Arabidopsis is susceptible to the cereal ear blight fungal pathogens *Fusarium graminearum* and *Fusarium culmorum*

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Abstract

The fungal pathogens *Fusarium graminearum* and *F. culmorum* cause ear blight disease on cereal crops worldwide. The disease lowers both grain quality and grain safety. Disease prevalence is increasing due to changes in cropping practices and the difficulties encountered by plant breeders when trying to introgress the polygene-based resistance. The molecular basis of resistance to *Fusarium* ear blight in cereal species is poorly understood. This is primarily due to the large size of cereal genomes and the expensive resources required to undertake gene function studies in cereals. We therefore explored the possibility of developing various model floral infection systems that would be more amenable to experimental manipulation and high-throughput gene function studies. The floral tissues of tobacco, tomato, soybean and *Arabidopsis* were inoculated with *Fusarium* conidia and this resulted in disease symptoms on anthers, anther filaments and petals in each plant species. However, only in *Arabidopsis* did this initial infection then spread into the developing siliques and seeds. A survey of 236 *Arabidopsis* ecotypes failed to identify a single genotype that was extremely resistant or susceptible to *Fusarium* floral infections. Three *Arabidopsis* floral mutants that failed to develop anthers and/or functional pollen (i.e. *agamous-1*, *apetala1–3* and *dad1*) were significantly less susceptible to *Fusarium* floral infection than wild type. Deoxynivalenol (DON) mycotoxin production was also detected in *Fusarium*-infected flowers at >1 ppm. This novel floral pathosystem for *Arabidopsis* appears to be highly representative of a serious cereal crop disease.

Keywords: *Fusarium* ear blight, *Gibberella zeae*, *Arabidopsis*, deoxynivalenol, flower disease.

Introduction

Fusarium ear blight infections of cereal crops (wheat, maize, barley and rye) annually cause considerable losses to grain quality and safety in numerous geographical regions (Parry *et al.*, 1995; http://www.scabusa.org). The disease is disseminated by both sexual and asexual spores released from the previous year's crop debris. *Fusarium* species synthesise a range of mycotoxins *in planta*. Those of particular concern to human health are the sesquiterpenoid epoxide trichothecenes, primarily deoxynivalenol (DON), 15-acetyl DON and nivalenol produced by *Fusarium graminearum* Schwabe (sexual stage *Gibberella zeae* (Schw.)) (Hohn *et al.*, 1998), *F. nivale* and *F. culmorum* and fumonisins produced by *F. moniliforme* and *F. proliferatum* that are primarily a problem on maize and rice crops (Nelson *et al.*, 1993; Ueno *et al.*, 1970).

Fusarium infection of cereal ears occurs primarily only once per year at anthesis (flowering). For wheat, barley and rye, this occurs when the anthers extrude from the spike. This permits hyphae arising from the air-borne fungal spores to enter either via colonising the anther and then the filament or to penetrate directly into the top of the exposed ovary as each floret (flower) opens to release the three anthers (Kang and Buchenauer, 2000; Pugh, 1933). For certain wheat cultivars, if the anthers have been carefully removed by physical emasculation, then the incidence of infections is dramatically reduced (Strange and Smith, 1971). Anthers and pollen contain two components highly stimulatory to *Fusarium* hyphal growth and mycelium branching, namely choline and glycine betaine (Strange *et al.*, 1974). *Fusarium* mycotoxins are produced shortly after hyphae invade floral tissue. These mycotoxins may or may not contribute to host tissue penetration by hyphae (Kang and Buchenauer, 1999; McCormick *et al.*, 1998). Highly aggressive but none or very low mycotoxin

producing *Fusarium* isolates can be readily recovered from infected field crops (Gang *et al.*, 1998; Miedaner *et al.*, 2000). Further colonisation of plant floral tissue involves a mixture of intercellular, intracellular, as well as extensive saprophytic aerial growth (Kang and Buchenauer, 2000; Pugh, 1933). Macroscopically, infected wheat, barley and rye ears prematurely turn a light brown colour and under moist conditions, masses of pink conidia are visible on the infected grains and the various outer ear tissues, i.e. glumes, lemma and palea. The disease does not cause a soft rot symptom; all infected tissues remain structurally intact. At harvest, the threshed grain is often shrivelled and has a 'tombstone appearance' (http://www.scabusa.org). Frequently, this grain is also internally infected with fungal hyphae lying quiescent beneath the seed testa until seed germination occurs. Non-invaded grains on a partially invaded ear/cob can also accumulate fungal mycotoxins (Hui *et al.*, 1997).

In wheat, there appears to be three main sources of unrelated resistant germplasm to *Fusarium* ear blight: exotics from Chinese/Japanese origin, for example Sumai-3 (Anderson *et al.*, 2001; Ban and Suenaga, 2000); exotics from Brazil, for example Frontana (Van Ginkel *et al.*, 1996) and numerous lines from central Europe known to be of a distinct breeding lineage (http://www.scabusa.org). Interestingly, all the resistant germplasm sources confer a defence response that is both *Fusarium* species non-specific and race non-specific within a single *Fusarium* species. However, only a poor correlation exists between resistance to ear blight and to a second disease symptom, namely a stem base rot that is also of frequent occurrence in cereal crops (Parry *et al.*, 1995). Some correlation between seedling resistance to the DON mycotoxin at the time of seed germination and resistance to ear infections has previously been reported (Buerstmayr *et al.*, 1997; Wang and Miller, 1988). However, similar results have not been observed by others (Bill Hollins, personal communication; Paul Nicholson, personal communication).

There is a great paucity in our understanding of the molecular control of the *Fusarium* ear infection process of cereal crop species and the identity of the key components of the plant resistance response leading to effective pathogen containment in a species and race-non-specific manner. Some molecular information exists on the regulation of mycotoxin production, but this is limited to the details of the steps in the specific biosynthetic pathways and various regulatory genes (Hohn *et al.*, 1998, 1999; Tag *et al.*, 2001). Others have commenced characterisation of the defence genes induced in both resistant and susceptible wheat ears following *Fusarium* infection (Pritsch *et al.*, 2001). In wheat, definitive plant molecular genetics experiments are difficult to carry out because of its large and hexaploid genome and the long plant generation time. Alternative diploid cereal model systems (i.e. rice and barley), although possibly appropriate, lack suitable resistant germplasm sources and also have long generation times. We therefore decided to explore whether a range of experimentally versatile dicotyledonous plant species could be exploited for high-throughput molecular genetic studies.

Fusarium symptomless infections have previously been noted on dicotyledonous weed species growing at the base of infected winter wheat crops in the UK. Jenkinson & Parry (1994) were able to recover five *Fusarium* species from the surface-sterilised stem bases of 14 different weed species that showed no evidence of macroscopic disease symptoms. These species were *F. avenaceum, F. culmorum, F. poae, F. sambucinum* and *F. graminearum,* cited in decreasing order of recovery frequency. Almost all of the *Fusarium* isolates recovered from the weed species were confirmed as also pathogenic to the stem bases of wheat seedlings. There is also a second earlier report of visible *F. graminearum* infections on the surface of detached, ripe tomato fruits following artificial inoculation of wounds (Crozier and Boothroyd, 1959). Other *Fusarium* species, for example *F. oxysporum* are excellent invaders and colonisers of the roots and stem bases of hundreds of dicotyledonous species (Agrios, 1997). *F. oxysporum* primarily attacks vascular tissues in upper parts of the plant from the initial root and stem base infections. The direct infection of *F. oxysporum* of wounded tomato fruits has been previously reported (Di Pietro *et al.*, 2001), but direct flower tissue infections have not.

In this paper, we describe experiments that show that the wheat-attacking fungal pathogens, *F. graminearum* and *F. culmorum*, can also attack the flowers of several dicotyledonous species including, tobacco, tomato, soybean and *Arabidopsis* to cause various disease symptoms. We also reveal that flowers lacking functional anthers, either by the use of physical ablation or genetic mutation, are highly resistant to *Fusarium* attack. From a screen of different *Arabidopsis* ecotype accessions, we did not recover any ecotypes that exhibited a high level of resistance to *Fusarium* floral infection. Through competitive ELISA experiments, we confirmed that DON mycotoxin accumulation occurs in infected *Arabidopsis* flowers. This novel floral pathosystem for *Arabidopsis* appears to be highly representative of a serious cereal crop disease. This compatible model system will now permit various follow-up molecular genetics and gene expression experiments to be performed to identify the defence signals and responses that restrict fungal hyphal colonisation *in planta*.

Results

Establishing the floral inoculation system

Various spray and droplet inoculation methods of flowers and leaf tissue of *Arabidopsis*, tobacco (*N. tabacum*),

tomato and soybean plants were attempted using a conidial spore suspension. In all experiments, control wheat ear inoculations were performed in parallel with the identical solution of spores and environmental conditions. To obtain floral disease symptoms, it was important to maintain the plants post-inoculation under continuous high humidity conditions, but ensure the plant roots did not become waterlogged, which greatly perturbs normal flower development and anther dehiscence. Also, a period of darkness for a minimum of 16 h immediately post-inoculation was required to ensure uniform floral infection. Fine spray inoculations of open flowers were always found to be more effective than small droplets because the later were frequently lost to variable extents through droplet run-off.

Inoculation of immature flowers or puncture wounded/ epidermis stripped/unwounded leaves did not result in the invasion of plant tissue. Microscopic observation of trypan blue–lactophenol-stained tissue taken at various times post-inoculation revealed that *Fusarium* conidia germinate on the leaf surface in the absence of anthers. However, the growth of the germ tube extended a maximum of 5–10 spore length and thereafter growth ceased and the surface hyphae collapsed. There were no obvious attempts to penetrate leaf tissue. When leaf infection tests were performed by mixing *Fusarium* conidia with detached anthers, only a mass of saprophytically growing surface mycelium developed (Figure 1a). Microscopic observations of stained tissue again revealed the absence of tissue penetration by hyphae in the presence of pollen.

The flower infection phenotypes on various solanaceous and leguminous plant species

Spray inoculation of conidia into the open flowers of *Arabidopsis* ecotype Columbia, tobacco cv. Petit Havana, tomato cv. Moneymaker and soybean cv. A3244 resulted in the development of aerial mycelia in association with anther and anther filament tissue within 2 days (Figure 1b,c and data not shown). There was a striking similarity in the appearance of disease symptoms on the floral tissue of these various dicotyledonous species and those observed on the inoculated ear of the susceptible wheat cv. Bobwhite (Figure 1d and as reported previously for wheat (Kang and Buchenauer, 2000; Pugh, 1933). Microscopic observation of trypan blue–lactophenol-stained tissue taken 0.5, 1 and 2 days post-inoculation of *Arabidopsis* flowers revealed that conidia germinated and grew on the anther surface. Hyphal entry into the anthers typically coincided with anther dehiscence. Subsequently, hyphal growth was predominantly intercellular between the pollen grains inside the anther sac, but there was also some evidence of intracellular growth through the anther sac wall. Considerable intercellular hyphal colonisation occurred within the anther filament (Figure 1e). Subsequently, in *Arabidopsis* flowers, the visible infection proceeded into the petals, ovary, sepal and peduncle tissue before reaching the main stem within another 2 or 3 days. By day 5 post-infection, the main stem within the flower head had dried and constricted and turned brown or grey. Necking over of the flower head at the point of stem invasion was frequently seen and the developing siliques exhibited grey or brown necrotic symptoms and appeared slightly shrivelled, both above and below the point of stem invasion (Figure 1b). Microscopic observations revealed both inter- and intracellular hypha colonisation of stem and silique tissue (Figure 1f,g) and colonisation of the immature seeds within infected siliques (Figure 1h,i). A considerable amount of aerial saprophytic mycelium extended above the surface of all the colonised plant tissues (Figure 1b,g). Over the comparable 2–5 days post-inoculation in wheat ears, the infection had spread beyond the colonisation of the extruded anthers into the corresponding spikelet and caused the outer glumes, lemma and palea tissues to turn pale brown. A mass of aerial hyphae extending 1–2 mm from the surface of the glumes was also visible (Figure 1d). By day 10 post-inoculation, the disease symptoms on *Arabidopsis* were maximal and the *Fusarium* infection did not spread down the main stem to invade the base of the plant. The formation of pink conidial masses were also evident on heavily infected floral tissue from day 6 onwards (Figure 1j). In wheat cv. Bobwhite, between 10 and 15 days are required for *Fusarium* to colonise the entire ear. Usually, these infections continue into the adjacent stem tissue, but these do not extend below the junction of the flag leaf. Pink conidial masses form day 12 onwards on infected spikelets and the entire ear senesced prematurely and turns pale brown by day 20 (Figure 1d).

When tobacco flowers were inoculated, a mass of aerial mycelium accumulated only in association with anthers by 2 days post-inoculation. Within another 2 days, the complete inside of the flower trumpet was filled with white mycelium (Figure 1c). This infection did not prevent the normal flower fertilisation process because when the flower petals and anthers senesced and dropped off, both macroscopic and microscopic examination revealed that the immature green seed pod below had not been invaded (Figure 1c). In tomato flowers, a similar infection phenotype was observed to that of tobacco. Due to the extreme waxiness of the anther cone of tomato flowers, a single anther was carefully removed with fine tweezers at the yellow or green stage and a droplet containing conidia was placed inside the remaining anther cone. Abundant aerial mycelium was evident with 2–3 days, but this did not affect the normal fertilisation process and green uninfected tomato fruits formed from each inoculated flower and the fruit calyx remained healthy. Spray inoculations of soybean flowers resulted in the colonisation of anthers and anther
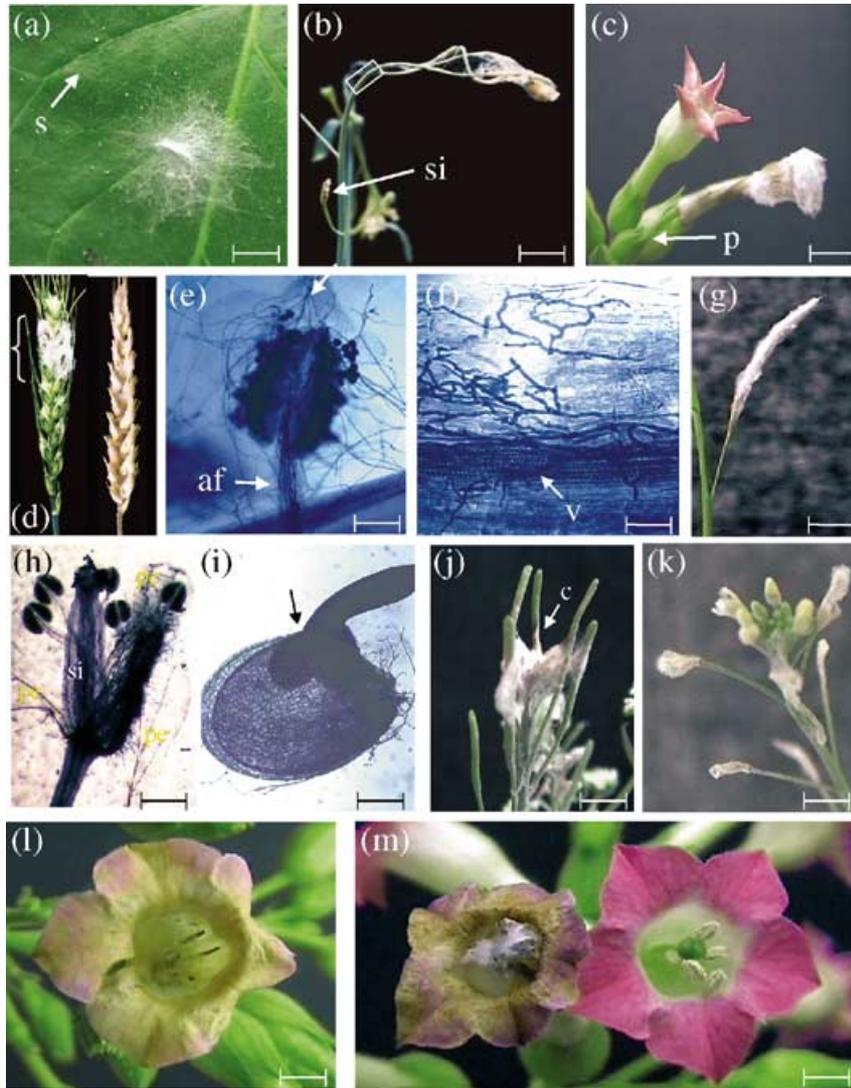
Figure 1. Various macroscopic and microscopic aspects of the *Fusarium graminearum* and *F. culmorum* disease phenotypes on floral and non-floral tissue of *Arabidopsis*, tobacco and wheat.

(a) Superficial growth of *F. graminearum* mycelium extending from a detached anther over the surface of a tobacco leaf. In contrast, at the leaf position indicated by S, a droplet of *Fusarium* conidia was placed and no hyphal growth is visible. Photograph taken 4 days post-inoculation.

(b) *Arabidopsis* floral tissue 5 days after spray inoculation with *F. graminearum* conidia. Extensive superfical mycelium covers the apical flowers and disease symptoms are evident on newly formed siliques (si). The white square indicates the stem tissue region examined by trypan blue–lactophenol staining and shown in panel (f).

(c) Tobacco flowers 4 days after spray inoculation with *F. graminearum* conidia. The petals are covered with mycelium and have senesced prematurely. At the base of the infected flower, the developing seed pod (p) remains disease-free.

(d) Wheat ears inoculated at anthesis and photographed at either 4 days (left) or 20 days (right) post-inoculation. Superficial mycelium is located at day 4 only where anthers were extruding from wheat florets. This region of the ear is highlighted by a white bracket. By 20 days post-inoculation, the entire wheat ear has senesced prematurely and turned brown.

(e,f,h,i) Photomicrograph taken of whole tissue mounts stained with lactophenol–typhan blue.

(e) *Arabidopsis* anther sacs and anther filaments (af) enveloped in *F. graminearum* mycelium, 2 days after inoculation of flowers with conidia. Both intercellular and intracellular fungal hyphal colonisation has occurred in both anther sac and filament tissues.

(f) Stem tissue sampled just below the point of visible infection highlighted in panel (b). Both intercellular and intracellular hyphal growth is evident in the parenchyma tissue. The host cells in close contact with the *Fusarium* mycelium have not accumulated the trypan blue stain and are therefore still alive (Keogh *et al.*, 1980). V, vascular tissue.

(g) An older *Arabidopsis* silique entirely covered with aerial mycelium, photographed 5 days after *F. graminearum* inoculation.

(h) A single *Arabidopsis* flower, 5 days after inoculation with *F. graminearum*. Extensive hyphal colonisation is present in both petal (pe) and silique tissues (si).

(i) An *Arabidopsis* seed dissected from the *Fusarium*-infected silique shown in panel (h). Fungal hyphae are seen extending over the surface of the seed testa. The arrow indicates the suspensor cell connecting the seed to the main vascular tissue in the silique.

(j) *Arabidopsis* ecotype Landsberg (La-er), 7 days after inoculation with *F. culmorum* conidia. Extensive mycelium is present at the flower apex and on the siliques. Pink asexual conidial sporulation is also visible (c).

filaments, but these infections never extend into the developing pods (data not shown).

Of the four dicotyledonous plant species tested, the disease symptoms forming post-*Fusarium*-infection were most extensive on *Arabidopsis* floral tissue, causing the developing seeds and stem tissue to become infected. The infections on tomato, tobacco and soybean flowers, although resulting in the formation of extensive aerial mycelium, did not proceed beyond floral tissues and did not cause seed infections.

The flower infection phenotype are different between the Arabidopsis *ecotypes Columbia and Landsberg erecta*

The research opportunities afforded by the *Arabidopsis* ecotypes Columbia Col-O and Landsberg erecta (La-er) are enormous (http://nasc.nott.ac.uk; http://arabidopsis.org). Therefore, we examined in detail whether there were differences in their *Fusarium* infection phenotypes. The initial inoculation experiments (described above) had already revealed considerable variation in the disease symptom phenotype manifested on the various floral tissues. Therefore, to assess quantitatively the degree of floral invasion, the infection process was divided into three subcomponent parts based upon organ type, i.e. flower infection (F) comprising of open flowers and closed buds, new siliques (NS) formed during the infection time course and the older siliques (OS) already present at the time of inoculation. Within each subcomponent, various degrees of infection were recognised based on macroscopic symptom assessments and follow-up microscopic observation of stained material (Table 1). The final *Fusarium–Arabidopsis* disease value (the FAD value) was calculated by adding the three subcomponent scores, i.e. individual $F + NS + OS$ scores = FAD value.

Interestingly, the La-er plants exhibited a statistically significant higher level of floral tissue disease than Col-O plants. The mean FAD value for La-er was 10.7 compared with 6.6 for Col-O when examined over 11 separate inoculation experiments and scored at day 6 or 7 (Table 2 and Figure 1j,k). When the three subcomponents of the total infection score were examined in detail, it was recognised that the La-er and Col-O flowers exhibited the identical infection symptoms on the new siliques, namely tissue drying and a pale grey or brown coloration. However, large differences in the levels of infection between La-er and Col-O were evident on the flowers and older siliques. Flower

Table 1 Description of the disease phenotypes used to quantify the three subcomponents of *Fusarium* infection of floral tissue on *Arabidopsis* plants

Organ	Score	Description of disease phenotypes
Flower (F)	0	Normal
	1	Aerial mycelium visible on flower
	3	Drying of flowers
	5	Stem constriction within flower head
New siliques[a] (NS)	0	Normal
Older siliques[b] (OS)	1	Aerial mycelium on silique surface
	3	Drying of silique surface
	5	Peduncle constriction or mycelium on peduncle or loss of siliques by disease travelling down stem
	7	Main stem constriction

Fusarium–Arabidopsis disease (FAD value) $= F + NS + OS$

The final FAD value is calculated by addition of the three subcomponent scores.
[a]Siliques formed during the disease time course.
[b]Siliques already formed at the time of inoculation. Both silique types are scored separately using the same scoring system.

infections in La-er were always maximum. All flowers were covered with abundant aerial mycelium, the flower petals and sepals had dried and taken on a grey or brown coloration, the supporting peduncle had also dried and constricted and these phenotypes extended into the adjacent stem tissue (Figure 1j). In comparison, the Col-O flowers showed various infection phenotypes over the 11 experiments. These ranged from only aerial mycelium on flowers (two experiments), drying and discoloration of flowers and sepals (five experiments) (Figure 1k), to all of the previous in association with some stem constriction (four experiments). This is reflected in the very high standard deviation (± 1.3) for the flower score of 3.0 on Col-0, whereas for the La-er plants in the same 11 experiments, there was a uniform response (Table 2). For the older silique infections on La-er, these were comparable with those of the newly formed siliques, and caused the siliques to dry and display a pale grey or brown colour (Figure 1j). In contrast, the older Col-O siliques only ever exhibited superficial aerial mycelium (Figure 1k). Inoculations using another isolate of *F. culmorum* and an isolate of *F. graminearum* confirmed that the infections on La-er were always greater than Col-O (data not shown).

Figure 1. continued

(k) *Arabidopsis* ecotype Columbia (Col-O), 7 days after inoculation with *F. culmorum* conidia. Some aerial mycelium is associated with the flower petals and disease symptoms are present on the immature siliques.

(l,m) Tobacco flowers 4 days after inoculation with *F. graminearum*. (l) an emasculated tobacco flower with brown discoloration of petals but no aerial mycelium; (m, left) a de-pistillated flower exhibiting extensive aerial hyphal colonisation of anthers and brown discoloration of petals and (m, right) a control non-inoculated tobacco flower at the identical physiological age to the inoculated flowers shown in (l) and (m).

Bars $= 2$ mm in (a, g); 4 mm in (b, j–m); 1 cm in (c); 100 μm in (e); 10 μm in (f); 500 μm in (h); 200 μm in (i).

Table 2 Component scores for *Fusarium culmorum* floral infections of Columbia (Col-O) and Landsberg erecta (La-er) *Arabidopsis* plants

Genotype	Organ	Experimental replica											Mean	SD
		1	2	3	4	5	6	7	8	9	10	11		
Col-O	Flower	2.4	4.4	4	0.9	3	3.2	0.9	2.3	4.1	4.8	2.8	3	1.3
	New silique	3	3	3	3	3	3	3	2.8	3	3	3	3	0.06
	Older silique	0	1.2	1.2	1.2	0	0	1.1	0.6	0.8	0.8	0	0.6	0.53
	Total FAD	5.4	8.6	8.2	5.1	6	6.2	4.9	5.7	7.9	8.5	5.8	6.6	1.4
		*	**	**	*	*	*	*	*	**	**	*		
La-er	Flower	5	5	5	5	5	5	5	5	5	5	5	5	0
	New silique	3	3	3	3	3	3	3	3	3	3	3	3	0
	Older silique	2.7	1.9	2.7	2.1	3.7	3.6	1.8	2.4	3	3.2	2.6	2.7	0.64
	Total FAD	10.7	9.9	10.7	10.1	11.7	11.6	9.8	10.4	11	11.2	10.6	10.7	0.64

*Col-O single experimental mean statistically below the overall experimental FAD mean.
**Col-O single experimental mean statistically above 2 SD from the overall mean of 6.6 ($P < 0.5$).

The inconsistent infection phenotype of the Col-O flowers to *Fusarium* is curious and was observed over a 1.5-year period. Despite many modifications to the inoculation protocol and changes in the fungal isolate used, stability of both the F and OS scores was never obtained. The variation evident in the older siliques scores of Col-O was very similar to that observed in the La-er older siliques, with SD values of 0.53 and 0.64, respectively. The large variation for the Col-O flower scores appears to have two main underlying causes. Firstly, open flowers at the time of inoculation, when infected, either become pollinated and are therefore scored as new siliques, or they remain as non-pollinated flowers attached to the flower peduncle by the aerial mycelium (Figure 1k). Secondly, the closed Col-O flower buds at the time of inoculation either open and exhibit disease symptoms, open and escape infection or remain closed and disease-free. Due to the different morphology of the La-er floral apex (discussed below), neither uninfected open flowers or uninfected closed buds were observed (Figure 1j).

When the combined data in Table 2 was explored in greater detail, an excellent positive correlation was evident between the overall level of floral disease in a single experiment (i.e. the FAD value) and the extent of disease symptoms on the flowers ($r = 0.93$). However, there was only a poor positive correlation between the total FAD value and the disease score of the older siliques ($r = 0.38$). No correlation was found between the disease levels on the flowers and older siliques ($r = 0.01$). Therefore, the limited infection levels on the older Col-O siliques are not caused from an overall lower infection level in a single experiment or from less fungal biomass on the flowers. In the same experimental series, the three subcomponents of the La-er FAD values remained consistent (Table 2). Overall, the total FAD values for Col-O was bimodal in distribution, with seven experiments giving mean FAD scores statistically below the overall mean and four experiments giving values

statistically above the mean (Table 2). For La-er, a normal distribution was obtained, with the only difference between experiments caused by the degree of colonisation of the older siliques.

The most plausible explanation for the higher infection phenotype of La-er compared to Col-O flower heads is the large difference in their flower head architecture. The La-er flower head is extremely compact, with immature siliques, open flowers and unopened buds in extremely close proximity to each other at the time of inoculation (Figure 1j). In comparison, the Col-O flower head is less compact with each open flower slightly more separated from the unopened flower buds. Also, a space of at least 4–6 mm separates an open flower from immature siliques at the time of inoculation (Figure 1k). These architectural differences will greatly affect the propensity of the *Fusarium* aerial mycelium formed at the initial site of flower infection to grow aerially into adjacent siliques and other flowers, and for the various infection sites to coalesce. The maximal aerial range of these *Fusarium* hyphae is around 2 mm for *Arabidopsis*, although considerable greater aerial extension was evident on wheat and tobacco. In addition, it is highly likely that the overall humidity within the tight La-er flower head could remain consistently higher than that in the more lax Col-O flower heads, even though high-humidity chambers were used throughout the experimental time course. *Fusarium* infections always proceed faster under very high humidity conditions because of the greater contribution of the aerial mycelium to initiate new infection sites (Martin Urban, unpublished data). We consider that the higher severity of infection on the older La-er siliques already formed at the time of infection could be the result of underlying genetic differences in the two ecotypes' abilities to respond to the *Fusarium* infection. However, because of the compact La-er flower head, the vertical growth of the older siliques causes them to come into contact with the abundant aerial mycelium. A high proportion of secondary

infections then take place, which could also elevate the overall disease score. Wheat cultivars with a compact and dense ear architecture are known to be generally more susceptible to *Fusarium* ear blight infection than those with loose and open ear morphologies (reviewed by Parry *et al.*, 1995).

Screening a diverse collection of Arabidopsis *ecotype for genetic variation in both flower and leaf infection phenotypes*

In total, 236 *Arabidopsis* ecotype accessions were spray inoculated with *Fusarium* when the primary bolt of each plant had at least two open flowers and no more than two siliques had already formed. A minimum of five inoculated plants per ecotype were assessed for visible flower, silique, stem and leaf infections, at either 7 or 8 days post-inoculation. Twenty-five ecotypes in the primary screen with FAD values below 3 or above 10 were re-grown and inoculated, with the population size increased to 12 flowering plants. None of these extreme FAD values re-confirmed during the secondary screen. We therefore conclude that all 236 *Arabidiposis* ecotypes are moderately susceptible to floral head infections. No ecotype exhibited visible leaf infection symptoms (http://www.iacr.ac.uk/ppi/staff/khkara.html).

The extent of Fusarium *floral infections is significantly affected by the presence and absence of functional anther components*

A key route for *Fusarium* to invade floral tissue involves the anthers. In wheat, when the anthers are carefully removed by physical emasculation, then the incidence of infections can be dramatically reduced (Strange and Smith, 1971). Two metabolites highly stimulatory to *Fusarium* hyphal growth and mycelium branching are found in abundance in anthers and pollen. Choline and glycine betaine (Strange *et al.*, 1974) have been suggested to be positive contributors to fungal pathogenicity and possibly explain why *F. graminearum* and *F. culmorum* invade flowers. Maximum cereal ear invasion always occurs at anthesis. We therefore wanted to explore whether there was a functional significance to the anther invasions observed on the four dicotyledonous species.

The large size of the tobacco flowers meant this plant species was the most experimentally tractable of the four to examine the effects of hand-emasculation and de-pistillation on the *Fusarium*-infection phenotype. When all the pollen sacs were removed from the ends of the anther filaments of tobacco flowers prior to pollen dehiscence, very little aerial *Fusarium* mycelium was evident on the remaining filament tissue post-inoculation and the petals turned brown (Figure 1l). Microscopic analyses revealed internal colonisation of petal tissue (data not shown). In contrast, when the pistil was removed prior to anther dehiscence and inoculation, the heavily infected flower remained alive for a few additional days, but no colonisation of the underlying pistil and unfertilised ovary and sepal tissues occurred (Figure 1m).

In *Arabidopsis*, a wide range of flower mutants exist that have either the entire floral whorls missing or present in duplicate, whilst other mutations result in no silique formation (http://nasc.nott.ac.uk; http://arabidopsis.org). We decided to examine the effects of the various genetic ablations of anther whorls' development or pollen function on the FAD values in preference to hand emasculation. Three floral mutants were selected for these experiments: *agamous-1* because it lacks the pistal and stamen whorls and instead possesses a double petal whorl (Yanofsky *et al.*, 1990); *apetala3-1* because it lacks both the stamen and petal whorls, and has instead double carpel and sepal whorls (Jack *et al.*, 1992). The *ag-1* and *ap3-1* mutants are both in the La-er ecotype backgrounds. The third mutant was the recently reported jasmonic acid mutant called *dad1*, present in the Ws-2 background (Ishiguro *et al.*, 2001). The *dad1* mutant has all the floral whorls intact, but has defective pollen dehiscence and filament hydration processes and the pollen is non-viable. In all three mutants, pollination does not occur. Instead residual, undeveloped ovules and pericarp tissue in a silique-like structure remain at the end of the flower peduncle following final petal and/or sepal senescence.

Interestingly, all three mutants showed significantly reduced *Fusarium* infection of flowers. For the *agamous-1* mutant, very little aerial mycelium formed and the flowers appeared normal. The individual flower infection score was La-er = 5 and *ag-1* = 1. Sometimes, slow petal senescence occurred in the *agamous-1* mutant under the high-humidity conditions and this caused saprophytic aerial mycelium to become visible only on the petals from day 5 onwards. However, this petal phenotype did not result in additional colonisation of other parts of the flower head. On the *apetala3-1* mutant, some aerial white mycelium was evident on the undeveloped ovary and calyx whorls by day 5. But, again no further disease symptoms or tissue browning ever occurred. The inoculation score of the flower phenotype for *ap3-1* = 1.0. We therefore conclude that the complete ablation of the anther tissue whorl prevents *Fusarium* hyphae from invading other tissues within the flower head.

The *dad1* mutant also exhibited a very low disease phenotype, with no macroscopically visible hyphae on flowers and minimal aerial hyphae on the surface of the non-fertilised siliques. The F + NS score for the wild-type Ws-2 ecotype = 4.2, whereas for *dad1* = 2.1. This result was not expected because the anther whorl is present, although non-functional. When inoculated *dad1* and wild-type Ws-2 flowers were examined microscopically over a 2-day time course extending to day 10, this revealed that almost no

extracellularly mycelial colonisation of the anther and anther filament has occurred on the *dad1* flowers and there was no evidence of attempted tissue invasion. In the Ws-2 flowers, extracellularly mycelial colonisation was extensive and other intercellular and intracellular hyphae were detected in the peduncle and silique tissues (data not shown). It would therefore appear that *Fusarium* hyphae lack the appropriate mechanism to penetrate the intact anther sac wall and do not use, as an alternative route, the 4–6 stomatal pores present near the junction with the anther filament. Also, the overall reduction in the density of hyphae associated with *dad1* anthers compared with Ws-2 may indicate a lower nutrient availability. Alternatively, it is possible that the intact anthers containing immature non-viable pollen grains either contain harmful pre-formed metabolites (phytoanticipins) (Morrissey and Osbourn, 1999) or are able to mount a successful active defence response at sites of attempted hyphal penetration. Microscopic analyses revealed no evidence of a hypersensitive cell death defence response or any specific modifications to the appearance of plant cell walls in either the anther sac or filament (data not shown).

The small amount of belated aerial mycelia evident on all three of the *Arabidopsis* flower mutants on the unfertilised and senescing calyx, siliques and petals (where present) never resulted in disease symptoms forming on the underlying peduncle or stem tissues. The formation of aerial mycelium on these tissues was to be expected because *Fusarium* has excellent saprophytic colonisation abilities, and can therefore utilise the considerable nutrients released from these tissues as each enters a senescence programme.

DON mycotoxin production during invasion of Arabidopsis flowers

A feature of many *F. graminearum* and *F. culmorum* invasions of wheat and barley ears and maize cobs is the production of mycotoxins (Hohn *et al.*, 1998). The trichothecene mycotoxin, deoxynivalenol (DON) and its precursor 15-acetyl deoxynivalenol are of concern for human food and animal feed safety (http://www.scabusa.org). The production of DON mycotoxins may contribute to the overall aggressiveness of naturally DON-producing *Fusarium* isolates to invade plant tissues. However, DON production is not a fundamental fungal pathogenicity factor (disease-causing component) (Harris *et al.*, 1999; Proctor *et al.*, 1995). DON production can also be induced under specific *in vitro* conditions that attempt to mimic *in planta* conditions, but DON and 15-acetyl is not synthesised when *F. culmorum* or *F. graminearum* are grown on more general culture media (Burmeister, 1971; Chen *et al.*, 2000).

We explored whether DON and 15-acetyl DON production occurred during *Fusarium* colonisation of *Arabidopsis*

floral tissue. Samples were taken at 8 days post-inoculation with two different *F. culmorum* isolates and one *F. graminearum* isolate known to produce DON mycotoxin when invading wheat ears (Claudia Heppner, unpublished). In both Col-O and La-er whole flowers, greater than 1 ppm DON was detected in all *Fusarium*-invaded flower tissue at 8 days post-inoculation. On Col-O flowers, the recovered values ranged between 1.22 and 1.42 ppm (SD = 0.10) whilst on La-er, a slightly wide range was evident from 1.21 to 2.27 ppm (SD = 0.53). From water only-inoculated Col-O and La-er floral tissue, DON levels of below 0.1 ppm were recorded. This level of background non-specific binding of plant components in the DON competitive ELISA is comparable to the values obtained for water only-inoculated Bobwhite wheat ears (C. Heppner, unpublished). When the two UK *F. culmorum* isolates were spray inoculated onto the anthesising ears of Bobwhite wheat growing in a field in Cambridgeshire, UK and subsequently mist irrigated to ensure a high level of visible infection, the DON levels recovered from the harvested and threshed grain ranged between 6 and 50 ppm (C. Heppner, unpublished). This indicated that the two *F. culmorum* isolates used in this study are moderate producers of DON. The large difference in the DON levels recovered from the wheat and *Arabidopsis* inoculation would appear to be caused by the total length of the infection period prior to assaying for DON levels. Only 8 days elapsed between inoculation and floral tissue harvest in *Arabidopsis*, whereas there are 50 days between inoculation and harvest for the wheat grain sample. In the later situation, considerably more time elapsed for mycotoxin biosynthesis and accumulation to occur.

The detection of the accumulation of DON and 15-acetyl DON mycotoxins in the *Fusarium*-infected flowers of both Col-O and La-er flowers indicates that the complete infection phenotype is manifested within *Arabidopsis* floral tissue.

Discussion

F. graminearum and *F. culmorum* are primarily considered to be pathogens of monocotyledonous cereal plant species and cause the serious ear blight disease (McMullen *et al.*, 1997). The combined data presented in this analysis clearly indicates that both fungal species are able to penetrate and invade the floral tissue of various dicotyledonous species and for *Arabidopsis*, these infections extend into stem tissue to cause severe disease symptoms, and the accumulation of DON mycotoxins and hyphae to associate with developing seeds inside infected siliques. This is a novel pathosystem for *Arabidopsis* and is the first to be described involving floral infection by any microbe. The lack of leaf tissue infections also highlights a frequently overlooked aspect of plant–microbe interactions, the phenomenon of organ specificity and/or tissue specificity for successful

microbial colonisation. Preliminary results using another wheat floral pathogen *Stagnospora (Septoria) nodorum* also indicates that this fungal species is able to invade and cause visible disease on tobacco flowers and *Arabidopsis* flowers, siliques and upper stem tissues (Martin Urban, personal communication).

Mode of infection

The mode of infection by *Fusarium* hyphae of the four dicotyledonous plant species, i.e. *Arabidopsis*, tobacco, tomato and soybean was very similar to that found in wheat and barley. Under high-humidity conditions, floral tissue was invaded but not leaf or lower stem tissues. The microscopic observations of the trypan blue–lactophenol-stained tissue indicate that the advancing hyphal front colonised living plant tissue, and therefore the pathogen is hemibiotrophic in these dicotyledonous plant species. Why *F. graminearum* and *F. culmorum* are primarily flower and stem base invaders is not known. This may be because of: (i) the local abundance of specific metabolites within the flowers, e.g. choline and glycine betaine; (ii) the excessive nutrients available post-fertilisation in the primarily sink tissues or (iii) tissues with no or highly reduced photosynthetic capacity have a different baseline primary metabolism. The extensive aerial mycelia that developed in association with anther tissue early in the infection was a striking feature of all five pathosystems. However, only when attacking the flowers of monocotyledonous cereal plants and *Arabidopsis* did the *Fusarium* infections spread into other floral tissues to eventually reach the stem and developing seeds. In tobacco, tomato and soybean, the infections were always contained within the open flowers and these infections were excised completely at flower senescence. It is currently unclear why these infections fail to spread further.

Within *Arabidopsis* floral tissue, both extensive intercellular and intracellular invasion occurred and this led to tissue drying and browning in association with asexual spore production. The same disease symptoms are seen in wheat and barley ears. However, unlike in wheat, the *Arabidopsis* infections invariably resulted in the diseased floral heads necking over at the point where the stem tissue became invaded, late in the time course. Infected wheat and barley ears remain vertical and firm even under periods of extended high humidity. We consider this major difference in disease symptoms to be primarily due to differences in the physiological age of the floral tissue invaded by the fungal hyphae and hence the degree of secondary cell wall modifications, i.e., lignification, that has occurred. At anthesis, the entire floral structure in both wheat and barley ear is approximately 30 days old. Whereas for *Arabidopsis*, at the onset of anthesis, the floral head is <10 days old and is considerably more supple.

The *Arabidopsis* floral mutants, *agamous-1*, *apetala3-1* and *dad1* confirm the need for the presence of both anthers and pollen function to permit the successful and extensive invasion of other floral tissues. An analysis of various male sterile barley genotypes (Matsui *et al.*, 2002) has revealed that lines producing sterile pollen were significantly more susceptible to *Fusarium* ear blight than sterile lines producing no pollen. The negative infection results with the *Arabidopsis dad1* mutant suggests that *Fusarium* may have difficulties penetrating non-dehiscing anther sacs. The emasculated tobacco flower results reveal that petal infections can take place even in the absence of pollen in this species. In contrast, another cereal floral fungal pathogen *Claviceps purpurea*, the causal agent of ergot disease, only enters floral tissue via the female stigmatic tissues (Mey *et al.*, 2002).

Resistance

Both *F. graminearum* and *F. culmorum* lack the ability to invade unwounded plant leaf tissue of both monocotyledonous and dicotyledonous plant species. No infection occurs even in the presence of detached anthers. Instead, infections by these two *Fusarium* species are confined to either the flowers or the stem bases. This tissue- or organ-specific resistance suggests one of three potential hypotheses: (i) specific signals are missing to trigger the onset of the *Fusarium* infection process; (ii) specific defence responses operate in the non-infected organs prior to, or during attempted penetration or (iii) in the predominantly non-photosynthetically active plant tissue, a full defence response cannot be manifested and this permits the fungal hyphae to gain entry. In wheat, *F. graminearum* infection of floral tissue triggers the induction of the typical set of defence-related genes (Pritsch *et al.*, 2001). Amongst the *Fusarium* genera, it is noticeable that most species occupy just specific tissue niches within the plant and do not attack every plant organ. For example, *Fusarium* wilt fungi such as *F. oxysporum* f sp. *lycopersici* are restricted to attacking the vascular tissue near the stem base (Agrios, 1997), whilst *F. oxysporum* f. sp. *matthiolae*, the only other *Fusarium* species reported to attack *Arabidopsis*, is primarily a stem base–root coloniser (Epple *et al.*, 1998).

The *Arabidopsis* ecotype screen had a disappointing outcome, with no extremely resistant or susceptible ecotypes identified. A similar outcome was found when a large number of *Arabidopsis* ecotyopes were inoculated with the economically important sugar beet cyst nematode species *Heterodera schactii*. Although statistically significant differences between La-er and Col-O infection phenotypes were repeatedly evident, these differences have probably more to do with overall floral architecture than inherent differences in their resistance response. One of the easiest ways to test this hypothesis is to transform the Landsberg

allele of the *erecta* gene into the Col-O background and repeat the *Fusarium* inoculation tests.

In wheat, two main types of germplasm resistance are recognised. Type 1 resistance prevents initial infection, whilst type 2 resistance operates post-infection to reduce the rate of secondary spread through the entire ear (reviewed by Parry *et al.*, 1995). The spray inoculation protocol undertaken in this study is very useful in trying to identify type 1 and type 2 resistance components together. However, to focus specifically on type 2 resistance (i.e., resistance to spread) would require the use of a single flower point inoculation technique. In wheat, a single spikelet inoculation technique is frequently used to identify germplasm exhibiting good type 2 resistance (Schroeder and Christensen, 1963).

Exploitation

This novel *Fusarium* floral pathosystem on *Arabidopsis* will now permit a study of various defence mutants and transgenics for their ability to restrict or enhance disease susceptibility and to alter symptomatology. A wide range of defence mutants already exist for *Arabidopsis* unlike wheat or maize. In parallel with these inoculation experiments, it will be important to determine whether the various genetic mutations that affect the levels of specific defence-signalling molecules, for example salicylic acid, jasmonic acid, ethylene and reactive oxygen species actually cause similar changes in floral tissues as reported for leaves. For most of the mutants, this type of information has not been reported. Large-scale gene expression array experiments could also be undertaken to examine the global changes in the transcriptome caused during *Fusarium* infection of floral tissue. These gene expression experiments could be extended to leaf tissue to determine whether plant cells actively respond during surface colonisation by non-penetrating *Fusarium* mycelium.

The biological complexity of the *Fusarium* infection process in floral tissue and the lack of specific infection structures at the site of initial host tissue penetration, i.e. appressoria formation means that any mutation screen would best be achieved by using a transformed isolate of *Fusarium* expressing both the green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter genes. This would permit an initial non-destructive and high-throughput analyses to be performed using UV to detect the presence of the green fluorescent protein within the fungal hyphae. This would be followed by GUS staining and light, epi-fluorescence and confocal microscopic analyses to evaluate the true extent of internal *Fusarium* colonisation. A recent study using a transgenic *Fusarium* strain constitutively expressing the GFP gene reporter was used to document *Fusarium* invasion of wheat ears (Pritsch *et al.*, 2001).

The detection of DON mycotoxin production within the *Fusarium*-infected floral tissue will also permit a detailed examination of the plant factors regulating mycotoxin biosynthesis. This type of analysis, which could be undertaken using monocotyledonous and/or dicotyledonous host plant species, is also best achieved using a transgenic *Fusarium* strain because the DON ELISA and GC–MS tests are both expensive and requires large amounts of infected plant tissue (>4 g). One suitable transgenic *Fusarium* strain to monitor DON induction could harbour a *Tri5* promoter: GFP-GUS reporter gene construct. The *Tri5* gene of *Fusarium* encodes for the enzyme trichodiene synthase, and this is the key regulatory step for DON mycotoxin production (Proctor *et al.*, 1995). By using a pTri5:GFP-GUS transgenic *Fusarium* isolate, the onset of DON biosynthesis can be pinpointed to specific infection stages and plant tissues. Then, commercial monoclonal antibodies to DON can be used on tissue sections to confirm associated DON production. By this approach, will it be possible to compare DON production in various mutant and transgenic *Arabidopsis* lines to that occurring in wheat and barley ears and maize cobs.

Experimental procedures

Fusarium *strains and media*

The two *F. culmorum* isolates used in this study were strains 97/7 and 98/11 which had been isolated from naturally infected wheat ears taken from the Trumpington Cambridge site in 1997 and 1998, respectively (Bill Hollins, personal communication). The *F. graminearum* isolate 16A originated from the USA, was isolated from infected wheat grain collected from a wheat grain elevator in Montana in 1997 and was a gift from Linda Lahman (Monsanto, St. Louis). All three isolates were capable of causing severe ear blight symptoms on susceptible wheat cultivars and to produce DON mycotoxins (M. Urban and C. Heppner, unpublished data).

Both *F. culmorum* and *F. graminearum* were propagated on SNA plates (synthetic nutrient poor agar) containing 0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4 \cdot 7 H_2O$, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 2% Bacto agar (Difco) supplemented with 200 ppm biotin and 200 ppm thiamine. For long-term storage in liquid nitrogen, conidiospore suspensions were prepared to a density of 10^7 spores ml^{-1} in 10% glycerol. Spore suspensions for plant inoculations were derived from culture plates which had been subcultured a maximum of four times. Conidiospores were harvested from 90 mm SNA agar plates after 10 days incubation by adding 5 ml sterile water and scraping off conidiospores with a spatula. Alternatively bubble cultures (Cappellini and Peterson, 1965) were established to create a greater volume of conidiospores. The spores were recovered by centrifuging at 3000 *g* for 6 min at 4°C, then re-suspended gently in growth media and stored for up to 7 days at 4°C prior to use. For plant inoculations, aliquots of spores were taken from the stock, recovered by centrifuging at 3000 *g* for 6 min at 4°C, washed once with sterile de-ionised water, centrifuged again and re-suspended in water plus 0.001% Silwet L-77 to give a final spore concentration of 1×10^5 spores ml^{-1}. Conidiospores were counted using a haemocytometer. All experiments

involving the *F. graminearum* isolate 16A were conducted in biological containment facilities under DEFRA licences PHL 39B/3819 (5/2001) and PHL 39A/3493 (01/2001).

Growth of plant material

Arabidopsis seed was sown into Levingtons F1 compost and kept in a propagator for 4 days at 4°C to ensure even germination. The pots of germinated seeds were then moved to a controlled environment growth room with a temperature of 20°C and a 12-h light/12-h dark cycle. Light was supplied by a mixture of metal halide and incandescent lamps to produce a fluence level of 171 microM at 64.5 W msq^{-1} at the plant surface. Ten days later, at the one leaf stage, seedlings were pricked into vacupots (24 units per tray), again into F1 compost. Plant pots were placed on capillary matting, which was kept moist at all times. Flowering commenced approximately 3 weeks after the seedling were pricking out. Under these environmental conditions, the full *ap3-1 Arabidopsis* mutant phenotype was always evident.

Tobacco seeds of cultivar Petit Havana, tomato seeds of cultivar Moneymaker (Cf-0) and soybean seeds of cultivar A3244 were sown in Levingtons F1 compost, and pricked out singly into Levingtons F2 compost at the 2–4 leaf stage into 10-cm pots. The plants were grown in a controlled environment growth room at 24°C during the 16-h day and 16°C at night. Light was supplied by a mixture of metal halide and incandescent lamps to produce a fluence level of 207 microM at 86.2 W msq^{-1} at the plant surface.

Wheat seeds of spring cultivar Bobwhite were sown in Levingtons C2 coarse potting compost for 2 months in a controlled environment growth room at 18°C during the 16-h day and 16°C during the 8-h night at 50% relative humidity. Light was supplied by a mixture of metal halide and incandescent lamps to produce a fluence level of 207 microM at 86.2 W msq^{-1} at the plant surface.

Plant inoculations

Only flowering plants were selected for inoculation. The *Arabidopsis* plants possessed an unbranched bolt with both open flowers on the terminal inflorescence and two to three developing siliques. Ten plants from each genotype were selected for each *Fusarium* inoculation, and a further two to three control plants to be sprayed with water. A permanent black marker pen was used to mark the position on the flower stem above which only open flowers were present and below which siliques had already set. Tobacco, tomato, soybean and wheat plants were selected just as flowering/anthesis commenced. Six plants of each species were selected for each experiment. One to three flowers per plant were inoculated. A randomised block experimental design was used to minimise experimental error and each experiment was conducted a minimum of three times.

For the inoculation of *Arabidopsis* flowers, a fine droplet spray applicator was used. The spore suspension was applied until droplet run-off had just commenced. After this, each inflorescence was re-inoculated with the inoculum dispensed this time from a medical nasal applicator, four puffs per flower head. Control plants were inoculated in the same way using de-ionised water. The inoculated plants were then kept in large plastic propagators at 100% relative humidity for the next 7 days. For the first 2 days, the chambers were shaded with capillary matting to exclude light. A similar dark procedure is used when inoculating wheat ears. Individual plants were scored for disease symptoms from day 2 onwards with the final disease score taken at day 7 post-inoculation.

For the inoculation of the tobacco flowers, soybean flowers and wheat ears, the inoculum was dispensed from the medical nasal applicator, four puffs per open flower and eight puffs per wheat ear. The anther cones of the tomato flowers are extremely waxy and this caused most spore droplets to be lost immediately through run-off. Therefore, a single segment of the tomato flower anther cone was removed by the use of a pair of fine forceps. Then, using a P10 Gilson pipette, 5 µl of spore suspension was added to the inside of each anther cone. After inoculation, all the plants were treated as described above for the *Arabidopsis* inoculations.

Disease scoring on *Arabidopsis*

To quantify accurately the levels of disease symptoms visible on the various *Arabidopsis* floral tissues, a numerical scoring system was devised. This is shown in Table 1. The disease phenotypes were assessed for three separate floral subcomponents, namely flowers (F) that were either open flowers or closed buds at inoculation, new siliques (NS) that were fully open flowers at inoculation (i.e. located above the permanent mark placed on the stem) and older siliques (OS) already present at inoculation. An increasing numerical score was used to quantify the abundance of aerial mycelium on a tissue surface (0, 1), as well as the increasing severity of the disease symptoms visible on plant tissue as the invasion process progresses (3–7). The intermediate scores of 2 and 4 (F), and 2, 4 and 6 (NS and OS) were reserved for when all the tissue on a single plant exhibited the disease phenotype described for the preceding score. For example, 100% drying of flowers received a score of 4. The final *Fusarium–Arabidopsis* disease (FAD) value was calculated by addition of the three subcomponent scores, i.e. F + NS + OS = FAD. *Arabidopsis* genotypes with FAD values of 3 and below were classified as exhibiting resistance to *Fusarium* ear blight whereas those with values of 10 and above were classified as susceptible.

Plant infection studies

Floral tissue was taken at 2, 3, 4, 5, 6 and 7 days after inoculation from each genotype/*Fusarium* isolate interaction. Conventional histochemical staining of fungal hyphae was performed using lactophenol–trypan blue and de-staining with chloral hydrate (Keogh *et al.*, 1980). Microscopic observations were made on a Carl Zeiss 'Axioskop 2' instrument under phase contrast and photomicrographs were prepared with Kodak Ektachrome 160 Tungsten films. Statistical analyses were conducted according to Snedecor and Cochran (1980) and using either the SAS statistical package or the data analysis tools in MS Excel.

DON determinations

Four grams of infected *Arabidopsis* floral tissue from La-er and Col-O plants was harvested 8 days post-*Fusarium*-inoculation, frozen in liquid nitrogen and stored at −80°C. Similar samples were collected from water-inoculated control plants. The frozen tissue samples were ground to a fine powder in a mortar using a pestle in the presence of liquid nitrogen. One gram of each sample was then re-suspended in 3 ml of water and thoroughly mixed using a polytron for 30 sec. The mixture was then incubated for 30 min at 30°C in a water bath. All solid parts were removed by centrifugation and the supernatants were analysed for mycotoxin content. Quantitative analysis of combined DON and its precursor 15-acetyl DON measurements were made using the commercially approved

competitive ELISA Veratox 5/5 kit (Adgen, Ayr, Scotland) and deploying a standard curve for DON ranging from 0.25 to 3.00 ppm. OD650 values were measured 5 min after the addition of the stop solution to the multiwells. To ensure accuracy, each biological same was quantified twice for combined DON and 15-acetyl DON levels.

Acknowledgements

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