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Enhanced mycotoxin production of a lipase-deficient *Fusarium graminearum* mutant correlates to toxin-related gene expression

Christian A. Voigt[1], Bianca von Scheidt[1], Attila Gácser[1], Helmut Kassner[2], Reinhard Lieberei[2], Wilhelm Schäfer[1,*] and Siegfried Salomon[1]
[1]*Department of Molecular Phytopathology and Genetics, University of Hamburg, Biocenter Klein Flottbek, Ohnhorststr. 18, D-22609, Hamburg, Germany;* [2]*Department of Ecology and Biology of Useful Plants, University of Hamburg, Biocenter Klein Flottbek, Ohnhorststr. 18, D-22609, Hamburg, Germany* **Author for correspondence (Phone: +49-40-42816-266; Fax: +49-40-42816-513; E-mail: schaefer@botanik.uni-hamburg.de)*

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Abstract

Fusarium graminearum causes important diseases of small-grain cereals and maize and produces several mycotoxins. Among them, deoxynivalenol (DON) and zearalenone (ZEA) can accumulate in feedstuffs and foods to health-threatening levels. Although DON is important for fungal virulence in wheat, disease severity in the field does not correlate with mycotoxin concentrations. We compared gene expression and mycotoxin production of lipase-deficient mutants (Δ*fgl1*), strongly reduced in virulence, and the respective wild-type isolate. Δ*fgl1* mutants exhibited up-regulated DON production during wheat head infection. On isolated wheat kernels, DON was only produced in low quantities, but higher in wild-type than in Δ*fgl1* mutants. In contrast, neither wild-type nor Δ*fgl1* mutants produced ZEA during wheat head infection. However, ZEA was clearly detectable on wheat kernels. Here, Δ*fgl1* mutants revealed a dramatically enhanced ZEA production. We could correlate the altered amounts of DON and ZEA directly with the expression of the toxin-related genes *Tri5* for DON and *PKS4* and *PKS13* for ZEA. Based on *Tri5* expression and the infection pattern of the wild-type and Δ*fgl1* mutants, we suggest that the transition zone of rachilla and rachis is important in the induction of DON synthesis. Gene expression studies indicate an involvement of the lipase FGL1 in regulation of 8 *PKS* genes and ZEA production.

Abbreviations: FHB – Fusarium head blight; DON – deoxynivalenol; ZEA – zearalenone; PKS – polyketide synthase; Dpi – days post-inoculation

Introduction

The fungal pathogen *Fusarium graminearum* (teleomorph *Gibberella zeae*) is the causal agent of Fusarium head blight (FHB) on wheat, barley, and other small-grain cereals and Fusarium cob rot on maize. FHB has gained increasing economic importance as it causes severe losses, especially during epidemic outbreaks (Goswami and Kistler, 2004). Not only yield is seriously reduced (Pomeranz et al., 1995), but also significant levels of mycotoxins hazardous to animals and humans contaminate *Fusarium*-infected grain and corn (Marasas et al., 1984). Several countries have therefore adopted or intend to legislate maximum limits for mycotoxins, among them the trichothecene deoxynivalenol (DON) and the polyketide zearalenone (ZEA) (van Egmond and Jonker,

2004). The European Commission introduced maximum limits for DON and ZEA in unprocessed cereals and cereal products intended for human consumption in July 2006 (Anon, 2005).

Mycotoxins are reported to be important for infection and possibly saprophytic growth under natural environmental conditions (Yoder, 1980; Desjardins et al., 1989; Lutz et al., 2003). A known example is the mycotoxin DON, which has an important function during infection. *Tri5* encodes a trichodiene synthase, the first enzyme in the trichothecene biosynthetic pathway. The inability of *Tri5* disruption mutants to produce DON significantly decreased their virulence in wheat (Proctor et al. 1995; Bai et al., 2001). During plant infection, *F. graminearum* spreads by systemic growth through the rachis from one spike to another (Kang and Buchenauer, 1999; Ribichich et al., 2000; Wanjiru et al., 2002). In contrast to wild-type, DON-deficient mutants could only infect spikelets at the point of inoculation (Bai et al., 2001).

The first indication of an involvement of secreted lipases in plant infection came from Comménil et al. (1998). They showed that supplementation of conidial suspensions with polyclonal antibodies against an extracellular lipase of *Botrytis cinerea* suppressed lesion formation on detached tomato leaves. Recently, we were able to show that the secreted lipase FGL1 of *F. graminearum* is a virulence factor required for infection of cereals and maize (Voigt et al., 2005). In contrast to the wild-type strain, infection with lipase-deficient mutants was restricted to directly inoculated spikelets. This indicates an involvement of the extracellular lipase FGL1 at later stages of invasive growth.

Since the lipase-deficient *F. graminearum* mutants exhibit a phenotype similar to the DON-deficient mutants, the aim of this study was to examine whether an inability to produce DON might cause the reduction in virulence of lipase-deficient mutants. Moreover, any changes in DON production of the lipase-deficient mutants would help to understand DON induction and production in *F. graminearum*. This should be seen against the background of observations showing that FHB reduction is not necessarily associated with a significant reduction in mycotoxin concentration (D'Mello et al., 1998; Magan et al., 2002). Hence, there is a growing concern that sub-lethal levels of particular fungicides may even lead to an increase in mycotoxin production. This might be associated with changes in mycotoxin induction due to reduced growth of the pathogen.

Even though *F. graminearum* does not require ZEA for infection of wheat (Gaffoor et al., 2005) and barley (Kim et al., 2005), this mycotoxin is a threat for stored grain (Wilson and Abramson, 1992). Incorrect storage conditions, e.g. moisture, can lead to increases in the mycotoxin content (Moss, 1984; Homdork et al., 2000). Similar to DON, almost nothing is known about induction mechanisms of ZEA synthesis. Therefore, the second aim of this study was to examine possible differences in ZEA production of the *F. graminearum* wild-type and the lipase-deficient mutants that might indicate induction mechanisms. Since the genetic background of the polyketide-derived metabolite ZEA was recently discovered (Gaffoor et al., 2005; Kim et al., 2005), it is possible to refer putative differences in ZEA production to differences in the expression of the ZEA-related polyketide synthase genes *PKS4* (= *ZEA1*) and *PKS13* (= *ZEA2*). Initial results prompted us to analyse the expression of the whole *PKS* gene family (Kroken et al., 2003; Gaffoor et al., 2005) of the wild-type and the lipase-deficient mutant on colonized wheat kernels.

Materials and methods

Fungal strains and culture conditions

Fusarium graminearum strain 8/1 was obtained from Dr. T. Miedaner (Miedaner et al., 2000). Lipase-deficient strains Δ*fgl1*-1/-2/-3 originated from our disruption of the secreted lipase FGL1 in *F. graminearum* (Voigt et al., 2005). Media, culture conditions, and induction of conidiation were performed according to Voigt et al. (2005).

Wheat infection

The high yielding, FHB-susceptible German spring wheat cultivar Nandu (Lochow-Petkus, Bergen-Wohlde, Germany) was chosen for wheat head infection assays. Seeds from this wheat cultivar were sown in soil at 21 °C, approximately 70% humidity, and a photoperiod of 16 h

(10,000 Lux) per day. Three weeks after germination, young plants (~8 plants per pot) were fertilized with 3×4 g of a NPK-fertilizer containing urea formaldehyde and magnesium [15-8-15-(2)] with trace elements (PlantoSan Compact, Spiess-Urania Chemicals, Hamburg, Germany). Infection was performed when plants reached anthesis (approximately 6–7 weeks after germination; Zadoks growth stages 65-69; Zadoks et al., 1974). A single spike was inoculated within the palea and lemma of two basal florets of two spikelets with a droplet of 10 μl water containing 200 conidia of *F. graminearum* wild-type or lipase-deficient strain Δfgl1-2 per spikelet. As a negative control, spikes were inoculated with pure water. The inoculated spikes were enclosed in small plastic bags during the first 3 days to ensure a high humidity for infection and to prevent cross-contamination of different *F. graminearum* isolates. The inoculated plants were incubated in a growth chamber at 21 °C with a 16 h photoperiod (10,000 Lux). For expression analysis inoculated spikelets were collected 1, 3, 7, and 14 days post-inoculation (dpi) and frozen in liquid nitrogen for subsequent RNA isolation. To detect mycotoxin production, inoculated wheat spikes were harvested 14 dpi (additionally 42 dpi for ZEA analysis), dried for 2 days at 90 °C, and milled in a commercial blender (MX 32, Braun, Frankfurt/Main, Germany) for 30s.

Induction of toxin production on isolated cereal kernels

The production of the mycotoxins DON and ZEA was induced on both wheat and maize kernels. Fifty grams of kernels and 50 ml water were autoclaved in a 500 ml Erlenmeyer flask. Autoclaved kernels were inoculated with 4×10^3 conidia from each of the following strains: *F. graminearum* wild-type and lipase-deficient strains Δfgl1-1,-2,-3. Uninoculated samples served as the control. Incubation was carried out at 28 °C in the dark for 1 week, followed by 2 weeks incubation at room temperature and diffuse day light. For expression analysis kernels were frozen in liquid nitrogen for subsequent RNA isolation. Samples were dried for 2 days at 90 °C and milled for subsequent mycotoxin detection as detailed above.

Expression analysis by quantitative real-time PCR

To study the expression of the *Tri5* gene and the *PKS* genes on isolated kernels and during wheat head infection, RNA was isolated with the Invisorb Spin Plant-RNA Mini Kit (Invitek, Berlin, Germany). For RT-PCR RevertAid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Roth, Germany) was used according to manufacturer's instructions. The resulting single stranded cDNA was then used as template in quantitative PCR real-time (qPCR). The single stranded cDNA was normalized in dependence of the level of constitutively expressed β-tubulin mRNA (Doohan et al., 1999). β-tubulin primers (TubbF and R) were found to be specific for *F. graminearum*. qPCRs were carried out with gene specific primers (Table 1) using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a volume of 25 μl. Quantification of gene expression was performed with gene specific cDNA fragments of known concentration. qPCR were run with the iCycler Thermal Cycler (Bio-Rad), using a programme consisting of an initial denaturation step for 3 min at 94 °C, followed by 60 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, followed by melting curve analysis to check specificity of product amplification.

On the basis of known *PKS* sequences (Kroken et al., 2003) gene specific primers were designed for *PKS1* (= *PKS8*, Gaffoor et al., 2005), *PKS2*, *PKS3*, *PKS4*, *PKS5*, *PKS6*, *PKS7*, *PKS9*, *PKS10*, *PKS11*, *PKS12*, *PKS13, PKS14* (= *PKS16*, Gaffoor et al., 2005), and *PKS15* (Table 1). Trichodiene synthase specific primers (Tri5F and Tri5R) were designed according to GenBank sequence U22464 (Proctor et al., 1995). qPCR were performed using normalized cDNA from *F. graminearum* wild-type and Δfgl1-2 mutant colonized wheat kernels. For *Tri5*, *PKS4* and *PKS13* qPCR were additionally performed for infected wheat heads and maize kernels.

The expression of the lipase *FGL1* during colonization of wheat kernels was performed by conventional PCR with primers comprising the complete *FGL1* ORF (Voigt et al., 2005). The previously normalized cDNA of wild-type and lipase-deficient strain Δ*fgl1–2* served as template (as described before). Similar to this, *PKS4*

Table 1. Primers used in quantitative real-time PCR

Identification	Sequence (5′ → 3′)	Accession no.[a]
TubbF	TGCTGTTCTGGTCGATCTTG	XM 389706
TubbR	GACGGAAGTTTGGACGTTG	
Tri5F	GCCTTCTCGAGTATATTCGATACC	U22464
Tri5R	GACCCAAACCATTCATACGACG	
PKS1F	GGCAGTCAACCAGGATACG	AH013707
PKS1R	AGGCCGCGAATAAGAGTTGAAA	
PKS2F	TCTTCTACTCGGATCTCTTCAG	AY495627
PKS2R	ACAGCATCATCAGTCGTCAAC	
PKS3F	CGCGCTGATTTGGATGATGACA	AY495628
PKS3R	AGCTGCGAGACCCCATTGACC	
PKS4F	GAAGCCTGGCGACCGTGTGAGTA	AY495629
PKS4R	CCACCAAGGCCTCCGACAAAGA	
PKS5F	CTTCTCGACCCGCAACCTCAAT	AY495630
PKS5R	CCAGGCGAGCTAGTTTCTTGATGC	
PKS6F	ATGGCCTGTGCAAGAAATC	AY495631
PKS6R	AACACCCGCCCACCAACCAG	
PKS7F	TGGAGTTCAGCGTTCATCACCTA	AY495632
PKS7R	CGTCTAACACTGCCGCGAATCTCA	
PKS9F	CATCTCGTAGTTATTGGGGGAAGT	AY495634
PKS9R	GCAGGAGACATCGATAGTAGCAGA	
PKS10F	GCGCAAGCAGACAACCAT	AY495635
PKS10R	TCGGAAGCCATACCCTGAG	
PKS11F	AGGCTGCTGGTTTTGAGGTG	AY495636
PKS11R	ATCGTCGCCGGTTAGTGTGG	
PKS12F	GAGATCGTGTTGGTGTGTTC	AY706311
PKS12R	GAAGCTTCCAAGATCTGTGAG	
PKS13F	GTAGTGAGCTGTATGAGACCA	AY495638
PKS13R	ATAGGATCCGAGTAGTTGG	
PKS14F	TTTTCTCTGGTGCTGGTAAGGAC	AH013706
PKS14R	CTCCGAAGAAGTTGGTGTGATGA	
PKS15F	GCCACTGCTTCGTCGTCTT	AY495640
PKS15R	CTCTCCCAGGCGTGTATCAG	

[a]Sequence data for primer design.
(F) Forward primer.
(R) Reverse primer.

expression analysis of wild-type colonized wheat kernels 7 dpi was performed with the primers PKS4F and R (Table 1).

Determination of mycelium amount in mycotoxin samples

To determine the amount of mycelium in inoculated wheat spikes and infected wheat kernels, cereal samples were ground in liquid nitrogen; 70–80 mg of ground samples were used for DNA isolation, which was performed according to the manufacturer's instructions of the NucleoSpin Plant Kit (Macherey & Nagel, Düren, Germany). Pure, ground *F. graminearum* wild-type mycelium was used as standard. Resultant DNA served as template in qPCR using β-tubulin primers (TubbF and R, Table 1). By calculating the number of β-tubulin gene copies per milligram mycelium of the standard and considering a 50% weight loss of dried inoculated kernels, determination of milligram mycelium per gram ground sample was possible. For maize samples, mycotoxin concentration was calculated as μg toxin per gram substrate used for extraction.

Deoxynivalenol (DON) detection

Extraction procedure of DON in cereals was done according to manufacturer's instructions of the

MycoSep 227 Trich + Columns (Romer Labs Diagnostic, Herzogenburg, Austria), only modifying the sample weight and volume of subsequent extraction steps. For DON detection, 1-(heptafluorobutyryl)imidazole (Fluka, Buchs, Switzerland) was used for derivatization according to Cohen and Lapointe (1982). DON standard (Sigma, St. Louis, MO, USA) solutions in the range of 250–4000 ng DON ml^{-1} 2,2,4-trimethylpentane were transferred into a test tube, dried in a nitrogen stream, derivatized like the cereal samples, and used for calibration. DON was analysed on a gas chromatograph (Hewlett-Packard Model 5890, Palo Alto, CA, USA) equipped with electron capture detector, controller and automatic injector 7673A (Hewlett-Packard), and integrator 3396A (Hewlett-Packard). A 30 m × 0.25 mm id fused silica capillary column coated with DB-1, 0.25 μm film thickness was used.

Zearalenone (ZEA) detection

Depending on sample size, 0.1–1 g of flour was extracted with 5 ml methanol/water (70:30 v/v) in an ultra-turrax (T25, Janke & Kunkel, Staufen, Germany) for 2 min. The extract was centrifuged in a labofuge (Heraeus Christ, Osterode, Germany) for 10 min at 5000 rpm. The supernatant was passed through a 0.45 μm filter (25 mm PTFE Multoclear, CS-Chromatographie Service, Langerwehe, Germany) and 20 μl were injected to the HPLC-system. HPLC-equipment consisted of an autosampler (Model 717 Plus, Waters, Milford, MA, USA) connected with a HPLC pump (pump 64, Knauer, Berlin, Germany) and a solution-degaser (Degassex DG-4400, Phenomenex, Torrance, CA, USA). The analytical column was a Polygosil 60-NH2, 250 mm × 4.6 mm, particle size 5 μm (Machery & Nagel), heated in an oven (Waters) to 30 °C. The flow rate of the mobile phase of acetonitrile/water (94:6 v/v) was 0.5 ml min^{-1} isocratic and the effluent was monitored at excitation wavelength λ_{ex} = 275 nm, emission wavelength λ_{em} = 470 nm with a fluorescence detector (F-1050 Fluorescence Spectrophotometer, Merck-Hitachi, Darmstadt, Germany). For calibration ZEA standard solutions (Sigma) in the range of 0.25–4 μg ml^{-1} ZEA were directly transferred into the HPLC-system.

Results

DON determination of colonized cereal kernels and infected wheat spikes

Spikes of intact wheat plants and wheat as well as maize kernels were inoculated with *F. graminearum* wild-type and lipase-deficient strains (Δ*fgl1*). DON concentration of kernels was determined 21 dpi and spikes 14 dpi. Mycotoxin production was normalized to the amount of mycelia present in the different tissues. Whereas mycelium development was comparable on isolated kernels, wild-type mycelia had doubled in size compared to mutant strains during infection of wheat spikes. Wheat kernels inoculated with Δ*fgl1* strains had DON concentrations about 50% that of kernels inoculated with the wild-type. DON was not present in control kernels (Figure 1A). Similar results were obtained for maize kernels showing a lower DON concentration for all tested lipase-deficient strains (Figure 1C).

In contrast to wheat and maize kernels, DON concentration was about 200% in Δ*fgl1* strain-infected wheat spikes than in wild-type infected spikes (Figure 1B). Fungal biomass of infected wheat spikes was determined by quantitative real-time PCR (qPCR). It was about 50% in Δ*fgl1* strain infected spikes compared to wild-type 14 dpi. Generally, DON concentrations were at least 250-fold higher in wheat spikes (ranging from 18.9 to 40.2 mg DON g^{-1} mycelium) as compared to wheat kernels (ranging from 0.028 to 0.069 mg DON g^{-1} mycelium) after fungal colonization.

Tri5 *gene expression after growth on cereal kernels and during infection of wheat spikes*

The concentration of DON depended on two factors: inoculum and substrate. To analyse whether DON concentration correlated with the expression of the *Tri5* gene, *Tri5* transcription was quantified. Normalized cDNA derived from colonized kernels 21 dpi and infected wheat spikes 1, 3, 7, and 14 dpi served as template in qPCR. The Δ*fgl1* strain revealed a *Tri5* expression reduction of almost 50% on wheat kernels as compared to *F. graminearum* wild-type infection (Figure 2A). On maize kernels, a 25% reduction was observed (Figure 2C), but the expression level was only 10%

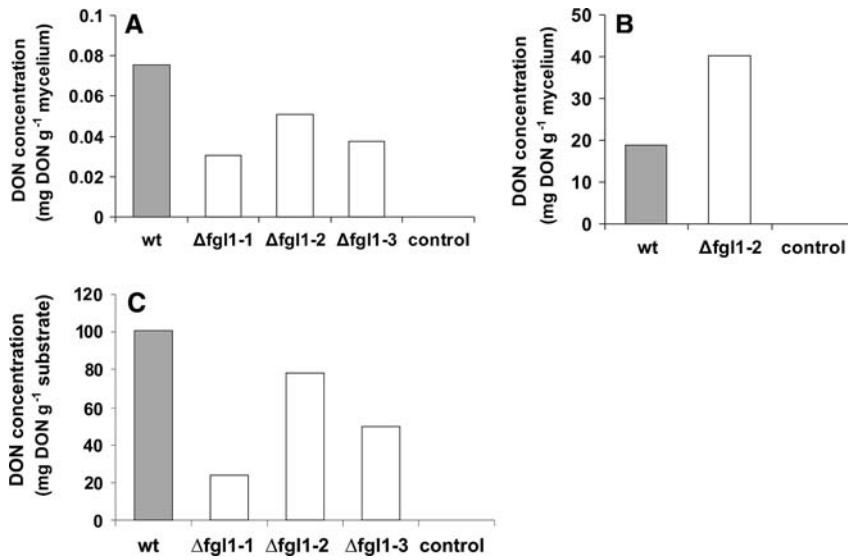

Figure 1. DON concentration of wheat kernels and spikes and maize kernels inoculated by *F. graminearum* wild-type (wt) and Δ*fgl1* strains (Δfgl1-1/-2/-3). (A) DON concentration of wheat kernels after inoculation with different *F. graminearum* strains incubated at 28 °C for 1 week and subsequently at RT for 2 weeks. Amount of fungal mycelium per gram substrate was determined via quantitative PCR. qPCR was performed with six replicates each. (B) DON concentration of inoculated wheat spikes of intact plants after incubation at 21 °C for 2 weeks. Determination of fungal mycelium as indicated in (A). (C) DON concentration of maize kernels after inoculation with different *F. graminearum* strains incubated as indicated in (A). DON concentration was quantified by gas chromatography. Measurements were repeated twice with two replicates each.

of that on wheat kernels. *In planta*, *Tri5* expression profile of the Δ*fgl1* strain completely differed from wild-type (Figure 2B). The expression was constant from 1 to 3 dpi and then strongly increased until 14 dpi. In contrast to this, wild-type reached highest *Tri5* expression 3 dpi with a subsequently decreased expression 7 dpi and unchanged expression level 14 dpi. At this time point, *Tri5* expression of the Δ*fgl1* strain was five times higher than of the wild-type. *Tri5* expression was about 1000-times greater on inoculated spikes than on wheat kernels.

ZEA determination of colonized cereal kernels and infected wheat spikes

ZEA concentration was determined in the same samples previously used for DON detection. Unlike DON production, ZEA production on wheat kernels was heavily increased for Δ*fgl1* strains as compared to the wild-type, resulting in a 10 to 12-fold higher ZEA concentration for strains Δ*fgl1*-1 and -3 and a more than 50-fold higher concentration for strain Δfgl1-2. Control kernels were free of ZEA (Figure 3A). A 7 to 10-fold higher ZEA concentration was determined for Δ*fgl1* strain-inoculated maize kernels (Figure 3B). ZEA was not detected during colonization of either wild-type and Δ*fgl1* strain-infected wheat spikes 14 and 42 dpi.

PKS4 *and* PKS13 *gene expression after growth on cereal kernels and during infection of wheat spikes*

To study whether ZEA-related gene expression correlated with measured ZEA concentration, the expression of the *PKS4* and the *PKS13* gene was quantified in qPCRs. The same normalized cDNA used for *Tri5* expression analysis before, served as template. *PKS4* expression in the lipase-deficient strain was detectable 21 dpi of wheat kernels, whereas no detectable expression of this gene was present in the wild-type (Figure 4A). A *PKS4* expression in the wild-type was detected 7 dpi of wheat kernels (Figure 4D). Analysis of cDNA derived from wild-type and Δfgl1-2-inoculated maize kernels showed a low expression of *PKS4* in the wild-type, but a

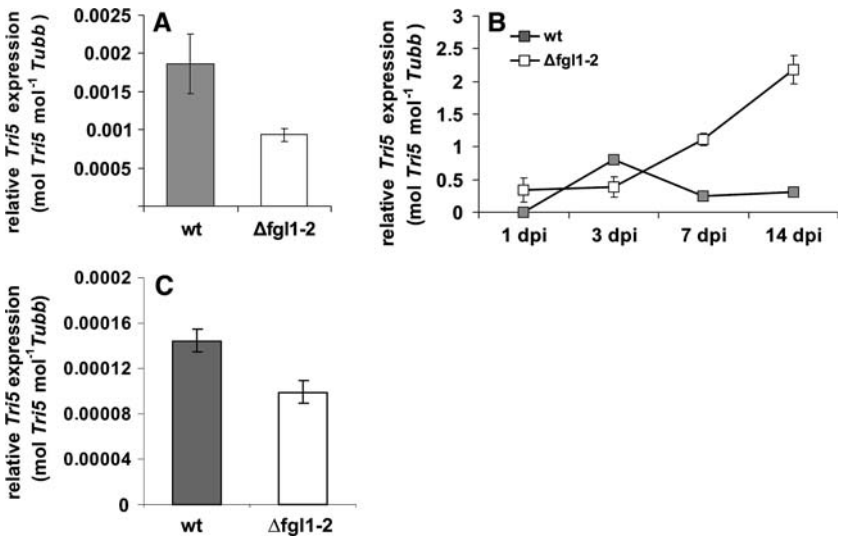

Figure 2. Tri5 gene expression of *F. graminearum* wild-type (wt) and Δ*fgl1* strain 2 (Δfgl1-2) during colonization of wheat and maize kernels and wheat head infection. (A) cDNA generated from isolated RNA of inoculated wheat kernels 21 dpi. (B) cDNA generated from isolated RNA of directly inoculated wheat spikelets 1, 3, 7, and 14 dpi of five independently infected wheat plants each. (C) cDNA generated from isolated RNA of inoculated maize kernels 21 dpi. All cDNA samples were normalized according to β-tubulin (*Tubb*) expression. Error bars indicate confidence interval with probability of error of $\alpha = 0.05$. qPCR was performed with eight replicates each.

10-times higher *PKS4* expression in the Δ*fgl1* strain (Figure 4F). Analysing the *PKS4* expression during infection of wheat spikes, no significant difference was detected between wild-type and Δfgl1-2. Both strains revealed a relatively low expression rate during infection (Figure 4B). *PKS4* expression of the Δ*fgl1* strain was 7-fold higher after colonization of wheat kernels as compared to wheat head infection. In contrast to *PKS4*, the *PKS13* expression of the Δ*fgl1* strain did not deviate from wild-type on colonized wheat kernels 21 dpi (Figure 4C). During infection of wheat spikes neither wild-type nor lipase-deficient mutant expressed *PKS13*.

Expression of the PKS gene family of wild-type and lipase deficient strain after growth on wheat kernels

The analysis of the *PKS4* and the *PKS13* genes in the wild-type and the lipase-deficient mutant revealed strong differences in the expression of *PKS4*, whereas *PKS13* expression was unchanged on colonized wheat kernels (Figure 4A, C). Since the *PKS* gene family comprises of 12 further known members, we were interested in determining, if the disruption of the lipase FGL1 affects only *PKS4* or if additional *PKS* genes are affected during colonization of wheat kernels. The lipase FGL1 was expressed by the wild-type on this substrate (Figure 4E). The expression level among different *PKS* genes varied highly within a given fungal isolate (Figure 5A). *PKS3*, *5*, *6* and *12* were expressed at a relatively low level (~0.001–0.002 mol *PKS* mol^{-1} *Tubb*; Figure 5B). In contrast, *PKS1* and *11* showed an approx. 100-times higher expression. Additionally, differences were detected between *PKS* gene expression levels of the *F. graminearum* wild-type and the lipase-deficient mutant (Figure 5A). The expression of 8 out of 14 *PKS* genes was deregulated in the mutant. *PKS2*, *11*, *12* and *14* exhibited decreased expression, whereas *PKS4*, *7*, *9* and *15* showed an elevated expression. Highest differences were determined for *PKS11*, revealing an expression that was more than twice as high in wild-type; and for *PKS7* and *PKS9* with 2.5 and nearly 4-times higher expression in the mutant strain, respectively. However, *PKS4* was the only *PKS* gene to be expressed in the lipase-deficient mutant.

Discussion

We assessed mycotoxin production of a highly virulent field strain and defined mutant strains

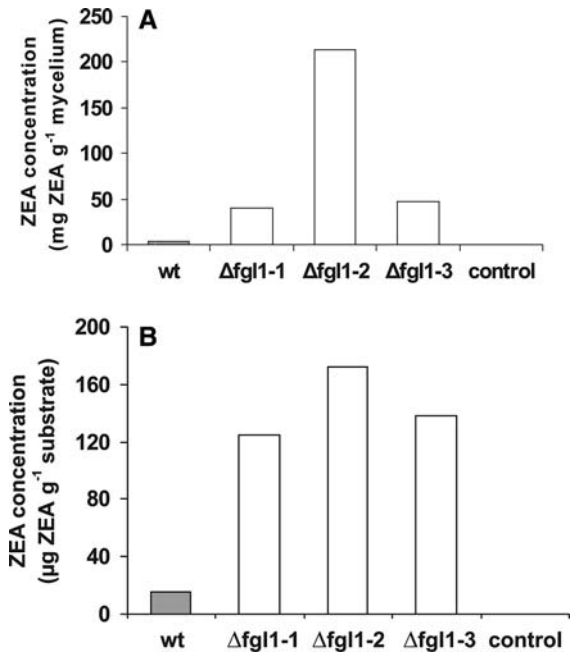

Figure 3. ZEA concentration of wheat and maize kernels inoculated by *F. graminearum* wild-type (wt) and Δ*fgl1* strains (Δfgl1-1/-2/-3). (A) Inoculated wheat kernels were incubated at 28 °C for 1 week and subsequently at RT for 2 weeks. Amount of fungal mycelium per g substrate was determined via quantitative PCR. qPCR was performed with six replicates each. (B) Inoculated maize kernels were incubated as indicated in (A). ZEA concentration was quantified by HPLC. Measurements were repeated twice with two replicates each.

(Δ*fgl1*), strongly reduced in virulence. These mutants differ from wild-type in the disruption of a single gene, which encodes the secreted lipase FGL1 (Voigt et al., 2005). We tested these near isogenic isolates for their ability to generate the mycotoxins DON and ZEA under different controlled conditions. Whereas DON constitutes an important virulence factor to wheat (Proctor et al., 1995; Bai et al., 2001), ZEA is not involved in virulence (Gaffoor et al., 2005; Kim et al., 2005) and its physiological function is still unknown. DON contamination is primarily associated with *Fusarium* infection in the field. However, inappropriate conditions during the storage of grain can result in further increases in DON content (Birzele et al., 2000; Homdork et al., 2000). In contrast to DON, ZEA is reported to be produced on grains (Wilson and Abramson, 1992). These observations correspond to our results. The wild-type strain produced DON predominantly during infection and to only a minor extent during growth on autoclaved grains (Figure 1A, B). ZEA, however, was produced at high concentration, only on grains (Figure 3A, B), but not during infection of wheat spikes. Since we tested the ZEA content of infected wheat heads 14 and 42 dpi, ZEA production at later stages of infection cannot be excluded. Matthäus et al. (2004) have shown that ZEA production of *F. culmorum* was not detectable until 44 dpi and strongly increased afterwards. Similar to ZEA, the red mycelial pigment aurofusarin is not required for the infection of wheat spikes (Malz et al., 2005). A possible function of this polyketide could derive from its bioactivity (Dvorska et al., 2002). Therefore, it has to be elucidated whether ZEA also exhibits similar bioactivity. These polyketides might be synthesized by *F. graminearum* to antagonise competing organisms during the saprophytic growth phase. DON synthesis during saprophytic growth might have a similar, specific function since general growth of *F. culmorum in vitro* was not affected by inhibited toxin production (Miller et al., 1996).

We found conditions that enhanced mycotoxin production in the lipase-deficient (Δ*fgl1*) mutants (Figures 1B, 3A, B). To get a better understanding of the increased DON production during wheat head infection, we quantified the expression of the *Tri5* gene. Unlike wild-type, the Δ*fgl1* mutants are unable to spread through the spike and produce typical FHB symptoms (Voigt et al., 2005). Although virulence was reduced, the production of DON was markedly increased during the limited infection of the spike (Figure 1B). The expression of the *Tri5* gene was shown to correlate with DON production *in vitro* (Doohan et al., 1999). Here, we determined that the expression of the *Tri5* gene in wild-type peaked 3 dpi with a subsequent reduction in transcriptional activity during the next stages of wheat spike colonization. In contrast to this, the Δ*fgl1* mutant exhibited a steady increase in *Tri5* transcription (Figure 2B). Thus, *Tri5* transcription not only correlated directly with the elevated DON production of the Δ*fgl1* mutant during plant infection, but also indicated a putative induction mechanism: during wild-type colonization, the *Tri5* expression is maximally induced approx. 3 dpi. At this point of infection, the wild-type reaches the transition zone of the rachilla and rachis and starts to enter the rachis. After 3 days, the expression decreases as the wild-type continues

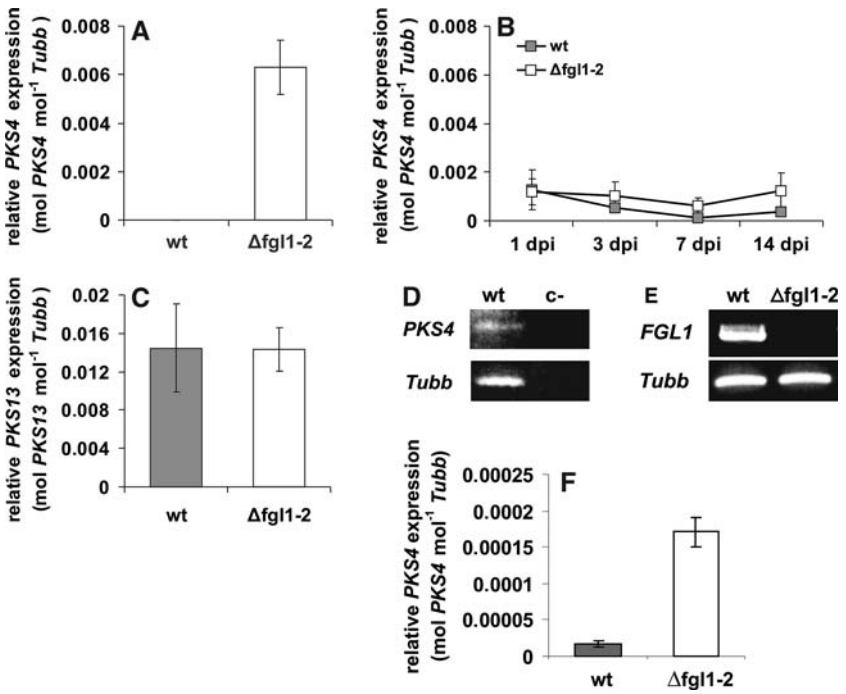

Figure 4. Gene expression analysis of *F. graminearum* wild-type (wt) and Δ*fgl1* strain 2 (Δfgl1-2) during colonization of wheat and maize kernels and wheat head infection. (A) *PKS4* expression of colonized wheat kernels. Template cDNA was generated from isolated RNA of inoculated wheat kernels 21 dpi. *PKS4* expression was not detectable in wild-type. (B) *PKS4* expression during wheat head infection. Template cDNA generated from isolated RNA of directly inoculated wheat spikelets 1, 3, 7, and 14 dpi of five independently infected wheat plants each. (C) *PKS13* expression of colonized wheat kernels. Template cDNA was used as described in (A). *PKS13* expression was not detectable during wheat head infection. (D) *PKS4* expression of colonized wheat kernels7 dpi with wt. β-tubulin (*Tubb*) expression served as expression control in conventional PCRs. Negative control (c−) was carried out with template cDNA of control kernels. (E) *FGL1* expression of colonized wheat kernels. Template cDNA was used as described in (A). β-tubulin (*Tubb*) expression served as expression control in conventional PCRs. (F) *PKS4* expression of colonized maize kernels. Template cDNA was generated from isolated RNA of inoculated maize kernels 21 dpi. (A–C + F) All cDNA samples were normalized according to β-tubulin (*Tubb*) expression. Error bars indicate confidence interval with probability of error of $\alpha = 0.05$. qPCR was performed with eight replicates each.

to grow inside the spike. In contrast to this, the infection of the lipase-deficient mutant is restricted to the directly inoculated spikelet and cannot enter the rachis (Voigt et al., 2005). The mutant remains in a state of constantly inducing *Tri5* expression (Figure 2B), resulting in increased DON production (Figure 1B). Hence, the transition zone of rachilla and rachis, or specific components of it, could constitute a strong inducer of DON synthesis in *F. graminearum*. Contrary to common belief, the high DON production of the lipase-deficient mutant was related to its reduced growth during infection (Yoder, 1980; Proctor et al., 1995). However, a DON-inducing effect of the transition zone would explain that the restricted growth of the fungus caused high DON concentration.

We hypothesize that *F. graminearum* strains with a low aggressiveness in the field, possibly through a mutated virulence factor, are trapped by plant defence mechanisms and are prevented from colonizing the spike effectively. In this case, fungal mycelium might primarily remain in the spikelet or other DON-inducing compartments of the spike. A particular fungicide application at sub-lethal level or incorrect time-points (Henriksen and Elen, 2005) might act similarly on the fungus. This would explain observations in the field that a FHB reduction is not necessarily associated with a significant reduction of the mycotoxin content (D'Mello et al., 1998; Magan et al., 2002).

In addition, our results demonstrate that DON alone is not sufficient for pathogenicity, although it represents an important virulence factor for

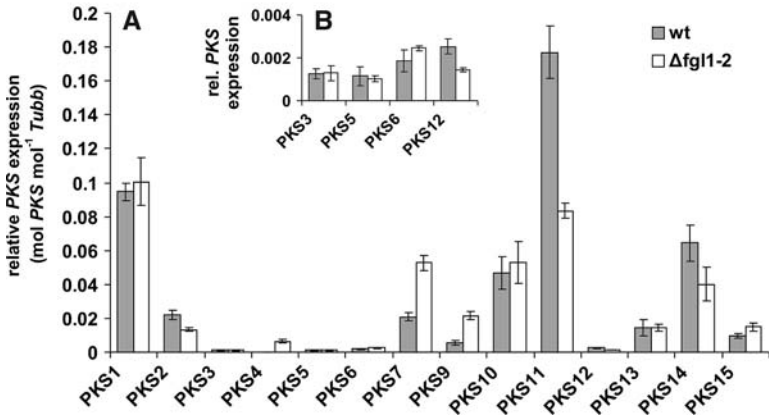

Figure 5. Expression analysis of *PKS* genes of *F. graminearum* colonized wheat kernels. (A) cDNA generated from isolated RNA of wheat kernels inoculated with *F. graminearum* wild-type (wt) and Δ*fgl1* strain 2 (Δfgl1-2) 21 dpi. All cDNA samples were normalized according to β-tubulin (*Tubb*) expression. Error bars indicate confidence interval with probability of error of $\alpha = 0.05$. qPCR was performed with eight replicates each. (B) Section of (A) with altered scale to illustrate the level of the weakly expressed genes *PKS3*, *5*, *6* and *12*.

wheat (Proctor et al., 1995; Bai et al., 2001). Even the overproduction of DON could not restore wild-type virulence in our virulence-reduced Δ*fgl1* mutants. This is in accordance with field experiments, in which DON production did not correlate with the general aggressiveness of *F. graminearum* field isolates (Miedaner et al., 2000).

During growth on autoclaved grains, the lipase-deficient mutants exhibited similar growth characteristics as the wild-type. We demonstrated this by biomass quantification via fungal DNA-content comparisons. However, the DON production of the Δ*fgl1* mutants was reduced to approx. 50% of the wild-type (Figure 1A). The differences in DON concentration corresponded to the transcription rate of the *Tri5* gene which had half the expression rate in the mutant compared to wild-type (Figure 2A). In general, we could show that the induction of DON production was low during saprophytic growth on harvested wheat kernels, but high during plant infection. This demonstrates, first, that specific factors present only during plant infection enhance *Tri5* expression. These factors seem to be absent during the colonization of kernels. Second, the increased DON production does not constitute an advantage for saprophytic growth. This is in accordance with the observation that DON production is not required for saprophytic growth on kernels (unpublished results).

In contrast to DON, ZEA production of the Δ*fgl1* mutants was dramatically enhanced during growth on kernels. One of the tested lipase-deficient mutants consistently showed an approx. 50-fold increase in ZEA concentration during growth on wheat kernels, whereas the two other mutant strains exhibited a 10 to 12-fold increase (Figure 3). The basis of this difference is unknown especially as we were unable to detect any other differences within these three mutants. On maize kernels, all three Δ*fgl1* mutants uniformly exhibited the same 7 to 10-fold increased ZEA concentration (Figure 3B). In general, the saprophytic growth on wheat kernels resembled the results obtained from maize kernels.

Recently, the polyketide synthases PKS4 and PKS13 were shown to be involved in ZEA synthesis (Gaffoor et al., 2005; Kim et al., 2005). We analysed the expression of these two genes 21 dpi of the kernels and could correlate the strongly elevated ZEA production of the lipase-deficient virulence mutant with the elevated expression of *PKS4*. *PKS4* was only expressed in the Δ*fgl1* mutant strain at this time point (Figure 4A). We did not detect a difference in *PKS13* gene expression on wheat kernels (Figure 4C). Therefore, the strongly elevated ZEA production of the Δ*fgl1* mutants seems to be caused solely by the continued expression of the *PKS4* gene in the mutant during later stages of colonization of

wheat kernels. A key role of this polyketide synthase in ZEA synthesis has been reported by Gaffoor et al. (2005) and Kim et al. (2005). Thus, the complete repression of the *PKS4* gene in wild-type at the measured time point of kernel colonization is sufficient to reduce the ZEA production as compared to the mutants, in spite of the consistent expression of the second key gene, *PKS13*. During wheat head infection, neither the Δ*fgl1* mutant strain nor wild-type produced ZEA. The genetic background for this is likely to be the complete *PKS13* repression during infection, confirming the expression analysis of Gaffoor et al. (2005). We detected *PKS4* expression in both strains without significant differences during wheat head colonization, however, at a relatively low level (Figure 4B). Our expression studies suggest a complex regulation of ZEA synthesis by expression or repression of only one of the two ZEA-related *PKS* genes. In wild-type, *PKS4* is repressed at later stages during colonization of autoclaved wheat kernels, and *PKS13* is repressed throughout wheat head infection.

However, *PKS4* and *13* were not the only *PKS* genes showing an altered expression after the deletion of the secreted lipase. We observed expression changes for the majority of the *PKS* genes. After 21 days of growth on kernels, four genes were up-regulated and an additional four genes were repressed in the Δ*fgl1* mutant compared to wild-type. The deletion of a single gene, coding for a secreted lipase, has therefore several seemingly unrelated consequences: (i) a reduction in virulence, (ii) an increase in the virulence factor DON during the reduced wheat head infection, (iii) an increase of the mycotoxin ZEA during saprophytic growth on kernels caused by the deregulation of the *PKS* genes.

Regarding the regulation of a whole *PKS* gene family, no direct link is known between a secreted lipase and polyketide synthesis. The lipase gene is, as many other genes, catabolically repressible and substrate inducible. We confirmed the expression of the lipase gene during saprophytic growth on kernels (Figure 4E). We hypothesize that plant material degraded by the fungal lipase regulates *PKS* gene expression. This would be in agreement with the model that we propose for induced DON production during wheat head infection: plant substances induce *Tri5* expression. However, the nature of these plant products has to be elucidated.

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References

Anon (2005) Commission regulation (EC) No. 856/2005 of 6 June 2005 amending regulation (EC) No. 466/2001 as regards *Fusarium* toxins. Official Journal of the European Union L 143: 3–8.

Bai G-H, Desjardins AE and Plattner RD (2001) Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153: 91–98.

Birzele B, Prange A and Krämer J (2000) Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation to storage parameters. Food Additives and Contaminants 17: 1027–1035.

Cohen H and Lapointe M (1982) Capillary gas–liquid chromatographic determination of vomitoxin in cereal grains. Journal - Association of Official Analytical Chemists. 65: 1429–1434.

Comménil P, Belingheri L and Dehorter B (1998) Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. Physiological and Molecular Plant Pathology 52: 1–14.

Desjardins AE, Spencer GF, Plattner RD and Beremand MN (1989) Furanocoumarin phytoalexins, trichothecene toxins and infection of *Pastinaca sativa* by *Fusarium sporotrichioides*. Phytopathology 79: 170–175.

D'Mello JPF, MacDonald AMC, Postel D, Dijksma WTP, Dujardin A and Placinta CM (1998) Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens. European Journal of Plant Pathology 104: 741–751.

Doohan FM, Weston G, Rezanoor HN, Parry DW and Nicholson P (1999) Development and use of a reverse transcription-PCR assay to study expression of *Tri5* by *Fusarium* species *in vitro* and *in planta*. Applied and Environmental Microbiology 65: 3850–3854.

Dvorska JE, Surai PF, Speake BK and Sparks NHC (2002) Antioxidant system of the developing quail embryo are compromised by mycotoxin aurofusarin. Comparative Biochemistry and Physiology. Toxicology & Pharmacology 131: 197–205.

Gaffoor I, Brown DW, Plattner R, Proctor RH, Qi W and Trail F (2005) Functional analysis of the polyketide synthase genes in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). Eukaryotic Cell 4: 1926–1933.

Goswami RS and Kistler H C (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5: 515–525.

Henriksen B and Elen O (2005) Natural *Fusarium* grain infection level in wheat, barley and oat after early application of fungicides and herbicides. Journal of Phytopathology 153: 214–220.

Homdork S and Fehrmann H and Beck R (2000) Influence of different storage conditions on the mycotoxin production and quality of Fusarium-infected wheat grain. Journal of Phytopathology 148: 7–15.

Kang Z and Buchenauer H (1999) Immunocytochemical localization of *Fusarium* toxins in infected wheat spikes by *Fusarium culmorum*. Physiological and Molecular Plant Pathology 55: 275–288.

Kim Y-T, Lee Y-R, Jin J, Han K-H, Kim H, Kim J-C, Lee T, Yun S-H and Lee Y-W (2005) Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zeae*. Molecular Microbiology 58: 1102–1113.

Kroken S, Glass NL, Taylor JW, Yoder OC and Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proceedings of the National Academy of Sciences USA 100: 15670–15675.

Lutz MP, Feichtinger G, Défago G and Duffy B (2003) Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. Applied and Environmental Microbiology 69: 3077–3084.

Magan N, Hope R, Colleate A and Baxter ES (2002) Relationship between growth and mycotoxin production by Fusarium species, biocides and environment. European Journal of Plant Pathology 108: 685–690.

Malz S, Grell MN, Thrane C, Maier FJ, Rosager P, Felk A, Albertsen KS, Salomon S, Bohn L, Schäfer W and Giese H (2005) Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. Fungal Genetics & Biology 42: 420–433.

Marasas WFO, Nelson PE and Toussoun TA (1984) Toxigenic Fusarium Species: Identity and Mycotoxicology, Pennsylvania State University Press, University Park, PA.

Matthäus K, Dänicke S, Vahjen W, Simon O, Wang J, Valenta H, Meyer K, Strumpf A, Ziesenib H and Flachowsky G (2004) Progression of mycotoxin and nutrient concentrations in wheat after inoculation with *Fusarium culmorum*. Archives of Animal Nutrition 58: 19–35.

Miedaner T, Reinbrecht C and Schilling AG (2000) Association among aggressiveness, fungal colonization, and mycotoxin production of 26 isolates of *Fusarium graminearum* in winter rye head blight. Journal of Plant Diseases Protection 107: 124–134.

Miller JD, Fielder DA, Dowd PF, Norton RA and Collins FW (1996) Isolation of 4-acetyl-benzoxazolin-2-one (4-ABOA) and diferuloylputrescine from an extract of gibberella ear rot-resistant corn that blocks mycotoxin biosynthesis, and the insect toxicity of 4-ABOA and related compounds. Biochemical Systematics and Ecology 24: 647–658.

Moss MO (1984) Conditions and factors influencing mycotoxin formation in the field and during storage of food. Chemistry & Industry 15: 533–536.

Pomeranz Y, Bechtel DB, Sauer DB and Seitz LM (1995) Fusarium head blight in cereal grains. Advances in Cereal Science and Technology 5: 373–397.

Proctor RH, Hohn TM and McCormick SP (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Molecular Plant–Microbe Interactions 8: 593–601.

Ribichich KF, Lopez SE and Vegetti AC (2000) Histopathological spikelet changes produced by *Fusarium graminearum* in susceptible and resistant wheat cultivars. Plant Disease 84: 794–802.

van Egmond HP and Jonker MA (2004) Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition Paper No. 81, Food and Agriculture Organization of the United Nations, Rome.

Voigt CA, Schäfer W and Salomon S (2005) A secreted lipase of *Fusarium graminearium* is a virulence factor required for infection of cereals. Plant Journal 42: 364–375.

Wanjiru WM, Zhensheng K and Buchenauer H (2002) Importance of cell wall degrading enzymes produced by *Fusarium graminearum* during infection of wheat heads. European Journal of Plant Pathology 108: 803–810.

Wilson DM and Abramson D (1992) Mycotoxins. In: Sauer DB (ed.) Storage of Cereal Grains and Their Products (pp. 341–392) American Association of Cereal Chemists, Inc, St. Paul, MN.

Yoder OC (1980) Toxins in pathogenesis. Annual Review of Phytopathology 18: 103–129.

Zadoks JC, Chang TT and Konzak DF (1974) A decimal code for the growth stages of cereals. Weed Research 14: 415–421.