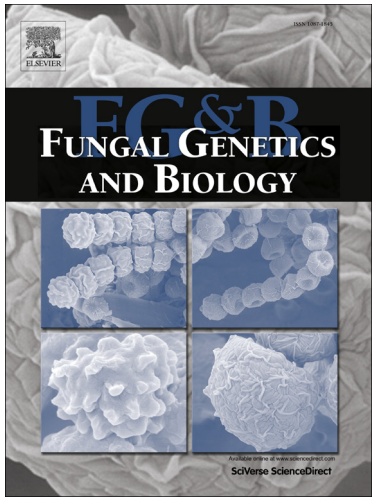

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A γ-lactamase from cereal infecting *Fusarium* spp. catalyses the first step in the degradation of the benzoxazolinone class of phytoalexins.

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Keywords

Phytoalexin, benzoxazolinone, phenoxazinone, *Fusarium pseudograminearum*, *Fusarium graminearum,* lactamase.

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Summary

The benzoxazolinone class of phytoalexins are released by wheat, maize, rye and other agriculturally important species in the Poaceae family upon pathogen attack. Benzoxazolinones show antimicrobial effects on plant pathogens, but certain fungi have evolved mechanisms to actively detoxify these compounds which may contribute to the virulence of the pathogens. In many *Fusarium* spp. a cluster of genes is thought to be involved in the detoxification of benzoxazolinones. However, only one enzyme encoded in the cluster has been unequivocally assigned a role in this process. The first step in the detoxification of benzoxazolinones in

Fusarium spp. involves the hydrolysis of a cyclic ester bond. This reaction is encoded by the *FDB1* locus in *F. verticillioides* but the underlying gene is yet to be cloned. We previously proposed that *FDB1* encodes a γ-lactamase, and here direct evidence for this is presented. Expression analyses in the important wheat pathogen *F. pseudograminearum* demonstrated that amongst the three predicted γ-lactamase genes only the one designated as *FDB1,* part of the proposed benzoxazolinone cluster in *F. pseudograminearum*, was strongly responsive to exogenous benzoxazolinone application. Analysis of independent *F. pseudograminearum* and *F. graminearum FDB1* gene deletion mutants, as well as biochemical assays, demonstrated that the γ-lactamase enzyme, encoded by *FDB1*, catalyses the first step in detoxification of benzoxazolinones. Overall, our results support the notion that *Fusarium* pathogens that cause crown rot and head blight on wheat have adopted strategies to overcome host-derived chemical defences.

Highlights

- Fdb1, a γ-lactamase, identified and functionally characterised in *Fusarium* spp.
- Fdb1 converts the benzoxazolinone class phytoalexins to aminophenols.
- Deletion of *FDB1* in *F. pseudograminearum* increased sensitivity to benzoxazolinones.

Abbreviations

BOA. Benzoxazolin-2-one.
MBOA. 6-methoxy-benzoxazolin-2-one, methoxylated BOA compound.
M_2BOA. 6,7-dimethoxy-2(3H)-benzoxazolinone.
2-AP. 2-aminophenol, the intermediate compound of BOA degradation.
2-APO. 2-amino-phenoxazin-3-one, the compound of spontaneous oxidation of 2-AP.
2-AMP. 5-methoxy-2-aminophenol, the intermediate compound of MBOA degradation.
2-AMPO. 2-amino-7-methoxy-3H-phenoxazin-3-one, the compound of resulting from spontaneous oxidation of 2-AMP.
HPMA. N-(2-hydroxyphenyl) malonamic acid, the final product of BOA detoxification.
HMPMA. N-(2-hydroxy-4-methoxyphenyl) malonamic acid, the final product of MBOA detoxification.
FDB. Fusarium Detoxification of Benzoxazolinone, employed in gene annotations and gene cluster identity.
FDB1. Genetic identity of Fdb1.
Fdb1. Protein transcribed from *FDB1.*
Δ*fdb1.* Mutant isolate with *FDB1* deletion.
Fp. *Fusarium pseudograminearum.*
Fg. *Fusarium graminearum.*
TLC. Thin layer chromatography, a method of separating and identifying metabolites.

1. Introduction

The production of phytoalexins (chemicals that have anti-pathogen or anti-pest activity) is a major component of the defence response of plants to various biotic stresses (Ahuja *et al.*, 2012). In turn, pathogens have typically evolved mechanisms to overcome these defences.

However, in most cases the specific mechanisms involved in fungal deactivation of phytoalexins are not well understood.

The benzoxazinoids, which are biosynthetically derived from indole, are a major class of plant defence compounds produced by various plants, such as Poaceae (Frey *et al.*, 2009). Benzoxazinoid precursors are stored in vacuoles as glucosides (Frey *et al.*, 2009) and are released upon pathogen or pest attack and subsequently de-glycosylated. Autocatalysis, liberating formic acid (Niemeyer, 1988), produces the active benzoxazolinones, which contain a nitrogenous hetero-pentacyclic ring (Maag *et al.*, 2014). The reactivity of the nitrogen group in this ring is considered to be the basis of benzoxazolinone toxicity (Hashimoto & Shudo, 1996). Benzoxazolinones are also exuded by roots into soil to perform allelochemical (Zheng *et al.*, 2010) and rhizosphere manipulation functions (Neal *et al.*, 2012). The two major benzoxazolinones found in wheat (*Triticum aestivum* L.) are 6-methoxy-benzoxazolin-2-one (MBOA) and benzoxazolin-2-one (BOA) (Villagrasa *et al.*, 2006).

A variety of organisms, including bacteria, fungi, insects and plant species, show tolerance to benzoxazolinones. Detoxification of benzoxazolinones can be achieved by at least two distinct mechanisms. Plants and insects appear to utilise conjugative detoxification mechanisms, which include O- and N- glycosylation of the hetero-pentacyclic ring (Schulz & Wieland, 1999), and conjugation to glutathione (Maag *et al.*, 2014; Schulz *et al.*, 2012; Wieland *et al.*, 1998). In contrast, fungi and bacteria use degradative detoxification mechanisms whereby the hetero-pentacyclic ring is opened and the product is either partially or completely catabolised (Chase *et al.*, 1991; Friebe *et al.*, 1998; Macias *et al.*, 2005).

Studies on detoxification mechanisms of benzoxazolinones have been conducted on a broad range of fungi including four endophytic fungi isolated from red aphelandra (*Aphelandra tetragona*) (Zikmundova *et al.*, 2002), as well as *Gaeumannomyces graminis* and *Fusarium culmorum*, pathogens of wheat, barley and oats (Friebe *et al.*, 1998), and *F. verticillioides*, a pathogen of maize (Glenn *et al.*, 2001). To overcome toxicity of benzoxazolinones, some *Fusarium* spp. detoxify these molecules via a ring opening reaction and decarboxylation (Glenn *et al.*, 2003) generating aminophenols, which are themselves toxic and require further metabolism. In *F. verticillioides* two distinct genetic loci are required for degradation of benzoxazolinones and aminophenols. The *Fusarium Detoxification of Benzoxazolinone 1* (*FDB1*) locus is hypothesised to encode an enzyme that catalyses the first step in detoxification of benzoxazolinones to the fungistatic aminophenols. Subsequent conjugation of an acyl group by Fdb2 encoded at the second locus forms non-toxic acids (Glenn & Bacon, 2009)(Figure 1).

We have previously shown that detoxification of aminophenols by a N-acyl transferase, Fdb2, in the *F. pseudograminearum* FDB gene cluster is important for virulence on wheat (Kettle *et al.*, 2015). However, the specific genetic identity of Fdb1, proposed to be a hydrolytic enzyme that performs transformation of benzoxazolinones, remains to be identified (Glenn *et al.*, 2003). Genomic comparisons of the FDB gene cluster in *F. pseudograminearum* to that in *F. verticillioides* (Glenn *et al.*, 2002) identified a single lactamase-encoding gene that was previously proposed (but not demonstrated) to encode the Fdb1 function (Kettle *et al.*, 2015).

In this study, the γ-lactamase encoding gene *FDB1* located in the main *FDB* gene cluster in *F. pseudograminearum* and *F. graminearum* (Kettle *et al.*, 2015) has been functionally characterised via both biochemical assays and gene knockouts. Our results show that Fdb1 acts

upon benzoxazolinones to produce aminophenols and is important for resistance of at least two *Fusarium* species to benzoxazolinone compounds.

2. Results

2.1 F. pseudograminearum FDB1 is transcriptionally responsive to exogenous BOA

We previously demonstrated that *FPSE_08124* (hereafter termed *FDB1*) was transcriptionally responsive to BOA treatment with a characteristic *FDB* gene cluster expression profile as measured by reverse transcriptase quantitative PCR (RT-qPCR) (Kettle *et al.*, 2015). To determine if there are other closely matching lactamase encoding genes that respond to BOA treatment, the *F. pseudograminearum* predicted proteome was searched for Fdb1 homologues using BLASTp. Two putative lactamase proteins with sequence similarity to Fdb1 were found; FPSE_08188 (59.7% query coverage, 41.7% amino acid identity, e-value 1.1×10^{-90}) and FPSE_08361 (58.3% query coverage, 39.24% amino acid identity, e-value 3.1×10^{-81}). The responsiveness of these putative lactamase encoding genes to BOA was investigated by RT-qPCR over a BOA treatment time-course relative to control samples treated with DMSO. As previously reported (Kettle *et al.*, 2015), *FDB1* expression increased two hours post induction (hpi) with BOA, peaking at ~400 fold at 12 hpi and then decreasing at 24 hpi. Expression from *FPSE_08188*, the closest match to Fdb1, could not be detected via RT-qPCR and *FPSE_08361* did not display as large an expression change as *FDB1* (Figure 2). In addition, the expression profile of *FPSE_08361* was not similar to the expression of *FDB1,* with a correlation coefficient of -0.06. Although *FPSE_08361* transiently responded to BOA, based on the stronger transcriptional responsiveness of *FDB1*, and genomic location of *FDB1* neighbouring *FDB2*, we concluded the putative lactamase-encoding gene in *F. pseudograminearum* was *FDB1* and this was investigated further.

2.2 Fdb1 processes BOA to 2-AP.

The genomic location in the *FDB* cluster, transcriptional responsiveness to BOA and predicted function make Fdb1 a prime candidate for catalysing the first step of benzoxazolinone degradation in multiple *Fusarium* species. The Fdb1 animo acid sequence has no recognisable export signal sequence assessed by SignalP (Petersen *et al.*, 2011) and is predicted to be cytoplasmic by WolF PSORT (Horton *et al.*, 2007). To test the enzymatic function of Fdb1, the *F. pseudograminearum* protein was heterologously expressed in *E. coli* and purified by nickel ion affinity chromatography (Figure 3A). In end point assays, purified Fdb1 was able to completely convert all BOA and MBOA to another product, as observed by both liquid chromatography and thin layer chromatography (TLC) (Supplementary Figure 1). In a BOA degradation enzymatic assay that utilised the spontaneous oxidation of the 2-AP to the coloured compound 2-amino-phenoxazin-3-one (2-APO) in the presence of copper, Fdb1 was determined to have a K_m of 0.19 ± 0.02 mM, a k_{cat} of 4.2 ± 0.1 (s^{-1}) and a catalytic efficiency (k_{cat}/K_m) of 2.2 ± 0.2 x 10^4 M^{-1} s^{-1} towards BOA as a substrate (Figure 3B).

2.3 Fdb1 is not essential for growth in axenic culture.

To confirm the involvement of Fdb1 in benzoxazolinone detoxification *in vivo*, *FDB1* was deleted from *F. pseudograminearum* isolate CS3096 and *F. graminearum* isolate CS3005. In *F. pseudograminearum* the entire *FDB1* gene (1242 bp) was replaced by a geneticin resistance gene cassette via homologous recombination. Transformants were initially screened using a triplex PCR assay and four independent mutants were identified (Figure 4A). Whole genome

sequencing of the mutant strains at approximately 25-fold coverage confirmed the absence of the entire gene in four independent mutants. Mapping reads to the parent CS3096 genome showed coverage consistent with an alteration limited to the *FDB1* locus (Figure 4B & Supplementary Figure 2). All other predicted genes in the genome showed some reads mapping to them, albeit not complete coverage in all cases. However incomplete coverage is to be expected for short read coverage at ~20-fold. Mapping reads to the predicted mutant locus sequence showed coverage consistent with a single insertion at the *FDB1* locus and absence of the plasmid backbone in only two mutants which were selected for the studies reported here (Figure 4B). The other two mutants had more than one copy of the transforming DNA including the vector backbone (Supplementary Figure 2). All mutants had comparable growth on media to the wild type with respect to hyphal growth on solid media (p-value ≥ 0.47) (Figure 5 & Supplementary Figure 3). In *F. graminearum*, the entire *FDB1* gene (*FGSG_00079*, 1242 bp) was replaced by a nourseothricin resistance cassette via homologous recombination and transformants screened using a triplex PCR assay (Supplementary Figure 4). Sequence data for Fdb1 mutants have been deposited in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) as Δ*fdb1*#1 (SRR2060957), Δ*fdb1*#2 (SRR2060958), Δ*fdb1*#3 (SRR2060959), and Δ*fdb1*#4 (SRR2060960).

2.4 Fdb1 is essential for tolerance to benzoxazolinones but not aminophenols in *F. pseudograminearum* and *F. graminearum.*

The relative ability of Δ*fdb1* mutants to grow in the presence of benzoxazolinones was tested on solid media in Petri plates. Compared to the parental strain, Δ*fdb1* strains showed significantly reduced growth at seven days on media amended with 0.5 mg mL^{-1} BOA and MBOA (BOA: p-value ≤ 3.16×10^{-3} , MBOA: p-value ≤ 1.95×10^{-2}, Figure 5A,B) and more unprocessed BOA and MBOA remained in media of knockouts than the parental strain (Figure 5C). Δ*fdb1* strains did not show significantly reduced growth at seven days on media amended with 0.2 mg mL^{-1} BOA compared to the parental strain (p-value ≥ 0.11, data not shown). There was no statistical difference observed between the independent mutants on any benzoxazolinone amended media conditions tested (p-value ≥ 0.18). Complementation of *F. pseudograminearum* Δ*fdb1#1* with the *FDB1* gene, including 971 bp upstream and 970 bp downstream of the coding sequence, restored benzoxazolinone detoxification (Figure 5) confirming the function of this gene.

The growth of a *F. graminearum FDB1* knockout grown on 0.5 mg mL^{-1} BOA amended media was also reduced compared to the parental strain (p-value ≤ 3.7×10^{-3}, Figure 6). Growth of the *F. graminearum FDB1* knockout on MBOA amended media was indistinguishable from the parental strain CS3005 (Figure 6A). The TLC profile comparing detoxification products of *FDB1* knockout with those of parental strain also showed no difference (Figure 6C). Unprocessed MBOA remained in media of both parental and Δ*fdb1* isolates with an absence of identifiable catabolic products. These results are consistent with our previous report that *F. graminearum* is unable to process MBOA (Kettle *et al.*, 2015). Complementation of the *F. graminearum FDB1* mutant with *FDB1* orthologues from *F. pseudograminearum* and *F. verticillioides* (*FVEG_08291*) remains to be conducted and is a future investigation in our laboratory.

In *F. pseudograminearum, F. graminearum* and *F. verticillioides*, processing of the intermediate of BOA degradation, 2-aminophenol (2-AP), is performed by Fdb2, an N-malonyltransferase (Glenn & Bacon, 2009; Kettle *et al.*, 2015). To determine whether Fdb1 influences aminophenol detoxification, we tested the relative ability of *FDB1* mutants to grow in the presence of 2-AP. As

expected, all *F. pseudograminearum* Δ*fdb1* strains maintained the ability to grow on media amended with 2-AP (Figure 7). The growth of *F. graminearum FDB1* knockouts grown on 0.5 mg mL^{-1} 2-AP amended media was also indistinguishable from the parental strain and 2-AP was completely removed from the media (Figure 6).

Although growth of the Δ*fdb1* strain of *F. graminearum* showed clear reductions in growth on BOA containing media, the mutant still maintained some ability to tolerate benzoxazolinones. Indeed at lower BOA concentrations such as 0.2 mg mL^{-1}, the growth difference between parent and mutant strains (tested with the *F. pseudograminearum* parent and Δ*fdb1* mutant strain) becomes less evident (Supplementary Figure 5). Together this suggests a secondary mechanism of BOA tolerance exists in these *Fusarium* spp. Also since BOA remains in the media after growth of the *F. graminearum Δfdb1* strain (Figure 6), this secondary mechanism is not expected to be degradative.

3. Discussion

In this study, we have shown, via biochemical assays and gene knockouts, that *F. pseudograminearum FPSE_08124* and *F. graminearum FGSG_00079* encode Fdb1, a γ-lactamases functioning in the benzoxazolinone catabolic pathway. Fdb1 is conserved across a number of *Fusarium* species, implying a broad ability to convert benzoxazolinones to aminophenols in these fungi (Kettle *et al.*, 2015). Whilst γ-lactamases have been isolated from soil- (Yang *et al.*, 2012) and legume-root nodulating bacteria (Zhu *et al.*, 2014), to our knowledge, this is the first functional characterisation of a fungal γ-lactamase.

In the absence of functional Fdb1, *F. pseudograminearum* could still tolerate 2-AP, consistent with the notion that Fdb2 catalyses the conversion of 2-AP to HPMA, which is downstream of the Fdb1 catalysed step in the detoxification pathway (Glenn & Bacon, 2009). The Δ*fdb1* mutants produced a compound that accumulated in 2-AP amended media. This compound was indistinguishable from that of the parental strain and is most likely N-(2-hydroxyphenyl) malonamic acid based on similar migration rates in TLC (Glenn & Bacon, 2009). In this study, other branch intermediates, such as 2-acetamidophenol in *F. verticillioides fdb1* mutants that have previously been clearly distinguished via TLC (Glenn *et al.*, 2003), were not observed in the *F. pseudograminearum* or *F. graminearum* isolates tested on either BOA or 2-AP amended media.

Fdb1 has been proposed to possibly have other substrates (Glenn & Bacon, 2009). As the proposed mechanism of hydrolysis of γ-lactamases is dependent on the carbonyl oxygen on the cyclic amide ring (Line *et al.*, 2004), it is expected that Fdb1 cannot contribute to tolerance to phytoalexins that do not possess this chemical structure. Therefore, testing the effect of Fdb1 on similarly structured plant defence compounds is required to determine whether Fdb1 would have a role in detoxification of other phytoalexins. For example, spirobrassinin produced by the mustard plant, *Brassica juncea* (Pedras *et al.*, 2009), or heliotropamide produced by *Heliotropium ovalifolium* (Guntern *et al.*, 2003) that is inhibitory to *F. graminearum* (Villarroel *et al.*, 2001) may be potential substrates for Fdb1 as they contain lactam or lactam-like rings. Other non-commercially available benzoxazolinones, such as 6,7-dimethoxy-2(3H)-benzoxazolinone (M_2BOA) produced by maize, were not tested.

The ability to detoxify benzoxazolinones in fungi has been used to define cereal pathogenic *Fusarium* spp. (Glenn *et al.*, 2001) and we have shown that this ability contributes towards virulence quantitatively (Kettle *et al.*, 2015). However the broader context of benzoxazolinone detoxification is, as yet, not fully explored. For example, collaborative detoxification has been observed between fungal isolates (Glenn *et al.*, 2002); the detoxification of other benzoxazolinones, such as M_2BOA (Kumar *et al.*, 1993), is yet to be associated with the FDB pathway; why *F. graminearum* and *F. culmorum* are unable to detoxify MBOA (Kettle *et al.*, 2015); and how the ability of individual species to detoxify benzoxazolinones for their own protection, which may impact on allelopathy functions, remains to be investigated. The latter of these is further complicated by the observations that auto-oxidised intermediates of benzoxazolinone degradation (ie the phenoxazinones) are the dominate allelochemicals in rye grown soils (Macias *et al.*, 2005). The plant-benzoxazolinone-aminophenol-microbiome interaction is required to holistically understand the role of benzoxazolinones in cereal crops. Virulence assays comparing the parental strains with the FDB1 mutants are yet to be performed.

The soil bacterium, *Acinetobacter calcoaceticus*, has been shown to degrade BOA to 2-APO, most likely via biocatalysed degradation to 2-AP with non-enzymatic oxidation of 2-AP to 2-APO (Chase *et al.*, 1991; Gagliardo & Chilton, 1992). However the responsible enzyme is yet to be identified. Moreover, to date γ-lactamases have only been identified from bacteria, unrelated to phytoalexin detoxification (Brabban *et al.*, 1996; Yang *et al.*, 2012; Zhu *et al.*, 2014). The confirmed role of the Fdb1 enzyme may assist identification of Fdb1 orthologs in other fungi, and potentially in soil-borne bacteria producing the phenoxazinones proposed as the phytotoxic compound in allelopathy associated with benzoxazolinone producing plants (Macias *et al.*, 2014). Additionally, the FDB1 protein sequence did not identify a signal sequence, supporting the observation of Glenn *et al.*, (Glenn *et al.*, 2001) where they found no evidence for an extracellular localisation of benzoxazolinone degradation.

Benzoxazolinone metabolism beyond malonamic acid accumulation has been reported for *F. verticillioides*, yet the genetic basis of this phenomenon remains to be fully understood (Glenn *et al.*, 2002). Additionally, 2-AP did not accumulate in BOA amended-media inoculated with *F. graminearum* CS3005 mutants lacking *FDB2* (Kettle *et al.*, 2015) suggesting that alternative 2-AP processing may be present in this isolate. Alternative aminophenol detoxification may avoid the toxic accumulation of 2-AP or its auto-oxidation product 2-APO (a phenoxazinone) and may additionally be beneficial for degradation of exogenous soil aminophenols and phenoxazinones. We propose that the genes surrounding *FDB1* and *FDB2* may be involved in subsequent metabolism of aminophenols, phenoxazinones and/or malonamic acids. Therefore, the genes flanking *FDB1* are our next target of investigation.

In conclusion, this work clearly identifies Fdb1, as the enzyme functioning in the first step of benzoxazolinone transformation and the production of the toxic aminophenols. Our understanding of the *FDB* gene cluster has improved, though the differential sensitivity and full metabolism of benzoxazolinones remains unclear. As the Fusarium crown rot disease is established by infection of wheat seedlings during emergence (Knight & Sutherland, 2013), seedling tissues that have heightened phytoalexin production may provide some protection against disease. As has been previously discussed (Etzerodt *et al.*, 2008; Niemeyer & Jerez, 1997), manipulation by either genetic modification or breeding for altered quantity or identity

of benzoxazolinone compounds (Jonczyk *et al.*, 2008) may contribute to enhanced crop protection in the future.

Experimental Procedures

4. 1 Fungal isolates and growth

All fungi were maintained on half strength potato dextrose agar (½ PDA). *F. pseudograminearum* isolate CS3096 and *F. graminearum* isolate CS3005 were selected for transformation (Akinsanmi *et al.*, 2004).

4.2 Vector construction and fungal transformation

The *F. pseudograminearum Fdb1* deletion vector was constructed as follows. *FDB1* promoter and downstream genomic regions were separately amplified using Phusion DNA polymerase (Thermo Fischer Scientific) using primers FpFDB1proF & FpFDB1proR and FpFDB1downF & FpFDB1downR (971 bp upstream and 970 bp downstream products). A neomycin phosphotransferase (*NPT*) cassette was amplified from pAN9.1 including the *Aspergillus nidulans gpdA* promoter (Gardiner *et al.*, 2009). The PCR products were assembled together, guided by homologous regions in the primers, in the order upstream, *NPT* and downstream amplification products into an *Hin*dIII-*Xba*I opened pYES2 vector using yeast strain BY4743 (Thermo Fischer Scientific) with transformation as previously described (Gietz & Schiestl, 2007). *FDB1* complementation vector was assembled from PCR amplified products from the *F. pseudograminearum FDB1* locus using primers FpFDB1proF & FpFDB1compR, a hygromycin B resistance cassette using primers hygF & hygR and an opened pYES2 vector. Primer sequences are given in Supplementary table 1. Fungal transformation was performed as previously described (Gardiner *et al.*, 2012). Mutants identified growing on 50 mg L^{-1} G418 (Sigma-Aldrich) or 50 mg L^{-1} hygromycin (Roche) selective media were reisolated from a single conidium and DNA extracts (Red Extract-N-Amp kit, Sigma-Aldrich) tested with a combination of three primers (FpFDB1A, FpFDB1B & FpFDB1C Supplementary table 1) to determine deletion of *FDB1*.

As whole genome sequencing is relatively inexpensive and the data generated can be used for multiple purposes (i.e. checking both if the target gene has been deleted without introduction of any background mutation), genome sequencing to further analyse the Δ*fdb1* strains was performed as previously described (Kettle *et al.*, 2015). CLCbio Genomics Workbench version 7.0 was used for mapping.

To delete *F. graminearum FDB1* the construct was synthesised as previously described (Kettle *et al.*, 2015). Briefly, 1000 bp of sequence immediately upstream of the *F. graminearum FDB1* start codon was followed by a gatgtccacgaggtctctctgtcaagtattcaaggccgcgtacgctgcaggtcgac barcode sequence, a nourseothricin resistance cassette and 993 bp of sequence immediately downstream of the *FDB1* stop codon. Transformants were selected on 50 mg L^{-1} nourseothricin sulfate (Werner Bioagents). A three primer (FgFDB1A, FgFDB1B & FgFDB1C; Supplementary table 1) PCR assay using Red Extract-N-Amp kit (Sigma-Aldrich) was used to identify transformants lacking *FGSG_00079* (the *F. graminearum* CS3005 strain annotation locus tag is *FG05_00079* (Gardiner *et al.*, 2014)).

4.3 Chemical sensitivity assays

100 g L^{-1} stock solutions of gramine, BOA, MBOA and 2-AP (Sigma Aldrich) were made in DMSO. Gramine, BOA, MBOA and 2-AP sensitivity was assessed using growth on solid media amended with the test compounds compared to solvent (DMSO) amended media in a method adapted from Martyniuk *et al* (2006) as described (Kettle *et al.*, 2015).

4.4 Thin layer chromatography

Benzoxazolinone and intermediates degradation by Fusarium species and Δ*fdb1* strains were assessed using the agar plug thin-layer chromatography (TLC) method developed by Glenn *et al*. (2001) and as previously described (Kettle *et al.*, 2015).

4.5 RT-qPCR

The BOA-treated *F. pseudograminearum* (CS3096) time-course assay used was as previously described (Kettle *et al.*, 2015). Four biological replicates were used for each treatment. RT-qPCR was performed as previously published (Gardiner *et al.*, 2009). Primer sequences are as previously described (Kettle *et al.*, 2015) and additionally in Supplementary table 1.

4.6 Purification of Fdb1

The Fdb1 expression construct was made with a PCR amplified product from *F. pseudograminearum* CS3096 gDNA using primers FpFbd1enzF & FpFdb1enzR ligated to an *Nhe*I-*Sac*I opened pET-28a(+) vector. Primer sequences are given in Supplementary table 1. Recombinant *E. coli* BL21(DE3)pLysS containing the *FDB1* construct was grown in Luria-Bertani media containing 34 μg mL^{-1} kanamycin at 37°C. The culture was induced with 0.2 mM IPTG when the optical density reached 0.6-0.8 at 600 nm. Cultures were grown overnight at 15°C while being shaken at 200 rpm. Cells were harvested by centrifugation at 10410×*g* for eight minutes and frozen at -20°C.

Pellets were thawed, resuspended in 20 mM HEPES, pH 8.5 and lysed by two passes through a cell disrupter (Constant Systems) at a pressure of 26 kPSI. Cellular debris was removed by centrifugation at 26895×*g* and filtered through a 0.45 μm filter. The clarified lysate was incubated in 50 mM sodium phosphate with 300 mM NaCl and 20 mM imidazole (pH 8.0) for one hour with NiNTA agarose (MCLAB) at 4°C. The resin was then poured into a column and washed with the above buffer. The protein was eluted by increasing the imidazole concentration to 250 mM. The buffer was changed to 50 mM sodium phosphate (pH 7.4) by dilutions in a stirred cell containing a YM10 filter (Amicon). The poly-histidine tag was removed by incubating the 1 mL aliquots of the protein with thrombin for 90 minutes at 25°C with 11% glycerol and 1 unit of thrombin. Thrombin was removed by incubation with *p*-aminobenzamidine-agarose and the cleaved histidine tag and any residual un-cleaved histidine-tagged protein was removed with a further incubation with NiNTA agarose. The buffer was then changed to 20 mM HEPES (pH 8.5) and the enzyme was aliquoted and stored at -80°C.

4.7 Assessment of purity, concentration and molecular mass

Protein concentration was assessed by the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) using Bovine Serum Albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using BOLT 4-12% Bis-Tris Plus gels and stained with SimplyBlue™ SafeStain (Life Technologies) to monitor purification and assess the cleavage of the 6xHis tag (Figure 3). SeeBlue® Plus2 was used at a standard with molecular weights between 4–250 kDa.

4.8 Fdb1 activity

The ability of purified Fdb1 to degrade BOA and MBOA *in vitro* was assessed by liquid chromatography. Fdb1 or buffer (control) was incubated with 300 µM BOA or MBOA for 15 minutes in one mL of 50 mM Tris pH 7.0. The reaction was quenched with concentrated HCl and 500 µL was subjected to analysis using an AKTA Pure 25 chromatography system (GE Healthcare) equipped with a Kinetex™ 5 µm EVO C18 column (150 x 4.6 mm, Phenomenex). Samples were eluted at a flow rate of 1.5 mL per minute using 0.1% formic acid (buffer A) and acetonitrile (buffer B) with the following gradient: 20% B for 2 minutes, followed by a linear gradient from 20% B to 70% B over 6 minutes, 3 minutes at 70% B and finishing with a equilibration at 20% B for 5 minutes. BOA was measured at 218 nm and MBOA at 288 nm.

The activity of Fdb1 was also assessed discontinuously by measuring the production of 2-APO. Varying concentrations of BOA were incubated with Fdb1 in 5 mL of 50 mM Tris pH 7.0. One mL aliquots were removed every 5 minutes and the reaction was quenched with 1 mM $CuCl_2$. In addition to stopping the enzymatic reaction the copper chloride acted as a catalyst to oxidize the 2-AP to 2-APO (Puiu, 2007). The reaction was allowed to fully oxidize for 150 minutes (no further oxidation occurred after 120 minutes) and the absorbance measured at 434 nm. The extinction coefficient of 2-APO was taken to be 23200 M^{-1} cm^{-1} (Puiu, 2007). Linear regression was used to determine the velocity of the reaction at each substrate concentration. The amount of 2-AP produced was determined from the stoichiometric ratio of 2:1 for each molecules of 2-APO produced. The data was fitted to the Michaelis-Menten equation by non-linear regression using GraphPad Prism 6.

The sequence of Fdb1 was analysed for a signal sequence using SignalP (Petersen *et al.*, 2011) and subcellular localisation using WolF PSORT (Horton *et al.*, 2007)."

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Figure 1. Fusarium detoxification of benzoxazolinone (FDB) pathway. Fdb1 metabolizes the benzoxazolinones, eg. benzoxazolin-2-one (BOA), to aminophenols, eg. 2-aminophenol (2-AP). Fdb2 metabolizes aminophenols via N- malonylation to non-toxic malonamic acids, eg. N-(2-hydroxyphenyl) malonamic acid (HPMA) (Glenn & Bacon, 2009). Grey arrow indicates spontaneous oxidation of 2-AP to 2-amino-phenoxazin-3-one (2-APO) (Gagliardo & Chilton, 1992). The figure was adapted from that presented in Glenn and Bacon (2009).

Figure 2. Transcriptional response of *FDB1* and another putative lactamase encoding gene (*FPSE_08361*) to benzoxazolin-2-one (BOA). Horizontal axes represent hours post inoculation (hpi) to BOA. Vertical axes represent expression fold change relative to solvent (DMSO) treated controls. Error bars represent the standard error of the mean for three biological replicates for BOA and DMSO controls at each time point. Data for *FDB1* (Kettle *et al.*, 2015) has been included for clarity.

Figure 3. Fdb1 purification and activity. (A) SDS-PAGE of *F. pseudograminearum* Fdb1 purified from *E. coli* BL21(DE3)pLysS. From left, molecular weight marker, Ni-affinity purified His-tagged Fdb1 and Fdb1 with the His tag removed by proteolytic cleavage. (B) Plot of a specific activity of Fdb1 utilizing BOA as a substrate.

Figure 4. *FDB1* deletion from *Fusarium pseudograminearum* CS3096. (A) Triplex PCR screen to detect successful gene deletion. The PCR assay used three primers (A, B and C) to initially screen transformants for successful homologous recombination. The assay was designed such that an intact *FDB1* locus yielded a smaller product than transformants containing the *FDB1* deletion cassette. CS3096 genomic DNA, and the plasmid used for targeted gene disruption were used as controls. Five transformants are shown; four knockouts and an ectopic insertion. (B) Sequence reads from the Δ*fdb1#1* and Δ*fdb1#3* mutants mapped to the sequence of the CS3096 predicted deletion locus, parental isolate's *FDB1* genomic region and plasmid used in transformation. Read coverage graphs are shown below each locus diagram.

Figure 5. *FDB1* is required for tolerance to benzoxazolinones in *F. pseudograminearum*. (A) Exemplar photographs of the parental *Fusarium pseudograminearum* strain CS3096 (WT) , Δ*fdb1*#1 and Δ*fdb1*#3 strains growing on half strength potato dextrose agar (½ PDA) amended with 0.5 mg mL^{-1} of benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) at 8 days post inoculation. DMSO was used as a control. Rings indicate daily growth. (B) Growth quantification of Δ*fdb1#1* and WT on ½ PDA amended with compounds as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. *t*-tests were performed comparing the parental strain and both Δ*fdb1* mutants on data collected at seven days post inoculation (dpi). Statistically significant differences were observed for both mutants compared to the parental strain at seven days growth on BOA and MBOA, but not DMSO. Error bars represent the standard error of the mean from three biological replicates. (C) Comparison of benzoxazolinone degradation via thin layer chromatography (TLC) by parental *F. pseudograminearum* CS3096 (WT) and Δ*fdb1#1* strain on amended ½ PDA. (D) Exemplar photographs of WT, Δ*fdb1* strain, and complemented Δ*fdb1::FDB1*#4 strain. Isolates growing on ½ PDA amended with 0.5 mg mL^{-1} of BOA at six days post inoculation. DMSO was used as a control. (E) Quantification of growth of WT, *Δfdb1#1* and Δ*fdb1::FDB1*#4 on ½ PDA amended media as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. Following five days of growth *t*-tests were performed comparing the parental strain and Δ*fdb1::FDB1*#4 complemented mutant. No statistically significant difference was observed for

the complemented mutant compared to the parental strain when growing on BOA (*p*-value 0.77). Error bars represent the standard error of the mean for three biological replicates. (F) Comparison of BOA degradation via TLC by isolates on amended ½ PDA at seven dpi. The BOA and MBOA metabolites are proposed to be N-(2-hydroxyphenyl) malonamic acid (HPMA) and N-(2-hydroxy-4-methoxyphenyl) malonamic acid (HMPMA), respectively (Glenn & Bacon, 2009).

Figure 6. *Fusarium graminearum FDB1* is required for tolerance to benzoxazolin-2-one (BOA) but not 2-aminophenol (2-AP). (A) Exemplar photographs of the parental *Fusarium graminearum* strain CS3005 (WT) and a Δ*fdb1* strain growing on half strength potato dextrose agar (½ PDA) amended with 0.5 mg mL^{-1} of BOA, 6-methoxy-benzoxazolin-2-one (MBOA) or 2-AP at 8 days post inoculation. Rings indicate daily growth. DMSO was used as a control. Rings indicate daily growth. (B) Quantification of growth of Δ*fdb1* and WT on ½ PDA amended media over four days. Vertical axes represent the percentage of growth relative to the DMSO control. Error bars represent the standard error of the mean from three biological replicates. (C) Comparison of benzoxazolinone degradation via thin layer chromatography (TLC) by parental *F. graminearum* CS3005 (WT) and a Δ*fdb1* strain on amended ½ PDA.

Figure 7. Fdb1 is not required for tolerance to 2-aminophenol (2-AP). (A) Exemplar photographs of the parental *Fusarium pseudograminearum* strain CS3096 (WT) and Δ*fdb1*#1 strain growing on half strength potato dextrose agar (½ PDA) amended with 0.2, 0.3 or 0.4 mg mL^{-1} of 2-AP at 6 days post inoculation. DMSO was used as a control. Rings indicate daily growth. (B) Quantification of growth of Δ*fdb1* and WT on ½ PDA amended media as indicated. Vertical axes represent the percentage of growth relative to the DMSO control. *t*-tests were performed comparing the parental strain and Δ*fdb1* mutants on data collected at six days post inoculation. No statistically significant differences were observed for the mutants compared to the parental strain when grown for six days on 0.2 mg mL^{-1} of 2-AP (*p*-value 0.98). Error bars represent the standard error of the mean for three replicates. (C) Comparison of 2-AP degradation via thin layer chromatography (TLC) by parental *F. pseudograminearum* CS3096 (WT) and Δ*fdb1* strains on amended ½ PDA.

Supplementary Figure 1. Degradation of benzoxazolinones by *F. pseudograminearum* Fdb1. (A) HPLC traces of BOA and MBOA degradation. (B) Degradation products of BOA and MBOA are proposed to be 2-AP and 5-methoxy-2-aminophenol (2-AMP), respectively (Glenn *et al.*, 2003). Products of BOA and MBOA degradation are clearly distinguishable on the via thin layer chromatography separation, presumed to be the spontaneously oxidized compounds, 2-amino-3H-phenoxazin-3-one (2-APO) and 2-amino-7-methoxy-3H-phenoxazin-3-one (2-AMPO), respectively. The different detection methods used for the HPLC (absorbance) and TLC (fluorescence) analyses are likely to account for the differing relative intensities of substrate and product observed using these different techniques for the Fdb1 assay.

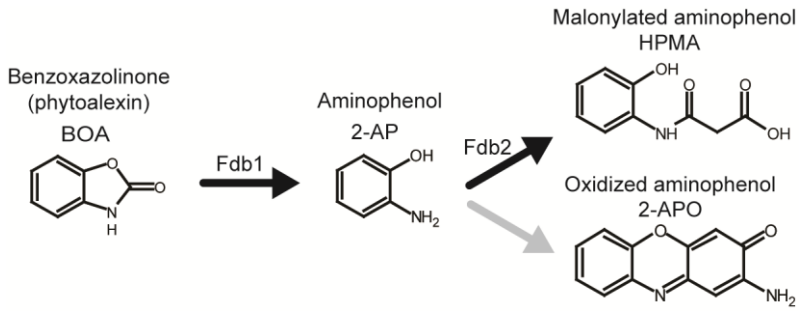
Supplementary Figure 2. Genome sequencing indicates presence of plasmid in Δ*fdb1 #2 & #4* knock-outs. Sequence reads from the Δ*fdb1*#2 & #4 mutant mapped to the predicted deletion locus, parental isolate's genomic region and the transformation plasmid. Representative read coverage graphs are shown below each locus diagram. Light grey shading of recombination fragment indicates homologous recombination area.

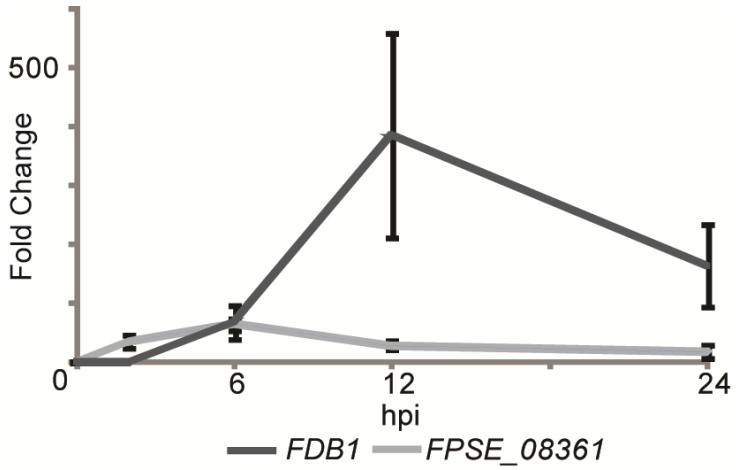
Supplementary Figure 3. All *Fusarium pseudograminearum* Fdb1 mutants are sensitive to both benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA). Exemplar photographs of the parental *F. pseudograminearum* strain CS3096 (WT) and all Δ*fdb1* strains

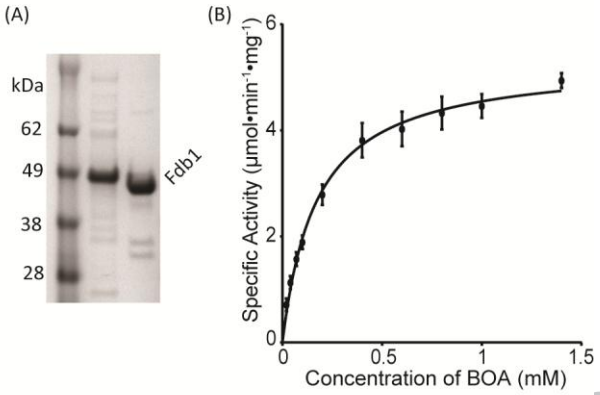
identified by PCR screen. Isolates growing on half strength potato dextrose agar (½ PDA) amended with 0.5 mg mL^{-1} of BOA and MBOA at 8 days post inoculation. DMSO was used as a control.

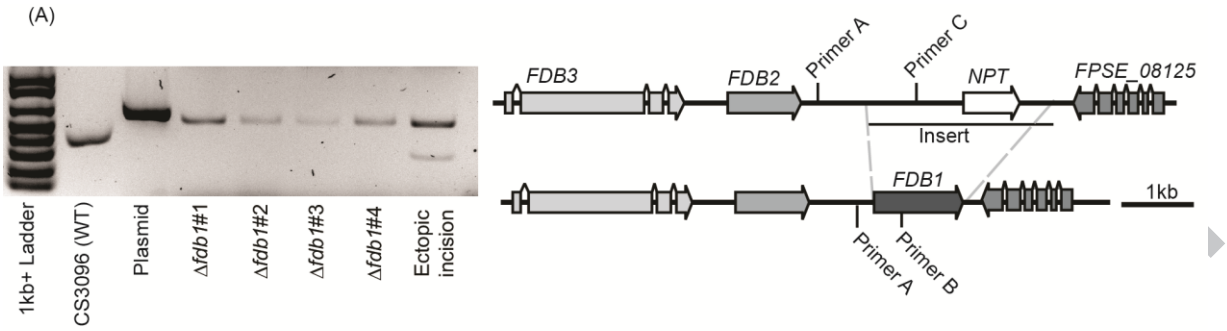
Supplementary Figure 4. *FDB1* deletion from *Fusarium graminearum* CS3005. (A) Triplex PCR screen to detect successful gene deletion. The PCR assay used three primers (FgFDB1A, B and C) to initially screen transformants for successful homologous recombination. The assay was designed such that an intact *FDB1* locus yielded a smaller product than transformants containing *FDB1* deletion cassette. Ten strains showing successful targeting of the gene are shown. CS3005 genomic DNA, no DNA template and the transformation plasmid used for targeted gene disruption were used as PCR controls. *F. graminearum* CS3005 strain annotation *FG05_00079* corresponds to the Ph-1 strain annotation *FGSG_00079* (Gardiner *et al.*, 2014).

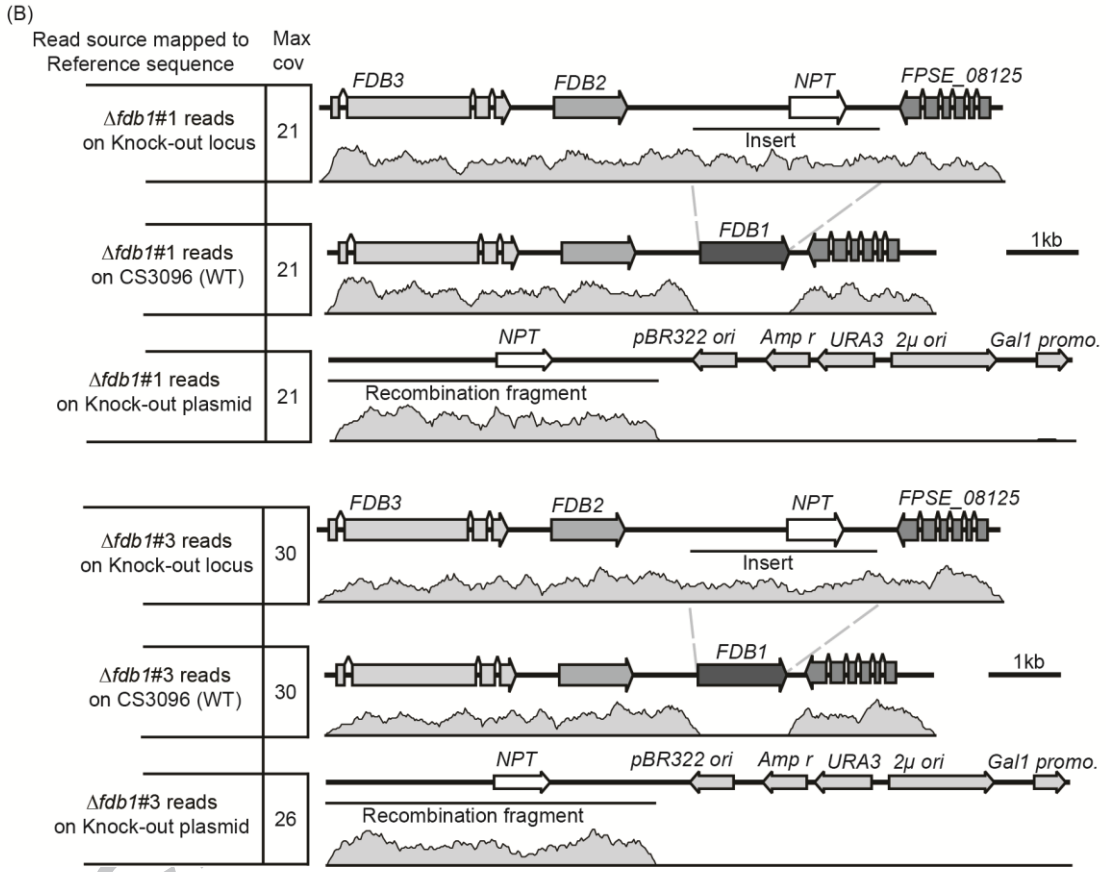
Supplementary Figure 5. *FDB1* is required for tolerance to benzoxazolinones in *F. pseudograminearum.* (A) Exemplar photographs of the parental *Fusarium pseudograminearum* strain CS3096 (WT) and the Δ*fdb1* strain growing on half strength potato dextrose agar (½ PDA) amended with 0.2 and 0.4 mg mL^{-1} BOA and MBOA at six days post inoculation (dpi). DMSO was used as a control. (B) Quantification of growth of Δ*fdb1* and WT on ½ PDA amended media as indicated. 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.

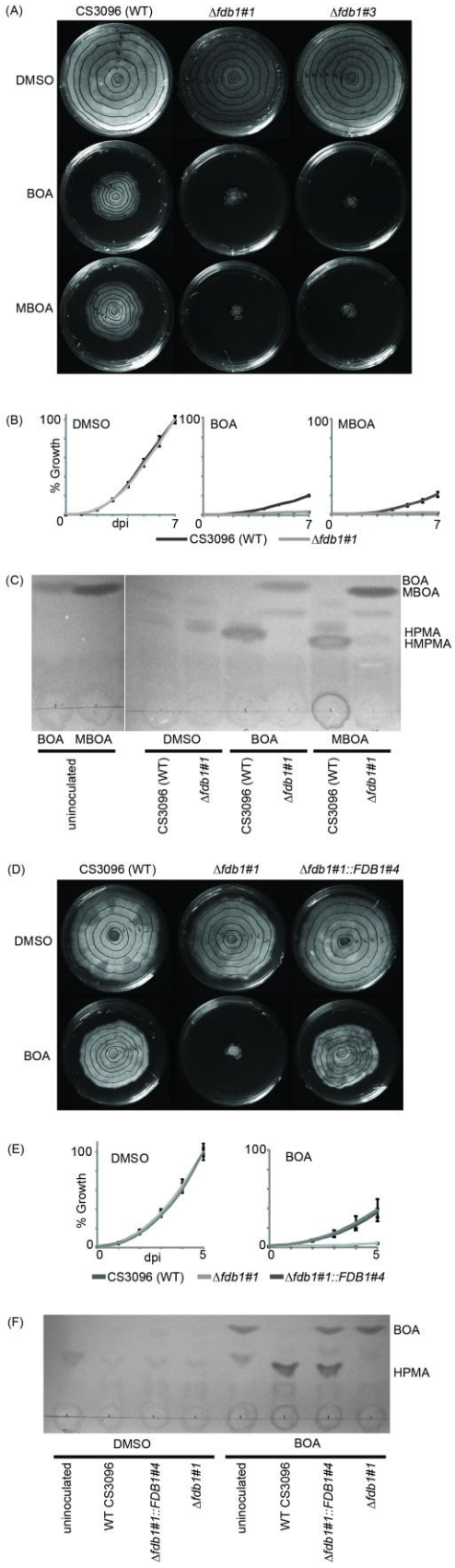


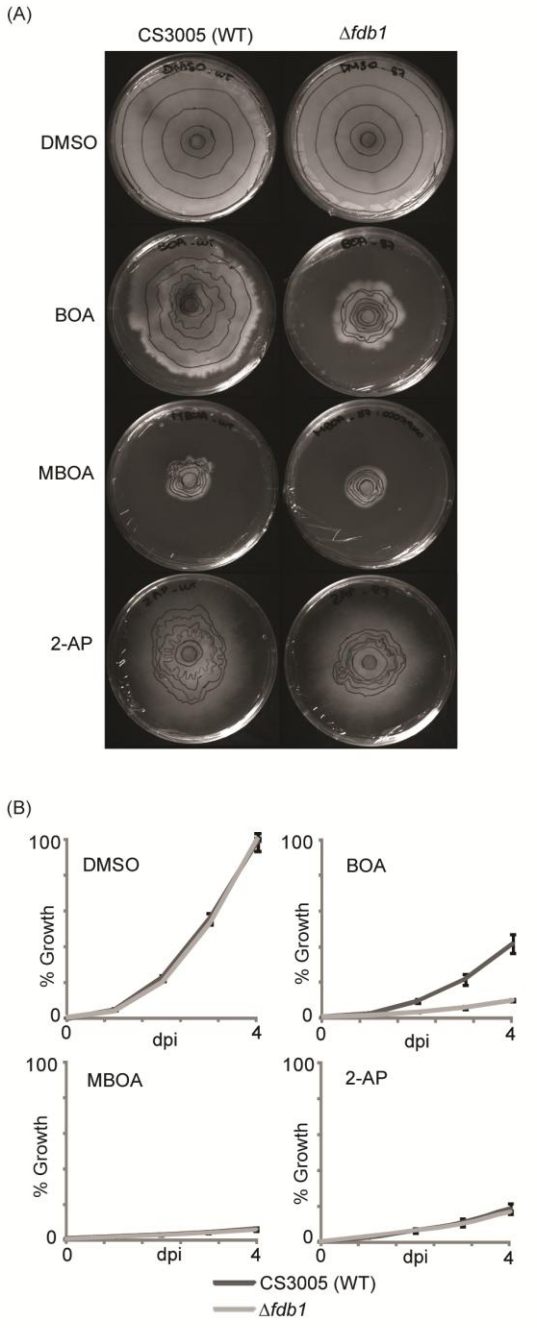


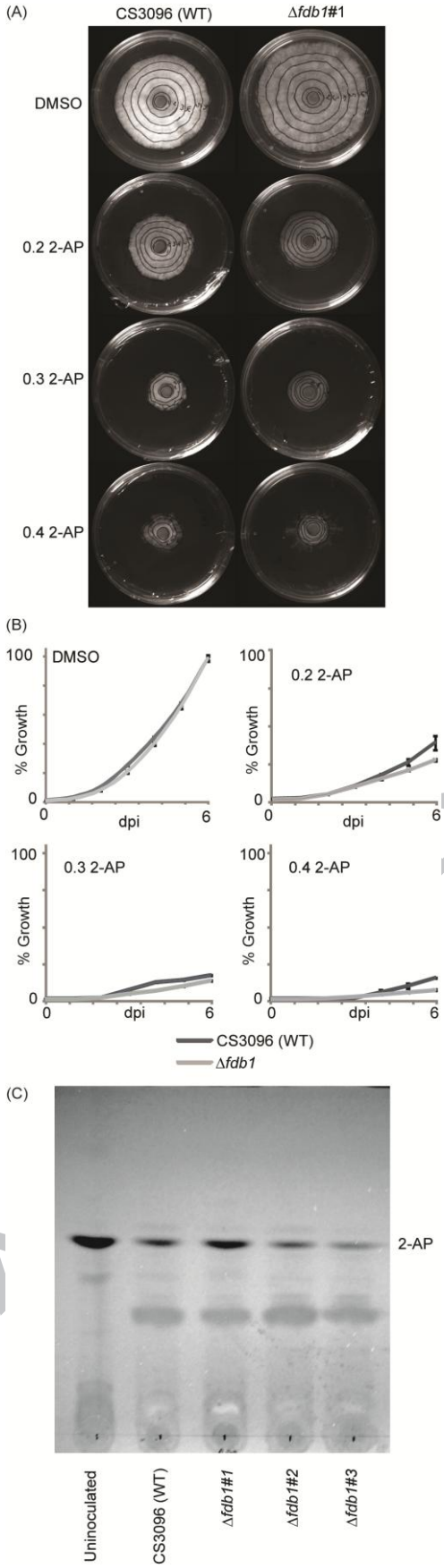












Highlights

- Fdb1, a γ-lactamase, identified and functionally characterised in *Fusarium* spp.
- Fdb1 converts the benzoxazolinone class phytoalexins to aminophenols.
- Deletion of *FDB1* in *F. pseudograminearum* increased sensitivity to benzoxazolinones.