New Phytologist

Fusarium graminearum gene deletion mutants *map1* and *tri5* reveal similarities and differences in the pathogenicity requirements to cause disease on Arabidopsis and wheat floral tissue

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Received: *1 August 2007*
Accepted: *11 November 2007*

Summary

• The Ascomycete pathogen *Fusarium graminearum* can infect all cereal species and lower grain yield, quality and safety. The fungus can also cause disease on *Arabidopsis thaliana*. In this study, the disease-causing ability of two *F. graminearum* mutants was analysed to further explore the parallels between the wheat (*Triticum aestivum*) and Arabidopsis floral pathosystems.

• Wild-type *F. graminearum* (strain PH-1) and two isogenic transformants lacking either the mitogen-activated protein kinase *MAP1* gene or the trichodiene synthase *TRI5* gene were individually spray- or point-inoculated onto Arabidopsis and wheat floral tissue. Disease development was quantitatively assessed both macroscopically and microscopically and deoxynivalenol (DON) mycotoxin concentrations determined by enzyme-linked immunosorbent assay (ELISA).

• Wild-type strain inoculations caused high levels of disease in both plant species and significant DON production. The *map1* mutant caused minimal disease and DON accumulation in both hosts. The *tri5* mutant, which is unable to produce DON, exhibited reduced pathogenicity on wheat ears, causing only discrete eye-shaped lesions on spikelets which failed to infect the rachis. By contrast, the *tri5* mutant retained full pathogenicity on Arabidopsis floral tissue.

• This study reveals that DON mycotoxin production is not required for *F. graminearum* to colonize Arabidopsis floral tissue.

Key words: *Arabidopsis thaliana*, deoxynivalenol, ear blight, *Fusarium graminearum*, *Gibberella zeae*, head scab, pathogenicity factor, *Triticum aestivum*. Gene ontology (GO) terms, GO:0009405 and GO:0044403.

New Phytologist (2008) **177**: 990–1000

doi: 10.1111/j.1469-8137.2007.02333.x

Introduction

Fusarium graminearum (teleomorph *Gibberella zeae*) is a filamentous Ascomycete capable of causing disease. Initially named *Gibberella saubinetti*, this pathogen emerged to cause damaging head scab epidemics in *c.* 1916 in the hard red spring wheat (*Triticum aestivum*) regions of the USA (Pugh & Johann, 1933). Although the severity and incidence of the disease in the USA diminished in subsequent decades, in the 1990s a global pandemic began to emerge. This has since seriously threatened many wheat, maize (*Zea mays*) and barley (*Hordeum vulgare*) growing regions in North America, Europe

and the Far East (Bai & Shaner, 2004). All small-grain cereal crops can become infected at anthesis, when either rain-splashed conidia or airborne ascospores, both arising from infected cereal stubble, alight on the floral tissue, germinate and infect. Within the next 10–20 d the spikelets are sequentially colonized by both intracellular and intercellular hyphae (Goswami & Kistler, 2004) and premature senescence of some or all of the floral tissue occurs. The grain subsequently produced is of reduced quality and/or size. When in association with plant tissue *F. graminearum* hyphae can produce several different B-type trichothecene mycotoxins, including deoxynivalenol (DON), nivalenol (NIV) and the acetylated derivatives 3-acetyl and 15-acetyl deoxynivalenol (3-ADON and 15-ADON) (Hohn *et al.*, 1998). These mycotoxins are harmful to eukaryotic cells because DON binds to the peptidyl transferase protein in the ribosomes and inhibits protein translation (Cundliffe *et al.*, 1974). In both the USA and Europe, there are now strictly enforced limits as to the concentrations of DON mycotoxins permitted in unprocessed grain as well as in processed foods and animal feed (European Commission Regulation No. 856/2005; advised by the US Food and Drug Administration; Jansen *et al.*, 2005). Control of head scab disease, referred to as ear blight disease in Europe, includes careful placement of cereals in cropping sequences (Parry *et al.*, 1995), ploughing in of infected stubble residues (Parry *et al.*, 1995), the timely application of azole-based fungicides to floral tissue (Nicholson *et al.*, 2003) and the use of quantitative trait loci-based semi-resistant cereal genotypes which reduce the rate of spread through the infected tissue (Somers *et al.*, 2005). However, disease control levels seldom exceed 50%. In the UK this disease is now a serious problem in one year in five, whereas in parts of the USA, China and continental Europe severe epidemics are an annual occurrence.

Previous studies have shown that *F. graminearum* and the related pathogenic species *F. culmorum* are able to infect the floral tissue (anthers, anther filaments, petals, sepals, siliques, pedicels and seed) of the model plant species *Arabidopsis thaliana* (Urban *et al.*, 2002). Disease levels on both flowers and siliques can be quantified using a Fusarium–Arabidopsis disease (FAD) scoring system. The robustness of this pathosystem has been verified by others (Van Hemelrijck *et al.*, 2006). In the Fusarium–Arabidopsis floral pathosystem, considerable DON mycotoxin production is detected by 8 d post-inoculation (dpi). Also, when anthers and functional pollen are absent in either wheat or Arabidopsis, disease levels are significantly reduced (Strange & Smith, 1971; Urban *et al.*, 2002). The Fusarium–Arabidopsis floral pathosystem is a highly tractable experimental interaction which can be used to explore the mechanisms conferring host defence and fungal virulence.

Molecular analysis of *Fusarium* virulence has been facilitated since the release of the *F. graminearum* genomic sequence in 2003 at the Broad Institute of MIT and Harvard (Cuomo *et al.*, 2007). Gene disruption/deletion studies have so far implicated 16 genes in virulence, encoding components of transcription, signal transduction, host-specific nutrition, host infection/colonization and trichothecene biosynthesis (http://www.phi-base.org version 3.0; Winnenburg *et al.*, 2007). Key signal transduction genes identified include a transducin beta-subunit gene, *TBL1* (Seong *et al.*, 2005), and genes encoding components of three different mitogen-activated protein (MAP) kinase signalling cascades, including the MAP kinase genes *MGV1* (Hou *et al.*, 2002), *FgHOG1/OS-2* (Ochiai *et al.*, 2007a,b; Ramamoorthy *et al.*, 2007) and *MAP1*/*GPMK1* (Jenczmionka *et al.*, 2003; Urban *et al.*, 2003; Ramamoorthy *et al.*, 2007). Of the three MAP kinase mutants, the *map1* mutants constructed from various strains are the most highly reduced in virulence on flowering wheat; hyphae are restricted to the site of inoculation, while still able to produce DON. The Map1 protein has been shown to be required for the early induction of different extracellular enzyme activities including a secreted *F. graminearum* lipase Fgl1 (Jenczmionka & Schafer, 2005; Voigt *et al.*, 2005). The latter is also essential for pathogenicity. Trichothecene mycotoxin production has been shown to contribute to virulence of *F. graminearum* towards wheat (Proctor *et al.*, 1995, 1997) and is required for successful rachis colonization through rachis internodes (Jansen *et al.*, 2005). DON biosynthesis requires ~15 biochemical steps (Desjardins, 2006). The initial committing step into the trichothecene biosynthetic pathway is catalysed by the enzyme trichodiene synthase (Supplementary material Fig. S1a) to produce the intermediate trichodiene (Proctor *et al.*, 1995; Hohn *et al.*, 1998; Tag *et al.*, 2000). Trichodiene synthase is encoded by the *TRI5* gene. DON production is not constitutive and is specifically induced *in planta*. In three *Fusarium* species, at least 10 of the trichothecene biosynthesis genes are located in close proximity to the *TRI5* gene, in a single gene cluster named the trichothecene (*TRI*) gene cluster (Kimura *et al.*, 2003). In *F. graminearum* (strain PH-1) this cluster is located on chromosome 2 (http://www.broad.mit.edu). However, four genes known to be required for trichothecene biosynthesis reside outside the *TRI* cluster (Alexander *et al.*, 2004).

A recent review has classified flower-infecting fungi into three groups based on a broad range of biological and life history attributes (Ngugi & Scherm, 2006). Briefly, these are unspecialized tissue colonizers (Group 1) and specialized tissue colonizers with either gynoecial entry (Group 2) or entry via the apical meristem (Group 3). Although the flower-infecting nonspecialist *F. graminearum* fits loosely into Group 1, this fungus exhibits two distinguishing features. First, annual infections are predominantly monocyclic, not polycyclic, and occur only once at anthesis (Paulitz *et al.*, 1999). Secondly, non-infected flowers within an infected inflorescence frequently fail to produce viable seed either because host-tissue constriction causes limited access to nutrients/water or because the developing seed has accumulated detrimentally high concentrations of mycotoxins (Savard *et al.*, 2000). The positioning of the mycotoxin-producing species *F. graminearum* in a distinct

Group 1 subgroup is clearly warranted. To date, only one species of *Fusarium* and two species of *Gibberella* have been placed into the Ngugi and Scherm classification scheme, whereas 17 related *Fusarium* species are capable of causing *Fusarium* ear blight disease.

To further understand the parallels between the *F. graminearum*–wheat and the *F. graminearum*–Arabidopsis floral pathosystems, infection of the susceptible Arabidopsis ecotype Landsberg *erecta* and susceptible wheat cultivar Bobwhite was explored in detail using the sequenced strain PH-1 and two isogenic single gene deletion mutants which exhibit reduced disease-causing ability on wheat floral tissue. The MAP kinase mutant *map1*, which can only infect wheat anthers (Urban *et al.*, 2003), caused minimal disease development on Arabidopsis flowers and siliques. Interestingly, the *F. graminearum tri5* mutant, which produces no DON mycotoxin, retains full pathogenicity on Arabidopsis floral tissue, whilst on wheat ears this strain exhibits a reduced virulence phenotype. These data reveal that *F. graminearum* does not require DON mycotoxin production for disease formation in Arabidopsis floral tissue.

Materials and Methods

Fusarium graminearum strains and culturing conditions

The *F. graminearum* strain PH-1 (NRRL 31084) originated from the USA and is a DON/15-ADON producer (Goswami & Kistler, 2005). *Fusarium graminearum* gene deletion mutants are PH-1 *map1* (MU58) and PH-1 *tri5* (MU102) (this study). Strains were routinely propagated on synthetic nutrient-deficient agar (SNA) and stored at −80°C as described previously (Urban *et al.*, 2002). To prepare fresh conidia in high amounts for plant inoculations, 7-d-old SNA plates were overlaid with 500 μl of sterile YPS medium (0.3% (w/v) yeast extract, 0.3% (w/v) bacto peptone and 20% (w/v) sucrose). Old conidia were re-suspended and discarded together with the YPS medium. Plates were then incubated for an additional 24 h. Conidia were re-suspended in water, filtered through miracloth (Calbiochem, La Jolla, CA, USA) and diluted in water to the appropriate concentration.

Experimental design, inoculation and statistical analysis

Arabidopsis growth conditions *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg *erecta* (L*er*-0) (stock ID NW20) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seed was sown into moist Levington's F2s compost and kept in the dark at 4°C for 4 d to ensure synchronous germination. Plants were grown in a controlled-environment growth room with a temperature of 23°C : 20°C and a 16 h light : 8 h dark cycle, 400 μmol m^{-2} s^{-1} and 70% humidity.

Fusarium–Arabidopsis floral inoculation and scoring of disease symptoms For floral spray inoculations, plants were selected with at least two open flowers and no more than three siliques present on their primary bolts. Each plant was sprayed with either 0.5 ml of 1×10^5 *Fusarium* conidia ml^{-1} or water as a mock control. This quantity of wild-type inoculum routinely generated uniform disease on all plants. To generate the optimum conditions for disease formation, inoculated plants were placed into racks placed above a 1 cm depth of water inside a perspex (10-mm-thick) inoculation box (length 100 cm, width 60 cm and height 60 cm) made at Rothamsted Research (Harpenden, UK). The sealed boxes were then placed in darkness for the first 16 h to encourage conidia germination and infection. Plants were assessed at 8 dpi using a numerical FAD scoring system for two floral subcomponents, namely flowers (F) which were buds at the time of inoculation and new siliques (NS) which were fully open flowers at inoculation. Old silique (OS) data were omitted from the calculation because younger plants were used for inoculation. In Supplementary material Table S1, this alternative FAD-individual floral component (FAD-I) quantitative scoring key is fully described.

For the silique wound inoculation experiments, older plants were selected with several full-size siliques. The stigma end of the silique had ~2 mm cut off with sterile scissors and a 2-μl droplet of inoculum containing ~200 conidia was placed on the wound.

Statistical analysis of Fusarium–Arabidopsis disease data For the Arabidopsis data sets, two sets of experiments were performed comparing disease severity scores between the *F. graminearum* wild-type strain PH-1 and either of the mutants *map1* and *tri5*. Each *F. graminearum* mutant data set consisted of three independently replicated experiments. For the statistical analysis, each *F. graminearum* mutant data set was analysed separately. An ANOVA was performed on the plot means for each group of plants and linear models were fitted using GenStat 8.0 (Payne *et al.*, 2005) with weighting according to the number of plants per mean, which ranged from four to 15.

Plant infection and pathogenicity tests on wheat Wheat (*Triticum aestivum* L.) plants of cultivar Bobwhite were grown and infected by point, spray or wound inoculation as previously described (Urban *et al.*, 2003). Infection was followed over a time period of 1–20 d. Disease progress was recorded as the number of visibly diseased spikelets divided by the number of infectable spikelets per wheat ear below and at the inoculation point. Each experiment was performed in triplicate.

Trichothecene mycotoxin analysis

The Arabidopsis tissues sampled for DON determination were taken from floral spray-inoculated plants at 8 dpi. The primary apical stem including buds, flowers, siliques and stem

tissue (no lateral branches or cauline leaves) was harvested into liquid nitrogen and stored at −80°C before processing. Typically, 6–12 apices were pooled and ground in liquid nitrogen. On average, 400 mg of sample tissue was diluted for use in the DON enzyme-linked immunosorbent (ELISA) kit. DON concentrations were calculated as parts per million (ppm) from fresh weight tissue. Whole wheat ears for mycotoxin analysis were inoculated and processed as described previously (Maier *et al.*, 2006), with slight modifications. Entire spikes were harvested at 20 dpi and ground to a fine powder in liquid nitrogen using a mortar and pestle.

The commercially available Ridascreen® Fast DON ELISA kit (R-Biopharm AG, Darmstadt, Germany) was used to quantify DON mycotoxin in *F. graminearum*-inoculated wheat spikes and Arabidopsis inflorescences. DON standards included in the kit were used to produce a dose–response curve which was fitted with a rectangular hyperbola. DON concentrations in samples were calculated within the standard curve using the software package GenStat® (Payne *et al.*, 2005). Samples with high DON concentrations > 6 ppm were diluted with water and re-evaluated until the recorded value fell within the range of the standard curve.

Microscopy and histochemical stains

A Leica MZFLIII dissecting microscope with UV light and a violet filter (excitation at 425/40 nm and emission at 475 nm) was used to visualize samples. A Zeiss Axiophot light microscope was used to examine samples at higher magnifications. For scanning electron microscopy, the sample was mounted on a cryo stub using embedding medium and prepared in the Gatan Alto 2100 (Gatan UK, Abingdon, UK) for examination in the Jeol 6360 LVSEM (Jeol UK Ltd, Welwyn Garden City, UK). For light microscopy, plants were stained with lactophenol-trypan blue (Koch & Slusarenko, 1990) by boiling for 1 min, de-staining in chloral hydrate and mounting in 70% (v/v) glycerol.

Results

The *map1* and *tri5* mutants in *F. graminearum* strain PH-1 exhibit reduced virulence on wheat ears

In order to compare the *F. graminearum* infection modes between Arabidopsis and wheat, gene deletion mutants for two well-known *F. graminearum* virulence genes were created in the sequenced strain PH-1. The *MAP1* gene (FG06385) encodes a MAP kinase and is required for infection and colonization of wheat. Using a similar strategy as described previously for the *F. graminearum* strain 16A (Urban *et al.*, 2003), a gene replacement mutant was created. The PH-1 *map1* mutant grew abundantly on wheat anthers, only occasionally caused abortion of wheat embryo development and could not spread into the rachis from the point-inoculated spikelet. These results are consistent with results reported previously (Jenczmionka *et al.*, 2003; Urban *et al.*, 2003).

The *TRI5* gene (FG03537) in *F. graminearum* controls mycotoxin biosynthesis (Supplementary material Fig. S1a). In order to test the involvement of DON/15-ADON mycotoxin in Arabidopsis infection, the *TRI5* gene was deleted using the split-marker technique (Catlett *et al.*, 2003) as described in Supplementary Text S1. The *tri5* mutant showed reduced virulence when inoculated into wheat florets and displayed phenotypes similar to those described previously (Proctor *et al.*, 1995; Jansen *et al.*, 2005). However, when wheat ears were spray-inoculated, characteristic eye-shaped lesions developed on outer glumes with a distinctive dark purple/black edge and a bleached white centre (Fig. 1a), and fungal infection of the diseased florets frequently prevented grain development (data not shown). When wheat ears were wound-inoculated, aerial mycelium developed on glume surfaces. The aerial *tri5* mycelium spread to neighbouring spikelets and again caused formation of eye-shaped lesions (Fig. 1b). By contrast, in the *map1* mutant only sparse aerial mycelium developed and the disease was contained within the inoculated spikelets.

Dissection and UV light microscopy were used to reveal that the *tri5* mutant was unable to grow through the rachis node tissue in a point-inoculated wheat spike. Such an *in planta* growth defect was reported previously for a similar gene mutation created in the German *F. graminearum* strain 8/1 when tested on susceptible wheat cultivar Nandu (Jansen *et al.*, 2005). This analysis also confirmed that the spread of the *tri5* mutant was blocked by the development of reinforced cell walls at the rachis node in the wheat cultivar Bobwhite (data not shown). In addition, this experiment revealed that *F. graminearum* wild-type inoculated wheat ears showed severe rachis bleaching and deposition of autofluorescent plant phenolic compounds within 5 dpi (Fig. 1c). By contrast, at this early time-point *map1*, *tri5* and mock-inoculated florets did not exhibit any visible deposition of phenolic compounds in rachis tissue.

DON production in Arabidopsis and wheat floral tissue inoculated with wild-type *F. graminearum*, the *map1* mutant or the *tri5* mutant

Wild-type strain PH-1 *F. graminearum* infections resulted in the accumulation of moderate DON mycotoxin concentrations in Arabidopsis but much higher concentrations in wheat ears (Table 1). In both species the concentrations of DON were above the regulated European threshold for cereals. Overall, the DON levels detected in both species were higher than previously reported for the wild-type strain 16A (Urban *et al.*, 2003). The *map1* mutant produced low concentrations of DON in both Arabidopsis and wheat. However, the differences between

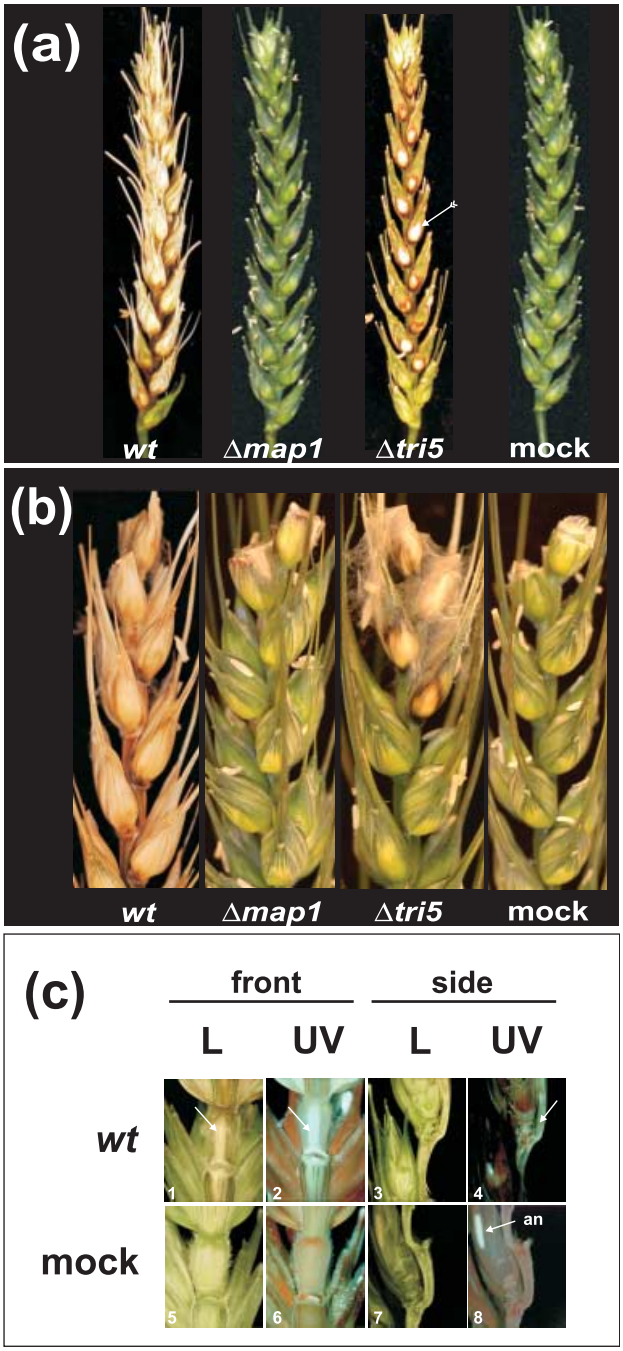

Fig. 1 Wheat (*Triticum aestivum*) ears infected with wild-type (*wt*) *Fusarium graminearum* PH-1, the mitogen-activated protein kinase Δ*map1* mutant or the trichodiene synthase Δ*tri5* mutant. (a) Ten days after spray inoculation with a conidial suspension (5×10^4 ml^{-1}) or mock inoculation with water as indicated. The Δ*tri5* fungus caused characteristic eye-shaped lesions (feathered arrow) on outer glumes of wheat spikelets but was unable to spread to adjacent rachis tissues. The Δ*map1* fungus was nonpathogenic and could only grow superficially on spikelet tissues and extruded anthers. (b) Wounded wheat ears droplet-inoculated with *F. graminearum* spore suspensions at 20 d post-inoculation. (c) Details at 5 dpi of the point-inoculated spikelet and adjacent rachilla and rachis tissues revealed by removing one spikelet. The left-hand pair of columns shows the front view, and the right-hand pair of columns the side view, observed under white light (L) and UV light (UV), as indicated. For the *wt*, the arrows denote where infection has led to rachis bleaching (panel 1) and deposition of blue-green autofluorescent compounds (panels 2 and 4). Healthy mock-inoculated tissues with a high chlorophyll content autofluoresced red under UV light (panels 6 and 8). An autofluorescent wheat anther (an) is indicated in panel 8.

Table 1 Deoxynivalenol (DON) mycotoxin analysis of *Fusarium graminearum* strain PH-1 (*wt*) and gene deletion mutants for trichodiene synthase (Δ*tri5*) and MAP kinase (Δ*map1*) following infection of Arabidopsis or wheat (*Triticum aestivum*) floral tissue

	DON production (ppm)[a]	
Fusarium genotype	Arabidopsis[b]	Wheat[e]
wt	2.8 ± 1.2[c]	270 ± 150[f]
Δ*map1*	0.2 ± 0.1	1.1 ± 0.7
Δ*tri5*	< 0.2[d]	< 0.2
Mock	< 0.2	< 0.2

[a]DON concentrations (ppm), based on plant fresh weight, were quantified by competitive enzyme-linked immunosorbent assay (ELISA).
[b]Spray-inoculated ecotype Landsberg *erecta*; the associated disease scores are given in Table 2. Combined floral and upper stem tissues were harvested at 8 d post-inoculation (dpi), pooled, frozen, ground and analysed for the presence of DON.
[c]Mean DON value ± SD obtained from three experiments for each individual mutant ($n = 3$) compared with the two controls ($n = 6$).
[d]DON value below the detection limit of 0.2 ppm.
[e]Every second spikelet of a spike of the wheat cultivar Bobwhite was inoculated with 200 conidia in a 10-μl droplet. Spikes were harvested 20 dpi, ground, and analysed for the presence of DON.
[f]DON value ± SD ($n = 3$).

the wild-type and *map1* mutant strain in wheat were particularly striking and indicate that the MAP kinase signalling cascade either directly or indirectly influences DON mycotoxin production. The DON concentrations produced in wheat by the *map1* mutant were lower than the European threshold for unprocessed grain of 1.25–1.75 ppm (European Commission Regulation No. 856/2005). The levels of DON produced in Arabidopsis with the *map1* mutant were only just above the detection threshold of < 0.2 ppm, with a range of values between < 0.2 and 0.3 ppm across three experiments. As anticipated, the *tri5* mutant was unable to produce DON mycotoxin in either Arabidopsis or wheat floral tissue. This latter result was consistent with previous reports (Proctor *et al.*, 1995; Desjardins *et al.*, 1996; Jansen *et al.*, 2005; Maier *et al.*, 2006). The higher DON concentrations detected in wheat compared with Arabidopsis were probably attributable to the much longer period of infection before tissue harvest (8 vs 20 d) and/or the fact that in wheat only alternate spikelets and not the entire floral tissue were inoculated, which permitted more seeds to develop and therefore accumulate DON.

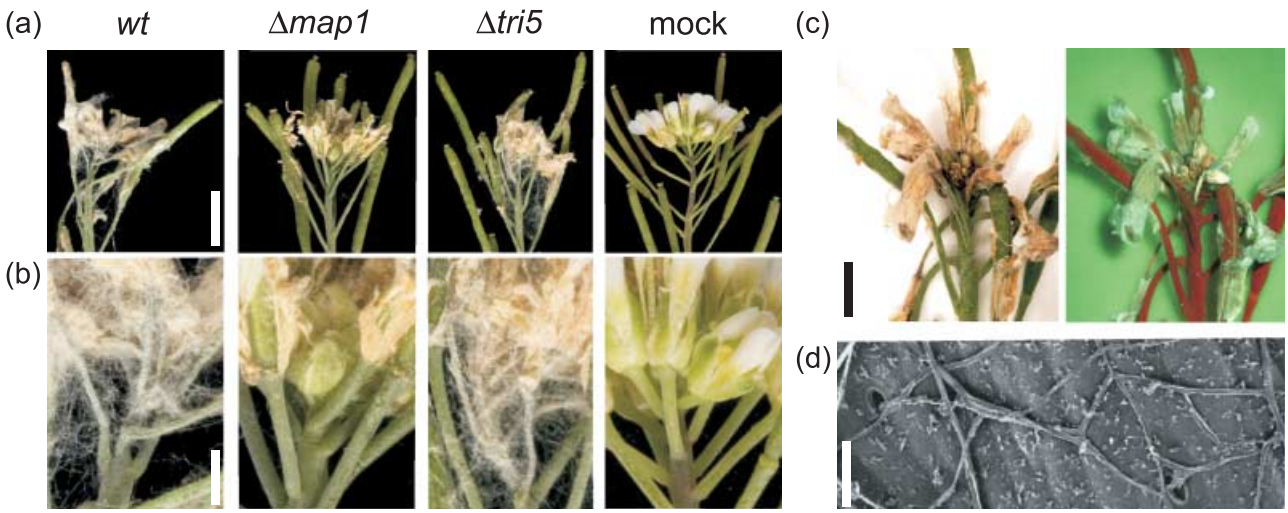

Fig. 2 Arabidopsis floral tissue infected with wild-type (*wt*) *Fusarium graminearum* PH-1, the mitogen-activated protein kinase Δ*map1* mutant or the trichodiene synthase Δ*tri5* mutant. (a) Floral disease 11 d post-inoculation (dpi) on Arabidopsis ecotype Landsberg *erecta* spray-inoculated with the indicated strains. (b) Close-up images from (a) highlighting the grey necrosis on the apical stem tissue evident in the *wt*- and Δ*tri5*-inoculated plants. (c) Floral disease 8 dpi with the *wt* strain viewed under light (left) and UV light (right). Necrotic tissues lack the red chlorophyll autofluorescence. (d) Scanning electron micrograph revealing *wt* fungal mycelium growing above the epidermal cell layer and through stomatal pores on silique tissue at 8 dpi. Bars: (a) 5 mm; (b) 1 mm; (c) 2 mm; (d) 25 μm.

Table 2 Arabidopsis ecotype Landsberg *erecta* mean disease scores 8 d post-inoculation (dpi) with wild-type (*wt*) and two different single gene deletion *Fusarium graminearum* mutants (mitogen-activated protein kinase Δ*map1* or trichodiene synthase Δ*tri5*)

	Fusarium graminearum genotype				*Fusarium graminearum* genotype			
Organ	*wt*	Δ*map1*	SEM	*P*-value	*wt*	Δ*tri5*	SEM	*P*-value
Flower	4.96	2.04	0.081	< 0.001	2.43[a]	3.00	0.174	0.554
New silique	6.42	1.11	0.098	< 0.001	3.19	3.42	0.251	0.060
n	93	93			72	72		

[a]A lower average disease score was observed in the Δ*tri5* comparative data set because one of the three experiments was scored at the earlier time-point of 6 dpi.
n, total number of plants per treatment per genotype.
The *wt*–Δ*map1* and *wt*–Δ*tri5* comparisons were performed separately.
In total, 25 and 6 degrees of freedom were used in the ANOVA for the Δ*map1* and Δ*tri5* data sets, respectively.

The *map1* mutant also exhibits reduced virulence on Arabidopsis floral tissue

To assist the reader in the interpretation of the inoculation results, a direct comparison between the indeterminate Arabidopsis floral apex and determinate wheat spike, along with the names of each floral structure, is given in Supplementary material Table S2.

Disease severities in both flowers and new siliques following spray inoculation were significantly lower with the *map1* mutant in comparison to infection levels with wild-type PH-1 (Table 2, Fig. 2). In the flowers the wild-type strain was able to cause stem constriction within the floral tissue, which resulted in a grey necrosis extending into the apical stem. By contrast, the *map1* mutant only developed aerial mycelium on sepals, petals and anthers, which was often accompanied by the drying of flowers. No stem constriction was evident. In the new siliques, the wild-type *F. graminearum* caused constriction of the pedicel and apical stem, whereas the *map1* mutant was again only able to grow aerially on the silique surface. In the mock-inoculated controls a purple coloration of stem and siliques was observed, which was not visible in the inoculated plants. The reason for this is unknown.

Localized trypan blue staining is indicative of host cell death (Koch & Slusarenko, 1990). Microscopic observations revealed that with the *map1* mutant some trypan blue-stained guard cells were visible on the pedicel and siliques, but not within

the noncolonized stem (data not shown). On the silique tissues, brown deposits were visible in the substomatal cavities neighbouring dead cells (data not shown). The *map1* hyphae were not observed growing within the green plant tissues.

Both the wild-type PH-1 strain and the *tri5* mutant exhibit full pathogenicity on Arabidopsis floral tissue

There were no statistically significant differences between disease severities of the wild-type *F. graminearum* strain PH-1 compared with the *tri5* mutant on either the Arabidopsis flowers or the new siliques (Table 2, Fig. 2). The extent of grey necrosis of the Arabidopsis tissue caused by *tri5* was the same as seen with PH-1. Also, the extensive visible aerial mycelium was the same between the mutant and wild-type strains. The *tri5* mutant caused wild-type levels of disease on Arabidopsis ecotype L*er*-0. This result indicates that Fusarium–Arabidopsis floral infection, colonization and symptom development are independent of DON mycotoxin production.

The wild-type fungus was able to cause cell death in the floral tissues as visualized under UV light (Fig. 2c) and with trypan blue staining (data not shown). Green chlorophyll-containing tissues autofluoresced red under UV light. By contrast, the necrotic buds, pedicel and apical stem tissues lacked chlorophyll and instead autofluoresced a green colour, possibly as a result of the deposition of host phenolic compounds. Multiple cell death sites were evident in the apical stem, pedicel and silique tissues. Extracellular hyphae were visible between the epidermal and upper mesophyll cell layers of the apical stem and pedicel tissues. Some but not all of the surrounding plant cells had accumulated the trypan blue stain. In each infected tissue, trypan blue staining was specific to the guard cells and neighbouring mesophyll cells, and no reaction was ever seen in the epidermal cell layer. In the silique tissues, faint brown deposits were visible surrounding dead cells. Hyphae were often seen protruding from some stomata in the colonized areas (Fig. 2d), which has previously been reported in Fusarium-infected Arabidopsis leaf tissues (Chen *et al.*, 2006).

Arabidopsis point inoculations of wounded siliques

A point inoculation technique was developed using apically wounded attached young siliques, ~10 mm in length. This permitted uniform disease progression and enabled a detailed study of the colonization of seeds, pedicels and stem. The *map1* mutant was again severely compromised in disease-causing ability, but did have the capacity to slowly grow through the interior of the silique (Fig. 3). The *tri5* mutant and wild-type strain had indistinguishable disease phenotypes and colonized tissues throughout the interior of the silique. Externally this was visible as a dark-brown necrotic ring, formed at the point of inoculation, moving down through the silique and leaving behind light-brown bleached tissue. Brown shrivelled seed surrounded by wild-type or *map1* mutant mycelium was seen to a greater or lesser extent, respectively. A general seed response to all strains was seen as a blue-green autofluorescence when viewed under a UV light source. This was brighter with the *map1* mutant, probably because of the slower rate of colonization and delayed seed death.

Colonization of the pedicel tissues was only just visible with the *map1* mutant by 11 dpi, and this coincided with the onset of natural senescence. By contrast, wild-type and *tri5* mycelium entered the pedicel from 7 dpi onwards and the colonized tissue autofluoresced blue-green. Disease progression of both the wild-type strain and the *tri5* mutant through the pedicel tissue towards the stem was slow during the period 11–14 dpi (~3 mm). On minimal agar plates, growth rates of 5 mm d^{-1} were typically observed (Urban *et al.*, 2003). *Fusarium graminearium* hyphae were not recovered from surface-sterilized stem tissues at 14 dpi (data not shown). These observations suggest that *F. graminearium* hyphae, whether producing or not producing DON mycotoxin, were able to colonize Arabidopsis pedicel tissues; however, entry into the pedicel base/mid-stem tissue was prevented by constitutive and/or induced host defence responses, the lack of a required susceptibility factor and/or an inability to suppress host defences.

Discussion

To further understand the parallels between the *F. graminearum*–wheat and *F. graminearum*–Arabidopsis floral pathosystems, inoculations were performed with two single gene deletion mutants with reduced ability to cause disease on wheat ears. The *tri5* mutant fails to produce DON mycotoxin, whereas the *map1* mutant is compromised in both initial penetration and tissue colonization. Arabidopsis infections, unlike wheat infections, were discovered to be DON independent: The *tri5* mutant retained full pathogenicity on Arabidopsis floral tissue. Arabidopsis infections with *map1* were similar to those of wheat ears, with minimal disease occurring on both intact and wounded floral tissue, and the ability to produce DON was reduced.

The modified FAD-I scoring system enabled a precise analysis of disease levels in individual floral subcomponents. The removal of disease assessments for the older siliques (OS) meant younger plants could be used for all experiments. The newly devised silique wounding point inoculation protocol provides an optimum environment for uniform and rapid *Fusarium* growth inside silique and pedicel tissue and evaluation of associated host responses. In the Fusarium–Arabidopsis pathosystem a high proportion of the *Fusarium* biomass was present as superficial aerial mycelium, and this therefore precluded the use of reverse transcriptase–polymerase chain reaction (RT-PCR) or quantitative PCR for fungal biomass quantification. The quantity of superficial mycelium was heavily influenced by the levels of humidity, therefore making a microscopic observation more accurate for assessment of this

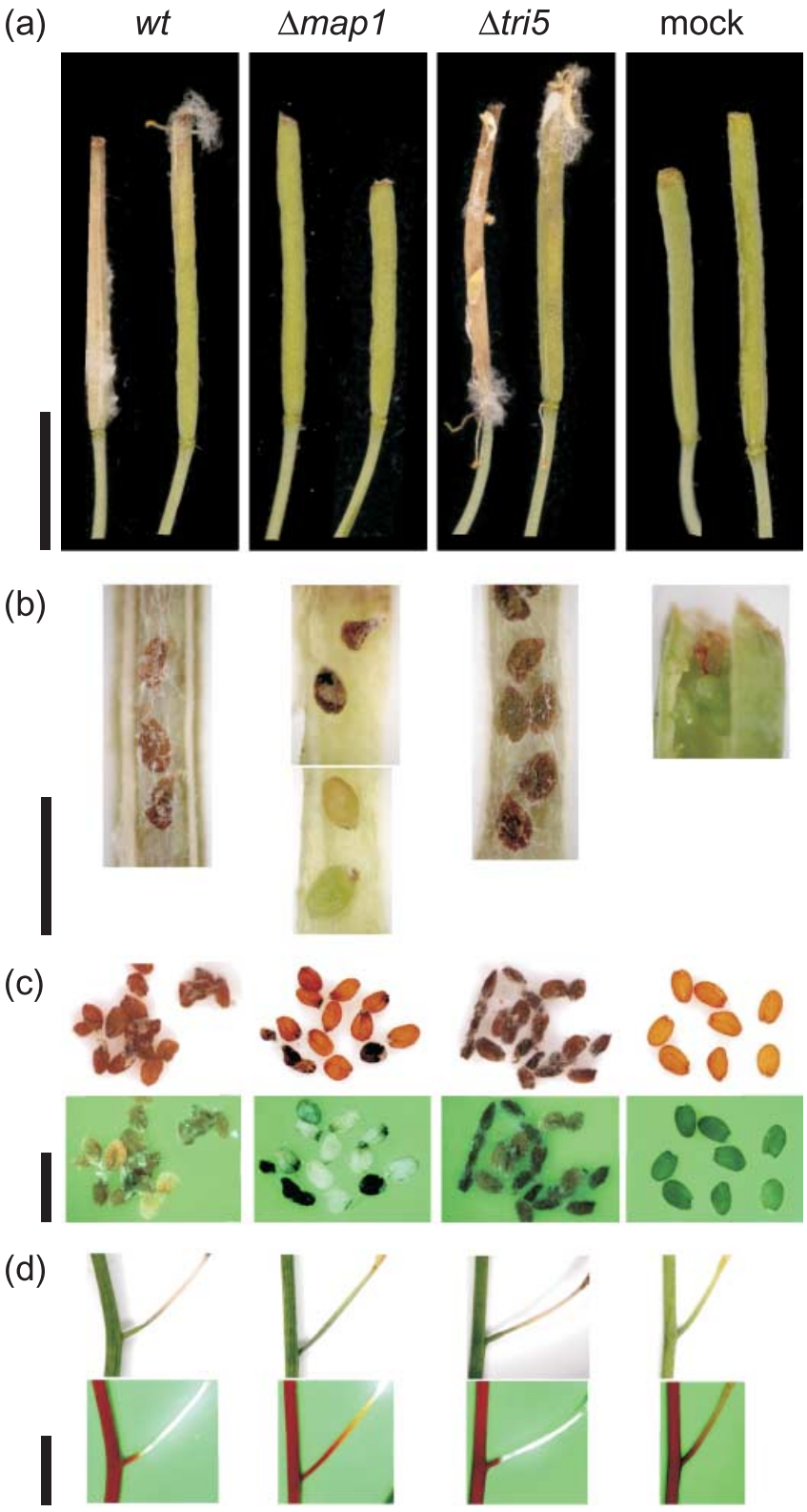

Fig. 3 Apically wounded Arabidopsis siliques point-inoculated with wild-type (*wt*) *Fusarium graminearum* PH-1, the mitogen-activated protein kinase Δ*map1* mutant or the trichodiene synthase Δ*tri5* mutant. (a) Siliques at 6 d post-inoculation (dpi). Two representative siliques are shown for each genotype. These represent the slight differences in disease progression evident within batches of *wt* and Δ*tri5* inoculations. Within 24 h, the silique on the right had symptoms equivalent to those on the left silique. *wt* and Δ*tri5* infections both caused a brown tissue discoloration and formed aerial mycelium on the silique surface. The Δ*map1* fungus caused a dark ring of necrosis and only a small tuft of aerial mycelium at the wound site while the silique valves remained green. No macroscopic disease symptoms were seen in the pedicels. (b) A valve from each silique was removed to visualize seed at 6 dpi. Dark-brown shrivelled seeds engulfed in mycelium were visible throughout the length of the *wt*- and Δ*tri5*-infected siliques. The Δ*map1*-inoculated siliques had dark-brown shrivelled seeds with mycelium at the site of wounding, followed by light-brown seeds and then unaffected green seeds with no visible hyphae in the rest of the silique. In the mock control, brown damaged seeds were visible only at the wound site, whilst the remaining unripe seeds were green. (c) Seeds recovered from inoculated siliques at 10–11 dpi, viewed under light (upper panel) and UV light (lower panel). The mock-inoculated seeds were ripe with a low level of autofluorescence. The Δ*map1* fungus continued to progress through the silique and a dark-brown area was visible on some light-brown seeds around the region of connection to the funiculus. The *wt* and Δ*tri5* strains caused extensive seed shrivelling. Under UV light, the light-brown seeds were highly autofluorescent irrespective of the fungal strain inoculated. This autofluorescence was most apparent with Δ*map1*. (d) Disease progression from the silique into the pedicel at 11 dpi viewed in the upper panel using light microscopy and in the lower panel under UV light. For the *wt* and Δ*tri5* strains, visible disease had spread from the silique into the pedicel, causing a brown discoloration. Under UV light the diseased pedicels autofluoresce blue-green, indicative of the host deposition of phenolic compounds. The red autofluorescence was from the chlorophyll present in healthy plant tissue. The Δ*map1* had also started to spread into the pedicel tissue. As the siliques ripened, host senescence in the pedicel of the mock control was visible as tissue yellowing (upper panel) and a lack of red autofluorescence but no blue-green autofluorescence (lower panel). Bars: (a, d) 5 mm; (b, c) 1 mm.

host–pathogen interaction. Transgenic strains harbouring a green fluorescent protein (GFP) reporter strain were also less than ideal for studying this interaction using intact floral tissue because of the high levels of autofluorescence detected in Arabidopsis and wheat anthers (see Fig. 1c, panel 8).

The first *tri5* strain unable to produce DON mycotoxin was constructed and revealed to cause reduced disease levels on wheat ears by Proctor *et al.* (1995). Since then, the role of DON in the disease-causing ability of *F. graminearum* has been found to vary depending on the host-specific infection and the tissue type under examination. These different experiments and their outcomes are presented in Supplementary material Table S3. In wheat, DON production is essential for the full virulence of *F. graminearum* on intact ear and stem tissue, irrespective of the strain or chemotype examined, whereas DON mycotoxins were found not to have a virulence role in the colonization of intact floral tissue of barley, maize and Arabidopsis or detached wheat floral tissue. When healthy Arabidopsis leaves were infiltrated with DON, plant cell death was only triggered at high concentrations (~30 ppm) (Nishiuchi *et al.*, 2006). This suggests that Arabidopsis leaf cells are relatively insensitive to the effects of DON. Only in detached and wounded Arabidopsis leaves has DON application been shown to slightly enhance *F. graminearum* disease levels following droplet inoculations (Chen *et al.*, 2006). However, as *Fusarium* is capable of aggressive saprophytic growth and detached leaves senesce, the value of this data set is questionable and needs to be re-addressed using an intact plant inoculation system.

Spray inoculation of wheat ears with the *tri5* mutant strain caused characteristic eye-shaped lesions to develop on glumes. Spray inoculation of wheat ears with the *tri5* mutant has to our knowledge not previously been reported. The eye-shaped lesions were strikingly similar to infections with fungal pathogens *Microdochium nivale* (Fig. 1a,b) and *Fusarium poae* (Jennings, 2005). *Microdochium nivale* is not known to produce mycotoxins, whereas *F. poae* is a mycotoxin producer. It is unclear why lesions with *F. poae* are eye-shaped. The centrally bleached lesions caused by the *tri5* mutant strain frequently develop on areas of the glumes that appear to show reduced chlorophyll content. Possibly this tissue is more susceptible to *Fusarium* attack.

A functional *F. graminearum* Map1 kinase signalling cascade is required for virulence/pathogenicity on both wheat and Arabidopsis. In an earlier study it was shown that a *map1* mutation in the *F. graminearum* strain 16A prevented colonization of tomato (*Lycopersicon esculentum*) fruit (Urban *et al.*, 2003). In the related pathogen *Fusarium oxysporum*, which causes vascular wilt disease on tomato and can also cause a vascular disease in immunocompromised mice, the *fmk1* MAP kinase deletion mutant was unable to infect tomato roots but shows full pathogenicity in mice. Thus it seems that the MAP kinase signalling cascade equivalent to Fus3/Kss1 MAP kinases in *Saccharomyces cerevisiae* is not an essential requirement for infection of all hosts (Di Pietro *et al.*, 2001; Ortoneda *et al.*, 2004).

The discovery that the Map1 signalling cascade is required for both Arabidopsis and wheat infection, while DON production is only essential for wheat infection, raises the question as to whether there are different classes of pathogenicity/virulence factors required for different host species and/or plant organs. For the rice blast pathogen *Magnaporthe grisea* it was shown that different pathogenicity genes are essential for leaf and root infection, with those compromised in appressorium formation only able to infect and colonize roots (Sesma & Osbourn, 2004). Besides the *MAP1* and *TRI5* genes, 14 other *F. graminearum* virulence genes have been identified (http://www.phi-base.org version 3.0; Winnenburg *et al.*, 2007). Most of these genes do not appear to affect *in vitro* growth or development. A systematic infection study of these gene deletion mutants on Arabidopsis would reveal if host-specific pathogen attack pathways exist in *F. graminearum*.

This study clearly revealed that *F. graminearum* was able to infect the Arabidopsis upper apical stem tissues but not the middle stem region (Figs 2b,c, 3d and Supplementary material Fig. S3), confirming what was observed previously (Urban *et al.*, 2002). There may be more basal defence in the mid-stem tissue than in the upper stem. Alternatively, light intensities, which influence defence, may be higher (Chappell & Hahlbrock, 1984). In wheat, various studies have previously noted that genotypes with lax ears exhibit lower disease levels than those with compact ears (Parry *et al.*, 1995).

The Fusarium–Arabidopsis floral pathosystem is ideally suited for molecular genetic investigations to study host resistance and fungal pathogenicity in parallel. For both species, full sequenced genomes are available. In addition, for Arabidopsis a vast collection of lines carrying single gene mutations can be accessed, a range of platforms are available for transcriptomic, proteomic and metabolomic analyses and already considerable data sets from healthy, biotic and abiotically stressed Arabidopsis plants have been archived for comparative experimental purposes (Zimmermann *et al.*, 2004). Although the genomic resources available for *F. graminearum* are more modest, the global community has in the past 10 yr already generated many single gene deletion mutants in various strains (www.phi-base.org) and extensive *in vitro* and *in vivo* Affymetrix array data sets are available (Guldener *et al.*, 2006). Obtaining a full genomic sequence for wheat is still a few years away, whilst creating and characterizing wheat mutant populations and generating stable wheat transformants are both still time- and resource-consuming activities compared with Arabidopsis. This study has revealed that full disease formation on Arabidopsis floral tissue can occur in the absence of DON mycotoxin production. By using a combination of transcriptomic-, proteomic- and metabolomic-based approaches with the *F. graminearum* wild-type and *tri5* mutant–Arabidopsis interaction, the DON-independent and DON-dependent events can be defined.

Acknowledgements

We would like to thank Ian Pearman and Julian Franklin for maintenance of controlled-environment facilities and Kerry Maguire for growing plants and recording data. Graham Shephard assisted with photographing the Arabidopsis floral tissues spray-inoculated with *F. graminearum* strains (Fig. 2a). Microscopy was performed in the Bioimaging facility at Rothamsted Research, and we thank Jean Devonshire for the SEM image. Salvador Gezan provided advice on experimental design and performed the statistical analysis. We are extremely grateful to colleagues at Rothamsted Research, Hai-Chun Jing, Rohan Lowe, John Lucas and Jason Rudd, for critical reading of earlier versions of the manuscript. All experiments involving *F. graminearum* strain PH-1, and isogenic mutants *map1* and *tri5* were conducted in biological containment facilities under DEFRA licence number PHL 174E/5543. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK. This study was additionally supported by a BBSRC responsive mode grant (BBS/B/12261).

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Construction of trichodiene synthase Δ*tri5* gene-replacement mutants.

Fig. S2 *In vitro* growth of *Fusarium graminearum* strains on diverse media.

Fig. S3 Apically wounded Arabidopsis siliques inoculated with wild-type (*wt*) *Fusarium graminearum* PH-1, the mitogen-activated protein kinase Δ*map1* mutant or the trichodiene synthase Δ*tri5* mutant.

Table S1 Description of the disease phenotype scoring system used to quantify the *Fusarium* infection of Arabidopsis floral tissue

Table S2 Arabidopsis and wheat floral anatomy

Table S3 Influence of host species, *Fusarium graminearum* strain and chemotype on deoxynivalenol (DON) requirement to cause disease

Text S1 Creation and evaluation of mitogen-activated protein kinase *map1* and trichodiene synthase *tri5* mutants in *F. graminearum*.

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