Low pH regulates the production of deoxynivalenol by *Fusarium graminearum*

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Fusarium graminearum, which causes the globally important head blight disease of wheat, is responsible for the production of the harmful mycotoxin deoxynivalenol (DON) in infected grain. The production of DON by *F. graminearum* occurs at much higher levels during infection than during axenic growth, and it is therefore important to understand how DON production is regulated in the fungus. Recently, we have identified amines as potent inducers of *in vitro* DON production in *F. graminearum*. Although amines strongly induced expression of the key DON biosynthesis gene *TRI5* and DON production to levels equivalent to those observed during infection, the timing of this induction suggested that other factors are also likely to be important for the regulation of DON biosynthesis. Here we demonstrate that low extracellular pH both promotes and is required for DON production in *F. graminearum*. A combination of low pH and amines results in significantly enhanced expression of the *TRI5* gene and increased DON production during axenic growth. A better understanding of DON production in *F. graminearum* would have implications in developing future toxin management strategies.

INTRODUCTION

Fusarium graminearum (*Gibberella zeae* Schwabe) and closely related *Fusarium* spp. cause fusarium head blight (FHB) in wheat (*Triticum aestivum* L.) and other small-grain cereals, and ear rot in maize (Goswami & Kistler, 2004). FHB is one of the most economically important diseases of wheat worldwide and causes yield losses, grain quality reduction and the contamination of grain with trichothecene mycotoxins (Goswami & Kistler, 2004). Trichothecenes such as deoxynivalenol (DON) are potent, non-specific inhibitors of eukaryotic protein synthesis and are produced by the fungus during infection as part of its virulence arsenal. Isolates of *F. graminearum* unable to produce trichothecenes show reduced aggressiveness towards wheat (Bai *et al.*, 2002; Jansen *et al.*, 2005; Proctor *et al.*, 1995). Trichothecenes are also harmful to humans and animals, and maximum allowable levels of trichothecenes in final food products are highly regulated (Anonymous, 2005). Therefore, understanding the regulation of mycotoxin production in the pathogen by endogenous and exogenous cues is important to enable the design of novel control strategies for *Fusarium*-incited diseases and also for better management of grain storage.

The conditions required for the production of many known fungal secondary metabolites appear very specific. For example, the production of fumonisin by *Fusarium*

verticillioides during infection of maize ears is triggered by amylopectin, a component of starch (Bluhm & Woloshuk, 2005). In *Aspergillus nidulans*, endogenous and exogenous oxylipids differentially regulate sterigmatocystin and penicillin production (Tsitsigiannis & Keller, 2006). A number of recent reports have also implicated various compounds, such as H₂O₂, NaCl, azole fungicides, magnesium, carbon source and a large number of phytochemicals, in both positive and negative regulation of trichothecene production *in vitro* (Boutigny *et al.*, 2008; Jiao *et al.*, 2008; Ochiai *et al.*, 2007; Pinson-Gadais *et al.*, 2008; Ponts *et al.*, 2006). However, in these studies the amount of DON produced in culture does not appear to approach the levels observed in infected heads, where concentrations can exceed 500 p.p.m. on a fresh weight basis (Boddu *et al.*, 2006; Mudge *et al.*, 2006). In *Fusarium sporotrichioides* and *F. graminearum*, amines, such as the plant stress metabolite putrescine, have been shown to be involved in the stimulation of trichothecene production *in vitro* to levels similar to those observed in infected plants (Gardiner *et al.*, 2009).

In addition to specific inducing molecules, the production of secondary metabolites is usually determined by generic factors and associated cellular regulatory systems, such as the global nitrogen, carbon and pH regulatory systems, common to most fungi (Yu & Keller, 2005). For example, nitrogen and carbon source and low pH play key roles in regulation of sterigmatocystin/aflatoxin production in *Aspergillus* spp. (Kachholz & Demain, 1983; Keller *et al.*, 1997a; Woloshuk *et al.*, 1997). In *A. nidulans*, low pH is

Abbreviations: DON, deoxynivalenol; RFU, relative fluorescence units.

Supplementary material is available with the online version of this paper.

required for sterigmatocystin production, whereas an alkaline pH is required for penicillin production by the same fungus (Keller *et al.*, 1997a; Shah *et al.*, 1991). Low pH is also conducive to the production of fumonisin by *Fusarium proliferatum* (Keller *et al.*, 1997b) and ochratoxin by *Aspergillus ochraceus* (O'Callaghan *et al.*, 2006). The regulation of secondary metabolism by pH in filamentous fungi is mediated by the highly conserved global pH regulatory system that consists of the transcriptional regulator PacC and a signal cascade (components of which are encoded by *palA*, *palB*, *palC*, *palF*, *palH* and *palI*) to mediate the activity of PacC (reviewed by Peñalva *et al.*, 2008). At acidic extracellular pH, the signalling cascade is off and the PacC protein remains in an inactive state. Upon sensing of an alkaline pH, signalling is activated, resulting in the proteolytic cleavage and activation of PacC, which then upregulates alkaline-responsive genes and represses acid-expressed genes. A functional global pH regulatory system is known to be required for the regulation of sterigmatocystin, penicillin and fumonisin production (Flaherty *et al.*, 2003; Keller *et al.*, 1997a; Shah *et al.*, 1991).

In this study, we used a condition profiling approach to characterize nutritional and/or environmental signals that influence the expression of the trichothecene biosynthesis gene *TRI5* and DON production in *F. graminearum* (Gardiner *et al.*, 2009). Previously, we identified amines as potent inducers of mycotoxin production in this fungus. Although amines induced *TRI5* expression and mycotoxin production *in vitro* to levels equivalent to those observed *in planta* during infection, maximal expression of *TRI5* was delayed by 2–3 days after exposure of the fungus to amines (Gardiner *et al.*, 2009), suggesting that amine-mediated induction of *TRI5* expression might be either due to, or influenced by, secondary effects. This prompted the investigation of other possible cues or signals to which *F. graminearum* may be responding to initiate DON production. Here we describe the role of extracellular pH in regulating DON production in *F. graminearum*.

METHODS

Culture conditions and fungal isolates. Isolate CS3005 of *F. graminearum* and its derivative (05T0017) containing the *GFP* gene regulated by the *TRI5* promoter were used for all experiments as previously described (Gardiner *et al.*, 2009). Fluorescence measured over time from the *TRI5-GFP* reporter strain was demonstrated to be an excellent predictor of DON production in *F. graminearum*, with a correlation coefficient of 0.82 ($P=4 \times 10^{-8}$) across 30 different media compositions (Gardiner *et al.*, 2009). The use of this method enabled many media combinations and time points to be studied.

All experiments were conducted in 96-well microtitre plates. Media consisted of 88 mM sucrose, 5 mM nitrogen source, 7.3 mM KH_2PO_4 , 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.7 mM KCl, 36 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 47 μM citric acid, 32 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.5 μM H_3BO_3 , 0.4 μM $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.03% Phytagel (Sigma) adjusted to pH 6.5 with NaOH. Phenotype Microarray 10 (PM10) plates with varying pH levels were obtained from Biolog. The proprietary buffer used in these plates is at a concentration of 60 mM but the exact composition of the well contents is undisclosed by

the manufacturer for commercial reasons. Wells in PM10 also contain 0.02% yeast extract (Biolog, personal communication). When the citrate buffer system was used, the various pH values were obtained by mixing different volumes of 1 M stock solutions of sodium citrate and citric acid to give a final concentration of 50 mM in the media.

Two types of experiments, termed 'growth' and 'induction', were used throughout the study. In growth experiments all test conditions or treatments were present in the media at the time of inoculation. For induction experiments, fungal cultures were grown from spores in media containing 5 mM glutamine as the sole nitrogen source as previously described (Gardiner *et al.*, 2009) for 2 days prior to addition of inducing (or control) compounds. For the putrescine induction time-course, cultures were grown in a single 96-well plate. Each replicate consisted of two wells pooled together. Culture filtrates were harvested for putrescine, DON and pH measurements by separating the mycelia with a pipette tip. This provided culture filtrate that after subsequent dilution was essentially free of fungal material for ELISA. The volume of medium recovered was less than the original volume of the medium ($2 \times 100 \mu\text{l}$) due to evaporation and fungal growth. This resulted in the detection of higher levels of putrescine than expected immediately after application of putrescine.

pH measurements. Culture filtrate pH was measured using a pH Boy-P2 pH meter according to the manufacturer's instructions (Shindengen Electric Manufacturing Co.). Filtrates were recovered from the sensor for polyamine and DON assays. pH measurements were made from culture filtrates of the parental strain in all cases.

Putrescine quantification. Putrescine quantification was carried out based on the method described by Marcé *et al.* (1995) with minor modifications. A known volume of culture filtrate (between 30 and 70 μl) was made up to 200 μl with a 5% perchloric acid solution; 40 μl of 50 μM diaminoheptane was added followed by 130 μl of a saturated sodium carbonate solution. Amines were derivatized with 260 μl of a 5 mg ml^{-1} dansyl chloride solution in acetone (Sigma), incubated at 70 °C for 10 min before 65 μl of a 100 mg ml^{-1} proline solution was added. To this solution, 325 μl toluene was added; samples were mixed for 30 s and the phases allowed to separate, after which 200 μl of the organic phase was dried under nitrogen and dissolved in 400 μl acetonitrile. Known volumes (and where required dilutions) of this solution was injected onto a Waters HPLC apparatus (600 controller, 717 autosampler and 2475 fluorescent detector) with a Phenomenex Luna 5 μ C18 100A column of 250 mm length and 4.6 mm internal diameter. The gradient used was identical to that described by Marcé *et al.* (1995). Putrescine levels were quantified in culture filtrates of the parental strain only.

DON assays. DON was measured using the Beacon Analytical System competitive ELISA as previously described (Gardiner *et al.*, 2009). DON was measured in culture filtrates of the parental strain only.

Estimation of *TRI5* expression using the *TRI5-GFP* strain. Fluorescence from the *TRI5-GFP* strain (05T0017) was measured using a fluorescent plate reader as previously described (Gardiner *et al.*, 2009). All data were compiled in Microsoft Excel. Results are expressed in relative fluorescence units (RFU), which are given as the fluorescence value for the *TRI5-GFP* strain less the background fluorescence of the parental strain cultured under identical conditions.

RESULTS

An extracellular pH change precedes induction of DON production by amines in *F. graminearum*

Previously, we reported, using both the *TRI5-GFP* reporter-based assay and quantitative real-time PCR

analysis, that exogenous application of amines to pre-grown hyphae induces *TRI5* expression, with maximal GFP levels reached at around 48–72 h post-induction (Gardiner *et al.*, 2009). To determine whether the putrescine was still present in the medium at the time of maximal fluorescence or whether it had been consumed by the fungus at this time, the concentration of putrescine was monitored during an induction experiment. Within 4 h after exposure to putrescine and prior to detection of *TRI5* expression (Fig. 1a), putrescine levels in the medium had more than halved, and by 24 h extracellular putrescine was undetectable (Fig. 1b). The pH of these cultures also showed an acidification (1.5 pH units) in the first 4 h after the addition of putrescine that correlated with the loss of putrescine from the medium (Fig. 1c). The drop in pH was not due to the putrescine addition itself, because immediately following putrescine addition the pH was the same as that of untreated cultures and putrescine

addition to fresh medium also did not change the pH (data not shown). The pH continued to lower to a minimum of 2.3 units at 24 h, which coincided with the first detection of both *TRI5* expression (Fig. 1a) and significant levels of DON in the culture medium (Fig. 1d).

Low extracellular pH induces DON production in the absence of amines

The observation of concomitant loss of putrescine, lowered pH and detection of DON during the induction time-course (Fig. 1b–d) suggested that the pH may be an important environmental factor for inducing DON production in *F. graminearum*. In a growth experiment, the Phenotype Microarray pH plates from Biolog were used to investigate the role of starting media pH on the expression of *TRI5* by measuring the activity of GFP under the control of the *TRI5* promoter. As shown in Fig. 2(a, b), low pH in

Fig. 1. Time-course of putrescine induction of DON production. (a) *TRI5* gene expression measured using the *TRI5*-GFP reporter strain, (b) putrescine levels, (c) extracellular pH and (d) DON concentration. The *F. graminearum* *TRI5*-GFP strain (a) or parental isolate CS3005 (b–d) was pre-grown in medium containing glutamine as the sole nitrogen source for 2 days prior to addition of putrescine. Data are the mean $\pm$ SEM of twelve (a) and four (b–d) biological replicates. pH was measured to one decimal place and almost no variability was observed between biological replicates; therefore error bars are not visible. hp ind, hours post-induction.

Fig. 2. (a) Expression of GFP regulated by the *TRI5* promoter in *F. graminearum* at different starting pH values. (b) Final DON concentration at 7 days post-inoculation (dp inoc) in media with different starting pHs. (c) Final pH values at 7 days post-inoculation in media of different starting pHs. (d) Growth of *F. graminearum* at different starting pH values measured by optical density 2 days post-inoculation. *F. graminearum* was grown in PM10 plates with media containing glutamine as the nitrogen source. Data for (b–d) are from parental isolate CS3005.

otherwise non-inducing media supported high levels of *TRI5* promoter activity and DON production. Furthermore, high levels of *TRI5* expression were observed at 2 days post-inoculation, the first time that fungal mycelia were visible in the well and much earlier than previously described (Gardiner *et al.*, 2009). The DON concentration when the fungus was grown in media buffered at pH 3.5 was almost 4000 p.p.m. (Fig. 2b), and close to double the maximum previously observed in culture filtrates of *F. graminearum* (Gardiner *et al.*, 2009). The final pH of all wells was measured, and in all cases, except the starting pH of 3.5, the final pH was in the range 7–8.5 units (Fig. 2c). Increased fluorescence and DON levels observed at low pH are not due to increases in fungal biomass. In fact, *F. graminearum* appears to grow more poorly under low pH conditions than at high or neutral pH (Fig. 2d).

Low pH regimes also triggered *TRI5* expression when applied to hyphae pre-grown in non-inducing media (see Supplementary Fig. S1, available with the online version of this paper). In this experiment, the initial growth medium was replaced with a medium buffered with citrate at various starting pHs. This experiment showed the pH required for maximal *TRI5* expression was between 2.4 and 3.1.

Amines and low pH synergistically induce the *TRI5* promoter

The potential for interaction between amine- and pH-mediated induction of *TRI5* expression was investigated in two separate experiments that made use of the PM10 plates from Biolog. These plates also contain a large number of treatments (mostly amino acids) buffered at either pH 4.5 or 9.5. Similar to the induction at low pH shown in Fig. 2(a), early expression of GFP was observed in a number of treatments buffered at pH 4.5. Of particular note was the very high level of GFP expression observed in the well containing ornithine at pH 4.5 (Fig. 3). The amino acids alanine, histidine and phenylalanine, not previously considered inducers of mycotoxin production (Gardiner *et al.*, 2009), were also moderate inducers of *TRI5* expression at this pH (Fig. 3). Despite the very high level of *TRI5*-GFP expression in the ornithine treatment, the level of DON at the end of the time-course was 1515 ± 327 p.p.m. This DON level is considerably lower

Fig. 3. *TRI5* gene expression in the presence of various nitrogen-containing compounds at pH 4.5 and 9.5. *F. graminearum* expressing GFP under the control of the *TRI5* promoter was grown in PM10 plates with media containing glutamine as the nitrogen source. The first two rows of the heat map (pH 3.5 and 4) are the same data as shown in Fig. 2(a). dp inoc, days post-inoculation. Data points are the mean of three biological replicates. The fluorescence for the ornithine-containing well (162 ± 8 RFU) at 2 days post-inoculation was assigned a value of 100% and all other values were relative to that value. Only treatments with a fluorescence value at any time point greater than 5% are shown. Full data tables are available as a supplementary file.

than the almost 4000 p.p.m. observed at pH 3.5, despite the fact that the fungus grown in the pH 3.5 treatment showed lower maximal fluorescence than the ornithine treatment. Only one treatment buffered at pH 9.5, cadaverine (another amine), showed some fluorescence, but this response was later than that of the low-pH treatments. Only 260 ± 90 p.p.m. DON accumulated in this cadaverine-containing medium. The final pH of culture filtrate from cadaverine-containing wells was 7.5 ± 0.07 , indicating that some acidification had occurred. As it was not possible to monitor pH throughout the time-course, it is possible that a pH lower than this, which was conducive to *TRI5* expression, was attained at some point during the time-course.

Previously agmatine was identified as the most potent inducer of DON production in *F. graminearum* (Gardiner *et al.*, 2009). Possible interactions between amine- and pH-mediated induction were tested here by supplying either glutamine or agmatine as sole nitrogen sources in PM10 plates. As shown in Fig. 4, agmatine greatly enhanced the expression of *TRI5-GFP* at low pH, suggesting that both low pH and amine signals are acting synergistically to induce *TRI5* expression. The difference in fluorescence observed with different nitrogen sources in this experiment is unlikely to be due to differences in fungal growth, as despite having a higher nitrogen content, agmatine supported similar levels of growth to those obtained with glutamine in this experiment (Supplementary Table S1).

Fungal-mediated acidification of the medium is required for *TRI5* induction

The rapid acidification of the growth medium following application of inducing compounds (Fig. 1c), which does not occur in the absence of the fungus, suggests that the fungus may be actively causing the pH drop. To determine whether this acidification is mediated by the fungus and necessary for induction, *TRI5* expression (using the *TRI5-GFP* strain) was monitored in media buffered at the non-inducing pH of ~ 6.5 with different buffering capacity. The

Fig. 4. Enhancement of expression of *TRI5* by agmatine at low pH. The *TRI5-GFP* strain of *F. graminearum* was grown in PM10 plates with defined media containing either glutamine or agmatine as the sole nitrogen source. Data are from a single replicate. dp inoc, days post-inoculation.

buffering capacity was altered using various concentrations of MES. As shown in Fig. 5(a), at higher buffer concentrations, the expression of *TRI5* was drastically reduced. There was only a minor effect of treatment on fungal growth (and no apparent correlation with MES concentration), which would not have affected the fluorescence measurements to any great extent (Supplementary Fig. S2). The final pH of these cultures was also measured; cultures grown in 0, 5 and 10 mM MES showed a pH below 3.5 while the cultures buffered with higher concentrations of MES showed much smaller reduction in pH (Fig. 5b), and much less fluorescence (Fig. 5a). The marked difference in total fluorescence between 5 and 10 mM MES, but a similar final pH value, is likely to be due to the low pH being attained later in the time-course in the 10 mM treatment than in the 5 mM treatment. This result further suggests that fungal-

mediated acidification plays a role in *TRI5* induction in *F. graminearum*.

DISCUSSION

Our interest in the potential for other factors to regulate trichothecene production was triggered by the relatively slow attainment of maximal *TRI5* expression following application of amine inducers of DON production to mycelia of *F. graminearum* (Gardiner *et al.*, 2009). To our knowledge, ambient pH has not previously been directly linked to regulation of trichothecene production. However, Miller *et al.* (1983) observed a drop in pH to 4.5 at the time of maximal DON production during a fermentation time-course with *F. graminearum*. Here we have demonstrated that extracellular pH is involved, in combination with the presence of amines, in the regulation of the expression of the *TRI5* promoter and DON production in *F. graminearum*. Previously we have shown that *TRI5* promoter activity driving the *GFP* reporter gene corresponds to the activity of the native *TRI5* gene (Gardiner *et al.*, 2009), which controls the key first step in trichothecene synthesis. Indeed the low-pH conditions in the media that led to high DON production also corresponded to those that gave high levels of GFP production from the *TRI5* promoter fusion (Fig. 2). It is expected that even under such acid extracellular conditions as reported here, *F. graminearum* would maintain its intracellular pH constant, as has been observed under extreme acidic conditions, including sudden pH changes, in other fungi (Bagar *et al.*, 2009), and it is probable that the GFP produced would remain in the cytoplasm. Therefore, the GFP-based reporter strain used here appears to provide a reliable indicator of *TRI5* expression. Other genes in the *TRI* gene cluster have been shown to be coordinately regulated with *TRI5*, and coordinate regulation of the *TRI5* cluster by pH is likely.

When fungi are grown on ammonium as the sole nitrogen source, acidification of the medium is observed (Morton & Macmillan, 1954), which may be attributable to the secretion of protons by a H^+ -ATPase (Jernejc & Legiša, 2001). Despite the acidification when ammonium is supplied as the sole nitrogen source, only low levels of DON are produced (Gardiner *et al.*, 2009). A similar acidification may occur when amines are supplied as the sole nitrogen source. The dramatic enhancement of *TRI5* expression in the presence of either ornithine (Fig. 3) or agmatine (Fig. 4) suggests that the pH and amine signals are acting synergistically on DON biosynthesis. However, our results also indicated that amines alone cannot induce DON production without the low-pH conditions (Fig. 5) and therefore it is possible that amines may also at least partially act through triggering an acidification of the extracellular environment.

The drop in pH observed prior to the onset of trichothecene biosynthesis may be required to release potential negative regulation by the global pH regulatory

Fig. 5. Effect of media buffer strength on *TRI5* gene expression (a) and final pH of the media (b). pH data were recorded from the parental CS3005 isolate. *TRI5* expression is a measure of total expression over a 7 day time-course where fluorescence was recorded every 24 h and the area under that expression curve used as an estimate of total *TRI5* expression. Data are the mean $\pm$ SEM of three biological replicates.

system. At alkaline pH, the transcription factor PacC acts as both an activator of alkali-expressed genes and a repressor of acid-expressed genes through the same consensus promoter recognition motif (Espeso & Arst, 2000; Tilburn *et al.*, 1995). In *F. verticillioides*, fumonisin production, which occurs at acidic pH, is likely to be negatively regulated by the PacC homologue (Flaherty *et al.*, 2003). In fact, the *F. graminearum* genome encodes all components of the pH signalling pathway, namely PacC, PalA, PalB, PalC, PalF, PalH and PalI (data not shown). Also, like many of the fumonisin biosynthetic genes, promoters of some of the *TRI* genes (including *TRI5* and *TRI6*) contain putative PacC-binding motifs (data not shown), through which the repressor activity of PacC at high pH could act. However, the exact function(s) of these putative sequences is unknown. Also, the short length of the consensus PacC-binding sequence indicates the possibility that these sequences found in *TRI* gene promoters may be due to random occurrences. Finally, in an Affymetrix experiment comparing DON-inducing conditions to non-inducing conditions, no differential expression of the genes encoding components of the pH signalling pathway was observed (D. M. Gardiner, K. Kazan & J. M. Manners, unpublished). Therefore, better understanding of the molecular mechanisms involved in pH regulation of DON biosynthesis in *F. graminearum* requires additional studies.

The rapid acidification of the growth medium suggests an active fungal response to the application of inducer. Whether or not extracellular acidification occurs *in planta* during interactions of *F. graminearum* with the host plant is yet to be determined. However, modification of the pH of host cells/tissues is common during fungal pathogenesis and this is thought to enhance the expression and/or activity of host cell-wall-degrading enzymes. For example, *Penicillium expansum* produces gluconic acid during infection of apples (Hadas *et al.*, 2007). *Sclerotinia sclerotiorum* and *Botrytis cinerea* both secrete oxalic acid, which has both pH-lowering and elicitor activities during pathogenesis (Kim *et al.*, 2008; Williamson *et al.*, 2007). Although an *in planta* requirement of an acidic pH for induction of DON production is unknown, the pH optimum of between 2.4 and 3.1 observed here for maximal DON induction is at the extreme end of the previously reported pH of plant apoplastic fluid (Yu *et al.*, 2000), and considerably different from the pH rise from 6 to 7.3 observed in wheat seedlings during crown rot disease caused by *Fusarium culmorum* (Aleandri *et al.*, 2007). However, accurate measurement of extracellular pH in complex tissues is difficult (Yu *et al.*, 2000), and the pH may not necessarily be homogeneous. For instance, during infection of tomato fruit by *Colletotrichum coccodes*, a pH gradient of 1.9 units was observed along the first 10 mm of the hyphae at the infection front (Alkan *et al.*, 2008). However, in light of the *in vitro* observations made here a thorough investigation of pH during infection is warranted.

In summary, we have shown that pH plays a central role in the regulation of DON production by *F. graminearum*. Presumably, changes in pH are sensed through components of the ambient pH regulatory system such as the products of the *pacC* and *pal* genes as in other fungi (Peñalva *et al.*, 2008). However, genetic dissection of this pathway in *F. graminearum* is yet to be carried out.

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