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MAP KINASE MGV1: A POTENTIAL SHARED CONTROL POINT OF BUTENOLIDE AND DEOXYNIVALENOL BIOSYNTHESIS IN *FUSARIUM GRAMINEARUM*

ABSTRACT

The mitogen-activated protein kinase (MAPK) MGV1 has been knocked out in *Fusarium graminearum* to produce the mutant *FgΔMGV1*. The mutant displays complementary phenotypes concerning deoxynivalenol (DON) and butenolide (BT) biosynthesis *in vitro*. In the rich medium 15-ADON accumulates are at low levels as detected by HPLC, whereas the accumulation of BT is substantial in *FgΔMGV1*. This is supported by the high expression of butenolide cluster genes in the mutant compared to the wild-type strain. Under nutrient-limiting conditions, where DON biosynthesis is normally favoured, the expression of genes of the trichothecene cluster does not differ between the two strains. However, the accumulation of 15-ADON is vastly different in *FgΔMGV1*. There is a reduction of 15-ADON accumulation with a concomitant accumulation of a novel compound. Although gene clusters comprising the synthesis of DON and BT have been identified, their regulation at the molecular level has not been fully elucidated. Since the expression levels of two regulatory genes from the trichothecene gene cluster and three regulatory genes from the butenolide gene cluster remained unchanged between WT and *FgΔMGV1*, we suggest that differential accumulation of both BT and DON biosynthesis is at least partially under post-transcriptional and/or post-translational control.

Key words: Gibberella zeae, scab

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INTRODUCTION

Fusarium graminearum is perhaps the most serious pathogen of wheat world wide, causing annual losses to Fusarium Head Blight in all temperate climates. Infected grain is severely damaged and is contaminated with mycotoxins (Placinta *et al*. 1999, Trail 2009). One of the most important of these from a regulatory point of view is deoxynivalenol (DON) and its modified derivatives. The biochemistry (Desjardins *et al*. 1993) and genetics (Brown *et al*. 2004) of DON biosynthesis have been studied extensively. Seven of the DON biosynthesis genes and their regulators (designated *Tri*) are clustered together and appear to be under the control of *Tri6*, which encodes a Cys_2His_2-type transcription factor (Proctor *et al*. 1995). Three other DON biosynthetic enzymes are encoded elsewhere in the genome. DON is phytotoxic and several lines of evidence suggest that it has an important role in virulence: elimination of DON through deletion mutagenesis has produced strains with reduced virulence. This was first demonstrated by Proctor *et al*. (1995b) who produced *tri5*$^{-}$ strains of *F. graminearum*, which lack trichodiene synthase and produce no DON. More recently Dyer *et al*. (2005) made *Tri14*$^{-}$ strains which accumulate DON *in vitro*, but not *in vivo*, however, the function of *Tri14* is not known. Significantly, both mutations significantly reduced disease severity in wheat. Thus, because of the likely role of DON in *F. graminearum* virulence, a complete understanding of DON synthesis and its regulation is necessary in the pursuit of FHB resistant wheat. Butenolide (BT) is a less well studied secondary metabolite of *F. graminearum*. This secondary metabolite has been associated with toxicity in cattle (Yates *et al*. 1969), and recent work suggests that its biosynthesis is also controlled from a gene cluster (Harris *et al*. 2007), however because of its lower toxicity, there are fewer problems associated with BT, and as a consequence its potential role in pathogenicity remains unknown.

Both of these mycotoxins can be produced *in vitro*. BT accumulates as nitrogen becomes limiting, but before the onset of DON accumulation (Miller and Blackwell 1986). The biochemical mechanisms controlling their biosynthesis remain largely unknown. Here we demonstrate that removal of the MAP kinase MGV1 through gene knock-out increases the quantity of BT synthesized, while decreasing the level of DON synthesis, suggesting that their production is regulated through a common biochemical pathway.

MATERIALS AND METHODS

Strains

F. graminearum Schwabe (teleomorph *Gibberella zeae* (Schw.)) Petch wild-type strain DAOM 233423 was grown in a two-stage medium as described previously (Miller and Blackwell 1986, Harris *et al*. 2007). Production of the deletion mutant ΔMGV1 was performed as described in Fig. 1.

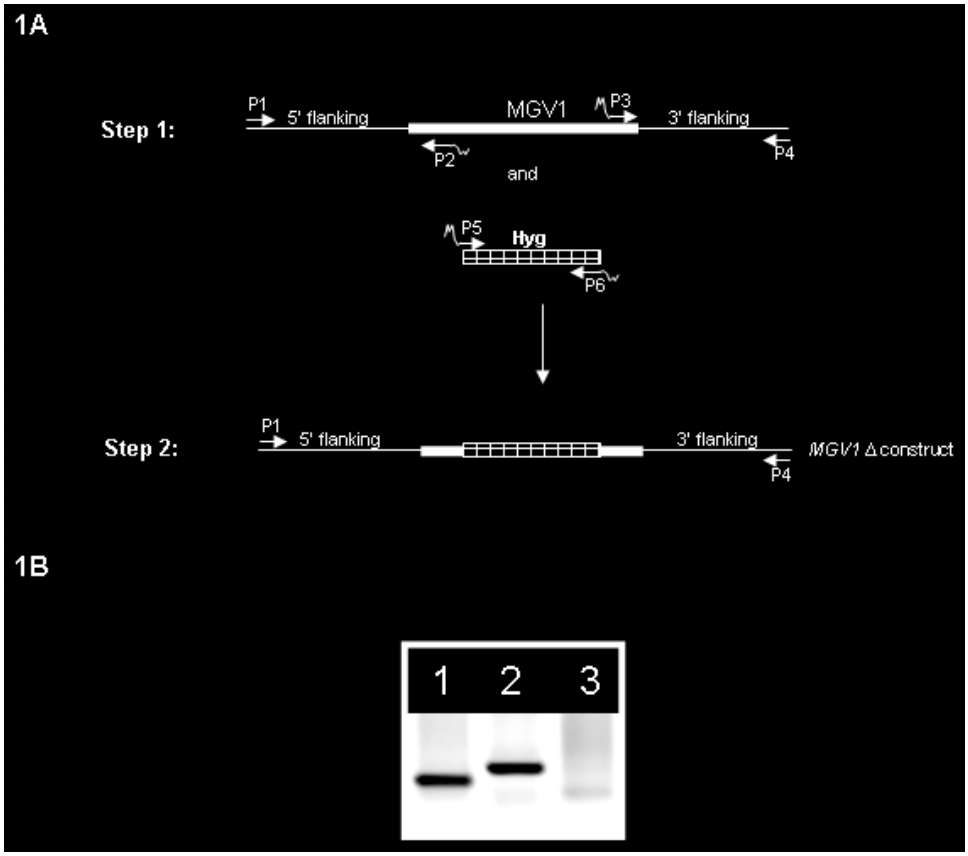

Fig. 1. Generation and characterization of an *MGV1* (*Fg10313*) deletion mutant (*FgΔMGV1)* in *F. graminearum* strain DAOM 233423.

(A). Homologous recombination was used to replace the trichothecene biosynthetic gene *MGV1* with a gene conferring resistance to hygromycin. Annealing sites of relevant PCR primers are indicated with arrows. Step one: PCR amplification of the 5' and 3' flanking regions of *MGV1* from the *F.graminearum* genomic DNA using the primer set P1 and P2, and P3 and P4 respectively. The primers P2 and P3 have an overhang sequence complementary to the Hygromycin gene. Primers P5 and P6 were used to amplify the hygromycin marker gene. The P5 primer has overhang sequence complementary to the 3' end of the 5' flanking region and P6 primer has overhang sequence complementary to the 5' end of the 3' flanking region. Step 2: Equimolar concentration of three PCR products from step 1 were combined and the primer pair P1 and P4 was used to make the final PCR product that was used to disrupt the *MGV1* gene. All PCR products were purified using a PCR purification kit (Qiagen, USA). PCR primer sequences are shown in Table 1.
(B). PCR-based confirmation of *MGV1* gene deletion in *F. graminearum*. Wild-type *F. graminearum* (Lane 1) the *FgΔMGV1* mutant (Lanes 2 and 3), were analyzed with primers designed to amplify the *MGV1* open reading frame (Primer set 1), the hygromycin resistance marker gene (primer set 2).

Trichothecene and Butenolide analysis by HPLC

To induce trichothecene and butenolide production in liquid culture, a two stage media protocol, modified from Miller and Blackwell (1986), was used. 2×10^4 spores were inoculated into 4 ml of first stage growth me-

dia and incubated in a multiwell culture tray (Falcon 6-well cat.#35304, ThermoFisher Scientific, USA). The culture trays were affixed to an orbital shaker, and incubated for 24 h in the dark at 170 rpm. Following 24 h growth, the mycelial solids were washed and resuspended in 4 ml of second stage media (pH 4.0)(Miller and Blackwell, 1986) and then returned to the 6-well culture trays. The mycelium was grown in second stage media under the same conditions. The supernatant was collected after 24 h for HPLC analysis and the mycelial solids were collected and stored at -80˚C for RNA/DNA analyses. Trichothecenes were separated reversed-phase HPLC (AKTA 10 purifier: GE Healthcare, Canada) with direct injection of 100 µl of the culture filtrate into a C_{18} column (150 × 4.6 mm, 5 µm bead Hypersil ODS: ThermoFisher Scientific, USA), using a methanol gradient from 15% (v/v) to 60% (v/v) over 25 min at a flow rate of 1 ml × min^{-1}. The eluant was monitored at 220 nm. 15-ADON is produced *in vitro* and would normally be converted to DON *in planta*. The retention time of BT is 2 min under these conditions.

Nucleic acid isolation and RT-PCR Analysis

The mycelial pellet isolated from the second stage media was ground to a powder in liquid nitrogen. Total RNA was isolated from *F. graminearum* mycelia using Trizol reagent (Invitrogen, USA). Approximately 0.25 g of ground mycelia was extracted in 1 ml of Trizol and the RNA was isolated according to manufacturer's instructions. An equal amount of ground mycelia was used to extract DNA (DNeasy plant mini kit: Qiagen, USA). cDNA for analysis by RT-PCR was synthesized from 2 µg total RNA using random hexamers with the Thermo script RT-PCR system (Invitrogen, USA). RT-PCR was were performed in a 25 µl reaction under the following conditions: 2 minutes at 94°C, followed by 37 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and 1 minute at 72°C, and final extension of 7 minutes at 72°C. Primers used for RT-PCR are listed in Table 1.

RESULTS AND DISCUSSION

The growth characteristics of the mutant *FgΔMGV1 in vitro* have been reported previously, and roles in some biological processes, such as in female fertility, heterokaryon formation and virulence, have been assigned to MGV1 (Hou *et al*. 2002). We confirmed that the *FgΔMGV1* mutant was not virulent on the susceptible wheat (*Triticum aestivum* L.) cultivar Roblin, noting that there were no symptoms on inoculated heads beyond necrosis limited to the injection site, as reported by Hou *et al*. (2002).

Table 1

Primers used in this study

Name	Sequence		Figure
P1	ATCTACTAGATATCGTCATATAC		1
P2	GATGTCAGGCCATTTTCATGGTGTATTTGTGTGGTGGTGTAG		1
P3	TGCCGACCGAACAAGAGCTGAGAGCTGCTACGCAAAGTAC		1
P4	ACCAGGAAGAGGAGAACATC		1
P5	CATACACCACCACACAAATACACCATGAAAATGGCCTGACATG		1
P6	GTACTTTGCGTAGCAGCTCTCAGCTCTTGTTCGGTCGGCA		1
Set 1 (MGV1)	CACCATGGGCGACCTACAAGGAC	(Forward)	4
	TCTTCGAGAAGCATCCAGGCC	(Reverse)	4
Set 2 (Hyg)	TGAAAATGGCCTGACATCAT	(F)	4
	AGCTCTTGTTCGGTCGGCATCTAC	(R)	4
Set 3 (Tri6)	CATGCCAAGGACTTTGTCCC	(F)	4
	GTGTATCCGCCTATAGTGAT	(R)	4
Set 4 (Tri10)	CCTTATACAGCTGGACGAGG	(F)	4
	CCATGGTGATCCGTCAAGTC	(R)	4
Set 5 (FG08079)	CAGGCCGAATTGGATGAAG	(F)	4
	CGTGTCGTCTCTCATGATC	(R)	4
Set 6 (FG08080)	TTGAGGAATGCCGTCAAG	(F)	4
	CAGATTCCAATAGACCCATT	(R)	4
Set 7 (FG08081)	CCTGTACTTCTTGCACTAC	(F)	4
	GAGTGTCGTCTGCTGCTTC	(R)	4
Set 8 (β-Tubulin)	CTGAGGCCGAGTCCAACAT	(F)	4
	AGGCGGCCGGTGATTTC	(R)	4

Secondary metabolites such as BT and trichothecenes such as 15-ADON accumulate only under nitrogen-limiting conditions in the wild-type *F. graminearum* (Miller and Blackwell 1986). The HPLC chromatograms of wild-type and *FgΔMGV1* media revealed that BT accumulated in the *FgΔMGV1* mutant already in the first-stage (rich) medium (Fig. 2). We also observed the a small accumulation of 15-ADON and an unknown metabolite. This accumulation suggests that the MAP kinase MGV1 is involved in the control of BT biosynthesis, possibly acting in a phosphorylation cascade terminating in the activation of one or more transcription factors that regulate the BT gene cluster. This is supported by the observed increase of BT gene cluster transcripts in *FgΔMGV1* (Fig. 4). The gene products of FG08079 (putative benzoate monooxygenase), FG08080 (putative regulatory protein) and FG08081.1 (putative 2OG-Fe(II) oxygenase family protein) have all been shown previously to be up-regulated in WT *F. graminearum* during nitrogen-limitation, under identical PCR conditions (Harris *et al*. 2007).

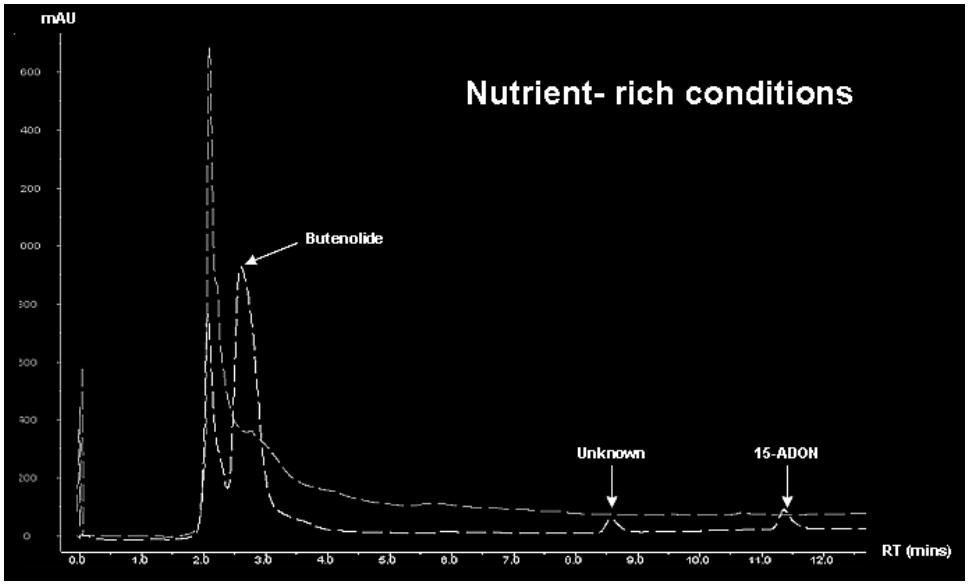

Fig. 2. *FgΔMGV1* produces BT under nutrient rich conditions in culture. Cultures of WT *F. graminearum* and *FgΔMGV1* were analyzed by HPLC after 24 hours of growth in first stage media. The peak corresponding to BT is indicated

In the second stage medium (where nitrogen is limiting), expression of both BT and the trichothecene gene cluster are induced in both Wild-type and *FgΔMGV1* strains (Fig. 4). Interestingly, BT accumulation is seen only in *FgΔMGV1* (Fig. 3). This is in contrast to published observations (Harris 2007), however, the growth conditions described here could account for the difference. Although the expression of the two regulatory genes of the trichothecene gene cluster (*Tri6* and *Tri10*) is the same between WT and *FgΔMGV1*, the accumulation of trichothecenes is markedly different between the two strains. Specifically, *FgΔMGV1* has reduced 15-ADON biosynthesis and there is an accumulation of another, yet uncharacterized metabolite in this mutant (Fig. 3).

Since the expression of regulatory genes is unchanged between *FgΔMGV1* and WT, we postulate that post-transcriptional and/or translational control is exerted by the MAP kinase MGV1. We have previously shown that staurosporine, a MAP kinase inhibitor, suppresses DON synthesis in wild-type *F. graminearum in vitro*, while okadaic acid, a phosphatase inhibitor, enhances DON synthesis under the same conditions (Rampitsch *et al*. 2010). However, the involvement of post-translational phosphorylation (or any other post-translational modification) directly in the regulation of the biosynthesis of either mycotoxin remains to be demonstrated.

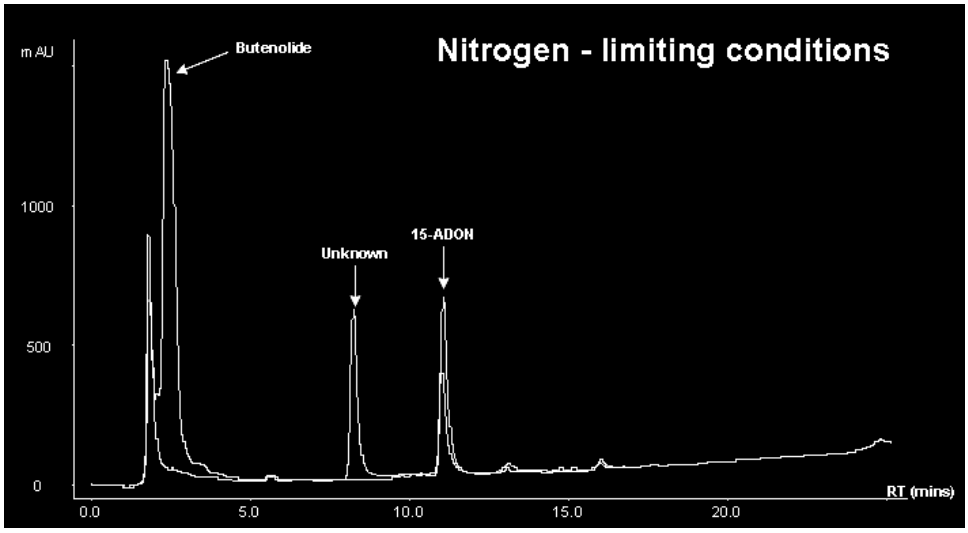

Fig. 3. *FgΔMGV1* accumulates BT and intermediates of trichothecenes under nitrogen limiting conditions in culture. Cultures of WT *F. graminearum* and *FgΔMGV1* were analyzed by HPLC after 24 hours of growth in the second stage media. The peak corresponding to BT and 15-DON and another intermediate is indicated

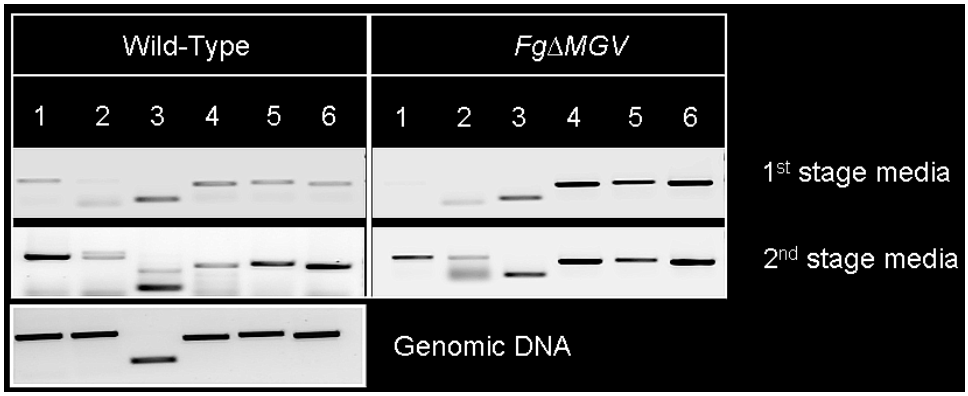

Fig. 4. RT-PCR analysis of trichothecene and butenolide pathway genes. cDNA from both WT and *FgΔMGV1* cultures growing in the first stage media and the second stage media were used for relative gene expression of trichothecene genes *Tri6* (lane 1), *Tri10* (lane 2) and butenolide genes Fg 08079 (lane 4), Fg 08080 (lane 5) and Fg 08081 (lane 6). β-Tubulin transcript (lane 3) was used as the internal control. Genomic DNA from WT *F. graminearum* was used to verify the integrity of the primers used in the RT-PCR analysis. For promer sequences see Table 1

CONCLUSION

Eliminating the MAP kinase gene *MGV1* in *F. graminearum* through gene knock-out results in a strain which is non virulent on susceptible wheat. We have demonstrated that this enzyme influences the regulation of both DON and BT biosynthesis *in vitro*, and we have confirmed these observations by comparing the expression profiles of the mutant *FgΔMGV1* with wild-type *F. graminearum*. Transcript abundance of genes from the BT and DON gene clusters corresponded with BT and DON abundance observed by HPLC, although the underlying control mechanisms governing the expression of these mycotoxins remains to be discovered.

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