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Localization, morphology and transcriptional profile of *Aspergillus flavus* during seed colonization

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SUMMARY

Aspergillus flavus is an opportunistic fungal pathogen that infects maize kernels pre-harvest, creating major human health concerns and causing substantial agricultural losses. Improved control strategies are needed, yet progress is hampered by the limited understanding of the mechanisms of infection. A series of studies were designed to investigate the localization, morphology and transcriptional profile of *A. flavus* during internal seed colonization. Results from these studies indicate that *A. flavus* is capable of infecting all tissues of the immature kernel by 96 h after infection. Mycelia were observed in and around the point of inoculation in the endosperm and were found growing down to the germ. At the endosperm–germ interface, hyphae appeared to differentiate and form a biofilm-like structure that surrounded the germ. The exact nature of this structure remains unclear, but is discussed. A custom-designed *A. flavus* Affymetrix GeneChip® microarray was used to monitor genome-wide transcription during pathogenicity. A total of 5061 genes were designated as being differentially expressed. Genes encoding secreted enzymes, transcription factors and secondary metabolite gene clusters were up-regulated and considered to be potential effector molecules responsible for disease in the kernel. Information gained from this study will aid in the development of strategies aimed at preventing or slowing down *A. flavus* colonization of the maize kernel.

INTRODUCTION

The infection of maize kernels by *Aspergillus flavus*, a fungal pathogen of maize, results in kernel deterioration and contamination with the carcinogenic mycotoxin aflatoxin (AF). Public exposure to feed and food containing AF in the USA is prevented through a rigorous, but expensive ($30–50 million per year), testing process mandated by the Grain Inspection, Packers and Stockyard Administration (GIPSA) (Robens and Cardwell, 2003). Diseased grain has a lower market value and may be destroyed if the AF concentration exceeds the action levels set by the US Food and Drug Administration (2000). In developing countries, AF poisoning is more common because of limited regulations and improper storage techniques (CDC, 2004).

Current management recommendations for reducing *A. flavus* disease are to maintain plant health and reduce plant stress throughout the growing season, especially during pollination and grainfill, and to plant regionally appropriate hybrids (Koenning and Payne, 1999; White, 1999). Although these practices are effective, often growers are faced with adverse environmental conditions, resulting in less than ideal growing conditions. New methods are needed that are more cost-effective and provide more proactive forms of resistance against seed pathogens. The goal of this study was to examine the response by *A. flavus* to interactions with the maize kernel to identify the mechanisms important in *A. flavus* pathogenesis.

The morphological and molecular changes that occur in *A. flavus*, an opportunistic pathogen, during maize kernel pathogenesis are poorly understood. Evidence suggests that the pathogen enters a nondamaged kernel through the pedicel end as the ear approaches maturity (Marsh and Payne, 1984; Payne *et al*., 1988b; Smart *et al*., 1990; Windham and Williams, 1998). However, *A. flavus* has been detected in younger, insect-damaged kernels (Anderson *et al*., 1975; Taubenhaus, 1920). After gaining entrance, *A. flavus* preferentially colonizes tissues high in oil, such as the germ and aleurone, with the highest concentration of AF occurring in the germ of infected kernels (Fennell *et al*., 1973; Jones *et al*., 1980; Keller *et al*., 1994; Smart *et al*., 1990). There has been some debate about the extent of endosperm colonization (Brown *et al*., 1995; Keller *et al*., 1994; Lillehoj *et al*., 1976; Smart *et al*., 1990).

The aim of this study was to investigate the localization, morphology and transcriptional profile of *A. flavus* during seed colonization. We addressed the first of these objectives by identifying the kernel tissue types infected by *A. flavus* over time, and the second by looking at the morphology of infectious hyphae within the different tissues of the kernel. Lastly, global gene expression was monitored using a custom-designed *A. flavus* Affymetrix

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GeneChip® microarray during *in vivo* growth. We conclude that *A. flavus* is capable of infecting all tissue types within the immature maize kernel, and find that a change in fungal morphology occurs at the endosperm–germ interface. An alteration in fungal gene expression was induced in *A. flavus* during the colonization of the living kernel. Those genes found to be up-regulated are considered as potential effectors. Collectively, these studies show that *A. flavus* infection of the maize kernel is more complex than previously suspected.

RESULTS

Aspergillus flavus colonizes all kernel tissues within 96 h of inoculation

Despite the efforts of several investigators, there is some uncertainty as to which tissue types of the maize kernel can be infected by *A. flavus* and how rapidly infection proceeds. To determine both the tissue specificity and rate of *A. flavus* infection after pin-mediated endosperm inoculation, the presence of the pathogen was monitored using polymerase chain reaction (PCR). *Aspergillus flavus* growth was successfully tracked inside the kernel when fungal DNA was positively detected by PCR in the endosperm, germ and pedicel tissues of inoculated kernels. DNA isolated from uninfected maize tissue and *A. flavus* grown in potato dextrose broth (PDB) were used to test the species specificity of the primers and served as positive controls (Fig. 1, Ctrl).

Aspergillus flavus was first detected in the endosperm tissue of diseased kernels as early as 24 h after inoculation (hai) and in the germ by 72 hai (Fig. 1, *A. flavus* rows). The removal of the germ from surrounding endosperm tissue became more difficult over time and, in some cases, *A. flavus* was macroscopically observed growing on the surface of the germ. *Aspergillus flavus* was detected in all three germ samples by 72 hai; however, it is unclear on the basis of these data alone whether the fungus was confined to the germ surface or had successfully penetrated and begun colonization. Although the pedicel tissue, which includes the kernel base, was the region farthest away from the site of inoculation, this region of the kernel was readily infected with *A. flavus* at 72–96 hai.

Aspergillus flavus undergoes a morphological change at the endosperm–germ interface

We performed a histological investigation to examine growth and morphological changes in *A. flavus* associated with kernel infection. *Aspergillus flavus* conidia were introduced into starchy endosperm tissue by inserting the inoculation pin into the crown of the kernel (Fig. 2a). Four days after inoculation, growth of *A. flavus in vivo* was observed by fungal-specific histological stains on 10-μm sections of diseased kernels, and hyphae could be differentiated from host cells. Endosperm colonization was predominantly at or around the site of inoculation. Often the process of inoculation created a cavity where the pin was inserted and a

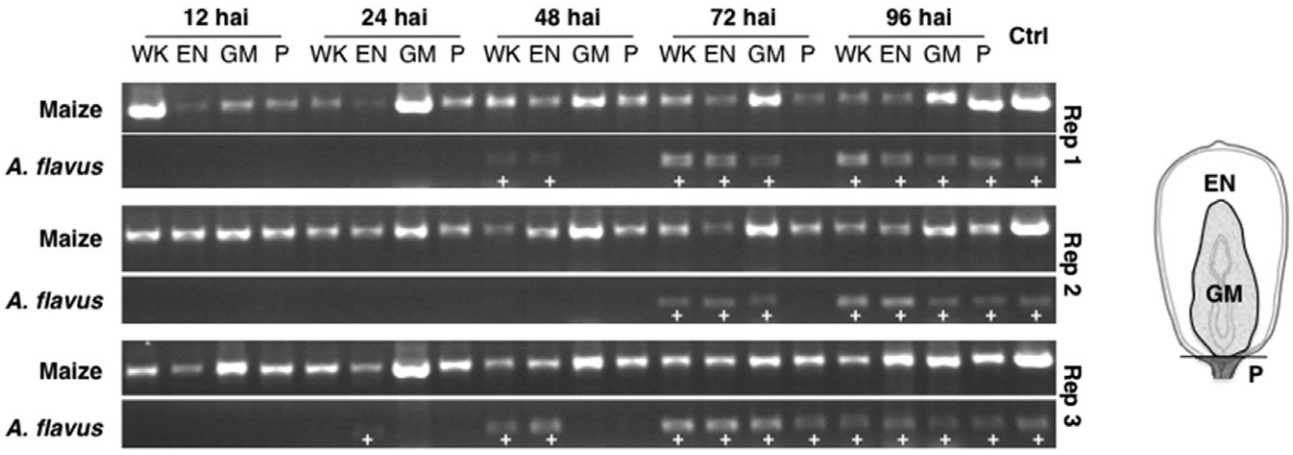

Fig. 1 An ethidium bromide-stained agarose gel of polymerase chain reaction (PCR) products amplified from DNA isolated from *Aspergillus flavus*-infected maize tissue. The DNA was tested for the presence of *A. flavus* from inoculated whole kernels (WK), endosperm (EN), germ (GM) and pedicel (P) tissues at 12, 24, 48, 72 and 96 h after inoculation (hai). The location of the maize tissues analysed is shown in the schematic drawing on the right. Maize-specific catalase primers effectively amplified the targeted PCR fragment from uninfected maize tissue DNA (Ctrl column, maize row), but failed to produce a band from DNA isolated from *A. flavus* grown in shake cultures (data not shown). The *A. flavus*-specific *gpdA* primers were capable of amplifying the product from the *A. flavus* shake culture DNA (Ctrl column, *A. flavus* row), but not from noninfected maize kernel DNA (data not shown). Positive detection of *A. flavus* within the diseased maize tissue is marked with a white '+'. The lack of amplicons in the *A. flavus gpdA* primer reactions (*A. flavus* rows, missing a '+' sign) is caused by the absence of fungal DNA in the samples assayed.

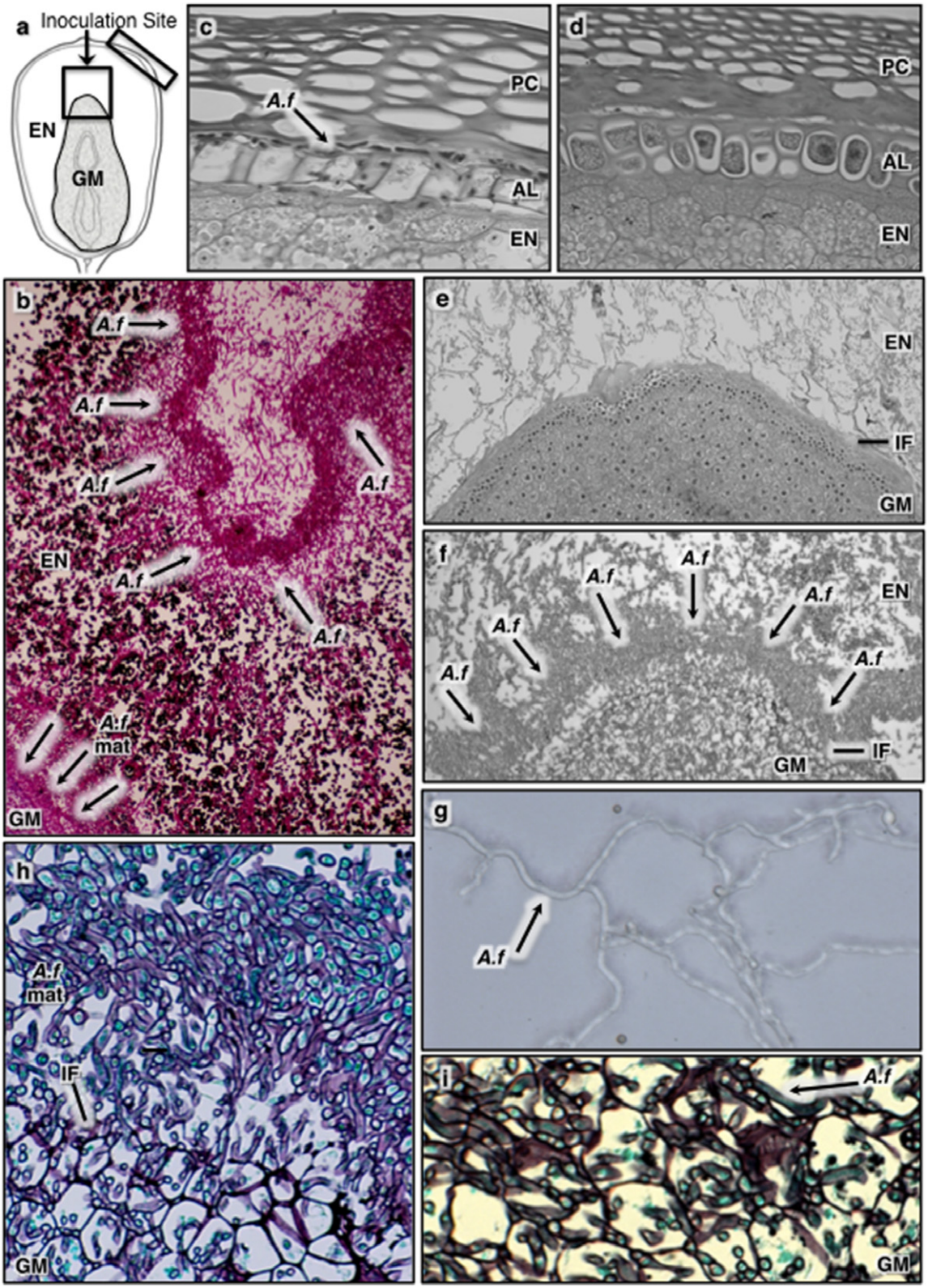

Fig. 2 Histology of the developing maize kernel 4 days after *Aspergillus flavus* pin-bar inoculation. The schematic drawing of the kernel (a) depicts the location of pin-bar inoculation (black arrow) and the kernel region (black box, □) from which the histology panels originate. White was added to the letters and arrow margins for easier viewing in the panels. *Aspergillus flavus* was observed (b) in the endosperm (EN) surrounding a cavity created by the inoculation pin and outside the germ (GM) in a unique *A. flavus* mat-like (*A.f* mat) structure. Hyphae were also found growing between the pericarp (PC) and aleurone (AL) (c) in which the AL cells in contact with *A. flavus* appear to be altered compared with cells in the noninoculated control (d). (e) Endosperm–germ interface (IF) in a noninoculated kernel. In the infected kernel, the *A.f* mat structure developed at the IF (f) and covered the germ tip. Hyphae within the *A.f* mat were morphologically distinct from *A. flavus* vegetative hyphae (g) and were highly branched, tightly intertwined and extended into the GM (h). Colonization of the GM by *A. flavus* was observed in highly infected kernels (i).

mass of mycelium was found surrounding or growing within this cavity (Fig. 2b). Conidiation was observed in a few of the cavities. The integrity of endosperm tissue was diminished in and around the infected area, but it is unclear whether this was a product of fungal degradation or part of the host's reaction to infection.

On the basis of the growth pattern observed at 96 hai, it can be inferred that *A. flavus* spread from the site of inoculation down to the germ through the starchy endosperm, although this was not always apparent as few kernel sections contained the narrow infection pathway extending from endosperm to the germ. Earlier studies have led to speculation that the fungus uses aleurone tissue to reach the germ. In this study, *A. flavus* was found between the aleurone and the pericarp cell layers (Fig. 2c) with infection heaviest near the inoculation site, but pockets of mycelium also occurred in more distal regions. Our observations were unable to confirm that *A. flavus* uses the aleurone as a means to access the germ; however, there was no evidence to indicate otherwise. Aleurone cells proximal to *A. flavus* hyphae lacked cytoplasm (Fig. 2c vs. 2d).

Hyphae at the endosperm–germ interface (Fig. 2e, noninoculated interface) were morphologically different from vegetative hyphae (Fig. 2f, h vs. 2g). *Aspergillus flavus* was not present at the endosperm–germ interface in every kernel surveyed, but, in kernels in which the fungus had made contact with the germ, it formed a mat-like structure composed of highly branched, tightly intertwined hyphae pressed up against the scutellum (Fig. 2f, h). This mat-like structure was seen predominantly at the germ apex, but, in heavily infected kernels, it extended from the tip to the base of the germ. This distinctive structure was observed in all kernels in which hyphae were able to penetrate and colonize germ tissue, although its formation did not guarantee germ infection. Hyphae within the germ appeared to originate from the mat-like structure (Fig. 2h). Invasion of the germ was mostly confined to the scutellum tissue (Fig. 2i), but, on occasion, *A. flavus* was also found in the embryo (not shown).

Aspergillus flavus undergoes significant transcriptional changes during the pathogenesis of maize kernels

As a part of this study, we performed global gene expression profiling of *A. flavus* during the pathogenesis of maize kernels. Reese *et al*. (2011), using detached kernels, reported significant transcriptional differences in *A. flavus* grown on kernels at different stages of development. To limit any effects of kernel age on fungal gene expression, we grouped expression values from *A. flavus*-inoculated blister (R2), milk (R3), dough (R4) and dent (R5) kernels into a single treatment before comparing them with the expression values from *A. flavus* inoculated into nonliving autoclaved kernels. This allowed for the identification of potential pathogenic effectors expressed in maize kernels regardless of the kernel's age.

An analysis of variance (ANOVA) identified 5061 of the 12 197 predicted *A. flavus* genes represented on the Affymetrix GeneChip® microarray to be differentially expressed during kernel pathogenesis (Table S1, see Supporting Information). Approximately 30% had a fold change of two or greater and, of these genes, 682 were up-regulated and 944 were down-regulated. Ninety of the differentially expressed genes from our study were the same *A. flavus* genes that had been designated previously by Reese *et al*. (2011) as being differentially expressed during the colonization of kernels at different stages of development.

The function of many of the differentially expressed gene products (>40%) is unknown and their annotation is listed as hypothetical. In all, 459 of the genes encoded a secretory signal peptide (bold; Table S1). Included amongst these were a cerato-platanin-like gene, three necrosis and ethylene-inducing protein (NEP)-like genes, an SMK (salt-mediated killer)-like toxin gene and a polygalacturonase *pecA* (P2c). Of these, 93 were annotated as extracellular hydrolytic enzymes. Predicted secretory proteins were both up- and down-regulated.

Other fungal genes differentially expressed in the *A. flavus*–maize interaction included 20 transcription factors (TFs), eight of which were up-regulated and 12 down-regulated (Table 1). Many were similar in sequence to other known fungal TFs, and they were found scattered throughout the eight *A. flavus* chromosomes (Fig. 3).

Next, we examined the expression profile for the 55 *A. flavus* secondary metabolite gene clusters to determine which clusters were expressed in *A. flavus* under pathogenic conditions. Secondary metabolite genes are located together on the chromosome as a cluster, typically near the telomeric ends. Each cluster contains a 'backbone' enzyme responsible for the first step in the biosynthesis of the metabolite. (Consult the *A. flavus* genome browser (http://www.Aspergillusflavus.org) for more information on the secondary metabolite clusters.)

Table 1 *Aspergillus flavus* transcription factors differentially expressed in kernel pathogenesis.

Up-regulated			Down-regulated		
NCBI gene identifier	Annotation	Map ID*	NCBI gene identifier	Annotation	Map ID*
AFLA_050250	bZIP transcription factor (CpcA)	a	AFLA_052030	Developmental regulatory protein (WetA)	i
AFLA_073870	C6 transcription factor (RegA)	b	AFLA_076320	Fungal-specific TF domain-containing protein	j
AFLA_031340	bZIP transcription factor (AtfA)	c	AFLA_082850	C2H2 transcription factor (BrlA)	k
AFLA_136410	Transcriptional regulator Medusa (MedA)	d	AFLA_087080	Fungal-specific TF domain-containing protein	l
AFLA_139360	AF transcription activator (AflR)	e	AFLA_124290	Fungal-specific TF domain-containing protein	m
AFLA_069140	SIR2 family histone deacetylase (Hst4)	f	AFLA_131640	HLH transcription factor (Hpa3)	n
AFLA_122570	C6 transcription factor	g	AFLA_112830	Fungal-specific TF domain-containing protein	o
AFLA_116680	PAS domain S-box family protein (white collar)	h	AFLA_005460	Cytochrome P450 monooxygenase (SirB-like)	p
			AFLA_001570	Fungal-specific TF domain-containing protein	q
			AFLA_093040	C6 transcription factor (NirA)	r
			AFLA_067910	HLH transcription factor	s
			AFLA_101920	Extracellular developmental signal biosynthesis protein (FluG)	t

NCBI, National Center for Biotechnology Information.

*Map ID denotes the chromosomal location of the TF in Fig. 3.

Fifty of the 55 predicted *A. flavus* secondary metabolite gene clusters had one or more genes differentially expressed during pathogenesis (Table 2). Of these clusters, 18 had ≥50% of the genes within the cluster, including the backbone enzyme, differentially expressed: clusters 11, 14, 18, 24, 27, 33, 51, 54 (AF) and 55 (cyclopiazonic acid) were up-regulated, and clusters 4, 5, 8, 26, 40, 41, 46, 48 and 50 were down-regulated (chromosome locations, Fig. 3). Clusters with ≥50% but near equal numbers of up- to down-regulated genes were not considered to be differentially expressed. The AF gene cluster (54) had 50% of the 34 predicted AF genes, including the backbone enzyme, differentially expressed as part of *A. flavus* disease development in the kernel. All 17 of the differentially regulated AF genes were up-regulated. AF was detected in inoculated kernels at all four developmental stages (data not shown).

Some genes from the aflatrem clusters were differentially expressed, but collectively failed to meet the above-mentioned criteria to be considered as differentially expressed. Aflatrem is a tremorgenic toxin that is produced by *A. flavus* (Wilson and Wilson, 1964) Two clusters (15 and 32) are necessary for the synthesis of aflatrem (Nicholson *et al*., 2009). In this study, cluster 15 (aflatrem) and cluster 32 had 58% and 62% of the cluster differentially expressed, respectively. The backbone enzyme for cluster 15, dimethylallyl tryptophan synthase, was down-regulated during infection. The other differentially expressed genes in cluster 15 (aflatrem) were an even mixture of both up-regulated (four genes) and down-regulated (two genes + backbone). As there were almost equal numbers of up- and down- regulated genes, cluster 15 was not considered to be differentially expressed. In contrast, all but two of the 13 differentially expressed genes in cluster 32 were up-regulated, including a NEP-like gene that had a fold change of 12.1 (Georgianna *et al*., 2010; Nicholson *et al*., 2009). Cluster 32 does not have a backbone enzyme. The aflatrem genes *atmG* and *atmC*, which are needed for aflatrem synthesis, were not differentially expressed.

DISCUSSION

Discrepancy exists in the literature as to whether or not *A. flavus* effectively colonizes all tissue types within the maize kernel (Brown *et al*., 1995; Keller *et al*., 1994; Lillehoj *et al*., 1976; Smart *et al*., 1990). We found that *A. flavus* is readily detected by PCR in the endosperm, germ and pedicel tissues of susceptible B73 kernels. The endosperm samples used in this study also contained aleurone tissue, but closer examination, as discussed later, revealed that the heaviest concentration of *A. flavus* was in the endosperm and not the aleurone.

These data largely support the findings of Brown *et al*. (1995) and Lillehoj *et al*. (1976). However, neither Smart *et al*. (1990) nor Keller *et al*. (1994) saw substantial endosperm colonization or mycotoxin production in the endosperm, respectively. We speculate that the incongruities between these studies are caused by variations in experimental design.

Differences in inoculation techniques would influence the route of infection and, potentially, disease severity by altering how the fungus enters the kernel and which tissue it comes into contact with initially. Although pin-bar inoculation ensures a higher success rate of infection in the field, it should be recognized that this method is invasive. Inoculation by pin-bar compromises the kernel's natural defences by penetrating the pericarp and aleurone layers, and forcing conidia directly into the endosperm. Although this method of inoculation may not reflect the method of hyphal entry in intact kernels, it closely simulates the infection process after insect damage, which is highly relevant in field-grown plants (Parsons and Munkvold, 2010).

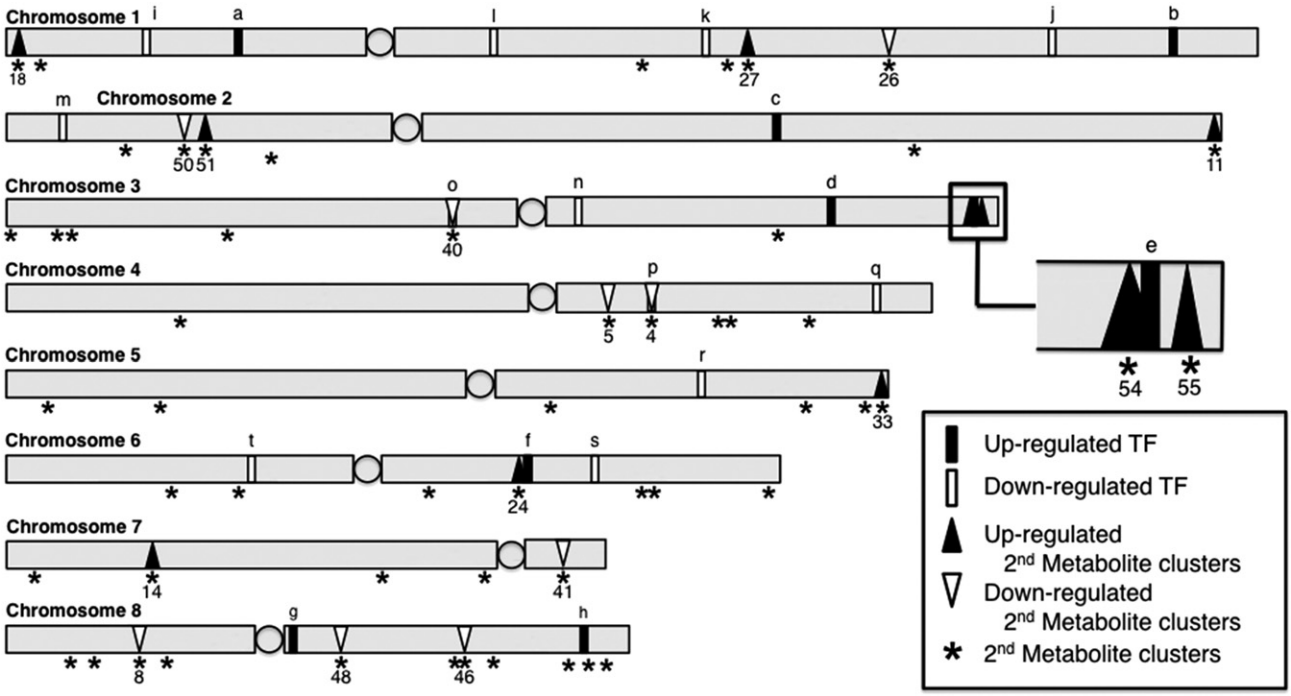

Fig. 3 The diagram illustrates the chromosomal location of the differentially expressed *Aspergillus flavus* transcription factors (TFs) (Table 1) and secondary metabolite gene clusters up- and down-regulated (Table 2, up and down arrows) during maize kernel colonization. Up-regulated TFs (map ID: a–h) are shown as filled vertical bars, and down-regulated TFs (map ID: i–t) as open vertical bars. All 55 predicted secondary metabolite clusters are depicted on the map as asterisks (*) positioned under the grey chromosome bars. Centromeres are represented as shaded circles on each of the chromosomes. Up-regulated secondary metabolite clusters and down-regulated clusters are denoted by filled and open triangles, respectively. The aflatoxin regulator *aflR* (map ID: e) is found within cluster 54, TF AFLA_112830 (map ID: o) in cluster 40 and TF AFLA_005460 (map ID: p) is part of cluster 4.

Keller *et al*. (1994) inoculated in a manner more similar to that used in our study, but infected mature instead of developing kernels. As maize kernels mature, the endosperm undergoes desiccation, which makes infection of the endosperm difficult by lowering water activity to a level unfavourable for fungal growth (Anderson *et al*., 1975; Gibson *et al*., 1994; Payne *et al*., 1988a). Higher amounts of water in developing kernels, such as those used in our study, are more conducive for endosperm colonization than the smaller amounts found in mature kernels. Also, Keller *et al*. (1994) followed growth by expression of the AF pathway intermediate, norsolorinic acid (NOR), and thus may not have observed NOR production in the endosperm, which is less conducive for AF formation.

These observational differences in tissue colonization emphasize how the method of entry and the developmental age of the kernel may significantly affect the route of colonization. Additional studies testing a range of inoculation techniques on different aged kernels would further confirm this assumption. Other variables, such as the overall health of the kernel and maize kernel genotype, should also be considered.

Results from the histological studies showed that *A. flavus* colonizes and uses endosperm tissue as a medium for growth down to the germ. These observations are in accordance with the findings of our PCR localization study.

Aspergillus flavus was also observed growing between the aleurone and pericarp layers, but growth was restricted to isolated patches of mycelium scattered throughout the kernel. The heaviest concentration of these mycelial patches was near the site of inoculation, and growth did not appear to extend down to the germ. In contrast, fungal hyphae traversing the endosperm from the point of inoculation to the germ were consistently observed in infected kernels, although colonization of the endosperm outside the site of inoculation was not extensive. This suggests that endosperm tissue supports the spread of *A. flavus* in the kernel, but might not be a preferred host tissue (compared with germ). Another possibility is that, after initial infection, the surrounding endosperm tissue becomes resistant to infection. Endosperm-specific resistance-associated proteins have been identified (Chen *et al*., 2007). However, it should be reiterated that the route of infection is probably influenced by the local environmental conditions under which infection takes place.

On reaching the germ, *A. flavus* undergoes dramatic morphological changes and forms a distinct mat-like structure of highly branched, intertwined hyphae at the germ–endosperm interface.

Table 2 Percentage of genes within the 55 *Aspergillus flavus* secondary metabolite clusters differentially expressed in kernel pathogenesis.

Cluster ID	Number of genes in cluster	Total %	Differentially expressed				Change in expression*
			Up-regulated (%)	+	Down regulated (%)	Backbone enzyme	
1	9	22	22		0	✗	
2	9	33	11		22	✗	
3	2	50	0		50	✗	
4	4	100	0		100	✓	↓
5	3	67	0		67	✓	↓
6	12	50	33		17	✓	
7	10	50	30		20	✓	
8	9	78	11		67	✓	↓
9	10	30	30		0	✗	
10	3	0	NA		NA	✗	
11	12	75	50		25	✓	↑
12	3	33	33		0	✗	
13	4	50	0		50	✗	
14	2	50	50		0	✓	↑
15	12	58	33		25	✓	
16	2	0	NA		NA	NA	
17	11	27	27		0	✓	
18	6	50	50		0	✓	↑
19	6	0	NA		NA	NA	
20	10	30	10		20	✓	
21	38	24	0		24	✗	
22	6	17	17		0	✗	
23	17	47	12		35	✓	
24	3	100	100		0	✓	↑
25	9	11	11		0	✗	
26	12	58	17		42	✓	↓
27	5	60	60		0	✓	↑
28	6	50	33		17	✗	
29	2	50	0		50	✗	
30	6	50	17		33	✓	
31	15	13	0		13	✗	
32	21	62	52		10	✗	
33	8	50	38		13	✓	↑
34	8	63	13		50	✗	
35	5	40	20		20	✗	
36	7	29	29		0	✓	
37	3	0	NA		NA	NA	
38	3	0	NA		NA	NA	
39	5	40	20		20	✗	
40	9	56	11		44	✓	↓
41	3	67	0		67	✓	↓
42	11	36	9		27	✓	
43	19	53	32		21	✓	
44	6	67	33		33	✓	
45	16	31	13		19	✗	
46	4	50	0		50	✓	↓
47	6	50	33		17	✓	
48	20	65	5		60	✓	↓
49	6	67	33		33	✓	
50	8	75	13		63	✓	↓
51	5	60	60		0	✓	↑
52	7	14	0		14	✗	
53	8	63	38		25	✓	
54	34	50	50		0	✓	↑
55	4	75	75		0	✓	↑

*Clusters with ≥50% of the genes differentially expressed during infection (including the backbone enzyme) are denoted with an arrow. An up arrow indicates the cluster was up-regulated and a down arrow that the cluster was down-regulated. Clusters with equal or near-equal percentages of up- and down-regulated genes were not marked even though greater than 50% of the cluster was differentially expressed. Cluster 15, aflatrem. Cluster 54, aflatoxin. Cluster 55, cyclopiazonic acid.

We hypothesize that this structure contributes to germ infection, but its presence does not guarantee germ infection. Although this structure has not been described previously in *A. flavus* infection of the kernel, there is some previous evidence that the endosperm–germ interface is an important region of the kernel in the interaction with seed-colonizing fungi (Anderson *et al*., 1975; Fennell *et al*., 1973; Freeman, 1904; Lillehoj *et al*., 1976; Manns and Adams, 1923). We suggest that this structure is analogous to the biofilm produced by *Aspergillus fumigatus* during *in vivo* infection of lung tissue (Loussert *et al*., 2010). *Aspergillus fumigatus* is a human pathogen capable of causing aspergillosis in immunosuppressed individuals. Biofilms are defined as groupings of microbes anchored to a nonmotile surface held together by an extracellular matrix (Ramage *et al*., 2009). Although biofilm formation is not exclusive to pathogens, it contributes to pathogenicity by enabling the pathogen to adhere tightly to the host and protects against host immune responses (Hall-Stoodley and Stoodley, 2009; Jabra-Rizk *et al*., 2004; Ramage *et al*., 2009).

Both physical and molecular evidence supports the conclusion that changes in fungal morphology during pathogenesis include biofilm-like formation. Physically, it was observed that separation of the germ from the kernel became more difficult as the severity of infection increased. The formation of this 'biofilm' could be responsible for the increased adhesion between these two tissues. Changes in fungal gene expression also support this theory. The TF *medusa* (*medA*) was found to be up-regulated during infection of maize kernels, and is discussed in further detail later. This TF is known to be a major regulator of biofilm production in *A. fumigatus* (Gravelat *et al*., 2010).

It is unclear at which stage of kernel development *A. flavus* infects maize. Reese *et al*. (2011) reported that kernel age significantly influences *A. flavus* gene expression. To minimize this effect, living maize kernels at different stages of development were inoculated. *Aspergillus flavus* expression values from these kernels were grouped into a single treatment and compared with gene expression from fungus grown in nonliving autoclaved kernels. Over 40% of the monitored *A. flavus* genes were differentially expressed in response to interactions with the living kernel. All but 90 of the fungal genes were expressed during infection, regardless of the kernel's age. Differentially expressed genes were broadly scattered across the genome and contained representatives from multiple functional categories. Unfortunately, a large portion of the transcripts found to be differentially expressed have not yet been annotated and their functions remain unknown.

Secretory proteins

Pathogens secrete a barrage of toxins and proteins, termed effectors, that help to establish and maintain disease in the host plant (de Jonge *et al*., 2011; Kale and Tyler, 2011). The examination of pathogen secretomes has been useful in identifying potential effectors (Guttman *et al*., 2002; Kamoun, 2006). *Aspergillus flavus* possesses several genes encoding extracellular secretory proteins expressed during kernel colonization. These include the up-regulation of two toxin genes: a cerato-platanin-like gene and an SMK-like toxin gene. Cerato-platanin is a phytotoxin produced by *Ceratocystis fimbriata* that elicits the synthesis of antimicrobial substances in the host (Pazzagli *et al*., 1999).

Also up-regulated were three NEP-like genes. These small proteins are expressed in pathogens during infection and can cause cellular death in plants (Dong *et al*., 2012; Küfner *et al*., 2009; Santhanam *et al*., 2013). It was unexpected to see increased transcription of NEP-like genes in *A. flavus* considering that these proteins are associated with necrosis in dicotyledonous, but not monocotyledonous, plants, such as maize (Bailey, 1995). The exact role of NEP proteins in the pathogenesis of monocotyledonous plants remains unclear (de Jonge *et al*., 2011). Curiously, one of the NEP-like genes up-regulated lies within the *A. flavus* secondary metabolite gene cluster 32, and another lies next to cluster 18 on chromosome 1.

A number (20%) of the secretory genes were annotated as encoding hydrolytic enzymes. *Aspergillus flavus* secretes hydrolases during saprobic growth to break down complex food sources (Luo *et al*., 2009). The up-regulation of secreted *A. flavus* hydrolase genes indicates that their production continues during pathogenesis. This was expected as hydrolases facilitate nutrient acquisition. It has long been suspected that extracellular hydrolases assist in the spread of disease by deconstructing host tissue (Kikot *et al*., 2009; Mellon *et al*., 2007; Schaller *et al*., 2005). Fakhoury and Woloshuk (1999) identified an α-amylase in *A. flavus* that was needed for endosperm colonization and allowed for growth down to the germ. In addition, Shieh *et al*. (1997) found an *A. flavus* polygalacturonase (*pecA*), a plant cell wall-degrading enzyme, important in the colonization of cotton bolls. The *pecA* gene, as well as several α-amylase and other cell wall-degrading enzyme-encoding genes, were up-regulated in this study. Yet, the down-regulation of some hydrolases during pathogenesis probably indicates that only specific enzymes or isozymes from this functional category are necessary for infection, or that the host's defence is repressing expression.

Transcription factors

Transcription factors (TFs) have a sizable impact on fungal development and metabolism as they regulate the transcription of multiple genes. They can also contribute to pathogenicity by inducing the expression of effector genes (Michielse *et al*., 2009; Zahiri *et al*., 2010). Our study identified *A. flavus* TFs differentially expressed during maize kernel pathogenesis. Several of these TFs have been well characterized in other *Aspergillus* species, which allows us to extrapolate their potential roles in *A. flavus* disease development.

Plant pathogens encounter a variety of environmental stressors and nutritional conditions during infection. For example, in response to pathogen attack, the plant produces antimicrobial reactive oxygen species (ROS) to prevent the spread of disease (Lamb and Dixon, 1997). In order to successfully infect the host, the pathogen must overcome this and other confronted obstacles. Two TFs previously shown to be involved in *Aspergillus* stress tolerance were found to be up-regulated in this study, and may indicate that *A. flavus* encounters and deals with environmental stressors during the colonization of the living maize kernel.

In *A. nidulans* and *A. oryzae*, the TF AtfA is important in stress tolerance, especially against ROS, by regulating the expression of stress-responsive genes (Balázs *et al*., 2010; Sakamoto *et al*., 2009). As mentioned, ROS are an important part of the plant defence response. Moreover, in *A. nidulans*, amino acid starvation activates the transcription of *cpcA*, the central regulator for the cross-pathway control system, and this, in turn, causes an increase in amino acid biosynthetic gene expression (Busch *et al*., 2003; Eckert *et al*., 2000; Hoffmann *et al*., 2001). This is relevant to *A. flavus* kernel colonization as amino acid availability is limited after the blister stage of development.

Increased transcription of *atfA* and *cpcA* would conceivably give *A. flavus* the ability to thrive in an environment that might otherwise be inhospitable. This ability to adapt to a rapidly changing local environment is probably very important in pathogenesis. In support of this, the *A. fumigatus cpcA* deletion mutant was less virulent in a murine model of pulmonary aspergillosis (Krappmann *et al*., 2004).

Changes in the environment can also trigger reproduction in fungi. Asexual sporulation in *A. nidulans* is influenced by nutrient starvation and other stressors (Adams *et al*., 1998). The TFs *fluG*, *brlA* and *wetA*, whose expression is required for asexual reproduction in *A. nidulans*, were down-regulated during *A. flavus* pathogenesis (Adams *et al*., 1998; Clutterbuck, 1969; Etxebeste *et al*., 2010). These data suggest that asexual reproduction is suppressed in *A. flavus*. Yet, the developmental modulator *medA*, which is also required for sporulation in *A. nidulans*, was, in contrast, more highly expressed in the colonization of the living kernel (Adams *et al*., 1998; Busby *et al*., 1996).

Gravelat *et al*. (2010) discovered that MedA is required for biofilm formation and full virulence in *A. fumigatus*. It is tempting to speculate that MedA serves a similar function in *A. flavus* during kernel pathogenesis and is involved in the formation of the mat-like structure at the endosperm–germ interface. At this time, it is unclear what is the role of this biofilm-like structure in kernel pathogenesis, but it may be important in germ infection. A *medA* homologue in the maize pathogen *Ustilago maydis* was also found to be required for full virulence (Chacko and Gold, 2012). Additional studies are needed to further examine the relationship between *medA* and the other asexual regulatory genes during the *in vivo* growth of *A. flavus*, especially during 'biofilm' formation.

Secondary metabolites

Fungal secondary metabolites are energetically expensive to synthesize and are not required for primary growth (Calvo *et al*., 2002; Keller *et al*., 2005). It is unclear what is the functional role of most fungal secondary metabolites, although it is thought that they protect the fungus against stressors encountered during growth.

This study identified 18 of the 55 predicted *A. flavus* secondary metabolite clusters as having the majority of the genes within the cluster (≥50% including the backbone enzyme) differentially expressed. Because of the condition under which the up-regulated clusters were found to be more highly expressed, it is tempting to assume that the up-regulated clusters (11, 14, 18, 24, 27, 33, 51, 54, and 55) aid in the colonization of the maize kernel.

Several fungal pathogens produce secondary metabolites when infecting a host (Osbourn, 2010). However, which secondary metabolites are produced *in vivo* and whether or not their presence contributes to pathogenicity are difficult to ascertain. The aforementioned increase in *A. flavus* secondary metabolite gene expression observed during pathogenesis may not be a pathogen-associated reaction, but rather a physiological response to changes in the environment. The environment within the plant is dynamic and changes in response to infection. Exposure to the new surroundings of the kernel may trigger the production of secondary metabolite expression in *A. flavus*. For example, AF synthesis is known to be influenced by many environmental variables, including temperature and pH (Calvo *et al*., 2002; Price *et al*., 2005).

Yet, secondary metabolites can function as effectors (Kwon-Chung and Sugui, 2009; Thines *et al*., 2006). Of the *A. flavus* clusters up-regulated during kernel pathogenesis, cluster 24 contains the *pes1* homologue, a known virulence factor in *A. fumigatus* (Reeves *et al*., 2006). Pes1 is a nonribosomal peptide synthetase and absence of expression leads to a reduction in *A. fumigatus* tolerance of oxidative stress. Clusters 18, 33 and 51 were also up-regulated. A previous report noted that these clusters are not expressed under standard laboratory testing conditions (Georgianna *et al*., 2010). This suggests that these metabolites serve a more specialized biological role in the fungus, which has yet to be determined.

It is unclear whether secondary metabolite gene clusters in *A. flavus* are involved in pathogenicity. However, this study has made known which clusters are expressed under pathogenic conditions. This information is important for future studies investigating *A. flavus* secondary metabolism (Brakhage and Schroeckh, 2011).

CONCLUSION

The experiments presented in this article expand our understanding of the localization, morphology and transcriptional state of

A. flavus during seed pathogenicity. This study has shown that *A. flavus* infection of the kernel is more complex and specialized than previously thought. It was observed that *A. flavus* undergoes a series of physical and transcriptional changes during pathogenicity. The formation of a biofilm-like structure shows that *A. flavus* is capable of responding to interactions with the living maize kernel. The benefits of this transformation are as yet unknown. The monitoring of the transcriptome during pathogenesis has provided a better understanding of the signals to which *A. flavus* is responding and how the fungus responds back. Changes in transcription would suggest that *A. flavus* encounters and deals with stressors during colonization. In addition, several types of effectors (i.e. toxins, secretory proteins, secondary metabolites) appear to be secreted as part of pathogenesis, which probably help to establish and maintain disease in the kernel. Together, these data indicate that *A. flavus* behaves similarly to other plant pathogens. Information acquired from these studies gives us a better understanding of *A. flavus* infection and can aid in the development of strategies aimed at reducing or eliminating *A. flavus* infection of maize.

EXPERIMENTAL PROCEDURES

Fungal growth and maize kernels

For inoculum, *A. flavus* strain NRRL 3357 was grown on potato dextrose agar (PDA) medium at 28 °C for 7–10 days. Conidial suspensions in 0.05% (v/v) Triton X-100 were adjusted to 1×10^6 spores/mL.

The maize inbred line B73 was grown in fields at the Central Crops Research Station in Clayton, NC, USA. Plants were hand pollinated and the dates of pollination were recorded to ensure that ears used in the study were at the same stage of development. At selected developmental stages, husks were pulled away from the ears and the kernels were inoculated with a pin-bar consisting of 18 straight pins embedded in an epoxy block. Pins were dipped into the *A. flavus* conidial suspension and inserted into the crown of the exposed kernels, introducing approximately 13 spores into the endosperm tissue. For each ear, four rows of kernels were inoculated and the husk was repositioned around the ear and held in place with a rubber band. A paper pollination bag (Lawson 402, Northfield, IL, USA) was placed over the inoculated ear and left until the ear was harvested.

Tracking *A. flavus* growth in the maize kernel

For this study, we used kernels inoculated at the late milk (R3) to early dough (R4) stage of development (approximately 22 days after pollination). Kernels were harvested 12, 24, 48, 72 and 96 hai. The kernels were carefully removed from the ear and stored on ice until the tissues were separated. Under sterile conditions, the pedicel regions of approximately 20 kernels, including the kernel base, were removed. The remainder of the kernel was processed further by separating the endosperm from the germ. The tissues were weighed, placed in a 20-mL vial (2.5 cm × 5 cm) and 4 mL of chilled methanol–water (60:40, v/v) per gram were added together with ceramic beads. A retrofitted reciprocating saw was used to macerate the tissues by two 30-s pulses. DNA was isolated using a cetyltrimethylammonium bromide (CTAB)-based method as follows. Five hundred microlitres of CTAB buffer (He *et al*., 2007) were added to 200 μL of kernel slurry and incubated at 65 °C for 30 min with occasional vortexing. Five hundred microlitres of chloroform–isoamyl alcohol (24:1, v/v) were added to the kernel mixture and the tube was subjected to centrifugation at 17 900 ***g*** for 5 min. The supernatant was transferred to a new tube containing 32 μL of 7.5 M ammonium acetate and 233 μL of 100% (v/v) isopropanol, and subjected to centrifugation as described previously. The DNA pellet was washed with 70% (v/v) ethanol, dried and resuspended in 30 μL of water. PCR was used to monitor the progression of *A. flavus* colonization within the different tissues of the kernel. Primers specific for *A. flavus* glyceraldehyde-3-phosphate dehydrogenase A (*gpdA*, AFLA_025100) were designed: *gpdA*_F, 5′-TCTGTTGTCGACCTCACCTG-3′; *gpdA*_R, 5′-GTCAATTTCAAGGGGTGGTG-3′; primers specific to a maize catalase gene (CAT1_F, 5′-GTCCAGACACCTGTTATTGTCCGT; CAT1_R, 5′-GAGGA AGGTGAACATGTGTAGGCT) were used to test maize DNA quality. The PCR programme was as follows: 95 °C for 4 min, [96 °C for 10 s, 55 °C for 10 s, 72 °C for 2.5 min] 40 cycles, 72 °C for 5 min. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide.

Histological studies

Late milk (R3) to early dough (R4) kernels harvested at 96 hai were cut longitudinally and placed in a fixation solution of 10% (w/v) neutral buffered formalin (Fisher Scientific, Pittsburgh, PA, USA, Cat. # SF100-4). A microtome was used to obtain 10-μm sections, which were stained according to Grocott's methenamine silver (GMS) or the periodic acid-Schiff (PAS) method (Grocott, 1955; Kligman and Mescon, 1950).

Whole-genome expression analysis

For this study, we used kernels inoculated at the blister (R2), milk (R3), dough (R4) and dent (R5) stages of development. To ensure that infection had occurred, only kernels with a mound of sporulating mycelium at the inoculation site were harvested. The kernels were harvested at 4 dai, except for dent kernels which were left in the field until sporulation occurred (6 dai). Approximately 100 inoculated kernels were carefully removed from five ears, flash frozen and stored at −80 °C until RNA could be extracted. Three replicates, each with 100 kernels, were collected at the four stages of development. AF was extracted and quantified using the protocol from Georgianna *et al*. (2010). Mature autoclaved B73 kernels severed as a nonliving control to the field kernels. These kernels were inoculated with a straight pin dipped into a suspension of 1×10^5 conidia/mL and placed in 20-mL vials (2.5 cm × 5 cm), incubated at 29 °C for 5 days and then stored at −80 °C until RNA could be extracted.

RNA was isolated as outlined in Smith *et al*. (2008) and the quality was checked before processing for array hybridization. A custom-designed *A. flavus* Affymetrix GeneChip® DNA microarray (AFAVUSa520391F), containing probe sets representing the 12 197 predicted *A. flavus* genes, was used to monitor *A. flavus* gene expression during kernel pathogenesis. Standard Affymetrix protocols were followed in the generation of biotinylated cRNA, the hybridization of the cRNA with the single-stranded DNA oligonucleotide probes and the scanning of the array. This procedure

was carried out at the Purdue Genomic Core Facility (http://www.genomics.purdue.edu) in West Lafayette, IN, USA.

Microarray analysis

The .CEL files generated from the Affymetrix GeneChip® DNA microarray scans were imported into dChip to calculate and extract expression values for each of the *A. flavus* probe sets (Li and Wong, 2001). The probe expression values were analysed without the inclusion of the mismatched probes to reduce variance and inaccurate intensity values associated with mismatched probes (Irizarry *et al*., 2003; Naef *et al*., 2002). Raw intensity values were imported into JMP Genomics (SAS, Cary, NC, USA) and $\log_2$ transformed. The expression profile for each of the arrays was reviewed using parallel plots. Based on the observed variation in distribution patterns amongst the arrays, the data were normalized using Loess normalization. The arrays were grouped into autoclaved kernel and field kernel treatment groups, and an ANOVA was performed to determine which genes were differentially expressed between treatment groups. A positive false discovery rate (pFDR) of 0.05 was used to control for the family-wise error rate (Storey, 2002).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 *Aspergillus flavus* genes differentially expressed during maize seed colonization.