenses after an initial microbe-associated molecular pattern-triggered activation. In contrast, studies of the hemibiotrophs suggest that they do not suppress plant defenses during the biotrophic phase, indicating that while there are similarities between the biotrophic phase of hemibiotrophs and biotrophic pathogens, the two lifestyles are not analogous. We performed transcriptomic, histological, and biochemical studies of the early events during the infection of maize (*Zea mays*) with *Colletotrichum graminicola*, a model pathosystem for the study of hemibiotrophy. Time-course experiments revealed that mRNAs of several defense-related genes, reactive oxygen species, and antimicrobial compounds all begin to accumulate early in the infection process and continue to accumulate during the biotrophic stage. We also discovered the production of maize-derived vesicular bodies containing hydrogen peroxide targeting the fungal hyphae. We describe the fungal respiratory burst during host infection, paralleled by superoxide ion production in specific fungal cells during the transition from biotrophy to a necrotrophic lifestyle. We also identified several novel putative fungal effectors and studied their expression during anthracnose development in maize. Our results demonstrate a strong induction of defense mechanisms occurring in maize cells during *C. graminicola* infection, even during the biotrophic development of the pathogen. We hypothesize that the switch to necrotrophic growth enables the fungus to evade the effects of the plant immune system and allows for full fungal pathogenicity.

Most of the agronomically important plants of the world are susceptible to members of the filamentous fungal genus *Colletotrichum*, the etiological agents of anthracnose disease or blight (Bailey and Jeger, 1978; Bergstrom and Nicholson, 1999; Perfect et al., 1999; Dickman, 2000; Latunde-Dada, 2001). Species of *Colletotrichum* employ diverse strategies for invading host tissue, ranging from intracellular hemibiotrophy to subcuticular/intramural necrotrophy (Bailey et al., 1992). *Colletotrichum graminicola*, the causal agent of anthracnose of maize (*Zea mays*), is a model for hemibiotrophic pathogens, those that begin their infection as biotrophic pathogens but later switch to a necrotrophic lifestyle (Bergstrom and Nicholson, 1999; Perfect et al., 1999; Münch et al., 2008; Crouch and Beirn, 2009). From ultrastructural studies of *C. graminicola*, we know that following appressorium formation, a thin penetration peg that invades the epidermal cell is formed. Later, within the epidermal cells, an enlarged, irregular primary hypha, also named an infection hypha, is developed (Politis and Wheeler, 1972; Mims and Vaillancourt, 2002). This primary hypha appears to grow biotrophically and can form one or more branches spreading to adjacent host cells. The fungus grows between the plant plasma membrane and the plant cell wall and spreads from cell to cell in this manner for about 36 to 48 h. This stage of infection is referred to as a biotrophic growth stage, since the penetrated host cells remain alive. This form of growth provides a very large surface area of host membrane relative to the fungus, allowing the fungus greater access to nutrients and signal exchanges with the host (Bergstrom and Nicholson, 1999; Perfect and Green, 2001; Panstruga, 2003; Micali et al., 2011).

Following the biotrophic growth phase, a switch to necrotrophic growth occurs, which is typified by the production of a large number of smaller diameter and

[1] This work was supported by the Ministerio de Ciencia e Innovación of Spain (grant no. AGL2008–03177/AGR) and the Junta de Castilla y León, Spain (grant no. SA–134A08), by the Spanish Ministry of Science and Innovation through the Juan de la Cierva (grant no. JCI–2009–05364 to W.A.V.), by the Ramón y Cajal Program (to M.R.T.), and by a Formación del Profesorado Universitario graduated fellowship (no. AP2009–2656 to J.M.S.M.), a Formación de Personal Investigador graduated fellowship (no. BES–2009–013920 to G.E.R.), and a Junta para la Ampliación de Estudios predoctoral scholarship (to L.P.R.) from the Consejo Superior de Investigaciones Científicas.

* Corresponding author; e-mail ssukno@usal.es.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Serenella A. Sukno (ssukno@usal.es).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.190397

regularly shaped hyphae, called secondary hyphae, that extensively colonize the intercellular and intracellular spaces of the tissue, causing the death of host cells prior to colonization (O'Connell et al., 1985; Bergstrom and Nicholson, 1999; Wharton et al., 2001; Mims and Vaillancourt, 2002). This process of disease development, called hemibiotrophy or facultative biotrophy, raises questions about the importance of lifestyle differences among fungi. It is expected that all these early events in anthracnose development are accompanied and regulated by the expression of stage-specific sets of genes. However, the physiological and molecular bases that determine (in both *C. graminicola* and maize cells) the outcome of this pathogenic interaction are unknown.

Interaction transcriptome studies are a popular method for understanding responses of both the pathogen and the host during the infection process (Birch and Kamoun, 2000; Wise et al., 2007; Mosquera et al., 2009; Kim et al., 2010). In general, plant cell reprogramming has been observed in several pathosystems, including maize-*Ustilago maydis*, rice (*Oryza sativa*)-*Magnaporthe oryzae*, and *Medicago truncatula*-*Colletotrichum trifolii* (Torregrosa et al., 2004; Doehlemann et al., 2008; Marcel et al., 2010). For instance, *U. maydis* is a biotrophic basidiomycete that infects maize plants, inducing host tumors, where the fungal cells proliferate in a biotrophic manner (Banuett, 1995). A transcriptional profiling of maize genes during *U. maydis* infection and tumor development revealed a complex cell reprogramming due to specific transcriptional and metabolic changes induced by the pathogen (Doehlemann et al., 2008). Those changes include a transient induction of defense mechanisms in maize that, with the onset of biotrophy at 24 h post infection (hpi), are suppressed. Microarray expression analysis revealed a transient induction of pathogenesis-related genes (PR genes), chitinases, and glucanases at 12 hpi that were repressed 24 h after the infection began. The plant cells initially recognize and respond to the presence of the invading pathogen. However, when *U. maydis* starts colonizing epidermal cells, the primary plant responses are attenuated. Similar patterns of plant defense gene induction, followed by suppression, have been observed in other biotrophic pathosystem species (Caldo et al., 2006; Doehlemann et al., 2008) and the hemibiotrophic *Mycosphaerella graminicola* (Adhikari et al., 2007). In contrast, a different behavior may be inferred during the infection of rice leaves by the hemibiotrophic fungus *M. oryzae*. Two independent transcriptional studies performed by two different groups suggest that transcripts for basal plant defense genes continued to increase in abundance during the infection process (Mosquera et al., 2009; Marcel et al., 2010). However, this conclusion still remains to be fully confirmed for this pathosystem.

The induction of plant genes in response to infection of *M. truncatula* by the hemibiotrophic pathogen *C. trifolii* was investigated by Torregrosa et al. (2004) using macroarray experiments. The expression assay included 22 defense-related genes, which mostly did not show a general trend in the expression pattern along the infection process. Only six of them (i.e. three chitinases, one superoxide dismutase, one peroxidase, and one glucanase) showed significant change in the expression level at one specific time point. This lack of consistency in the response of the defense mechanisms was in agreement with the well-accepted concept that defense responses are delayed and not very intense in susceptible plants (Métraux et al., 2009).

The Arabidopsis (*Arabidopsis thaliana*)-*Colletotrichum higginsianum* pathosystem has also been investigated previously at the transcriptomic level. Arabidopsis expression studies were used to investigate the defense reactions and their relationship with jasmonic acid, salicylic acid, and ethylene signaling pathways (Narusaka et al., 2004). More recently, Takahara et al. (2009) performed a transcriptional study of *C. higginsianum* primary hyphae extracted from infected Arabidopsis leaves. In that report, 161 unigenes from *C. higginsianum* were identified. Expression assays on a selected group of genes further identified six fungal genes specifically expressed during the biotrophic stage. Also, in the case of the *C. graminicola*-maize pathosystem, infected plant cells were laser microdissected and transcriptional profiling of fungal RNA was performed by microarray analysis (Tang et al., 2006). The authors detected more than 400 fungal genes up-regulated at early stages of infection, but the identities of these genes have not been reported. Previous studies on the *C. graminicola*-maize pathosystem described the use of a cDNA subtractive sequential protocol to identify genes expressed at early stages of anthracnose development, which led to the identification of only 13 genes (six from plants, three fungal genes, and four transcripts of unknown origin) differentially expressed (Sugui and Deising, 2002). In general, subtractive protocols have not been successful in the identification of fungal genes at early stages of infection due to the low-level representation of fungal transcripts in RNA samples extracted from infected tissue. This is believed to be the crucial limitation in the study of some plant-microbe interactions and fungal expression profiling at early stages of plant colonization.

One important feature that is induced once a pathogen is recognized by the host plant is an army of defense mechanisms that counteract the attack. Plants depend on several defense mechanisms to attack microbial intruders that threaten their physiology. The production of reactive oxygen species (ROS), primarily in the form of superoxide and hydrogen peroxide (H_2O_2), at the penetration site is one of the most rapid plant defense reactions after pathogen attack (Apostol et al., 1989). Apoplastic peroxidases can also use the H_2O_2 in reactions involved in the synthesis of lignin and other phenolic compounds that act as additional antimicrobial barriers (Torres and Dangl, 2005).

Many plants respond to fungal attack with the accumulation of ROS as a means to arrest fungal growth. ROS production was demonstrated to be one of the earliest cytologically detectable responses to

restrict infection by various fungal species (Mellersh et al., 2002). Mellersh et al. (2002) described a comprehensive study on the relevance of plant defense mechanisms for fungal penetration in plant-fungus interactions. That article highlighted the importance of H_2O_2, superoxide, and phenolic compounds to prevent penetration and restrict fungal growth. In contrast, fungi have developed biochemical mechanisms to overcome elevated levels of intracellular ROS (van Kan, 2006). The methods employed to control ROS can include detoxification or scavenging (Rolke et al., 2004; Lev et al., 2005; Voegele et al., 2005; Molina and Kahmann, 2007). In addition, recent reports describe an important role of ROS produced by the fungus in virulence and development in some fungi (Heller and Tudzynski, 2011). These varying responses during the plant's interaction with different fungal pathogens prompted us to further study and characterize the cytological and molecular aspects of the infection of maize leaves by the hemibiotrophic pathogen *C. graminicola*.

In the past decade, enormous progress has been made in our understanding of pathosystems involving biotrophic and necrotrophic fungal species. However, our understanding of the molecular and biochemical mechanisms mediating plant infection by hemibiotrophic fungi is still very limited. In this article, we present a histological, metabolic, and transcriptional study of the maize-*C. graminicola* pathosystem during early stages of anthracnose leaf blight development. Suppression subtractive hybridization experiments at early infection stages, 48 and 72 hpi, led us to identify more than 200 differentially expressed genes from maize plants and 50 genes expressed in *C. graminicola*. The findings presented in this article provide novel targets to further study genetic and biochemical factors involved in leaf blight development in maize plants.

We also present evidence for the induction of a number of plant defense responses through cytological, biochemical, and molecular analyses. Based on the transcriptional data together with the detection of ROS and phenolic compounds, we conclude that, unlike biotrophs, the hemibiotroph *C. graminicola*, during its biotrophic stage, is unable to suppress many of the plant defense mechanisms that are typically suppressed by biotrophic pathogens. The switch to a necrotrophic lifestyle, which enables the fungus to kill plant tissue before it is colonized, may allow it to avoid direct contact with the plant-produced defense responses and continue its pathogenicity program.

RESULTS

Characterization of Disease Progress

As fungal development in planta may vary depending on environmental and/or infection conditions, we performed experiments to identify the most appropriate time points for our assays. The course of the infection was followed using confocal imaging on maize leaves infected with a GFP-tagged strain of *C. graminicola* (Sukno et al., 2008). Microscopic analysis showed that under our working conditions, the fungus follows the well-established stages of infection and structure differentiation. At 12 hpi, conidia could be observed germinating. At 24 hpi, mature, melanized appressoria could be found on the leaf surface. Host penetration, evidenced by the formation of penetration pegs, occurred between 24 and 36 hpi. During this time, we also observed the formation of primary hyphae within the infected cells, which is consistent with the establishment of the biotrophic stage. From 36 to 60 hpi, we continue to see the development of primary hyphae, which spread to adjacent cells (Fig. 1, A and B). After 60 hpi, secondary hyphae begin to develop from the primary hyphae and proliferate throughout the plant tissue. By 72 hpi, symptoms of

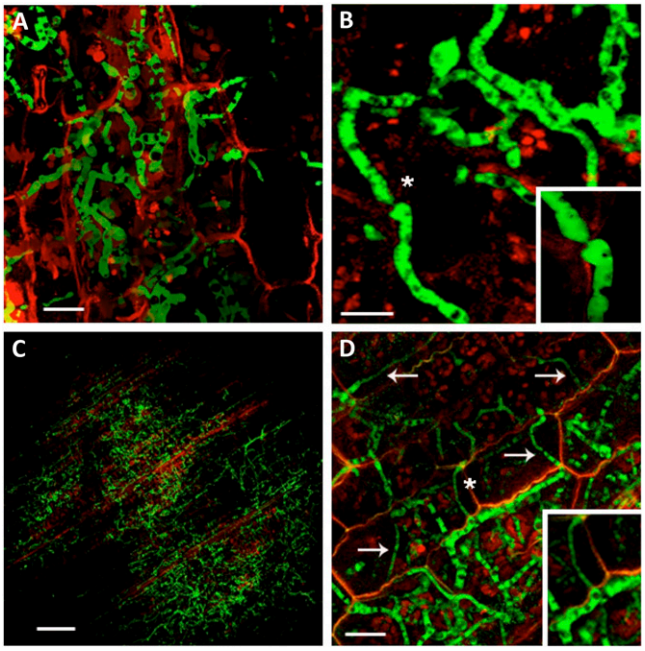

Figure 1. Microscopic analysis of *C. graminicola* infection sites on maize leaves inoculated for the preparation of SSH libraries. A and B, Micrographs taken 48 hpi. A, Biotrophic primary hyphae can be identified by their irregular, globular morphology. Bar = 75 μm. B, Closeup view showing the biotrophic lifestyle, as the primary hypha is moving to a second cell. Bar = 50 μm. The inset shows a magnification of the section indicated by the asterisk, showing the biotrophic hyphae of *C. graminicola* growing intracellulary and becoming constricted as they passed through the cell wall through specific points of contact with other cells (possibly plasmodesmata). C and D, Micrographs obtained 72 hpi. C, Massive fungal growth and necrotrophic development is evident. Bar = 350 μm. D, The secondary hyphae are evident by their regular, narrower, and elongated shape. Bar = 70 μm. The inset shows a magnification of the section indicated by the asterisk, showing the switch to the necrotrophic lifestyle by the development of secondary hyphae. Micrographs were obtained on a Leica TCS SP2 laser scanning microscope.

anthracnosis are observed, including necrotic lesions surrounded by a yellow halo and massive secondary hyphae development (Fig. 1, C and D). Using this time line, we focused our studies on the 48-hpi time point, at which time only biotrophic hyphae are present, and the 72-hpi time point, when necrotrophic hyphae are predominant.

Antimicrobial Compounds and Cell Wall Metabolism in Infected Maize Leaves

One of the first defense barriers that pathogens encounter in plants is the production of antimicrobial compounds, such as phenolic compounds related to cell wall metabolism and ROS (Lamb and Dixon, 1997; Heath, 2000). To have a better understanding of the biochemical changes induced in maize plants during biotrophic and necrotrophic development of *C. graminicola*, we monitored the activation of plant defense mechanisms in a time-course experiment during a compatible interaction. As we wanted to test in vivo responses induced in the plant without the interference caused by leaf detachment (Liu et al., 2007), we only infected leaves on intact plants. Increased levels of phenolic compounds were induced in maize leaves upon infection with *C. graminicola*. The activation of defense mechanisms was evidenced by the presence of autofluorescence in infected cells 48 hpi (Fig. 2A). This observation was further confirmed by quantitative data showing increased levels of *p*-hydroxycinnamic acid and phytoalexins 48 hpi (Fig. 2B). The increased metabolism of phenolic compounds and lignin deposition in infected tissue was further demonstrated after toluidine blue staining 72 hpi (Fig. 2C). These results are in agreement with increased levels of mRNA encoding for Phe ammonia lyase, a key enzyme involved in the biosynthesis of *p*-hydroxycinnamic acid and derivative phenolic compounds acting as building blocks for lignin and several flavonoids, such as phytoalexins and anthocyanins (data not shown).

C. graminicola and Maize Produce Superoxide and Peroxide, Respectively, during the Infection Processes

To further investigate defense mechanisms during maize leaf blight development, we sought to investigate the production of ROS in a time-course experiment collecting samples every 12 h up to 72 hpi. The production of peroxide and superoxide in infected samples was determined by in situ oxidation of diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively. The results revealed a strong and continuous ROS production in maize cells as *C. graminicola* infection progressed. The main ROS com-

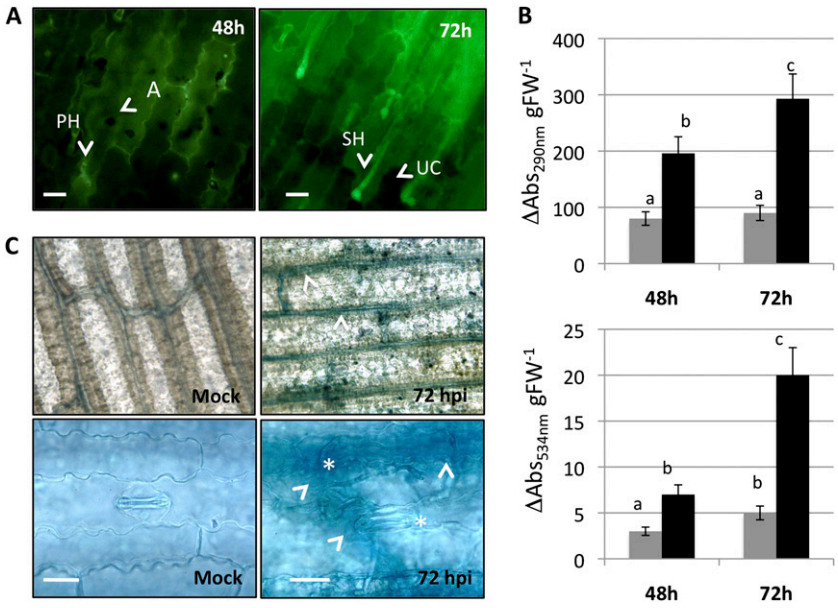

Figure 2. Production of antimicrobial compounds associated with plant cell wall metabolism during *C. graminicola* infection. A, Micrographs showing green autofluorescence in maize cells 48 and 72 hpi after infection. Mock-inoculated plants did not show any green autofluorescence at these time points. A, Appressorium; PH, primary hyphae; SH, secondary hyphae; UC, uninfected cell. B, Quantitative determination of anthocyanins (A_{534}) and hydroxycinnamic acid derivatives (A_{290}) 48 and 72 hpi. Gray and black bars represent control and infected leaves, respectively. The values shown are averages of two independent experiments ± SE. In each panel, bars with different letters differ significantly according to Tukey's HSD test at a significance level of 1%. FW, Fresh weight. C, Lignification and cell wall deposition determined after toluidine blue O staining. The bottom panels represent closeup views of the micrographs in the top panels. Arrowheads indicate strong staining of the vascular bundle 72 hpi with *C. graminicola*. Asterisks indicate fungal hyphae. Bars = 75 μm.

pound produced by maize was in the form of peroxide (Fig. 3). The accumulation of peroxide was evident 36 hpi as the reduction of DAB near the plant cell membrane, beneath fungal appressoria, and surrounding the penetration peg (Supplemental Fig. S1A). With the progression of fungal infection, plant cells started producing vesicular bodies (positive for DAB staining) targeted toward the fungal hyphae (Fig. 3). The production of vesicular bodies in response to *C. graminicola* in the plant cells was more evident 72 hpi. At this time point, secondary hyphae are heavily covered by the plant-derived vesicles (Fig. 3, E and F). Interestingly, the production of such vesicles was also observed in the uninfected maize cells surrounding the penetration sites (Supplemental Fig. S1B). These observations confirmed that these vesicles are of plant origin, and it is likely that uninfected plant cells surrounding the penetration sites are anticipating the fungal attack.

The production of superoxide ions was also assayed as the oxidation and precipitation of NBT in infected maize tissue. The samples tested were collected every 12 h up to 72 hpi. The results suggest that the plant cells are not producing superoxide at any of the time points assayed, or at least at the detection limits of NBT (data not shown). However, in these assays, the production of superoxide ions was detected in some specific fungal cells 60 and 72 hpi (Fig. 4; Supplemental Fig. S1C). The staining protocol yielded positive reactions in tips of hyphae approaching the border of the plant cells and preparing to cross into the neighboring cell. Staining was also observed in fungal hyphae beginning to colonize a second cell, where the presence of superoxide was restricted to the hyphal tips in contact with the plant cell border (Fig. 4). In contrast, hyphae growing in vitro under saprophytic conditions did not show this specific and confined pattern of superoxide production (data not shown). These results suggest that a localized oxidative burst may be part of the strategy of the fast-growing fungal hyphae to pass through plant cell wall/membranes.

Construction and Analysis of Two Subtractive Suppression Hybridization cDNA Libraries

In view of the cytological and biochemical changes induced during the infection process, we sought to identify differentially expressed plant and fungal genes during the progress of infection. We prepared two subtractive suppression hybridizations (SSH) using *C. graminicola*-infected and mock-inoculated maize leaves at 48 and 72 hpi. The use of this technology has presented many limitations for the study of early stages of infection, due to the low representation of mRNA from the pathogen compared with that of the host. Thus, to increase the efficiency of the fungal cDNA recovery in our libraries, we inoculated approximately 50 7.5-μL spots (3×10^5 spores mL^{-1}) on the leaves, and a leaf disc of 5 mm containing the infected tissue was recovered with a cork borer at the different time points. We followed the infection process using a GFP-tagged strain of *C. graminicola* developed previously (Sukno et al., 2008).

The time points selected for our subtracted libraries correspond to the biotrophic stage and to the switch from biotrophic to the necrotrophic stage of disease development, and samples were collected at 48 and 72 hpi, respectively (Fig. 1). We prepared mRNA samples from approximately 3,500 (48-hpi samples) and 2,000 (72-hpi samples) infected leaf discs collected in three independent inoculation experiments. RNA integrity was assayed on agarose gels, and RT-PCR assays were conducted to detect the amplification of the β-tubulin gene from *C. graminicola* in total RNA samples (Supplemental Fig. S2). After this control, two subtracted libraries were constructed using the 48- or 72-hpi RNA samples.

A total of 309 and 348 clones from the 48- and 72-h libraries were sequenced, respectively (Table I). From all the identified clones, 50 sequences were mapped to genes encoded in the *C. graminicola* genome. Of these 50 genes, 13 cDNAs have homologs deposited in PHI-base, a database that catalogs functionally characterized virulence genes from pathogenic fungi (Baldwin

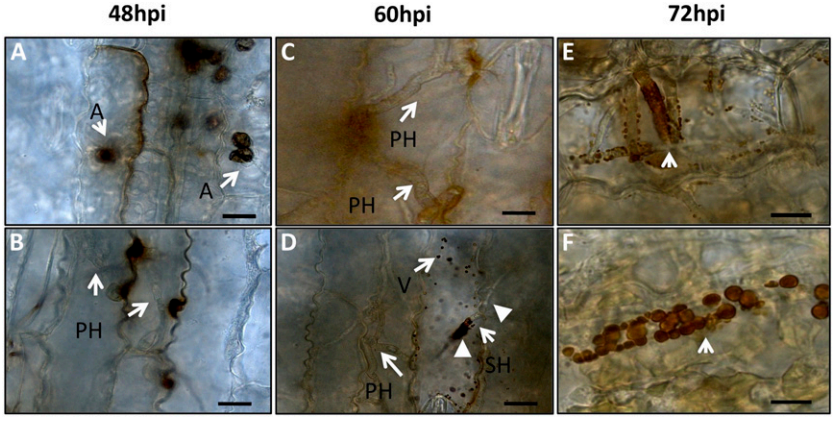

Figure 3. ROS produced in maize leaves during *C. graminicola* infection. Infection sites collected 48, 60, and 72 hpi were assayed for H_2O_2 production by DAB staining. A and B, Samples collected 48 hpi showing DAB staining in infected cells. A DAB-positive reaction is evident in the border of plant cells and beneath appressoria. C and D, Samples collected 60 hpi showing the presence of vesicle-like structures in the cytosol and surrounding fungal hyphae. E and F, At 72 hpi, the presence of vesicles is evident inside the plant cell and closely surrounding fungal hyphae (arrowheads). A, Appressorium; PH, primary hyphae; V, vesicle. Bars = 75 μm (A–D) and 10 μm (E and F).

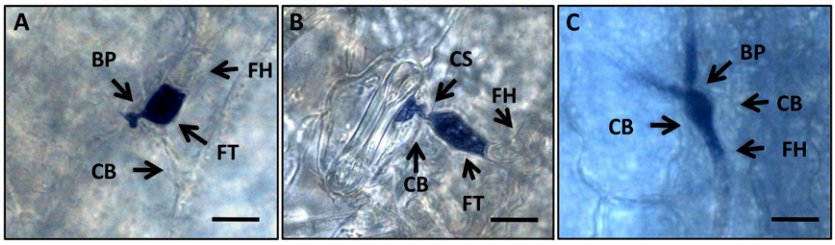

Figure 4. ROS produced by *C. graminicola* during maize infection. Infection sites collected 72 hpi were tested for superoxide accumulation by NBT staining. A and B, *C. graminicola* hyphae tips in contact with the plant cell boundaries and preparing to cross into a neighboring cell. C, Fungal hyphae branched at the crossing site and superoxide accumulation in the fungal cells in contact with the plant cell membrane. BP, Hyphal branching point; CB, plant cell boundary; CS, fungal crossing site; FH, fungal hyphae; FT, fungal tip. Bars = 80 μm.

et al., 2006; Supplemental Table S1), although none have homology to other genes previously described to be expressed in *C. graminicola* during pathogenesis. Despite that 10.7% of the proteins encoded in *C. graminicola* are predicted to be extracellular (M.R. Thon and S.A. Sukno, unpublished data), the results obtained here revealed that our libraries were enriched in secreted proteins (18% of them contained putative secretion signals), which could act as effector proteins or secreted pathogenicity factors.

In general, the most highly represented functional categories corresponded to hypothetical proteins, energy, protein metabolism, and transport (22%, 18%, 14%, and 5% of the total fungal genes, respectively). The discovery of more than 20 nonfunctionally characterized fungal genes being expressed during anthracnose development in maize brings to light potential pathogenicity factors for future functional experiments.

We also identified 216 plant genes differentially expressed at these time points (Supplemental Table S2). A large number of the plant genes differentially regulated are involved in the regulation of maize defense mechanisms, signal transduction, cell cycle, and metabolism (Fig. 5). The most outstanding difference is related to genes involved in signaling and transport, being 4.5 times more represented at the 48-hpi time point. This observation is in agreement with maize cell biochemical reprogramming to adjust to the new metabolic scenario imposed by the biotrophic fungal hypha. When *C. graminicola* initiates the necrotrophic program, it causes interference with the signaling and transport pathways and enhances the expression of carbohydrate metabolism-related messengers (Fig. 5). However, it is also possible that the changes in gene expression, in both situations, are necessary to cope with the increased protein turnover in the plant cell during the pathogenic invasion.

About 15% of the maize genes identified in the SSH libraries are related to plant defense mechanisms (Supplemental Table S2). In particular, we highlight the identification of PR1, PR5, chitinases, glucanases, and a barwin-related protein in both libraries, all of them well-known pathogenesis-related genes in plants (Wu et al., 1994; Morris et al., 1998; Muthukrishnan et al., 2001; Torregrosa et al., 2004; Zhu et al., 2006). In addition, defense-related genes previously described in other pathosystems were also identified by the SSH libraries. A homolog to a transcription factor endowed with protein phosphatase activity, previously described in tobacco (*Nicotiana tabacum*) plants as induced in response to viral attack, was detected among the cDNAs cloned 72 hpi (GRMZM2G108147). This protein was described as being involved in the regulation of the expression of genes involved in plant cell wall metabolism. A protein containing a Leu-rich region and a nucleotide-binding domain was also identified 48 hpi. In many pathosystems, proteins with similar features (NBS-LRR proteins) have been described as involved in the pathogen-recognition mechanisms and defense activation (DeYoung and Innes, 2006). A Bax-inhibitor 1 homolog (GRMZM2G479608) and plant cell death-related genes (lethal leaf spot [ZmLls]) were also identified in the libraries (Supplemental Table S2). It is also important to highlight the presence of genes related to plant carbohydrate partitioning, such as starch and Suc metabolism. In agreement, a previous report suggested the importance of carbon partition in maize cells, as the sink activity of the leaf tissue is affected by anthracnose development (Behr et al., 2010). Our libraries show the induction of the Suc transporter SUT1 (GRMZM2G034302) in the infection sites, which would lead to increased Suc mobilization during the fungal infection.

Table I. *Summary of SSH libraries*

Parameter	Hours after Inoculation	
	48	72
Sequenced clones	309	348
Total unigenes	160	107
Plant unigenes	153	63
Fungal unigenes	7	44
Library redundancy[a]	22%	40%

[a] Calculated according to Cramer et al. (2006).

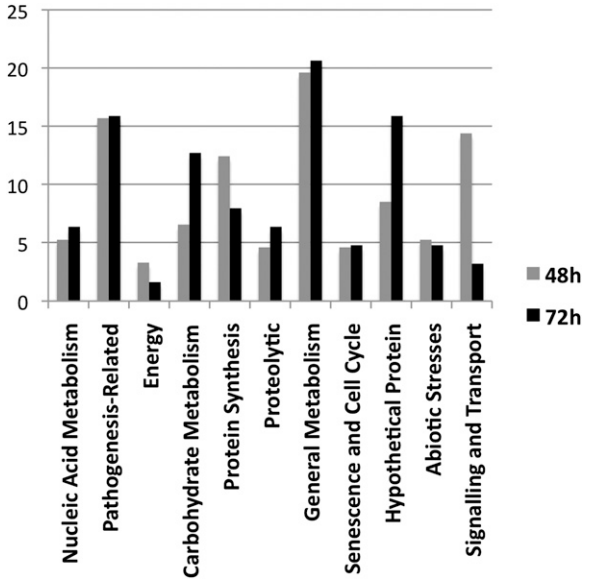

Figure 5. Functional categories of maize genes identified in SSH libraries. The cDNA sequences were used to identify the corresponding genes in the maize genome using BLAST, and the protein sequences were analyzed with InterProScan. The functional categories were manually assigned based on InterProScan results and information about the gene in the GenBank database. The vertical axis represents the percentage of genes in each functional category.

Plant Defense Mechanisms Are Induced during *C. graminicola* Infection of Maize Plants

The fact that pathogenesis-related genes are detected in the 48-hpi library suggests that, contrary to other pathosystems (Caldo et al., 2006; Jones and Dangl, 2006; Doehlemann et al., 2008), *C. graminicola* is unable to suppress the expression of classical defense-related genes at early stages of infection (including the biotrophic stage).

To confirm the up-regulation of defense mechanisms of maize at early stages of *C. graminicola* infection, we studied the mRNA levels of ZmPR1, ZmPR5, ZmPR4b, ZmP21 (a blight-associated β1-3 glucanase), class I acid chitinase (ZmAChit), wound-induced Ser protease inhibitor (ZmWind), and a lethal leaf spot protein (ZmLls). To test the rate of mRNA accumulation for this set of genes, maize plants were inoculated with 20 infection sites per leaf and samples were collected every 12 h up to 72 h for northern-blot assays.

The results confirmed that maize defense mechanisms are not down-regulated during the early stages of anthracnose establishment. In contrast, the induction of ZmPR1, ZmPR5, ZmPR4b, and ZmAChit was detected as early as 12 hpi, with a continuous increase in mRNA levels during the infection progress (Fig. 6). The expression of the other genes tested was also induced between 48 and 60 hpi. The densitometry analysis of the signals detected on the blots supports these observations (Fig. 6). The northern-blot experiments also confirmed that genes identified in both libraries are highly expressed at 48 and 72 hpi. In contrast, the expression of ZmP21 was detected 60 hpi, which is consistent with the fact that its transcripts were identified in the 72-hpi library.

The analysis of signal intensity reveals that during *C. graminicola* infection of maize plants, there is an immediate activation of plant defense mechanisms. Based on the gene expression assays, we can speculate that the maximum defense activation state is reached approximately 60 hpi, the time when a clear differentiation of secondary hyphae of *C. graminicola* can be observed (Supplemental Fig. S3).

The Effect of Exogenous Abscisic Acid in the Lifestyle Change of *C. graminicola*

Our assays revealed an up-regulation of genes related to pathogen attack as well as several abscisic acid (ABA)-responsive genes, such as GRMZM2G052100, GRMZM2G145461, and ZmAChit (Figs. 1–6; Supplemental Table S2). ABA treatment has been reported to increase the susceptibility of pepper (*Capsicum annuum*) fruits to anthracnose (Hwang et al., 2008). Based on the observation by Hwang et al. (2008) as well as on the presence of ABA-responsive genes in our libraries, we hypothesize that ABA may alter plant defense mechanisms and contribute to anthracnose development in maize. To further explore the influence of ABA on plant defense, we tested the effect of exogenous ABA on the *C. graminicola* infection process and the expression of defense genes in maize leaves. Maize plants were infected with 20 droplets containing 650 spores each on the adaxial side of the maize leaf. Once the spores germinated and initiated host penetration (24 hpi), leaves were sprayed with 2 mL (and watered with 5 mL) of a 100 mM solution of ABA. Control plants, consisting of noninfected plants and infected plants, were treated with sterile water instead of the ABA solution.

After the treatments, anthracnose development was evaluated in leaves, and the timing for the fungal switch to the necrotrophic lifestyle was monitored. Since *C. graminicola* growing on the leaves were in contact with ABA, we corroborated in vitro whether this compound exerts any effect on fungal growth. A spore suspension of *C. graminicola* was inoculated in the center of potato dextrose agar (PDA) plates supplemented with 10 or 100 mM ABA. Colony size was measured during 4 d, and no difference was found between the treatments and controls (no ABA addition), suggesting that ABA per se has no apparent effect on *C. graminicola* growth (Supplemental Fig. S4).

The in planta experiment revealed that ABA treatment caused profound effects on the timing of secondary hyphae development, lesion formation, and lesion growth (Fig. 7). Microscopic analysis showed that 85% of the infection sites displayed a massive development of secondary hyphae 48 hpi on ABA-treated plants, while the fungal hyphae in the control plants continued their biotrophic program (Fig. 7A).

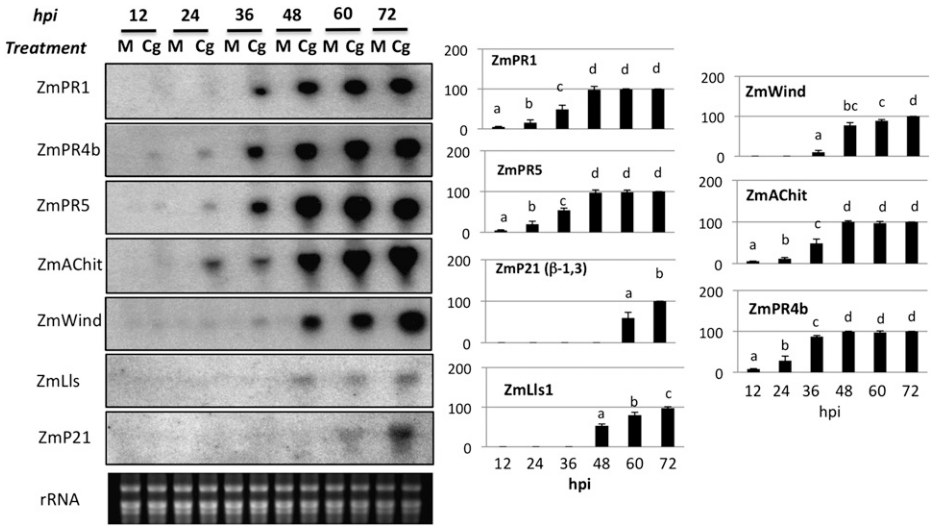

Figure 6. Time-course experiment to determine the mRNA level of maize genes during *C. graminicola* infection. Maize leaves were collected every 12 h (up to 72 h) after infection with *C. graminicola* spores. Total RNA samples were blotted and hybridized with the corresponding probes. Signal intensities were quantified using the MCID analysis software, and the values are shown in the plots. The values were normalized to the signal intensity at 72 hpi. The values shown are averages of two independent experiments with SE bars. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 1%. M, RNA samples from mock-inoculated leaves; Cg, RNA samples from leaves infected with *C. graminicola*.

Also, in order to have an estimation of the degree of disease progress, at 48 hpi we estimated a disease index on a scale of 0 to 3 (0 = no evidence of symptoms, 3 = symptoms indicating chlorosis and maceration; Supplemental Fig. S5). The quantitative analysis indicated a 3 times higher disease index in ABA-treated plants (Fig. 7B). These differences in disease incidence were also observed when the lesion size was compared 72 hpi (data not shown). In parallel, gene expression analysis revealed no suppression of a set of defense-related genes. In fact, ABA displayed an additive effect on the expression of two of the resistance-related genes (PR1 and ZmChitI) and ZmWind (wounding induced) at early stages of *C. graminicola* infection (Fig. 7C; Supplemental Fig. S6). Together, northern-blot assays and microscopic analyses indicate that ABA signaling affects the expression of some resistance genes and that the increased susceptibility to *Colletotrichum* infection in maize is due to a premature transition to the necrotrophic lifestyle.

C. graminicola Genes Expressed during Maize Infection as Novel Candidates for Pathogenicity Factors

Fungal genes identified in the SSH libraries are expected to be the most highly expressed in the fungus. We identified a total of 50 *C. graminicola* genes being expressed during early anthracnose development. Among those genes, we found that 16 of them were annotated as hypothetical proteins in the *C. graminicola* genome database (Supplemental Table S1). However, sequence analysis and BLAST searches revealed that six of them display similarity to functionally characterized genes, but 10 genes still remain to be functionally characterized (Table II). Of the 10 hypothetical proteins, eight have homologs only in fungal species and two (GLRG_08002 and GLRG_06140) are unique to *C. graminicola*. Further sequence analysis revealed that four of them (GLRG_00597, GLRG_02577, GLRG_04925, and GLRG_05464) contain putative signals for secretion or retention in the plasma membrane. BLAST searches on public databases revealed that seven of the sequences are highly conserved mostly in plant pathogenic species. It is likely that the functions of these novel 10 genes are related to *C. graminicola* pathogenicity and anthracnose disease development.

C. graminicola Differentially Expressed Genes

Histological studies revealed an active production of ROS in both the plant and the fungal cells (Figs. 2 and 3), but no fungal ROS-producing or -detoxifying genes were detected in the SSH libraries. However, we did detect five fungal genes related to respiration (Supplemental Table S3). In part, ROS are side products of electron transport during respiration, and an elevated respiration rate might contribute to the accumulation of superoxide ions detected by NBT staining (Fig. 3). In consequence, using the same RNA samples as in the northern blotting (Fig. 6), we assayed the expression profiles of the genes putatively involved in respiration. GLRG_00654, GLRG_03787, GLRG_06039, GLRG_07547, GLRG_08990, and GLRG_10407. With the exception of GLRG_06039, the expression profiles confirmed the importance of fungal respiration at early stages of anthracnose development. According to the RT-PCR assays, the highest accumulation of mRNA for the respiration-related genes was detected in infection sites collected 60 hpi (Fig. 8A). This expression

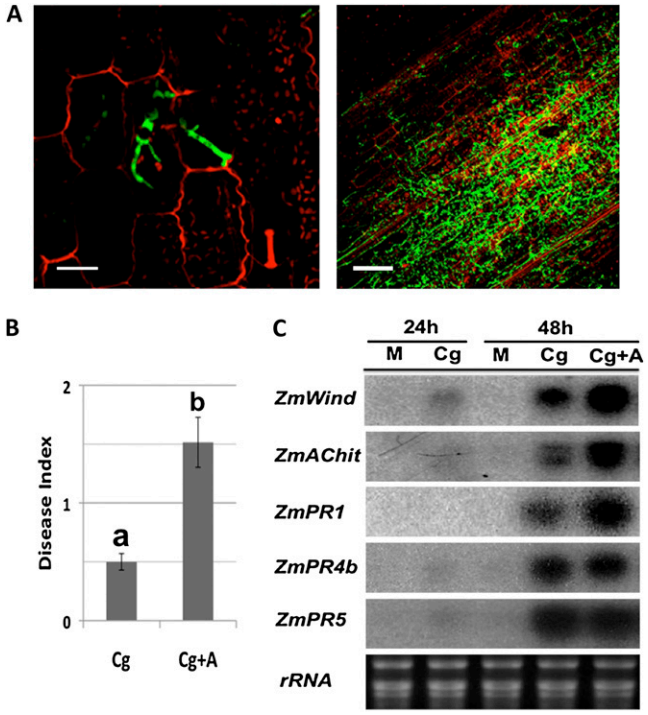

Figure 7. Anthracnose progress in maize plants treated with ABA. Maize plants were treated with 0.1 mM ABA 24 hpi, and disease development was evaluated 24 h later. A, Confocal microscopy of the infection sites 48 hpi in control plants (left panel; bar = 80 μm) and treated plants (right panel; bar = 300 μm). B, The disease index was determined in control and treated plants 48 hpi. The bars represent averages of two experiments with SE bars. Bars with different letters differ significantly according to Tukey's HSD test at a significance level of 1%. C, Northern blotting of defense-related genes in control and ABA-treated plants 24 and 48 hpi. M, RNA samples from mock-inoculated leaves; Cg, RNA samples from leaves infected with *C. graminicola*; Cg+A, RNA samples from leaves infected with *C. graminicola* and treated with ABA.

pattern suggests that a respiration burst occurs during the early stages of anthracnose development. In fact, the highest gene expression is correlated with the detection of superoxide ions in fungal hyphae (Fig. 3). Regardless of whether fungus-produced ROS is a side product of increased respiratory activity or is specifically produced during infection, these results highlight the important role of fungus-produced ROS in the disease development program of *C. graminicola*.

A significant number of fungal genes encoding hypothetical and unknown proteins have been identified in the libraries. We selected three of them to confirm their in planta expression profile: GLRG_06140 and GLRG_08002 were chosen, as they are two hypothetical proteins exclusive of *C. graminicola*; and GLRG_00597, a hypothetical small protein containing a putative secretion signal. We also characterized the expression pattern of GLRG_06543, encoding a putative secreted metalloprotease named fungalysin (belonging to the metalloprotease family M36), which in animal pathogenic fungal species has been suggested as important for pathogenicity (Rosenblum et al., 2008; Mathy et al., 2010).

The fungalysin and GLRG_00597 displayed a particular expression pattern with a maximum accumulation of mRNA simultaneously with the switch to the necrotrophic lifestyle (Fig. 8C). The *C. graminicola*-specific gene GLRG_06140 is highly expressed at very early stages of infection (during biotrophic growth), as its expression was first detected 36 hpi with maximum expression at 48 hpi (Fig. 8B). The expression of gene GLRG_8002 begins at 24 hpi but appears to be strongly up-regulated by 36 hpi and continues to be expressed after the switch to necrotrophy. These results confirm the functional expression of these hypothetical proteins, and their differential expression during the infection process suggests their involvement in specific processes at early stages of anthracnose development.

DISCUSSION

In this article, we investigated the development of anthracnose, one of the most destructive plant diseases worldwide (Bailey and Jeger, 1978; Perfect et al., 1999; Dickman, 2000; Prusky et al., 2000; Latunde-Dada, 2001). Like many species of *Colletotrichum*, *C. graminicola* is a hemibiotroph, and during the initial stage of infection, it colonizes host tissue intracellularly without causing host cell death. Most biotrophs achieve biotrophic growth by actively suppressing the disease responses of the plant. To understand whether this is also the case for *C. graminicola* and to further characterize the early stages of this interaction, we performed histological studies and transcriptional profiling on infected tissue at early stages of fungal infection. Our results highlight important metabolic changes in the plant and the fungus, the induction of plant defense mechanisms during the infection process (including the biotrophic stage), the involvement of plant- and fungus-produced ROS, and the participation of ABA-signaling pathways in the responses triggered in maize during *Colletotrichum* infection.

The general foliar resistance response of maize to *C. graminicola* involves a non-cultivar-specific biosynthesis of phenolic compounds and lignin deposition (Lyons et al., 1990; Bergstrom and Nicholson, 1999). In agreement, our histological studies revealed the activation of defense mechanisms during the interaction of *C. graminicola* with leaves of a highly susceptible maize inbred line. These observations included the accumulation of antimicrobial compounds such as phytoalexins, phenylpropanoid intermediates, and H_2O_2 during biotrophic growth of *C. graminicola* (Figs. 2 and 3). Based on our results, we conclude that, unlike biotrophic pathogens, *C. graminicola* induces classical plant defense responses, even during the biotrophic stage of development. Since *C. graminicola* is still able to cause disease, even in the presence of an active defense response from the plant, we hypothesize that

Table II. *Uncharacterized C. graminicola genes expressed during pathogenesis*
Boldface data indicate sequences unique to *C. graminicola*.

GLRG No.	Genome Annotation	InterPro	Homolog	GenBank Accession No.
48-hpi library				
08002	**Hypothetical protein**	**No match**	**No match**	**No match**
72-hpi library				
00597	Hypothetical protein	No match	*Nectria haematococca*	XP_003053926
05251	Hypothetical protein	RGP1	*A. fumigatus*	XP_755570
10581	Hypothetical protein	No match	*Verticilium albo-atrum*	XP_003000779
00894	Hypothetical protein	No match	*N. haematococca*	XP_003053961
02035	Hypothetical protein	DNA binding	*V. albo-atrum*	XP_003005657
02577	Hypothetical protein	No match	*Aspergillus oryzae*	XP_001819804
03767	Hypothetical protein	No match	*N. haematococca*	XP_003051842
04232	Hypothetical protein	Protein kinase SNF1	*Fusarium oxysporum*	AF420488_1
04925	Hypothetical protein	No match	*M. oryzae*	XP_364632
05464	Hypothetical protein	No match	*Plasmodium falciparum*	XP_001348500
07547	Hypothetical protein	Methyl citrate synthase	*N. hematococca*	CAZ64274
10807	Hypothetical protein	TSG24 family	*Paracoccidioides brasiliensis*	XP_002792213
05185	Hypothetical protein	No match	*V. albo-atrum*	XP_003004080
06140	**Hypothetical protein**	**No match**	**No match**	**No match**
10108	Hypothetical protein	HSP70	*V. albo-atrum*	XP_003007453

plant defenses are either partially activated or there are other responses not reported by the markers we used in our experiments that are suppressed during infection.

To identify metabolic changes and differentially expressed genes at early stages of anthracnose development in a highly susceptible maize line (Mo940), two SSH libraries were constructed using mRNA samples prepared 48 and 72 hpi. Previous attempts to use this methodology at early stages of infection had only limited success due to the low representation of pathogen mRNA in the samples (Sugui and Deising, 2002; Bittner-Eddy et al., 2003). However, we developed a strategy to increase the fungal mRNA ratio in the samples, which consisted of the manual recovery of the infection sites for further mRNA preparations. In addition to this enrichment process, to increase the reliability of our results we also avoided the use of detached leaves that could introduce artifacts due to cross talk between senescence-, wounding-, and pathogen-related signaling pathways (Politis and Wheeler, 1973; Mims and Vaillancourt, 2002; Liu et al., 2007).

We identified more than 200 genes that are differentially expressed in maize during the early stages of *C. graminicola* infection (Fig. 5; Supplemental Table S2). The efficiency of subtraction and the use of SSH in cloning differentially expressed genes was confirmed, since highly expressed maize genes such as ribulose-1,5-biphosphate carboxylase and chlorophyll *a/b*-binding protein were not retrieved in the libraries. Also, gene expression profiles of a set of selected genes further validated the identification of cDNAs from differentially expressed maize genes during *C. graminicola* infection (Fig. 6).

It is interesting to highlight the finding of a number of cDNAs involved in signal transduction and the regulation of the maize cell cycle and defense mechanisms (Fig. 5). A significant number of maize cDNAs encoding proteins related to protein folding and turnover (heat shock proteins, proteosome-associated and ubiquitination-related factors) were identified in our libraries. This observation demonstrates that plant protein stability is compromised during the pathogenic attack, and the recycling of protein might supply resources for the synthesis of plant disease-related proteins. Also, several maize genes involved in gene expression regulation and signal transduction that are probably important for anthracnose development were identified. They include various proteins belonging to the MYB, NAC, and zinc-finger family of transcription factors, protein phosphatases, and protein kinases. Protein members belonging to these families of regulatory factors have been related to cellular morphogenesis, plant defense activation, diverse development processes, and responses mediated by ABA signaling pathways (Seki et al., 2002; Rabbani et al., 2003; Buchanan et al., 2005; Supplemental Table S4). The genes encoding putative proteins involved in the signaling networks provide a starting point for the further biochemical characterization of responses induced in maize during anthracnose leaf blight development.

It is of particular interest to highlight the induction of defense-related genes at early stages of infection, beginning at 12 hpi, and during the infection process, including the whole biotrophic stage. *C. graminicola* initially colonizes the plant leaf biotrophically and later switches to a necrotrophic lifestyle. It has been well established that biotrophic pathogens such as *Uromyces vignae* and *U. maydis* as well as hemibiotrophs such as *M. graminicola*, during the biotrophic phase, must suppress or attenuate host defenses to be able to parasitize the invaded host cells (Panstruga, 2003; Caldo et al., 2006; Jones and Dangl, 2006; Adhikari et al., 2007; Doehlemann et al., 2008; Eich-

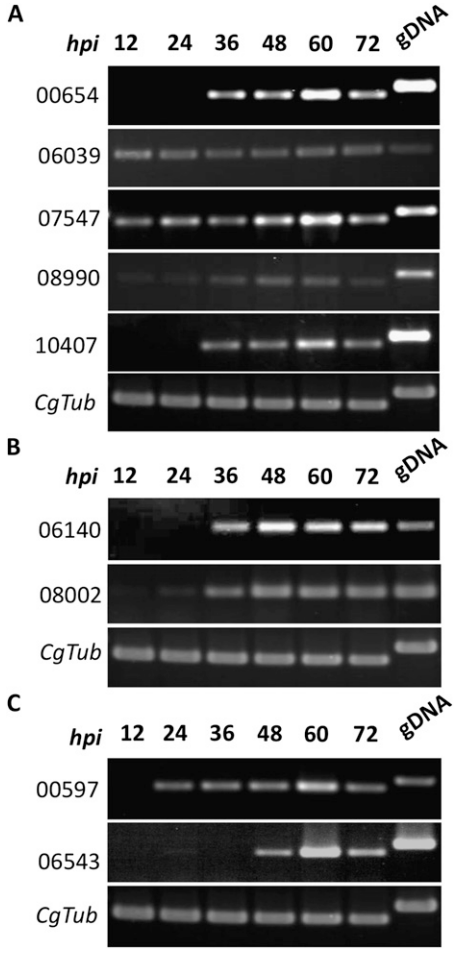

Figure 8. Time-course expression of *C. graminicola* genes identified in the SSH libraries. Due to the low representation of fungal mRNA in the samples, semiquantitative RT-PCR assays were conducted to test the expression of selected fungal genes. The amount of total RNA used in each PCR was adjusted to the amount needed to provide equal amplification levels of *C. graminicola* β-tubulin (*CgTub*) in all samples. PCR products were visualized after electrophoresis on agarose gels and ethidium bromide staining. A, RT-PCR products corresponding to genes involved in respiration. B, Genes encoding proteins of unknown function. C, Putative secreted effectors GLRG00597 and fungalysin (GLRG06543). The numbers next to each panel represent the Broad Institute-assigned identifier numbers in the genome annotation. The number of cycles of PCR was optimized to be in the linear amplification range of each gene. These assays were performed three times using RNA samples from three independent experiments.

mann and Hückelhoven, 2008). Microarray studies revealed that in maize infected with *U. maydis*, there is a transient activation of defense mechanisms (including the up-regulation of 34 defense-related genes) 12 hpi that are later attenuated upon the establishment of biotrophic growth (Doehlemann et al., 2008). However, transcriptomic studies on a hemibiotrophic interaction, between *C. trifolii* and *M. truncatula*, revealed no major differences in defense gene expression during the early stages of infection, up to 72 hpi (Torregrosa et al., 2004).

The *M. oryzae*-rice pathosystem establishes a hemibiotrophic interaction that has been well studied and characterized at the molecular and histological levels. Two independent transcriptional studies, performed by two different groups, suggest that during foliar infection, basal defense-related gene transcripts continued to increase in abundance during the infection process (including biotrophic and necrotrophic stages; Mosquera et al., 2009; Marcel et al., 2010). We show that, in contrast to the biotrophic maize pathogen *U. maydis* (Doehlemann et al., 2008), from early stages of this hemibiotrophic interaction (even before host penetration) defense-related genes are activated and their expression increases during disease development (Fig. 6). The set of genes tested in our time-course expression experiment included PR1, PR4b, PR5, a chitinase, and β1-3 glucanase, whose homologs are up-regulated in rice upon *M. oryzae* infection (Mosquera et al., 2009; Marcel et al., 2010) but repressed upon establishment of the biotrophic growth of *U. maydis* (Doehlemann et al., 2008). Together, our results and those reported by Doehlemann et al. (2008) demonstrate that maize leaves respond differentially to biotrophic and hemibiotrophic pathogens, and a similar behavior might be extrapolated for rice based on the results reported by Mosquera et al. (2009) and Marcel et al. (2010).

The involvement of phytohormones in plant-pathogen interactions has been studied in many pathosystems (Bari and Jones, 2009). Several reports have specifically suggested the involvement of ABA in the regulation of plant defenses (Agrawal et al., 2001; Jiang and Zhang, 2001; Anderson et al., 2004; Ameline-Torregrosa et al., 2006; Schmidt et al., 2008; Wang et al., 2012). Microarray analysis to study the effects of ABA on gene expression in Arabidopsis, rice, and sorghum (*Sorghum bicolor*) plants revealed that a set of defense-related genes are induced after treatment with the phytohormone (Seki et al., 2002; Rabbani et al., 2003; Buchanan et al., 2005). In agreement, a class I acid chitinase from maize is induced in maize callus cocultured with *Aspergillus flavus* or 100 mM ABA (Wu et al., 1994). In this article, we present evidence that ABA-responsive genes are induced in maize during the early stages of anthracnose development (Figs. 6 and 7; Supplemental Table S4). In particular, the results depicted in Figure 7 suggest an additive effect of both *C. graminicola* infection and ABA treatment on the induction of PR1, ZmAChit, and ZmWind genes.

The involvement of ABA in anthracnose development had been previously demonstrated in *Colletotrichum acutatum*-infected pepper fruits, where the enhanced susceptibility of the host was shown to be correlated with high ABA concentrations (Hwang et al., 2008). We obtained similar results in the *C. graminicola*-maize pathosystem, where an increased susceptibility in leaves to anthracnose development was observed after treatment with the phytohormone (Fig. 7). Microscopic analysis revealed that the physiological changes induced in maize by ABA caused a premature switch to the production of secondary hyphae initiating an early necrotrophic lifestyle (Fig.

7). Thus, the increased disease response caused by ABA is correlated with a premature switch to necrotrophy and increased disease.

The most outstanding discovery in the histological studies was the detection of maize-produced vesicles loaded with H_2O_2, as indicated by DAB staining (Fig. 3). The production of these vesicular bodies was paralleled by the induction of the expression of seven maize genes (including ras-like GTPase, Rab-GTPase, and a SNARE-like superfamily protein) involved in the regulation of vesicle sorting and transport (Supplemental Table S2). Recent findings in eukaryote organisms, including plants, showed that vesicle trafficking plays an important role in stress responses (Cavalli et al., 2001; Kargul et al., 2001; Levine et al., 2001; Mazel et al., 2004). One of the major vesicle-trafficking pathways in plants is the transport of vesicles to a central lytic vacuole (Okita and Rogers, 1996; Vitale and Raikhel, 1999). It was demonstrated that a member of the SNARE-like superfamily in Arabidopsis is involved in the formation of H_2O_2-containing megavesicles in response to salt stress (Leshem et al., 2006). Many strategies to cope with abiotic stresses are shared with pathogen defense mechanisms, and this also seems to be the case for the vesicle-mediated accumulation of H_2O_2. This was also evident during the infection of barley (*Hordeum vulgare*) plants by the biotrophic fungus *Blumeria graminis*, where multivesicular bodies containing H_2O_2 were demonstrated as a plant cell wall-associated defense mechanism (An et al., 2006). In contrast to barley, maize plants developed an oxidative vesicle system targeted against *C. graminicola*, and the effect of the vesicles on the fungus still remains to be fully investigated.

In plants, the accumulation of ROS in the infection sites is one of the primary responses during pathogen attacks (Apostol et al., 1989; Nürnberger et al., 2004). To survive in harsh environments and successfully invade host cells, pathogens had to develop mechanisms to scavenge ROS and protect against ROS-induced damage (Miller and Britigan, 1997; Moye-Rowley, 2003; Apel and Hirt, 2004; Lev et al., 2005; Molina and Kahmann, 2007; Temme and Tudzynski, 2009; Guo et al., 2010; Williams et al., 2011). Not much is known about ROS detoxification in *Colletotrichum* species or plant defense mechanisms during host infection and anthracnose development. The expression assays presented in Figure 6 and the timing of the developmental process of *C. graminicola* on maize leaves (Bergstrom and Nicholson, 1999; Münch et al., 2008; Supplemental Fig. S4) suggest a correlation between the timing of the highest activation of defense mechanisms and the switch to the necrotrophic lifestyle. Also, our experiments suggest that the developmental program of *C. graminicola* may depend on the physiological status of the plant (Figs. 6 and 7). As the fungus does not suppress the classical defense pathways, the plant cells create a highly defensive environment at very early stages of the infection, generating oxidative vesicles and inducing defense-related genes. In consequence, the biotrophic hyphae are exposed to detrimental conditions that would constrain host colonization. However, the ability of *C. graminicola* to differentiate secondary hyphae allows the fungus to kill plant cells rather than growing within living plant cells. This change in the lifestyle of the fungus may enable it to escape direct contact with living plant tissues and the defense molecules that they produce. Thus, the switch to necrotrophic growth may be seen as a mechanism to avoid direct contact with defense compounds, including the ROS induced in the host. This observation is further supported by the high susceptibility of primary hyphae to plant defenses. Mutants of *C. graminicola*, *Colletotrichum lindemuthianum*, and *C. higginsianum*, impaired in the switch from biotrophy to necrotrophy, only produced primary hyphae and were not able to colonize more than a limited number of cells in the host, and no anthracnose development was evident (Dufresne et al., 2000; Thon et al., 2002; Huser et al., 2009). In those cases, plant defenses do successfully restrict the growth of *C. graminicola* and *C. higginsianum* mutants arrested in the biotrophic stage. We speculate that the inability to differentiate secondary hyphae does not allow the fungus to kill the host cells and that the plant defense mechanisms succeed in limiting biotrophic hyphae to spread into the host.

In addition to the 10 sequences with homologs annotated in PHI-base as pathogenicity factors, the fungal genes identified in the SSH libraries also include 16 genes encoding hypothetical proteins conserved in pathogenic fungi. Two of them (GLRG_08002 and GLRG_06140) are unique to *C. graminicola*, as no homolog was detected in public databases. These novel genes, induced at early stages of infection (Fig. 8), may represent novel pathogenicity factors for future functional characterization.

We identified nine secreted proteins that could act as effectors during the early stages of anthracnose development. Effectors are proteins secreted by the pathogens that can interfere with the host metabolism and are important for pathogenicity (Kamoun, 2007; Ellis et al., 2009). The expression of one such effector candidate, GLRG_00597, was evident at 24 hpi and further increased as infection progressed, with a peak of expression 60 hpi. A secretion signal was identified with a cleavage site between resides 20 and 21. The gene is predicted to encode a mature protein rich in Gly, Ala, and Ser (24%, 14.4%, and 9.6%, respectively) with a calculated mass of 28,377 D. Another structural feature is the presence of a repetition of seven GGS motifs, which can also be found in a pathogenicity factor from *M. oryzae* (MGG_11899; PHI: 773; Jeon et al., 2007). Among the candidate effectors, we also found the metalloprotease fungalysin (GLRG_06543). This is a single-copy gene whose expression is enhanced during the switch to the necrotrophic lifestyle, with a maximum expression level that occurs simultaneously with the switch in lifestyle (60 hpi). Fungalysin belongs to a family of zinc-dependent proteases

that has been suggested as part of host invasion mechanisms by *Aspergillus fumigatus* and *Microsporum canis* (Brouta et al., 2002; Jousson et al., 2004; Rosenblum et al., 2008; Mathy et al., 2010). In fungi, extracellular proteases usually serve two roles. The first is general proteolysis, providing nutrients that are taken up by the fungus, and the second is degrading host tissues, enabling the fungal mycelium to expand into the substrate. GLRG_06543 has no clear homology to genes that are known to be effectors in plant pathogens, but other metallopeptidases are known to be fungal effectors, most notably Avr-Pita of *M. oryzae* (Jia et al., 2000; Orbach et al., 2000). Together, the involvement of fungalysin in the pathogenicity of animal fungal species and the characteristic expression pattern in *C. graminicola* suggest an important role of this enzyme for fungal infection and anthracnose development. Further experiments, such as the development of mutant strains and functional characterization of this gene, are being conducted to elucidate the role of fungalysin during maize anthracnose development.

The *C. graminicola* genes identified in this study include a set of putative respiration-related genes, suggesting a respiratory burst during the maize infection. While these genes are expressed during the entire infection process, there is a peak of expression 60 hpi that coincides with the change in lifestyle according to histological studies (Supplemental Fig. S3). More importantly, assays to detect ROS accumulation revealed the specific accumulation of superoxide ions with increased concentrations at hyphal sites preparing to pass through the plant plasma membrane and cell wall (Fig. 3). The detection of this confined accumulation of ROS is observed simultaneously with the peak of expression of respiration-related genes. This physiological behavior of the fungus can be attributed to an enhanced energy requirement to support the change in lifestyle, and the production of superoxide to colonize neighboring uninfected plant cells might be necessary for the necrotrophic hyphae to disrupt plant membranes and cell walls (Dhindsa et al., 1981; Vianello and Macrì, 1991; Van Breusegem and Dat, 2006).

The accumulation of ROS in fungal tips was also evident in the endophytic association of *Epichloe festuca* and ryegrass (*Lolium perenne*), where the mutualistic association, and the fungal growth inside the plant, are controlled by a localized burst of ROS at fungal tips (Tanaka et al., 2006). Also, the phytopathogenic species *Botrytis cinerea* and *M. oryzae* generate spatiotemporal spike accumulations of ROS, such as the accumulation of superoxide in hyphal tips of *B. cinerea* and appressoria of *M. oryzae* during plant pathogenesis (Egan et al., 2007; for review, see Heller and Tudzynski, 2011). Recent reports suggest that fungal Nox genes are responsible for the specific and localized accumulation of ROS (Heller and Tudzynski, 2011). Even though in the *C. graminicola* genome there are two putative *nox* genes (GLRG_02946 and GLRG_09327), neither of them was identified among the cDNAs cloned in the libraries. However, the differential expression of respiration-related genes demonstrated by RT-PCR assays was paralleled by the accumulation of superoxide in *C. graminicola* hyphal tips (Fig. 3). In part, the accumulated superoxide compounds could be by-products of enhanced mitochondrial activity and respiration rates. Future work will lead us to a better understanding of ROS generation and Nox protein function in *C. graminicola* pathogenesis.

In this article, we present a comprehensive study on the development of anthracnose, one of the most devastating plant diseases affecting agriculture. The study presented in this article not only contributes to our understanding of the molecular bases that rule maize leaf blight anthracnose but also sheds some light on the biochemical process activated in a hemibiotrophic interaction. Our findings uncover novel areas of research that will allow a better understanding of hemibiotrophic interactions, the regulation of changes in fungal lifestyles, host responses, and plant disease development.

MATERIALS AND METHODS

Maize Plants and *Colletotrichum graminicola* Growth

C. graminicola wild-type strain M1.001-BH (also reported as CgM2; Forgery et al., 1978) and its derivative GFP-tagged strain (Sukno et al., 2008) were used for the experiments presented in this article. Cultures were maintained at 23°C on PDA medium (Difco Laboratories) with continuous illumination under white fluorescent light. Liquid cultures were incubated with orbital shaking in Fries' medium (complete medium; Vaillancourt and Hanau, 1992) or minimal medium supplemented with 1% Suc (Horbach et al., 2009).

In Vivo Quantitative Analysis of Anthracnose Development on Maize Plants

The *C. graminicola* cultures used for maize (*Zea mays*) infection assays were grown for 15 to 20 d on PDA as described previously (Sukno et al., 2008). Conidia were recovered from plates, filtered using cheesecloth, and washed three times in sterile distilled water. Conidia were counted using a hemacytometer, and the spore suspension was adjusted to 6.5×10^4 conidia mL^{-1}. To inoculate plant leaves, Tween 20 was added to the spore suspension to a final concentration of 0.005%.

The highly susceptible maize inbred line Mo940 (Warren, 1975; Nicholson and Warren, 1976) was cultured in a greenhouse for 2 weeks (V3 developmental stage) in Ray Leach Cone-Tainers (approximately 5 cm × 15 cm; Stuewe and Sons). For inoculations, the plants were placed on their side on trays and the leaves were taped onto moist paper towels. *C. graminicola* was inoculated on the third leaf from each plant, placing 10-μL droplets containing 650 conidia on the adaxial side (away from the midvein). The position of each infection site was marked for future reference. The trays were sealed with plastic wrap to preserve moisture and incubated for 18 h at 23°C. After incubation, the plastic wrap was removed, the plants were left undisturbed for several hours to allow the droplets to dry, and then the plants were returned to their upright position and transferred to a growth chamber (25°C, 50% humidity, and 600 $\mu E\ m^{-2}\ s^{-1}$). Using this assay, quantitative differences in virulence can be measured by recording disease index and lesion development and tested using a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test (http://faculty.vassar.edu/lowry/VassarStats.html).

Infection Progress after ABA Treatment of Maize Plants

To assess the effects of ABA on the developmental program of *C. graminicola* and the progress of anthracnose disease, we induced the expression of plant defense mechanisms by the application of 100 mM ABA (Sigma-

Aldrich; Wu et al., 1994). A solution of ABA was sprayed directly on maize leaves 24 hpi, and plants were further watered with 5 mL of the same solution. Mock treatments were performed on control plants, where water was used instead of the ABA solution. The treatments were repeated 48 hpi. Disease progress was monitored 48 and 72 hpi as mentioned above, and fungal growth was followed by microscopic observations.

At earlier stages of development (up to 48 hpi), the anthracnose progress was measured using an arbitrary scale ranging from 0 to 3 (no symptom observed to chlorotic infection sites with early necrotic symptoms, respectively). The arbitrary scale is exemplified in Supplemental Figure S5. Each treatment, consisting of 20 spots per leaf per plant, was replicated three times, and the experiment was repeated two times. The results were tested using a one-way ANOVA followed by Tukey's HSD test.

Preparation of RNA Samples and Library Construction

For the construction of subtractive libraries, maize plants were inoculated as described above but using a spore suspension of 3×10^5 spores mL^{-1}. In this way, we increased the representation of fungal mRNA in the samples. Total RNA from infection sites was extracted using TRIZOL reagent (Gibco-BRL) following the protocol provided by the manufacturer. RNA quality was verified after electrophoresis on agarose gels with ethidium bromide staining.

Poly(A^+) RNA from samples collected 48 or 72 hpi was used as the driver and poly(A^+) RNA from mock-inoculated leaves was used as the tester for the construction of two SSH libraries. For this purpose, the PCR-select cDNA subtraction kit (BD Biosciences Clontech) was used following the manufacturer's instructions. Amplified fragments after a second round of PCR (using subtracted cDNA as a template) were ligated into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5a cells. The resulting clones were sequenced to determine sequence identity.

cDNA sequences were assembled into contigs using Geneious software (http://www.geneious.com/). To identify the full-length gene model for each transcript, the assembled sequences were aligned to the predicted gene models for the genomes of *C. graminicola* and maize (http://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html and http://www.maizesequence.org/index.html, respectively). The protein sequence of each gene model was searched for conserved domains with InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and for similarity to functionally characterized proteins deposited in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) database. The *C. graminicola* sequences were also compared with the Pathogen-Host Interactions database of experimentally verified pathogenicity genes (PHI-base; Baldwin et al., 2006). Putative secretion signals and transmembrane helices were predicted using SignalP and TMHMM (Krogh et al., 2001; Bendtsen et al., 2004).

Nucleic Acid Blotting and Hybridizations

Northern-blot analyses were performed using Hybond-N^+ membranes (Amersham Biosciences) according to the manufacturer's suggestions. Probes for northern-blot assays were obtained after *Eco*RI digestion of plasmid generated after cloning the products of the subtractive libraries, which had been sequenced. The purified probes were radioactively labeled with [^{32}P] dCTP using the RadPrime DNA Labeling System (Invitrogen) and then used for blot hybridization using ULTRAhyb Ultrasensitive Hybridization buffer (Ambion) following the protocol recommended by the manufacturer. Signal intensities were quantified using the MCID analysis software and tested with a one-way ANOVA followed by Tukey's HSD test.

RT and PCR Assays

cDNA synthesis was performed using 5 μg of total RNA, Moloney murine leukemia virus-reverse transcriptase (Promega), and oligo(dT) primers. Prior to the RT, RNA samples had been treated with Turbo DNA-Free DNase (Ambion) to remove trace amounts of genomic DNA.

For fungal gene expression analysis, semiquantitative RT-PCR experiments were carried out. Following RT of RNA, PCR was performed using specific primers for each gene assayed. The amplification of the constitutively expressed tubulin and GAPc genes from *C. graminicola* and maize, respectively, was used as loading and RT controls. PCR was performed in the linear range of product amplification that is between 30 and 35 cycles, due to the low amount of fungal mRNA in the samples. To confirm the absence of genomic DNA contamination, RT-PCR assays were performed in reactions where the reverse transcriptase was omitted. PCR products were visualized after electrophoresis on agarose gels and staining with ethidium bromide. Primers used for the PCR are listed in Supplemental Table S5.

Histochemical Analysis of Compatible Maize-*Colletotrichum* Interactions

Infection sites were assessed for the production of H_2O_2, superoxide ions, and phenolic compounds ascribed as part of plant defense mechanisms in response to pathogen attack. To detect peroxide, leaf pieces were vacuum infiltrated for 5 min with 2 mg mL^{-1} DAB dissolved in 0.1 M phosphate buffer, pH 6.8 (Thordal-Christensen et al., 1997). After infiltration, the samples were incubated 1 h at 25°C in darkness. The DAB solution was removed, and the leaf pieces were washed with distilled water. To stop the reaction and fix the tissue, the samples were boiled for 5 min in 96% ethanol. To detect superoxide ions, a similar staining protocol as for DAB was followed, but using a NBT solution (0.5 mg mL^{-1} in the same buffer) as the staining solution.

Toluidine blue O has been widely used for the detection of phenolic compounds and the lignification of plant tissue (O'Brien et al., 1964). To visualize phenolic compounds and lignin deposition, infection sites were fixed/cleared after 5 min of boiling in 95% ethanol. The samples were incubated overnight in fresh ethanol, and the following day the samples were infiltrated for 1 min with a 0.5 mg mL^{-1} toluidine blue O solution prepared in 0.1 M phosphate buffer, pH 6.8. The stained tissue was washed with tap water and mounted for microscopic observation.

Extraction and Quantification of Phenolic Compounds

The production of phenolic compounds was initially monitored by fluorescence microscopy. To determine the total content of phenolic compounds in *C. graminicola*-infected maize leaves, leaf samples were extracted with 0.1% HCl using 90% methanol as solvent. The protocol was followed according to Chirinos et al. (2007). The content of anthocyanins and hydroxycinnamic acid was determined spectrophotometrically (A_{534} and A_{290}, respectively; Doehlemann et al., 2008). The results were tested with a one-way ANOVA followed by Tukey's HSD test.

Microscopy Analysis and Image Processing

Microscopic analysis was performed using a Leica DMLB fluorescence microscope, a Leica DG300F digital camera, and Leica IM1000 software. This instrument was used for bright-field and fluorescence microscopy to assess phenolic compounds in infection sites, as well as for GFP detection. GFP was excited at 588 nm, and green fluorescence was detected at 610 nm. A Leica TCS SP2 laser scanning spectral confocal microscope was used to determine the progress of the fungal infection in tissues used for RNA extraction. GFP was excited at 480 nm, and green fluorescence was detected at 530 nm. Projections were generated from adjusted individual channels in the image stacks using Leica Confocal Software LCS version 2.61.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Information Figure S1. Detection of ROS in infection sites.

Supplemental Information Figure S2. Detection of *C. graminicola* mRNA in samples for subtractive libraries.

Supplemental Information Figure S3. In planta development of *C. graminicola* 60 hpi.

Supplemental Information Figure S4. Growth of *C. graminicola* in the presence of ABA.

Supplemental Information Figure S5. Disease index analysis.

Supplemental Information Figure S6. Densitometric analysis of Northern blotting results.

Supplemental Table S1. *C. graminicola* genes identified in the subtracted libraries.

Supplemental Table S2. Maize genes identified in the subtractive libraries.

Supplemental Table S3. *C. graminicola* genes involved in respiration.

Supplemental Table S4. Plant genes induced in response to ABA.

Supplemental Table S5. List of primers used in this study.

ACKNOWLEDGMENTS

We thank Dr. María Dolores García Pedrajas for critical discussions in building the SSH libraries and Tomas Velazco Criado for valuable assistance in greenhouse work.

Received November 2, 2011; accepted January 11, 2012; published January 12, 2012.

LITERATURE CITED

Adhikari TB, Balaji B, Breeden J, Goodwin SB (2007) Resistance of wheat to *Mycosphaerella graminicola* involves early and late peaks of gene expression. Physiol Plant Pathol **71:** 55–68

Agrawal GK, Rakwal R, Jwa N-S, Agrawal VP (2001) Signalling molecules and blast pathogen attack activates rice OsPR1a and OsPR1b genes: a model illustrating components participating during defence/stress response. Plant Physiol Biochem **39:** 1095–1103

Ameline-Torregrosa C, Dumas B, Krajinski F, Esquerre-Tugaye M-T, Jacquet C (2006) Transcriptomic approaches to unravel plant-pathogen interactions in legumes. Euphytica **147:** 25–36

An Q, Hückelhoven R, Kogel K-H, van Bel AJE (2006) Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cell Microbiol **8:** 1009–1019

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16:** 3460–3479

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373–399

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **90:** 109–116

Bailey JA, Jeger M, editors (1978) Colletotrichum: Biology, Pathology and Control. CAB International, Wallingford, UK

Bailey JA, O'Connell RJ, Pring RJ, Nash C (1992) Infection strategies of *Colletotrichum* species. *In* JA Bailey, MJ Jeger, eds, Colletotrichum: Biology, Pathology and Control. CAB International, Wallingford, UK, pp 88–120

Baldwin TK, Winnenburg R, Urban M, Rawlings C, Koehler J, Hammond-Kosack KE (2006) The Pathogen-Host Interactions database (PHI-base) provides insights into generic and novel themes of pathogenicity. Mol Plant Microbe Interact **19:** 1451–1462

Banuett F (1995) Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. Annu Rev Genet **29:** 179–208

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol **69:** 473–488

Behr M, Humbeck K, Hause G, Deising HB, Wirsel SGR (2010) The hemibiotroph *Colletotrichum graminicola* locally induces photosynthetically active green islands but globally accelerates senescence on aging maize leaves. Mol Plant Microbe Interact **23:** 879–892

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:** 783–795

Bergstrom GC, Nicholson RL (1999) The biology of corn anthracnose. Plant Dis **83:** 596–608

Birch PRJ, Kamoun S (2000) Studying interaction transcriptomes: coordinated analyses of gene expression during plant-microorganism interactions. *In* R Wood, ed, New Technologies for Life Sciences: A Trends Guide. Elsevier Science, New York, pp 77–82

Bittner-Eddy PD, Allen RL, Rehmany AP, Birch P, Beynon JL (2003) Use of suppression subtractive hybridization to identify downy mildew genes expressed during infection of *Arabidopsis thaliana*. Mol Plant Pathol **4:** 501–507

Brouta F, Descamps F, Monod M, Vermout S, Losson B, Mignon B (2002) Secreted metalloprotease gene family of *Microsporum canis*. Infect Immun **70:** 5676–5683

Buchanan CD, Lim S, Salzman RA, Kagiampakis I, Morishige DT, Weers BD, Klein RR, Pratt LH, Cordonnier-Pratt M-M, Klein PE, et al (2005) Sorghum bicolor's transcriptome response to dehydration, high salinity and ABA. Plant Mol Biol **58:** 699–720

Caldo RA, Nettleton D, Peng J, Wise RP (2006) Stage-specific suppression of basal defense discriminates barley plants containing fast- and delayed-acting Mla powdery mildew resistance alleles. Mol Plant Microbe Interact **19:** 939–947

Cavalli V, Vilbois F, Corti M, Marcote MJ, Tamura K, Karin M, Arkinstall S, Gruenberg J (2001) The stress-induced MAP kinase p38 regulates endocytic trafficking via the GDI:Rab5 complex. Mol Cell **7:** 421–432

Chirinos R, Rogez H, Campos D, Pedreschi R, Larondelle Y (2007) Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. Separ Purif Tech **55:** 217–225

Cramer RA, La Rota CM, Cho Y, Thon M, Craven KD, Knudson DL, Mitchell TK, Lawrence CB (2006) Bioinformatic analysis of expressed sequence tags derived from a compatible *Alternaria brassicicola -Brassica oleracea* interaction. Mol Plant Pathol **7:** 113–124

Crouch JA, Beirn LA (2009) Anthracnose of cereals and grasses. Fungal Divers **39:** 19–44

DeYoung BJ, Innes RW (2006) Plant NBS-LRR proteins in pathogen sensing and host defense. Nat Immunol **7:** 1243–1249

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot **32:** 93–101

Dickman MB (2000) *Colletotrichum*. *In* JW Kronstad, ed, Fungal Pathology. Kluwer Academic, Dordrecht, The Netherlands, pp 127–148

Doehlemann G, Wahl R, Horst RJ, Voll LM, Usadel B, Poree F, Stitt M, Pons-Kühnemann J, Sonnewald U, Kahmann R, et al (2008) Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. Plant J **56:** 181–195

Dufresne M, Perfect S, Pellier A-L, Bailey JA, Langin T (2000) A GAL4-like protein is involved in the switch between biotrophic and necrotrophic phases of the infection process of *Colletotrichum lindemuthianum* on common bean. Plant Cell **12:** 1579–1590

Egan MJ, Wang Z-Y, Jones MA, Smirnoff N, Talbot NJ (2007) Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proc Natl Acad Sci USA **104:** 11772–11777

Eichmann R, Hückelhoven R (2008) Accommodation of powdery mildew fungi in intact plant cells. J Plant Physiol **165:** 5–18

Ellis JG, Rafiqi M, Gan P, Chakrabarti A, Dodds PN (2009) Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. Curr Opin Plant Biol **12:** 399–405

Forgery WM, Blanco MH, Loegering WQ (1978) *Differences in pathological capabilities and host specificity of Colletotrichum graminicola on Zea mays* (*maize*). Plant Dis Rep **62:** 573–576

Guo M, Guo W, Chen Y, Dong S, Zhang X, Zhang H, Song W, Wang W, Wang Q, Lv R, et al (2010) The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. Mol Plant Microbe Interact **23:** 1053–1068

Heath MC (2000) Hypersensitive response-related death. Plant Mol Biol **44:** 321–334

Heller J, Tudzynski P (2011) Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annu Rev Phytopathol **49:** 369–390

Horbach R, Graf A, Weihmann F, Antelo L, Mathea S, Liermann JC, Opatz T, Thines E, Aguirre J, Deising HB (2009) Sfp-type 4′-phosphopantetheinyl transferase is indispensable for fungal pathogenicity. Plant Cell **21:** 3379–3396

Huser A, Takahara H, Schmalenbach W, O'Connell R (2009) Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. Mol Plant Microbe Interact **22:** 143–156

Hwang S, Kim J, Kim YH, Kim HT (2008) ABA increases susceptibility of

pepper fruits to infection of anthracnose by *Collectotrichum acutatum*. Plant Pathol J **24:** 400–406

Jeon J, Park SY, Chi MH, Choi J, Park J, Rho HS, Kim S, Goh J, Yoo S, Choi J, et al (2007) Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. Nat Genet **39:** 561–565

Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J **19:** 4004–4014

Jiang M, Zhang J (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. Plant Cell Physiol **42:** 1265–1273

Jones JDG, Dangl JL (2006) The plant immune system. Nature **444:** 323–329

Jousson O, Léchenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M, Monod M (2004) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology **150:** 301–310

Kamoun S (2007) Groovy times: filamentous pathogen effectors revealed. Curr Opin Plant Biol **10:** 358–365

Kargul J, Gansel X, Tyrrell M, Sticher L, Blatt MR (2001) Protein-binding partners of the tobacco syntaxin NtSyr1. FEBS Lett **508:** 253–258

Kim S, Park J, Park S-Y, Mitchell TK, Lee Y-H (2010) Identification and analysis of in planta expressed genes of *Magnaporthe oryzae*. BMC Genomics **11:** 104

Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol **305:** 567–580

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275

Latunde-Dada AO (2001) *Colletotrichum*: tales of forcible entry, stealth, transient confinement and breakout. Mol Plant Pathol **2:** 187–198

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A (2006) Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H_2O_2-containing vesicles with tonoplast and increased salt tolerance. Proc Natl Acad Sci USA **103:** 18008–18013

Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC, Horwitz BA (2005) Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. Eukaryot Cell **4:** 443–454

Levine A, Belenghi B, Damari-Weisler H, Granot D (2001) Vesicle-associated membrane protein of Arabidopsis suppresses Bax-induced apoptosis in yeast downstream of oxidative burst. J Biol Chem **276:** 46284–46289

Liu G, Kennedy R, Greenshields DL, Peng G, Forseille L, Selvaraj G, Wei Y (2007) Detached and attached Arabidopsis leaf assays reveal distinctive defense responses against hemibiotrophic *Colletotrichum* spp. Mol Plant Microbe Interact **20:** 1308–1319

Lyons PC, Wood KV, Nicholson RL (1990) Caffeoyl ester accumulation in corn leaves inoculated with fungal pathogens. Phytochemistry **29:** 97–101

Marcel S, Sawers R, Oakeley E, Angliker H, Paszkowski U (2010) Tissue-adapted invasion strategies of the rice blast fungus *Magnaporthe oryzae*. Plant Cell **22:** 3177–3187

Mathy A, Baldo A, Schoofs L, Cambier L, Defaweux V, Tabart J, Maréchal F, Symoens F, Mignon B (2010) Fungalysin and dipeptidyl-peptidase gene transcription in *Microsporum canis* strains isolated from symptomatic and asymptomatic cats. Vet Microbiol **146:** 179–182

Mazel A, Leshem Y, Tiwari BS, Levine A (2004) Induction of salt and osmotic stress tolerance by overexpression of an intracellular vesicle trafficking protein AtRab7 (AtRabG3e). Plant Physiol **134:** 118–128

Mellersh DG, Foulds IV, Higgins VJ, Heath MC (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. Plant J **29:** 257–268

Métraux J-P, Jackson RW, Schnettler E, Goldbach RW (2009) Plant pathogens as suppressors of host defense. Adv Bot Res **51:** 39–89

Micali CO, Neumann U, Grunewald D, Panstruga R, O'Connell R (2011) Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. Cell Microbiol **13:** 210–226

Miller RA, Britigan BE (1997) Role of oxidants in microbial pathophysiology. Clin Microbiol Rev **10:** 1–18

Mims CW, Vaillancourt LJ (2002) Ultrastructural characterization of infection and colonization of maize leaves by *Colletotrichum graminicola*, and by a *C. graminicola* pathogenicity mutant. Phytopathology **92:** 803–812

Molina L, Kahmann R (2007) An *Ustilago maydis* gene involved in H_2O_2 detoxification is required for virulence. Plant Cell **19:** 2293–2309

Morris SW, Vernooij B, Titatarn S, Starrett M, Thomas S, Wiltse CC, Frederiksen RA, Bhandhufalck A, Hulbert S, Uknes S (1998) Induced resistance responses in maize. Mol Plant Microbe Interact **11:** 643–658

Mosquera G, Giraldo MC, Khang CH, Coughlan S, Valent B (2009) Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. Plant Cell **21:** 1273–1290

Moye-Rowley WS (2003) Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. Eukaryot Cell **2:** 381–389

Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB (2008) The hemibiotrophic lifestyle of *Colletotrichum* species. J Plant Physiol **165:** 41–51

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2001) Pathogenesis-related proteins and their genes in cereals. Plant Cell Tissue Organ Cult **64:** 2–3

Narusaka Y, Narusaka M, Park P, Kubo Y, Hirayama T, Seki M, Shiraishi T, Ishida J, Nakashima M, Enju A, et al (2004) RCH1, a locus in Arabidopsis that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Mol Plant Microbe Interact **17:** 749–762

Nicholson RL, Warren HL (1976) Criteria for evaluation of resistance to maize anthracnose. Phytopathology **66:** 86–90

Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev **198:** 249–266

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma **59:** 367–373

O'Connell RJ, Bailey JA, Richmond DV (1985) Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Physiol Plant Pathol **27:** 75–98

Okita TW, Rogers JC (1996) Compartmentation of proteins in the endomembrane system of plant cells. Annu Rev Plant Physiol Plant Mol Biol **47:** 327–350

Orbach MJ, Farrall L, Sweigard JA, Chumley FG, Valent B (2000) A telomeric avirulence gene determines efficacy for the rice blast resistance gene Pi-ta. Plant Cell **12:** 2019–2032

Panstruga R (2003) Establishing compatibility between plants and obligate biotrophic pathogens. Curr Opin Plant Biol **6:** 320–326

Perfect SE, Green JR (2001) Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. Mol Plant Pathol **2:** 101–108

Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999) *Colletotrichum*: a model genus for studies on pathology and fungal-plant interactions. Fungal Genet Biol **27:** 186–198

Politis DJ, Wheeler H (1972) The perfect stage of *Colletotrichum graminicola*. Plant Disease Rep **56:** 1026–1027

Politis DJ, Wheeler H (1973) Ultrastructural study of penetration of maize leaves by *Colletotrichum graminicola*. Physiol Plant Pathol **3:** 465–468

Prusky D, Kobiler I, Ardi R, Beno-Moalem D, Yakoby N, Keen NT (2000) Resistance mechanisms of subtropical fruits to *Colletotrichum gloeosporioides*. *In* D Prusky, S Freeman, MB Dickman, eds, Colletotrichum: Host Specificity, Pathology, and Host-Pathogen Interaction. American Phytopathological Society, St Paul, pp 232–244

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol **133:** 1755–1767

Rolke Y, Liu S, Quidde T, Williamson B, Schouten A, Weltring K-M, Siewers V, Tenberge KB, Tudzynski B, Tudzynski P (2004) Functional analysis of H_2O_2-generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. Mol Plant Pathol **5:** 17–27

Rosenblum EB, Stajich JE, Maddox N, Eisen MB (2008) Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. Proc Natl Acad Sci USA **105:** 17034–17039

Schmidt K, Pflugmacher M, Klages S, Mäser A, Mock A, Stahl DJ (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of

the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet. Mol Plant Pathol **9:** 661–673

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, et al (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Funct Integr Genomics **2:** 282–291

Sugui JA, Deising HB (2002) Isolation of infection-specific sequence tags expressed during early stages of maize anthracnose disease development. Mol Plant Pathol **3:** 197–203

Sukno SA, García VM, Shaw BD, Thon MR (2008) Root infection and systemic colonization of maize by *Colletotrichum graminicola*. Appl Environ Microbiol **74:** 823–832

Takahara H, Dolf A, Endl E, O'Connell R (2009) Flow cytometric purification of *Colletotrichum higginsianum* biotrophic hyphae from Arabidopsis leaves for stage-specific transcriptome analysis. Plant J **59:** 672–683

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell **18:** 1052–1066

Tang W, Coughlan S, Crane E, Beatty M, Duvick J (2006) The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Mol Plant Microbe Interact **19:** 1240–1250

Temme N, Tudzynski P (2009) Does *Botrytis cinerea* ignore H_2O_2-induced oxidative stress during infection? Characterization of *Botrytis* activator protein 1. Mol Plant Microbe Interact **22:** 987–998

Thon MR, Nuckles EM, Takach JE, Vaillancourt LJ (2002) CPR1: a gene encoding a putative signal peptidase that functions in pathogenicity of Colletotrichum graminicola to maize. Mol Plant Microbe Interact **15:** 120–128

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J **11:** 1187–1194

Torregrosa C, Cluzet S, Fournier J, Huguet T, Gamas P, Prospéri J-M, Esquerré-Tugayé M-T, Dumas B, Jacquet C (2004) Cytological, genetic, and molecular analysis to characterize compatible and incompatible interactions between *Medicago truncatula* and *Colletotrichum trifolii*. Mol Plant Microbe Interact **17:** 909–920

Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8:** 397–403

Vaillancourt LJ, Hanau RM (1992) Genetic and morphological comparisons of *Glomerella* (*Colletotrichum*) isolates from maize and from sorghum. Exp Mycol **16:** 219–229

Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. Plant Physiol **141:** 384–390

van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci **11:** 247–253

Vianello A, Macrì F (1991) Generation of superoxide anion and hydrogen peroxide at the surface of plant cells. J Bioenerg Biomembr **23:** 409–423

Vitale A, Raikhel NV (1999) What do proteins need to reach different vacuoles? Trends Plant Sci **4:** 149–155

Voegele RT, Hahn M, Lohaus G, Link T, Heiser I, Mendgen K (2005) Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. Plant Physiol **137:** 190–198

Wang J-M, Liu H-Y, Xu H-M, Li M, Kang Z-S (2012) Analysis of differential transcriptional profiling in wheat infected by *Blumeria graminis* f. sp. *tritici* using GeneChip. Mol Biol Rep **39:** 381–387

Warren HL (1975) Field reaction of corn inbreds to *Colletotrichum graminicola*. Plant Dis Rep **59:** 767–769

Wharton PS, Julian AM, O'Connell RJ (2001) Ultrastructure of the infection of *Sorghum bicolor* by *Colletotrichum sublineolum*. Phytopathology **91:** 149–158

Williams B, Kabbage M, Kim H-J, Britt R, Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathog **7:** e1002107

Wise RP, Moscou MJ, Bogdanove AJ, Whitham SA (2007) Transcript profiling in host-pathogen interactions. Annu Rev Phytopathol **45:** 329–369

Wu S, Kriz AL, Widholm JM (1994) Molecular analysis of two cDNA clones encoding acidic class I chitinase in maize. Plant Physiol **105:** 1097–1105

Zhu T, Song F, Zheng Z (2006) Molecular characterization of the rice pathogenesis-related protein, OsPR-4b, and its antifungal activity against *Rhizoctonia solani*. J Phytopathol **154:** 378–384