Amylopectin Induces Fumonisin B₁ Production by *Fusarium verticillioides* During Colonization of Maize Kernels

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Fusarium verticillioides, a fungal pathogen of maize, produces fumonisin mycotoxins that adversely affect human and animal health. Basic questions remain unanswered regarding the interactions between the host plant and the fungus that lead to the accumulation of fumonisins in maize kernels. In this study, we evaluated the role of kernel endosperm composition in regulating fumonisin B₁ (FB₁) biosynthesis. We found that kernels lacking starch due to physiological immaturity did not accumulate FB₁. Quantitative polymerase chain reaction analysis indicated that kernel development also affected the expression of fungal genes involved in FB₁ biosynthesis, starch metabolism, and nitrogen regulation. A mutant strain of *F. verticillioides* with a disrupted α -amylase gene was impaired in its ability to produce FB₁ on starchy kernels, and both the wild-type and mutant strains produced significantly less FB₁ on a high-amylose kernel mutant of maize. When grown on a defined medium with amylose as the sole carbon source, the wild-type strain produced only trace amounts of FB₁, but it produced large amounts of FB₁ when grown on amylopectin or dextrin, a product of amylopectin hydrolysis. We conclude that amylopectin induces FB₁ production in *F. verticillioides*. This study provides new insight regarding the interaction between the fungus and maize kernel during pathogenesis and highlights important areas that need further study.

One of the most ubiquitous pathogens of maize, *Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland), is also a prolific producer of fumonisin mycotoxins. Fumonisins comprise a class of structurally related molecules characterized by two tricarballic acid side chains esterified to an aminopentol backbone with one or more hydroxyl groups (Gelderblom et al. 1988). More than 20 distinct fumonisins have been described (Rheeder et al. 2002), but fumonisin B₁ (FB₁) is the most commonly encountered. FB₁ is carcinogenic in rats (Howard et al. 2001) and affects sphingolipid metabolism by inhibiting ceramide synthase (Desai et al. 2002). Ingestion of FB₁ by equids can lead to leukoencephalomalacia (Joffe 1986). In humans, ingestion of FB₁ has been linked to cancer and birth defects (Etzel 2002). Because of the risks associated with their presence in food and grains, fumonisins have drawn the attention of regulatory agencies throughout the world (Marasas et al. 2000).

The current knowledge about FB₁ biosynthesis by *F. verticillioides* is derived from the cluster of 15 *FUM* genes that

constitute the structural genes of the fumonisin biosynthetic pathway (Proctor et al. 1999, 2003). The cluster does not contain regulatory genes, and experimental evidence indicates that the regulation of fumonisin biosynthesis is complex, involving nitrogen metabolism, pH, and the biochemical composition of the maize kernel. A few regulatory genes in *F. verticillioides* have been shown to affect FB₁ biosynthesis, such as *PAC1* (a pH regulatory gene), *FCC1* (a C-type cyclin), and *ZFR1* (a zinc finger regulatory gene) (Flaherty et al. 2003; Flaherty and Woloshuk 2004; Shim and Woloshuk 2001). However, the molecular signals that control fumonisin biosynthesis are not clearly understood.

This study focuses on FB₁ production by *F. verticillioides* during colonization of maize kernels. The typical mature kernel consists primarily of two tissues, the protein- and lipid-rich tissues of the embryo (germ) and the starchy endosperm. Shim and associates (2003) showed that FB₁ production is greater in degermed kernels than in germ tissue. The stage of kernel development also is known to influence production of FB₁; immature kernels support less FB₁ production than mature kernels (Warfield and Gilchrist 1999). The objective of our study was to obtain a better understanding of how colonization of these tissues of the maize kernel by *F. verticillioides* influences FB₁ biosynthesis. Kernels were inoculated with the pathogen at various stages of kernel development, and FB₁ accumulation was correlated with the formation of starch in the kernels. Colonization of maize kernel mutants that accumulate abnormal forms of starch indicated that a high-amylose mutant accumulated low amounts of FB₁. To explore the role of starch metabolism in kernel colonization and FB₁ production, we cloned the α -amylase gene (*AMY1*) in *F. verticillioides* and generated an *AMY1*-disruption mutant. The mutant colonized maize kernels but produced low levels of FB₁. Additional experiments with the wild-type strain indicated that digestion of starch, specifically amylopectin, is crucial for FB₁ production by *F. verticillioides*.

RESULTS

The effect of kernel development on FB₁ production and gene expression is not due to differences in fungal growth.

Previous studies indicated that mature maize kernels supported higher levels of FB₁ accumulation in laboratory and field conditions (Bush et al. 2004; Warfield and Gilchrist 1999). The goal of our study was to characterize growth and gene expression during kernel development. We collected maize kernels at various developmental stages and tested their ability to support growth and FB₁ production by *F. verticillioides*. Based on visual assessments and ergosterol measure-

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ments, the fungus grew well on all kernel stages (Fig. 1A). The fungus grew most rapidly on dough-stage kernels, with growth peaking 4 days after inoculation. Growth on blister- and milk-stage kernels followed a similar pattern but reached the maximum 6 days after inoculation. In contrast, ergosterol content in dent kernels showed a linear increase during the course of the 10-day experiment. Significant FB₁ production occurred only in the colonized dent kernels (Fig. 1B). The increase in FB₁ paralleled growth (ergosterol content) in dent kernels, accumulating to over 200 µg of FB₁/g of kernel at 10 days after inoculation. At the same time point, only trace amounts of FB₁ were detected in milk- and dough-stage kernels, and no FB₁ was detected in blister-stage kernels (Fig. 1B). Additionally, we measured changes in the pH of the kernel extracts during colo-

Fig. 1. Effects of maize kernel development on **A**, fungal growth, **B**, fumonisin B₁ (FB₁) accumulation, and **C**, change in kernel pH. Stages: blister (R2) = open squares, milk (R3) = diamonds, dough (R4) = circles, and dent (R5) = triangles. Initial pH values ranged from 6.1 to 6.5. Data are presented as mean values obtained from analyzing three cultures, with error bars representing the standard error of the mean.

nization (Fig. 1C). Before inoculation, the pH of the kernels ranged from 6.1 to 6.4, and the pH of uninoculated kernels changed little over the course of 10 days (data not shown). At 10 days after inoculation, the pH of blister- and milk-stage kernels increased by 2 and 1 pH units, respectively. The pH of dough-stage kernels changed very little after 10 days of fungal growth, whereas the pH of inoculated dent-stage kernels decreased by more than 1.5 units (Fig. 1C).

Expression of four fungal genes (*FUM8*, *FUM12*, *AREA*, and *AMY1*) was normalized to growth (*TUB2* expression) 4 days after inoculation on each of the kernel stages (Table 1). Transcription of the two FB₁ biosynthetic genes (*FUM8* and *FUM12*) dramatically increased in the fungus growing on advanced stages of kernel development, which indicated that the induction (or derepression) of *FUM* genes was uncoupled from differences in growth. A similar, although less dramatic, pattern was observed for *AREA*, the nitrogen metabolism regulatory gene of *F. verticillioides* (Tudzynski et al. 1999). We also observed a 20-fold higher level of expression of *AMY1*, a putative α -amylase encoding gene, in colonized dent-stage kernels compared with blister-stage kernels (Table 1). This observation was consistent with data obtained from total starch analysis of developing kernels, which indicated that dent-stage kernels had a starch content of 70% compared with 58% for dough kernels, 20% for milk kernels, and 12% for blister kernels.

Kernel starch composition affects FB₁ accumulation.

To determine whether maize starch or a specific component of maize starch directly induces FB₁ biosynthesis, we utilized four mutants of maize with abnormal kernel starch composition. We evaluated the ability of the wild-type strain to grow, produce FB₁, and affect kernel pH in the four maize mutants. We found that the amount and type of starch present in maize kernels significantly influenced the ability of *F. verticillioides* to produce FB₁ (Table 2). Total starch analysis indicated that the mutant kernels were composed of 38 to 45% starch by dry weight compared with 72% for the conventional hybrid (Table 2), a reduction most likely explained by the relatively small size of the endosperms of the kernel mutants (data not shown). Growth of the wild-type strain was not inhibited on the kernel mutants, but the fungus produced less FB₁ in all four of the maize mutants compared with the conventional maize hybrid (Table 2). Remarkably, the amount of FB₁ detected in *amylose extender1* kernels was very low, reaching less than 10% of the amount observed in the conventional hybrid. Additionally, *amylose extender1* was the only kernel mutant for which the pH increased after colonization by the wild-type strain (Table 2).

An *AMY1*-disruption strain colonizes kernels but is impaired in FB₁ production.

By catalyzing the saccharification of starch, α -amylase is the primary starch utilization enzyme of filamentous fungi. In *F. verticillioides*, we cloned a 1,380-bp gene, designated *AMY1* (GenBank number DQ143884), which encodes a putative α -amylase. *AMY1* shares 83% identity with a hypothetical α -amylase (FG03842.1) of *F. graminearum* and is highly homologous to previously characterized α -amylases, including Taka-amylase A of *Aspergillus oryzae* (GenBank number M33218) and amy-CS2 of *Cryptococcus* sp. (GenBank number D83541). *AMY1* corresponds to TC30364, a tentative consensus sequence alignment in the TIGR *F. verticillioides* Gene Index. A BLAST search of the *F. verticillioides* genome indicated that *AMY1* is a single-copy gene, which was confirmed by Southern analysis (data not shown). We disrupted *AMY1* in the wild-type strain to produce a Δ *amy1* mutant. The pheno-

type of the $\Delta amy1$ strain resembled the wild type in all aspects of growth and development, except that the mutant was inhibited in its ability to digest starch (Fig. 2).

To investigate the role of α -amylase in FB_1 production, we evaluated the ability of the mutant strain to grow, produce FB_1 , and change the pH of mature kernels and kernel tissues of a conventional maize hybrid. On whole kernels, growth of the $\Delta amy1$ strain was comparable with the wild type, but the $\Delta amy1$ strain produced less FB_1 (Table 2). Whole-kernel acidification by the $\Delta amy1$ strain was only slightly less than the wild type (Table 2). When grown on the low-starch tissue of the germ, the $\Delta amy1$ strain closely resembled the wild type. The mutant produced low amounts of FB_1 and caused tissue alkalization (Table 2). However, growth of the mutant was three times greater on germ tissue than on whole kernels. When grown on starch-rich degermed kernels, the mutant's phenotype was more dramatic. The $\Delta amy1$ strain produced

over 100-fold less FB_1 , grew threefold less (Table 2), and failed to produce significant amounts of a red pigment frequently observed during kernel colonization by the wild-type strain (data not shown). Additionally, growth of the wild-type strain on degermed kernels led to significant kernel acidification (decreased by 1.3 pH units), whereas growth of the $\Delta amy1$ mutant on degermed kernels resulted in kernel alkalization (increase of 2 pH units).

We also evaluated the ability of the $\Delta amy1$ strain to grow, produce FB_1 , and affect pH in the four kernel mutants to determine whether impaired starch metabolism would magnify the effect of abnormal starch accumulation on FB_1 production. On *sh2* kernels, which contain glucose in place of starch, growth and production of FB_1 by the $\Delta amy1$ strain were similar to the wild type. On *sul/se1* and *wx1* kernels, both of which contain starch, growth of the $\Delta amy1$ strain was similar to the wild type, but FB_1 accumulation was significantly reduced. Re-

Table 1. Effect of kernel development on fungal gene expression^a

Fungal gene	Stage of kernel development			
	Blister (R2)	Milk (R3)	Dough (R4)	Dent (R5)
<i>FUM8</i>	nd	1.0 (0.6 – 1.7)	35 (23 – 37)	180 (140 – 270)
<i>FUM12</i>	nd	1.0 (0.4 – 2.3)	480 (350 – 670)	2,800 (1,900 – 3,900)
<i>AREA</i>	1.0 (0.7 – 1.4)	3.7 (2.3 – 6.1)	8.6 (6.8 – 11)	14 (10 – 20)
<i>AMY1</i>	1.0 (0.7 – 1.4)	2.3 (1.2 – 4.3)	7.5 (6.4 – 8.7)	20 (14 – 28)

^a Real-time quantitative polymerase chain reaction was performed with SYBR-green as the fluorescent reporter with the expression of each gene normalized to an endogenous reference (*TUB2*). Data represent fold differences in expression. The relative expression of each gene was calculated as $2^{\Delta\Delta Ct}$. The range of expression for each gene = $(2^{-\Delta\Delta Ct - 2} - 2^{-\Delta\Delta Ct + s})$, where s = the standard deviation of the $\Delta\Delta Ct$ value; nd = not detected.

Table 2. Effect of kernel tissue and genotype on fungal growth, fumonisin B₁ (FB_1) production, and change in kernel pH^a

Kernel type ^b	Starch (%)	Wild type			$\Delta amy1$		
		FB_1 ^c	Ergosterol ^d	pH (Δ pH) ^e	FB_1	Ergosterol	pH (Δ pH)
5322	72	470 ± 33	120 ± 42	4.2 ± 0.1 (–1.2)	150 ± 20	150 ± 27	4.8 ± 0.1 (–0.6)
5322 (germ)	8.6	19 ± 7	290 ± 8	7.6 ± 0.1 (+2.0)	6 ± 4	526 ± 95	7.7 ± 0.1 (+2.1)
5322 (degermed)	78	590 ± 86	190 ± 13	3.9 ± 0 (–1.3)	5 ± 2	62 ± 12	7.1 ± 0 (+1.9)
<i>sh2</i>	38	170 ± 22	180 ± 23	5.4 ± 0.3 (0)	210 ± 95	210 ± 6	5.1 ± 0.2 (–0.3)
<i>sul/se1</i>	43	300 ± 29	107 ± 15	4.7 ± 0 (–0.8)	100 ± 17	90 ± 22	5 ± 0.1 (–0.5)
<i>wx1</i>	41	290 ± 56	190 ± 69	4.5 ± 0.1 (–0.6)	78 ± 18	250 ± 40	5.6 ± 0.4 (+0.5)
<i>ae1</i>	45	30 ± 3	220 ± 27	6.7 ± 0 (+1.5)	4 ± 1	100 ± 12	8.0 ± 0 (+2.8)

^a All measurements were taken after 10 days of growth.

^b All kernels and tissues were analyzed at the R6 stage of development (maturity).

^c As measured in μ g of FB_1 /g of maize (fresh weight), $\pm$ standard error.

^d As measured in μ g of ergosterol/g of maize (fresh weight), $\pm$ standard error.

^e Δ pH = (the pH of the uninoculated material) – (the average pH after 10 days of growth).

Fig. 2. Growth and production of α -amylase by the **A**, wild-type and **B**, $\Delta amy1$ strains of *Fusarium verticillioides*. Fungi were grown for 3 days on starch medium and then stained with an iodine solution; the unstained halo under the wild-type strain indicates digestion of starch.

markably, on high-amylose *ae1* kernels, the $\Delta amy1$ strain grew less than the wild type, produced less FB₁, and raised the pH of kernels more than the wild-type strain.

Amylopectin induces FB₁ biosynthesis.

Maize starch consists of two polymers, amylose and amylopectin. To explore the effect of these individual components on FB₁ biosynthesis, we grew the wild-type strain for 10 days in liquid medium provided with amylose, amylopectin, one of three products of starch hydrolysis (maltose, glucose, or dextrin), or dextran, an α -1,6 branched glucoside. The fungus grew on all carbon sources provided (Table 3), although cultures provided with amylose grew somewhat less than cultures provided with other carbon sources. The carbon source had a strong influence on the amount of FB₁ produced. Cultures provided with amylopectin produced 40- to 50-fold more FB₁ than cultures containing amylose or maltose, and 5- to 10-fold greater levels than cultures containing glucose or dextran (Table 3). Interestingly, cultures provided with dextrin produced nearly as much FB₁ as cultures provided with amylopectin, suggesting that the higher level of FB₁ observed in these cultures resulted from a specific induction of FB₁ biosynthesis rather than a higher rate of growth.

DISCUSSION

Maize kernel development has been divided into six distinct stages, through which kernels undergo changes in levels of amino acids, lipids, proteins, nucleic acids, and carbohydrates (Ingle et al. 1965). The focus of this study was to determine how kernel development and composition affect FB₁ biosynthesis during kernel colonization. In nature, *F. verticillioides* has the opportunity to infect maize kernels at all six stages of development. The best evidence of this is a study by Bush and associates (2004), who observed naturally occurring kernel infections as early as the milk stage of development. We found that the fungus grew well on kernels from all stages of development, although dent and mature kernels supported the highest levels of growth. We also observed that kernel development affected FB₁ production by the fungus. Ten days after inoculation, dent and mature kernels accumulated the highest levels of toxin, whereas no FB₁ was detected in blister-stage kernels. This finding was similar to that of Warfield and Gilchrist (1999), who observed 140- to 2,000-fold differences in FB₁ accumulation between blister- and dent-stage kernels, with a low level of FB₁ in blister kernels after 15 days of fungal growth. Under field conditions, FB₁ was detected only in dent stage kernels regardless of whether ears were naturally infected or inoculated during the blister stage (Bush et al. 2004). Given that blister, milk, and dough stages of development support low levels of FB₁ production in laboratory conditions of near-ideal temperature and moisture, the absence of FB₁ in field conditions may underscore the importance of environmental

factors such as temperature and moisture on fungal growth and subsequent production of FB₁. Additionally, kernel development is highly dynamic and progresses rapidly from the blister to dent stage of development, thus providing a potentially narrow window to document production of FB₁ at an early stage of development under field conditions.

The effect of kernel development on the expression of *FUM8* and *FUM12* further indicates that kernels in early stages of development support low levels of FB₁ production. Transcription of *FUM8* and *FUM12* increases during the course of kernel development, reaching a maximum level at the dent stage, which presumably continues through kernel maturity. The transcriptional activation of genes involved in FB₁ biosynthesis and the subsequent accumulation of FB₁ in kernels seemingly is dependent on fungal perception of an appropriate kernel environment, which suggests that kernel development plays a uniquely important role in regulating FB₁ biosynthesis. The challenge is to identify the specific cue or cues that activate *FUM* gene expression during kernel development.

One of the most striking changes during kernel development is the rapid accumulation of starch as kernels mature. The starch-rich endosperm of mature kernels supports significantly higher levels of FB₁ production by *F. verticillioides* than the protein- and lipid-rich tissues of the embryo (Shim et al., 2003). Expression analysis of the α -amylase gene *AMY1* during colonization of kernels indicated that it is induced in dough- and dent-stage kernels, which have correspondingly elevated levels of starch (Ingle et al. 1965). A $\Delta amy1$ mutant strain produced significantly less FB₁ on mature kernels than the wild type, although growth of the two strains was similar. On germ tissue, the $\Delta amy1$ strain grew approximately twice as much as the wild type, and both strains produced low levels of FB₁. In contrast, the mutant grew threefold less than the wild type on endosperm tissue and produced 100-fold less FB₁. These results are interesting because they suggest that whole-kernel levels of growth and FB₁ production result from the changes in composition of individual tissues.

Amylose and amylopectin each have a unique set of physical properties and macromolecular organization (Ball et al. 1998; Myers et al. 2000). In recent years, numerous mutants with abnormal patterns of starch accumulation have been identified in maize. We evaluated four kernel mutants (*sh2*, *sul1/se1*, *wx1*, and *ae1*) for their ability to support FB₁ biosynthesis. Mutant *sh2* encodes ADP-glucopyrophosphorylase, which mediates the rate-limiting step in starch formation; disruption of *sh2* leads to the accumulation of glucose rather than starch in mature kernels (Bhave et al. 1990). Presumably, fungal colonization of *sh2* kernels is largely independent of starch catabolism, which would explain why growth and FB₁ levels were similar between the wild-type and the $\Delta amy1$ strains. In both *sul1/se1* and *wx1* kernels, the wild-type strain grew better than the $\Delta amy1$ strain and produced higher levels of FB₁. Kernels harboring the *sul1* and *se1* mutations accumulate high levels of glucose during milk and dough stages of development; however, by maturity, they contain starch as a carbohydrate reserve (Schultz and Juvik 2004). Mutant *wx1* kernels are characterized by the accumulation of amylopectin rather than amylose (James et al. 2003). We hypothesize that the differences in FB₁ production and growth between the two fungal strains on *sul1/se1* and *wx1* kernels are due to higher levels of starch catabolism by the wild-type strain. Kernels of the *ae1* mutant accumulate amylose rather than amylopectin (James et al. 2003). Surprisingly, *ae1* kernels inoculated with wild-type *F. verticillioides* supported a low level of FB₁ accumulation, although growth was similar to that observed on other kernel types and tissues. The effects of the *ae1* mutation on FB₁

Table 3. Growth and production of fumonisin B₁ (FB₁) in defined liquid media^a

Carbon source	Ergosterol ^b	FB ₁ ^c
Amylopectin	3.1 ± 0.5	100 ± 40
Amylose	0.8 ± 0.1	2 ± 1
Glucose	4.5 ± 0.6	25 ± 10
Maltose	3.6 ± 0.7	1 ± 0.3
Dextrin	3.5 ± 0.2	70 ± 20
Dextran	4.2 ± 0.1	10 ± 2

^a The wild-type strain was grown for 10 days at 28°C with shaking.

^b As measured in µg of ergosterol/culture (10 ml), ± standard error.

^c As measured in µg of FB₁/culture (10 ml), ± standard error.

accumulation were even more dramatic in the *Δamy1* strain, with very low levels of FB₁ detected and a growth rate approximately one half the rate of the wild type. Starch in *ae1* mutants of maize is resistant to hydrolysis, which is most likely explained by an altered granule organization (Evans and Thompson 2004). We hypothesize that the starch in *ae1* kernels is resistant to fungal digestion, which is exacerbated by the disruption of α -amylase activity in the *Δamy1* strain. Unable to effectively utilize starch in *ae1* kernels, the fungus may turn to less favorable carbon sources such as lipids or proteins.

To further explore the effect of individual components of maize starch on FB₁ biosynthesis, we performed experiments with defined media in which only the carbon source was adjusted. We found that amylopectin, which accumulates during the dent stage of kernel development, supported a high level of FB₁ production. Interestingly, amylose supported a much lower level of FB₁, which is consistent with our observations with high-amylose maize kernels. Furthermore, two end products of starch hydrolysis, glucose and maltose, supported lower levels of FB₁ production than amylopectin, indicating that decreased digestibility of amylose is not solely responsible for the low FB₁ levels. Structurally, the presence of α -1,6 branches is one of the properties that distinguishes amylopectin from amylose. Dextrin, an α -1,6 glucoside that is an intermediate product of amylopectin hydrolysis, supported higher levels of FB₁ than glucose or maltose. Growth on the three substrates was comparable, suggesting that α -1,6 glucosides may induce FB₁ biosynthesis. However, cultures growing on dextran, which consists of glucose molecules linked by α -1,6 and α -1,3 branches, produced levels of FB₁ comparable with glucose or maltose. Further research is needed to determine the exact mechanism by which amylopectin induces FB₁ biosynthesis.

The factors regulating FB₁ production during colonization of maize kernels by *F. verticillioides* are complex, and may include pH and nitrogen metabolite repression. Low pH is favorable for FB₁ biosynthesis, whereas high pH is repressive (Keller et al. 1997). High levels of starch in dent-stage kernels would provide an abundant supply of glucose to the fungus; metabolism of glucose by fungi is known to cause acidification of culture media, a process facilitated by proton export via a plasma membrane H⁺-ATPase (Brandao et al. 1992). Additionally, the *Δamy1* strain was not able to acidify endosperm tissues, which further supports the hypothesis that starch metabolism is directly responsible for kernel acidification during colonization by *F. verticillioides*. However, it is difficult to determine whether the acidification of dent stage kernels is a by-product of fungal metabolism or a prerequisite for FB₁ production. In dent-stage kernels 4 days after inoculation, before whole-kernel pH has decreased more than one unit, FB₁ accumulation is significantly higher than in blister, milk, and dough stages, and expression of genes involved in FB₁ production is significantly increased. Nevertheless, whole-kernel measurements of pH do not reflect what may be occurring in the kernel microenvironment; it is possible that localized decreases in pH influence the induction of FB₁ biosynthesis.

The potential effects of nitrogen metabolism on FB₁ biosynthesis also must be considered in the maize kernel environment. When a preferred nitrogen source such as ammonia is abundant, FB₁ biosynthesis is repressed in *F. verticillioides* (Shim and Woloshuk 1999). Nitrogen repression of FB₁ biosynthesis presumably is alleviated by AREA, a positive-acting DNA-binding protein that regulates the expression of genes involved in utilization of secondary nitrogen sources and, in some cases, secondary metabolism in filamentous fungi. Wang and Larkins (2001), who evaluated endosperm tissues of two inbred lines at 20, 30, 40, and 50 days after pollination, dem-

onstrated that levels of free amino acids are highest at the earliest time point, which corresponds to the milk stage of kernel development. At 4 days after inoculation, expression of AREA was 14-fold less in blister kernels than in dent kernels, and remained significantly lower throughout the course of the experiment (data not shown). Nitrogen repression of *FUM* genes could explain the low levels of FB₁ and *FUM* gene transcription observed on blister and milk kernels despite the fact that these stages contain high levels of sucrose, which is a favorable substrate for FB₁ production.

We hypothesize that, when a mature maize kernel is colonized, metabolism of amylopectin induces FB₁ biosynthesis, possibly through uptake or perception of α -1,6 linked glucosides such as dextrin. The low amounts of amylopectin in the earliest stages of development and in some maize mutants may be below the threshold required to induce appreciable levels of FB₁. A more complete model of the interaction requires understanding the phenomenon of kernel acidification or alkalinization during colonization. The uptake and subsequent utilization of sugars derived from starch in endosperm tissue may drive kernel acidification, which in turn is conducive for FB₁ biosynthesis. Furthermore, metabolism of lipids or proteins in the germ may lead to kernel alkalinization, which is repressive for FB₁ biosynthesis. Finally, a model must take into account the potential effects of nitrogen repression, especially when the fungus infects early stages of kernel development or germ tissue. At the present time, many aspects of the interaction remain highly speculative, especially the events at the plasma membrane interface responsible for the sensing and uptake of sugars, as well as the sensing and modulation of kernel pH. Furthermore, the components of the signal transduction network that mediate the interaction between the maize kernel and the regulation of FB₁ biosynthesis remain poorly understood.

MATERIALS AND METHODS

Maize hybrids.

Beck's maize hybrids 5727 and 5322 were planted in April 2003. We collected open-pollinated ears from each hybrid at the blister (R2), milk (R3), dough (R4), dent (R5), and mature (R6) stages of development. Ears were selected arbitrarily from interior rows of the plots and were rejected for use if they contained visible fungal contamination or insect damage. Maize mutants *shrunk2* (*sh2*), *waxy1* (*wx1*), and *amylose extender1* (*ae1*) were obtained from the Maize Genomics Co-operative Stock Center (University of Illinois, Champaign, IL, U.S.A.) and were planted in May 2004. Plants were self-pollinated and bagged to prevent cross-pollination; ears were collected at the R6 stage of development. Kernels of genotype *sugary1/sugary enhancer1* (*su1/se1*) were kindly provided by Dr. Cliff Weil (Purdue University). All maize lines were grown at the Purdue University Crop Diagnostic Training and Research Center near West Lafayette, IN, U.S.A.

Fungal strain and culture conditions.

F. verticillioides strain 7600 (wild type; Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City, KS, U.S.A.), a prolific producer of fumonisins, was used for all kernel inoculations. Working cultures were maintained on one-quarter-strength potato dextrose agar (B & D, Sparks, MD, U.S.A.). Conidia were collected by rinsing plates with sterile water and quantified with a hemacytometer. Fresh kernels from each developmental stage of hybrids 5727 and 5322 were removed from ears with pedicels intact and were surface sterilized by immersion in 20% chlorine bleach for 10 min, followed by three rinses in sterile water. Kernels were placed in sterile glass scintillation vials (RPI, Mt. Prospect, IL,

U.S.A.), wounded with an 18.5-gauge needle, and inoculated with conidia (1×10^5) of *F. verticillioides*. For genotypes *sh2*, *sul1/se1*, *wx1*, and *ae1*, kernels were soaked in water, autoclaved in scintillation vials, and inoculated with conidia (1×10^5). For all kernel types, vials were incubated at 28°C for 0, 2, 4, 6, or 10 days. After incubation, vials were flash frozen in liquid N₂ and stored at -80°C. Before analysis, the contents were transferred to a chilled mortar and ground to a fine powder.

BSAL liquid media consisted of 3 g of KH₂PO₄, 500 mg of MgSO₄, 5 g of NaCl, 1 g of bovine serum albumin, and 20 g of a carbon source consisting of either glucose, maltose, amylose, amylopectin, dextrin, or dextran per liter. Media (10 ml) were dispensed in sterile 50-ml Erlenmeyer flasks, inoculated with conidia (1×10^5), and incubated with shaking for 10 days at 28°C. Three flasks were prepared for each medium, and the experiment was performed twice.

Fungal growth.

The ergosterol content of kernels or liquid cultures was analyzed as a quantitative measure of fungal growth. Ground kernels (100 to 400 mg) were extracted overnight at room temperature in 2:1 chloroform:methanol. Liquid cultures (10 ml) were pelleted by centrifugation and extracted in 5 ml of 2:1 chloroform:methanol. Ergosterol analysis was performed on a high-performance liquid chromatography (HPLC) system with UV detection at 282 nm (Beckman Coulter, Fullerton, CA, U.S.A.) and a 4.6-U ODS column (200 by 4.6 mm; Beckman Coulter). Compounds were eluted with 100% methanol at a flow rate of 1.0 ml/min. Quantities were determined by comparing peak areas of samples to a standard curve generated from HPLC-grade ergosterol (Sigma-Aldrich, St. Louis).

FB₁ extraction and analysis.

Ground kernels (100 to 400 mg) were extracted in 2 ml of 1:1 acetonitrile/water for 1 h at room temperature with shaking. For clean-up, crude extracts were passed over an equilibrated C18 column (Agilent Technologies, Palo Alto, CA, U.S.A.) following the protocol of Rice and associates (1995). Liquid cultures were processed by centrifugation (10 min at 1,500 × g) and 5 ml of the supernatant was passed over equilibrated C18 columns. Before analysis by liquid chromatography, samples were derivatized with 0-phthalaldehyde as described by Rice and associates (1995). Analysis was performed on an HPLC system equipped with a 5U C18 column (150 by 4.6 mm; Alltech, Deerfield, IL, U.S.A.) and a fluorescence detector (Shimadzu, Kyoto, Japan) set at 335-nm excitation, 440-nm emission. Quantities were determined by comparing peak area of samples to a standard curve generated by analysis of HPLC-grade FB₁ (Sigma-Aldrich).

pH analysis of kernels.

Ground kernels (0.5 g) were suspended in 5 ml of deionized H₂O in a 50-ml conical vial, and pH was measured with a standard pH electrode (VWR Scientific, West Chester, PA, U.S.A.). Three samples were analyzed for each time point.

Quantitative polymerase chain reaction.

Total RNA was extracted from ground kernels and cDNA was generated for polymerase chain reaction (PCR) as described by Flaherty and associates (2003). Guidelines provided by Applied Biosystems (Foster City, CA, U.S.A.) were used to design primers for the following sequences: *AREA*, (GenBank number Y11006, forward primer 5' TGGACCTGGTGCCTCA TCAAT 3', reverse primer 5' CATCGATACTGGCACATCTC CTC 3'), *FUM8* (GenBank number AF155773, forward primer 5' TTGGACCCAGCTCTGCCA 3', reverse primer 5' TGAGA AGGATGTTTCATGACGCC 3'), *FUM12* (GenBank number

AF155773, forward primer 5' GCAGCGTACTACTCCGAC TTG 3', reverse primer 5' GATAAGCCAGATTCTGTCCGA TGC 3'), *AMY1* (GenBank number DQ143884, forward primer 5' GGCATGGGATTTGATGCTATTTGG 3', reverse primer 5' GTCATGAGCAGATTTGACAAGACTC 3'), and *TUB2* (GenBank number U27303, forward primer 5' TGCTCATTTCCAA GATCCGCG 3', reverse primer 5' GTAGTTGAGGTCACCGT AGGAGG 3'). All primers were obtained from Integrated DNA Technologies (Coralville, IA, U.S.A.). Reactions were performed in an ABI 7700 Sequence Detection System (Applied Biosystems) and data were collected with Sequence Detector Software (version 1.7; Applied Biosystems). Each reaction consisted of the following: 10 µl of QuantiTect SYBR-green PCR Master mix (Qiagen, Valencia, CA, U.S.A.), forward and reverse primers (500 nM each), cDNA template, and nuclease-free water (Sigma-Aldrich) to a final volume of 20 µl. PCR cycling conditions consisted of 2 min at 50°C (1 cycle), 10 min at 95°C (1 cycle), 15 s at 95°C, and 1 min at 60°C (40 cycles). Expression of genes was measured in triplicate. To verify that the efficiencies of the target and reference reactions were approximately equal, reactions were performed using the primers for each gene or *TUB2* with serial dilutions of cDNA as template. After verifying that the efficiencies of the primers were acceptable, expression levels were calculated by the comparative cycle threshold method (Applied Biosystems) with *TUB2* as the endogenous reference for normalization.

Starch analysis.

Total starch analysis was performed by the amyloglucosidase/ α -amylase method (AOAC method 996.11) with the Total Starch assay kit from Megazyme (Wicklow, Ireland). Kernels were dried at 80°C for 24 h before analysis. All kernels were pretreated to remove glucose and maltosaccharides as described in the method protocol.

Cloning and targeted disruption

of the α -amylase gene of *F. verticillioides*.

A 674-bp expressed sequence tag (EST) encoding a putative fungal α -amylase was obtained from Robert Proctor (United States Department of Agriculture-Agricultural Research Service, Peoria, IL, U.S.A.). From the EST, PCR primers were designed according to the guidelines for genome-walker PCR (Clontech; BD Biosciences; San Jose, CA, U.S.A.). Genome-walker PCR products were subcloned into the pGEM T Easy vector (Promega Corp., Madison, WI, U.S.A.) and sequenced at the Purdue University Sequencing Center (West Lafayette, IN, U.S.A.). From the sequence information, we identified a gene (*AMY1*) highly homologous to other fungal α -amylase genes.

To disrupt *AMY1*, a gene-replacement vector (pAMYKO) was constructed containing the hygromycin resistance cassette from pCB1003 (Fungal Genetics Stock Center) flanked on one side by 726 bp of sequence upstream of the start codon of *AMY1*, and on the other side with 750 bp of sequence beginning 100 bp after the predicted stop codon of *AMY1*. For transformation, we performed PCR with pAMYKO as template to amplify the 2.8-kb region of the vector containing the flanking sequences of *AMY1* separated by the hygromycin resistance cassette. DNA for transformation was concentrated with Microcon YM-50 microcentrifuge filters (Millipore, Bedford, MA, U.S.A.). Wild-type protoplasts were transformed as described by Proctor and associates (1999) and plated on regeneration media (Flaherty et al. 2003) with hygromycin B (Sigma-Aldrich) at 150 µg/ml. Hygromycin-resistant transformants were screened for homologous recombination events by PCR and verified by Southern analysis (Sambrook et al. 1989). To determine the ability of the $\Delta amy1$ mutant to metabolize starch,

the strain was grown on starch plates (0.3 M NaNO₃, 6 mM K₂HPO₄, 4 mM MgSO₄, 7 mM KCl, 66 µM FeSO₄, and 2% amylose) and stained with an iodine solution (0.5 % iodine and 5% potassium iodine).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Institute for Genomic Research (TIGR) website: www.tigr.org
Broad Institute website: www.broad.mit.edu