BDM1, a phosducin-like gene of *Fusarium graminearum*, is involved in virulence during infection of wheat and maize

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SUMMARY

Fusarium graminearum is a common pathogen of wheat and maize throughout the world. Despite recent advances in the elucidation of the genetic basis of virulence, significant gaps in the regulatory network underlying pathogenesis remain to be filled. In particular, little is known at the molecular level about the overlap among mechanisms of pathogenicity on maize and wheat. G-protein signalling has been implicated in pathogenesis in *F. graminearum*, although the underlying mechanisms are not fully understood. In this study, we investigated the involvement of a putative phosducin-like gene (*BDM1*) in growth, development and pathogenesis in *F. graminearum*. Targeted deletion of *BDM1* revealed roles in sexual and asexual sporulation, germ tube development, hyphal branching and mycelial morphology. During pathogenesis, *BDM1* is required for wild-type levels of colonization of maize silk tissue and stalks, but is dispensable for the colonization of kernels. The deletion of *BDM1* also reduced the virulence of *F. graminearum* during the infection of wheat seedlings and heads, resulting in a significant reduction in fungal biomass and a delayed spread of visual symptom expression (i.e. bleaching in heads). Furthermore, *BDM1* is required for wild-type levels of deoxynivalenol biosynthesis during the infection of wheat heads and maize silks. In summation, *BDM1* is one of the few genes characterized to date in *F. graminearum* involved in virulence during infection of both maize and wheat. Thus, the functional characterization of *BDM1* has established a new regulatory link between pathogenesis in maize and wheat, and provides a genetic resource through which the regulatory networks underlying virulence in *F. graminearum* can be further elucidated.

INTRODUCTION

Fusarium graminearum (teleomorph: *Gibberella zeae*) is a globally distributed pathogen of cereal crops, such as wheat, barley and maize (Bai and Shaner, 2004; De Nijs *et al.*, 1996; White, 1999). *Fusarium graminearum* is capable of infecting various tissues in a wide variety of host species. For example, in wheat and barley, *F. graminearum* causes Fusarium head blight (FHB), seedling

blight and foot (root) rot (Bushnell *et al.*, 2003; Mathre, 1997); in maize, *F. graminearum* causes Gibberella ear rot, Gibberella stalk rot, Fusarium root rot and seedling blight (White, 1999). Each of these diseases causes a distinct set of problems in an agronomic context. For example, seedling and root rots result in poor stand establishment, stalk rots cause lodging of mature plants, which complicates harvesting and reduces yields, and kernel infections lead to reduced yields and grain quality. In field conditions, *F. graminearum* reproduces asexually by forming macroconidia and sexually by forming ascospores, either of which can serve as primary and secondary inocula for infection (Goswami and Kistler, 2004). In addition, *F. graminearum* overwinters in soil by forming chlamydospores or as mycelia in plant debris (Markell and Francl, 2003). The ability of the pathogen to overwinter in multiple forms, produce sexual and asexual spores in abundance and attack multiple tissues on a wide range of crop species makes *F. graminearum* difficult to control in most agricultural settings.

Despite the obvious physiological differences between wheat and maize inflorescences, some basic similarities are shared between the infection processes underlying FHB and Gibberella ear rot. During the infection of wheat, macroconidia or ascospores are deposited by wind or rain splash on flowering spikelets, whereupon they gain entry to the host by colonizing protruding anthers or through natural openings (Bushnell *et al.*, 2003). The propensity of the pathogen to colonize anthers is well documented (Miller *et al.*, 2004), and the protrusion of anthers from spikelets during anthesis has been positively correlated with *F. graminearum* infection (Strange and Smith, 1971). After gaining access to the spikelet, the fungus preferentially colonizes ovaries and stigmas of florets before moving to the rachis and/or exterior of floret tissue (Miller *et al.*, 2004). The mycotoxins deoxynivalenol (DON) and nivalenol (NIV) are produced predominantly during the colonization of developing kernels and rachis tissue (Ilgen *et al.*, 2009; Savard *et al.*, 2000). During the infection of maize, macroconidia or ascospores are deposited on maize silks protruding from husks and, somewhat analogously to the colonization of anthers during head blight, the fungus grows down the silks to gain access to developing maize kernels (Reid *et al.*, 1992a,b). The fungus then penetrates maize kernels either by growing through the silk scar or directly through the pericarp (Sutton, 1982), and subsequently produces mycotoxins, such as DON and NIV, during kernel colonization (Maier *et al.*, 2006). In both pathosystems, access to seeds is primarily gained through the colonization of protruding floral

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organs, and mycotoxins accumulate in the developing grains (Goswami and Kistler, 2004). Some of the environmental cues may be conserved across the pathosystems. For example, *F. graminearum* displays a high affinity for pollen during the infection of both wheat and maize (Miller *et al.*, 2004; Naik and Busch, 1978; Ribichich *et al.*, 2000), and the underlying mechanisms of perception and downstream signalling could plausibly be conserved during the colonization of multiple hosts. Thus, one approach to create novel management strategies for *F. graminearum* on a broad range of hosts and tissues is to target mechanisms of virulence that are conserved during the infection of multiple hosts. To this end, it is particularly important to obtain a molecular-level understanding of how the fungus senses specific aspects of the plant environment during the infection of wheat or maize, how these signals trigger pathogenicity-related pathways and how the induction of these pathways is maintained during the progression of disease.

A major concern associated with diseases caused by *F. graminearum* is the accumulation of mycotoxins, such as DON, NIV and zearalenone (ZEA), in infected grains. Although a clear link between ZEA biosynthesis and virulence has proven to be elusive (Lysøe *et al.*, 2008), the trichothecene mycotoxins DON and NIV are thought to play a significant role in *Fusarium* diseases of cereals (Bai and Shaner, 2004; Desjardins, 2003). In the past decade, the trichothecene biosynthesis pathway has been studied extensively and the biosynthetic gene cluster has been identified (Kimura *et al.*, 2003; Proctor *et al.*, 1995; Ward *et al.*, 2002). Targeted disruption of a key trichothecene biosynthetic gene (*TRI5*) and functional analyses of the resulting mutant indicated that DON is a virulence factor during head blight of wheat (Desjardins *et al.*, 1996), and subsequent studies have indicated that at least one important mechanism of host defence involves the detoxification of DON (Berthiller *et al.*, 2009; Lemmens *et al.*, 2005). However, whether or not DON functions as a virulence factor during infection of maize is less clear. For example, Harris *et al.* (1999) reported that trichothecenes are virulence factors in Gibberella ear rot, whereas Adams and Hart (1989) and Maier *et al.* (2006) found that DON was not a component of virulence during infection of maize by *F. graminearum*. According to Maier *et al.* (2006), after disruption of the gene encoding trichodiene synthase (*TRI5*) in three field isolates of *F. graminearum*, only mutants generated from the NIV-producing strain caused less disease than their wild-type progenitor strain. These data indicate that the role of trichothecenes in the virulence of *F. graminearum* is complex and unresolved, and may rely on the genotype- or tissue-specific response of the host as well as on the chemotype of the pathogen.

Recently, significant discoveries have been made regarding the genetic basis of pathogenesis in *F. graminearum*, particularly in the identification of genes regulating FHB of wheat and barley. Several regulatory genes involved in FHB have been identified and characterized, including *MGV1* and *GPMK1/MAP1*, which encode

mitogen-activated protein kinases (MAPKs) (Hou *et al.*, 2002; Jenczmionka *et al.*, 2003; Urban *et al.*, 2003); *CID1*, encoding a C-type cyclin (Zhou *et al.*, 2010); *Pac1*, encoding the PacC transcription factor (Merhej *et al.*, 2011); and *RAS2*, which encodes a Ras guanosine triphosphatase (GTPase) (Bluhm *et al.*, 2007). In addition, downstream components have been identified, such as *FGL1*, which encodes a secreted lipase that functions as a virulence factor during the infection of wheat (Voigt *et al.*, 2005). In *F. graminearum*, many of the functionally characterized genes known to be involved in pathogenicity play a role in two somewhat interwoven signalling pathways: G-protein-coupled receptor signalling and MAPK cascades (Liu *et al.*, 2010). In many plant pathogenic fungi, G-protein-associated signalling regulates virulence by transducing various environmental stimuli (Bolker, 1998). Heterotrimeric ($\alpha\beta\gamma$) guanosine triphosphate (GTP)-binding proteins are coupled to specific categories of receptors that are activated by specific environmental stimuli. In the presence of a stimulus, GTP is exchanged for guanosine diphosphate (GDP), which induces the dissociation of the subunits as α -GTP and a $\beta\gamma$ heterodimer, and activates downstream signalling components, such as MAPK signalling pathways (Cabrera-Vera *et al.*, 2003; Li *et al.*, 2007; Liu *et al.*, 2010). Genes encoding $G\alpha$ and $G\beta$ subunits of heterotrimeric G-protein complexes have been functionally characterized in numerous plant pathogenic *Fusarium* species (Delgado-Jarana *et al.*, 2005; Jain *et al.*, 2005; Sagaram *et al.*, 2006; Sagaram and Shim, 2007; Yu *et al.*, 2008), and have been demonstrated to regulate various components of pathogenicity, including virulence and secondary metabolism. In *F. graminearum*, Yu *et al.* (2008) identified three putative $G\alpha$ subunits (GzGPA1, GzGPA2 and GzGPA3) and one $G\beta$ subunit (GzGPB1) in the *F. graminearum* genome. Functional analyses of these genes indicated that GzGPA1 is necessary for normal sexual reproduction, and both $G\alpha$ GzGPA1 and $G\beta$ GzGPB1 negatively regulate DON biosynthesis. However, the regulatory links between G-protein signalling and downstream components of pathogenicity have not been fully established, and the role of G-protein signalling during the infection of maize has not been studied extensively.

Phosducin and phosducin-like proteins are known modulators of downstream signalling from $G\beta\gamma$ complexes (Flanary *et al.*, 2000; Miles *et al.*, 1993; Schulz, 2001). These proteins are generally considered to be positive regulators of G-protein signalling because they are required for the sequential assembly of the $G\beta\gamma$ dimer in conjunction with the cytosolic chaperonin complex (Lukov *et al.*, 2006). In the chestnut blight fungus *Cryphonectria parasitica*, *bdm-1* (beta disruption mimic factor 1) is required for wild-type levels of asexual sporulation and virulence (Kasahara *et al.*, 2000). The product of the *bdm-1* gene has been described recently as a phosducin-like protein, the phosphorylation of which is required for virulence and $G\beta$ subunit stability in *C. parasitica* (Salamon *et al.*, 2010). Based on these studies, we predicted that an orthologue of *bdm-1* in *F. graminearum* could serve as a sig-

nalling intermediary between G-protein signalling and downstream components involved in pathogenesis.

The objective of this study was to expand the current working model of how G-protein signalling regulates pathogenesis in *F. graminearum*, with an emphasis on the identification of the genes regulating virulence on both wheat and maize. To this end, an orthologue of *bdm-1* of *C. parasitica* was disrupted in *F. graminearum* and the phenotypes of the disruption mutant were analysed in the context of growth, development and pathogenesis on wheat and maize.

RESULTS

Identification and targeted deletion of *BDM1* in *F. graminearum*

To identify a putative orthologue of *bdm-1* of *C. parasitica* in *F. graminearum*, a homology-based search (tblastn) of the *F. graminearum* genome was performed using the coding region of *bdm-1* from *C. parasitica* as the query. This resulted in the identification of a single predicted gene (locus FGSG_04105; designated *BDM1*) in the genome of *F. graminearum* that was predicted to encode a protein highly similar (expected value, 0.0; 54% amino acid identity) to the protein encoded by *bdm-1* of *C. parasitica*. Based on conceptual translation, *BDM1* of *F. graminearum* is 921 nucleotides in length, is predicted to contain one intron of 51 nucleotides and encodes a protein of 289 amino acids. Although no expressed sequence tag (EST) data were available for *BDM1* in the Fusarium Comparative Genome Database (<http://www.broadinstitute.org>), the annotation indicated the existence of short untranslated regions at both the 5' and 3' ends of the transcript. In addition, according to microarray data deposited in the PLEXdb database, FGSG_04105 (*BDM1*) is moderately up-regulated and detected by 72 h after infection of barley (Güldenier *et al.*, 2006), and by 48–96 h after infection of wheat (Lysøe *et al.*, 2011), indicating that it may be involved in plant infection.

For functional characterization, *BDM1* was targeted for deletion in a wild-type strain (PH-1) of *F. graminearum* via homologous recombination (Fig. S1A, see Supporting Information). First, transformants were screened by polymerase chain reaction (PCR) to identify mutants in which the deletion construct had been successfully integrated into the *BDM1* locus (Fig. S1B); this led to the identification of two independent deletion mutants of *BDM1* (designated $\Delta bdm1-1$ and $\Delta bdm1-3$). Then, Southern blots were analysed to confirm that the mutants: (i) contained a single copy of the gene disruption cassette in the genome; (ii) that the gene disruption had integrated specifically into the *BDM1* locus; and (iii) that the wild-type copy of *BDM1* could not be detected in the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants. Based on the number and predicted size of the bands that hybridized with a hygromycin-

specific probe, we concluded that the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants contained a single copy of the gene disruption cassette in their genomes, and that the size of the hybridizing band was consistent with targeted integration into the *BDM1* locus (Fig. S1C). Consistent with targeted deletion of *BDM1*, a *BDM1*-specific probe did not hybridize to the genomes of the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants (Fig. S1C). Together, these results confirmed the successful deletion of *BDM1* in two independent mutants.

To complement the deletion of *BDM1*, a wild-type copy of the gene was co-transformed into the $\Delta bdm1-3$ mutant together with a selectable marker for geneticin resistance. Putative complemented strains were initially identified via PCR (Fig. S1B), followed by Southern analyses (Fig. S1C). Strain *bdm1-3Comp* was demonstrated to contain a single-copy re-introduction of *BDM1*.

Deletion of *BDM1* affects germ tube development and colony morphology

To assess whether *BDM1* plays a role in the growth and development of *F. graminearum*, the wild-type, $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants, and the complemented strain were analysed in a variety of culture conditions. On potato dextrose agar (PDA) and V8 agar media, radial growth of the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants was consistent, albeit slightly less than that of the wild-type or *bdm1-3Comp* strain, and the deletion mutants formed a denser, more compact mycelium (Fig. 1). More pronounced differences in growth were observed in germinating spores. Although the morphology (i.e. shape, size) of macroconidia was not affected by the disruption of *BDM1* (Fig. 2), nor were delays in germination observed (data not shown), the patterns of germ tube emergence and germ tube morphology were consistently different in the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants compared with the wild-type or *bdm1-3Comp* strain. Specifically, although the germ tubes of the wild-type and *bdm1-3Comp* strain began to branch within 12 h after germination, the germ tubes of the $\Delta bdm1$ mutants were almost universally linear and unbranched, and generally lacked branches for an additional 6–12 h (Fig. 2). This delay in branching led to an unusual colony morphology in the $\Delta bdm1$ mutants by 48 h after germination compared with the wild-type or *bdm1-3Comp* strain (Fig. 2). Branching in the $\Delta bdm1$ mutants was more angular, with less flocculent aerial hyphae than in the wild-type or complemented strain, and gave rise to a distinctive, knotted mycelium with a highly irregular colony margin. However, growth beyond 48 h was radial and, by 5–6 days after germination, mycelia were asymmetrically circular, with slightly irregular margins (e.g. Fig. 1). These observations are in agreement with published expression data from Plexdb (Seong *et al.*, 2008), where expression of FGSG_04105 (*BDM1*) was moderately increased during conidial germination at 2 and 8 h, but decreased by two-fold at 24 h.

Fig. 1 Growth of the wild-type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and *bdm1-3Comp* strain on carrot agar (CA) and V8 agar. Photographs were taken after 3 days of growth.

Fig. 2 Deletion of *BDM1* affected the germination of macroconidia and early stages of hyphal development. Macroconidia of the designated strains were allowed to germinate on 0.2% potato dextrose agar (PDA) and hyphal development was observed at 0, 12, 18 and 48 h after inoculation. Scale bars: 0 h, 10 µm; 12 h, 100 µm; 18 h, 100 µm; 48 h, 1 cm.

***BDM1* is involved in sexual and asexual reproduction**

In *F. graminearum*, asexual (conidia) and sexual (ascospores) spores play important roles in dispersal, secondary infections and survival during disease cycles. To determine whether *BDM1* regulates asexual or sexual sporulation, we quantified the amount of conidia produced in cultures of the $\Delta bdm1$ mutants and also evaluated the ability of the mutants to form perithecia. In culture conditions highly conducive for conidiation, the wild-type and *bdm1-3Comp* strain produced comparable amounts of macroconidia, whereas the two independent $\Delta bdm1$ mutants produced

approximately three-fold fewer conidia than the wild-type strain (Fig. 3A). The reduction in conidia did not appear to result from a reduction in the number of conidiogenous cells, but rather from a reduction in the number of conidia produced per conidiophore (Fig. 3B). This result indicates that the reduction in conidiation observed in the $\Delta bdm1$ mutants results from impairment at a specific stage of conidiation, rather than from morphological pleiotropy.

As a homothallic fungus, *F. graminearum* readily produces perithecia and ascospores under suitable culture conditions. In this study, the deletion of *BDM1* essentially abolished the formation of

Fig. 3 Asexual sporulation of the wild-type strain PH-1 of *Fusarium graminearum*, mutants $\Delta bdm1-1$ and $\Delta bdm1-3$, and complemented strain *bdm1-3Comp* on Spezieller Nährstoffarmer Agar (SNA) medium after 7 days. (A) The wild-type strain produced approximately three times as many conidia as the mutants. (B) The reduction in conidia appears to result from a reduction in the number of conidia produced per conidiophore. Scale bars, 25 μ m. Error bars indicate standard deviations of the sample means.

perithecia in the two independent $\Delta bdm1$ mutants (Fig. 4). Taken with the requirement of *BDM1* for wild-type levels of conidiation, this finding reveals that *BDM1* plays an important role in both sexual and asexual reproduction.

***BDM1* is involved in virulence during infection of maize ears and stalks**

During typical infections of maize ears, ascospores or conidia of *F. graminearum* germinate on silks that emerge from the tip of the husk, and the pathogen colonizes the silks to gain access to kernels through the silk scar (Reid *et al.*, 1992a,b). However, insect damage and mechanical injury (e.g. hail damage) can provide the pathogen with an alternative route to infect kernels directly (Munkvold *et al.*, 1997). To assess the role of *BDM1* during the infection of maize ears, we inoculated silks and kernels with the $\Delta bdm1$ mutants. Deletion of *BDM1* impaired significantly the colonization of maize silks (Fig. 5A); by 7 days after inoculation, the $\Delta bdm1$ mutants grew approximately three-fold less than the wild-

type or complemented strain (Fig. 5B). However, direct infection of developing maize kernels with the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants revealed no substantial difference in kernel colonization compared with the wild-type and complemented strains (Fig. S2). These results indicate that *BDM1* regulates the infection of maize ears via silk tissue, but plays a minor role in kernel colonization.

As a result of substantial morphological and physiological differences between maize kernels and stalks, it is unclear to what extent genes that regulate kernel infections also regulate stalk infections, and vice versa. In this study, stalk rot assays with the wild-type, $\Delta bdm1$ mutants and complemented strain revealed a consistent reduction in lesion size induced by the mutant strains (Fig. 6), thus implicating *BDM1* in the regulation of pathogenesis on distinct tissues of maize.

***BDM1* is involved in virulence during infection of wheat**

In wheat, *F. graminearum* causes head blight, which is characterized by the bleaching of heads after infection as a result of colonization of the rachis by the pathogen. To assess the role of *BDM1* in head blight, we first inoculated spikelets of individual wheat heads directly with the wild-type, $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants and the complemented strain. Disease symptoms were evaluated daily from 5 to 15 days after infection. For all strains, disease symptoms first became visible approximately 5 days after infection, and were manifested as slight discoloration of the glume tissue at the site of infection (data not shown). By 7 days after infection, a qualitative difference in symptom development could be observed, as symptoms became visible on adjacent spikelets in heads inoculated with the wild-type or *bdm1-3Comp* strain, whereas no spread was visible in heads inoculated with the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants (Fig. 7A). From 7 to 15 days after infection, the number of diseased spikelets in heads inoculated with the wild-type or complemented strain increased rapidly (Fig. 7A), culminating in the spread of disease symptoms to all spikelets in the head by 15 days after inoculation (Fig. S3A). However, the spread of disease symptoms was significantly slower in heads infected with either the $\Delta bdm1-1$ or $\Delta bdm1-3$ mutant (Fig. 7A). Interestingly, at 10 days after inoculation, the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants had colonized the rachis tissue adjoining inoculated spikelets, but appeared to be unable to colonize the nodes of adjoining spikelets and thus to spread from the rachis to reach adjoining spikelets on the head (Fig. S3B). The reduced ability of the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants to spread within wheat heads indicates that *BDM1* plays an important regulatory function during the progression of head blight after initial colonization.

To further evaluate the role of *BDM1* in early infection events, we spray inoculated individual wheat heads with the wild-type, $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants and the complemented strain,

Fig. 4 Deletion of *BDM1* in *Fusarium graminearum* impaired sexual reproduction. Photographs show representative self-mating plates of the wild type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and complemented strain *bdm1-3Comp*. Perithecia were produced by strains PH-1 and *bdm1-3Comp*, but not by either of the $\Delta bdm1$ mutants.

and evaluated disease symptoms daily from 4 to 7 days after inoculation. Spray inoculations differ from direct, single spikelet inoculations in that every floret in each head theoretically has an equal chance of being infected, and water-borne/air-borne deposition of conidia on flowering heads in spray inoculations is more representative of naturally occurring inocula in field conditions. By 5 days after inoculation, a pronounced difference in disease development was observed among the strains; the percentages of diseased spikelets in heads inoculated with the wild-type or *bdm1-3Comp* strain were three to four times higher than in heads inoculated with the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants (Fig. 7B). Taken together, direct spikelet inoculations and spray inoculations indicate that *BDM1* plays an important regulatory function during the early stages of pathogenesis and, possibly, in the spread of the pathogen during colonization of the rachis, whereas disruption of the gene has little effect on spikelet colonization in cases in which infection succeeds.

As with maize, *F. graminearum* is capable of causing multiple diseases of wheat. Seedling blight of wheat is particularly troublesome from an agronomic perspective because of decreased germination and seedling emergence, as well as post-emergence seedling death, all of which contribute to poor stand establishment. To determine whether *BDM1* was involved in seedling blight, we inoculated wheat seeds with the mutant, wild-type and complemented strains and evaluated symptoms for 7 days. The wild-type and complemented strains colonized wheat seeds aggressively, with visible signs of colonization observed as early as 48 h after inoculation (data not shown). By 7 days, large amounts of mycelia of the wild-type and complemented strains were visible on seeds and roots, and hypocotyls were blighted or failed to emerge (Fig. S4). In contrast, the $\Delta bdm1$ mutants were much less virulent than the wild-type or complemented strains on wheat

seeds; comparatively small numbers of seeds showed symptoms of infection, which did not increase during the course of the experiment, as observed in the control strains (Fig. 8). In addition, seedlings infected with the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants showed substantially reduced symptoms, specifically less necrosis of root and shoot tissues (Fig. S4). In summation, these results indicate that *BDM1* is involved during diverse components of pathogenesis on wheat heads, as well as in seedling and root rot of wheat.

***BDM1* is involved in DON production in wheat and maize**

To evaluate the possible relationships between *BDM1* and DON biosynthesis, we assessed the ability of the mutant strains to grow and produce DON during growth on maize silks and during colonization of wheat and maize kernels. For each strain, the amount of DON measured in plant tissue was normalized to the amount of fungal biomass in order to account for differences in fungal growth. After 10 days of growth on maize silks, DON biosynthesis was decreased in the $\Delta bdm1$ mutants by approximately 50% compared with the wild-type or complemented strain (Fig. 9A). Similarly, in colonized wheat kernels evaluated 42 days after flowering, deletion of *BDM1* reduced DON levels by approximately 50% (Fig. 9B). However, 7 days after direct inoculation of maize kernels, DON levels were not significantly different among the wild-type, $\Delta bdm1$ mutants and complemented strain (Fig. 9C). Together, these data indicate that reductions in DON biosynthesis as a result of *BDM1* are conditional on host tissue, rather than constitutively impaired, which implicates *BDM1* in sensing the environmental cues underlying the induction of DON production.

Fig. 5 Deletion of *BDM1* decreased virulence on maize silks. (A) Small blocks of potato dextrose agar (PDA) cultures of the wild-type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and complemented strain *bdm1-3Comp* were placed on freshly cut ends of maize silks. Photographs were taken 7 days after inoculation. (B) Discoloration was reduced significantly in mutants $\Delta bdm1-1$ and $\Delta bdm1-3$ compared with the wild-type and complemented strain. Error bars indicate standard deviations of the sample means.

To evaluate the possible relationships between *BDM1* and DON biosynthesis *in vitro*, the wild-type, $\Delta bdm1$ mutants and complemented strain were grown on autoclaved, nonviable rice grains. The $\Delta bdm1$ mutants produced higher levels of DON than did the wild-type strain (Fig. S5A), and also grew slightly more than the wild-type strain (Fig. S5B) on nonviable rice grains. When levels of DON biosynthesis were normalized to the amount of fungal biomass, the $\Delta bdm1$ mutants produced higher levels of DON per

unit of growth than did the wild-type strain (Fig. S5C). This observation further confirms a role for *BDM1* in the regulation of DON biosynthesis in response to environmental cues, such as nutrient availability.

DISCUSSION

BDM1 of *F. graminearum* was initially identified as a putative orthologue of *bdm-1* of *C. parasitica*, the causal agent of chestnut blight. Kasahara *et al.* (2000) have reported previously that deletion of *bdm-1* in *C. parasitica* results in a flat colony morphology on PDA, a significant reduction in virulence on chestnut stems and reductions in hyphal branching, pigmentation and asexual sporulation. In the current study, disruption of *BDM1* in *F. graminearum* resulted in analogous phenotypes with regard to hyphal branching, asexual spore production and virulence on different tissues of two distinct hosts. We did not observe as pronounced an impact on colony morphology in *BDM1* deletion mutants of *F. graminearum* as Kasahara *et al.* (2000) observed after the deletion of *bdm-1* of *C. parasitica*. However, the $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants of *F. graminearum* were somewhat inhibited in radial growth on defined media and produced thicker, more condensed mycelia than the wild-type. Thus, based on the level of amino acid conservation, copy number in the genome and phenotypic similarities observed on targeted disruption, *BDM1* of *F. graminearum* appears to be a true orthologue of *bdm-1* of *C. parasitica*. In the context of what is currently known about G-protein signalling in *F. graminearum*, our observations that the deletion of *BDM1* did not impair growth and development, beyond irregular patterns of germ tube development and hyphal branching, were consistent with analyses of deletion mutants of the $G\beta$ subunit (encoded by *GzGPB1*) in *G. zeae* (Yu *et al.*, 2008). Furthermore, the reduced subapical branching observed on disruption of *BDM1* appears to be common among $G\beta$ subunit deletions in many plant pathogenic fungi, including *Fusarium oxysporum*, *Fusarium verticillioides* and *C. parasitica* (Delgado-Jarana *et al.*, 2005; Kasahara *et al.*, 2000; Sagaram *et al.*, 2006). In addition, this phenotype appears to be unique to $G\beta$ subunit deletions among components of G-protein signalling and, to our knowledge, has not been reported in published descriptions of other pathogenicity genes in *F. graminearum*. Thus, this phenotype may be of use in future *in vitro* genetic screens to rapidly identify other signalling components that influence G-protein signalling in *F. graminearum*.

The involvement of *BDM1* in the pathogenesis of both wheat and maize—and in the infection of distinct tissues/developmental stages of both hosts—raises interesting questions about how mechanisms of pathogenesis overlap and converge in *F. graminearum* at the molecular level. The disruption of *BDM1* reduced the infection of wheat and corn via floral tissues, as determined by spray inoculations of wheat heads and direct inoculations of maize silks. The preference for *F. graminearum* to infect

Fig. 6 Deletion of *BDM1* decreased the virulence of *Fusarium graminearum* on maize stalks. Photographs show representative stalks of 10-week-old maize plants inoculated with the wild type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant or complemented strain *bdm1-3Comp*, 14 days after inoculation. For a negative control, sterile water was injected into the wounded stalks. The mutant strains consistently induced smaller lesions than the wild-type or complemented strain.

developing kernels via anthers is well documented (e.g. Boshoff *et al.*, 1999; Pugh *et al.*, 1932, 1933), and we predict that *BDM1* is involved in the colonization of anther tissue and/or directional growth towards the developing ovaries. During the infection of maize silks, *F. graminearum* typically grows randomly on silks protruding from husks (distal to kernels) for a variable period of time before orienting growth along silks towards kernels (Miller *et al.*, 2007). We predict that an as yet unknown molecular mechanism is required for the transition from random to directed growth during silk colonization, and is likely to require the perception of an environmental cue (or cues) specific to silks. Interestingly, the disruption of *BDM1* did not affect the initial, random colonization of silk tissue, but did impair the ability of the fungus to grow directionally along silk tissues. Furthermore, the inability of the $\Delta bdm1$ mutants to colonize maize silks longitudinally was indistinguishable from the colonization defects exhibited by a $\Delta ras2$ mutant (Bluhm *et al.*, 2007), which suggests a possible regulatory interaction between *BDM1* and MAPK signalling during the colonization of silks. Furthermore, we determined that the $\Delta bdm1$ mutants also produced nearly 50% less DON than the wild-type during the colonization of silk tissues. However, whether or not DON functions as a virulence factor during growth on maize silks has not been demonstrated conclusively, and, to our knowledge, our study is the first to report DON levels during silk colonization. Based on these observations, we hypothesize that *BDM1* is required for the perception and/or transduction of specific environmental signals specific to, or characteristic of, floral organs in wheat and maize, and interacts directly or indirectly with MAPK signal transduction cascades to activate downstream components of virulence.

Although disruption of *BDM1* reduced DON biosynthesis in maize silks and wheat kernels, the underlying mechanism is not clear, nor is the full significance of the reduction during the infection of maize. One possibility is a direct regulatory relationship, in which *BDM1* is a positive regulator of DON biosynthesis.

In this scenario, environmental signals that induce DON biosynthesis would be transduced through G-protein-mediated signalling networks, and *BDM1* would be required for the activation of downstream effectors that ultimately induce *TRI* gene expression. However, this model is complicated by observations that other components of the G-protein signalling network in *F. graminearum* are negative regulators of DON biosynthesis, including *GzGPA1* (one of three $G\alpha$ encoding genes in *G. zeae*) and *GzGPB1* ($G\beta$ subunit in *G. zeae*) (Yu *et al.*, 2008). It is important to note, however, that direct comparisons between the findings of Yu *et al.* (2008) and our observations regarding *BDM1* may not be applicable. In our study, DON was measured *in planta* and normalized to fungal growth, whereas, in Yu *et al.* (2008), DON was measured in culture media and was not directly normalized to fungal growth. This explanation is further supported by a recent report that two *F. verticillioides* mutants involved in G-protein signalling (*DflbA2* and *DrgsB*), grown on autoclaved, nonviable maize kernels, produced significantly higher levels of fumonisin B₁ compared with the wild-type strain, but, when grown on living maize kernels, the mutants produced significantly lower levels of fumonisin B₁ compared with the wild-type (Mukherjee *et al.*, 2011). Indeed, in our study, after assessing DON biosynthesis *in vitro* and normalizing levels to fungal growth, a similar scenario was observed. Therefore, a second plausible possibility is that G-protein signalling is not a primary regulator of DON biosynthesis, but, instead, regulates the ability to perceive other environmental regulators of secondary metabolism in *F. graminearum*, such as pH, carbohydrate availability or nitrogen availability (Lengeler *et al.*, 2000; Yu and Keller, 2005). Further dissection of the genetic linkage between *BDM1* and DON biosynthesis is required to elucidate the underlying regulatory mechanism.

At the molecular level, the most probable explanation for the deficiencies in pathogenesis caused by the disruption of *BDM1* is that the Bdm1 protein is involved in the induction and/or sustain-

Fig. 7 Deletion of *BDM1* decreased the spread and initial infection of *Fusarium graminearum* on wheat heads. (A) Graph depicts the development of head blight on wheat heads inoculated with the wild type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and complemented strain *bdm1-3Comp*. The spread of disease symptoms beyond the point of inoculation was significantly slower in heads infected with either the $\Delta bdm1-1$ or $\Delta bdm1-3$ mutant. (B) Mutants were reduced in their ability to infect and colonize maturing florets and to cause necrosis on wheat heads. Error bars indicate standard deviations of the sample means.

ability of specific signalling pathways underlying virulence. Phosducin and phosducin-like proteins are known to regulate heterotrimeric G-protein activity by altering GTPase activity, which consecutively influences the signalling machinery that controls fungal growth, virulence and secondary metabolism (Flanary *et al.*, 2000; Miles *et al.*, 1993; Schulz, 2001). G-protein-mediated signalling is often associated with responsiveness to environmental stimuli (Li *et al.*, 2007). In order to integrate multiple (and, at times, conflicting) signals during infection of host tissue, *F. graminearum* probably relies on a finely tuned, complex network of signal transduction to perceive a wide variety of environmental signals. In this model, *BDM1* could belong to a functionally redundant group of signalling intermediaries that

Fig. 8 Deletion of *BDM1* decreased the virulence of *Fusarium graminearum* on wheat seedlings. Ten surface-disinfested wheat seeds were placed on blotter paper in a Petri plate and inoculated with *F. graminearum* macroconidia of the wild-type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant or complemented strain *bdm1-3Comp*. Sterile water was applied to the control plate. Mutants were reduced in their ability to colonize germinating seeds and to cause necrosis on developing seedlings. Error bars indicate standard deviations of the sample means.

modulate the expression of specific virulence factors, as the dynamic process of pathogenesis unfolds, by modulating G-protein signalling. As our results suggest, disruption of *BDM1* slows the development of several diseases of wheat and maize, but does not abolish the ability of the pathogen to infect, and does not fundamentally change the patterns of disease progression (e.g. Figs 5B and 7A). Consistent with this explanation, Liu *et al.* (2010) have suggested that MAPK signal transduction in *F. graminearum* is integrated with G-protein signalling, specifically through direct interaction of the G protein β subunit with RAS proteins, which, in turn, activates MAPK proteins. To refine the understanding of specific downstream effectors that may be regulated by *BDM1*, comparative gene expression profiling between the $\Delta bdm1$ mutant and the wild-type during the colonization of specific tissues would be highly informative, as would the further elucidation of regulatory links between MAPK signal transduction and G-protein-mediated signalling.

In summary, *BDM1* is involved in distinct and diverse aspects of virulence in *F. graminearum* during the colonization of host plants, and thus provides a novel genetic foothold from which the molecular mechanisms underlying pathogenicity can be unravelled. In addition, as a global regulator of sporulation, *BDM1* plays an important role in the dissemination of *F. graminearum*, a critical component of the disease cycle. The versatility of *F. graminearum*—specifically, the ability to cause multiple, distinct diseases on separate hosts—complicates the molecular dissection of pathogenesis. Considering the physiological and

Fig. 9 Deoxynivalenol (DON) production by the wild-type strain PH-1 of *Fusarium graminearum*, mutants $\Delta bdm1-1$ and $\Delta bdm1-3$, and complemented strain *bdm1-3Comp* in maize silks after 7 days (A), mature wheat kernels (B) and maize kernels after 7 days (C). The amount of measured DON was normalized to the amount of measured ergosterol. Error bars indicate standard deviations of the sample means.

morphological differences between wheat and maize, the involvement of *BDM1* in virulence on both hosts strongly suggests that at least some of the signalling mechanisms and/or environmental cues underlying pathogenesis in *F. graminearum* are global in nature. Furthermore, the involvement of *BDM1* orthologues in pathogenesis in taxonomically diverse fungal pathogens could

reveal a broadly conserved regulatory mechanism underlying plant–microbe interactions among filamentous fungi.

EXPERIMENTAL PROCEDURES

Fungal strains, media and culture conditions

Fusarium graminearum wild-type strain PH-1 (NRRL 31084), the mutants $\Delta bdm1-1$ and $\Delta bdm1-3$, and the complemented strain *bdm1-3Comp* were cultured at 25 °C on PDA (B&D, Sparks, MD, USA), V8 agar, Spezieller Nährstoffarmer Agar (SNA) for conidiation and carrot agar for induction of sexual development (Leslie and Summerell, 2006). To quantify conidiation, agar cylinders were removed with a cork borer (diameter, 1 cm) from an SNA culture 7 days after incubation, placed in a test-tube with 1 mL of sterile water and vortexed to displace the conidia, which were quantified with a haemocytometer. For the production of macroconidia, fungal cultures were incubated in liquid carboxymethyl cellulose (CMC) medium as described by Hou *et al.* (2002). Cultures for genomic DNA extraction were grown in YEPD (0.3% yeast extract, 1% peptone, 2% dextrose) medium at 28 °C with shaking at 120 rpm for 3 days. Wild-type strain PH-1, the complemented strain and all mutants generated in this study were preserved by cutting and immersing small squares of mycelium from PDA in 25% glycerol and stored at –80 °C as part of the fungal culture collection at Bluhm's laboratory. For microscopic examinations of conidial germination, spore suspensions were placed on 0.2 × PDA plates. Spores were allowed to germinate at room temperature, and microscopic observations of unstained and untreated spores and hyphae were performed with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL, USA).

Nucleic acid manipulations

Bacterial plasmids were isolated with a Qiagen miniprep DNA purification system (Qiagen, Valencia, CA, USA). PCR primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Isolation of protoplasts from *F. graminearum* and transformation were performed as described previously (Zhou *et al.*, 2010). Standard procedures were followed for the isolation of fungal genomic DNA and Southern blotting (Zhou *et al.*, 2010).

Deletion and complementation of *BDM1*

To delete *BDM1* in *F. graminearum*, a DNA fragment consisting of a selective resistance gene cassette (*HPH*, hygromycin B phosphotransferase), fused between the 5' and 3' flanking sequences of *BDM1*, was generated by double-joint PCR (Yu *et al.*, 2004). To accomplish this, DNA fragments corresponding to the 5' (976 bp) and 3' (706 bp) regions flanking the open reading frame of *BDM1* were amplified from genomic DNA with the primers *BDM1*-F1 (5'-TCTTCACGGTCAATGATGAACACG-3')/*BDM1*-R2 (5'-TTGACCTCCACTAGCTCCAGCCAAGCCTATCGTCTGGAAGG TGTGG-3') and *BDM1*-F3 (5'-GAATAGAGTAGATGCCGACCGCGGGTTCAG GACTGCTAGATCCCTTGG-3')/*BDM1*-R4 (5'-CGTAAGAGTACAGTAGCCCGT ACC-3'), respectively. The *HPH* cassette was amplified from pCB1003 (Fungal Genetics Stock Center, Kansas City, KS, USA) with primers HYG5 (5'-GGCTTGCTGGAGCTAGTGGAGGTCAA-3') and HYG3 (5'-GAACCC GCGGTGCGCATCTACTTATTC-3'). Sequences complementary to primers

HYG5 and HYG3 were incorporated into the 5' ends of primers *BDM1*-R2 and *BDM1*-F3 (given in *italic* above) to facilitate fusion of the three PCR products. For double-joint PCR, the 5' flanking region of *BDM1*, the *HPH* cassette and the 3' flanking region of *BDM1* were fused as described by Yu *et al.* (2004). Finally, the fusion product was amplified with nested primers *BDM1*-NF (5'-ATGTCCAAGCACCAAGGTATCAA-3') and *BDM1*-NR (5'-TTGCGATGATGACCTCGTCC-3') to create a 2.6-kb product containing the *HPH* cassette fused to the *BDM1* flanking regions. PCR products were precipitated and transformed into the wild-type strain of *F. graminearum* as described by Zhou *et al.* (2010). To complement the $\Delta bdm1-3$ mutant, a PCR product containing the wild-type copy of *BDM1* was amplified with primers *BDM1*-A1 (5'-CCGACTAATAGCAGTAGTAGCAGCA-3') and *BDM1*-R5 (5'-ACCTCCATAGCCGTACCACT-3') from strain PH-1 (NRRL 31084), and co-transformed into the $\Delta bdm1-3$ mutant with plasmid pKSGEN, which confers resistance to geneticin (Flaherty *et al.*, 2003). Transformants were screened by PCR with primers *BDM1*-A1 (5'-CCGACTAATAGCAGTAGTAGCAGCA-3')/*HPH*-HB (5'-CCCAAGCATCCAAATGAAAAAGCCT-3') and *BDM1*-P1F (5'-ATTGCCGACGCAGAACTA-3')/*BDM1*-P1R (5'-GTGCTTGCTCGATAGTGGCA-3') for the deletion region, respectively. The complemented strain was screened for resistance to geneticin (75 µg/mL) and confirmed by PCR with primers *BDM1*-A1/*HPH*-HB and *BDM1*-P1F/*BDM1*-P1R, as mentioned above. Targeted deletion of *BDM1* and complementation of the $\Delta bdm1-3$ mutant were confirmed by Southern analysis by probing for the presence of the *BDM1* open reading frame. Genomic DNA was digested with *EcoRV* and hybridized with the *BDM1*-specific probe 1 (a PCR product amplified with primers *BDM1*-P1F/*BDM1*-P1R) and with the *HPH*-specific probe 2 [a PCR product amplified with primers *HPH*-P2F (5'-GCTGATCCCATGTGTATCCTGG-3')/*HPH*-P2R (5'-CAGCTATTACCCGACGACATATCC-3')].

Assessment of pathogenicity on maize

To evaluate the infection and colonization of maize silks, a modified method of Seong *et al.* (2005) was used in this study. Briefly, fresh maize silks were collected from young maize ears of Pioneer hybrid 33D47 and sliced into 8-cm fragments. Five to eight maize silks were aligned evenly in bundles on Whatman no. 1 filter paper in 9-cm-diameter Petri plates and soaked with 1.5 mL of sterile distilled water. The lower ends of maize silks within each bundle were covered with a 5-mm² block of *F. graminearum* PDA culture. For a negative control, a sterile block of PDA was placed over the ends of silks. The infection of maize silks was scored by the extent of discoloration and colonization after incubation at room temperature in a moisture chamber for 5–7 days. Three bundles were placed into each Petri plate and each strain was evaluated in two Petri plates, resulting in six bundles per strain. The experiment was repeated twice. Seven days after inoculation, silk tissue was frozen in liquid N₂, ground to a fine powder and stored at –80 °C for DON and ergosterol analysis.

To assess the infection and colonization of maize kernels, seeds of maize hybrid 33D47 (Pioneer) were sown and self-pollinated in a glasshouse in 15-cm pots, and ears were removed at the dough stage of kernel development (growth stage R4, approximately 28 days after pollination; Ritchie *et al.*, 1993). All procedures, including inoculation and incubation, were performed essentially as described by Kim and Woloshuk (2011) with minor modification of the incubation period lasting up to 7 days. To assess DON and ergosterol levels, ears were inoculated individually with the

wild-type, $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant or the *bdm1-3Comp* strain. On each ear, four to five rows of kernels were punctured with a pin bar, and subsequently inoculated with macroconidia (1×10^3 per kernel). To measure DON and ergosterol levels, five inoculated kernels were selected arbitrarily and removed from a single ear inoculated with a single strain; four individually inoculated ears were analysed for each strain, thus resulting in four samples analysed per strain, each consisting of five kernels. For each sample, kernels were pooled, ground with liquid N₂ to a fine powder and stored at –80 °C until analysis. The entire experiment was repeated twice with similar results.

To evaluate the infection and colonization of maize stalks, a method described by Thon *et al.* (2000) was modified. Briefly, maize hybrid 33D47 (Pioneer) was grown in a glasshouse and inoculated when tassels emerged (growth stage VT, approximately 8 weeks after germination; Ritchie *et al.*, 1993). Stalks were wounded with a sterile toothpick and 10 µL of a suspension of macroconidia (10^6 in 1 mL) was injected into the internodes of maize stalks with a PB600-1 repeating dispenser (Hamilton Co., Reno, NV, USA). For a negative control, 10 µL of sterile water were injected into the wounded stalk. Wound sites were covered with Parafilm M Barrier Film (SPI Supplies, West Chester, PA, USA) and plants were incubated in a glasshouse at 19–25 °C with a 14-h photoperiod. After 14 days, the stalks were split open longitudinally to evaluate the extent of stalk rot. The assay consisted of three independent replicates (maize plants) for each strain, and the experiment was repeated twice.

Assessment of pathogenicity on wheat

A modified method of Mesterházy (1995) was used to evaluate the pathogenicity of fungal strains on wheat seedlings. Briefly, 10 surface-disinfested wheat seeds of cultivar Bess were placed on blotter paper in 9-cm-diameter Petri plates and inoculated with 5 mL of a suspension of macroconidia (10^5 in 1 mL). Petri plates were stored in clear plastic containers at room temperature and the number of visually diseased seedlings was counted 4–7 days later. Control seedlings were inoculated with 5 mL of sterile water per Petri plate. Each strain was replicated five times, resulting in the evaluation of 50 seedlings per strain, and the entire experiment was repeated twice.

To assess pathogenicity on flowering wheat heads, the modified method of Seong *et al.* (2005) was followed. Approximately 8-week-old plants of spring wheat cv. Norm were selected for point or spray inoculations at the flowering stage. For point inoculations, the eighth full-sized spikelet from the base of the head was injected (without wounding floral tissue) with 10 µL of a suspension of macroconidia (10^6 in 1 mL) with a PB600-1 repeating dispenser. The control heads were inoculated with 10 µL of sterile water. For spray inoculations, plants were inoculated at the flowering stage when anthers were visible on 5–10 spikelets per head. Each head was uniformly sprayed with approximately 0.6 mL of inoculum (10^5 macroconidia in 1 mL) using an atomizer (G-R Manufacturing Co., Manhattan, KS, USA). To promote infection and disease development, individual heads were enclosed in plastic bags immediately after inoculation, and the plants were incubated in a growth chamber at 18–25 °C with a 16-h photoperiod. The bags were removed after 48 h. Disease symptoms were evaluated daily from 5 to 15 days after infection, and the number of spikelets blighted on each head was counted. The point inoculation experiment consisted of nine plants per strain; the experiment was conducted

three times. At maturity, approximately 6 weeks after flowering, five wheat spikes were randomly chosen per strain, kernels were harvested by hand, ground to a fine powder and used for DON and ergosterol analysis. The spray inoculation experiment consisted of five plants per strain and the experiment was conducted twice.

Analysis of ergosterol and DON production

Ergosterol levels were measured in inoculated maize silks and wheat or maize kernels as described by Bluhm and Woloshuk (2005) with slight modifications. Briefly, ergosterol was extracted from ground kernels or silk tissue (100–500 mg) overnight at room temperature in 2 : 1 chloroform–methanol (1–2 mL). Ergosterol analysis was performed on a high-performance liquid chromatography (HPLC) system with photodiode array detection at 282 nm (Shimadzu, Columbia, MD, USA) and a 4.6- μ ODS column (250 mm $\times$ 4.6 mm; Phenomenex, Torrance, CA, USA). Compounds were eluted with 100% methanol at a flow rate of 1.0 mL/min. Quantities were determined by comparing peak areas of samples with a standard curve generated from HPLC-grade ergosterol (Sigma-Aldrich, St. Louis, MO, USA).

Levels of DON were analysed with an AgraQuant DON ELISA Kit (Romer Labs Inc., Union, MO, USA) according to the manufacturer's instructions, or by gas chromatography as follows. Rice grains (5 g) were autoclaved with 3 mL of H₂O in sterile scintillation vials, inoculated with 5×10^4 conidia in 1 mL of H₂O and incubated in the dark at room temperature for 16 days. Rice grains were ground under liquid N₂ and 0.5 g was extracted with 2 mL of acetonitrile–water (84 : 16, v/v) with shaking for 10 h. A 100- μ L aliquot of the extract in a 2-mL autosampler vial (National Scientific, Rockwood, TN, USA) was evaporated under a stream of N₂ at room temperature. The dry residue was derivatized in 100 μ L of N-trimethylsilylimidazole (TMSI)–trimethylchlorosilane (TMCS) (100 : 1, v/v) for 30 min at 37 °C. The derivatized product was mixed with 100 μ L of iso-octane and 200 μ L of H₂O, and then vortexed thoroughly. Aqueous and organic phases were separated by centrifugation at 200 g for 2 min. The organic top phase was collected in a 250- μ L microvolume insert (National Scientific) and placed in an emptied autosampler vial. Gas chromatography was performed according to Schothorst and Jekel (2001) with a GC 2010 gas chromatograph (Shimadzu) and a Zebron ZB-5 (Phenomenex) column (30 m, 0.25 mm ID, 0.1 μ m film thickness) with a minor modification. One microlitre of each standard and sample was injected splitless at 340 °C with helium used as carrier gas. The column temperature was increased from 120 to 180 °C at a rate of 30 °C/min. After holding at 180 °C for 3 min, the temperature was increased to 300 °C at a rate of 5 °C/min. The temperature was held at 300 °C for 6 min, and then increased to 330 °C at 30 °C/min and held for 10 min. The retention time for DON was 11.42 min. Quantification was performed by comparing the peak areas of the samples with those of an external standard of DON (Sigma).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The *BDM1* gene in *Fusarium graminearum* and the generation of the $\Delta bdm1$ mutants. (A) A hygromycin resistance cassette (*HPH*) was inserted into the *BDM1* gene via split-marker homologous recombination to create a $\Delta bdm1$ strain. For complementation, protoplasts of $\Delta bdm1$ were co-transformed with a vector harbouring a geneticin resistance cassette and the wild-type copy of *BDM1*. Probes 1 and 2 are polymerase chain reaction (PCR) products amplified with primers P1F/P1R and P2F/P2R, respectively. The presence or absence of *BDM1* was verified by PCR (B) and Southern blot analysis (C) in the wild-type strain PH-1 (lane 1), $\Delta bdm1-1$ and $\Delta bdm1-3$ mutants (lanes 2 and 3) and the *bdm1-3Comp* strain (lane 4). Genomic DNA was digested with *EcoRV* and hybridized with *BDM1*-specific probe 1 (left) or *HPH*-specific probe 2 (right).

Fig. S2 *BDM1* is dispensable for maize kernel colonization. Photographs show representative ear rows and dissected kernels 4 days after inoculation with the wild type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and complemented strain *bdm1-3Comp*. For a negative control, sterile water was injected into the maize kernels at the soft-dough stage. The mutant strains readily colonized surface and endosperm portions of maize kernels in a similar manner to the wild-type or complemented strain.

Fig. S3 Photographs depict the development of head blight on wheat heads inoculated with the wild-type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant and complemented strain *bdm1-3Comp*. For a negative control, sterile water was injected into (A, B) or sprayed onto (C) florets on separate heads. (A) The spread of disease symptoms beyond the point of inoculation was significantly slower in heads infected with either the $\Delta bdm1-1$ or $\Delta bdm1-3$ mutant. Photographs were taken at 15 days post-inoculation. Arrows indicate the point of inoculation. (B) The colonization of the rachis and adjacent spikelets was reduced significantly in heads infected with either the $\Delta bdm1-1$ or $\Delta bdm1-3$ mutant. Photographs were taken at 10 days post-inoculation. (C) Initial infection and bleaching symptoms of wheat spikelets at 5 days post-inoculation.

Fig. S4 Deletion of *BDM1* decreased the virulence of *Fusarium graminearum* on wheat seedlings. Ten surface-disinfested wheat seeds were placed on blotter paper in a Petri plate and inoculated with *F. graminearum* macroconidia of the wild type (PH-1), $\Delta bdm1-1$ mutant, $\Delta bdm1-3$ mutant or the complemented strain *bdm1-3Comp*. Sterile water was applied to the control plate. Photographs were taken at 7 days post-inoculation. Mutants were reduced in their ability to colonize germinating seeds and cause necrosis on developing seedlings.

Fig. S5 Production of deoxynivalenol (DON) (A), ergosterol (B) and DON/ergosterol ratio (C) by the wild-type strain PH-1, mutants $\Delta bdm1-1$ and $\Delta bdm1-3$, and complemented strain *bdm1-3Comp* of *Fusarium graminearum* in nonviable autoclaved rice grains after 16 days. Error bars indicate standard deviations of the sample means.

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