The bZIP Protein MeaB Mediates Virulence Attributes in Aspergillus flavus

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Abstract

LaeA is a fungal specific virulence factor of both plant and human pathogenic fungi. Transcriptional profiles of laeA mutants have been successfully exploited to identify regulatory mechanisms of secondary metabolism in fungi; here we use laeA mutants as tools to elucidate virulence attributes in Aspergillus flavus. Microarray expression profiles of ΔlaeA and over-expression laeA (OE:laeA) were compared to wild type A. flavus. Strikingly, several nitrogen metabolism genes are oppositely mis-regulated in the ΔlaeA and OE:laeA mutants. One of the nitrogen regulatory genes, the bZIP encoding meaB, is up-regulated in ΔlaeA. Significantly, over-expression of meaB (OE:meaB) phenocopies the decreased virulence attributes of a ΔlaeA phenotype including decreased colonization of host seed, reduced lipase activity and loss of aflatoxin B1 production in seed. However, a double knock-down of laeA and meaB (KD:laeA,meaB) demonstrated that KD:laeA,meaB closely resembled ΔlaeA rather than wild type or ΔmeaB in growth, aflatoxin biosynthesis and sclerotia production thus suggesting that meaB does not contribute to the ΔlaeA phenotype. MeaB and LaeA appear to be part of regulatory networks that allow them to have both shared and distinct roles in fungal biology.

Introduction

Aspergillus flavus is an opportunistic phytopathogen that colonizes oil-rich seeds such as maize, peanuts, and treenuts before and after harvest [1,2]. This fungus is infamous for the production of polyketide-derived carcinogenic and mutagenic secondary metabolites known as aflatoxins. Aflatoxins cause aflatoxicosis resulting from ingesting high levels of aflatoxin-contaminated food or feed, and long-term exposure can lead to liver carcinoma [1,3]. There are few pre-harvest controls, but successful post-harvest measures deployed to control A. flavus and aflatoxin contamination include controlling temperature and moisture levels of stored grain. Control in pre-harvest venues requires an understanding of A. flavus mechanisms involved in A. flavus infection of host tissue. One of the few proteins found to function in virulence in A. flavus is LaeA, as well as its interactor, VeA [4].

In previous research LaeA and VeA were found to regulate developmental processes in A. flavus including sclerotial, conidial and aflatoxin production [4–6]. LaeA is a global regulator of secondary metabolism in filamentous fungi, including Aspergillus, Penicillium, Fusarium and Cochliobolus species [7–10]. LaeA is located in the nucleus where it partners with VeA and another protein called VeLB to form what is known as the Velvet Complex [11]. VeA is a light regulated protein controlling spore development and secondary metabolism [12]. Both VeA and LaeA have been found to be pathogenicity factors in the plant pathogens A. flavus, F. fujikuroi, F. graminearum and C. heterosporus [4,6,8,10,13] and LaeA in invasive aspergillosis in the human pathogen A. fumigatus [7].

The central role of LaeA in secondary metabolic synthesis has lent itself well to using LaeA mutants as tools to uncover mechanisms in their regulation [14,15]. For instance, one recent study identified a novel bZIP transcriptional factor termed RsmA (restorer of secondary metabolism A) that partially restored sterigmatocystin in both ΔlaeA and Δeol mutants in A. nidulans [16,17]. bZIP proteins are eukaryotic transcription factors well described in yeasts and, increasingly, in filamentous fungi [18,19]. The identification of RsmA through the ΔlaeA mutagenesis screen served to emphasize the recently recognized role of bZIP proteins in fungal secondary metabolism. For example, the bZIPs AtfB and Aoyap1 have been reported to positively and negatively regulate aflatoxin in A. parasiticus respectively [20,21], Aoyap1 negatively...
regulates ochratoxin in A. ochraceus [22], and MeaB negatively regulates bikaverin in a nitrogen dependent manner in F. fujikuroi [23]. MeaB has also been found important in transmitting a conserved nitrogen-responsive pathway to control infectious growth in the vascular wilt pathogen, F. oxysporum [24].

Considering the usefulness of examining LaeA mutants in elucidating cellular processes governing secondary metabolism in fungi, it seemed that LaeA could also serve as a tool to uncover virulence mechanisms in fungi. A previous transcriptional profiling study aimed at viewing global secondary metabolism regulation in A. flavus utilized both ΔlaeA and OE:laeA mutants to examine expression of this species’ 55 secondary metabolite gene clusters [15]. We hypothesized that this profiling data could also provide clues to A. flavus virulence pathways as the ΔlaeA strain is reduced in its ability to colonize host seed [4,6]. Re-examination of the array set showed that several GO categories other than secondary metabolism are regulated by LaeA, including many genes involved in nitrogen metabolism. In particular, the nitrogen regulatory bZIP gene, meaB, is up-regulated in the ΔlaeA background strain. We examined both meaB over-expression and deletion strains for pathogenicity attributes. Whereas loss of meaB did not affect seed pathogenesis, the OE:meaB strain phenocopied several hypovirulent ΔlaeA traits including impairment in seed colonization, lipase activity and aflatoxin production in seed. However, a strain with both laeA and meaB depleted by RNAi does not restore wild type phenotype but resembles ΔlaeA for growth, aflatoxin biosynthesis and sclerotia production.

Results

Nitrate Metabolic Genes are Mis-regulated in laeA Mutants

Georgianna et al. [15] examined 28 diverse conditions for the regulation of 55 secondary metabolic gene clusters in A. flavus. Nine of these conditions compared transcriptional profiles of wild type, ΔlaeA and OE:laeA strains grown in three different environments (liquid shake at 6 and 24 hours or stationary growth at 24 hours). Not unexpectedly, LaeA was found to be a positive regulator of many of the secondary metabolic clusters including aflatoxin. Re-examination of this data showed that several other GO categories were highly regulated by LaeA. Notably, some genes involved in nitrate utilization were oppositely expressed in the ΔlaeA and OE:laeA strains. In general, nitrate metabolism genes were down regulated in ΔlaeA but up-regulated in OE:laeA (Table 1). A decrease in niaD expression was also observed in the ΔlaeA mutant (Figure 1). Additionally, one of the negative nitrogen regulatory proteins previously characterized in fungi, MecA, was up-regulated in the ΔlaeA strain under sclerotial induction conditions (Table 1). Expression of two other global nitrogen regulators, nmoA and anaA, were not statistically different in these conditions.

Creation of meaB Mutants

Both meaB deletion and over-expression (OE) strains were created through standard transformation processes. Twenty transformants were obtained from transformation of the A. flavus OE:meaB allele and two transformants, TSA15.13 and 15.18, were found correct using PCR, sequencing, Southern, and Northern analysis (Figure S1A). The A. flavus meaB allele was replaced with either the A. fumigatus pyrG allele (2 correct strains, TSA14.13 and 14.18, out of twenty transformants, Figure S1B) or the A. parasiticus pyrG allele (3 correct strains, TSA19.4, 19.7, and 19.8, out of 20 transformants, Figure S1C). Both pyrG alleles were used as replacement markers as we found the A. fumigatus pyrG replacement required uracil and uridine supplementation for normal growth (Figure S2). However, the same was found to be true for the A. parasiticus pyrG replacement strains (data not shown).

These results could indicate a mutation in both pyrG genes, a marker gene effect (e.g. pyrG is not expressed to full levels at this locus), or that loss of meaB affects pyrimidine metabolism. To determine which of these possibilities was most likely, both pyrG replacement genes were sequenced and found to be intact. Next, knock-down (KD) meaB mutants were created using RNA silencing technology and one representative transformant named TSA23.15 was assessed (Figure S1D). The KD strain also required uracil and uridine supplementation for normal growth despite placement of pyrG at a different locus (Figure S2). The OE:meaB strain, using the same marker gene, did not require uracil and uridine supplementation. To account for the supplementation needed by the meaB deletion strains, all growth media experiments were carried out with uracil and uridine supplementation. The mutant strains chosen for the following studies were TSA15.18 (OE:meaB) and TSA14.13 (ΔmeaB).

meaB Affects A. Flavus Virulence

MeaB has been reported as critical in transmitting nitrogen signaling through a MAPK cascade in controlling infectious growth of the vascular wilt pathogen F. oxysporum [24]. Specifically, deletion of meaB in this fungus allowed fungal invasion of host tissue during ammonium repressive conditions when normally the fungus is unable to colonize host tissue, hence presenting an enhanced aggressiveness in this particular environment. Here, virulence of A. flavus meaB mutants were tested directly by assessing growth on peanut seed and indirectly through observation of lipase activity, a strong indicator of degradative powers required for seed invasion.

As shown in Figure 2A and 2B, the OE:meaB strain was crippled in its ability to colonize and sporulate on host seed. Although the ΔmeaB strains showed some impairment in growth media without supplemented uracil and uridine (Figure S2), they grew equally well on seed as wild type, thus suggesting all nutritional needs of the ΔmeaB strain were met by the host seed. At 3 days, ΔmeaB and wild type strains started to conidiate on the seed surface (data not shown). At 5 days, these strains had colonized the entire surface of the seed whereas the over expression strain was delayed in conidiation and, moreover, grew aerially with fluffy mycelia (Figure 2A). These visual results were reflected by conidial counts in which sporulation was significantly decreased in the OE:meaB strain (Figure 2B).

The poor seed colonization by OE:meaB was similar to that described for the ΔlaeA mutant [4]. We also assessed two other parameters of seed infection impacted by laeA loss. First, we assessed the ability of the strain to degrade the lipase substrate

\[ \text{WT} \quad \Delta \text{meaB} \quad OE: \text{meaB} \quad \Delta \text{laeA} \quad OE: \text{laeA} \]

(Figure 1. Northern analysis of Aspergillus flavus velvet complex mutants. VeA and LaeA mutants [4] grown in liquid GMM conditions under dark for 48 hours at 250 rpm. Note increase and decrease of niaD expression in the laeA deletion (ΔlaeA TJW71.1) and over-expression (OE:laeA TJW79.13) respectively. ΔveA (TSA1.54) also shows decreased niaD expression. OE:veA = TSA2.46. doi:10.1371/journal.pone.0074030.g001)
glycerol tributyrate and found, like the \( \Delta laeA \) mutant, \( OE::meaB \) showed significantly less lipase activity than either wild type or the \( \Delta meaB \) strains (Figure 2C). Finally we assessed aflatoxin B1 production in the infected seed and observed a visual loss of B1 production by this strain (Figure 2D). Both the decreased lipase activity and loss of aflatoxin B1 production recapitulated that of the \( \Delta laeA \) strain.

**Depletion of meaB in a laeA Knockdown Background does not Restore Aflatoxin or Sclerotial Production**

The virulence data supported a view that LaeA may indeed act through MeaB. To more thoroughly address this hypothesis, we generated knock-down mutants in which both \( laeA \) and \( meaB \) were depleted by RNAi technology (\( KD::laeA,meaB \)). Nine transformants were confirmed to be correct by Northern and Southern analysis (Figure S3). RNAi mutants can display different phenotypes depending on the level of gene repression, so of the nine that were correct, we selected four for subsequent experiments (TKJA20.1, 20.4, 20.8, and 20.12). These strains and their controls were assessed for their ability to produce aflatoxin and sclerotia with the thought that both parameters could be restored in the \( KD \) strains.

Aflatoxin synthesis in *A. flavus* has been linked to nitrogen metabolism as nitrate medium is generally regarded as a poor substrate for aflatoxin synthesis [25–29]. In contrast, ammonium as nitrogen source is reported to support high levels of aflatoxin metabolism (e.g. bikaverin production, [23]) and regulation of protein not only in nitrogen metabolism but also secondary cascades [24] in *Fusarium* species. The finding that meaB is an *A. flavus* Virulence Factor

| Accession # | Gene | Sclerotia | \( \Delta laeA \) 6 hr Liquid | \( \Delta laeA \) 24 hr Liquid | OE::laeA 6 hr Liquid | OE::laeA 24 hr Liquid | \( \Delta meaB \) 6 hr Liquid | \( \Delta meaB \) 24 hr Liquid | OE::meaB 6 hr Liquid | OE::meaB 24 hr Liquid |
|-------------|------|-----------|-----------------------------|-----------------------------|---------------------|---------------------|-----------------------------|-----------------------------|---------------------|---------------------|
| AFLA_018790 | AFL2G_03510.2 | crnA | – | + | NS | NS | – | + | – | – |
| AFLA_018800 | AFL2G_03511.2 | niA | – | + | – | NS | + | – | – | – |
| AFLA_018810 | AFL2G_03512.2 | niD | – | + | – | + | – | + | – | + |
| AFLA_049870 | AFL2G_10206.2 | areA | NS | NS | NS | NS | NS | NS | NS | NS |
| AFLA_005620 | AFL2G_09875.2 | nmrA | NS | NS | NS | NS | NS | NS | NS | NS |
| AFLA_031790 | AFL2G_03512.2 | meaB | + | NS | NS | NS | NS | NS | NS | NS |

Data summarized from [15].

= decreased expression in \( laeA \) mutant versus wild type.

= increased expression in \( laeA \) mutant versus wild type.

NS = not significant difference in expression in \( laeA \) mutant versus wild type.

crnA: nitrate transporter, niA: nitrite reductase, niD: nitrate reductase, areA: GATA transcriptional activator, nmrA: nitrogen metabolite repression regulator, meaB: methylammonium resistant B.

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Because our results from the \( KD \) strains did not support the hypothesis that the \( \Delta laeA \) phenotype was mediated by MeaB over-expression, we were curious if this non-remediation would also be exhibited on growth on nitrogen sources and toxic analogs [23,30]. The \( OE::meaB \) deletion phenotype was the same as those described earlier for *A. nidulans* and *F. fujikuroi* mutants where \( meaB \) loss resulted in repressed growth on medium amended with the toxic nitrate analog chlorate but enhanced growth on methyl ammonium medium (Figure 5). Growth of the \( \Delta meaB \) strain was also restricted on nitrate and nitrite media. As predicted, the \( OE::meaB \) strain showed enhanced growth over \( \Delta meaB \) on chlorate medium but a near inability to grow on methylammonium medium. Both \( OE::laeA \) and \( \Delta laeA \) strains grew nearly equivalent to wild type on all media tested, except for \( OE::laeA \) which exhibited repressed growth on chlorate medium. All four isolates of \( KD::laeA,meaB \) exhibited similar growth patterns to those of the \( \Delta laeA \) mutant and not the \( \Delta meaB \) mutant on the different nitrogen sources.

**Discussion**

The meaB gene was described in 1996 in a study looking for genes involved in nitrogen metabolite repression in *A. nidulans* [31]. The protein was further defined as a regulatory factor in *A. nidulans* where it was proposed to activate NmrA [30], a repressor of the GATA factor AreA known to regulate several genes required for nitrate utilization [32,33]. From these studies both MeaB and NmrA were regarded as repressive nitrogen regulatory proteins. Another study, however, has recently shown nmrA expression in *A. nidulans* and *F. fujikuroi* is not meaB dependent [23]. All three proteins, MeaB, NmrA and AreA are conserved in filamentous fungi, and recent studies with MeaB have implicated a role for this protein not only in nitrogen metabolism but also secondary metabolism (e.g. bikaverin production, [23]) and regulation of virulence cascades [24] in *Fusarium* species. The finding that meaB...
and nitrate metabolism genes were regulated in *A. flavus* by *laeA* in a microarray study [15], a conserved virulence factor in pathogenic fungi [4,6,8,10,13], led us to ask if any aspects of virulence in *A. flavus* were mediated by MeaB. Specifically, *meaB* expression was significantly upregulated in the *A. flavus ΔlaeA* strain leading us to hypothesize that OE::meaB might be responsible, to some extent, for the ΔlaeA phenotype.

Deletion of *laeA* leads to several striking phenotypes contributing to decreased virulence on host seed including decreased colonization as reflected by lower conidial production, an impairment in lipase activity and, finally, loss of aflatoxin production on seed [4,6]. All three of these characteristics were also displayed by the OE::meaB mutant (Figure 2). This data suggested that *meaB* over-expression in the ΔlaeA strain might contribute to the decreased virulence of the ΔlaeA strain. However, our characterization of the KD::laeA,meaB strain argues against this hypothesis, as the KD::laeA,meaB strains closely mimic the ΔlaeA phenotype and not the ΔmeaB or wild type phenotypes for nitrogen utilization and ability to produce sclerotia and aflatoxin. This implies that, despite the connection by microarray, the roles of LaeA and MeaB are distinct in *A. flavus*.

The only other study assessing *meaB* impact on pathogenicity is with the tomato pathogen *F. oxysporum* in which a ΔmeaB, but not an OE::meaB, strain was assessed for a role in virulence [24]. In the *F. oxysporum* study, loss of *meaB* resulted in increased virulence in ammonium supplemented pathogenicity assays. The authors of that study suggested that MeaB normally inhibits SteA activation of a MAPK signaling pathway in *F. oxysporum*, and that loss of this inhibition could explain the enhanced virulence of the ΔmeaB strain. Whereas there was no evidence of increased virulence of the *A. flavus* ΔmeaB strain, the OE::meaB strain was reduced in virulence. We found an opposite regulation of *steA* by *meaB* in *A.

![Figure 2. Pathogenicity of *A. flavus* meaB mutants.](doi:10.1371/journal.pone.0074030.g002)
flavus (Figure S4) suggesting that the impact of meaB on virulence in A. flavus does not work through the same signaling pathways as F. oxysporum. However, it is possible that steA over-expression governed some of the OE::meaB phenotypes described in this study. SteA has been characterized in several Aspergillus spp. In A. oryzae [34], a species now considered as a clade of A. flavus [35], over-expression of steA led to restricted vegetative growth in A. oryzae and may explain, in part, the somewhat restricted vegetative growth of the A. flavus OE::meaB strain on media. In A. nidulans steA is required for sexual development [35] and while we observed that the A. flavus strain OE::meaB produced equivalent mass of sclerotia (the analogous A. flavus structure to cleistothecia) to wild type, the sclerotia appeared larger and were clumped together on the plate instead of evenly distributed like wild type (Figure 4). Further suggesting that MeaB may be involved in sclerotia formation, two of the four KD::laeA,meaB isolates were able to restore a small amount of sclerotia in comparison to the complete loss of sclerotia in KD::laeA. However, it is not known whether this is due to different levels of gene repression – of either laeA or meaB - or varying off-target effects among the isolates.

The requirement for uracil and uridine supplementation in the D meaB and KD::meaB strains was of note. A position effect for pyrG function has been previously described [36,37] and may explain the observations in this study. However, because the pyrG gene located to a different position in the KD strain than in the deletion strains, yet the KD mutant still required supplementation and the OE::meaB strain (where pyrG located to the same region as in the D meaB strains) did not require supplementation, it is possible that MeaB could be involved in pyrimidine metabolism. Although there is no report for a requirement for MeaB in pyrimidine synthesis in the literature, another nitrogen global regulator, AreA, has been connected with pyrimidine metabolism [38]. Whatever the mechanism underlying the requirement for supplementation in growth medium, there was no observable impact on growth of the deletion strain on seed.

In summary, through LaeA microarray sleuthing, we have identified MeaB as an important controller of A. flavus virulence and toxin attributes including seed colonization and aflatoxin synthesis on seed. However, it appears that meaB plays little – if any – role in the pleiotropic effects of laeA loss on fungal biology as LaeA and MeaB exhibit distinct roles in growth and development. This finding is in contrast to another study where a LaeA regulated gene, nosA, was found to mediate the decreased radial growth and delayed conidial germination observed in A. fumigatus D::laeA [39]. Considering that upwards of 10% of fungal genome is regulated by LaeA, it is likely that only a subset of the LaeA regulated genes would impact the D::laeA phenotype when deleted or overexpressed in this background.
MeaB is an *A. flavus* Virulence Factor

Materials and Methods

Microarray Data

The microarray data used for this study has been published and is deposited in the Gene Expression Omnibus (GEO) database under accession number GSE15435 [15].

Fungal Strains and Growth Conditions

*Aspergillus flavus* strains used and created in this study are listed in Table 2. All strains were maintained as glycerol stocks and grown on glucose minimal media (GMM, [40]) amended with appropriate supplements for *A. flavus* spore production.

Creation of Fungal Strains

**Aspergillus flavus meaB over-expression and deletion constructs.** An over-expression *A. flavus meaB* (AFLA_051790, also called AFL2G_02367.2) cassette was created by using the primers listed in Table 3. To create meaB over-expression cassettes, 4 PCR fragments were created and fused in this order: 1.5 kb of meaB upstream fragment, 1.97 kb *A. fumigatus* pyrG, 1.5 kb *A. nidulans* gpdA promoter, and 1.5 kb meaB open reading frame. First, the A. fumigatus pyrG and A. nidulans gpdA PCR fragments were fused and gpdAp::pyrGFor and A. nidulans gpdA(p)Rev primers for the gpdA fragment. Next, the meaB upstream flank, OEAfmeaB5FRev and OEAfmeaB5FRev, and the meaB open reading frame, OEAfmeaB5FRev and OEAfmeaB3FRev, PCR fragments were fused to the A. fumigatus pyrG:A. nidulans gpdA promoter PCR fragment. The final construct was confirmed with restriction endonuclease and sequencing.

Aspergillus flavus meaB deletion cassettes were created where meaB was replaced with *A. fumigatus* pyrG or *A. parasiticus* pyrG. These were created by PCR amplifying 1.5 kb of the meaB upstream fragment from NRRL3357 gDNA, 1.97 kb *A. fumigatus* pyrG from AF293 gDNA or 3 kb *A. parasiticus* pyrG from *A. parasiticus* genome, and 1.5 kb of the meaB downstream fragment from NRRL3357 gDNA using the following primers: OEAfmeaB3FRev and OEAfmeaB5FRev or KOAfmeaB3FRevAP, *A. parasiticus* pyrGFor and Rev, *A. fumigatus* pyrGFor and Rev, KOAfmeaB3FFor or KOAfmeaB3FForAP and Rev. Three PCR fragments were fused as previously described [41]. The final constructs were confirmed with restriction endonuclease and then transformed into *A. flavus* NRRL 3357.5.

**RNAi constructs.** A RNAi silencing construct to down regulate meaB expression in *A. flavus* was generated in the following manner. First, a 498 bp PCR fragment of *A. flavus* meaB was amplified from wild type gDNA using AFKDmeyaB3F For and 5FRev, and this was then inserted into the NolI and AscI site of pTMH44.2 through quick-change method [42] to create pSA16.5. After confirming the sequence and direction of the meaB insert using PCR with primers with pTMH44seq1 For and seq2Rev, the same PCR fragment was amplified with AFKDMeyaB3F For and Rev from wild type gDNA and ligated into the NotI and BamHI site of pSA16.5 to create pSA17.3. This transformation vector was confirmed by PCR with primers pTMH44seq1 For and seq2Rev, endonuclease digestion, and sequencing using pTMH44seq2 For and seq2Rev.

RNAi technology was also used to create a strain with both *lacA* and meaB transcripts depleted. A 342 bp fragment of *A. flavus* lacA was amplified with primers KS H-N-lacA F and KS lacA-meaB R with HindIII and NolI sites at the 5’ end, and a 3’ tail that overlaps with meaB. A 474 bp fragment of meaB was amplified with primers KS lacA-meaB F and KS meaB-A-B R. This fragment had a 5’ tail that overlaps with lacA and 3’ AscI and BamHI sites. The two fragments were joined by PCR, and the resulting construct was digested with AscI and NolI and ligated into pTMH44.2. This resulted in the plasmid pKJA38.6. The lacA-meaB construct was then digested with NolI and BamHI and ligated into pKJA38.6 to generate pKJA39.1. *A. parasiticus* pyrG was cut from pJW663.4 [43] using EcoRI and then inserted into the EcoRI site of pKJA39.1 to create pKJA40.1. PCR was used to confirm each step of construction.

**Transformation and strain confirmation.** Fungal protoplast preparation and transformation were carried out using a polyethylene glycol method [44]. Protoplasts were mixed with 6 μg of the constructed PCR cassette described above. Over-expression transformants were confirmed by PCR using *A. nidulans* gpdAFor and AFOEmeaB3FRev. Southern analysis was used to confirm correct transformants. Probes were created with AFOEmeaB3FFor and AFOEmeaB3FRev from *A. flavus* NRRL3357 wild type gDNA for Southern analysis. *A. flavus* meaB deletant

Figure 5. Growth of *A. flavus* meaB mutants on different nitrogen sources. Plates containing GMM with no nitrogen, GMM with 10 mM sodium nitrate, GMM with 10 mM ammonium chloride, GMM with 10 mM ammonium chloride plus 30 mM sodium nitrite, GMM with 10 mM ammonium chloride plus 200 mM potassium chloride, or GMM with 10 mM sodium nitrate plus 100 mM methylammonium chloride were inoculated with the indicated strains of *A. flavus* and grown for 3 days at 29 C. All media was supplemented with uracil and uridine.
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strains were created by adding 5 μg of the pyrG replacement vectors described above with protoplasts of A. flavus NRRL3357.5 and plated on minimal medium containing no supplements. The twenty mutants were examined by PCR and further confirmed by Southern analysis with OEAFlaeA3FFor and OEAFlaeA3FRev primers. The lack, meaB double knockdown strain was created by mixing pKJA40.1 with protoplasts of A. flavus NRRL3357.5 and plating on minimal medium. Transformants were first confirmed by Northern blot. For this, 106 spores/mL were inoculated into 50 mL GMM and shaken for 24 hours at 225 rpm at 30°C. RNA was extracted using the Trizol method (Invitrogen), and the blots were hybridized with gene fragments amplified from gDNA of NRRL3357 for Northern analysis. Blots were hybridized with gene fragments amplified from gDNA of NRRL3357 for the characteristic laeA, meaB. All isolates that exhibited the characteristic smear indicating degraded transcript were probed by Southern analysis with OEAFmeaB5FFor and KOAFmeaB3FRev primers. The lack, meaB double knockdown experiment, the fungi still grew and sufficient mycelium was obtained for Northern analysis. Blots were hybridized with gene fragments amplified from gDNA of NRRL3357 for A. flavus using the primers OEAFmeaB3FFor and KOAFmeaB3FRev for A. flavus meaB, NAFlaeAFor and NAFlaeARev for A. flavus laeA. All other Northern primers were listed on Table 3 as indicated by a N (Northern). Detection of signals was carried out with a Phosphor-imager-SI (Molecular Dynamics).

### Pathogenicity Tests

**Lipase activity.** To test for lipase activity, lipase medium (0.5% mycological peptone, 0.3% yeast extract in 1% agar containing 0.1% glyceryl tributyrinate) was used in a sterile test tube. Lipase medium was overlaid with 100 μl of 106 conidia/ml for each strain. Tubes were incubated at 29°C in continuous light. Measurements of the clearing zone, indicative of lipase activity, were taken at days 5. Experiment was repeated two times with five biological replications.

**Seed infections.** Mature live peanut seeds (*Arachis hypogaea*) were used to measure pathogenicity of meaB mutants in *A. flavus* following previously described methods [4]. Briefly, the peanuts were prepared by removing the testa using the fingers after soaking in tap water for 5 min. The two cotyledons of each seed were separated and the embryo carefully removed without damaging the cotyledon tissue. After sterilization, 20 peanut cotyledons were inoculated with 105 spores/cotyledons suspensions of each strain as well as a water control (mock inoculation) and incubated for 5 days at 29°C in continuous light.

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### Table 2. Fungal strains and plasmids used in this study.

| Strains     | Genotype       | References |
|-------------|----------------|------------|
| Aspergillus flavus |                |            |
| NRRL3357    | pyrG           | [45]       |
| NRRL3357.5  | ΔveaA:A. fum pyrG | [4]       |
| TSA 1.54    | ΔveaA:A. fum pyrG | [4]       |
| TSA 2.46    | A. fum pyrG, veA | [4]       |
| TJW 7.1     | ΔlaeA:A. fum pyrG | [6]       |
| TJW 79.12   | ΔlaeA: A. fum pyrG, niaD-, niaD, laeA | [6]       |
| TSA 2.8     | A. fum pyrG, veA, niaD, laeA | [4]       |
| TSA 14.13   | ΔmeaB:A. fum pyrG | This study |
| TSA 15.18   | A. fum pyrG:A. nidulans gpdA(p): meaB | This study |
| TSA 19.4    | ΔmeaB:A. parasiticus pyrG | This study |
| TSA 23.15   | pyrG, ITR meaB, A. fumigatus pyrG | This study |
| TKJA20.1    | pyrG, ITR meaA, A. fumigatus pyrG | This study |
| TKJA20.4    | pyrG, ITR meaA, A. fumigatus pyrG | This study |
| TKJA20.8    | pyrG, ITR meaA, A. fumigatus pyrG | This study |
| TKJA20.12   | pyrG, ITR meaA, A. fumigatus pyrG | This study |
| plasmid     |                |            |
| pJW 24      | A. parasiticus pyrG | [46]       |
| pJW44.2     | gpdA(p):gfp:trpC(t) | [47]       |
| pJW66.3     | gpdA(p):pppA:pppB:pppC:pppC:pppB:pppA:trpC(t), A. fum pyrG | [43]       |
| pSA 17.3    | gpdA(p):meaB:meaB:meaB:trpC(t) | This study |
| pKJA 38.6   | gpdA(p):meaB:meaA:meaB:trpC(t) | This study |
| pKJA 39.1   | gpdA(p):meaB:meaA:meaA:meaB:meaB:trpC(t) | This study |
| pKJA 40.1   | gpdA(p):meaB:meaA:meaA:meaA:meaB:meaB:trpC(t), A. fum pyrG | This study |

A. fum = A. fumigatus.
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MeaB is an A. flavus Virulence Factor
30 min in a rotary shaker at 50 rpm. Peanut cotyledons were incubated for 5 days for peanut cv TR96 (harvested from Stephenville, Texas in 2008) at 29°C in dark conditions. The filter paper was moistened daily. All seed experiments were repeated two times with three biological replications.

**Aflatoxin extraction from seed.** Inoculated peanut cotyledons were immediately processed to extract aflatoxin, as described in Table 3.

Table 3. Oligonucleotides used in this study.

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| A. nidulans gpdA(p)For | AAG GCT TG GGC CGC TGC GTT GGT T                                               |
| gpdA(p)::pyrG For  | GTG ACG ACA ATA CCT CCC GAC ACC TGG CAT CCG GAT GTC GAA GGC TTG            |
| A. nidulans gpdA(p) Rev | CAT GGT GAT GTC TGC TCA AG                                                   |
| A. fumigatus pyrG For  | TGCCCTAAACATGCTTCTTC                                                      |
| A. fumigatus pyrG Rev   | CAAGGTATCGGGAGGT                                                         |
| ANOEmeaB5For        | CCTTCTCTGCGATGACATCGG                                                     |
| ANOEmeaB5FRev       | AAGACACCCTTGCGGATGTTGCGTGGGCCACTGCTGTTGG                                  |
| ANOEmeaB3FFor       | GAGGGTGAGAGACATGTTGAGGCA GCTTCCGCTTCCTGATTG T                              |
| ANOEmeaB3FRev       | GAGGGTGAGAGACATGTTGAGGCA GCTTCCGCTTCCTGATTG T                              |
| OEAfmeaB5For        | CCAAGGCAAGACATGTTGAGGCA GCTTCCGCTTCCTGATTG T                              |
| OEAfmeaB5FRev       | GATGATCTCTCTCATTTTCTGG                                                     |
| OEAfmeaB3FnestedFor | GGATCGCGGATGTTGCGTGAAGAGGCA                                                 |
| KOAfmeaB3FnestedRev | CGCTTCTCCCAACAAAAATGCATTC                                                 |
| KOAfmeaB5FRev AP    | GATGATCTCGTCAACAAATGAGTGGGTTATGGGGAAGGTATATC                                |
| KOAfmeaB3FFor AP    | CCACACCCTTGCGGAGGCA GCTTCCGCTTCCTGATTG T                                  |
| pTMH44seq1For      | CTATCCTCATATCCCTCCT                                                      |
| pTMH44seq1rev      | GTGGCCAGAAGATGTTTCCATCC                                                  |
| pTMH44seq2For      | CAAGGTGAGAGACATGTTGAGGCA GCTTCCGCTTCCTGATTG T                              |
| pTMH44seq2Rev      | CCATTTGCTCAACCTGGAGGCA                                                    |
| gpdA(p) intFor scr  | GTGGAGAAGGCTGTTGCGTGGTGCAG                                              |
| NAFmmA For scr     | CAACAGAAGACATGTTGCGTGGTGCAG                                              |
| NAFmmA Rev scr     | CAACATCCTGCTGGAGGCA CATACC                                                |
| NAFlaeA For        | CCTGTATGATGATGATGATGATGATGAC                                               |
| NAFlaeA Rev        | GACAGCGAAAGATGAGGAAGGACCATC                                               |
| NAFactin For       | GAAGCCGCGTGAATGCTCCCToring                                                  |
| NAFactin Rev       | ACAGTCCAGGCGTGATCC                                                       |
| NAFniaD For        | AGAGATCTCGAGAGGACCTG                                                     |
| NAFniaD Rev        | GGTCTCTAGACATCATCC                                                       |
| NAFsteA For        | CTCGGTGAGGCGATATTGCGTGG                                                   |
| NAFsteRev          | GAAATGAGGCGTCTGGAGATTTG                                                   |
| KS H-N-laeA F      | TATAAAGCTTCCATGAGGAGGTTCTGGA AAAACAGGCC                                    |
| KS laeA-meaB R     | ATTTCAACACGGCTCTCTCTCGTGAGGCTGAGATGCTCGATTG T                              |
| KS laeA-meaB F     | TTTTCCGCCAATCGACTGAGGCTGAGATTGATGCTGAGGACCCC AAAAAAAGAGGAGC                |
| KS meaB-A-B R      | TATAGGATTCCGCGGCGGCGGAGAATTGAGGAGC                                        |
| KS laeA int F      | CAGAGCGTCTGTTGCGTGG                                                      |
| KS laeA int R      | TTTTCCGCCAATCGACTGAGGCTGAGATTGATGCTGAGGACCCC AAAAAAAGAGGAGC                |
| KS meaB int F      | GAAGCAAGGTAACGCTGAGG                                                     |
| KS meaB int R      | ACAGCTACGTGCTCCTCATG                                                     |
| KS JP-M13 F        | GTAAAACAGCGGCGAGG                                                        |
| KS JP-M13 R        | GAAACAGCTATGAGG                                                         |

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before [4]. The extracts from inoculated peanut cotyledons were dried for three days and then re-suspended in 300 μl of chloroform and 10 μl of each extract was separated on a silica gel TLC plate using the chloroform:acetone (95:5 v/v) solvent system. Extractions were repeated two times with three biological replications.

**Growth on Different Nitrogen Sources**

To assess ability of the *meaB* mutants to utilize different nitrogen sources, colony growth was assessed on the following media: GMM containing no nitrogen source, GMM with 10 mM sodium nitrate, GMM with 10 mM ammonium chloride, GMM with 10 mM ammonium chloride plus 30 mM sodium nitrate, GMM with 10 mM ammonium chloride plus 200 mM potassium chloride, and GMM with 10 mM sodium nitrate plus 100 mM methylammonium chloride [23,30]. Uracil and uridine were added to all media at 5 mM each. 1 μL containing 10^5 spores was point inoculated on 40 mL of media and incubated for 3 days at 29°C. The experiment was performed with four replicates and repeated two times.

**Aflatoxin Analysis**

Flasks containing 25 mL of either GMM with no nitrogen, standard GMM (70.6 mM sodium nitrate), or GMM with 70.6 mM ammonium chloride were inoculated with 10^8 conidia/mL of each strain and inoculated with shaking at 250 rpm at 29°C. After 48 h, 10 mL of chloroform was added to each flask, and samples were mixed gently at room temperature for 30 minutes. The lower layer (chloroform) was transferred to clean TLC plates (Whatman, Maidstone, England). TLC plates were developed using chloroform:acetone (95:5, vol/vol) solvent system and visualized under 254 nm light. Each strain was grown in duplicate, and the entire experiment was repeated two times.

**Sclerotial Assays**

Sclerotial formation was measured for fungal strains following previously described methods [4]. Briefly, 10 ml of GMM media containing 1.6% agar and 2% sorbitol was overlaid with 3 mL of GMM media with 103 spores. The exposed sclerotia were then collected, lyophilized, and allowed to dry for 3 days, then resuspended in 25 to 30 hours at 250 rpm at 29°C. To visualize sclerotium formation, plates were sprayed with 70% ethanol to kill and wash away conidia. The exposed sclerotia were then collected, lyophilized, and weighed (dry weight per plate). Sclerotial weight was determined by using four replicates.

**Statistical Analysis**

Statistical differences were analyzed using the JMP software package (version 9.0.2, SAS Institute, Inc, Cary, NC).

**Supporting Information**

**Figure S1**  Diagram of creation of *meaB* mutants and identification of mutants by Southern analysis. Transformants were screened by at least two different endonucleases. Asterisks show the correct mutants. Bold (deletion and over-expression) and dashed (RNAi silencing knock-down) lines on wild type (WT) locus indicate the radioactive probe sites. (A) Over-expression of *A. flavus meaB* with *A. nidulans gpdA* promoter: N: *Nde I* (3925 bp for WT, 6137 bp and 4304 bp for OE::meaB mutants). (B) Deletion of *A. flavus meaB*, replaced with the *A. fumigatus pyrG* S: *Sph I* (4202 bp and 3896 bp for WT, 9414 bp for *AmeaB* mutant). (C) Deletion of *A. flavus meaB*, replaced with *A. parasiticus pyrG* S: *Sph I* (4202 bp and 3896 bp for WT, 9467 bp for *AmeaB* mutant). (D) Knockdown of *A. flavus meaB* through RNAi technology. X: *XmaI* (3998 bp for WT, 3998 bp and extra copy from RNA silencing plasmid, pSA17.3).

**Figure S2** *Aspergillus flavus meaB* deletion and KD strains require uracil and uridine supplementation for optimal growth on laboratory medium. *AmeaB* = gene deletion, OE::meaB = over-expression of *meaB*, KDmeaB = knock down *meaB*, +UU = supplementation with uracil and uridine.

**Figure S3** Confirmation of simultaneous deletion of *A. flavus lacI* and *meaB*. (A) Diagram of portion of plasmid pKAJ40.1 used to delete both *A. flavus lacI* and *meaB*. The *A. nidulans* constitutive gpdA promoter (gpdA(p)) drives expression of inverted copies of lacI and meaB gene fragments, which are separated by a short spacer. The *A. nidulans* trpC terminator (trpC(t)) stops transcription. “S” indicates the location of the Std site, and the thick black bar above gpdA(p) represents where the probe that was used for Southern analysis hybridizes. (B) 10^6 spores per mL from each of twelve transformants were inoculated into 50 mL GMM and shaken for 30 hours at 250 rpm at 29°C. RNA was extracted and probed by Northern blot with gene fragments corresponding to lacI (left blot) and meaB (right blot). Ribosomal RNA bands are shown below each lane. Lanes marked with an asterisk indicate transformants that are undergoing degradation of lacI and meaB based on the smearing pattern. (C) Southern analysis was carried out for correct isolates from B). DNA was cut with StdI and probed with a fragment corresponding to a portion of the *A. nidulans gpdA(p) to generate one band per copy of integrated plasmid. The wild type (WT) and parental strain (P, NRRL3357.5) were probed as well. (D) Knockdown of *A. nidulans meaB* through RNAi technology. X: *XmaI* (3998 bp for WT, 3998 bp and extra copy from RNA silencing plasmid, pSA17.3).

**Figure S4** Figure 4. Northern analysis of steA in *A. flavus meaB* mutants grown for 48 hours in different nitrogen sources. WT = NRRL3357; AmeaB = TSA14.13; OE::meaB = TSA15.18. All media was supplemented with uracil and uridine. Ribosomal RNA (rRNA) is shown as the loading control. Probes are written on the left.

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**Author Contributions**

Conceived and designed the experiments: SA WBY NPK KJA. Performed the experiments: SA WBY SF AC KJA. Analyzed the data: SA NPK KJA. Contributed reagents/materials/analysis tools: NPK. Wrote the paper: SA WBY NPK KJA.
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