Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*

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Contributed by Diter von Wettstein, September 28, 2005

*Fusarium* head blight epidemics in wheat and barley cause heavy economic losses to farmers due to yield decreases and production of mycotoxin that renders the grain useless for flour and malt products. No highly resistant cultivars are available at present. Hyphae of germinating fungal spores use different paths of infection: After germination at the extruded tip of an ovary, the hyphae travel along the epicarp in the space between the lemma and palea. Hyphae reaching the rachis proceed to axially located developing kernels. Using a constitutively green-fluorescence protein-expressing *Fusarium* wild-type strain, and its knockout mutant, preventing trichothecene synthesis, we demonstrate that trichothecenes are not a virulence factor during infection through the fruit coat. In the absence of trichothecenes, the fungus is blocked by the development of secondary wall thickenings in the rachis node of *Nandu* wheat, a defense inhibited by the mycotoxin. In barley hyphae of both wild-type and the trichothecene knockout mutant, are inhibited at the rachis node and rachilla, limiting infection of adjacent florets through the phloem and along the surface of the rachis. Effective resistance to *Fusarium* head blight requires expression of genes that combat these different pathways of infection.

*Fusarium* head blight (FHB) or scab of barley and wheat is considered the worst plant disease in the U.S. since the stem rust epidemics of the 1950s (1). The economic losses caused by scab epidemics during 1993–1998 for wheat and barley farmers in the Midwest are estimated at $3 billion. The epidemics led to barley yield decreases from 75.4 to 46.5 buhshels per acre in North Dakota and from 76.2 to 60.2 bushels per acre in Minnesota. Wheat yields dropped in the two states by 48% and 39%, respectively (2). In addition, a majority of the crop is rejected by the industry, which tolerate less than 1 ppm of the *Fusarium*-produced mycotoxin deoxynivalenol (DON, vomitoxin) (3) in flour products and 0 ppm in melting barley (4). Despite considerable breeding efforts, no highly resistant cultivars of barley and wheat have been obtained so far (5). Recently infection patterns in developing spikes and caryopses of barley and wheat have been studied at the tissue or cellular level (6, 7). In barley, *Fusarium graminearum* strain expressing GFP with an *Aurobasidium pullulans* translation elongating factor promoter (8, 9) has been used to study the infection pattern of intact or detached spikes (10). It demonstrated rapid colonization of brush hairs at the extruded tip of the ovary and growth of the fungus along the epicarp at the space between the lemma and palea. Infection occurred through the pericarp, but in confirmation of earlier work, hyphal growth was halted at the testa for considerable time before the aleurone and endosperm was invaded (11).

Extensive ultrastructural and immunocytochemical analyses have been performed on wheat florets injected with spore suspensions of *Fusarium culmorum* and *F. graminearum* between the lemma and palea. By immunogold labeling with antisera against DON and 3-acytelydeoxynivalenol, the trichothecenes were identified in the cytosol, mitochondria, vacuoles, and the cell wall of the hyphae. In wheat, the toxins were detected in association with cytosolic ribosomes, chloroplasts, plasma-lemma, cell walls, and vacuoles. Toxins were transported apically in xylem and phloem of the rachis to distal uninfected florets (12). With the aid of antibodies reacting with cellulose, xylan, and pectin, it was shown that the cell walls of infected host cells were degraded. This finding provides evidence for the release of enzymes from the pathogen for digestion of cell walls at early stages of infection (13, 14). Labeling of wheat 1,3-β-glucanase and chitinase with antibodies against the tobacco enzymes indicated that these enzymes were induced during infection in the cultivar Arina, considered to be partially resistant to *Fusarium* (15). Arina also contained higher amounts of hydroxyproline-rich glycoproteins and cell wall bound thionins upon infection with *Fusarium* than the more susceptible cultivar Agent.

More detailed studies are required to evaluate the possibilities for targeting metabolites or the elimination of mycotoxins or for using defense genes known to be effective against various necrotrophic or biotrophic pathogens. In the present investigation, we explored, with wheat and barley, whether the infection with *F. graminearum* exhibits a short biotrophic phase before the necrotrophic phase, as is characteristic for hemibiotrophic pathogens. Toward this end, we carried out experiments by infecting isolated caryopses with strains marked with GFP. We followed the infection process at 48, 72, and 96 h after infection (hai) by confocal fluorescent microscopy in the wheat cv. *Nandu*, barley cv. Chevon, which is considered to be partially resistant to FHB (16), and two *mlo*-genotypes resistant backcross lines (BCIngrid-mlo5, BCPallas-mlo5) as well as their parent genotypes Ingrid and Pallas. These two *mlo*-genotypes are highly sensitive to the hemibiotrophic fungi *Bipolaris sorokiniana* (17) and *Magnaporthe grisea* (18).

Two main types of partial resistance to FHB are distinguished in wheat: type I inhibits initial infection of single florets, whereas type II diminishes spreading of the infection within the spikes (19). Considerable type II resistance is found in many barley varieties (20), although spreading occurs in barley under field conditions (21). Type II resistance was compared in the wheat cv. *Nandu* and barley cv. *Chevon* by injection of spore suspension into single developing florets of intact spikes. We used two GFP-expressing strains of *F. graminearum*, wild type strain WT-GFP and mutant strain tri5-GFP. Such mutant strains are incapable of producing trichothecenes because of disruption of

Conflict of interest statement: No conflicts declared.

Abbreviations: FHB, *Fusarium* head blight; DON, deoxynivalenol; hai, hours after infection; dai, days after infection.

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the trichodiene synthase gene TRI5 and exhibit reduced virulence on wheat and winter rye in field trials (10, 22). The use of the two strains allowed determination of the role of trichothecenes as virulence factors of F. graminearum in the wheat and barley lines studied.

Materials and Methods

Supporting Information. For further details, see Supporting Text and Figs. 6–8, which are published as supporting information on the PNAS web site.

Transformation of F. graminearum. Plasmid pIGPAPA (23) was used for generation of transformants of F. graminearum containing the GFP gene and the wild-type TRI5 gene. (Fig. 7A). To obtain efficient integration of the inactivated transgene into the genome of F. graminearum strain 8/H20862 (24) with single crossover events (25), vector pTri5ko carrying a truncated tri5 coding region was constructed. The truncated tri5 with a unique restriction site and flanking HindIII and XbaI sites (Fig. 7B) was cloned into the pGEM-T vector (Promega) and then transferred into plasmid pAN7–1 (26) to yield pTri5ko. This plasmid also carries the hygromycin phosphotransferase gene (HPH) under the control of the GPDA promoter conferring resistance to the antibiotic hygromycin (Fig. 7C). Protoplasts of F. graminearum were cotransformed with linearized pTri5ko and pIGPAPA according to Maier et al. (25). The transformants were cultivated on complete medium plates containing 100 μg/ml hygromycin B (Duchefa, Haarlem, The Netherlands) and tested by Southern hybridization for insertion of the transgene. Two kinds of transformants were selected: class I, those with ectopic integration of the GFP-carrying pIGPAPA (Fig. 7D); and class II, tri5 disruption events with additional integration of pIGPAPA inserted into the backbone of the disrupting vector before integration (Fig. 7E). Three mutants of each class were tested for

![Fig. 1](image-url) Confocal laser microscopy images of the infection of epicarp (48 hai, A–F) and inner tissues of the caryopsis (72 hai, G–K) of wheat and barley by different GFP-expressing strains of F. graminearum. (A–C) Hyphae of WT- GFP in epicarp cells of wheat cv. Nandu. (A) Hyphae showing appressorium-like structures. (B) Constricted parts of hyphae traversing the cell wall through a pit. (C) Intercellular growth of hyphae. (D and E) Infection of wheat cv. Nandu with tri5-GFP. Contents of infected cells are disintegrated; arrow in E indicates infection pore. (F) Infection of barley cv. Ingrid with WT-GFP. Penetration of cell walls occurs preferentially at junction regions. (G) Cross section of barley caryopsis cv. Ingrid infected with tri5-GFP. Fungal growth is restricted to the hypodermis. (H) Cross section of wheat caryopsis cv. Nandu infected with WT-GFP. Arrowheads indicate fungal mycelium in the gap between hypodermis and cross cells. (I and J) Cross sections of barley caryopsis BCPallas-mlo5 infected with WT-GFP showing disintegration of aleurone and endosperm cells (j). Extensive generation of macroconidia on the surface of necrotic caryopsis was observed (J). (K) Cross section of barley caryopsis BCPallas-mlo5 infected with tri5-GFP exhibiting destruction of cell walls and content of hypodermis cells. (Scale bars, 10 μm in A–G and 50 μm in H–L.) a, aleurone; c, cross cells; e, endosperm; h, hypodermis; t, testa.
their pathogenicity and trichothecene production. All mutants of class I showed wild-type FHB symptoms and DON production. All class II mutants showed a strongly reduced pathogenicity in wheat and no production of DON.

**Fungi, Plants, and Growth Conditions.** The wild-type strain 8/1 of *F. graminearum* used for transformation was isolated from naturally infected plants and has been investigated in field trials (24). Induction of conidiation was carried out on SNA-plates (27) incubated at 18°C under near-UV and white light (TLD 36 W-08; TL 40 W-33 RS; Philips) with a 12-h photoperiod for 7–14 days. Conidia were harvested from the plates with a sterile glass rod and sterile water. Macroconidia of all strains were stored as aqueous suspensions at −70°C. For DNA extraction, the wild-type strain or transformants were cultured at 28°C in liquid complete medium with shaking (28).

Barley (*Hordeum vulgare* L.) lines Ingrid, Pallas, and their near isogenic lines BCIngrid-*mlo5* (P22) and BCIngrid-*mlo5* (122) were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Erlangen-Nuernberg, Germany). The six-rowed barley cultivar Chevron was provided by Uwe Sonnewald (Friedrich-Alexander University, Erlangen-Nuernberg, Germany). Spring wheat cultivar Nandu was purchased from Lochow-Petkus (Bergen-Wohle, Germany). Plants were grown in the greenhouse at 18–20°C, 60% relative humidity, and a photoperiod of 16 h (60 μmol m−2 s−1 photon flux density).

**Caryopsis Inoculation.** Spikes of wheat cv. Nandu and barley lines Chevron, Ingrid, Pallas, BCIngrid-*mlo5*, and BCIngrid-*mlo5* were harvested at anthesis. Isolated caryopses were disinfected by incubation in 6% sodium hypochlorite for 40 min, freed from glume, lemma, and palea, and placed on 0.5% (vol/vol) phytagar (Gibco/BRL, Invitrogen Life Technologies, Karlsruhe, Germany). The dorsal side of each caryopsis was covered with 10 μl of a suspension containing 1,500 macroconidia per ml of WT-GFP or *tri5*-GFP, respectively. After inoculation, the caryopses were incubated in a growth chamber at 22°C, 16 h light period, for up to 4 days. Three independent inoculation experiments were performed, and at least three caryopses infected with either of the two *F. graminearum* strains were examined per experiment and time point.

**Spike Inoculation.** Spikes of wheat cv. Nandu and barley cv. Chevron were inoculated with WT-GFP and *tri5*-GFP as described by Voigt et al. (29). For wheat, 10 μl of a suspension containing 2 × 10^7 macroconidia per ml and for barley 5 μl of a 2-fold concentrated macroconidia suspension was applied. 

**Results**

**Progress of Infection with Isolated Caryopses.** We analyzed the infection course of the GFP-expressing *F. graminearum* wild-type strain WT-GFP and its mutant strain *tri5*-GFP, which is incapable of synthesizing trichothecenes, on isolated wheat and barley caryopses. Spikes of wheat cv. Nandu, barley cv. Chevron, and the barley lines Ingrid, Pallas, BCIngrid-*mlo5*, and BCIngrid-*mlo5* were harvested at anthesis. After disinfection, separated caryopses were inoculated by pipetting a macroconidia suspension of either WT-GFP or *tri5*-GFP on the dorsal side of the caryopses. Both fungal strains showed extensive growth on the wheat and barley kernels, and mycelium covered the caryopses completely at 3 dai (data not shown). At 2 dai, growth of WT-GFP and *tri5*-GFP was analyzed in epicarps that had been stripped off the pericarp (fruit coat). Occasionally, the hyphae of both fungi developed mats in the subcuticular space before penetrating the underlying cell wall. As depicted in Fig. 1, the intracellular hyphae attacking the epicarp cells of wheat and barley displayed multiple appressoria-like structures and penetrated through pits or pores into the cytosol of the individual cells (Fig. 1 A–F). Occasionally intercellular growth of hyphae was observed (Fig. 1C). Penetrated host cells showed plasmosis (Fig. 1 D–F) or strong autofluorescence of the cell walls (Fig. 1F), indicating that they underwent cell death. No marked differences between WT-GFP and *tri5*-GFP were observed in term of stages of infection. The stages described occurred both in wheat and barley.
Three and four days after infection, fungal structures were present in inner layers of the pericarp, the testa (seed coat), aleurone, and endosperm in cross sections of infected caryopses (Fig. 1 G–K). Extensive growth of both fungal strains was visible inside the hypodermis, and newly formed macroconidia were present on the surface of the caryopses of wheat cv. Nandu and the barley lines BCIngrid-mlo5 (Fig. 1J) and BC-Pallas-mlo5 on day 3. Generation of macroconidia was first observed on the other genotypes on day 4.

**Comparison of the Different Genotypes.** A comparison of the progress of *F. graminearum* infection at 72 hai through the caryopses of the different barley genotypes infected with the two strains is presented in Fig. 2A. No significant difference is observed for the speed with which the wild-type and trichothecene-deficient fungus traversed the pericarp, cross cells, testa, and aleurone tissues to reach the endosperm in the different barley genotypes. An exception was the slower penetration of the wild type compared to the mutant fungus in the variety Chevron. There is a distinct increase in the speed at which the aleurone and endosperm was infected in the two *mlo5*-lines compared to their isogenic parent lines. Aleurone and endosperm cells were disintegrated in BCIngrid-mlo5 and BCPallas-mlo5 (Fig. 1I) and large lesions were found in the hypodermis (Fig. 1K). This finding demonstrates an increased susceptibility of barley caryopses to *F. graminearum* infection in the presence of the mutated MLO plant defense regulator protein (30). Additionally, the two Ingrid lines were more susceptible than the two Pallas lines. At 72 hai no difference was observed for the wheat cv. Nandu regarding the pathogenicity of the wild-type and trichothecene-deficient fungus (Fig. 2A). The susceptibility of cv. Nandu was comparable to that of the two barley *mlo*-lines. In all infected caryopses of cv. Nandu, fungal mycelium was visible in gaps between the hypodermis and cross cells (Fig. 2H). This growth characteristic was not observed in barley and could be confirmed independently when caryopses of wheat cv. Bobwhite were infected with *F. graminearum* (C.J., unpublished data). The progression of infection was subsequently analyzed at 96 hai in the barley genotypes (Fig. 2B). Again there was no significant difference in the progress of infection when wild-type and trichothecene-disrupted fungus were compared. In a much larger portion of the sections the aleurone and endosperm had been reached by the infecting hyphae in the two *mlo5*-mutant genotypes compared to their near-isogenic parent lines. Again slower progress of the infection in cv. Chevron than in Ingrid and Pallas was visible at 96 hai.

**Spreading of Infection Within Spikes from One Floret to Another in Wheat and Barley.** In the wheat cv. Nandu injection of florets with the trichothecene-deficient fungus led to its containment in the rachis node at the bottom of the floret (Fig. 3D, see Fig. 8 for identification of organs). A larger magnification of these areas revealed a heavy cell wall thickening that formed a cavity containing accumulated hyphae, and arose probably by synthesis of pectins, xylans, or cellulose (Fig. 4A). When cv. Nandu was infected with the wild-type *F. graminearum*, large amounts of hyphae were seen moving through the rachis at 6 dai (Fig. 3B) and thickened cell walls were not formed (Fig. 4B). The movement of the GFP labeled *F. graminearum* wild-type strain intracellularly occurred in wheat cv. Nandu through the elements of the vascular bundles (Fig. 5A and B). In the parenchyma cells the fungus grew in the apoplastic space (Fig. 5C). After extensive accumulation of the pathogen close to the epidermis of the rachis, the mycelium was released through stomata (Fig. 5D). In contrast to wheat, *F. graminearum* hyphae of both wild type and the trichothecene knockout mutant were inhibited in barley at the rachis node and rachilla (Fig. 3G and H) limiting infection of adjacent florets through the phloem and along the surface of the rachis through infection of trichomes (Fig. 6). Thus, an enhanced defense was not observed by elimination of the mycotoxin.
wild-type F. graminearum wheat, cell death is induced (Fig. 1). The cuticle and the walls of the epicarp. As soon as the fungus appears to be living only as long as the fungus grows between the pericarp to the testa, aleurone, and endosperm, the cells tests of the progress of infection from the epicarp through the rachis node showing the heavily increased thickness of the cell walls in wheat, preventing the trichothecene deficient mutant of F. graminearum tri5-GFP from entering the rachis (12 dai). (B) Heavy infection of the rachis node by wild-type F. graminearum (6 dai, WT-GFP). (Scale bars, 50 μm.)

Discussion

The combined use of GFP labeled wild-type and trichothecene-deficient F. graminearum strains in certain barley and wheat genotypes has provided the following information: In tests of the progress of infection from the epicarp through the pericarp to the testa, aleurone, and endosperm, the cells appear to be living only as long as the fungus grows between the cuticle and the walls of the epicarp. As soon as the fungus enters into the cytosol of the epipcarp cells in both barley and wheat, cell death is induced (Fig. 1 A–F). Thus, an initial biotrophic phase of Fusarium in head blight cannot be identified. The progress of infection through the fruit coat of barley cv. Chevron is slower than in the two other cultivars (Ingrid and Pallas, Fig. 2 A and B). As Chevron is considered to exhibit partial type I resistance (16), the test with isolated caryopses may be used to further elucidate the mechanism for this type of resistance. The barley mlo5 mutants providing complete resistance to the biotrophic barley powdery mildew fungus (Blumeria graminis f. sp. hordei) were more susceptible to F. graminearum on the basis of the speed of the necrotrophic fungus to traverse the fruit coat to the aleurone and endosperm. Additionally the inactivation of the MLO protein promoted the cell wall destruction by F. graminearum as evidenced by early appearance of holes in the hypodermis (Fig. 1A). This observation is reminiscent of the enhanced susceptibility of barley mlo mutant lines against the hemibiotrophic fungi B. sorokiniana (17) and M. grisea (18). Clear evidence was obtained that the trichothecenes do not act as virulence factors in the fruit coat tissues of the barley and wheat lines investigated (Fig. 2 A and B) in agreement with the analysis by Bai et al. (31) regarding the initial infection of wheat florets by injection of spore suspension.

Previous investigations have demonstrated the action of DON in promoting the spreading of F. graminearum in wheat from one infected floret to neighboring ones by hyphal growth through the rachis internodes (10). The present investigation demonstrates that, in the absence of trichothecene production, the Fusarium fungus is prevented in wheat from moving into the rachis by the development of strong cell wall fortifications in the rachis node (Figs. 3 and 4). This defense response is inhibited by trichothecenes. In barley, the Fusarium hyphae are inhibited at the rachis node and rachilla irrespective of the presence or absence of the mycotoxin, explaining the considerable type II diminished spreading of the infection within the spike, which occurs primarily by hyphal growth and infection on the surface of the rachis. This inhibition is not accompanied by the development of cell wall thickenings, thus indicating a different mechanism.

In a recent investigation, constitutive (over)expression of trichothecene 3-O-acetyltransferase in wheat conferred partial protection against spread of scab in greenhouse tests (32). Transformants of cv. Bobwhite with the TRI101 gene of F. sporotrichioides under the control of the ubiquitin promoter were produced. A transformant with trichothecene 3-O-acetyltransferase activity in the endosperm and glumes showed partial protection against spread of FHB. The effect of the trichothecene 3-O-acetyltransferase was the inactivation of DON and T-2 toxin as virulence factors by acetylation. This finding indicates that the induced defense response observed in our study is inhibited by the deacetylated trichothecenes.

Detoxification of trichothecenes does not prevent the infection of the caryopses through the degenerating anthers and growth of the fungus between lemma or palea and the epicarp in both wheat and barley. Infection through the epicarp leads to early cell death and rapid growth through the different layers of the fruit coat independent of the production of trichothecenes. Therefore, expression of proteins or low molecular weight compounds inhibiting the growth of the pathogen in the different tissue layers of the fruit coat are required for effective and durable resistance. Our finding that the penetration by F. graminearum is slower in cv. Chevron than in other barley lines suggests using transcriptome analysis of infected caryopses of Chevron and more susceptible varieties to identify genes that are differentially regulated in Chevron. The greater sensitivity of mutant mlo barley lines to F. graminearum suggests that overexpression of wild-type Mlo (30) and other cell death regulators, like HvBI-1 (33), in the epicarp, pericarp, and testa using the available fruit coat specific gene promoters of the germin family (34), could confer enhanced resistance to Fusarium. A further approach is sug-
gested by the observation that *F. graminearum* secrets enzymes degrading xylans, pectins, and cellulose of the host cell walls (13, 14). Expression of specific inhibitors like the wheat TAXI (35) or XIP (36) xylanase inhibitors or polygalacturonase inhibitor proteins (37) might prevent the penetration of the fungus through the fruit coat tissues of developing wheat and barley kernels.

Although the breeding aims for preventing infection through the fruit coat are identical in wheat and barley, different avenues have to be explored for obtaining type II resistance against spread of the infection within a spike. Wheat transformants detoxifying DON and other trichothecenes either by expressing trichothecene 3-O-acetyltransferase or by glycosylation of the C3 position with a UDP-glycosyltransferase (38) provide the means of investigating in detail whether the inhibition of the cell wall thickening can be reversed by detoxification of the mycotoxins through the host. Further aims are to prevent growth of the hyphae in the apoplastic space and in the vascular bundles of the rachis, possibly by modification of cell walls. In barley, the focus will be on preventing hyphal penetration of the rachis from the outside, especially through the trichomes, by expressing inhibitors with trichome specific gene promoters.

This work was supported by the Genome Analysis of the Biological System of Plants program of the Federal Ministry of Education and Research (BMBF), Grants GABI-Agrotec (to K.-H.K. and W.S.).

Figure 8