Degradation of the benzoxazolinone class of phytoalexins is important for virulence of *Fusarium pseudograminearum* towards wheat

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SUMMARY

Wheat, maize, rye and certain other agriculturally important species in the Poaceae family produce the benzoxazolinone class of phytoalexins on pest and pathogen attack. Benzoxazolinones can inhibit the growth of pathogens. However, certain fungi can actively detoxify these compounds. Despite this, a clear link between the ability to detoxify benzoxazolinones and pathogen virulence has not been shown. Here, through comparative genome analysis of several *Fusarium* species, we have identified a conserved genomic region around the *FDB2* gene encoding an *N*-malonyltransferase enzyme known to be involved in benzoxazolinone degradation in the maize pathogen *Fusarium verticillioides*. Expression analyses demonstrated that a cluster of nine genes was responsive to exogenous benzoxazolinone in the important wheat pathogen *Fusarium pseudograminearum*. The analysis of independent *F. pseudograminearum* *FDB2* knockouts and complementation of the knockout with *FDB2* homologues from *F. graminearum* and *F. verticillioides* confirmed that the *N*-malonyltransferase enzyme encoded by this gene is central to the detoxification of benzoxazolinones, and that *Fdb2* contributes quantitatively to virulence towards wheat in head blight inoculation assays. This contrasts with previous observations in *F. verticillioides*, where no effect of *FDB2* mutations on pathogen virulence towards maize was observed. Overall, our results demonstrate that the detoxification of benzoxazolinones is a strategy adopted by wheat-infecting *F. pseudograminearum* to overcome host-derived chemical defences.

Keywords: benzoxazolinone, crown rot, *Fusarium graminearum*, *Fusarium pseudograminearum*, head blight, phenoxazinone, phytoalexin.

INTRODUCTION

Fusarium-induced diseases of cereal crops, including *Fusarium* head blight (FHB) (Goswami and Kistler, 2004), *Fusarium* crown rot (Moya-Elizondo, 2013) and *Fusarium* root rot, adversely affect food and feed production, and threaten both food safety and security (Chakraborty and Newton, 2011). The most significant causal agents of these diseases in Australia, *Fusarium pseudograminearum*, *F. graminearum* and *F. culmorum* (Hogg *et al.*, 2010), cause substantial losses (~AU\$100 million per annum) in Australian cereal production (Murray and Brennan, 2009, 2010). Although *F. graminearum* and *F. culmorum* are the major pathogenic species in North America, Europe and northern Asia, in Australia, *F. pseudograminearum* is the most prevalent pathogen species causing *Fusarium* diseases on cereals (Akinsanmi *et al.*, 2004).

Plant-produced chemical compounds play an important role in defence against pathogen attack. Importantly, such compounds produced by a plant may differ between species of the same genus and even within the same species (Morrissey and Osbourn, 1999). The plant-produced defence chemicals can be broadly classified as phytoalexins that are synthesized *de novo* in response to pathogen attack, and phytoanticipins that are either present before pathogen attack or produced from pre-existing components following pathogen attack (VanEtten *et al.*, 1994). Some plant defence compounds, such as the red clover (*Trifolium pratense*) root antimicrobial maackiain and the Poaceae family benzoxazinoids, can be categorized into both groups. Indeed, both of these defence chemicals have been proposed to be stored in plant vacuoles as compartmentalized phytoanticipin glucosides and activated in damaged plant tissue by glucosidases to aglycones (Niemeyer, 2009; VanEtten *et al.*, 1994). Wheat, maize, rye and certain other agriculturally important species in the Poaceae family produce the benzoxazolinone class of phytoalexins on pest and pathogen attack. The benzoxazinoids have been shown to increase in concentration following pathogen attack

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(Oikawa *et al.*, 2004), and in response to defence-eliciting compounds (Oikawa *et al.*, 2001) in maize, suggesting that these compounds are phytoalexins.

So far, only a small number of fungal resistance mechanisms to plant phytoalexins and phytoanticipins have been discovered and, in some instances, have been shown to be associated with pathogen virulence (Morrissey and Osbourn, 1999). Some well-characterized phytoanticipin examples include fungal resistance mechanisms to avenacin, α -tomatine and pisatin, produced by oat (*Avena sativa*), tomato (*Solanum lycopersicum*) and pea (*Pisum sativum*), respectively. Detoxification of avenacin by *Gaeumannomyces graminis* var. *avenae* involves the extracellular fungal enzyme avenacinase, which deglycosylates avenacins (Crombie *et al.*, 1986). Avenacinase contributes to the virulence of *G. graminis* var. *avenae* (Osbourn *et al.*, 1995). Deglycosylated avenacin products have also been identified from *F. avenaceum*; however, the detoxification mechanism is unknown (Crombie *et al.*, 1986). The steroidal glycoalkaloid α -tomatine, which is similar in structure to avenacin, can be detoxified by tomatinase from *Septoria lycopersici* via a deglycosylation reaction (Osbourn *et al.*, 1995). *Botrytis cinerea* xylosyl hydrolase (Quidde *et al.*, 1998) and *F. oxysporum* tomatinase (Roldan-Arjona *et al.*, 1999) can also detoxify tomatine; however, the chemical detoxification cleavage sites and mechanisms of action differ between these pathogens. Tomatinase has been shown to contribute to virulence in *F. oxysporum* f. sp. *lycopersici* and *Cladosporium fulvum* (Okmen *et al.*, 2013; Pareja-Jaime *et al.*, 2008). The legume phytoalexin pisatin is modified by both degradation via demethylation and ABC transporter-mediated export in *F. solani* f. sp. *pisi*, with both activities contributing to fungal virulence (Coleman *et al.*, 2011; VanEtten *et al.*, 1980).

Benzoxazolinones, naturally occurring hydroxamic compounds, were first isolated from wheat and maize over half a century ago (Wahlroos and Virtanen, 1959) and have been shown to have allelopathic (Singh *et al.*, 2009), fungicidal (Martyniuk *et al.*, 2006), insecticidal (Escobar *et al.*, 1999) and mutagenic (Hashimoto and Shudo, 1996) activities. The mode of action of these compounds was attributed to *N*-covalent bonding with nucleic and amino acids (Hashimoto and Shudo, 1996). Outside the key role of these hydroxamic acids in cereal defence (Niemeyer, 2009), other wheat phytoalexins and fungal resistance to wheat defence chemicals are largely unknown. Benzoxazinone production is at its highest during the early germination of wheat seedlings, with fungistatic concentrations of benzoxazinones and benzoxazolinones present in leaf tissue (Copaja *et al.*, 1999), suggesting that these compounds can actively contribute to plant defence during germination and early seedling establishment. As *Fusarium* crown rot can result from infection of wheat seedlings during emergence (Knight and Sutherland, 2013), heightened benzoxazolinone production in

the seedling tissue could provide some protection against this disease. A positive correlation between benzoxazinoid content of ears and FHB resistance has also been reported in Danish wheat cultivars (Soltoft *et al.*, 2008). It is currently unknown, however, whether the head blight- and crown rot-causing *Fusarium* species, such as *F. pseudograminearum*, are either insensitive to these compounds or able to overcome their antimicrobial activity.

The maize pathogen *F. verticillioides* can degrade the two predominant maize benzoxazolinones [benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA)] via the toxic phenoxazinone intermediates that are subsequently converted to non-toxic malonamic acid conjugates by the *N*-malonyltransferase encoded by the *FDB2* gene (Fig. 1A) (Glenn and Bacon, 2009; Glenn *et al.*, 2002, 2003). The proposed benzoxazolinone detoxification pathway in *F. verticillioides*, via *N*-acylation, may directly overcome the proposed reactivity of the nitrogen group (Hashimoto and Shudo, 1996). Given the diverse biological benzoxazolinone activities, and their release by the plant during pathogen attack, the ability to deal with these toxic defence compounds has been considered to be important for pathogen infectivity. Surprisingly, however, mutants of *F. verticillioides* unable to process benzoxazolinones showed unaltered virulence towards maize, suggesting that these detoxification pathways were not required for virulence in this interaction (Glenn *et al.*, 2002). However, the importance of these compounds against other fungal cereal pathogens remains to be tested.

In this study, we have identified a conserved gene cluster with at least one gene involved in benzoxazolinone detoxification across several cereal-infecting *Fusarium* spp., and functionally characterized the *N*-malonyltransferase-encoding gene (*FDB2*) located in this cluster, via gene knockouts, in *F. pseudograminearum* and *F. graminearum*. Our results, using FHB assays in wheat, show that benzoxazolinone detoxification by *F. pseudograminearum* contributes to virulence.

RESULTS

A gene cluster involved in benzoxazolinone detoxification is conserved in multiple *Fusarium* species

An *N*-malonyltransferase encoded by *FDB2* has been identified as necessary for BOA detoxification in *F. verticillioides* (Glenn and Bacon, 2009). To determine whether other fusaria share the same benzoxazolinone detoxification pathway as *F. verticillioides*, the genome sequences of seven other *Fusarium* species were searched for genes encoding Fdb2 homologues. Fdb2 had BLASTP matches in *F. pseudograminearum*, *F. graminearum*, *F. culmorum*, *F. oxysporum*, *F. fujikuroi*, *F. acuminatum* and *F. sp.* FIESC5 (an

Fig. 1 (A) *Fusarium* detoxification of benzoxazinone (FDB) pathway. The phytoalexin 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) degrades to the phytoalexin benzoxazinone-2-one (BOA). Fdb1 metabolizes the benzoxazinone BOA to the phenoxazinone 2-aminophenol (2-AP). Fdb2 metabolizes 2-AP via *N*-malonylation to *N*-(2-hydroxyphenyl) malonic acid (HPMA) (Glenn and Bacon, 2009). The grey arrow indicates a non-enzymatic reaction. The figure was adapted from that presented in Glenn and Bacon (2009). (B) The conservation and gene arrangement of the FDB region in *Fusarium pseudograminearum*, *F. graminearum*, *F. culmorum*, *F. verticillioides*, *F. fujikuroi* and *F. oxysporum*. *Fusarium acuminatum*, *F. sp.* FIESC5 and *F. solani* f. sp. *pisi* showed limited matches to the genes in the cluster. Probable orthologous genes are given in identical colours from reciprocal best BLASTp hit matches. T.F., transcription factor.

unnamed species in the *F. incarnatum-equiseti* species complex). The *F. pseudograminearum* Fdb2 homologue FPSE_08123 (72% amino acid identity covering the entire length; e-value = 1×10^{-150}) is hereafter also called Fdb2.

In addition to *FDB2*, the genes surrounding *FDB2*, from *FPSE_08122* (hereafter called *FDB3*) to *FPSE_08127*, are all conserved in a sequential arrangement across most of the fusaria examined (Fig. 1B), with the exception of *F. acuminatum* and *F. sp. FIESC5*, whereas, in *F. verticillioides*, a single gene translocation is present. The potential functions of the proteins encoded by the six conserved genes in *F. pseudograminearum*, in gene identification order, are: *FDB3*, encoding a GAL4-like Zn(II)₂Cys₆ transcription factor proposed to regulate the *FDB* cluster or other responses to a benzoxazolinone signal (Glenn and Bacon, 2009); *FDB2*, encoding an *N*-malonyltransferase; *FPSE_08124*, encoding a lactamase probably performing the function of Fdb1 (see below); *FPSE_08125*, encoding an aldo-keto reductase possibly associated with a ketone component of BOA and MBOA degradation; *FPSE_08126*, encoding an esterase; and *FPSE_08127*, encoding a transmembrane transporter proposed to shuttle metabolites of benzoxazolinone degradation. In addition, the intron-exon structure was conserved for all six genes in fusaria that contained this gene cluster. In *F. acuminatum*, *F. solani* f. sp. *pisi* and *F. sp. FIESC5*, the gene cluster appears to be absent. Interestingly, the cluster was not restricted to pathogenic *Fusarium* of cereals or monocots in which benzoxazolinones are known to be produced. The genome of the tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* also contains an intact *FDB* gene cluster. Outside of the fusaria, the corn leaf blight *Setosphaeria turcica* (strain Et28A) draft genome (Condon *et al.*, 2013) contains genes that show best reciprocal BLASTp matches to Fdb3, Fdb2 and *FPSE_08124*, identified as *Settudraft_160635*, *Settudraft_108270* and *Settudraft_158560*, respectively.

In *F. verticillioides*, possibly as a result of translocation, the 1241-nucleotide lactamase-encoding gene (*FPSE_08124* homologue; *FVEG_08291*; 58.73% amino acid identity over the full length of the protein; e-value = 5×10^{-124}) is found on a chromosome different from that containing the main *FDB* cluster (*FVEG_12635* to *FVEG_12640*). However, within the main cluster in *F. verticillioides*, a 285-nucleotide remnant (*FVEG_12637*) of this lactamase-encoding gene is found (Fig. 1B). Although this latter sequence is also annotated as a lactamase, it is likely that the encoded protein is non-functional (Fig. S1, see Supporting Information). Interestingly, two distinct genetic loci are required for BOA detoxification in *F. verticillioides*, but the gene for the first step in the pathway is yet to be identified (Glenn and Bacon, 2009). Benzoxazolinone detoxification involves the opening of a γ -lactam ring and this function is likely to be performed by a lactamase (Glenn and Bacon, 2009), making *FPSE_08124* and its orthologues in other fusaria probable candidates for Fdb1 function.

***Fusarium* species associated with cereal diseases show different sensitivity to benzoxazolinones**

The conservation of the *FDB* gene cluster in various fusaria suggests that these organisms may also be able to degrade benzoxazolinones by similar mechanisms. To investigate whether different *Fusarium* spp. can detoxify the two quantitatively most abundant benzoxazolinones (BOA and MBOA) found in grasses, the growth of *F. pseudograminearum* (CS3096), *F. graminearum* (CS3179), *F. culmorum* (CS7071), *Fusarium* sp. FIESC-5 (CS3069) and *F. verticillioides* (BRIP 149534) was monitored over 5 days in the presence of these compounds at 0.5 mg/mL. This concentration is similar to that previously used in a study with *F. graminearum* (Gardiner *et al.*, 2013). In addition, thin layer chromatography (TLC) was used to investigate MBOA and BOA degradation by these pathogens. *Fusarium pseudograminearum* and *F. verticillioides* showed similar growth rates, sensitivity and degradation profiles on TLC for BOA and MBOA (Figs 2 and 3A), suggesting that BOA was degraded to *N*-(2-hydroxyphenyl) malonamic acid (HMPA) and MBOA was degraded to *N*-(2-hydroxy-4-methoxyphenyl) malonamic acid (HMPMA) by *F. pseudograminearum*. The BOA and MBOA degradation profiles by *F. verticillioides* have been described previously (Glenn and Bacon, 2009; Yue *et al.*, 1998). In this study, comparisons of degradation profiles on TLC between *F. pseudograminearum* and *F. verticillioides* were employed to tentatively identify intermediate and catabolic compounds, as chemical standards are not commercially available for HMPA, HMPMA, 2-amino-7-methoxy-3*H*-phenoxazin-3-one (2-AMPO) and 2-amino-3*H*-phenoxazin-3-one (2-APO). It should also be noted that 2-aminophenol (2-AP), the first known intermediate in BOA degradation, spontaneously oxidizes to 2-APO (Gagliardo and Chilton, 1992).

Two additional fusaria pathogenic on wheat, *F. graminearum* and *F. culmorum*, and the weakly pathogenic *F. sp. FIESC5* isolated from wheat, were also investigated for their response to benzoxazolinones (Fig. 2). *Fusarium graminearum* and *F. culmorum* were able to grow on BOA-amended medium, comparable with the growth rates of *F. pseudograminearum* and *F. verticillioides* on BOA. However, in contrast with *F. pseudograminearum* and *F. verticillioides* growth rates on MBOA, substantially reduced growth on MBOA-amended medium was observed for *F. graminearum* and *F. culmorum* (Fig. 2). *F. culmorum*, but not *F. graminearum*, has been reported previously to have differential sensitivity to BOA and MBOA compounds (Friebe *et al.*, 1998). The benzoxazolinone degradation analysis (Fig. 3B) conducted showed that, although BOA is degraded by *F. culmorum* and completely removed from media without an identifiable accumulated product by *F. graminearum*, MBOA appears to be mostly undegraded by these pathogens, with an accumulation of an unidentified compound. MBOA sensitivity was observed for four different isolates [CS3179 (Fig. 2), CS3005,

Fig. 2 Relative growth of wheat-associated fusaria in the presence of benzoxazolin-2-one (BOA), 6-methoxy-benzoxazolin-2-one (MBOA) and 2-aminophenol (2-AP). (A) Example photographs of *Fusarium pseudograminearum* (CS3096), *F. culmorum* (CS7071), *F. verticillioides* (BRIP 149534), *F. graminearum* (CS3179) and *F. sp. FIESC5* (CS3069) grown on benzoxazolinones- and BOA detoxification intermediate (2-AP)-amended half-strength potato dextrose agar (PDA). Rings marked on the plates indicate the daily hyphal growth edge positions. *Fusarium graminearum* (CS3179) marked at 2 and 5 days post-inoculation (dpi) only. All compounds were tested at 0.5 mg/mL, with an equal percentage (v/v) of dimethylsulfoxide (DMSO) used as a control. (B) Quantification of relative growth rates of five *Fusarium* spp. when grown for 5 days on half-strength PDA amended with BOA, MBOA, 2-AP or DMSO only. Error bars represent the standard error of the mean for five biological replicates.

Fig. 3 Analysis of benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) degradation via thin layer chromatography (TLC). (A) BOA and MBOA are presumed to be degraded to *N*-(2-hydroxyphenyl) malonamic acid (HPMA) and *N*-(2-hydroxy-4-methoxyphenyl) malonamic acid (HMPMA), respectively, in both *Fusarium pseudograminearum* and *F. verticillioides* based on comparisons with previously described TLC profiles (Glenn and Bacon, 2009). (B) *Fusarium graminearum* and *F. culmorum* are unable to degrade MBOA as efficiently as *F. pseudograminearum*. *Fusarium culmorum* and *F. graminearum* also accumulate an unknown compound, presumably 2-amino-7-methoxy-3*H*-phenoxazin-3-one (2-AMPO), an oxidation product of the MBOA intermediate compound 5-methoxy-2-aminophenol (2-AMP) (Glenn *et al.*, 2003).

CS3386 and Ph1 (data not shown)] of *F. graminearum*. The differential sensitivity of these *Fusarium* species to benzoxazolinones is quite surprising, considering that *F. graminearum*, *F. culmorum* and *F. pseudograminearum* are genetically similar (O'Donnell *et al.*, 2013) and commonly isolated from wheat plants with FHB or *Fusarium* crown rot (Obanor *et al.*, 2013). An ability to grow on MBOA- and BOA-, but not 2-AP-amended medium, was shown for *F. sp.* FIESC5 (Fig. 2). 2-AP was fungistatic towards *F. sp.* FIESC5 at 0.5 mg/mL, with the isolate surviving for at least 4 days on this medium without growth (Fig. 2). The corresponding BOA and MBOA detoxification TLC plate for *F. sp.* FIESC5 (Fig. S2, see Sup-

porting Information) showed a different profile from that of the other fusaria, consistent with the gene cluster being missing in this species (Fig. 1B).

The *FDB* cluster genes show significant co-expression patterns after BOA treatment

Comparative genome analyses suggest that the *Fusarium* BOA detoxification cluster contains six genes (Fig. 1B). In *F. graminearum*, functionally associated co-linear genes and compact gene clusters containing a transcription factor, a compo-

sition that matches the *FDB* cluster, often show similar expression patterns during pathogenesis, development and stress responses (Lawler *et al.*, 2013). To determine whether the genes in the putative *FDB* cluster are co-expressed, their expression was assessed by reverse transcriptase-quantitative polymerase chain reactions (RT-qPCRs) at multiple time points after application of BOA to *F. pseudograminearum*. Control samples were treated with dimethylsulfoxide (DMSO) used to dissolve BOA. The general expression profile observed for most genes in the cluster was that of increased expression commencing at six hours post-inoculation (hpi) with BOA, further increasing to a peak over 200-fold at 12 hpi and returning towards pretreatment levels at 24 hpi. The expression profiles of *FPSE_08118* to *FPSE_08127* inclusive were similar to the expression of *FDB2*, with correlation coefficients ranging from 0.94 to 0.99 (Fig. 4). Although the *FPSE_08118* induction profile showed a correlation coefficient of 0.96 with that of *FDB2*, this gene is not considered to be part of the *FDB* cluster as its induction levels (~12-fold at 12 h) were minor compared with other genes in the cluster predicted to encode biochemical functions. Genes (*FPSE_08117* and *FPSE_08128*) within the vicinity of the *FDB* cluster showed no significant correlation (coefficients in the range 0.02–0.06) to *FDB2* after BOA treatment, suggesting that they are unlikely to be part of the *FDB* cluster in *F. pseudograminearum*.

The co-expression profile observed suggests that the gene cluster in *F. pseudograminearum* should be extended beyond the sequential homologous region observed across *Fusarium* species to include *FPSE_08119* encoding a carrier protein, *FPSE_08120* encoding an acyl-CoA transferase and *FPSE_08121* encoding a Zn(II)₂Cys₆ transcription factor. These additional genes have no reciprocal best BLAST hits in *F. graminearum* or *F. culmorum*, but have orthologous genes in *F. oxysporum* and *F. verticillioides* (Gardiner *et al.*, 2012). *Fusarium graminearum* appears to have a genomic inversion of the six genes in its *FDB* cluster compared with adjacent genes, relative to the *F. pseudograminearum* *FDB* cluster and surrounding gene arrangement (Fig. 1). Furthermore, the genes immediately adjacent to *F. graminearum* *FDB3*, *FGSG_00082* to *FGSG_00086*, have no matching homologues in the *F. pseudograminearum* CS3096 genome.

To determine whether the BOA induction profile seen in this gene locus was specific to the Fdb2 *N*-acyltransferase, and did not include other acyltransferases, the *F. pseudograminearum* predicted proteome was searched for Fdb2 homologues using BLASTp. Three *N*-acyltransferase proteins (*FPSE_07946*, *FPSE_11041* and *FPSE_08077*) with sequence similarity to Fdb2 were found. These *N*-acyltransferases may also have the potential to function in BOA degradation. However, none of the genes encoding the proteins displayed expression changes similar to *FDB2*, suggesting that regulation by BOA is specific to *FDB2* (Fig. S3, see Supporting Information) and that Fdb2 alone provides the *N*-acyltransferase function in BOA degradation.

Fdb2 is not essential for growth in axenic culture

To confirm the involvement of Fdb2 in BOA detoxification, the gene was deleted from *F. pseudograminearum* isolate CS3096. The entire *FDB2* gene (1032 bp) was replaced by a geneticin resistance gene cassette via homologous recombination. Transformants were initially screened using a triplex PCR assay (Fig. 5A) and two independent mutants were identified. Whole-genome sequencing of the mutant strains at approximately four-fold coverage confirmed the absence of the entire gene. Mapping of the reads to the *F. pseudograminearum* CS3096 genome (Gardiner *et al.*, 2012) showed clear deletion of *FDB2* in both mutants (Fig. 5B). In addition, mapping reads to the predicted mutant locus showed coverage consistent with a single insertion at the *FDB2* locus (Fig. 5B). Mutants were indistinguishable from the wild-type (WT) with respect to hyphal growth in liquid culture (Fig. 5C) or on solid media (Fig. 6A). *FDB2* (*FGSG_00080*) was also deleted from *F. graminearum* strain CS3005 and the growth of these mutants was indistinguishable from that of the parental strain (Fig. S4A, see Supporting Information).

Fdb2 is essential for tolerance to benzoxazolinones in *Fusarium* spp.

FDB2 encodes a predicted *N*-malonyltransferase; in *F. verticillioides*, the Fdb2 homologue has been demonstrated to catalyse the phenoxazinone intermediate in benzoxazolinone detoxification (Glenn and Bacon, 2009). To determine whether Fdb2 is similarly involved in benzoxazolinone detoxification, we tested the relative ability of *FDB2* mutants to grow in the presence of benzoxazolinones. Compared with the parental strain, both *F. pseudograminearum* $\Delta fdb2$ strains showed significantly reduced growth on medium amended with BOA or MBOA and no growth on 2-AP (Fig. 6B). There was no statistically significant difference observed between the two independent mutants on any medium conditions tested. *Fusarium graminearum* *FDB2* mutants also showed significantly reduced growth on BOA-amended medium (Fig. S4). However, in contrast with *F. pseudograminearum*, the *F. graminearum* *FDB2* mutant did not show an additional reduction in growth in the presence of MBOA (Fig. S4), consistent with the greater MBOA sensitivity of *F. graminearum*.

Coupled with growth rates on MBOA-, BOA- and 2-AP-amended half-strength potato dextrose agar (PDA), TLC was used to assess the degradation of MBOA and BOA, and the accumulation of intermediate products, in the medium. Greater amounts of unprocessed BOA and MBOA remained in the medium of knock-outs than of the parental strain (Fig. 6C). Based on the TLC analysis, the $\Delta fdb2$ strains showed an excessive accumulation of 2-APO when grown on BOA-amended medium (Fig. 6C), consistent with observations in *F. verticillioides* (Glenn *et al.*, 2002). As 2-AP oxi-

Fig. 4 Expression of the *Fusarium pseudograminearum* (CS3096) *FDB* gene cluster in response to benzoxazolin-2-one (BOA) exposure. Horizontal axes represent hours post-exposure to BOA. Vertical axes represent expression relative to solvent-treated controls. Vertical axes were set to a maximum value of 1000-fold, except for genes encoding transcription factors (*FPSE_08121* and *FPSE_08122*), which showed lower relative induction levels. Error bars represent the propagated standard error of the mean for three biological replicates for BOA and dimethylsulfoxide (DMSO) controls at each time point. Shaded genes indicate the proposed gene cluster limits based on the gene expression profiles.

gradation to 2-APO is a spontaneous process (Oancea and Puiu, 2003), the accumulation of 2-APO eventuates from the failure of the *FDB2* knockouts to *N*-malonylate 2-AP, which would otherwise prevent this oxidation. However, in contrast with *F. pseudograminearum*, no 2-APO accumulation was evident when *F. graminearum* *FDB2* knockouts were grown on BOA-amended medium (Fig. S4), suggesting that *F. graminearum* may have an additional pathway for processing the 2-AP intermediate when it is derived from the activity of *Fdb1*, but not when 2-AP is amended into the medium, which is likely to oxidize to 2-APO prior to the action of such a hypothetical alternative pathway.

Complementation of the *F. pseudograminearum* *FDB2* mutant with *FDB2* orthologues from *F. graminearum* (*FPSE_00080*) and *F. verticillioides* (*FVEG_12636*) restored the ability of the mutant to tolerate BOA, MBOA (Fig. 7) and 2-AP (Fig. S5, see Supporting Information), and produced degradation profiles on TLC identical to those of the *F. pseudograminearum* parental strain CS3096.

To investigate the specificity of *Fdb2* in the detoxification of benzoxazolinone compounds, we further tested the transformants on another indole-derived cereal defence metabolite, 3(dimethyl-amino-methyl)-indole (gramine) (Smith, 1977). Both gramine and benzoxazoids are synthesized from tryptophan in the *Hordeum* genus (Grun *et al.*, 2005). Both the parental *F. pseudograminearum* (WT) and $\Delta fdb2$ strains showed similar growth reductions on 0.5 mg/mL gramine-amended medium (Fig. S6, see Supporting Information). This result indicates that *Fdb2* is specific to benzoxazolinone detoxification.

Fdb2 contributes to pathogen virulence

Fusarium pseudograminearum can cause FHB of wheat (Obanor *et al.*, 2013). Therefore, the importance of *Fdb2* for pathogen virulence was assessed by single floret inoculation. Both $\Delta fdb2$ isolates showed a clear reduction in virulence compared with the parental strain. Both spikelet discoloration and necrosis progression along the rachis were significantly reduced in heads inoculated with the mutant strains (Fig. 8). Independent replication of this experiment confirmed the reduced virulence phenotype of these mutants (data not shown). This virulence reduction suggests the importance of benzoxazolinones in wheat heads as a mode of defence.

Fig. 5 *FDB2* deletion from *Fusarium pseudograminearum*. (A) Triplex polymerase chain reaction (PCR) screen to detect successful gene deletion. The PCR assay used three primers Fp (A, B and C) to initially screen transformants for successful gene deletion. The assay was designed such that an intact *FDB2* locus yielded a smaller product than transformants with successful *FDB2* deletion. CS3096 gDNA and the plasmid used for targeted gene disruption were employed as controls. (B) Sequence reads from the $\Delta fdb2\#1$ and $\Delta fdb2\#2$ mutants mapped to the predicted deletion locus and parental isolate's *FDB2* genomic region. Read coverage graphs are shown below each locus diagram. (C) Growth of CS3096 (parental strain) and $\Delta fdb2$ mutants over a 102-h time course in half-strength potato dextrose broth 96-well plate assay [at 102 h post-inoculation (hpi): CS3096 vs. $\Delta fdb2\#1$, $P = 0.5$; CS3096 vs. $\Delta fdb2\#2$, $P = 0.27$]. Error bars represent standard error of the mean for eight replicate wells. OD, optical density.

DISCUSSION

In this study, we identified and functionally characterized *FDB2* from the wheat pathogen *F. pseudograminearum*. Analyses of *FDB2* gene knockouts and complemented strains with two individual *FDB2* homologues from other *Fusarium* spp. showed that Fdb2 is essential for the detoxification of BOA, MBOA and 2-AP. However, the quantitative reduction in growth observed for *FDB2* mutants on BOA and MBOA in *F. pseudograminearum* (and BOA alone in *F. graminearum*) suggests that, although the *FDB* cluster is the major mechanism of tolerance to benzoxazinoids in these species, other mechanisms are also likely to be acting. As shown here, in *F. pseudograminearum*, Fdb2 contributes to pathogen

virulence on wheat. The detoxification of BOA by the maize pathogen *F. verticillioides* also requires Fdb2 (Glenn and Bacon, 2009), but Fdb2 does not contribute to the virulence of this pathogen towards maize (Glenn *et al.*, 2002). Therefore, this is the first report showing that benzoxazinone detoxification contributes significantly to the virulence of a *Fusarium* spp. towards cereal hosts known to produce these defence compounds.

McGary *et al.* (2013) have suggested that genes encoding multiple enzymes of the catabolic and associated metabolic pathways for detoxification of antifungal compounds are physically linked in fungi. The benzoxazinone detoxification pathway in fungi proceeds via the toxic intermediate 2-AP (Glenn and Bacon, 2009), and our description of the largely conserved nature of the

Fig. 6 *Fdb2* is required for tolerance to benzoxazolinones. (A) Example photographs of the parental *Fusarium pseudograminearum* strain CS3096 (WT) and a $\Delta fdb2$ strain growing on half-strength potato dextrose agar (PDA) amended with 0.5 mg/mL of benzoxazolin-2-one (BOA), 6-methoxy-benzoxazolin-2-one (MBOA) or 2-aminophenol (2-AP) at 6 days post-inoculation (dpi). Dimethylsulfoxide (DMSO) was used as a control. (B) Quantification of growth of $\Delta fdb2\#1$ (representative of both mutants) and WT on half-strength PDA amended medium as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. *t*-tests were performed comparing the parental strain and *FDB2* mutants for the data collected at 5 dpi. Statistically significant differences were observed for both mutants compared with the parental strain when growing on BOA ($P \leq 9.76 \times 10^{-5}$), MBOA ($P \leq 5.69 \times 10^{-4}$) and 2-AP ($P \leq 6.15 \times 10^{-2}$), but not DMSO ($P \geq 0.44$). Error bars represent the standard error of the mean for four biological replicates. (C) Comparison of benzoxazolinone degradation via thin layer chromatography (TLC) by parental *F. pseudograminearum* CS3096 (WT) and $\Delta fdb2$ strains on amended half-strength PDA. 2-APO, 2-amino-3H-phenoxazin-3-one.

single *FDB* gene cluster in most fusaria examined is consistent with this phenomenon. In contrast, genes responsible for BOA detoxification in *F. verticillioides* are known to be located at two distinct genetic loci, one of which encodes an as yet unidentified enzyme that catalyses the opening of the lactam ring (Glenn *et al.*, 2002). However, the *F. pseudograminearum FDB* cluster contains a gene (*FPSE_08124*) that is likely to encode this function (Fig. 1B). The gene order conservation within this cluster in most other fusaria suggests that the *F. pseudograminearum FDB* cluster organization is more ancient than that in *F. verticillioides*. It is likely that, in *F. verticillioides*, the probable lactamase-encoding orthologue *FVEG_08291* has translocated out of this region. Although *F. sp. FIESC5* grew on BOA and MBOA, 2-AP appeared fungistatic to this isolate (Fig. 2). This suggests a different route for detoxification of BOA in this species, as 2-AP tolerance is required for the *FDB* pathway intermediate produced by *Fdb1*. Indeed, our analyses show that *F. sp. FIESC5* isolate CS3069 does not contain the *FDB* gene cluster (Fig. 1B), supporting the existence of an alternative benzoxazolinone detoxification pathway that does not operate via a 2-AP intermediate in this organism. The presence of a putative benzoxazolinone detoxification gene cluster in *F. oxysporum* f. sp. *lycopersici*, the tomato wilt pathogen, suggests that an ability to overcome these compounds may provide advantages to soil-living fungi that are not directly pathogenic on cereals.

Outside of the *Fusarium* genus, resistance to the corn leaf blight pathogen *S. turcica* was reduced on maize unable to produce cyclic hydroxamic acids (Couture *et al.*, 1971). Best reciprocal BLASTp matches for three members of the *FDB* cluster were found in *S. turcica*, suggesting that this species may detoxify benzoxazolinones in a manner similar to that found in *Fusarium* spp. In contrast, other grass pathogens, such as *Puccinia graminis* var. *tritici*, *Puccinia triticina*, *G. graminis* var. *tritici*, *Cochliobolus*

Fig. 7 Complementation of *FDB2* in the *Fusarium pseudograminearum* $\Delta fdb2$ isolate. (A) Example photographs of the parental *F. pseudograminearum* strain CS3096 (WT), $\Delta fdb2$ and complemented strains $\Delta fdb2::FDB2$, $\Delta fdb2::FGSG_00080$ and $\Delta fdb2::FVEG_12636$ growing on half-strength potato dextrose agar (PDA) amended with 0.5 mg/mL of benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) at 5 days post-inoculation (dpi). Dimethylsulfoxide (DMSO) was used as a control. (B) Quantification of growth of *F. pseudograminearum* parental CS3096 (WT), $\Delta fdb2$ and $\Delta fdb2$ complemented strains $\Delta fdb2::FDB2$, $\Delta fdb2::FGSG_00080$ and $\Delta fdb2::FVEG_12636$ on half-strength PDA amended medium as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. Error bars represent the standard error of the mean for four biological replicates. Two repetitions produced similar results. (C) Analysis of BOA and MBOA degradation via thin layer chromatography (TLC) of complemented $\Delta fdb2$ strains $\Delta fdb2::FDB2$, $\Delta fdb2::FGSG_00080$ and $\Delta fdb2::FVEG_12636$. BOA and MBOA are presumed to be degraded to *N*-(2-hydroxyphenyl) malonic acid (HPMA) and *N*-(2-hydroxy-4-methoxyphenyl) malonic acid (HMPMA), respectively, in both *F. pseudograminearum* parental strain and restored complemented $\Delta fdb2$ isolates. Degradation products based on comparisons with previously described *F. verticillioides* TLC profiles (Glenn and Bacon, 2009).

sativus, *Cochliobolus heterostrophus* and *Stagonospora nodorum*, do not possess any gene that shows best reciprocal BLASTP matches to any members of the *FDB* cluster (Gardiner *et al.*, 2012). However, *G. graminis* var. *tritici* is able to detoxify BOA to HPMA (Friebe *et al.*, 1998), yet is sensitive to 2-AP and 2-APO (Martyniuk *et al.*, 2006). Similarly, *St. nodorum* is able to tolerate BOA (Du Fall and Solomon, 2013). Presumably, the mechanism(s) for benzoxazinoid detoxification or tolerance in these fungi is different from that in fusaria. Other detoxification mechanisms certainly exist in nature. For example, weeds employ highly active transporters and glutathione-S-transferases to defend themselves against benzoxazinoids (Schulz *et al.*, 2013).

Importantly, a role of benzoxazinones in wheat defence against fungal pathogens has not been demonstrated directly previously. Here, we provide evidence using the *F. pseudograminearum* *FDB2* knockouts that benzoxazalinones play a role in wheat head blight resistance. In spite of the inability of *F. graminearum* to metabolize MBOA, the primary cause of FHB worldwide, the alteration of levels of different benzoxazolinones in wheat may be a route to increased disease resistance. However, such alterations should be tempered with other agricultural considerations, such as the persistence, toxicity and concentration of benzoxazinoids and metabolites in soil (Etzerodt *et al.*, 2008). Of note, the wheat enzymes and respective genes responsible for the methoxylation of the benzoxazolinone precursor 2,4-hydroxy-1,4-benzoxazin-3-one 2,4-dihydroxy-1,4-benzoxazin-3-one-2-O- β -d-glucopyranoside (DIBOA-Glc), which could be used to increase the abundance of MBOA, are yet to be identified (Sue *et al.*, 2011).

Fusarium graminearum and *F. culmorum* showed enhanced sensitivity to MBOA, but *F. pseudograminearum* did not (Fig. 2), despite their relatively close phylogenetic relationships (O'Donnell *et al.*, 2013) and the ability of these pathogens to cause the same diseases on wheat. The underlying cause of this differential MBOA sensitivity is unknown. One possibility is that one of the conserved cluster genes in these fungi has an altered biochemical function. In spite of the inability of *F. graminearum* to process MBOA, the complementation experiments using the *F. graminearum* *FDB2* homologue into the *F. pseudograminearum* $\Delta fdb2$ mutant restored the processing of both BOA and MBOA, indicating that MBOA sensitivity in *F. graminearum* is independent of *FDB2*. Alternatively,

the additional BOA-responsive genes in the *F. pseudograminearum* cluster, three (FPSE_08119 to FPSE_08121) of which also have reciprocal best BLAST hits in *F. verticillioides* but not *F. graminearum* or *F. culmorum*, could be responsible for the differential sensitivities observed. These three genes have considerably higher conservation between *F. pseudograminearum* and *F. verticillioides* than the other genes in the cluster (~97% identity compared with other cluster genes at ~80% at the amino acid level). Biochemical investigation of each co-expressed enzyme in the cluster is required to unequivocally determine their roles in benzoxazolinone detoxification.

In summary, the need to establish durable resistance to *Fusarium*-induced diseases of wheat has prompted research into the understanding of the virulence mechanisms of these pathogens. In the long term, this understanding will be required to enable improved crop protection strategies. This work clearly demonstrates that detoxification of the plant-derived benzoxazalinones is required for successful infection of cereals that produce these compounds, demonstrating directly, for the first time, the role of these compounds in the host response to fungal infection. Correlations between higher benzoxazinoid concentrations and both FHB resistance (Soltoft *et al.*, 2008) and yield in wheat (Silva *et al.*, 2006) have been reported using a small number of cultivars. Wheat cultivars show significant differences in the total amount and proportions of benzoxazinoids found in their roots (Stochmal *et al.*, 2006; Villagrasa *et al.*, 2006), leaves and grain (Villagrasa *et al.*, 2006), although again only a small number of cultivars have been analysed. Although the correlation between benzoxazalinone levels and disease resistance has not been examined for *Fusarium* crown and root rot diseases, these defence compounds may also have an effect on these diseases if their concentrations are increased via traditional or molecular breeding approaches.

EXPERIMENTAL PROCEDURES

Genomic comparisons

Reciprocal best BLASTP matches were obtained from previously published data (Gardiner *et al.*, 2012), or determined as described previously

Fig. 8 The role of Fdb2 in wheat head blight infection. (A) Representative images of mock, parental CS3096 (WT) and $\Delta fdb2$ strains in head infection assay. (B) Number of diseased spikelets as a measure of virulence during head blight infection by *Fusarium pseudograminearum* parental strain CS3096 (WT) and $\Delta fdb2$ mutant strains. Error bars indicate standard error of the mean for ≥ 22 replicates (P values: $\Delta fdb2\#1$ vs. WT, 7.5×10^{-7} ; $\Delta fdb2\#2$ vs. WT, 2.7×10^{-5} ; $\Delta fdb2\#1$ vs. $\Delta fdb2\#2$, 0.14). (C) Number of diseased rachis sections as a measure of virulence during head blight infection by *Fusarium pseudograminearum* parental strain CS3096 (WT) and $\Delta FDB2$ mutant strains. Error bars indicate standard error of the mean for ≥ 22 replicates (P values: $\Delta fdb2\#1$ vs. WT, 1.3×10^{-7} ; $\Delta fdb2\#2$ vs. WT, 2.0×10^{-5} ; $\Delta fdb2\#1$ vs. $\Delta fdb2\#2$, 0.09). Asterisks show $P < 0.001$.

(Gardiner *et al.*, 2012). Four additional genomes were examined in the current study: *F. culmorum* (CS7071) (unpublished, EMBL accession CBMH000000000), *F. sp. FIESC5* (CS3069), *F. acuminatum* (CS5907) (Moolhuijzen *et al.*, 2013) and *F. fujikuroi* (IMI58289) (Wiemann *et al.*, 2013).

Chemicals, fungal isolates and growth

BOA, MBOA and 2-AP were obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO to give a stock solution of 100 g/L. *Fusarium pseudograminearum* isolate CS3096 and *F. graminearum* isolate CS3005 were selected for transformation (Akinsanmi *et al.*, 2004). Other isolates were obtained from the collection stored at the Commonwealth Scientific and Industrial Research Organization (CSIRO) Agriculture Flagship, Brisbane, Qld, Australia. These were: *F. graminearum* Ph1 (Cuomo *et al.*, 2007) obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas City, KS, USA (<http://www.fgsc.net/>) as FGSC #9075, CS3179 and CS3386 (Akinsanmi *et al.*, 2006); *F. culmorum* isolate CS7071; *F. sp. FIESC5* isolate CS3069; and *F. acuminatum* isolate CS5907 (Moolhuijzen *et al.*, 2013). *Fusarium verticillioides* isolate BRIP 149534 was obtained from the Queensland Plant Pathology Herbarium, Brisbane, Qld, Australia (Shivas *et al.*, 2006). The fungi were maintained on half-strength potato dextrose broth (PDB) (BD Difco, North Ryde, NSW, Australia) plus 1% agar.

Generation and analysis of mutant strains

The vector for deletion of *FDB2* in *F. pseudograminearum* was constructed as follows. *FDB2* was amplified using Phusion DNA polymerase (Finnzymes, Vantaa, Finland) employing primers FpFDB2F and FpFDB2R including surrounding genomic regions (887 bp upstream and 2128 bp downstream), A-tailed with *Taq* DNA polymerase and cloned into pCR8-GW/TOPO (Life Technologies, Carlsbad, CA, USA). The entire *FDB2* coding sequence was replaced in this construct using lambda phage-mediated recombination in *Escherichia coli* strain DY380 with a neomycin phosphotransferase (*npt*) cassette amplified from pAN9.1 including the *Aspergillus nidulans gpdA* promoter, as described previously (Gardiner *et al.*, 2009). Primer sequences are given in Table S1 (see Supporting Information). Fungal transformation was performed as described previously (Gardiner *et al.*, 2012). Mutants identified growing on G418 (Sigma-Aldrich) selective medium were single spored, and DNA extracts (Red Extract-N-Amp kit, Sigma-Aldrich) were subjected to PCR analysis with a combination of three primers (Fig. 5A) to determine the deletion of *FDB2*.

To further analyse the $\Delta fdb2$ strains, whole-genome sequencing was performed using an Illumina MiSeq with a NexteraXT library preparation kit (Illumina, San Diego, CA, USA). Paired-end 250-nucleotide sequence reads were imported into CLCbio (Prismet, Aarhus, Denmark) Genomics Workbench version 6.01, trimmed (quality limit, 0.01; ambiguous nucleotides removed; sequences shorter than 20 nucleotides removed) and mapped (0.8 length fraction; 0.8 similarity) to the predicted knockout locus and parental strain's (CS3096) genome.

Mycelial growth rates were measured using a microtitre plate growth assay over a 102-h time course, as described previously (Gardiner *et al.*, 2012).

To delete *FDB2* from *F. graminearum*, a construct was synthesized by GenScript (Piscataway, NJ, USA) containing 1000 bp of sequence immedi-

ately upstream of the *FDB2* start codon, followed by the following 56-bp sequence (GATGTCCACGAGGTCTCTAAGACCTTAGGCGGCTCATTCGTACGCTGCAGGTCGAC), a nourseothricin resistance cassette driven by an *Aspergillus nidulans* *trpC* promoter, corresponding to nucleotide positions 437–1387 of GenBank accession AY631958.2, and 993 nucleotides of the sequence immediately downstream of the *FDB2* stop codon. The flanking sequences were derived from the genome of the Australian *F. graminearum* isolate CS3005 (Gardiner *et al.*, 2014). This synthesized fragment was cloned into pUC57 and a PCR product generated with M13f and M13r primers was used for fungal transformation as described previously (Desmond *et al.*, 2008). Transformants were selected on 50 mg/L nourseothricin sulfate (Werner BioAgents, Cospeda, Jena, Germany). A three-primer (FgA, FgB and FgC, Table S1) PCR assay employing a Red Extract-N-Amp kit (Sigma-Aldrich) was used to identify transformants lacking *FGSG_00080*.

Complementation of mutants

The vectors for complementation of the *F. pseudograminearum* $\Delta fdb2$ strain with *FDB2* homologues from various *Fusarium* spp. were constructed as follows. *FDB2* homologues were separately amplified using Phusion DNA polymerase (Finnzymes) employing primers pYES2+FpFDB2compF and HygR+FpFDB2compR for *F. pseudograminearum* *FDB2*, pYES2+FgFDB2compF and HygR+FgFDB2compR for *F. graminearum* *FDB2* (*FGSG_00080*), and pYES2+FvFDB2compF and HygR+FvFDB2compR for *F. verticillioide*s *FDB2* (*FVEG_12636*). A hygromycin B resistance cassette (*hygR*) was amplified from a pUChp plasmid using primers HygR-pUChpF and HygR-pUChpR. Yeast-mediated homologous recombination was used to assemble the constructs employing 30-bp homologous regions in the primers, in the order *FDB2* and *hygR* amplification products, into pYES2 cut with *HindIII* and *XbaI* using yeast strain BY4743 (Thermo Fischer Scientific, Scoresby, VIC, Australia). Yeast transformation was carried out as described previously (Gietz and Schiestl, 2007) with plasmids recovered from the resulting colonies using a Zymoprep miniprep II kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Fungal transformation was performed as described previously (Gardiner *et al.*, 2012). Mutants identified growing on hygromycin B (Sigma-Aldrich) selective medium were isolated from a single conidium, and DNA extracts made from the subsequent culture (Red Extract-N-Amp kit, Sigma-Aldrich) were subjected to PCR analysis with primers (FpFDB2compF and FpFDB2compR for *F. pseudograminearum* *FDB2*, FvFDB2compF and FvFDB2compF for *F. verticillioide*s *FDB2*, and FgFDB2compF and FgFDB2compF for *F. graminearum* *FDB2*) to determine the insertion of *FDB2*. The primer sequences are given in Table S1.

FHB assays

Wheat cultivar Kennedy seeds were obtained from the collection stored at CSIRO Agriculture Flagship, Brisbane, Qld, Australia. Plants were grown in a controlled environment facility (CSIRO Agriculture Flagship, Brisbane, Qld, Australia) maintained at 20 °C and 70% relative humidity during a 15-h photoperiod and at 85% relative humidity at 15 °C during darkness in a 1:1 sand and peat mix with added Osmocote fertilizer in 15-cm pots. At mid-anthesis, a single central spikelet was inoculated with 10 μ L of a 1×10^6 spores/mL suspension obtained from filtered and washed 7-day-old

quarter-strength Campbell's V8 juice (pH 6.5) shake culture. Sterile water used in the re-suspension of the spores was employed for mock inoculations. Inoculated heads were covered for 3 days with small, pre-wet, clip-lock luncheon bags, after which they were replaced with white glassine paper bags for the remaining time. Heads were removed at 21 days post-inoculation (dpi) and dissected to determine the number of spikelets and rachis internodes displaying browning symptoms. Significance of difference was determined between the parental strain (CS3096) and $\Delta fdb2$ strains using Student's *t*-tests. The experiment was conducted twice.

Chemical sensitivity assays

Sensitivity to BOA, MBOA and 2-AP was assessed using growth on solid medium amended with the test compounds compared with medium amended with solvent (DMSO) alone in a method adapted from Martyniuk *et al.* (2006). All compounds were tested at 0.5 mg/mL in half-strength PDA medium in 90-mm Petri dishes. Plates were inoculated in the centre with agar plugs from the growing edge of a fungal colony growing on unamended half-strength PDA plates using an inverted 1-mL pipette tip. The plates were incubated in darkness at 28 °C and the colony diameter was measured daily by drawing a line around the growing edge until the dish confines were reached. Following 5 days of growth, an image was captured using a Gel logic 212 Pro system (Carestream Health, New Haven, CT, USA) and growth rings were used to calculate the colony area employing a Carestream molecular imaging system. The percentage of inhibition was calculated in relation to the DMSO control, subtracting the initial agar plug diameter from the mean measured colony area.

To assess the fungistatic activity of benzoxazolinones and derivatives, agar plugs from the growing edge of inoculated unamended half-strength PDA plates were placed on half-strength PDA medium plates amended with 0.5 mg/mL concentrations of the test compounds. At 24 h, agar plugs not exhibiting growth were removed and placed on unamended half-strength PDA plates and assessed for growth.

TLC

Transformation of benzoxazolinone and intermediates by *Fusarium* species and transformants was assessed using the agar plug TLC method developed by Glenn *et al.* (2001). In brief, using an inverted 1-mL pipette tip, a single plug of agar was taken from culture plates next to the site of initial inoculation containing amended half-strength PDA and spotted with mycelia upwards onto a TLC Silica gel 60 F₂₅₄ sheet (Merck, Darmstadt, Germany). The spot contents and chemical standards were migrated with toluene–ethyl acetate–formic acid (50:40:10). The dried plates were photographed under UV light from a Gibco BRL UV trans-illuminator with a Canon IXUS 870IS digital camera (Canon, Chichibu, STM, Japan).

RT-qPCR

Fusarium pseudograminearum (CS3096) was cultured for 3 days in half-strength potato dextrose broth (PDB). The medium was then amended to a final concentration of 1 mg/mL BOA or equivalent percentage (v/v) of DMSO. Mycelia continued to grow throughout the experiment. Mycelia were harvested at 0, 2, 6, 12 and 24 h by rapid vacuum filtration with

miracloth (Merck), capturing the mycelia, followed by immediate liquid nitrogen submersion. After freeze-drying the samples and milling by ball-beating in a Retsch (Haan, NRW, Germany) MM 400 mill (30 Hz for 30 s) using a single 3-mm ball bearing in a 2-mL tube, total RNA was extracted using Trizol (Life Technologies) according to the manufacturer's instructions. Three biological replicates were used for each time point and treatment.

RT-qPCR was performed as described previously (Gardiner *et al.*, 2009) using normalization to β -tubulin expression and relative quantification to control (DMSO) treatments. Melt curve analysis indicated single amplification products. Three RT-qPCR technical replicates were tested for each sample. Primer sequences are described in Table S2 (see Supporting Information). Paired Student's *t*-tests were used to determine which treatment differences were statistically significant.

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REFERENCES

- Akinsanmi, O.A., Mitter, V., Simpfendorfer, S., Backhouse, D. and Chakraborty, S. (2004) Identity and pathogenicity of *Fusarium* spp. isolated from wheat fields in Queensland and northern New South Wales. *Aust. J. Agric. Res.* **55**, 97–107.
- Akinsanmi, O.A., Backhouse, D., Simpfendorfer, S. and Chakraborty, S. (2006) Genetic diversity of Australian *Fusarium graminearum* and *F. pseudograminearum*. *Plant Pathol.* **55**, 494–504.
- Chakraborty, S. and Newton, A.C. (2011) Climate change, plant diseases and food security: an overview. *Plant Pathol.* **60**, 2–14.
- Coleman, J.J., White, G.J., Rodriguez-Carres, M. and VanEtten, H.D. (2011) An ABC transporter and a cytochrome P450 of *Nectria haematococca* MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. *Mol. Plant–Microbe Interact.* **24**, 368–376.
- Condon, B.J., Leng, Y.Q., Wu, D.L., Bushley, K.E., Ohm, R.A., Otiillar, R., Martin, J., Schackwitz, W., Grimwood, J., MohdZainudin, N., Xue, C.S., Wang, R., Manning, V.A., Dhillon, B., Tu, Z.J., Steffenson, B.J., Salamov, A., Sun, H., Lowry, S., LaButti, K., Han, J., Copeland, A., Lindquist, E., Barry, K., Schmutz, J., Baker, S.E., Ciuffetti, L.M., Grigoriev, I.V., Zhong, S. and Turgeon, B.G. (2013) Comparative genome structure, secondary metabolite, and effector coding capacity across *Cochliobolus* pathogens. *PLoS Genet.* **9**, e1003233.
- Copaja, S.V., Nicol, D. and Wratten, S.D. (1999) Accumulation of hydroxamic acids during wheat germination. *Phytochemistry*, **50**, 17–24.
- Couture, R.M., Routley, D.G. and Dunn, G.M. (1971) Role of cyclic hydroxamic acids in monogenic resistance of maize to *Helminthosporium turcicum*. *Physiol. Plant Pathol.* **1**, 515–521.
- Crombie, W.M.L., Crombie, L., Green, J.B. and Lucas, J.A. (1986) Pathogenicity of 'Take-all' fungus to oats: its relationship to the concentration and detoxification of the four avenacins. *Phytochemistry*, **25**, 2075–2083.
- Cuomo, C.A., Guldener, U., Xu, J.R., Trail, F., Turgeon, B.G., Di Pietro, A., Walton, J.D., Ma, L.J., Baker, S.E., Rep, M., Adam, G., Antoniw, J., Baldwin, T., Calvo, S., Chang, Y.L., Decaprio, D., Gale, L.R., Gnerre, S., Goswami, R.S., Hammond-Kosack, K., Harris, L.J., Hilburn, K., Kennell, J.C., Kroken, S., Magnuson, J.K., Mannhaupt, G., Mauceli, E., Mewes, H.W., Mitterbauer, R., Muehlbauer, G., Munsterkotter, M., Nelson, D., O'Donnell, K., Ouellet, T., Qi, W., Quesneville, H., Roncero, M.I., Seong, K.Y., Tetko, I.V., Urban, M., Waalwijk, C., Ward, T.J., Yao, J., Birren, B.W. and Kistler, H.C. (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science*, **317**, 1400–1402.
- Desmond, O.J., Manners, J.M., Stephens, A.E., Maclean, D.J., Schenk, P.M., Gardiner, D.M., Munn, A.L. and Kazan, K. (2008) The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. *Mol. Plant Pathol.* **9**, 435–445.
- Du Fall, L.A. and Solomon, P.S. (2013) The necrotrophic effector SnToxA induces the synthesis of a novel phytoalexin in wheat. *New Phytol.* **200**, 185–200.
- Escobar, C.A., Sicker, D. and Niemeyer, H.M. (1999) Evaluation of DIMBOA analogs as antifeedants and antibiotics towards the aphid *Sitobion avenae* in artificial diets. *J. Chem. Ecol.* **25**, 1543–1554.
- Etzerodt, T., Mortensen, A.G. and Fomsgaard, I.S. (2008) Transformation kinetics of 6-methoxybenzoxazolin-2-one in soil. *J. Environ. Sci. Health B*, **43**, 1–7.
- Friebe, A., Vilich, V., Hennig, L., Kluge, M. and Sicker, D. (1998) Detoxification of benzoxazolinone allelochemicals from wheat by *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, and *Fusarium culmorum*. *Appl. Environ. Microbiol.* **64**, 2386–2391.
- Gagliardo, R.W. and Chilton, W.S. (1992) Soil transformation of 2(3H)-benzoxazolinone of rye into phytotoxic 2-amino-3H-phenoxazin-3-one. *J. Chem. Ecol.* **18**, 1683–1691.
- Gardiner, D.M., Kazan, K. and Manners, J.M. (2009) Novel genes of *Fusarium graminearum* that negatively regulate deoxynivalenol production and virulence. *Mol. Plant–Microbe Interact.* **22**, 1588–1600.
- Gardiner, D.M., McDonald, M.C., Covarelli, L., Solomon, P.S., Rusu, A.G., Marshall, M., Kazan, K., Chakraborty, S., McDonald, B.A. and Manners, J.M. (2012) Comparative pathogenomics reveals horizontally acquired novel virulence genes in fungi infecting cereal hosts. *PLoS Pathog.* **8**, e1002952.
- Gardiner, D.M., Stephens, A.E., Munn, A.L. and Manners, J.M. (2013) An ABC pleiotropic drug resistance transporter of *Fusarium graminearum* with a role in crown and root diseases of wheat. *FEMS Microbiol. Lett.* **348**, 36–45.
- Gardiner, D.M., Stiller, J. and Kazan, K. (2014) Genome sequence of *Fusarium graminearum* isolate CS3005. *Genome Announcements*, **2**, e00227–14.
- Gietz, R.D. and Schiestl, R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2**, 31–34.
- Glenn, A.E. and Bacon, C.W. (2009) *FDB2* encodes a member of the arylamine N-acetyltransferase family and is necessary for biotransformation of benzoxazolinones by *Fusarium verticillioides*. *J. Appl. Microbiol.* **107**, 657–671.
- Glenn, A.E., Hinton, D.M., Yates, I.E. and Bacon, C.W. (2001) Detoxification of corn antimicrobial compounds as the basis for isolating *Fusarium verticillioides* and some other *Fusarium* species from corn. *Appl. Environ. Microbiol.* **67**, 2973–2981.
- Glenn, A.E., Gold, S.E. and Bacon, C.W. (2002) *Fdb1* and *Fdb2*, *Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from corn. *Mol. Plant–Microbe Interact.* **15**, 91–101.
- Glenn, A.E., Meredith, F.I., Morrison, W.H. and Bacon, C.W. (2003) Identification of intermediate and branch metabolites resulting from biotransformation of 2-benzoxazolinone by *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **69**, 3165–3169.
- Goswami, R.S. and Kistler, H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* **5**, 515–525.
- Grun, S., Frey, M. and Gierl, A. (2005) Evolution of the indole alkaloid biosynthesis in the genus *Hordeum*: distribution of gramine and DIBOA and isolation of the benzoxazinoid biosynthesis genes from *Hordeum lechleri*. *Phytochemistry*, **66**, 1264–1272.
- Hashimoto, Y. and Shudo, K. (1996) Chemistry of biologically active benzoxazinoids. *Phytochemistry*, **43**, 551–559.
- Hogg, A.C., Johnston, R.H., Johnston, J.A., Klouser, L., Kephart, K.D. and Dyer, A.T. (2010) Monitoring fusarium crown rot populations in spring wheat residues using quantitative real-time polymerase chain reaction. *Phytopathology*, **100**, 49–57.
- Knight, N.L. and Sutherland, M.W. (2013) Spread of *Fusarium pseudograminearum* in wheat seedling tissues from a single inoculation point. *Australas. Plant Pathol.* **42**, 609–615.
- Lawler, K., Hammond-Kosack, K., Brazma, A. and Coulson, R.M.R. (2013) Genomic clustering and co-regulation of transcriptional networks in the pathogenic fungus *Fusarium graminearum*. *BMC Syst. Biol.* **7**, 2–16.
- Martyniuk, S., Stochmal, A., Macias, F.A., Marin, D. and Oleszek, W. (2006) Effects of some benzoxazinoids on in vitro growth of *Cephalosporium gramineum* and other fungi pathogenic to cereals and on *Cephalosporium* stripe of winter wheat. *J. Agric. Food Chem.* **54**, 1036–1039.
- McGary, K.L., Slot, J.C. and Rokas, A. (2013) Physical linkage of metabolic genes in fungi is an adaptation against the accumulation of toxic intermediate compounds. *Proc. Natl. Acad. Sci. USA*, **110**, 11 481–11 486.

- Moolhuijzen, P.M., Manners, J.M., Wilcox, S.A., Bellgard, M.I. and Gardiner, D.M. (2013) Genome sequences of six wheat-infecting *Fusarium* species isolates. *Genome Announcements*, **1**, e00670–13.
- Morrissey, J.P. and Osbourn, A.E. (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* **63**, 708–724.
- Moya-Elizondo, E.A. (2013) *Fusarium* crown rot disease: biology, interactions, management and function as a possible sensor of global climate change. *Cienc. Invest. Agraria*, **40**, 235–252.
- Murray, G.M. and Brennan, J.P. (2009) Estimating disease losses to the Australian wheat industry. *Australas. Plant Pathol.* **38**, 558–570.
- Murray, G.M. and Brennan, J.P. (2010) Estimating disease losses to the Australian barley industry. *Australas. Plant Pathol.* **39**, 85–96.
- Niemeyer, H.M. (2009) Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-3(4H)-one: key defense chemicals of cereals. *J. Agric. Food Chem.* **57**, 1677–1696.
- Oancea, D. and Puiu, M. (2003) Temperature and pH effects on the kinetics of 2-aminophenol auto-oxidation in aqueous solution. *Cent. Eur. J. Chem.* **1**, 233–241.
- Obanor, F., Neate, S., Simpfordorfer, S., Sabburg, R., Wilson, P. and Chakraborty, S. (2013) *Fusarium graminearum* and *Fusarium pseudograminearum* caused the 2010 head blight epidemics in Australia. *Plant Pathol.* **62**, 79–91.
- O'Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J., Frandsen, R.J., Lysoe, E., Rehner, S.A., Aoki, T., Robert, V.A., Crous, P.W., Groenewald, J.Z., Kang, S. and Geiser, D.M. (2013) Phylogenetic analyses of *RPB1* and *RPB2* support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genet. Biol.* **52**, 20–31.
- Oikawa, A., Ishihara, A., Hasegawa, M., Kodama, O. and Iwamura, H. (2001) Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. *Phytochemistry*, **56**, 669–675.
- Oikawa, A., Ishihara, A., Tanaka, C., Mori, N., Tsuda, M. and Iwamura, H. (2004) Accumulation of HDMBOA-Glc is induced by biotic stresses prior to the release of MBOA in maize leaves. *Phytochemistry*, **65**, 2995–3001.
- Okmen, B., Etalo, D.W., Joosten, M.H.A.J., Bouwmeester, H.J., de Vos, R.C.H., Collemare, J. and de Wit, P.J.G.M. (2013) Detoxification of tomatine by *Cladosporium fulvum* is required for full virulence on tomato. *New Phytol.* **198**, 1203–1214.
- Osbourne, A., Bowyer, P., Lunness, P., Clarke, B. and Daniels, M. (1995) Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify different host plant saponins. *Mol. Plant-Microbe Interact.* **8**, 971–978.
- Pareja-Jaime, Y., Roncero, M.I.G. and Ruiz-Roldan, M.C. (2008) Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* is required for full virulence on tomato plants. *Mol. Plant-Microbe Interact.* **21**, 728–736.
- Quide, T., Osbourne, A.E. and Tudzynski, P. (1998) Detoxification of alpha-tomatine by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* **52**, 151–165.
- Roldan-Arjona, T., Perez-Espinosa, A. and Ruiz-Rubio, M. (1999) Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* defines a new class of saponinases. *Mol. Plant-Microbe Interact.* **12**, 852–861.
- Schulz, M., Marocco, A., Tabaglio, V., Macias, F.A. and Molinillo, J.M.G. (2013) Benzoxazinoids in rye allelopathy—from discovery to application in sustainable weed control and organic farming. *J. Chem. Ecol.* **39**, 154–174.
- Shivas, R.G., Beasley, D.R., Pascoe, I.G., Cunnington, J.H., Pitkethley, R.N. and Priest, M.J. (2006) Specimen-based databases of Australian plant pathogens: past, present and future. *Australas. Plant Pathol.* **35**, 195–198.
- Silva, H., Copaja, S.V., Bravo, H.R. and Argandona, V.H. (2006) Relationship between grain yield, osmotic adjustment and benzoxazinone content in *Triticum aestivum* L. cultivars. *Z. Naturforsch. C*, **61**, 704–708.
- Singh, P., Shrivastava, A.K., Suman, A., Arya, N., Tiwari, P., Rai, R.K., Singh, J. and Singh, A.K. (2009) Allelopathic effects of hydroxamic acids from sugarcane leaves on germination and growth of crops. *Allelopath. J.* **23**, 203–212.
- Smith, T.A. (1977) Tryptamine and related compounds in plants. *Phytochemistry*, **16**, 171–175.
- Soltøft, M., Jørgensen, L.N., Svensmark, B. and Fomsgaard, I.S. (2008) Benzoxazinoid concentrations show correlation with *Fusarium* head blight resistance in Danish wheat varieties. *Biochem. Syst. Ecol.* **36**, 245–259.
- Stochmal, A., Kus, J., Martyniuk, S. and Oleszek, W. (2006) Concentration of benzoxazinoids in roots of field-grown wheat (*Triticum aestivum* L.) varieties. *J. Agric. Food Chem.* **54**, 1016–1022.
- Sue, M., Nakamura, C. and Nomura, T. (2011) Dispersed benzoxazinone gene cluster: molecular characterization and chromosomal localization of glucosyltransferase and glucosidase genes in wheat and rye. *Plant Physiol.* **157**, 985–997.
- VanEtten, H.D., Matthews, P.S., Tegtmeyer, K.J., Dietert, M.F. and Stein, J.I. (1980) The association of pisatin tolerance and demethylation with virulence on pea in *Nectria haematococca*. *Physiol. Plant Pathol.* **16**, 257–268.
- VanEtten, H.D., Mansfield, J.W., Bailey, J.A. and Farmer, E.E. (1994) Two classes of plant antibiotics: phytoalexins versus 'phytoanticipins'. *Plant Cell*, **6**, 1191–1192.
- Villagrasa, M., Guillamon, M., Labandeira, A., Taberner, A., Eljarrat, E. and Barcelo, D. (2006) Benzoxazinoid allelochemicals in wheat: distribution among foliage, roots, and seeds. *J. Agric. Food Chem.* **54**, 1009–1015.
- Wahlroos, O. and Virtanen, A.I. (1959) The precursors of 6-methoxy-benzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand.* **13**, 1906–1908.
- Wiemann, P., Sieber, C.M.K., Von Barga, K.W., Studt, L., Niehaus, E.M., Espino, J.J., Huss, K., Michielse, C.B., Albermann, S., Wagner, D., Bergner, S.V., Connolly, L.R., Fischer, A., Reuter, G., Kleigrew, K., Bald, T., Wingfield, B.D., Ophir, R., Freeman, S., Hippler, M., Smith, K.M., Brown, D.W., Proctor, R.H., Munsterkotter, M., Freitag, M., Humpf, H.U., Guldener, U. and Tudzynski, B. (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. *PLoS Pathog.* **9**, e1003475.
- Yue, Q., Bacon, C.W. and Richardson, M.D. (1998) Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. *Phytochemistry*, **48**, 451–454.

SUPPORTING INFORMATION

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

Fig. S1 Amino acid sequence alignment of the predicted lactamase proteins from *Fusarium pseudograminearum* FPSE_08124 and *F. verticillioides* FVEG_12637 and a possible remnant gene, encoding FVEG_08291.

Fig. S2 Benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) degradation analysis via thin-layer chromatography (TLC) for *Fusarium pseudograminearum* and *F. sp.* FIESC5 on BOA- or MBOA-amended half-strength potato dextrose agar (PDA). The production of compounds by *F. sp.* FIESC5 clearly does not match the *N*-(2-hydroxyphenyl) malonamic acid (HPMA) and *N*-(2-hydroxy-4-methoxyphenyl) malonamic acid (HMPMA) generated by *F. pseudograminearum*. Irrelevant lanes have been removed from the image. DMSO, dimethylsulfoxide.

Fig. S3 Relative expression of the *Fusarium pseudograminearum* (CS3096) *N*-acyltransferase genes with sequence similarity to Fdb2 [FPSE_07946 (92% query coverage; 35% amino acid identity; e-value 3×10^{-53}), FPSE_11041 (92% query coverage; 32% amino acid identity; e-value 8×10^{-43}) and FPSE_08077 (92% query coverage; 30% amino acid identity; e-value 2×10^{-35})] in response to benzoxazolin-2-one (BOA) exposure. Horizontal axes represent hours post-inoculation (hpi) by BOA. Vertical axes represent expression relative to solvent-treated controls. Error bars represent the propagated standard error of the mean for three biological replicates for BOA and dimethylsulfoxide (DMSO) controls at each time point.

Fig. S4 *Fusarium graminearum* Fdb2 is required for tolerance to benzoxazolinones. (A) Example photographs of the parental *Fusarium graminearum* strain CS3005 (wild-type, WT) and a $\Delta fdb2$ strain growing on half-strength potato dextrose agar

(PDA) amended with 0.5 mg/mL of benzoxazolin-2-one (BOA), 6-methoxy-benzoxazolin-2-one (MBOA) or 2-aminophenol (2-AP) at 4 days post-inoculation (dpi). Dimethylsulfoxide (DMSO) was used as a control. (B) Quantification of growth of $\Delta fdb2$ and WT on half-strength PDA amended media as indicated following 4 days of growth. Vertical axes represent the percentage of growth relative to the DMSO control. Error bars represent the standard error of the mean for three biological replicates. (C) Comparison of benzoxazolinone degradation via thin layer chromatography (TLC) by parental *Fusarium graminearum* CS3005 (WT) and $\Delta fdb2$ strains on amended half-strength PDA. 2-APO, 2-amino-3H-phenoxazin-3-one.

Fig. S5 Complementation of *FDB2* in the *Fusarium pseudograminearum* $\Delta fdb2$ isolate. (A) Example photographs of the parental *F. pseudograminearum* strain CS3096 (wild-type, WT), $\Delta fdb2$ and complemented $\Delta fdb2::FDB2$, $\Delta fdb2::FGSG_00080$ and $\Delta fdb2::FVEG_12636$ strains growing on half-strength potato dextrose agar (PDA) amended with 0.2–0.4 mg/mL of 2-aminophenol (2-AP) at 6 days post-inoculation (dpi). Dimethylsulfoxide (DMSO) was used as a control. (B) Quantification of growth of *F. pseudograminearum* parental strain CS3096 (WT), $\Delta fdb2$ and $\Delta fdb2$ complemented strains $\Delta fdb2::FDB2$, $\Delta fdb2::FGSG_00080$

and $\Delta fdb2::FVEG_12636$ on half-strength PDA amended medium as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. Error bars represent the standard error of the mean for four biological replicates. Two repetitions produced similar results.

Fig. S6 *Fdb2* is specific to benzoxazolinone detoxification. (A) Example photographs of the parental *Fusarium pseudograminearum* strain CS3096 (wild-type, WT) and a $\Delta fdb2$ strain growing on half-strength potato dextrose agar (PDA) amended with 0.5 mg/mL of gramine at 5 days post-inoculation (dpi). Dimethylsulfoxide (DMSO) was used as a control. (B) Quantification of growth of $\Delta fdb2$ (data from both mutants pooled) and WT on half-strength PDA-amended medium as indicated. Vertical axis represents the percentage of growth relative to the DMSO control. $P \geq 0.27$. Error bars represent the standard error of the mean for four biological replicates.

Table S1 Primers used in this study for transformation construct generation and polymerase chain reaction (PCR) screening of transformants.

Table S2 Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) primers used in this study.