Running Head: Anthracnose development and defense mechanisms in maize

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Research area: Plants Interacting with Other Organisms
Plant defense mechanisms are activated during biotrophic and necrotrophic development of *Colletotrichum graminicola* in maize

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Footnotes

Financial Source:

This research was supported by funds from the Ministerio de Ciencia e Innovación (MICINN) of Spain (grant AGL2008-03177/AGR) and by the Junta de Castilla y León, Spain (grant SA-134A08).

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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 MAMP 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 with C. graminicola, a model pathosystem for the study of hemibiotrophy. Time-course experiments revealed that mRNA 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 H₂O₂ targeting the fungal hyphae. We describe fungal respiratory burst during host infection, paralleled by superoxide ion production in specific fungal cells during the transition from biotrophy into necrotrophic lifestyle. We also identified several novel putative fungal effectors and study 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 allow for full fungal pathogenicity.
INTRODUCTION

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*, causal agent of anthracnose of maize, 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 plant cell wall and spreads from cell to cell in this manner for about 36 to 48 hours. 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., 2010).

Following the biotrophic growth phase, a switch into necrotrophic growth occurs, which is typified by the production of a large number of smaller diameter and regularly shaped hyphae, called secondary hyphae that extensively colonize the inter- and intracellular spaces of the tissue, causing 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 the 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-Magnaporthe oryzae and Medicago trunculata-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 (Bauuett, 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, 24 hpi, are suppressed. Microarray expression analysis revealed transient induction of pathogenesis related genes (PR genes), chitinases and glucanases at 12 hpi that were repressed 24 h after the infection begun. 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 pathosystems 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 Magnaporthe 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 Medicago truncatula by the hemibiotrophic pathogen Colletotrichum trifolli was investigated by Torregrosa and collaborators using macroarray experiments (Torregrosa et al., 2004). 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 a significant change in the expression level, at one specific time-point. This lack of consistency in 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-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 (Takahara et al., 2009) performed a transcriptional study of *C.
higginsianum* primary hyphae extracted from infected *Arabidopsis* leaves. In this 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 identity of these gene
has not been reported. Previous studies on *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 plant, 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 the
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 event 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
H$_2$O$_2$, 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$_2$O$_2$, 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 and colleagues (2006) described a comprehensive study on the relevance of plant defense mechanisms for fungal penetration in plant-fungal interactions. The article highlighted the importance of $\text{H}_2\text{O}_2$, 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-Colletotrichum graminicola pathosystem during early stages of anthracnose leaf blight development. Suppression subtractive hybridization experiments at early infection stages, 48 and 72 h post infection (hpi), lead 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 analysis. 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 into necrotrophic lifestyle, that 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 hours post inoculation (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. 1A-B). After 60 hpi, secondary hyphae begin to develop from the primary hyphae and proliferate throughout the plant tissue. By 72 hpi, symptoms of antracnosis are observed, including necrotic lesions surrounded by a yellow halo and massive secondary hyphae development (Fig. 1C-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 phenylalanine ammonium 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 DAB and NBT, respectively. The results revealed a strong and continuous ROS production in maize cells as *C. graminicola* infection progressed. The main ROS compound 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 Information Fig. 1A). With the progression of fungal infection, plant cells started producing vesicular bodies (positive for DAB staining) targeted toward the fungal hyphae (Fig. 3A-F). The production of vesicular bodies in response to *C. graminicola* in the plant cells was more evident 72 hpi. In this time-point secondary hyphae are heavily covered by the plant derived-vesicles (Fig. 3 E, F). Interestingly, the production of such vesicles was also observed in the uninfected maize cells surrounding the penetration sites (Supplemental Information Fig. 1B). 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 and up to 72 hpi. The results suggest that the plant cells are not producing superoxide in 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 (Supplemental Information Fig. 1C, Fig. 4). 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 infections due to the low representation of mRNA from the pathogen compared to 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 (3x10^5 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 green fluorescent protein-tagged strain of C. graminicola previously developed (Sukno et al., 2008).

The time-points selected for our subtracted libraries correspond to the biotrophic stage and to the switch from biotrophic into the necrotrophic stage of disease development and samples were collected at 48 and 72 hpi, respectively (Fig. 1). We prepared mRNA samples from approximately 3500 (48 hpi samples) and 2000 (72 hpi samples) infected leaf discs collected in three independent inoculation experiments. RNA integrity was assayed on agarose gels and RT-PCR assay were conducted to detect the amplification of the beta-tubulin gene from C. graminicola in total RNA samples (Supplemental Information Fig. 2). 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 72h libraries were sequenced, respectively (Table 1). From all the identified clones, 50 sequences were mapped to genes encoded in *C. graminicola* genome. Of these 50 genes, 13 cDNAs have homologues deposited in the PHI-base, a database that catalogues functionally characterized virulence genes from pathogenic fungi (Baldwin et al., 2006) Supplemental Information Table 1) 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 (Thon et al., unpublished results), 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 non-functionally 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 Information Table 2). 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 cells 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 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 Information Table 2). 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 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
leucine-rich 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 Information Table 2). It is also important to highlight the presence of
genes related to plant carbohydrate partitioning, such as starch and sucrose 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 sucrose transporter SUT1
(GRMZM2G034302) in the infection sites, which would lead to an increased sucrose
mobilization during the fungal infection.

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 serine 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 12h
up to 72h for Northern blotting 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 support these observations (Fig. 6). The Northern blotting 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 the 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 Information Fig. 3).

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

Our assays revealed an up-regulation of genes related to pathogen attack as well as several ABA-responsive genes such as GRMZM2G052100, GRMZM2G145461, and ZmAChit) (Fig. 1-6, Supplemental Information Table 2). ABA treatment has been reported to increase susceptibility of pepper fruits to anthracnose (Hwang et al., 2008). Based on the observation by Hwang, et al (2008) as well as 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 defenses, we tested the effect of exogenous ABA on the *C. graminicola* infection process and 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 100mM solution of ABA. Control plants, consisting of non-infected plants and infected plants, were treated with sterile water instead of ABA solution.

After the treatments, anthracnose development was evaluated in leaves and the timing for the fungal switch into 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 PDA plates supplemented with 10 or 100
mM ABA. Colony size was measured during four days 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 Information Fig. 4).

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). 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 Information Fig. 5). The quantitative analysis indicated a three-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 three of the resistance-related genes (PR1 and ZmChitI) and ZmWind (wounding-induced) at early stages of C. graminicola infection (Fig. 7C, Supplemental Information Fig. 6). Together, Northern blotting assays and microscopic analysis 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 into 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 C. graminicola genome database (Supplemental Information Table 1). 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 2). Of the 10 hypothetical proteins, eight have homologues 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 database revealed that seven of the sequences are highly conserved mostly in plant pathogenic species. It is likely that the function of these novel 10 genes is 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 (Fig. 2, 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 Information Table 3). 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 exemption 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 were detected in infection sites collected 60 hpi (Fig. 8A). This expression 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 fungal-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 into 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 upregulated 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, 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 fungal-produced reactive oxygen species, 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 hydrogen peroxide during biotrophic growth of *C. graminicola* (Fig. 2, 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 plant defenses are either partially activated or that 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 (Supplemental Information Table 2, Figure 5). 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 binding 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 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 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 Information Table 4). The genes encoding putative proteins involved in the signaling networks provide a starting point for 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. \textit{C. graminicola} initially colonizes the plant leaf biotrophically and later switches into a necrotrophic lifestyle. It has been well established that biotrophic pathogens such as \textit{Uromyces vignae} and \textit{U. maydis} as well as hemibiotrophs such as \textit{Mycosphaerella 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; Eichmann and Hückelhoven, 2008). Microarray studies revealed that in maize infected with \textit{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 \textit{Colletotrichum trifolii} and \textit{Medicago trunculata}, revealed no major differences in defense gene expression during the early stages of infection, up to 72 hpi (Torregrosa et al., 2004).

The \textit{M. oryzae}\textemdash 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 biotrophic maize pathogen \textit{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 \textit{M. oryzae}
infection (Mosquera et al., 2009; Marcel et al., 2010), but repressed upon establishment
of biotrophic growth of *U. maydis* (Doehlemann et al., 2008). Together our results and
those reported by Doehlemann et al. demonstrate that maize leaves respond
differentially to biotrophic and hemibiotrophic pathogens, and a similar behavior might
be extrapolated for rice based on the results by Mosquera et al. and Marcel et al.
(Mosquera et al., 2009; Marcel et al., 2010)

The involvement of phytohormones in plant-pathogen interactions has been studied
in many pathosystems (Bari and Jones, 2008). 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., 2011). Microarray analysis to study effects of ABA
on gene expression in *Arabidopsis*, rice and sorghum 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 co-cultured 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 (Fig. 6, 7;
Supplemental Information Table 4). 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 on anthracnose development had been previously
demonstrated in *C. acutatum*-infected pepper fruits where 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 on maize by ABA caused a premature switch into
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$_2$O$_2$, 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 SNARE-like
superfamily protein) involved in the regulation of vesicle sorting and transport (Supplemental Information Table 2). 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$_2$O$_2$-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$_2$O$_2$. This was also evident during the infection of barley plants by the biotrophic fungus *Blumeria graminis*, where multivesicular bodies containing H$_2$O$_2$ 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 remain 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 Information Figure 4) suggest a correlation between the timing of the highest activation of defense mechanisms and the switch into necrotrophic lifestyle. Also, our experiments suggest that the developmental program of *C. graminicola* may depend on the physiological status of the plant (Fig. 6, 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, C. lindemuthianum and C. higginsianum, impaired in the switch from biotrophy to necrotrophy, only produced primary hyphae and were not able to colonize further 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 the plant defense mechanisms succeed in limiting biotrophic hyphae to spread into the host.

In addition to the 10 sequences with homologs annotated in the pathogen-host interaction database (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 residues 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 molecular weight of 28377 Da. 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, Park et al., 2007). Among the candidate effectors, we also found a metalloprotease fungalysin (GLRG_06543). This is a single-copy gene whose expression is enhanced
during the switch into the necrotrophic lifestyle, with a maximum expression level that occurs simultaneously with the switch in lifestyle (60 hpi). Fungalysin belongs to a family of Zn-dependent proteases that has been suggested as part of host invasion mechanisms by *A. 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 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 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 includes 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 Information Fig. 3). 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 Macri, 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, where the mutualistic association, and fungal growth inside the plant, is controlled by a localized burst of ROS at fungal tips (Tanaka et al., 2006). Also, the phytopathogenic species *B. cinerea* and *M. oryzae*
generate spatiotemporal spike accumulations of ROS, such as the accumulation of superoxide in hyphal tips of \textit{B. cinerea} and appressoria of \textit{M. oryzae} during plant pathogenesis (Egan et al., 2007; reviewed by 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 \textit{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 \textit{C. graminicola} hyphal tips (Fig. 3). In part, the accumulated superoxide compounds could be byproducts of enhanced mitochondrial activity and respiration rates. Future work will lead us to a better understanding of ROS generation and Nox protein function in \textit{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 into 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.
MATERIAL AND METHODS

Maize Plants and *Colletotrichum graminicola* growth

*Colletotrichum 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 potato dextrose agar medium (PDA; Difco Laboratories, Detroit, MI) 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% sucrose (Horbach et al., 2009).

*In vivo* Quantitative Analysis of Anthracnose Development on Maize Plants

The *C. graminicola* cultures used for maize infection assays were grown for 15-20 days on PDA as previously described (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 $6.5 \times 10^4$ 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 two-weeks (V3 developmental stage) in Ray Leach Cone-Tainers (approx. 5 cm x 15 cm) (Stuewe and Sons, Corvallis, Oregon). For inoculations, the plants were placed on their side in 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 μE m$^{-2}$s$^{-1}$). Using this assay, quantitative differences in virulence can be measured by recording disease index and the lesion development and tested using a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test (http://faculty.vassar.edu/lwory/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 100mM 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 also 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 Information Figure 5). Each treatment, consisting of 20 spots per leaf per one 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 3x10^5 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 and ethidium bromide staining.

Poly (A^+) RNA from samples collected 48 or 72 hpi were used as the driver and poly (A^+) RNA from mock-inoculated leaves were used as the tester for the construction of two subtractive suppressive hybridization libraries (SSH). For this purpose the PCR-select™ cDNA subtraction kit (BD Biosciences Clontech, Palo Alto, CA, USA) 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, France) 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 NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) database. The C. graminicola sequences were also compared to 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 blotting analyses were performed using Hybond-N+ membranes (Amersham Biosciences) according to the manufacturer’s suggestions. Probes for Northern-blot assays were obtained after EcoRI-digestion of plasmid generated after cloning the products of the subtractive libraries, which had been sequenced. The purified probes were radioactively labeled with [32P]dCTP using RadPrime DNA Labelling System (Invitrogen) and then used for blot hybridization using ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion, Austin Texas) following the protocol recommended by the manufacturer. Signal intensities were quantified using the MCID analysis software (Mering, Germany) and tested with a one-way ANOVA, followed by Tukey's HSD test.

**Reverse Transcription and PCR assays**

cDNA synthesis was performed using 5 μg of total RNA, Moloney Murine Leukaemia Virus-Reverse Transcriptase (MMLV-RT®, Promega) and oligo-dT primers. Previous to the reverse transcription, RNA samples had been treated with Turbo DNA-Free DNAse (Ambion, Austin Texas) to remove trace amounts of genomic DNA.

For fungal gene expression analysis, semiquantitative RT-PCR experiments were carried out. Following reverse transcription of RNA, PCR reactions were performed
using specific primers for each gene assayed. The amplification of the constitutively
expressed tubulin and GAPc genes from *C. graminicola* and maize, respectively, were
used as loading and RT controls. PCR reactions were 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 contaminations 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 reactions are listed in Supplemental
Information Table 5.

**Histochemical Analysis of Compatible Maize-Colletotrichum Interactions**

Infection sites were assessed for production of H$_2$O$_2$ (hydrogen peroxide) O$_2^-$
(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 a 2 mg mL$^{-1}$ dianinobenzidine (DAB) dissolved in
0.1 M phosphate buffer pH 6.8 (Thordal-Christensen et al., 1997). After infiltration, the
samples were incubated at 1h 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 Nitrotetrazolium Blue (NBT)
solution (0.5 mg mL$^{-1}$ in the same buffer) as the staining solution.

The use of Toluidine Blue O (TBO) has been widely used for detection of phenolic
compounds and lignification of plant tissue (O’Brien et al., 1964). To visualize phenolic
compounds and lignin deposition, infection sites were fixed-cleared after 5 min 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$^{-1}$ TBO 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 fluorescence microscopy. GFP was exited 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 exited 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 v2.61.

Acknowledgments

WAV was supported by the Spanish Ministry of Science and Innovation through the
Juan de la Cierva (JCI-2009-05364) Postdoctoral program and MRT was supported by
the Ramón y Cajal Program. JMSM FPU graduated fellowship (AP2009-2656)
program, GER FPI (BES-2009-013920) graduated fellowship, and LPR is a recipient of
a JAE predoctoral scholarship from CSIC. 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.
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Legend to Figures

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Supplemental Information Figure 1. Reactive oxygen species (ROS) produced by maize cells and *C. graminicola* at early stages of anthracnose development. **A-B)** Infection sites collected 36 and 48 hpi were assayed for H$_2$O$_2$ production by DAB staining. **C)** Reactive oxygen species produced by *C. graminicola* 48 and 60 hpi of maize leaves. Infection sites were tested for superoxide accumulation by NBT staining. *C. graminicola* hyphae tips in contact with the plant cell boundaries and preparing to cross into a neighboring cell yielded positive reaction 60 hpi (Arrowheads). A, appresorium; V, Vesicle-like structures; NC, non-infected neighbor cell; P, positive reaction. Scale bars, 40 μm.

Supplemental Information Figure 2. Detection of mRNA produced by *C. graminicola* in total RNA samples used for the construction of the subtractive libraries. Semi-quantitative RT-PCR assays were conducted to test the presence of mRNA for *Colletotrichum* beta-tubulin (*CgTub*) and *ZmGapC* in samples collected 48 and 72 hpi. PCR products were visualized after electrophoresis on agarose gel and ethidium bromide staining.

Supplemental Information Figure 3. Micrograph showing hyphal development 60 hpi inside a plant cell. This picture was taken in samples used to test for H$_2$O$_2$ production by DAB staining. The transition from primary into secondary hyphae inside maize cells 60 hpi is evident. PH, primary hyphae; SH, secondary hyphae. Scale bar, 60 μm.

Supplemental Information Figure 4. Effect of ABA on *C. graminicola* growth on PDA. *Colletotrichum graminicola* spore suspensions were inoculated in the center of Petri dishes containing PDA supplemented with 10 or 100 mM ABA. Control dishes (PDA) represent samples inoculated on PDA with no ABA addition. Radial growth was measured daily and the area of the colony was compared. The values presented in the bars represent mean ± SD of three independent experiments.

Supplemental Information Figure 5. Disease index determination after ABA treatment. Disease development in response to ABA was quantified according to an arbitrary scale from 0 to 3, were 0 represent no symptom detected and 3 was assigned to
infection sites were chlorosis and necrosis was evident. The figure shows representative pictures for the disease progress, indicated form 0 to 3.

Supplemental Information Figure 6. Bar plots depicting quantitative data of the signals detected in the films corresponding to Northern blot results presented in Figure 7. The signals were quantified through densitometric analyses using MCID Analysis software. The values are expressed as percentage of expression normalized to the signal intensity determined 72 hpi (considered as 100%). The values shown are the average 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%.
Table 1. Summary of SSH libraries.

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<th>Hours after inoculation</th>
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<tr>
<td></td>
<td>48h</td>
<td>72h</td>
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<tr>
<td>Sequenced clones</td>
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<td>348</td>
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<tr>
<td>Total unigenes</td>
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<tr>
<td>Library redundancy (a)</td>
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(a) Calculated according to Cramer et al., 2006
Table 2. Uncharacterized *C. graminicola* genes expressed during pathogenesis.

Bold-case, indicate sequence unique to *C. graminicola*

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<th>GLRG</th>
<th>Genome Annotation</th>
<th>InterPro</th>
<th>Homolog</th>
<th>GenBank Accession #</th>
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<td>08002 Hypothetical protein</td>
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<td>No match</td>
<td>No match</td>
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