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The Fdb3 transcription factor of the *Fusarium Detoxification of Benzoxazolinone* gene cluster is required for MBOA but not BOA degradation in *Fusarium pseudograminearum*

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ABSTRACT

A number of cereals produce the benzoxazolinone class of phytoalexins. Fusarium species pathogenic towards these hosts can typically degrade these compounds via an aminophenol intermediate, and the ability to do so is encoded by a group of genes found in the *Fusarium Detoxification of Benzoxazolinone* (FDB) cluster. A zinc finger transcription factor encoded by one of the *FDB* cluster genes (FDB3) has been proposed to regulate the expression of other genes in the cluster and hence is potentially involved in benzoxazolinone degradation. Herein we show that Fdb3 is essential for the ability of *Fusarium pseudograminearum* to efficiently detoxify the predominant wheat benzoxazolinone, 6-methoxy-benzoxazolin-2-one (MBOA), but not benzoxazoline-2-one (BOA). Furthermore, additional genes thought to be part of the *FDB* gene cluster, based upon transcriptional response to benzoxazolinones, are regulated by Fdb3. However, deletion mutants for these latter genes remain capable of benzoxazolinone degradation, suggesting that they are not essential for this process.

1. Introduction

In response to pathogen infection, wheat, rye and maize release benzoxazolinones, a class of phytoalexins with anti-fungal and anti-insect activities (Niemeyer, 1988). Investigations into these defence compounds have now spanned over 50 years since the first identification of 6-methoxy-benzoxazolin-2-one (MBOA) (Wahlroos and Virtanen, 1959), the major benzoxazolinone produced by wheat (Villagrasa et al., 2006).

Certain fungi pathogenic on wheat can degrade benzoxazolinones (Friebe et al., 1998), suggesting that these pathogens have evolved to overcome host defences. In *Fusarium* species that infect cereals, tolerance to benzoxazolinones occurs via active detoxification (Glenn et al., 2001). Much of the earlier research on the detoxification of benzoxazolinones by *Fusarium* spp. has been conducted in Fusarium *verticillioides* (Glenn et al., 2002). The detoxification of benzoxazolinones to non-toxic products is facilitated by the Fdb1 and Fdb2 enzymes (Glenn and Bacon, 2009; Kettle et al., 2015b) (Fig. 1). In most *Fusarium* spp. examined to date, the genes that encode Fdb1 and Fdb2 are arranged in the *Fusarium Detoxification of Benzoxazolinone* (*FDB*) gene cluster, which also includes *FDB3,* a $Zn(II)_2Cys_6$ transcription factor encoding gene. In contrast, in *F. verticillioides, FDB1* and *FDB2* are located at two distinct loci (Glenn et al., 2002) that contain individual transcription factors. Despite the largely conserved composition of the *FDB* cluster in most *Fusarium* spp., the genomic neighbourhood around this cluster seems to be extensively rearranged (Gardiner et al., 2012; Kettle et al., 2015a), as exemplified by the comparison between *Fusarium pseudograminearum* and *Fusarium graminearum*, where a local inversion of six genes including *FDB1* and *FDB2,* was identified (Kettle et al., 2015a). Additionally, differential sensitivities to different benzoxazolinones have been observed in these *Fusarium* spp. (Kettle et al., 2015a). For instance, *F. pseudograminearum* can grow on relatively high concentrations of both benzoxazoline-2-one (BOA) and MBOA, whereas *F. graminearum* and *Fusarium culmorum* can only grow on BOA but not on MBOA (Kettle et al., 2015a).

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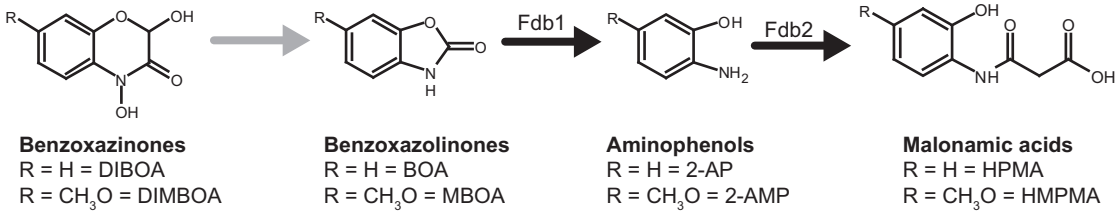

Fig. 1. Fusarium detoxification of benzoxazolinone (FDB) pathway. The benzoxazinone phytoanticipins are non-enzymatically converted to the benzoxazolinone phytoalexins (Niemeyer, 1988). Fdb1 catalyses the conversion of benzoxazolinones to aminophenols and Fdb2 subsequently converts these via N-malonylation to malonamic acids (Glenn and Bacon, 2009). Grey arrow indicates a non-enzymatic reaction. The figure was adapted from Glenn and Bacon (2009). DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; BOA, 2-benzoxazolinone, MBOA, 6-methoxy-2-benzoxazolinone; 2-AP, 2-aminophenol; 2-AMP, 2-amino-5-methoxyphenol; HPMA, N-[2-hydroxyphenyl]malonamic acid; HMPMA, N-[2-hydroxy-4-methoxyphenyl]malonamic acid.

Transcription factors play central roles in co-ordinating the responses of fungi to external stimuli (Yin and Keller, 2011). Zn $(II)_2Cys_6$ transcription factors are unique to fungi and have been associated with specialised regulatory functions such as mycotoxin production in *Leptosphaeria maculans* (Fox et al., 2008) and *F. verticillioides* (Flaherty and Woloshuk, 2004), hyphal tip apical dominance in *F. graminearum* (Zhao et al., 2011) and cellulosic degradation in *Aspergillus* spp. (Tani et al., 2014). A $Zn(II)_2Cys_6$ transcription factor has also been reported to perform non-DNA-binding co-activation, as a regulator of nitrogen metabolism in *Aspergillus nidulans* (Downes et al., 2014).

The *F. verticillioides FDB3* quantitatively contributes to efficient BOA detoxification but was not absolutely required (Glenn and Bacon, 2009). *FDB3* is the only transcription factor encoded in the cluster in *F. verticillioides*. In contrast, in the *F. pseudograminearum FDB* gene cluster, two genes were proposed to encode $Zn(II)_2Cys_6$ transcription factors (Kettle et al., 2015a). Of these, *FPSE_08122* (hereafter called *FDB3*) is present in all *Fusarium* species that also have *FDB1* and *FDB2*. Homologs of *FPSE_08121* are found in *F. verticillioides* and *Fusarium oxysporum* f. sp. *lycopersici* (*FVEG_17758* and *FOXG_17365*, respectively) but not other *Fusarium* spp. Whilst both of these *F. pseudograminearum* genes encode putative $Zn(II)_2Cys_6$ type transcription factors, their products share only 20% amino acid identity to each other.

In this work, Fdb3 and FPSE_08121, the two proposed $Zn(II)_2Cys_6$ transcription factors encoded by the *FDB* gene cluster, were characterised using gene deletion mutants. We have shown that similarly to its ortholog in *F. verticillioides*, Fdb3 quantitatively contributes to tolerance to BOA in *F. pseudograminearum* (Glenn and Bacon, 2009). We also found that Fdb3 is essential for degradation of the methoxylated benzoxazolinone, MBOA. Deletion of *FPSE_08121* did not interrupt detoxification of benzoxazolinones, but reduced growth of the mutants on BOA and MBOA, suggesting a quantitative but not essential role for this gene as a regulator of benzoxazolinone tolerance. In addition, the BOA responsive genes flanking *FDB1*, *FDB2* and *FDB3* (Kettle et al., 2015a), have been characterised for their roles in tolerance to benzoxazolinones.

2. Results

2.1. Fdb3 is not essential for fungal growth in axenic culture

F. verticillioides FDB3 is dispensable for growth (Glenn and Bacon, 2009) and a *F. graminearum FDB3* (*FGSG_00081*) mutant was not associated with loss of pathogenesis in wheat head blight assays (Son et al., 2011). In *F. pseudograminearum* this gene appears to be present in all strains analysed to date (Moolhuijzen et al., 2013). To test if Fdb3 is also dispensable for growth in *F. pseudograminearum,* a mutant strain was created in isolate CS3096 by replacing the entire *FDB3* gene with a geneticin resistance gene cassette via homologous recombination. Transformants were initially screened using a triplex PCR assay (Fig. 2A) and a single mutant was obtained and further characterised. Whole genome sequencing of the mutant strain at ~23-fold coverage and mapping of the reads to the *F. pseudograminearum* CS3096 genome (Gardiner et al., 2012) showed precise deletion of *FDB3* from the genome (Fig. 2B). Mapping reads to the predicted mutant locus showed coverage consistent with both a single insertion of the neomycin phosphotransferase gene cassette at the *FDB3* locus and the homology flanks used to guide homologous recombination being present in single copies. In addition, mapping reads to the plasmid sequence used in transformation showed an absence of the plasmid backbone (Fig. 2B). Together, these read mapping analyses are consistent with a single insertion and no additional copies of the transforming DNA in the genome of the mutant. The mutant displayed comparable growth to the wild type with respect to hyphal growth on half strength potato dextrose agar (½ PDA) containing DMSO as a control (WT vs *Δfdb3* at 5 days: *p*-value 0.15, Fig. 2C).

2.2. Fdb3 controls transcription of genes at the FDB gene cluster in response to MBOA

In a previous study (Kettle et al., 2015a), we tentatively defined the boundaries of the *F. pseudograminearum FDB* gene cluster by transcriptional expression profiling at multiple time points after application of BOA. This analysis indicated that in addition to *FDB1*, *FDB2* and *FDB3*, the transcriptionally defined cluster included six other genes. Of these, *FPSE_08119* encodes a putative carrier protein; *FPSE_08120* encodes a putative acyl-CoA transferase; *FPSE_08125* encodes a putative aldo–keto reductase; *FPSE_08126* encodes a putative esterase; and *FPSE_08127* encodes a putative transmembrane transporter.

Exposure of *F. pseudograminearum* to MBOA caused significant up-regulation of *FPSE_08119*, *FPSE_08120*, *FDB1, FDB2, FDB3* and *FPSE_08125* with greater fold-changes than those observed with BOA treatment at six hours post-induction (Fig. 3) (p-value $\leqslant 2.1 \times 10^{-2}$). *FPSE_08118* (which was not originally considered part of the cluster (Kettle et al., 2015a)), *FPSE_08121*, *FPSE_08126*, and *FPSE_08127* were also upregulated by MBOA but their magnitude of the response was not statistically different from that observed in response to BOA. The cluster was also responsive to 2-AP treatment (Supp. Fig. 1).

To determine if Fdb3 acts as a positive regulator of the putative *FDB* cluster, the expression of the nine cluster genes plus *FPSE_08118* was compared between the progenitor and *Δfdb3* strains under BOA and MBOA treatment. This analysis revealed that the magnitude of the induction observed for nine of the ten genes was significantly reduced in response to MBOA treatment in the *Δfdb3* mutant compared to the progenitor strain (p-value $\leqslant 2 \times 10^{-2}$) (Fig. 3). The inducibility for the remaining gene (*FPSE_08126*) was close to being significant ($p = 0.053$). The strong