The Fusarium mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat.

Author

Desmond, Olivia J, Manners, John M, Stephens, Amber E, MaClean, Donald J, Schenk, Peer M, Gardiner, Donald M, Munn, Alan L, Kazan, Kemal

Published

2008

Journal Title

Molecular Plant Pathology

DOI

10.1111/J.1364-3703.2008.00475.X

The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat.

Olivia J. Desmond[a,b,c], John M. Manners[a], Amber E. Stephens[a,d], Donald J. Maclean[b], Peer M. Schenk[c], Donald M. Gardiner[a], Alan L. Munn[d,e], Kemal Kazan[a,*]

[a]CSIRO Plant Industry, Queensland Bioscience Precinct, 306 Carmody Road, St. Lucia, Brisbane 4067, Australia

[b]School of Molecular and Microbial Sciences and [c]School of Integrative Biology, University of Queensland, St Lucia, Queensland 4072, Australia

[d]Institute for Molecular Bioscience and ARC Special Research Centre for Functional and Applied Genomics, University of Queensland, St Lucia, Queensland 4072

[e]School of Biomedical Sciences, University of Queensland, St Lucia, Queensland 4072

* Corresponding author. Tel.: +61-07 3214 2678; fax: +61-07 3214 2920.

E-mail address: kemal.kazan@csiro.au

Keywords: pathogenicity, host-pathogen interaction, secondary metabolite, vomitoxin, filamentous fungi, crop spoilage, antioxidant, apoptosis, gene induction, defence proteins

Summary

Fusarium species infect cereal crops all over the world and cause the important diseases Fusarium head blight and crown rot in wheat. *Fusarium* pathogens reduce yield and some species also produce trichothecene mycotoxins, such as deoxynivalenol (DON), during infection. These toxins play roles in pathogenesis on wheat and have serious health effects if present in grain consumed by humans or animals. In this study, the response of wheat tissue to DON has been investigated. Infusion of wheat leaves with DON induced hydrogen peroxide production within 6h followed by cell death within 24h that was accompanied by DNA laddering, a hallmark of programmed cell death. In addition, real-time PCR analysis revealed that DON treatment rapidly induced transcription of a number of defence genes in a concentration-dependent manner. Co-treatment with DON and the antioxidant ascorbic acid reduced these responses suggesting their induction may be at least partially mediated by reactive oxygen species (ROS), commonly known to be signalling molecules in plants. Wheat defence genes were more highly expressed in wheat stems inoculated with a DON producing fungal strain than those inoculated with a DON-nonproducing mutant, but only at a late stage of infection. Taken together, results are consistent with a model where DON production during infection of wheat induces ROS, which on one hand may stimulate programmed host cell death assisting necrotrophic fungal growth, but on the other hand the ROS may contribute to the induction of anti-microbial host defences.

Introduction

Many plant pathogenic fungi produce secondary metabolites including mycotoxins during the infection process. Mycotoxins, such as the trichothecene deoxynivalenol (DON) produced by some *Fusarium* species, are not always essential for initiating disease but are often linked with increased aggressiveness (Desjardins *et al.*, 1995). The effect of DON on cell function has been most extensively studied in animal cells in attempts to understand its toxicity (Pestka *et al.*, 2004, Rocha *et al.*, 2005). These studies have resulted in a model where trichothecenes inhibit protein synthesis by binding to the 60S ribosomal subunit, activating a cell signalling program that results in apoptosis. In conjunction with this, there is an induction of cytokine-regulated gene expression resulting in an inflammatory response. In macrophages, as well as T and B cells of the mammalian immune system, these complex effects mean that trichothecenes such as DON can be either immunostimulatory or immunosuppressive depending on dosage, duration and frequency of exposure (Pestka et al., 2004).

Fusarium head blight (FHB) and crown rot (CR) are both diseases of wheat caused by several *Fusarium* species, including *F. graminearum (Fg)* and *F. pseudograminearum (Fp)* (Akinsanmi *et al.*, 2004). The major difference between these diseases is the site and timing of infection, with FHB infecting wheat heads around the time of anthesis, and CR infecting stem base and crown tissue at all growth stages. DON is produced during both FHB and CR diseases (Mudge *et al.*, 2007, Blaney & Dodman, 2002) and this is a major concern due to its toxicity to humans and animals following consumption of contaminated grain (Larsen *et al.*, 2004).

The role of DON and other trichothecene mycotoxins during pathogenesis has been analysed using mutant fungal strains that do not produce toxin (Hohn & Desjardins, 1992). Studies on a strain of *Fg* that has a mutation in the *Tri5* gene encoding a DON biosynthetic enzyme have

shown that *Fg* strains unable to produce DON are less aggressive during FHB in both wheat and barley (Langevin *et al.*, 2004, Boddu *et al.*, in press). More specifically in wheat, the mycotoxin appears to be necessary for fungal passage from infected florets into the rachis from where it can further colonise the head (Jansen *et al.*, 2005). These DON-nonproducing *Fg* strains are unable to prevent thickening of host cell walls after penetration and so their movement from the point of infection is hindered (Jansen et al., 2005, Bai *et al.*, 2002, Maier *et al.*, 2006). There is also evidence that FHB resistant wheat genotypes accumulate far less DON than susceptible ones (Goswami & Kistler, 2005, Wilde & Miedaner, 2006). For FHB, it therefore appears that occurrence of disease may not be dependent on the toxin, but DON production does affect disease levels, and so, improving resistance to DON may improve resistance to the disease. The role of DON during CR is less well defined than for FHB, but it appears to be necessary for fungal colonisation of upper stem nodes following infection at the crown and stem base (Mudge et al., 2007).

Several studies have shown that fungal derived toxins can elicit responses in plants that have aspects in common with well-known pathogen-induced responses. It has recently been reported that another trichothecene produced by some *Fusaria*, the *T*-2 toxin, induces hydrogen peroxide (H_2O_2) production, inhibits protein synthesis and stimulates cell death in the non-host plant *A. thaliana* at concentrations as low as 0.4 mg L^{-1} (Nishiuchi *et al.*, 2006). This study also showed that DON can induce defences in *A. thaliana* plants but only at the higher concentration of 30 mg L^{-1}. In barley, a comparison of gene expression induced by WT and a DON-nonproducing strain of *F. graminearum* revealed that DON is responsible for the induction of genes encoding proteins involved in ubiquitination and programmed cell death (PCD) among others (Boddu et al., in press). In wheat, differential display analysis has been used to demonstrate that DON affects transcript levels of a few specific host genes in roots including peroxidase genes (Ansari

et al., 2007). However, the physiological and molecular responses of wheat cells to DON exposure have not been described.

We aimed to assess the effects of DON on wheat defence responses and observed the production of H_2O_2, a well-known signalling molecule, followed by cell death, a phenomenon frequently observed in mammalian cell lines exposed to DON (Pestka et al., 2004). In addition, DON induced a range of well-known defence genes and interestingly a DON-producing fungal strain induced higher levels of defence transcripts than a DON-nonproducing mutant during disease development. Both cell death and defence gene induction was reduced by co-treatment of DON and an anti-oxidant that would scavenge free radicals like H_2O_2. These results suggest that DON produced during *Fusarium*-related diseases may play a role in the activation of wheat defence responses and cell death, at least partially via the signalling molecule H_2O_2.

Results

DON elicits ROS production, gDNA laddering and cell death in wheat

Wheat stem tissue was infiltrated with a solution of 100 or 200 mg L^{-1} DON resulting in the production of localised H_2O_2 microbursts (Levine *et al.*, 1994) within 6h after treatment (Fig. 1A-C, Table 1, Supp. Fig. 1). Co-infiltration of DON with 40 mM ascorbic acid, an anti-oxidant capable of scavenging reactive oxygen species, or 4 mg L^{-1} cycloheximide, a eukaryotic protein synthesis inhibitor, reduced the amount of H_2O_2 produced (Table 1, Supp. Fig. 1). Trypan blue staining to reveal cell death was negative at this 6h time point (data not shown). By 24h post infiltration, H_2O_2 staining was no longer visible in DON infiltrated tissue (data not shown), however the tissue stained positively for cell death, primarily in stomatal guard cells but also in mesophyll cells of samples treated with 200 mg L^{-1} DON (Fig.1D-F, Table 1, Supp. Fig. 1) . Again, co-infiltration with ascorbic acid reduced cell death and co-infiltration with cycloheximide prevented cell death (Table 1, Supp. Fig. 1).

To further investigate the mechanism behind DON-induced cell death, genomic DNA collected from wheat stems 24h after infiltration was analysed for cleavage products by gel electrophoresis. Shown in Figure 1G, a distinct DNA laddering pattern was observed in DON-treated leaves when compared to mock-treated controls. Similar to cell death visualised using trypan blue staining, DNA laddering appeared to be reduced when tissue was co-infiltrated with ascorbic acid. This effect of ascorbic acid on DNA laddering was observed in two independent experiments. Visually quantifying differences between these DNA laddering profiles was subjective, therefore to quantify the results, the total number of pixels in each lane was assessed using the software ImageJ. This was then compared to the number of pixels in the region of the image showing only degraded DNA (<5000 bp). This method for quantification showed that DNA from mock treated samples was only 5% degraded, while that from DON treated samples

was 66% degraded and combining ascorbic acid with the DON treatment reduced degradation to 29%. DON induced DNA laddering as observed here indicates induction of a host-mediated PCD process (Ryerson & Heath, 1996).

Fp and *Fg* are closely related species that are both able to cause CR and FHB in wheat, and both pathogen species produce DON (Mudge et al., 2007). Tissue inoculated with *Fp* spores was studied in detail and revealed H_2O_2 production and cell death indicating defences induced by purified DON are consistent with those induced during a natural CR infection. DON has previously been shown to be produced during CR at levels >100 mg L^{-1} when *Fp* is the causal agent (Mudge et al., 2007). Within 7 days after inoculation of wheat, using a detached leaf assay, intense H_2O_2 production was observed in some stomatal guard cells that were in close proximity to *Fp* spores (Fig. 2A). DAB staining also revealed the presence of H_2O_2 in some fungal spores (Fig. 2A inset), a phenomenon previously associated with pathogenicity in other host-pathogen interactions (Egan *et al.*, 2007). Host cell death of *Fp* inoculated wheat tissue was observed at multiple infection points and spread in a way that seemed to follow vascular tissue (Fig. 2B, and Supp. Fig. 2). Stomatal guard cells showed the most intense and widespread staining for cell death among those cells surrounding infection points. Preliminary examination of *Fg* infection sites indicated similar host responses to those observed with *Fp* (data not shown).

Induction of wheat defence gene transcripts and proteins by DON

We have previously shown that drop inoculation of wheat with *Fp* spores leads to the induction of a suite of defence gene transcripts including *PR1.1, PR2* (*β,1-3 glucanase*), *PR3* (*chitinase*), *PR4* (*wheatwin*), *PR5* (*thaumatin-like protein*), *PR10, peroxidase* and *germin-like* (Desmond *et al.*, 2005). Presented here, a similar drop treatment of wheat seedlings with a solution of DON

also induced expression of these well-known defence genes. Figure 3A shows transcript induction by DON, generally in a concentration dependent manner, within the range of 1-100 mg L^{-1}. Treatment with 0.1 mg L^{-1} DON did not induce defence gene expression (data not shown). Interestingly, the most highly induced genes were *germin-like* and *peroxidase*, both genes implicated in reactive oxygen metabolism.

DON is known to be a potent inhibitor of eukaryotic protein synthesis at concentrations well below 100 mg L^{-1} (Nishiuchi et al., 2006). Therefore, a western blot to assess protein levels was performed on 100 mg L^{-1} DON-treated wheat samples using PR2 (β-1,3-glucanase) and PR3 (chitinase) specific antibodies. Again, ImageJ was used to quantify pixels for each band and protein abundance as a percentage of Rubisco is shown for each band in Figure 3B. PR2 and PR3 are clearly more abundant both visually and quantitatively in both *Fp*-inoculated and DON-treated samples compared to controls.

Reactive oxygen molecules like H_2O_2 are known to have a role in plant defence signalling. The influence of these reactive oxygen molecules on DON-induced defence gene induction was investigated by comparing samples treated with DON alone or DON combined with ascorbic acid. Figure 4 shows that the presence of ascorbic acid reduced the average induction of defence genes at both 6h and 24h after treatment. Statistical analysis showed this reduction was significant ($p < 0.05$) for *PR3*, *peroxidase*, and *PR2*. These results suggest that reactive oxygen molecules elicited by DON may have a role in the induction of defence gene transcription.

Differential defence gene induction by a DON-nonproducing fungal strain at late stages of CR disease development

To test whether DON may play a role during CR infection of wheat we assessed fungal biomass and the levels of defence gene transcripts in wheat stem tissue inoculated with either the transgenic DON-nonproducing strain of *Fg* (*Fg* Δ*Tri5*) or its WT DON-producing parental strain. *Fg* was specifically used for this particular study because its facile transformation system and published genome sequence enabled the construction of the *Tri5* deletion strain. Inoculation of detached leaves with *Fg* Δ*Tri5* resulted in similar H_2O_2 production and cell death responses as for tissues inoculated with the wild type *Fg* and *Fp* strains at early stages of infection (data not shown, Fig. 1; Supp. Fig 1). Results also indicated that wheat defence gene transcript levels were unchanged between tissues inoculated with these two strains at 1,2 and 14 days post-inoculation. However, a significant reduction in defence transcript levels (Fig. 5A) and fungal biomass (Fig. 5B) was observed in wheat stem tissue inoculated with the *Fg* Δ*Tri5* strain at 28 days post-inoculation.

Discussion

Previous work has shown that production of DON plays an important part in fungal colonisation of host tissues in both FHB and CR caused by the fungal pathogens *Fg* and *Fp* (Jansen et al., 2005, Langevin et al., 2004, Maier et al., 2006, Mudge et al., 2007). Interestingly, the work described here indicates that DON can also induce a range of classical plant defence responses in wheat, including the production of ROS that may be at least partially responsible for the induction of host defence gene transcripts, their protein products, and PCD. This suggests that DON may on the one hand assist necrotrophic growth of the pathogen by promoting host cell death, but on the other hand, DON may also stimulate an anti-microbial defence response in the host. These contrasting effects of DON may influence the rate and extent of disease development of FHB and CR diseases of wheat.

Previous characterisation of responses induced in Arabidopsis following exposure to a range of trichothecenes, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol and DON, has shown that all of these toxins can cause cell death and the induction of defence gene transcripts (Masuda *et al.*, 2007, Nishiuchi et al., 2006). Compared to these other trichothecenes, DON was a relatively weak inducer and only caused these effects at concentrations such as 29.6 mg L^{-1} (Nishiuchi et al., 2006). In addition, it was demonstrated that protein synthesis, assessed by measuring the incorporation of [^{3}H]-leucine into proteins of an Arabidopsis cell suspension, was 50% inhibited by 1.5 mg L^{-1} DON. Although the accumulation of defence proteins was not studied in Arabidopsis, it was suggested that DON may be acting as a protein synthesis inhibitor at concentrations below the threshold required to activate Arabidopsis defence responses (Nishiuchi et al., 2006). Consistent with these studies, our work showed that in wheat, a concentration-dependent induction of defence gene transcripts occurred, with larger effects on defence gene induction, including defence protein accumulation, at higher

concentrations (100 mg L^{-1}), which are known to inhibit protein synthesis in wheat (Miller & Ewen, 1997, Rocha et al., 2005). In our experiments, we applied DON droplets to the surface of wheat tissue and it would have diffused into surrounding tissue. Thus, defence gene transcript and protein induction may have occurred in plant cells prior to DON reaching levels that inhibit protein synthesis. It is not clear from current studies if it is specifically DON that is detected by host cells leading to the induction of defence transcripts or if non-specific cellular stress caused by this toxin leads to the release of endogenous signals that activate defence responses in surrounding cells.

The most highly transcriptionally induced genes following DON treatment were the *germin-like* and the *peroxidase* genes, both of which are related to reactive oxygen metabolism (Zimmermann *et al.*, 2006, Liu *et al.*, 2005). These functional associations are consistent with the production of H_2O_2 that was also observed in wheat cells following DON infiltration. Application of the anti-oxidant ascorbate, together with DON, significantly reduced the level of transcriptional activation of the *peroxidase* gene by DON, as well as that of several other defence genes. The effects of ascorbic acid on the activity of DON are unknown, however it is likely that reactive oxygen production in response to DON is at least partially responsible for the observed induction of host defence genes.

In this study we demonstrated that DON causes cell death in wheat and this was associated with genomic DNA laddering, a hallmark of PCD in plants and other eukaryotes (Tada *et al.*, 2001, Ryerson & Heath, 1996). PCD is known to be an active process that requires de novo protein synthesis (Tada et al., 2001) and treatment with DON combined with cycloheximide, a eukaryotic protein synthesis inhibitor, prevented cell death 24h after treatment. The mechanisms involved in this protective effect of cycloheximide are unknown, however it is

possible that synthesis of a specific protein associated with DON-inducible cell death is inhibited by cycloheximide. If this is the case, it suggests that the cell death observed here is actively undertaken by host cells and is likely to be PCD rather than necrosis.

H_2O_2 is known to induce PCD in plant cells, (Houot *et al.*, 2001, Yoda *et al.*, 2006) and interestingly, we observed that co-infiltration of DON and ascorbic acid reduced genomic DNA laddering and cell death, further suggesting that DON elicitation of reactive oxygen species may be an important signalling event that stimulates PCD. Necrotrophic fungal pathogens often produce toxins to induce PCD during infection (Navarre & Wolpert, 1999, Tada et al., 2001). The toxin fumonisin, produced by pathogens such as *F. verticillioides* and *F. proliferatum* (Munkvold *et al.*, 1999), has been demonstrated to induce cell death by depletion of extracellular ATP (Chivasa *et al.*, 2005). Stimulation of cell death by mycotoxins would release nutrients facilitating necrotrophic fungal growth as well as spread throughout the host. This functional characteristic of DON is consistent with the reduced ability of DON-nonproducing mutant *Fusarium* strains to infect spikelets surrounding the point of infection during FHB (Jansen et al., 2005).

Host cell death and H_2O_2 production all appeared to require the application of relatively high concentrations of DON. Measurements of DON in inoculated crown and head tissues of wheat following inoculation with *F. graminearum* have shown that DON can accumulate to physiological levels on a fresh weight basis in excess of 100 $\mu g\ g^{-1}$ (Mudge et al., 2007). However, these high concentrations of DON in infected tissues occur at relatively late stages after stem inoculation (14-28 days) and it is therefore likely that host responses to DON such as PCD are associated with the later stages of CR disease when visible lesions develop. We were able to investigate directly whether DON has a role in inducing wheat defence responses during

CR disease development using a *Tri5* deletion mutant of *Fg.* Defence gene induction was not significantly different between the two strains within the first 14 days post-inoculation and it was only at 28 days post-inoculation that induction of several defence genes was lower in tissue inoculated with the *Fg* Δ*Tri5* compared to WT. This is consistent with previous observations where infection on wheat stems by the wild type *Fg* resulted in DON levels of approximately 300 μg g^{-1} fresh wt at 28 days post-inoculation (Mudge et al., 2007). According to the results presented herein, these levels should be capable of inducing plant defence genes. However, it was also at this time point that significantly less fungal biomass was observed for the *Fg* Δ*Tri5* strain compared to WT, and so it is unclear if reduced fungus or a lack of DON is influencing defence gene expression at this later stage of infection. Recently, Ansari *et al*. (2007) also reported that a small suite of genes in wheat heads were inducible by DON, but there was no difference in the expression of these genes in wheat heads during the early stages post-inoculation with a DON-nonproducing mutant of *Fg* and a wild type strain (Ansari et al., 2007). So even though we saw several parallels between wheat responses induced by DON and those induced following inoculation with DON-producing *Fp* and *Fg,* it is likely that these fungal pathogens may produce many other elicitors of host responses, and DON is not likely to be a primary determinant of the host defence responses during early stages of fungal infection.

During later stages of disease, DON production may elicit defences such as the accumulation of the chitinase and glucanase proteins studied here. It may be that the effectiveness of these defence proteins is one of the factors determining fungal colonisation rates and therefore quantitative resistance responses. A recent study has indeed suggested that transgenic wheat plants expressing *PR2* (*β-1-3 glucanase*) showed reduced FHB development (Mackintosh *et al.*, 2007). Our experiments suggest that the level of production of DON affects wheat cellular responses in a way that could either promote necrotrophic fungal growth by initiating PCD or

reduce fungal growth by triggering defence gene expression and accumulation of anti-microbial proteins. Interplay between these responses may contribute to both resistance and susceptibility in different wheat cultivars.

Experimental Procedures

Fusarium pseudograminearum isolate, plant material and inoculation procedure

Spore suspensions of the aggressive *Fp* isolate CS3069 from the CSIRO collection were cultured as previously described (Desmond et al., 2005). The commercial variety of hexaploid wheat (*Triticum aestivum*) cv. Kennedy, was grown in glasshouse conditions as previously described (Desmond et al., 2005). Inoculations were performed using a detached leaf assay where leaves of 2-week old plants were cut into segments ~4cm long and placed on moist tissue in a Petri dish and then 10 μL drops of spore solution were evenly spaced along the leaves ~0.5cm apart. Samples were collected ~7 days later for DAB and trypan blue staining.

Infiltration of wheat tissue for DAB and trypan blue staining

Wheat leaf segments from 2-week old plants were infiltrated with the following solutions: 1) 100 mg L^{-1} DON, 2) 200 mg L^{-1} DON, 3) 100 mg L^{-1} DON and 7 g L^{-1} ascorbic acid, 4) 100 mg L^{-1} DON and 4 mg L^{-1} cycloheximide 5) distilled water. Infiltration was performed by covering tissue with treatment solutions and applying a vacuum for 15-20 mins. Stem segments were then left in solution for ~10 mins before being removed and placed on moist filter paper in a Petri dish. After infiltration, samples were collected 6h and 24h later and stained using DAB to reveal H_2O_2 production and trypan blue to reveal cell death.

DAB and trypan blue staining of inoculated and infiltrated tissue

DAB staining to reveal H_2O_2 was carried out using the DAB Liquid Substrate System (Sigma) according to the manufacturer's directions. Tissue was de-stained by boiling tissue in 90% EtOH for 1 min and viewed using a light microscope. Trypan blue staining was performed to reveal dead plant cells using the procedure described by (Belenghi *et al.*, 2003) with a further

dilution of the stock solution described with 2 volumes of 96% ethanol. Tissue was immersed and boiled in the trypan blue solution for 1 min and left to stain overnight before de-staining with chloral hydrate and viewing using a Leica MZ16FA stereo light microscope.

Infiltration of wheat tissue for DNA cleavage analysis

Wheat stem segments ~2cm long, collected from 2-week old Kennedy plants, were infiltrated using the same procedure described above with treatments of 100 mg L^{-1} DON and a combination of 100 mg L^{-1} DON and 7 mg L^{-1} ascorbic acid. DNA degradation was assessed by extracting gDNA 24 h after infiltration as previously described (Kazan *et al.*, 1993), followed by electrophoresis through a 1% agarose gel made with TAE buffer and stained with ethidium bromide. Digital analysis of the gel images was performed using the software ImageJ. Firstly the image was converted to 8-bit binary and the threshold was set from 0-160. Total pixels was estimated for each lane using the Analyse Particles function with default settings and this was compared to the number of pixels in each lane that were below the 5000 bp band from the 1kb+ DNA ladder (Invitrogen).

Drop treatment with DON and ascorbic acid for gene expression analysis

Deoxynivalenol (Sigma, St Louis, MO, USA) was initially dissolved to 10 g L^{-1} in 100% methanol and then further diluted with water to final concentrations of 100 mg L^{-1}, 10 mg L^{-1} and 1 mg L^{-1}. For treatments of DON combined with an antioxidant, a 100 mg L^{-1} DON plus 7 g L^{-1} ascorbic acid solution (pH~7) was used. All treatment solutions also contained 0.05% Tween 20. Wheat plants were treated by placing a 10 µl drop onto the stem base using the same method previously described for *Fp* inoculation (Desmond et al., 2005).

Gene expression analysis by reverse transcriptase quantitative PCR

Tissue samples were collected for three independent biological replicates 1 day after treatment with DON or DON combined with ascorbic acid, followed by RNA isolation and cDNA synthesis as previously described (Desmond et al., 2005). Real-time quantitative PCR conditions, including primer details and analysis of results, have been previously described (Desmond et al., 2005).

Defence protein expression analysis by protein extraction and western blot

Total protein was extracted from ~200 mg of frozen wheat stem base tissue from 2-week old seedlings as described (Jayaraj *et al.*, 2004), with the following alterations: 1 tablet of complete protease inhibitor (Roche, Mannheim Germany) was added freshly to 50 mL of extraction buffer instead of PMSF. After tissue homogenisation in the extraction buffer, samples were left on ice for 15 mins. Protein concentration was determined using the bicinchoninic acid microtitre plate assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Proteins (75 μg) were separated using a Nu-Page 4-12% bis-tris pre-cast gel (Invitrogen, Carlsbad, CA, USA) electrophoresed in a Novex Mini-Cell (Invitrogen) and transferred to a nitrocellulose membrane using an XCell II Blot module (Invitrogen) followed by protein detection, all done according to the manufacturer's instructions. For size determination, the SeeBlue Plus2 (Invitrogen) pre-stained standard was included in all gels. Primary antibodies were kindly donated by S. Muthukrishnan, Kansas State University, KS, USA. Secondary antibodies were detected by incubating blots in 1-step NBT/BCIP (Pierce) for 5-10 mins before stopping the reaction with water. To quantify protein levels ImageJ was again used to count pixels for each band and levels relative to rubisco were determined. ImageJ settings included a threshold of 10-116 and the Analyse Particle function was run with default settings.

Generation of *Fg Tri5* deletion lines

Fusarium graminearum isolate CS3005 (Akinsanmi *et al.*, 2006) was used as a parent to construct the *Fg* Δ*Tri5* strain. The 2.8 kb of the *Tri5* genomic locus was amplified (AATCTATCAGTGCTTAAATGCAGTTCC and TACGTAGGCCGCGCAGAGGTCAGTA), cloned into pCR8/GW-TOPO (Invitrogen) and the *Hin*dIII-*Nco*I fragment of the *Tri5* coding sequence replaced with the hygromycin phosphotransferase cassette from pUChph, leaving two flanks of 1.2 kb. The construct was introduced into CS3005 protoplasts generated from approximately 1×10^7 conidia germinated overnight in 200 mL of TB3 media (0.3% yeast extract, 0.3% casamino acids, and 20% sucrose) at 20°C with shaking at 150 rpm. Germlings were collected on miracloth and washed with 1 M sorbitol before being resuspended in 20 mL of 1 M sorbitol containing 5 U chitinase (Sigma-Aldrich, St Louis, MO, USA), 500 mg Driselase (Sigma-Aldrich, St Louis, MO, USA) and 200 mg lysing enzymes (Sigma-Aldrich, St Louis, MO, USA), and incubated 1-2 hours at 28°C to release protoplasts. Protoplasts were collected by centrifugation at 2600×g, 4°C, washed three times with ice cold STC (20% sucrose, 50 mM Tris/HCl pH 8, 50 mM $CaCl_2$). To a 200 µL aliquot of STC containing 2×10^7 protoplasts 10 µg circular plasmid DNA was added, incubated for 20 minutes at room temperature, then 1 mL of 40% PEG_{4000}, 10 mM Tris HCl pH 8, 50 mM $CaCl_2$ was added, incubated for 20 minutes at room temperature and then 5 mL of TB3 media was added and the tubes incubated overnight at room temperature with gentle agitation. Three mL of the transformation mixture was embedded in 30 mL regeneration media (0.2% yeast extract, 0.2% casamino acids, 0.55% low melt agarose, and 27% sucrose) and set in 14 cm Petri dishes, incubated overnight and then overlaid with 30 mL of regeneration media containing hygromycin (Roche, Penzberg, Germany) at 400 mg L^{-1}. After 5-7 days of growth at room temperature in the dark, transformants were transferred to individual plates containing

hygromycin (200 mg L^{-1}), allowed to grow, single spored then checked for insertion of the construct by PCR (data not shown).

Inoculation of wheat with *Fg*

Wheat (cv. Kennedy) was grown as described above. Seedlings were inoculated with the wild type *F. graminearum* isolate CS3005 and the *Fg* Δ*Tri5* strain in parallel at the stem base as described (Mitter *et al.*, 2006).

Estimation of fungal biomass in wheat stems inoculated with *Fg* Δ *Tri5* and parent *Fg*

Samples were collected 1, 2, 14 and 28 days post-inoculation by collecting stem from the crown to the first leaf. Each time point consisted of three biological replicates taken in parallel and each replicate was a pool of 8 stem bases. DNA was extracted from the samples using a QIAGEN DNeasy® Plant Mini Kit according to the manufacturer's instructions. Real-time quantitative PCR was performed as described above using *Fg rRNA18S* and wheat *actin* primers (Mudge et al., 2007). Internal PCR amplification efficiencies were calculated by using the program LinRegPCR 7.5 (Ramakers *et al.*, 2003) and absolute DNA amplification was calculated by the average efficiencies raised to the negative crossing threshold. *Fg* biomass was estimated as the absolute amplification of fungal DNA relative to the absolute amplification of wheat DNA.

Analysis of gene expression in *Fg* inoculated tissue

RNA was extracted from the same samples used for estimating fungal biomass followed by cDNA synthesis as described above. RT-qPCR was performed to assess defence gene expression using primers, procedures, and analysis described above.

Acknowledgements

The authors would like to thank Dr Bruno Dombretch and Anca Rusu for assistance with western blot procedures and Dr S. Muthukrishnan for supplying protein anti-bodies, Dr Anne Rae for assistance with staining procedures, and the Grains Research and Development Corporation for supplying a postgraduate scholarship to OJD.

References

Akinsanmi, O. A., Backhouse, D., Simpfendorfer, S. and Chakraborty, S. (2006) Genetic diversity of Australian Fusarium graminearum and F. pseudograminearum. *Plant Pathology,* **55,** 494-504.

Akinsanmi, O. A., Mitter, V., Simpfendorfer, S., Backhouse, D. and Chakraborty, S. (2004) Identity and pathogenicity of *Fusarium* spp. isolated from wheat fields in Queensland and northern New South Wales. *Aust J Agr Res,* **55,** 97-107.

Ansari, K. I., Walter, S., Brennan, J. M., Lemmens, M., Kessans, S., McGahern, A., *et al.* (2007) Retrotransposon and gene activation in wheat in response to mycotoxigenic and non-mycotoxigenic-associated *Fusarium* stress. *Theor Appl Genet,* **114,** 927-937.

Bai, G. H., Desjardins, A. E. and Plattner, R. D. (2002) Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia,* **153,** 91-98.

Belenghi, B., Acconcia, F., Trovato, M., Perazzolli, M., Bocedi, A., Polticelli, F., *et al.* (2003) AtCYS1, a cystatin from *Arabidopsis thaliana,* suppresses hypersensitive cell death. *Eur J Biochem,* **270,** 2593-2604.

Blaney, B. J. and Dodman, R. L. (2002) Production of zearalenone, deoxynivalenol, nivalenol, and acetylated derivatives by Australian isolates of *Fusarium graminearum* and *F-pseudograminearum* in relation to source and culturing conditions. *Aust J Agr Res,* **53,** 1317-1326.

Boddu, J., Cho, S. and Muehlbauer, G. J. (in press) Transcriptome analysis of trichothecene-induced gene expression in barley. *Mol Plant Microbe Interact.*

Chivasa, S., Ndimba, B. K., Simon, W. J., Lindsey, K. and Slabas, A. R. (2005) Extracellular ATP functions as an endogenous external metabolite regulating plant cell viability. *Plant Cell,* **17,** 3019-3034.

Desjardins, A., Plattner, R., Nelsen, T. and Leslie, J. (1995) Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. *Appl Environ Microbiol,* **61,** 79-86.

Desmond, O. J., Edgar, C. I., Manners, J. M., Maclean, D. J., Schenk, P. M. and Kazan, K. (2005) Methyl jasmonate induced gene expression in wheat delays symptom development by the crown rot pathogen *Fusarium pseudograminearum*. *Physiol Mol Plant P,* **67,** 171-179.

Egan, M. J., Wang, Z. Y., Jones, M. A., Smirnoff, N. and Talbot, N. J. (2007) Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *PNAS,* **104,** 11772-11777.

Goswami, R. S. and Kistler, H. C. (2005) Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice. *Phytopathology,* **95,** 1397-1404.

Hohn, T. M. and Desjardins, A. E. (1992) Isolation and Gene Disruption of the Tox5 Gene Encoding Trichodiene Synthase in *Gibberella pulicaris*. *Mol Plant-Microbe Interact,* **5,** 249-256.

Houot, V., Etienne, P., Petitot, A.-S., Barbier, S., Blein, J.-P. and Suty, L. (2001) Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. *J. Exp. Bot.,* **52,** 1721-1730.

Jansen, C., von Wettstein, D., Schafer, W., Kogel, K.-H., Felk, A. and Maier, F. J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *P Natl Acad Sci USA,* **102,** 16892-16897.

Jayaraj, J., Muthukrishnan, S., Liang, G. H. and Velazhahan, R. (2004) Jasmonic acid and salicylic acid induce accumulation of beta-1,3-glucanase and thaumatin-like proteins in

wheat and enhance resistance against *Stagonospora nodorum*. *Biol Plantarum,* **48,** 425-430.

Kazan, K., Manners, J. M. and Cameron, D. F. (1993) Genetic-variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. *Theor Appl Genet,* **85,** 882-888.

Langevin, F., Eudes, F. and Comeau, A. (2004) Effect of trichothecenes produced by *Fusarium graminearum* during Fusarium head blight development in six cereal species. *Eur J Plant Pathol,* **110,** 735-746.

Larsen, J. C., Hunt, J., Perrin, I. and Ruckenbauer, P. (2004) Workshop on trichothecenes with a focus on DON: summary report. *Toxicol Lett,* **153,** 1-22.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell,* **79,** 583-593.

Liu, G., Sheng, X., Greenshields, D. L., Ogieglo, A., Kaminskyj, S., Selvaraj, G., *et al.* (2005) Profiling of wheat class III peroxidase genes derived from powdery mildew-attacked epidermis reveals distinct sequence-associated expression patterns. *Mol. Plant-Microbe Interact.,* **18,** 730-741.

Mackintosh, C., Lewis, J., Radmer, L. E., Shin, S., Heinen, S. J., Smith, L. A., *et al.* (2007) Overexpression of defense response genes in transgenic wheat enhances resistance to Fusarium head blight. *Plant Cell Rep,* **26,** 479-488.

Maier, F. J., Miedaner, T., Hadeler, B., Felk, A., Salomon, S., Lemmens, M., *et al.* (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. *Mol Plant Pathol,* **7,** 449-461.

Masuda, D., Ishida, M., Yamaguchi, K., Yamaguchi, I., Kimura, M. and Nishiuchi, T. (2007) Phytotoxic effects of trichothecenes on the growth and morphology of Arabidopsis thaliana. *J. Exp. Bot.,* **58,** 1617-1626.

Miller, J. D. and Ewen, M. A. (1997) Toxic effects of deoxynivalenol on ribosomes and tissues of the spring wheat cultivars Frontana and Casavant. *Nat Toxins,* **5,** 234-237.

Mitter, V., Zhang, M. C., Liu, C. J., Ghosh, R. and Chakraborty, S. (2006) A high throughput glasshouse bioassay to detect crown rot resistance in wheat germplasm. *Plant Pathol,* **55,** 433-441.

Mudge, A. M., Dill-Macky, R., Dong, Y., Gardiner, D. M., White, R. G. and Manners, J. M. (2007) A role for the mycotoxin deoxynivalenol in stem colonisation during crown rot disease of wheat caused by Fusarium graminearum and F. pseudograminearum *Physiol Mol Plant P,* **69,** 73-85.

Munkvold, G. P., Hellmich, R. L. and Rice, L. G. (1999) Comparison of fumonisin concentrations in kernels of transgenic Bt maize hybrids and nontransgenic hybrids. *Plant Dis,* **83,** 130-138.

Navarre, D. A. and Wolpert, T. J. (1999) Victorin induction of an apoptotic/senescence-like response in oats. *Plant Cell,* **11,** 237-249.

Nishiuchi, T., Masuda, D., Nakashita, H., Ichimura, K., Shinozaki, K., Yoshida, S., *et al.* (2006) *Fusarium* phytotoxin trichothecenes have an elicitor-like activity in *Arabidopsis thaliana*, but the activity differed significantly among their molecular species. *Mol. Plant-Microbe Interact.,* **19,** 512-520.

Pestka, J. J., Zhou, H. R., Moon, Y. and Chung, Y. J. (2004) Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicol Lett* **153,** 61-73.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. and Moorman, A. F. M. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters,* **339,** 62-66.

Rocha, O., Ansari, K. and Doohan, F. M. (2005) Effects of trichothecene mycotoxins on eukaryotic cells: A review. *Food Addit Contam,* **22,** 369-378.

Ryerson, D. E. and Heath, M. C. (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell,* **8,** 393-402.

Tada, Y., Hata, S., Takata, Y., Nakayashiki, H., Tosa, Y. and Mayama, S. (2001) Induction and signaling of an apoptotic response typified by DNA laddering in the defence response of oats to infection and elicitors. *Mol Plant Microbe Interact,* **14,** 477-486.

Wilde, F. and Miedaner, T. (2006) Selection for Fusarium head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat. *Plant Breeding,* **125,** 96-98.

Yoda, H., Hiroi, Y. and Sano, H. (2006) Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. *Plant Physiol.,* **142,** 193-206.

Zimmermann, G., Baumlein, H., Mock, H.-P., Himmelbach, A. and Schweizer, P. (2006) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. *Plant Physiol.,* **142,** 181-192.

Figure Legends

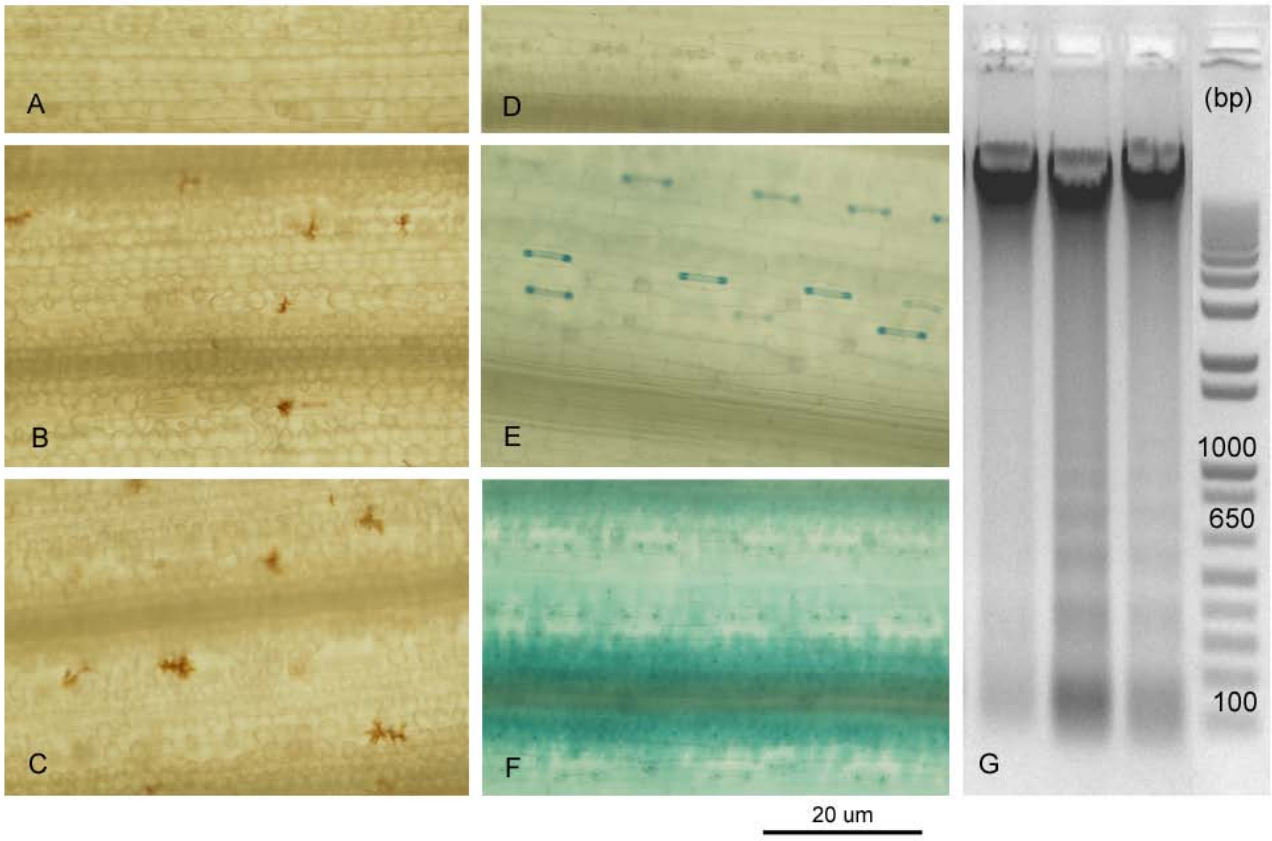

Figure 1. Infiltration of wheat tissue with DON resulted in H_2O_2 production, cell death and DNA laddering. DAB stained wheat leaf tissue from two-week old seedlings showing H_2O_2 production at 6 h after (A) mock infiltration, (B) 100 mg L^{-1} DON infiltration, and (C) 200 mg L^{-1} DON infiltration. Trypan blue stained wheat leaf tissue 24h after (D) mock infiltration, (E) 100 mg L^{-1} DON infiltration, and (F) 200 mg L^{-1} DON infiltration. (G) Genomic DNA laddering in wheat 24h after infiltration with mock solution shown in the first lane, 100 mg L^{-1} DON shown in the second lane, 100 mg L^{-1} DON combined with 7 g L^{-1} ascorbic acid shown in the third lane and the 1 kb+ DNA size ladder (Invitrogen) in the fourth lane. Further images of tissue from all infiltration treatments are shown in Supp. Fig. 1.

Table 1

Summary of H_2O_2 and cell death observed in infiltrated wheat leaf tissue stained with DAB 6h after treatment and stained with trypan blue 24h after treatment. Scale: **+ some stomata affected or sporadic** H_2O_2 **microbursts in some tissue; ++ most stomata affected or many** H_2O_2 **microbursts in some tissue; +++ all stomata affected or many microbursts in all tissue; ++++ widespread tissue affected including stomata and mesophyll cells.** Further images of tissue described in this table are shown in Supp. Fig. 1.

	H_2O_2 - 6h	Cell death - 24h
Mock	-	+
100 mg L^{-1} DON	+++	++
200 mg L^{-1} DON	+++	++++
100 mg L^{-1} DON + 7 g L^{-1} AA	++	+
100 mg L^{-1} DON + 4 mg L^{-1} CHX	++	-

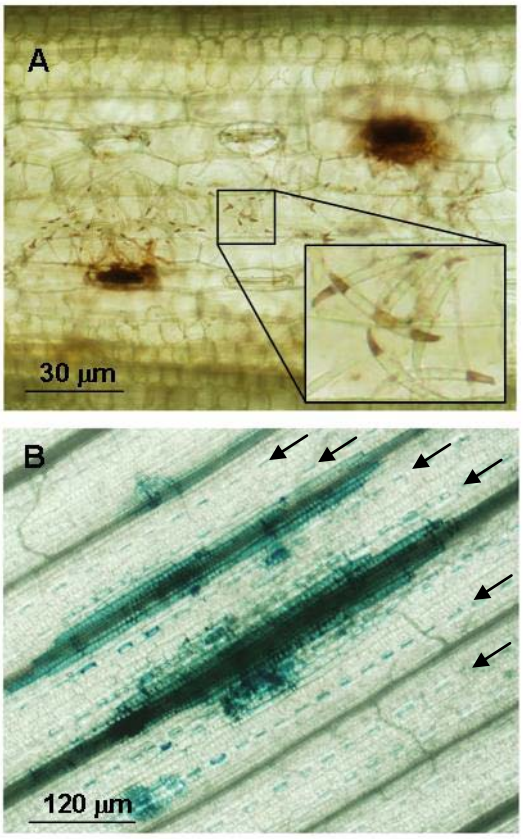

Figure 2

H_2O_2 production and cell death was observed in wheat leaves after inoculation with *F. pseudograminearum* spores. (A) Wheat leaf tissue was inoculated using a detached leaf assay stained using DAB 7 days later. H_2O_2 was visible in stomata that were in close proximity to spores and also in the spore tips (inset). (B) Trypan blue staining of inoculated leaf tissue showed cell death seemed to follow vascular tissue and was most wide-spread in stomatal guard cells. Rows of affected stomatal guard cells that surround the primary infection site are indicated by arrows. Further images of cell death in inoculated tissue are shown in Supp. Fig. 2.

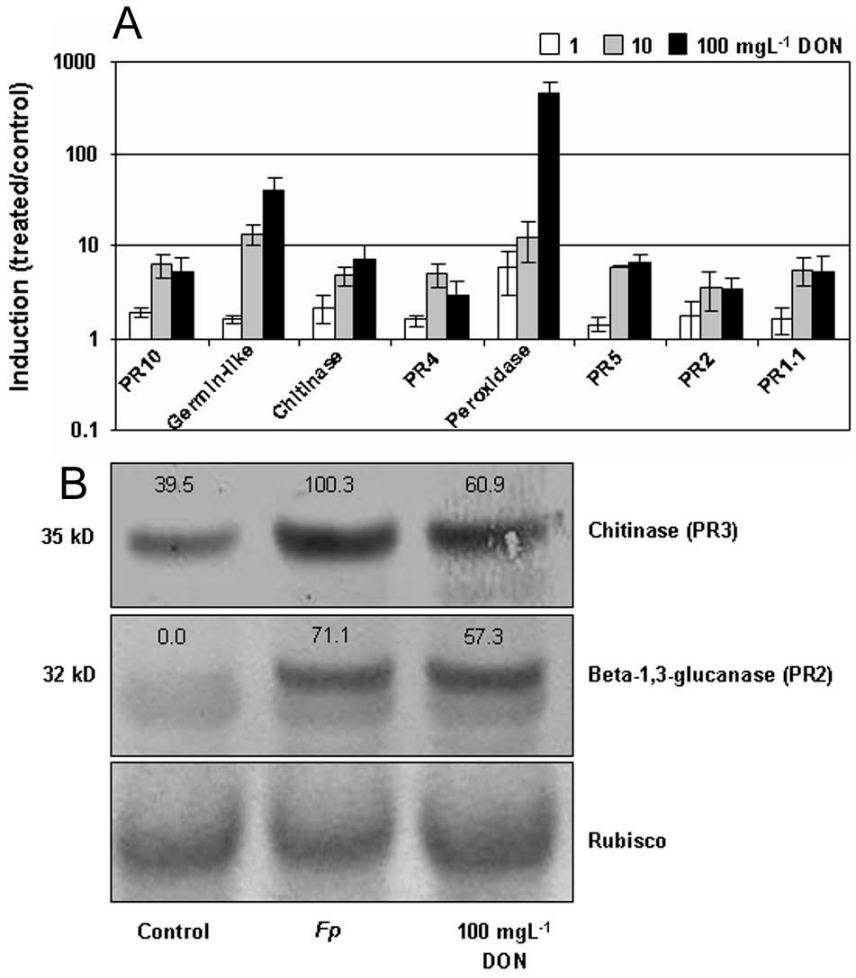

Figure 3. (A) Induction of defence gene expression in two-week old seedlings at 1 day after treatment with 1, 10, and 100 mg L^{-1} DON. Columns represent average induction ratios (±SE; n=3) of gene transcripts in treated compared to mock-treated and are plotted on a logarithmic scale. (B) Western blot analysis of total protein extracted from wheat tissue using β-1,3-glucanase (PR2) and chitinase (PR3) antibodies 2 days after mock treatment, *F. pseudograminearum* inoculation, or 100 mg L^{-1} DON treatment. Lower panel shows Rubisco stained with Ponceau red to show protein loading. The numbers above bands for chitinase and β-1,3-glucanase indicate protein levels as a percentage of rubisco. Total protein (20 μg) was separated on a 4-12% polyacrylamide gradient gel. Protein molecular masses are indicated on the left.

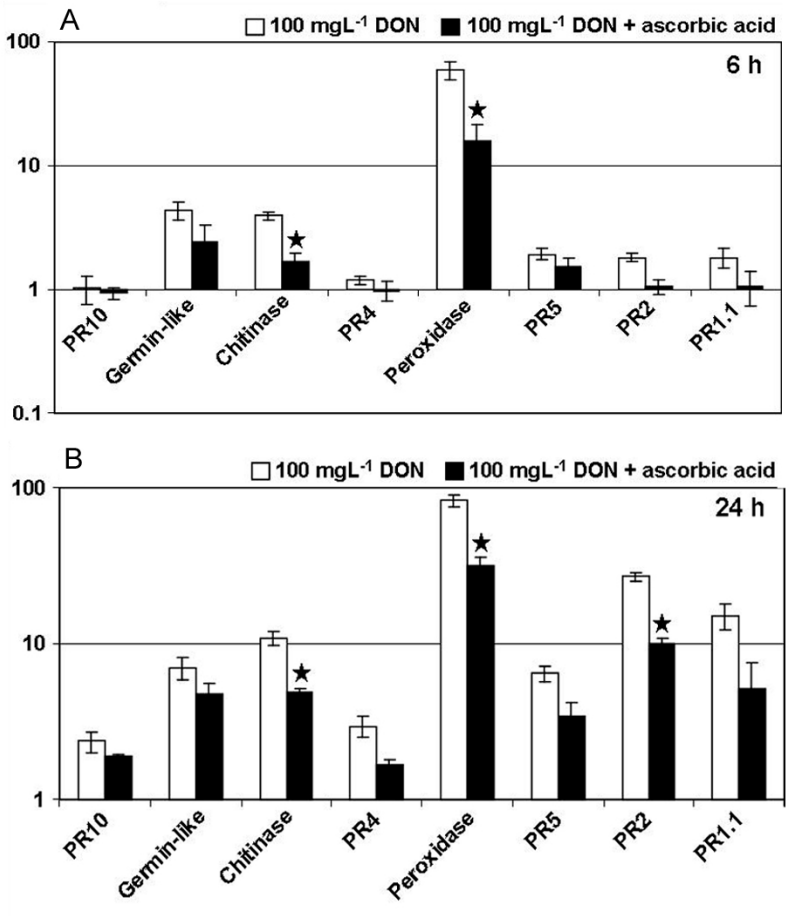

Figure 4. Induction of defence gene expression in two-week-old wheat seedlings (A) 6h and (B) 24h after treatment with 100 mg L^{-1} DON and a combination of 100 mg L^{-1} DON and 7 g L^{-1} ascorbic acid. Columns represent average induction ratios (±SE; n=3) of gene transcripts in treated compared to mock-treated plants and are plotted on a logarithmic scale. Statistically significant differences in gene induction (Student's t-test, $p < 0.05$) resulting from treatments including ascorbic acid are indicated by a star.

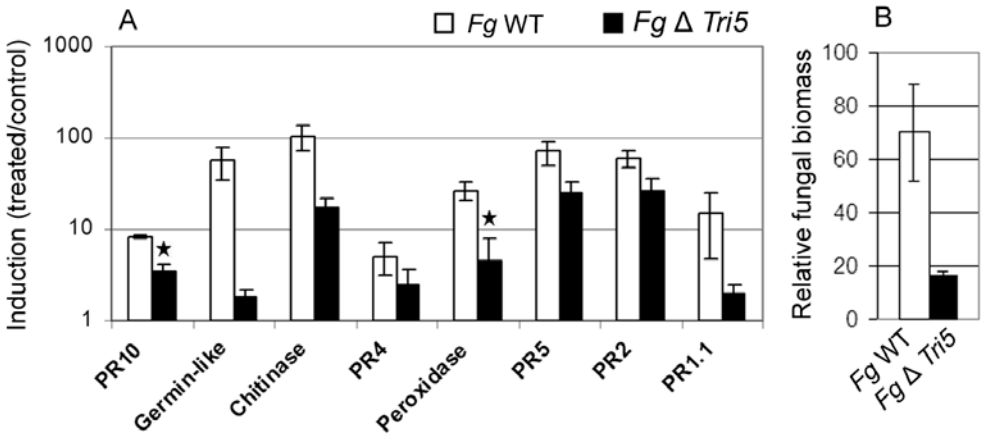

Figure 5: (A) Induction of defence gene expression 28 days after inoculation with the DON-nonproducing *F. graminearum Tri5* deletion line (*Fg* Δ*Tri5*) and WT. Columns represent average induction ratios (±SE; n=3) of gene transcripts in treated compared to mock-treated plants and are plotted on a logarithmic scale. Statistically significant differences in gene induction (Student's t-test, $p < 0.05$) resulting from treatments including ascorbic acid are indicated by a star. (B) Fungal biomass 28 days after inoculation with the DON-nonproducing *F. graminearum Tri5* deletion line and WT. Columns represent average amplification of *Fg rRNA18S* relative to wheat *actin* (±SE; n=3).