Tissue-specific gene expression in maize seeds during colonization by *Aspergillus flavus* and *Fusarium verticillioides*

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SUMMARY

Aspergillus flavus and *Fusarium verticillioides* are fungal pathogens that colonize maize kernels and produce the harmful mycotoxins aflatoxin and fumonisin, respectively. Management practice based on potential host resistance to reduce contamination by these mycotoxins has proven difficult, resulting in the need for a better understanding of the infection process by these fungi and the response of maize seeds to infection. In this study, we followed the colonization of seeds by histological methods and the transcriptional changes of two maize defence-related genes in specific seed tissues by RNA *in situ* hybridization. Maize kernels were inoculated with either *A. flavus* or *F. verticillioides* 21–22 days after pollination, and harvested at 4, 12, 24, 48, 72, 96 and 120 h post-inoculation. The fungi colonized all tissues of maize seed, but differed in their interactions with aleurone and germ tissues. RNA *in situ* hybridization showed the induction of the maize *pathogenesis-related protein, maize seed (PRms)* gene in the aleurone and scutellum on infection by either fungus. Transcripts of the maize sucrose synthase-encoding gene, *shrunk-1 (Sh1)*, were observed in the embryo of non-infected kernels, but were induced on infection by each fungus in the aleurone and scutellum. By comparing histological and RNA *in situ* hybridization results from adjacent serial sections, we found that the transcripts of these two genes accumulated in tissue prior to the arrival of the advancing pathogens in the seeds. A knowledge of the patterns of colonization and tissue-specific gene expression in response to these fungi will be helpful in the development of resistance.

Keywords: *Aspergillus flavus*, *Fusarium verticillioides*, histology, maize, *PRms*, RNA *in situ* hybridization, *Sh1*.

INTRODUCTION

Maize (*Zea mays* L.), one of the most economically important and widely grown crops, is used for human food, livestock feed and

alcohol production. Maize ear rots and mycotoxin contamination caused by *Aspergillus flavus* and *Fusarium verticillioides* are chronic problems in the USA and all over the world (Bush *et al.*, 2004; CAST, 2003; Johansson *et al.*, 2006; Munkvold, 2003; Payne, 1992; Woloshuk and Shim, 2013).

Aspergillus flavus is an opportunistic fungal pathogen (Payne, 1992) capable of infecting immature maize kernels under favourable conditions, which include high temperature and water stress (Diener *et al.*, 1987; Horn *et al.*, 2009; Jones *et al.*, 1980; Payne, 1992; Payne *et al.*, 1988; Widstrom *et al.*, 2003b). This fungus can invade maize ears through the silk channel or other openings in the husks (Marsh and Payne, 1984). Once in the ear, the fungus colonizes kernel surfaces and enters kernels either through the pedicel region or through wounds created by insect or mechanical injury of the pericarp (Anderson *et al.*, 1975; Hesseltine *et al.*, 1976; Lee *et al.*, 1980; Lillehoj *et al.*, 1975; Payne, 1983; Smart *et al.*, 1990; Widstrom *et al.*, 2003a). Inside the kernel, the fungus can colonize all tissue types, with the most extensive colonization occurring in the germ (Dolezal *et al.*, 2013; Fennell *et al.*, 1973; Keller *et al.*, 1994; Smart *et al.*, 1990).

Unlike *A. flavus*, *F. verticillioides* can exist as an endophyte in maize plants, becoming pathogenic under conditions that are not completely understood (Bacon *et al.*, 2008; Cao *et al.*, 2013; Munkvold *et al.*, 1997; Parsons and Munkvold, 2010a, b, 2012). This fungus enters the ear through the ear shank or silk channel of the ear, infects kernels and causes ear rots (Bacon *et al.*, 2008; Duncan and Howard, 2010). Insect feeding and mechanical damage of the kernel also facilitate invasion of *F. verticillioides* (Koehler, 1942; Maiorano *et al.*, 2009; Munkvold, 2003; Parsons and Munkvold, 2010a, b, 2012; Sobek and Munkvold, 1999; Warfield and Davis, 1996).

Genetic resistance to aflatoxin and fumonisin accumulation has been identified in maize breeding populations, but resistance is polygenic and subject to large gene $\times$ environment interactions. The lack of good markers for phenotyping further hampers the development of agronomically adapted maize genotypes with resistance to these diseases (Brown *et al.*, 2013; Bryła *et al.*, 2013; Eller *et al.*, 2008; Moreno and Kang, 1999; Warburton *et al.*, 2013; Warburton and Williams, 2014; Zila *et al.*, 2013). Maize plants

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have evolved shared mechanisms to defend against ear rot fungi (Mideros *et al.*, 2014), and Robertson-Hoyt *et al.* (2007) showed, through marker assisted selection, that at least some of the genes associated with the plant defence response to *A. flavus* and *F. verticillioides* infection are genetically linked.

Proteomic and transcriptomic analyses have identified changes in protein production during the infection of maize seeds by *A. flavus* and *F. verticillioides* (Brown *et al.*, 2013; Campos-Bermudez *et al.*, 2013; Dolezal *et al.*, 2013, 2014; Lanubile *et al.*, 2012). The biological activity of many of these proteins is unknown, but a few are known to have antifungal activity, or to be involved in host defence. These include pathogenesis-related (PR) proteins, lipoxygenases, α -amylase inhibitors, ribosome-inactivating proteins (RIPs) and zeamatin (Bravo *et al.*, 2003; Brown *et al.*, 2010; Fakhoury and Woloshuk, 2001; Guo *et al.*, 1997; Kelley *et al.*, 2012; Lanubile *et al.*, 2012; Moore *et al.*, 2004; Murillo *et al.*, 1999; Nielsen *et al.*, 2001; Wilson *et al.*, 2001; Zila *et al.*, 2013).

To gain additional insights into the interaction between these two pathogens and maize kernels, we used histology to follow the colonization of developing maize kernels by each fungus from 4 to 120 h post-inoculation (hpi). We compared the colonization of maize kernels by the two pathogens under the same field conditions. Concurrently, RNA *in situ* hybridization was used to monitor the temporal and spatial expression pattern of two maize genes, *PRms* (pathogenesis-related protein, maize seed; accession number, X54325; gene id, AC205274.3_FG001) and *sh1* (shrunken-1; synonym, *Sucrose-UDP glucosyltransferase 1*; accession number, NM_001279762; gene id, GRMZM2G089713). The two genes were selected to monitor the timing and localization of the host response to infection by the two pathogens. The expression of PRms, one of the better studied PR proteins in maize, was monitored as a marker for defence signalling in maize. *Sh1* was selected because earlier studies had shown its expression to be induced in response to infection by *A. flavus* (Dolezal *et al.*, 2014), and because changes in carbohydrate metabolism often occur in plants during pathogen attack (Berger *et al.*, 2004; Botanga *et al.*, 2012; Doehlemann *et al.*, 2008; Dolezal *et al.*, 2014; Göhre *et al.*, 2012; Santi *et al.*, 2013). Through the observation of fungal colonization and the expression of the two maize genes in serial histological sections of the maize kernels, we found *PRms* and *Sh1* to be expressed in a tissue-specific fashion before visible fungal colonization.

RESULTS

Colonization of maize kernels by *A. flavus* and *F. verticillioides*

Maize kernels were mock inoculated (Fig. 1a) or inoculated with *A. flavus* or *F. verticillioides* by wounding with a needle (Fig. 1c,d).

Kernels were collected at 4, 12, 24, 48, 72, 96 and 120 hpi. Figure 1e–j shows the location of *A. flavus* and *F. verticillioides* in maize kernels at 96 hpi. The localization of the fungi within the seeds at 48, 72 and 96 hpi is summarized by the schematic drawings in Fig. 2j–l. Initial colonization by these two fungi was observed in the aleurone and endosperm at the site of inoculation at 48 hpi (Figs 2j, S1a,b, see Supporting Information). Mycelia and conidia of *A. flavus* were observed in the aleurone, endosperm and germ at 72 hpi (Fig. 2k). In contrast, *F. verticillioides* was detected only in the aleurone and endosperm at 72 hpi (Fig. 2k). Both fungi were observed in all kernel tissues by 96 hpi (Fig. 2l).

Extensive disruption of the cytoplasm and nuclei was observed in the aleurone colonized by *A. flavus* (Fig. 1e), whereas many aleurone cells were either intact or only partially destroyed by *F. verticillioides* (Figs 1f, S1d). Only in the later stages of infection by *F. verticillioides* was substantial degradation of the aleurone observed (Fig. S1f). Both fungi extensively colonized and degraded tissues of the endosperm, creating cavities that often contained mycelia and conidia (Figs 1j, S1i,j). In some areas, these fungi were observed throughout the endosperm without obvious degradation of host tissue (Figs 1h, S1e,h).

In this study, little colonization was observed in the embryo tissue. At 96 hpi, both *A. flavus* and *F. verticillioides* colonization was observed in the scutellum of only a few kernels (Figs 2l, S1e,g). Instead, *A. flavus* often formed a biofilm-like structure at the endosperm–scutellum interface (Figs 1g, S1e), as reported by Dolezal *et al.* (2013). In many cases, the biofilm-like structure covered a large area of the scutellum without evidence of germ colonization (Fig. 1g). A similar biofilm-like structure was not observed in *F. verticillioides*-infected kernels (Fig. 1h). Rarely was *A. flavus* found in the tip of the scutellum, and this occurred only in those kernels with obvious damage to the germ (Fig. S1g).

Aspergillus flavus colonization of the basal endosperm transfer layer (BETL) was observed at 96 hpi (Fig. 1i). *Fusarium verticillioides* hyphae were not observed in the BETL, but were observed in the aleurone and endosperm near the BETL region at 96 hpi (Fig. 1j).

Tissue-specific gene expression of *PRms*

RNA *in situ* hybridization was conducted to assess the timing and localization of host expression of *PRms* in response to the two pathogens. By 48 hpi, transcripts of *PRms* were observed in aleurone cells during *A. flavus* colonization of the seed, and were detected in the scutellum by 72 hpi (Fig. 2c,e,g). In *F. verticillioides*-infected kernels, *PRms* transcripts were detected in the aleurone and scutellum at 48 hpi (Fig. 2f). In the scutellum, transcripts of this gene were predominantly detected at the tip and in the glandular layer (Fig. 2c,d,g). *PRms* transcripts were also observed within the scutellum (Fig. 2c,d). During *F. verticillioides* infection, *PRms* transcripts were often observed in the scutellum

Fig. 1 *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv) colonization in maize kernels at 96 h post-inoculation. Kernels were wound inoculated with Triton X-100 (a), Af (c) or Fv (d). Kernel sections were stained with safranin and fast green (b and e–j). No fungal colonization was observed in the aleurone (Al) of a mock-inoculated kernel (b). Af colonized and destroyed the Al (e), whereas Fv colonized around the intact Al (f). Af colonized at the endosperm (En)–scutellum (Sc) interface forming a biofilm-like structure and creating cavities (Ca) in the En (g). Fv ramified through the En near the Sc (h). Af colonized in the basal endosperm transfer layer (BETL) (i). Fv colonized in the basal Al and En near the BETL and formed a Ca in the En (j). Arrows denote fungal colonization. Scale bars: (a, c, d) 3 mm; (b, e, f) 30 μ m; (g–i) 50 μ m; (j) 200 μ m.

Fig. 2 Localization of pathogenesis-related protein, maize seed (PRms) transcripts (purple signal) in maize kernels during *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv) colonization. No PRms transcripts were observed in the aleurone (Al), endosperm (En) or scutellum (Sc) of mock-inoculated kernels (a, b). PRms transcripts were localized in kernel sections at 96 h post-inoculation (hpi; c–i). PRms transcripts were present in the Al, Sc tip and glandular layer (Gl) during both Af (c, i) and Fv (d) infection. PRms transcripts were observed in the Al (e) and Gl (g) during Af infection. PRms transcripts were observed in the Al (f) and Sc near the basal endosperm transfer layer (BETL; h) during Fv infection. (j–l) Schematic drawings of longitudinal kernel sections showing colonization by Af (green dots) and Fv (orange dots), as well as the presence of PRms transcripts (purple signal) over time. At 48 hpi (j), Af and Fv colonized at the inoculation site; PRms transcripts were localized in the Al. At 72 hpi (k), Af colonized in the Al, En and Sc; Fv colonized only in the Al and En; PRms transcripts were localized in the Al, Sc tip and Gl. At 96 hpi (l), Af colonized in the Al, En, Sc, BETL and at the En–Sc interface with the formation of a biofilm-like structure; Fv colonized in the Al, En and Sc; PRms transcripts were localized in the Al, Sc tip and Gl. Arrows denote the presence of PRms transcripts. Scale bars: (a, b, e–h) 30 µm; (c, d) 200 µm; (i) 1 mm.

near the BETL (Fig. 2h). No *PRms* transcripts were observed in mock-inoculated kernels (Fig. 2a,b) or kernels collected at 4, 12 and 24 hpi (data not shown). No *PRms* gene expression signal was observed in the sections hybridized with the control probe (Fig. 4c,f,i).

Tissue-specific gene expression of *Sh1*

RNA *in situ* hybridization indicated that the expression of *Sh1* was altered on infection by *A. flavus* and *F. verticillioides*. *Sh1* transcripts were observed in some aleurone cells in response to *A. flavus* infection at 48 hpi (data not shown). At 72 and 96 hpi, *Sh1* transcripts were detected in the aleurone and scutellum (Fig. 3c,e,g). However, during *F. verticillioides* infection, *Sh1* transcripts were observed in the aleurone at 24 hpi (data not shown), before fungal colonization was observed in any tissue of the kernel. At 48, 72 and 96 hpi, this gene was expressed in the aleurone and scutellum (Fig. 3d,f). It was also expressed in the scutellum near the BETL region at 72 and 96 hpi (Fig. 3h). Transcripts of *Sh1* were observed in the scutellum near the BETL during *A. flavus* infection at 120 hpi (data not shown). In both *A. flavus* and *F. verticillioides* kernels, *Sh1* was often induced at the scutellum tip and in the glandular layer.

Unlike *PRms*, *Sh1* transcripts were observed in the embryo of some mock-inoculated (Fig. 3i) and non-wounded kernels (data not shown). However, *Sh1* was not expressed in the aleurone (Fig. 3a) and scutellum (Fig. 3b) of either mock-inoculated or non-wounded kernels. Likewise, no *Sh1* signal was observed in the sections hybridized with the control probe (Fig. 3j). Thus, *Sh1* was expressed in the embryo independent of fungal infection, but its expression in the aleurone and scutellum was induced on colonization of the kernel by each of these two fungi.

Tissue-specific gene expression of *PRms* and *Sh1* before fungal colonization

By comparing histological and RNA *in situ* hybridization results from adjacent serial sections, we observed both *PRms* (Fig. 4a–f) and *Sh1* (data not shown) transcripts in tissues lacking visible fungal colonization. These results indicate that kernel tissue responds to fungal invasion in advance of visible fungal colonization. Co-localization of the fungus with *PRms* and *Sh1*, as measured by *in situ* hybridization, was sometimes observed in scutellum tissue, but neither *PRms* nor *Sh1* was detected in the aleurone cells colonized by either fungus.

Quantification of *PRms* and *Sh1* gene expression

Gene expression levels of *PRms* and *Sh1* were quantified by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR; Fig. 5). *PRms* transcripts were not detectable in non-

wounded kernels or in inoculated kernels collected at 4 and 12 hpi (Fig. 5a). However, both fungi activated the expression of this gene at 24, 48 and 72 hpi. Compared with the samples collected at 24 and 48 hpi, expression levels of *PRms* increased at 72 hpi on infection by both fungi. This finding agrees with the RNA *in situ* hybridization results. qRT-PCR analysis showed no elevation of transcript accumulation of *Sh1* during fungal infection (Fig. 5b).

DISCUSSION

Aspergillus flavus and *F. verticillioides* differ in their colonization of maize seed tissues

Aspergillus flavus and *F. verticillioides*, two ear rot pathogens exhibiting different tropic behaviour, differed in their pathogenesis of the aleurone and scutellum, two metabolically active tissues of maize seed. *Aspergillus flavus* rapidly colonized the aleurone and destroyed the structure of the cells. In the later stages of colonization, the cells collapsed and the resulting space was replaced by fungal mycelium (Figs 1e, S2c). In contrast, the aleurone cells colonized by *F. verticillioides* remained structurally intact during initial colonization (Fig. 1e,f). Some cells had visible mycelium of the fungus within the cells (Fig. S1d), but the cells of the aleurone did not collapse until later in the infection process. Others (Bacon *et al.*, 2008; Duncan and Howard, (2010) have suggested that endophytic growth of *F. verticillioides* in the aleurone occurs only in non-symptomatic maize kernels, whereas starburst symptoms and visible rot are associated with disease. Starburst symptoms on inoculated kernels were not observed in our studies as a consequence of infection by *F. verticillioides*, perhaps because of the stage of kernel maturity. Our observations suggest that extensive colonization of kernels by *F. verticillioides* can occur without visible macroscopic symptoms of rot. This supports the observations by Bush *et al.* (2004) which showed high concentrations of fumonisin in visibly sound kernels.

Although both fungi grew extensively in infected cells of the aleurone, this colonization was localized; the fungi began to preferentially colonize the endosperm rather than continuing to colonize the aleurone. This was surprising given the nutrient-rich status of the aleurone (Bethke *et al.*, 1998). Perhaps defence responses in the aleurone limit the growth of these fungi.

Aspergillus flavus also differed from *F. verticillioides* in the colonization of the scutellum. *Aspergillus flavus* mycelium was rarely observed within the scutellum before the formation of a biofilm-like structure at the endosperm–scutellum interface (Fig. 1g), as reported by Dolezal *et al.* (2013). This specialized structure of *A. flavus* resembles the biofilm formed by *A. fumigatus* in the human lung (Loussert *et al.*, 2010). It was not possible to determine from this study whether or not the structure is absolutely required for colonization of the germ, but the prevalence of this structure at the interface of the scutellum prior to infection argues

Fig. 3 Localization of *shrunken-1* (*Sh1*) transcripts (purple signal) in mock-, *Aspergillus flavus* (*Af*)- and *Fusarium verticillioides* (*Fv*)-inoculated maize kernels at 72 h post-inoculation. No *Sh1* transcripts were observed in the aleurone (Al), endosperm (En) or scutellum (Sc) of mock-inoculated kernels (a, b). *Sh1* transcripts were observed in the Al (c), outermost layer of Sc (e) and inner areas of Sc (g) during *Af* infection. *Sh1* transcripts were detected in the Al (d), Sc and embryo (Em) (f), and Sc near the basal endosperm transfer layer (BETL; h), during *Fv* infection. *Sh1* transcripts were observed in the Em of the mock-inoculated kernel (i). No signal was observed in the Em of the mock-inoculated kernel section hybridized with the control probe (j). Scale bars: (a–d, g, h) 30 μ m; (e, f, i, j) 200 μ m.

Fig. 4 Activation of *pathogenesis-related protein, maize seed (PRms)* gene expression (purple signal) before visible colonization by *Aspergillus flavus* (*Af*) and *Fusarium verticillioides* (*Fv*) at 72 h post-inoculation. Adjacent serial sections of an *Af*-inoculated kernel were stained with safranin and fast green (a), hybridized with *PRms* probe (b) or control probes (c). *PRms* transcripts were detected in the aleurone (Al) and scutellum (Sc) tip of the kernel inoculated with *Af* (b). Adjacent serial sections of an *Fv*-inoculated kernel were stained with safranin and fast green (d), hybridized with *PRms* probe (e) or control probes (f). *PRms* transcripts were detected in the aleurone and scutellum tip of the kernel inoculated with *Fv* (e). Adjacent serial sections of a mock-inoculated kernel were stained with safranin and fast green (g), hybridized with *PRms* probe (h) or control probes (i). No *PRms* transcripts were detected in the mock-inoculated kernel (h). No fungal colonization was detected in *Af*- (a), *Fv*- (d) or mock (g)-inoculated kernels. No signal was observed in the sections hybridized with the control probes (c, f, i). Scale bars: 50 μ m.

that it may either be required for infection or needed for resistance to toxic compounds within, or secreted by, the scutellum. The scutellum is metabolically active and is known to secrete hydrolases and defence-associated compounds (Guo *et al.*, 1999). No such structure was observed during colonization by *F. verticillioides*.

We found no striking differences in the colonization of the endosperm by the two fungi. Both fungi were detected in the endosperm at the inoculation site at 48 hpi, and reached all tissues at 96 hpi (Fig. 1). Previous studies have shown that *A. flavus* is detected by PCR assays in the endosperm as early as 24 hpi and in the germ at 72 hpi (Dolezal *et al.*, 2013); however, in our study, neither microscopic nor macroscopic fungal colonization was observed in inoculated kernels until 48 hpi.

***Aspergillus flavus* and *F. verticillioides* induce tissue-specific gene expression before visible fungal colonization**

The staining of serial histological sections for visualization of fungal colonization or for RNA *in situ* hybridization allowed for the observation of fungal colonization and the expression of *PRms* and *Sh1* in adjacent tissue sections during the colonization of maize kernels. We found *PRms* to be expressed in the aleurone and scutellum of infected kernels in advance of visible colonization by either fungus. Using cellular and subcellular immunolocalization, Murillo *et al.* (1999) also showed that the PRms protein accumulated in the aleurone and inner parenchyma cells of the scutellum of germinating maize seeds early after infection

Fig. 5 Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of *pathogenesis-related protein, maize seed (PRms)* (a) and *shrunken-1 (Sh1)* (b) gene expression in maize kernels during infection by *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv). Expression levels were normalized by the maize gene GRMZM2G024838 encoding the structural component of maize ribosomes, and were relative to the non-wounded kernels. hpi, hours post-inoculation.

by *F. verticillioides*. Although they were able to visualize PRms accumulation in the scutellum at the site of infection by *F. verticillioides*, they did not report the accumulation of PRms in these tissues in advance of colonization. They did observe PRms localization in papillae in advance of colonization by *F. verticillioides*.

Similar to PRms, *Sh1* was expressed in the aleurone and scutellum of infected seeds in advance of visible colonization by either fungus. Unlike PRms, *Sh1* was expressed in the embryo of seeds independent of fungal colonization; *Sh1* was also detected in the embryo of non-wounded kernels and mock-inoculated kernels. Using a histochemical enzyme assay to localize the Sh1 enzyme, Wittich and Vreugdenhil (1998) found maize Sh1 to be activated in the aleurone, endosperm and embryo during seed development. The differences observed between the two studies may be a result of differences in kernel maturity, detection techniques or the genotype of maize used.

Sh1 was expressed earlier in the aleurone in response to *F. verticillioides* than to *A. flavus*, even though there was more

visible mycelium of *A. flavus* than of *F. verticillioides*. Moreover, *Sh1* was detected earlier than PRms in the aleurone and scutellum near the BETL during *F. verticillioides* infection. Transcript accumulation of both PRms and *Sh1* in response to the two fungi was highest at the tip of the scutellum. Colonization by these two fungi was rarely observed in the scutellum tip, perhaps because of an accumulation of these defence-related compounds in this region.

Possible roles of maize PRms and Sh1 in response to *A. flavus* and *F. verticillioides*

Maize plants have complex regulatory networks that respond to pathogen attack (Brown *et al.*, 2010; Chen *et al.*, 2007; Denancé *et al.*, 2013; Dolezal *et al.*, 2014; Guo *et al.*, 1999; Royo *et al.*, 2006; Warburton *et al.*, 2013; Wu *et al.*, 2014). We have shown that *A. flavus* and *F. verticillioides* induce transcriptional changes in genes associated with the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) defence signalling pathways (Dolezal *et al.*, 2014; Shu, 2014). Also induced in response to infection are mRNAs encoding several PR proteins, including PRms. PRms expression is associated with increased resistance to several plant pathogens (Campos-Soriano and San Segundo, 2009; Cordero *et al.*, 1994; Gómez-Ariza *et al.*, 2007; Murillo *et al.*, 2003). As an example, transgenic rice constitutively expressing PRms is more resistant to *Magnaporthe oryzae*, *F. verticillioides*, *Helminthosporium oryzae* and *Erwinia chrysanthemi* (Gómez-Ariza *et al.*, 2007).

Increased resistance associated with PRms appears to be mediated, in part, by the induction of host resistance priming (Campos-Soriano and San Segundo, 2009; Cordero *et al.*, 1994; Gómez-Ariza *et al.*, 2007; Murillo *et al.*, 2003). Gómez-Ariza *et al.* (2007) found that the PR genes *OsPR1b*, *PBZ1* and *Sci1* in rice were expressed earlier and to a greater level in plants with constitutive expression of PRms on infection by *M. oryzae*. The authors also found that rice plants transgenic for PRms accumulated higher levels of sucrose and showed increased resistance to *M. oryzae* (Gómez-Ariza *et al.*, 2007). Thus, PRms appears to act as a defence regulator to modulate resistance in maize plants (Gómez-Ariza *et al.*, 2007; Huffaker *et al.*, 2011; Murillo *et al.*, 2001). We also found PRms to be highly expressed in developing maize kernels in response to infection by *A. flavus* (Dolezal *et al.*, 2014) and *F. verticillioides* (Shu, 2014). In our studies, we showed the induction of PRms in two tissues that are metabolically active during fungal colonization. Modulation of PRms or other defence compounds in these tissues could be part of a strategy for the control of these ear rot fungi.

Sh1 catalyses the reversible conversion of sucrose and uridine diphosphate (UDP) into fructose and UDP-glucose (Xu *et al.*, 1989). Fructose and UDP-glucose are important substrates for various metabolic pathways, including cell wall synthesis and respiratory pathways (Delmer and Amor, 1995; Huber and Huber, 1996). *Sh1* is not essential for sucrose synthesis in maize kernels

(Cobb and Hannah, 1988), but overexpression of this gene results in elevated levels of starch (Jiang *et al.*, 2013), indicating an important role of *Sh1* in starch biosynthesis.

Sh1, whose expression increases during kernel development (Azama *et al.*, 2003), is highly regulated in maize, and expressed in response to specific developmental and environmental stimuli, such as anaerobic stress (Clancy and Hannah, 2002; Hauptmann *et al.*, 1988; Maas *et al.*, 1991; McCarty *et al.*, 1986; Vasil *et al.*, 1989). Maize *Sh1* is localized to both mitochondria and nuclei in maize seedlings (Subbaiah *et al.*, 2007).

The role played by *Sh1* in host defence is not yet clear. Infection of plants by several fungi causes changes in hexose partitioning and accumulation. Up-regulation of *Sh1* was observed in maize leaves on *Ustilago maydis* infection, and sucrose contents were increased slightly in this interaction (Doehlemann *et al.*, 2008). *Sh1* has also been associated with plant–microbe interactions in other plant species (Fraissinet-Tachet *et al.*, 1998; Langlois-Meurinne *et al.*, 2005; Ma *et al.*, 2010; van de Mortel *et al.*, 2012; O'Donnell *et al.*, 1998; Poppenberger *et al.*, 2003, 2006). The induction of *Sh1* might enhance the sucrose metabolic pathway and provide substrates for the synthesis of defence compounds (Cobb and Hannah, 1988; Delmer and Amor, 1995; Huber and Huber, 1996). We have shown that *A. flavus* causes a shift in hexose metabolism away from starch synthesis and into other pathways of carbon metabolism, such as the shikimate and pentose phosphate pathways, two pathways that lead to the production of defence-related compounds (Dolezal *et al.*, 2014). These observations, together with the evidence that sucrose is involved in defence priming (Berger *et al.*, 2004; Santi *et al.*, 2013), argue that sugars play important roles in host defence.

Our studies show that *A. flavus* and *F. verticillioides* colonize tissues of maize kernels in a predictable pattern and establish relationships with the seed that would be expected for other well-described necrotrophs. Further, these two fungi induce PRms, a characterized resistance-associated protein, in the aleurone and scutellum in advance of visible fungal colonization. Taken together, these data argue that enhanced resistance to these fungi could be achieved by the manipulation of known pathways of host defence. The greatest challenge to the deployment of resistance genes will be the achievement of the expression of these genes during conditions of plant stress, which favour colonization and mycotoxin production by the two fungi.

CONCLUSION

Safranin and fast green staining of tissue sections, which allowed the sensitive detection of fungal mycelium and conidia within the maize seeds, coupled with RNA *in situ* hybridization, which has the ability to detect both rare and abundant transcripts, allowed us to monitor both the location and temporal pattern of host gene expression in relation to colonization by two important ear rot-

causing fungi of maize. *Aspergillus flavus* grew more quickly and caused more cell death in the early stages of colonization. Mycelium of *F. verticillioides*, but not that of *A. flavus*, was observed growing adjacent to intact aleurone cells. Further, infection of the scutellum by *A. flavus* was often preceded by the formation of a biofilm-like structure and, in some cases, *A. flavus* was observed growing among intact cells of the scutellum. No specialized mycelial structures were observed for *F. verticillioides*.

Maize *PRms* and *Sh1* genes were expressed in tissues before fungal mycelium was visible, indicating that tissues respond to infection before colonization. Expression of *PRms* was restricted to cells in the aleurone and scutellum, with the greatest expression in the outer layer of the scutellum and at the tip of the scutellum. *Sh1* expression was also induced in cells of the aleurone and scutellum in response to infection. Unlike *PRms*, *Sh1* was expressed in the embryo of non-infected kernels. Both of these genes have been associated with resistance to plant diseases (Casacuberta *et al.*, 1991, 1992; Dolezal, 2010; Gómez-Ariza *et al.*, 2007; Huffaker *et al.*, 2011; Murillo *et al.*, 1999, 2001) and may play a role in resistance to these fungi. No evidence was found that the host–parasite interactions involving these fungi differ appreciably from well-characterized host–pathogen interactions. Thus, sustainable host resistance to the fungi should be possible.

EXPERIMENTAL PROCEDURES

Plant and fungal materials

Maize inbred line B73 was grown at the Central Crops Research Station near Clayton, NC, USA. Maize ears were hand pollinated and covered with pollination bags. Fungal strains (*A. flavus* NRRL 3357, *F. verticillioides* n16) were grown on potato dextrose agar (PDA) plates at 28 °C for 5 days. Conidia were harvested from culture plates with sterile distilled water containing 0.05% (v/v) Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of conidia was determined with a haemocytometer (Hausser Scientific, Horsham, PA, USA), and the conidial suspension was diluted to 1×10^6 conidia/mL for plant inoculations.

Fungal inoculation and tissue collection

Maize ears free of visible disease symptoms or insect damage were inoculated in the field at 21–22 days after pollination with either *A. flavus* or *F. verticillioides*. Kernels were wounded with a needle that had been dipped into a conidial suspension. Kernels for the mock treatment were inoculated with sterile distilled water containing 0.05% (v/v) Triton X-100. Inoculated kernels were collected at 4, 12, 24, 48, 72, 96 and 120 hpi. Corresponding mock-inoculated and non-wounded kernels were collected at 4, 12, 24, 48, 72, 96 and 120 hpi and used as negative controls. Three ears were harvested for each treatment as biological replicates. Kernels for histology and RNA *in situ* hybridization studies were harvested and processed immediately. Kernels collected for RNA extraction and qRT-PCR studies were frozen in liquid nitrogen immediately, and then stored at –80 °C.

Table 1 Microwave fixation, dehydration and embedding steps to process maize kernels.

Step	Chemical medium	Time	Temperature (°C)	Wattage (watts)	Vacuum (Hg)
Fixation	Modified FAA*	2 h	35	650	20
Dehydration	70% flex	30 min	32	650	20
	80% flex	30 min	32	650	20
	95% flex	30 min	32	650	20
	100% flex	30 min	32	650	20
	100% flex	30 min	32	650	20
	1:1 (v/v) flex : xylene	30 min	32	650	20
	1:1 (v/v) flex : xylene	30 min	32	650	20
	100% xylene	30 min	32	650	20
	100% xylene	30 min	32	650	20
	1:1 (v/v) xylene : paraffin	30 min	65	650	20
	1:1 (v/v) xylene : paraffin	30 min	65	650	20
Embedding	100% paraffin	1 h	65	650	20
	100% paraffin	1 h	65	650	20

*Modified FAA fixative is 40% (v/v) distilled H₂O, 45% (v/v) methanol, 10% (v/v) formaldehyde and 5% (v/v) glacial acetic acid.

Tissue fixation, dehydration and embedding

Kernels for histology and RNA *in situ* hybridization were collected in tissue-embedding capsules (Fisher). Kernels were fixed, dehydrated and embedded in paraffin using a microwave oven (Pelco, Clovis, CA, USA) according to a protocol modified from Livingston *et al.* (2009, 2013; Table 1). The paraffin blocks were sectioned with an RM2255 microtome (Leica Microsystems, Wetzlar, Germany). Ten-micrometre-thick sections were mounted on microscope slides (Gold Seal, Thermo Fisher Scientific) for histology staining. Adjacent 20-µm sections were mounted on Probe microscope slides (Fisher) for RNA *in situ* hybridization. Slides were dried at 35 °C on a hot plate overnight and stored at room temperature.

Tissue staining

Kernel sections were stained with safranin and fast green to allow for the differentiation of maize and fungal tissues (Livingston *et al.*, 2009, 2013; Table 2). Stained sections were mounted in Permount (Thermo Fisher Scientific) and covered with coverslips.

RNA extraction and probe cloning

Eight kernels from individual ears were pooled and ground in liquid nitrogen with a mortar and pestle. Ground tissue was added to 0.75 mL of tris(hydroxymethyl)aminomethane (Tris)-saturated phenol, pH 6.6 (Fisher), and homogenized for 2 min. Samples were then dissolved in Tris-ethylenediaminetetraacetic acid (Tris-EDTA, Thermo Fisher Scientific) buffer, pH 8.0 (ACROS Organics, Thermo Fisher Scientific), extracted with 5:1 (v/v) acid phenol : chloroform, pH 4.5 (Thermo Fisher Scientific), and precipitated with ice-cold 100% ethanol (ACROS Organics) overnight. Total RNA was further purified with an RNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The quantity and quality of RNA were analysed using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was treated with DNase (Promega, Madison, WI, USA), and cDNA synthesis was performed using the first-strand cDNA synthesis kit (Fermentas, Thermo Fisher Scientific) according to the manufacturer's instructions.

Table 2 Safranin and fast green staining protocol for sections of maize kernels at room temperature.

Step	Chemical medium	Time
Paraffin removal	100% xylene	20 min
Rehydration	100% ethanol	10 s
	95% (v/v) ethanol	10 s
	70% (v/v) ethanol	10 s
	50% (v/v) ethanol	10 s
Staining	Safranin	2 h
Dehydration	50% (v/v) ethanol	10 s
	70% (v/v) ethanol	10 s
	95% (v/v) ethanol	10 s
	100% ethanol	10 s
Counterstaining	Fast green	1 min
Post-staining	100% xylene	1 min

RT-PCR was conducted with *PRms-1* and *Sh1* primer sets to amplify *PRms* and *Sh1* probe sequences, respectively (Table 3). cDNA from *A. flavus*-inoculated kernels collected at 96 hpi was used to amplify *PRms* and *Sh1* probe sequences. RT-PCR was performed using Ex Taq (Chemicon, Otsu, Shiga, Japan). The conditions used for RT-PCR were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min 30 s, and then followed by a final extension step performed at 72 °C for 7 min. The PCR products were resolved through a 0.8% (w/v) agarose gel and cleaned using a QIAquick PCR purification kit (Qiagen). The purified *PRms* and *Sh1* PCR products were inserted into the dual promoter vector PCR[®]II-TOPO[®] (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol, and sequenced. The vectors carrying *PRms* and *Sh1* probe sequences were linearized by the restriction enzymes *NcoI* and *ApaI*, respectively. The antisense and sense (control) probes were transcribed from the linearized vectors using the Riboprobe[®] System-SP6 and -T7 transcription kits (Promega) following the manufacturer's instructions.

Gene (gene id)	Primer usage	Primers 5'–3'
<i>PRms</i> (AC205274.3_FG001)	Probe	PRms-1F: TACAATGGAGGCATCCAACA PRms-1R: CATTGATCGCAGGCACAAT
	qRT-PCR	PRms-2F: TACAATGGAGGCATCCAACA PRms-2R: CTGTTTGGGGAGTGAGGTA
<i>Sh1</i> (GRMZM2G089713)	Probe	Sh1-1F: TGGAGTAGCCTGCGTTCTACG Sh1-1R: TGCAGCCAATTCTACCAT
	qRT-PCR	Sh1-2F: GGAGTAGCCTGCGTTCTACG Sh1-2R: GTCATGTGCAGGCCAGATA
<i>Ribosome structural component gene</i> (GRMZM2G024838)	qRT-PCR	Rib-F: GGCTTGCTTAAAGGAAGGT
		Rib-R: TCAGTCCAACCTCCAGAATGG

Table 3 Primers used in this study.

RNA *in situ* hybridization

RNA *in situ* hybridization was carried out according to previously described protocols (Franks *et al.*, 2002; Lincoln *et al.*, 1994; Long and Barton, 1998). The hybridization temperature was 65 °C. The sense probes of each gene were included as negative controls. Mock-inoculated kernels were hybridized with both antisense and sense probes as biological controls.

Microscopy

Images of sections stained with safranin and fast green, as well as sections hybridized with RNA probes, were collected on an Eclipse E600 light microscope (Nikon, Tokyo, Japan), captured on an Infinity1-3C digital camera and analysed with Infinity Analyze imaging software (Lumenera, Ottawa, Ontario, Canada).

qRT-PCR expression analysis

cDNAs from either *A. flavus*- or *F. verticillioides*-inoculated kernels collected at 4, 12, 24, 48 and 72 hpi were used for qRT-PCR studies. The primers used for qRT-PCR are listed in Table 3. qRT-PCR was performed using a SYBR® Green kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The conditions used for qRT-PCR were as follows: 96 °C for 5 min, followed by 35 cycles of 96 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, plate read, 78 °C for 20 s, plate read, and then followed by a final extension step performed at 72 °C for 10 min. The expression levels of GRMZM2G024838, a structural component of maize ribosomes, were used for normalization. Data were analysed by the comparative C_T method with the amount of target given by the calibrator $2^{-\Delta\Delta C_T}$ (Schmittgen and Livak, 2008).

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SUPPORTING INFORMATION

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

Fig. S1 *Aspergillus flavus* (Af) and *Fusarium verticillioides* (Fv) colonization in maize kernels. Kernel sections were stained with safranin and fast green. Af (a) and Fv (b) colonized at the inoculation site at 48 h post-inoculation (hpi). Af typically colonized and destroyed the aleurone (Al) (c), whereas Fv often colonized in the intact Al (d) and destroyed the Al at a later infection stage (f). Af colonized at the endosperm (En)–scutellum (Sc) interface forming a biofilm-like structure and colonizing the Sc (e). Af colonized the Sc tip of a kernel with obvious damage to the germ (g). Fv colonized the En (h). Af (i) and Fv (j) colonized the En, creating cavities (Ca) in the En (j). Arrows denote fungal colonization. Scale bars: (a, b, g, h) 200 µm; (c, d, f, j) 30 µm; (e, i) 50 µm.