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Reduced Virulence of *Gibberella zeae* Caused by Disruption of a Trichothecene Toxin Biosynthetic Gene

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The production of trichothecene mycotoxins by some plant pathogenic species of *Fusarium* is thought to contribute to their virulence. *Gibberella zeae* (*F. graminearum*) is an important cereal pathogen that produces the trichothecene deoxynivalenol. To determine if trichothecene production contributes to the virulence of *G. zeae*, we generated trichothecene-deficient mutants of the fungus by gene disruption. The disrupted gene, *Tri5*, encodes the enzyme trichodiene synthase, which catalyzes the first step in trichothecene biosynthesis. To disrupt *Tri5*, *G. zeae* was transformed with a plasmid carrying a doubly truncated copy of the *Tri5* coding region interrupted by a hygromycin B resistance gene. *Tri5*$^-$ transformants were selected by screening for the inability to produce trichothecenes and by Southern blot analysis. *Tri5*$^-$ strains exhibited reduced virulence on seedlings of Wheaton wheat and common winter rye, but wild-type virulence on seedlings of Golden Bantam maize. On Caldwell and Marshall wheat and Porter oat seedlings, *Tri5*$^-$ strains were inconsistent in causing less disease than their wild-type progenitor strain. Head blight developed more slowly on Wheaton when inoculated with *Tri5*$^-$ mutants than when inoculated with wild-type strains. These results suggest that trichothecene production contributes to the virulence of *G. zeae* on some hosts.

Gibberella zeae (Schwein.) Petch (anamorph *Fusarium graminearum*) is an important pathogen of a number cereal crops in many areas of the world. The fungus is remarkable in the diversity of plants and tissues on which it incites disease. *G. zeae* causes head and seedling blight of small grains such as wheat and rye, ear and stalk rot of maize, stem rot of carnation, and seedling blight and root rot of a number of other plant species, including beans, clover, and tomato (Dickson 1923; Nelson et al. 1975; Cook 1980, 1981; Kommedahl and Windels 1981). In the case of head blight and ear rot, *G. zeae* not only reduces the yield and quality of infected grain but also produces a number of mycotoxins, including zearalenone and the trichothecene deoxynivalenol (DON) in infected grain (Cook 1980, 1981).

Trichothecenes are sesquiterpenoid epoxides produced by certain species of the fungal genera *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* and by at least two species of the plant genus *Baccharis* (Jarvis et al. 1991; Sharma and Kim 1991). These toxins are potent inhibitors of protein synthesis in eukaryotic cells but have a number of toxic affects on intact cells and purified mitochondria (Sharma and Kim 1991). In addition, trichothecenes have been implicated in incidents of mycotoxicosis in humans and animals (Marasas et al. 1984). Although the role of these toxins in the ecology of the fungi that produce them is unclear, there is a growing body of evidence indicating that trichothecenes can enhance the virulence of some plant-pathogenic species of *Fusarium* on some hosts. For example, trichothecene nonproducing strains of *G. pulicaris* (anamorph *F. sambucinum*), generated by disruption of the trichothecene biosynthetic gene *Tri5*, exhibited reduced virulence on parsnip root (Desjardins et al. 1992). Similarly, trichothecene nonproducing mutants of *F. sporotrichioides*, generated by UV irradiation, were also less virulent on parsnip than their wild-type progenitor strain (Desjardins et al. 1989).

There is also indirect evidence to suggest that trichothecenes may contribute to the virulence of *G. zeae*. DON and its acetylated derivatives, such as 3- and 15-acetyldeoxynivalenol (3ADON and 15ADON) are phytotoxic. They inhibit the growth of wheat seedlings, coleoptile segments, embryos, and calli (Bruins et al. 1993; Wang and Miller 1988). DON and 15ADON induced lesions on wheat leaves and the severity of the lesions was positively correlated with head blight susceptibility (Scholbrock et al. 1992). In addition, trichothecenes tend to accumulate to higher concentrations in the kernels of more susceptible wheat than more resistant wheat (Snijders and Perkowski 1990; Atanassov et al. 1994). Finally, there is some evidence for a positive correlation between head blight resistance and the ability of wheat to metabolize DON (Miller and Arnison 1986). However, it appears that DON production may not be required for pathogenicity, since some field isolates of *G. zeae* that were unable to produce DON and 3ADON in culture were pathogenic on wheat, rye, and triticale seedlings (Manka et al. 1985). In addition, when protoplast fusions were done between a DON produc-

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ing, pathogenic strain and a DON nonproducing, nonpathogenic strain, strains that were DON nonproducers but pathogenic on maize and carnation were regenerated (Adams and Hart 1989).

Several genes involved in trichothecene biosynthesis in *F. sporotrichioides* have been cloned (Hohn and Beremand 1989; Hohn et al. 1993). One of these genes, *Tri5* (previously designated *Tox5*), encodes the enzyme trichodiene synthase, which catalyzes the cyclization of farnesyl pyrophosphate to trichodiene (Fig. 1). The high degree of sequence homology between *Tri5* from *F. sporotrichioides* and *G. pulicaris* facilitated the cloning and subsequent disruption of *Tri5* from the latter fungus (Hohn and Desjardins 1992). *Tri5* is an excellent candidate for study of trichothecene function, because the conversion of farnesyl pyrophosphate to trichodiene is the step at which trichothecene biosynthesis branches from general isoprenoid metabolism. As a result, strains carrying a disrupted *Tri5* do not produce trichothecenes or their biosynthetic intermediates (Hohn and Desjardins 1992).

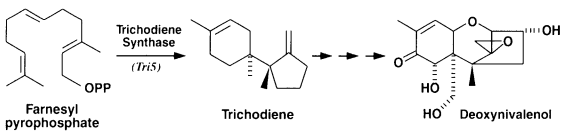

Fig. 1. Biosynthetic pathway of deoxynivalenol. This scheme omits the numerous biosynthetic steps between trichodiene and deoxynivalenol. Abbreviation: OPP, pyrophosphate.

The objective of the present study was to determine whether the ability to produce trichothecenes contributes to virulence in *G. zeae*. This was accomplished by cloning the *Tri5* coding region from *G. zeae* and generating trichothecene nonproducing mutants by gene disruption.

RESULTS

Amplification of the *Tri5* coding region.

A DNA fragment 1,186 base pairs long was amplified from the genomic DNA template of *G. zeae* strain W-8 using oligonucleotides O63 and O178. Sequence analysis of this fragment indicated that it consisted of an 1125 nt open reading frame (ORF) that was interrupted by a 59 nt intron. The intron was identified by sequence motifs homologous to the 5′, internal, and 3′ consensus sequences for fungal introns (Gurr et al. 1987). In addition, the intron within the *G. zeae* ORF is in the same position as the introns in *Tri5* from *F. sporotrichioides* and *G. pulicaris* (Hohn and Beremand 1989; Hohn and Desjardins 1992). The nucleotide sequence of the

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Gz   MENFPTEYFLNTSVRLLEYIRYRDSNYTREERIENLHYAYNKAAHHFAQP    50
Gp   MENFPTEYFLNTSVRLLEYIRYRDSNYTREERIENLHYAYNKAAHHFAQP    50
Fs   MENFPTEYFLNTTVRLLEYIRYRDSNYTREERIENLHYAYNKAAHHFAQP    50
     ************.************************************

Gz   RQQQMLKVDPKRLQASLQTIVGMVVYSWAKVSKECMADLSIHYTYTLVLD   100
Gp   RQQQLLKVDPKRLQASLQTIVGMVVYSWAKVSKECMADLSIHYTYTLVLD   100
Fs   RQQQLLKVDPKRLQASLQTIVGMVVYSWAKVSKECMADLSIHYTYTLVLD   100
     ****.*********************************************

Gz   DSSDDPHPAMLNYFDDLQAGREQAHPWWALVNEHFPNVLRHFGPFCSLNL   150
Gp   DSSDDPYPAMMNYFNDLQAGREQAHPWWALVNEHFPNVLRHFGPFCSLNL   150
Fs   DSKDDPYPTMVNYFDDLQAGREQAHPWWALVNEHFPNVLRHFGPFCSLNL   150
     **.***.*.*.***.************************************

Gz   IRSTMDFFEGCWIEQYNFGGFPGSDDYPQFLRRMNGLGHCVGASLWPKDL   200
Gp   IRSTLDFFEGCWIEQYNFGGFPGSHDYPQFLRRMNGLGHCVGASLWPKEQ   200
Fs   IRSTLDFFEGCWIEQYNFGGFPGSHDYPQFLRRMNGLGHCVGASLWPKEQ   200
     ****.******************.**********************.

Gz   FDERKHFLEITSAVAQMENWMVWVNDLMSFYKEFDDERDQISLVKNFVTC   250
Gp   FDERGLFLEITSAIAQMENWMVWVNDLMSFYKEFDDERDQISLVKNYVVS   250
Fs   FNERSLFLEITSAIAQMENWMVWVNDLMSFYKEFDDERDQISLVKNYVVS   250
     *.**  *******.*********************************.*..

Gz   HEITLDEALEKLTQETLHSSKQMVAVFSDKDPQVMDTIECFMHGYVTWHL   300
Gp   DEITLHEALEKLTQDTLHSSKQMVAVFSEKDPQVMDTIECFMHGYVTWHL   300
Fs   DEISLHEALEKLTQDTLHSSKQMVAVFSDKDPQVMDTIECFMHGYVTWHL   300
     .**.*.********.*************.**********************

Gz   CDARYRLHEIYEKVKDQDTEDAKKFCKFFEQAANVGAVAPSEWAYPQVAQ   350
Gp   CDHRYRLNEIYEKVKGQKTEDAEKFCKFYEQAANVGAVSPSEWAYPPIAQ   350
Fs   CDRRYRLSEIYEKVKEEKTEDAQKFCKFYEQAANVGAVSPSEWAYPPVAQ   350
     ** **** ********...*****.*****.*********.********.**

Gz   LANVRAKD--------DVKEAQKPILSSIELVE   375
Gp   LANIRTKDVKDLKDVKDLKEIQKPLLSSIELVE   383
Fs   LANVRSKDVK---------EVQKPFLSSIELVE   374
     ***.*.**           * ***.********
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Fig. 2. Alignment of deduced amino acid sequences from *Tri5* of *Gibberella zeae* (Gz), *G. pulicaris* (Gp), and *F. sporotrichioides* (Fs). At a given position, identical sequences are indicated by (*), conservative amino acid differences are indicated by (·). The Gp and Fs sequences are from Hohn and Desjardins (1992) and Hohn and Beremand (1988), respectively. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL databank with accession number U22464.

ORF had 85.8 and 86.4% homology to the *Tri5* coding regions of *F. sporotrichioides* and *G. pulicaris*, respectively (Hohn and Beremand 1989; Hohn and Desjardins 1992), while the deduced amino acid sequence had 90.9 and 91.2% homology to the trichodiene synthases of *F. sporotrichioides* and *G. pulicaris*, respectively (Fig. 2).

Transformation-mediated disruption of *Tri5*.

The transformation vector pGZTS4-1 (Fig. 3) was constructed to facilitate gene disruption via either one (additive disruption) or two (gene replacement) homologous recombination events between the vector and the resident *Tri5* in *G. zeae*. To permit disruption through a single recombination event, the *Tri5* coding region present in pGZTS4-1 was truncated at the 5′ end, by deletion of the adenosine nucleotide in the initiation codon, and at the 3′ end, by deletion of the last 87 nucleotides. During the transformation process, a single homologous recombination event between pGZTS4-1 and the resident *Tri5* in *G. zeae* should generate two truncated, and presumably nonfunctional, copies of the *Tri5* coding region (Shortle et al. 1982). One copy of the coding region should lack the adenosine nucleotide of the initiation codon and the other copy should lack the last 87 nucleotides at the 3′ end of the coding region. To facilitate disruption via two recombination events, the *Tri5* coding region in pGZTS4-1 was interrupted by insertion of the Promoter 1-*hyg*B (P1-*hyg*B) marker between nucleotides 576 and 577 of the coding region. Thus, two homologous recombination events between pGZTS4-1 and the *Tri5* in *G. zeae* should result in replacement of the wild-type *Tri5* coding region with a copy of *Tri5* interrupted by the P1-*hyg*B insert.

Following transformation of wild-type *G. zeae* field isolate Z3639, 42 hygromycin B resistant transformants were recovered and analyzed for their ability to produce DON and its acetylated derivatives (e.g., 15ADON). Nine transformants (GZT4, GZT9, GZT18, GZT26, GZT27, GZT33, GZT34, GZT36, and GZT40) that did not produce these trichothecenes were identified. Genomic DNA from these transformants was digested with either *Stu*I or *Xmn*I and subjected to Southern blot analysis. *Stu*I does not cut within the transformation vector pGZTS4-1, while *Xmn*I cuts once at nt 674 of the *Tri5* coding region (Fig. 3). Southern analysis indicated that five classes of trichothecene nonproducing transformants were recovered. The first class consisted of transformants GZT33 and GZT36. In Southern analyses, DNA from these strains yielded hybridization patterns that were consistent with the integration of a single copy of the entire transformation vector at the *Tri5* locus via a single homologous recombination event (Fig. 4A and B, lane 2). The structure of the *Tri5* locus in these two transformants should include two copies of the *Tri5* coding region separated from one another by the pCR1000 sequence from the transformation vector (Fig. 4C, map 2). One copy of the coding region should be truncated at the 3′ end and the other at the 5′ end. The latter copy should also contain the P1-*hyg*B insert. The second class of trichothecene nonproducing transformants consisted of GZT9, GZT18, and GZT27. Hybridization patterns of DNA from these three transformants were consistent with the integration of two copies of pGZTS4-1 at the *Tri5* locus (Fig. 4A, lane 4; B, lane 3; C, map 3). The third class of transformants included GZT26 only. Hybridization patterns of DNA from this transformant were consistent with the integration of two or more copies of pGZTS4-1 at the *Tri5* locus and a subsequent rearrangement that resulted in a construct consisting of two copies of the *Tri5* coding region separated from one another by a copy of the pCR1000 sequence (Fig. 4A, lane 3; B, lane 4; C, map 4). Both copies of the coding region should have the P1-*hyg*B insert; however, the copy upstream from the pCR1000 sequence should have the 3′ truncation while the copy downstream from pCR1000 should have the 5′ truncation (Fig. 4C, map 4). The fourth-class of transformants included GZT40 only. The hybridization patterns of genomic DNA from GZT40 were consistent with replacement of the resident copy of the *Tri5* coding region with one containing the P1-*hyg*B insert (Fig. 4A and B, lane 5) presumably via two homologous recombination events between pGZTS4-1 and *Tri5* in *G. zeae*. Thus, the structure of the *Tri5* locus in this transformant should consist of a single copy of the *Tri5* coding region containing the P1-*hyg*B insert but with intact 5′ and 3′ ends (Fig. 4C, map 5). The fifth class of trichothecene nonproducing transformants included GZT4 and GZT34. Surprisingly, the hybridization patterns of DNA from these transformants were consistent with ectopic integration of pGZTS4-1 (Fig. 4A and B, lane 6).

The disruption of *Tri5* was probably the most stable in transformants GZT26 and GZT40 since there is no obvious mechanism by which reversion to wild-type *Tri5* could occur in these two strains. In contrast, the structures of the mutant *Tri5* loci in class 1 and 2 transformants can revert to the wild type via homologous recombination (May 1992). Transformants GZT26 and GZT40 and the wild-type progenitor strain, Z3639, had the same morphology and pigmentation and similar growth rates on V-8, YEP, and potato-dextrose agar media. Similarly, no consistent differences were observed in the production of macroconidia on V8 agar and perithecia on carrot agar between GZT26, GZT40, and Z3639. Because these transformants appeared similar to wild type with respect to growth and morphological characteristics in addition to

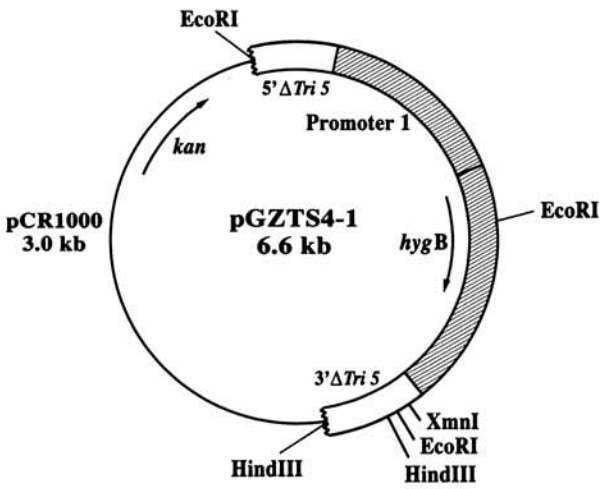

Fig. 3. Map of transformation vector (pGZTS4-1) used to disrupt *Tri5* in *Gibberella zeae*. Abbreviations: 5′ *Tri5*, truncated 5′ half of *Tri5* coding region (nt 2-576); 3′ *Tri5*, truncated 3′ half of *Tri5* coding region (nt 577 to 1098); *hyg*B, hygromycin B phosphotransferase coding region; Promoter 1, Promoter 1 from *Cochliobolus heterostrophus*; *kan*, kanamycin phosphotransferase gene.

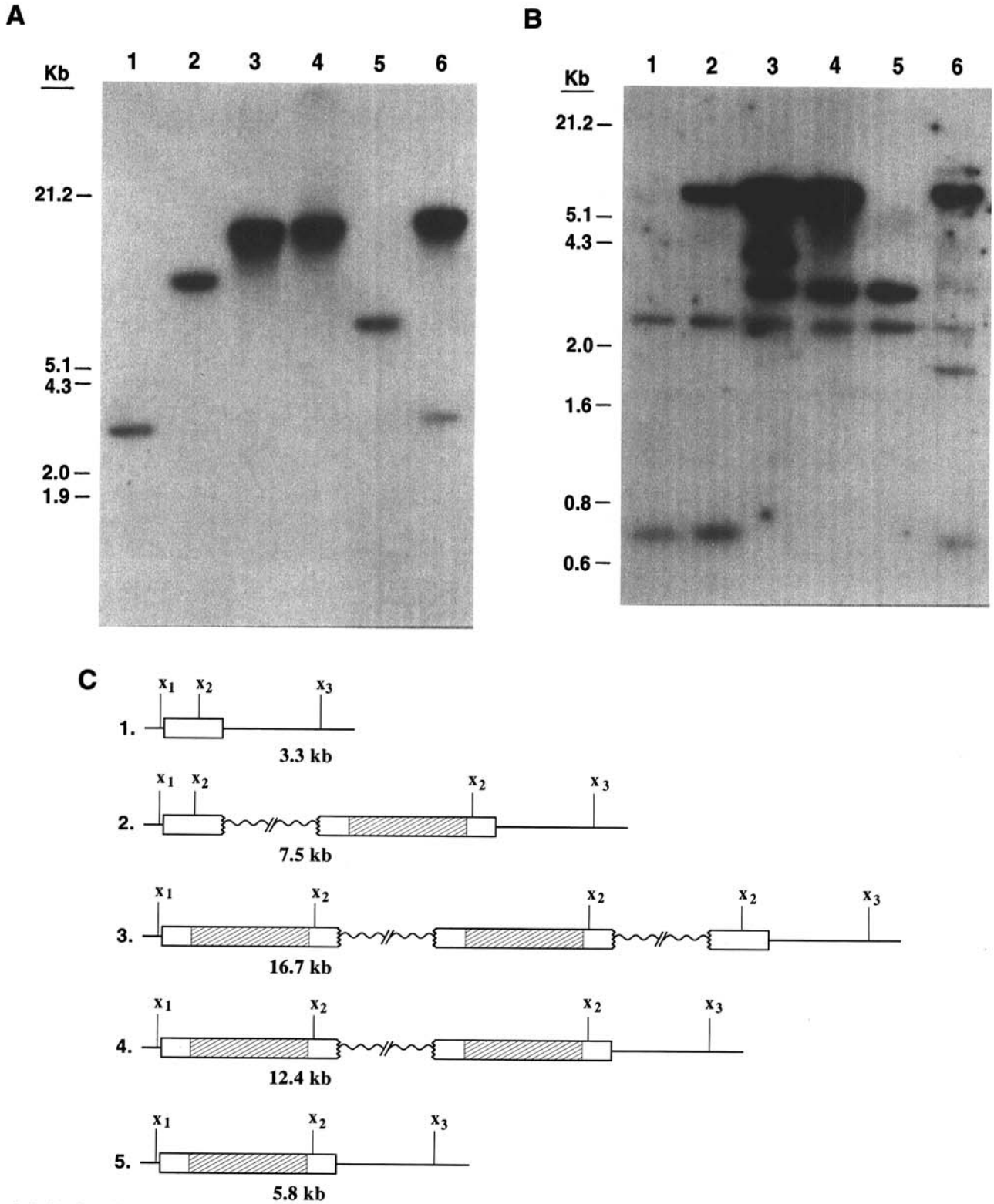

Fig. 4. **A,** Southern blot analysis of *Gibberella zeae* genomic DNA digested with restriction enzyme *Stu*I. Lanes: 1, wild-type strain Z3639; lanes 2 to 6, trichothecene-nonproducing transformants GZT33, GZT26, GZT27, GZT40, and GZT4, respectively. **B,** Southern blot analysis of *Xmn*I digested *G. zeae* genomic DNA. Lanes, 1, Z3639; 2 to 6, GZT33, GZT27, GZT26, GZT40, and GZT4, respectively. Both blots were probed with ^{32}P-labeled DNA corresponding to the *G. zeae Tri5* coding region. **C,** Restriction maps of *Tri5* loci in wild type and trichothecene nonproducing transformants of *G. zeae*. The maps are based on the interpretation of the Southern blot data from **A** and **B.** Maps: 1, wild-type *Tri5* in strain Z3639; 2 to 5, *Tri5* in transformants GZT33, GZT27, GZT26, and GZT40, respectively. The white boxes represent the *Tri5* coding region. Jagged ends of white boxes represent truncated ends of the *Tri5* coding region. Hatched boxes represent the P1-*hyg*B insert within the *Tri5* coding region. Wavy lines correspond to sequences derived from pCR1000. X_1 and X_3 are *Xmn*I sites situated upstream and downstream, respectively, of the *Tri5* coding region. X_2 is an *Xmn*I site located at nt 674 of the wild-type *Tri5* coding region. The kb values indicate the length of each map from X_1 to X_3.

having stable *Tri5* disruptions, GZT26 and GZT40 were selected and used in virulence assays to determine whether disruption of *Tri5* affected the virulence of *G. zeae*.

Virulence of *Tri5*⁻ transformants.

One cultivar each of maize, oats, and rye and three wheat cultivars were employed in seedling virulence assays to examine the virulence of the wild-type progenitor strain Z3639 and the trichothecene nonproducing (*Tri5*⁻) transformants GZT26 and GZT40. Disease severity was assessed in terms of seedling emergence and growth of seedlings once they had emerged.

On Golden Bantam maize, no significant differences were observed in emergence or seedling growth when seeds were inoculated with Z3639 or the *Tri5*⁻ transformants (Table 1). In contrast, the *Tri5*⁻ mutants did not reduce emergence of Porter oats seedlings as much as Z3639. However, there were no significant differences in the effect of the wild type and *Tri5*⁻ strains on growth of oat seedlings once they had emerged (Table 1). *Tri5*⁻ mutants were less virulent on rye than the wild type. The mutants did not reduce emergence or growth of rye seedlings as much as strain Z3639 (Table 1). With respect to wheat, differences in virulence between the wild type and *Tri5*⁻ strains varied depending on the cultivar examined and on the method used to assess disease. On varieties Caldwell and Marshall, there were not consistent differences in emergence from mutant and wild-type inoculated seeds (Table 1). However, once seedlings had emerged, the growth of mutant inoculated seedlings was greater than the growth of wild-type inoculated seedlings. In contrast, the *Tri5*⁻ mutants caused less disease on Wheaton wheat than the wild-type strain, both in terms of emergence and growth.

Trichothecene production was examined in Golden Bantam maize and Caldwell wheat. As expected, no trichothecenes were detected in water inoculated and *Tri5*⁻ transformant inoculated seedlings. However, concentrations of TriAc-DON as high as 113 μg/g fresh weight were detected in the leaves of seedlings infected with wild-type strain Z3639 (Fig. 5).

Head blight assays were conducted to determine if the *Tri5*⁻ mutants GZT26 and GZT40 also exhibited reduced virulence on Wheaton wheat heads. This assay was repeated four times and included the trichothecene-producing (*Tri*⁺) transformant GZT12. There was considerable variation in disease symptoms within individual treatments and in the rate of disease development in the different experiments. However, the general tendency was for disease to develop more slowly on the heads inoculated with *Tri5*⁻ strains than on those inoculated with *Tri5*⁺ strains (Fig. 6). In three out of the four trials, head blight was significantly ($P < 0.05$) less on GZT26 and GZT40 (*Tri5*⁻) inoculated heads than on GZT12 and Z3639 (*Tri5*⁺) inoculated heads at 10 (data not shown) and 14 days after inoculation (Table 2). However, in a fourth experiment (assay 2), differences between disease on *Tri5*⁻ and *Tri5*⁺ inoculated heads were not always statistically significant (Table 2). Generally, any differences in head blight between *Tri5*⁻ and *Tri5*⁺ inoculated heads were no longer apparent by 21 days after inoculation (Fig. 6).

DISCUSSION

The 1,186-bp fragment amplified from *G. zeae* was identified as the *Tri5* coding region based on the high degree of sequence homology between its nucleotide and deduced amino acid sequences and those sequences for *Tri5* from *F. sporotrichioides* and *G. pulicaris* (Hohn and Beremand 1989; Hohn and Desjardins 1992). The greatest variability between the deduced amino acid sequence from *G. zeae* and the sequences from the other two fungi occurs at the carboxy terminal end. However, the *G. pulicaris* and *F. sporotrichioides* sequences also differ substantially from one another in this region (Fig. 2).

Transformation-mediated gene disruption was used to generate *Tri5*⁻ mutants of *G. zeae*. These mutants did not produce trichothecenes under conditions conducive to trichothecene production by wild-type *G. zeae*. The transformation vector, pGZTS4-1, was constructed to facilitate both additive and gene replacement type disruption. Two homologous recombination events, one upstream and the other downstream from the P1-*hyg*B insert, between the vector and the *G. zeae* genome resulted in replacement of the *Tri5* coding region by a copy of the coding region with the P1-*hyg*B insert in a manner similar to that described by Rothstein (1983) for yeast. A single homologous recombination event between the transformation vector and the *G. zeae* genome resulted in the

Table 1. Seedling blight severity on six cereal hosts caused by trichothecene producing (Z3639) and nonproducing (GZT26 and GZT40) strains of *Gibberella zeae*

				Wheat		
Inoculum	**Maize**	**Oats**	**Rye**	**Caldwell**	**Marshall**	**Wheaton**
Percent seedling emergence[a]						
Water	82 a	100 a	88 a	92 a	98 a	91 a
GZT26	66 b	85 b	72 b	79 b	85 ab	74 b
GZT40	74 ab	92 ab	80 ab	79 b	90 a	74 b
Z3639	64 b	78 c	55 c	78 b	72 b	50 c
Seedling height (cm)[a]						
Water	22.1 a	18.6 a	15.4 a	17.7 a	21.3 a	19.3 a
GZT26	11.5 b	17.1 b	12.3 b	16.3 b	18.9 b	18.8 a
GZT40	11.6 b	16.4 b	13.1 b	16.5 ab	18.9 b	16.1 b
Z3639	16.4 b	16.4 b	9.3 c	14.3 c	13.8 c	12.5 c

[a] Values are means from two (oats, rye, and Marshall) or three (maize, Caldwell, and Wheaton) experiments. Each experiment included three or four pots for each inoculum type. Ten seeds were sown in each pot. For the analysis of emergence, one replicate was one pot with 10 seeds. In the seedling height analysis one replicate was an individual seedling and values of 0 (i.e., ungerminated seeds) were omitted from the analysis. Data for each host plant were analyzed separately by one-way analysis of variance. The overall *F*-tests for treatments were significant at $P < 0.01$. Within a column, means followed by different letters are significantly different ($P < 0.05$) by *t*-tests of least squares means.

generation of two truncated copies of the *Tri5* coding region, essentially in the same manner as the additive gene disruption described by Shortle et al. (1982) for yeast. Transformant GZT40 appears to have resulted from gene replacement while transformants GZT33 and GZT36 appear to have resulted from additive disruption of the *Tri5* coding region. The second and third classes of transformants probably resulted from an initial single homologous recombination event (additive disruption) and subsequent integration of an additional copy, and in the case of GZT26 possibly more, of the transformation vector. In addition, GZT26 presumably had undergone rearrangements leading to the loss of part of at least one copy of the transformation vector. These data demonstrate that *G. zeae* is amenable to gene disruption by either the replacement or additive procedures. The replacement disruption was less frequent than additive disruption, perhaps because the former requires two homologous recombination events while the latter requires only one such event.

Transformants GZT26 and GZT40 were used as *Tri5*$^-$ strains in the virulence assays because they were considered to be the most stable transformants. That is, the structure of the *Tri5* locus in these strains was such that it could not recombine to produce a wild-type gene (May 1992). Although there was considerable variation in disease development in the head blight assays, the general trend was that disease developed more slowly in heads inoculated with the *Tri5*$^-$ strains than in heads inoculated with the *Tri5*$^+$ strains (Z3639 and GZT12). The results of seedling virulence assays indicate that disruption of *Tri5* causes a small but consistent reduction in virulence of *G. zeae* on some hosts (i.e., Wheaton wheat and common winter rye), a small inconsistent reduction in virulence on other hosts (i.e., Caldwell and Marshall wheat and Porter oats), and no effect on still another host (i.e., Golden Bantam maize). These results are consistent with those obtained with *G. pulicaris* in which *Tri5* had been disrupted. Trichothecene nonproducing strains of *G. pulicaris* were less virulent than the wild type on parsnip root, but exhibited wild-type virulence on potato tubers (Desjardins et al. 1992). The wild-type levels of virulence of the *Tri5*$^-$ strains of *G. zeae* on the maize seedlings are also consistent with the high levels of virulence on maize ears of trichothecene nonproducing strains of the fungus that were regenerated from protoplast fusions of a trichothecene producing and a nonproducing strain (Adams and Hart 1989).

Because *Tri5* disruption leads to an inability to produce trichothecenes, the data presented here suggest that trichothecene production contributes to the virulence of *G. zeae*. Although the reduction in virulence of the *Tri5*$^-$ strains is small, their ability to compete against more virulent wild-type strains under more natural conditions could be severely limited. Less virulent strains may be less able to colonize host tissue and to produce inoculum for further infection. Whether or not this occurs could be tested by inoculating plants with a mixture of conidia from *Tri5*$^+$ and *Tri5*$^-$ strains and characterizing strains isolated from the resulting infected tissue.

It is possible that disruption of *Tri5* could result in physiological changes, other than the loss of trichothecene production, that could affect virulence. For example, the lack of trichodiene synthase in *Tri5*$^-$ mutants could result in accumulation of farnesyl pyrophosphate or diversion of excess farnesyl pyrophosphate into other biosynthetic pathways. This in turn could affect the physiology of *G. zeae* and, as a result, virulence. Alternatively, it is possible that the inability of *Tri5*$^-$ mutants to produce trichodiene-derived compounds other than trichothecenes caused the reduction in virulence of GZT26 and GZT40. Such compounds could include the

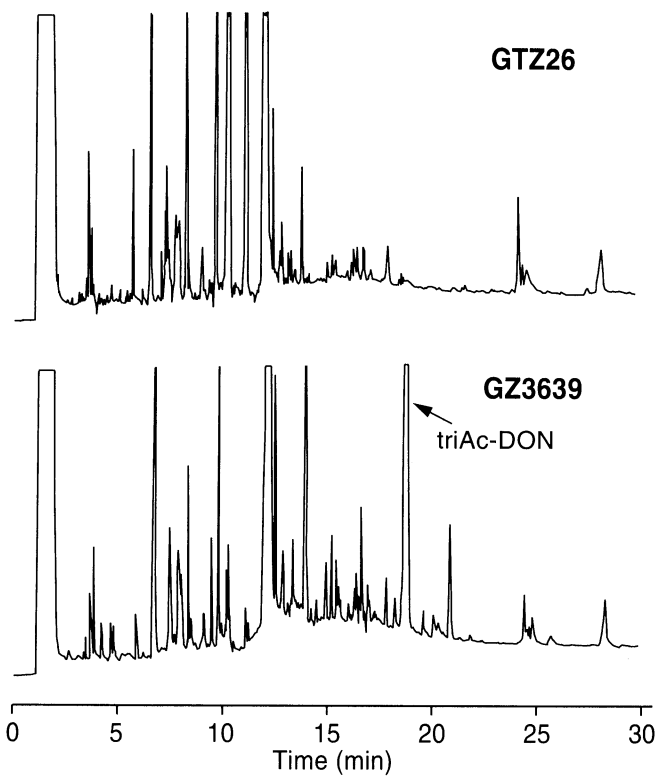

Fig. 5. Gas liquid chromatograms of ethyl acetate extracts from Golden Bantam maize seedlings infected with wild-type strain Z3639 and the trichothecene deficient transformant GZT26. TriAc-DON is triacetyldeoxynivalenol.

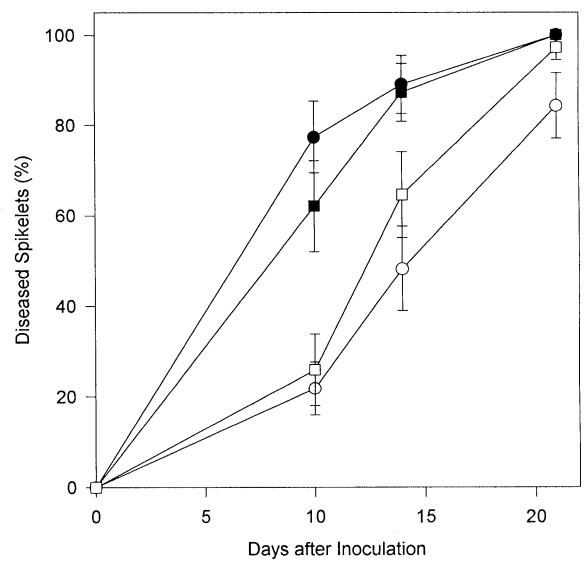

Fig. 6. Disease progress of head blight on Wheaton wheat cultivar infected with trichothecene producing strains Z3639 (●) and GZT12 (■) and trichothecene deficient strains GZT26 (⁰) and GZT40 (□) of *G. zeae*. Error bars represent standard error values.

trichodiene-derived apotrichothecenes that have been reported from *Fusarium* (Greenhalgh et al. 1989).

A limitation of the transformation-mediated gene disruption approach to study pathogenicity is that the transformation process itself can cause genetic alterations in the transformed organism in addition to the targeted gene disruption. Thus, it is conceivable that changes in trichothecene production or virulence could result from alterations in the genome caused by the transformation process rather than disruption of *Tri5*. This may have been the case with transformants GZT4 and GZT34, which had a wild-type *Tri5*, according to Southern analysis, but did not produce trichothecenes. The possibility that the reduced virulence of GZT26 and GZT40 is due to an untargeted transformation effect has not been ruled out; however, we have indirect evidence that this is not the case. First, the *Tri5*$^+$ transformant GZT12 was as virulent as the wild-type progenitor strain Z3639 in the head blight assays. Thus, transformation itself or the presence of the transformation vector does not always cause a reduction in virulence. Second, GZT26 and GZT40 exhibited very similar phenotypes with respect to the amount of disease they caused on all the hosts plants examined. For example, both of these *Tri5*$^-$ transformants exhibited wild-type virulence on Golden Bantam seedlings but reduced virulence on Wheaton seedlings and heads. Thus, the genetic change that affected the virulence of these strains was probably similar. It seems more plausible that this change was the targeted *Tri5* disruption rather than some untargeted change. Nevertheless, a more definitive test is required to demonstrate unequivocally that *Tri5* disruption is the cause of the reduced virulence of GZT26 and GZT40. An appropriate test would be to restore trichothecene production by the introduction of the wild-type *Tri5* into a *Tri5*$^-$ strain and then to determine if wild-type levels of virulence are also restored in the resulting transformants.

The data presented here add to the growing body of evidence indicating that trichothecenes play a role in the pathogenesis of *Fusarium*. There are two common features, with respect to the role of trichothecenes in pathogenesis, that have become apparent from the data obtained from gene disruption and UV mutagenesis. First, trichothecenes appear to exhibit some degree of host specificity. That is, the inability to produce trichothecenes is associated with a reduction in virulence on some hosts but does not affect virulence on other hosts (Desjardins et al. 1992; Adams and Hart 1989). Second, trichothecenes appear to act as virulence factors, in that the inability to produce trichothecenes is associated with reduced virulence rather than a complete loss of pathogenicity (Desjardins et al. 1992).

Table 2. Trichothecene production and disease severity incited by trichothecene producing (Z3639 and GZT12) and nonproducing (GZT26 and GZT40) strains of *Gibberella zeae* on Wheaton wheat heads

Strain	Trichothecene production[a]	Percent symptomatic spikelets[b]			
		Assay 1	Assay 2	Assay 3	Assay 4
Z3639	163	49.8 a	91.5 a	89.0 a	100.0 a
GZT12	88	ND	86.0 ab	87.2 a	96.3 a
GZT26	0	7.0 b	60.8 bc	48.3 b	74.4 b
GZT40	0	6.7 b	52.3 c	64.6 b	54.6 c

[a] mg TriAc-DON/g corn meal after one week of incubation.

[b] Data are means of percent symptomatic spikelets per head at 14 days after inoculation. There were 15 to 30 heads per treatment in each assay. Data were analyzed by one-way analysis of variance and the overall *F*-tests for treatments were significant at $P < 0.01$. Within a column, means followed by different letters are significantly different at $P < 0.05$ according to *t*-tests of least squares means. ND indicates not determined.

MATERIALS AND METHODS

Fungal strains and media.

G. zeae field isolate Z3639 was isolated from scabby wheat by R. L. Bowden (Kansas State University) and isolate W-8 (Hart et al. 1982) was kindly provided by L. P. Hart (Michigan State University). The fungus was routinely grown on V8 juice agar (Tuite 1969) at 25°C under white and near UV fluorescent lights for 12 h/day.

Nucleic acid manipulations.

Fungal genomic DNA isolation and Southern blot analyses were done as described previously (Hohn and Desjardins 1992). DNA probes were labeled with [α-^{32}P]dCTP (DuPont NEN, Boston MA) using the Prime-a-Gene labeling system (Promega, Madison, WI). The sequence of both DNA strands was determined using the Taq DyeDeoxy Terminator (Applied Biosystems, Foster City, CA) method as described by the manufacturer. Both synthetic oligonucleotides specific to the region of *G. zeae* DNA under study and commercially available oligonucleotides were employed as sequencing primers.

Amplification and cloning of *Tri5* coding region.

The *Tri5* coding region was amplified by the polymerase chain reaction (PCR) from *G. zeae* genomic DNA. PCR primers consisted of oligonucleotide O63 (5′-TGGAGAACTTTCCCACC-3′), which represents nucleotides 2 through 18 of the *F. sporotrichioides Tri5* coding region (Hohn and Beremand 1989), and oligonucleotide O178 (5′-TCAYTCCACTAGCTC-3′), which was designed based on the reverse complements of the last 15 nucleotides of the *G. pulicaris* (Hohn and Desjardins 1992) and *F. sporotrichioides* (Hohn and Beremand 1989) *Tri5* coding regions. PCR conditions were as described previously (Proctor and Hohn 1993) except that the annealing temperature was 48°C. The amplification product was subjected to the GENECLEAN protocol (BIO 101, La Jolla CA) and cloned into the PCR cloning vector pCR1000 (Invitrogen, San Diego, CA) following the instructions of the manufacturer, except that *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) was employed.

Construction of transformation vector.

A truncated copy of the *Tri5* coding region was amplified by PCR from *G. zeae* genomic DNA. PCR primers consisted of oligonucleotides O63 (see above) and O57 (5′-GCCC-ATGGTGGATANGCCCAYTC-3′), which contains the reverse complement of nucleotides 1083 through 1098 of the *G. zeae Tri5* coding region preceded by an *Nco*I site (underlined). The amplified fragment was subjected to GENECLEAN and cloned into pCR1000 to yield plasmid pGZTS1-1. An inverse PCR procedure modified from Hemsley et al. (1989) was used to introduce an *Eco*47III site between nucleotides 575 and 576 of the truncated *Tri5* coding region in pGZTS1-1. The primers for inverse PCR were 5′ phosphory-

lated oligonucleotides O176 (5′-CTGGGAATCCTC-CAAAGTTG-′3) which represented the reverse complement of nt 557 to 576 of the *G. zeae Tri5* coding region, and O177 (5′-GGAGCGCTGATCTGATGACTACCCTC-3′), which corresponds to nt 577 to 594 of the *G. zeae Tri5* coding region preceded by a *Eco*47III site (underlined). Fifteen microliters of the inverse PCR product was treated with 5 units of Klenow in the presence of dNTPs. The product of this reaction was subjected to electrophoresis on a 1% SeaPlaque Gel (FMC BioProducts, Rockland, ME). The single visible band corresponding to approximately 4 kb was cut from the gel and 5 µl of the melted band was added to a ligation mix to generate a circularized plasmid that was designated pGZTS3-1. A 2,494-bp *Xba*I–*Hind*III fill-in fragment from pUCH1 was inserted at the *Eco*47III site of pGZTS3-1 to yield the transformation vector, pGZTS4-1 (Fig. 3). The *Xba*I–*Hind*III fragment from pUCH1 carries the *E. coli* hygromycin phosphotransferase gene (*hyg*B) fused downstream from Promoter 1 from *Cochliobolus heterostrophus* (Turgeon et al. 1987). This chimeric gene facilitates the selection of transformants of *Fusarium* by their resistance to the antibiotic hygromycin B (Hohn and Desjardins 1992).

Transformation.

Protoplast formation and the transformation protocol for *G. zeae* were done as described previously for *G. pulicaris* (Hohn and Desjardins 1992; Salch and Beremand 1993) except that the final concentration of hygromycin B in the 1% water agar overlay was 262 µg/ml.

Virulence assays.

Seedling blight and head blight assays were employed to evaluate the virulence of *G. zeae*. In both assays, inoculum consisted of macroconidia of individual strains of *G. zeae* diluted in water to 1×10^5 conidia per milliliter. Inoculum was prepared by washing the macroconidia from V8 juice agar cultures grown under white and near UV fluorescent lights for 7 to 10 days. The following host plants were employed in seedling assays: spring wheat cultivars Marshall, and Wheaton, kindly provided by R. Bush (University of Minnesota); winter wheat cultivar Caldwell (Kelly's Seed Co., Peoria IL); and Golden Bantam maize, Porter oats, and common winter rye (Johnny's Selected Seeds, Albion, ME). In seedling assays, seeds were surface sterilized by washing in 0.5% sodium hypochlorite solution for approximately 1 min and rinsed four times with sterile distilled water. The seeds were soaked in a suspension of macroconidia for approximately 10 min and then sown in vermiculite contained in 10-cm plastic pots (10 seeds per pot). Prior to sowing, the pots were filled approximately three quarter full with vermiculite and set in 2 to 4 cm of water until the top of the vermiculite was wet. After sowing, seeds were covered with an additional 1 to 2 cm of vermiculite and pots were placed individually into plastic bags and incubated in a G10 environmental growth chamber (EGC, Chagrin Falls, OH) at 22°C with 16 h light and 8 h dark. After approximately 1 week the pots were removed from the bags, and after 2 weeks, disease was evaluated by 1) counting the number of seedlings that emerged in each pot and 2) measuring plant height (distance from seed to top of the furthest extended leaf). Controls were treated as described above except that the seeds were soaked in sterile water. Data were analyzed using the general linear models procedure (SAS Institute, Cary, NC) and significant differences between treatments were determined by Student's *t*-test comparisons of least squares means at $P < 0.05$. Treatment by experiment interactions were tested and found not to be significant ($P > 0.05$); thus, data from different experiments were combined in the statistical analyses and presentation of results.

In head blight assays, Wheaton wheat seeds were surface sterilized as described above and sown in Redi-Earth (Grace Sierra, Milpitas, CA) in 15-cm plastic pots. The pots were placed in the growth chamber at 22°C with 16 h light and 8 h dark. After emergence, plants were culled to three per pot. At anthesis, heads were inoculated by injecting approximately 20 µl of inoculum between the lemma and palea of one floret near the middle of each head. In each experiment, heads were inoculated with each strain of *G. zeae* tested. Heads that were left uninoculated or that were inoculated with sterile water were included in all experiments. Disease was evaluated by counting the number of symptomatic and asymptomatic spikelets on each inoculated head and from this calculating the percentage of spikelets on each head that were symptomatic. Symptoms consisted of premature whitening and in some cases necrosis of spikelets. Each head constituted a replicate and there were 15 to 30 heads per treatment (i.e., inoculum type) in each experiment. Data were analyzed with the general linear models procedure (SAS Institute, Cary, NC) and significant differences between treatments were determined by Student's *t*-test comparisons of least squares means at $P < 0.05$. Means were determined by calculating average percent of symptomatic spikelets for all heads in a treatment.

Trichothecene analysis.

The ability of *G. zeae* to produce trichothecenes was assessed from cultures grown on yellow cornmeal. For the analysis, DON and DON-related trichothecenes (e.g., 3ADON and 15ADON) in culture extracts were converted to triacetyldeoxynivalenol (TriAc-DON) by an acetic anhydride-pyridine derivitization (Schuda et al. 1984). Thus, DON and DON-related compounds were detected as a single peak by gas liquid chromatography.

Conidia were washed with water from 7- to 10-day-old V8 juice agar plates and added to an autoclaved mixture of 25 g of cornmeal and 10 ml of water. After 1 week of incubation at 28°C in the dark, the cornmeal culture was extracted twice with 50 ml of ethyl acetate. The combined extracts were concentrated under vacuum and then taken to dryness under a stream of nitrogen. After the residue was dissolved in 1 ml of ethyl acetate, 100 µl was removed, dried under a stream of nitrogen and acetylated overnight with 2 ml of acetic anhydride and pyridine (1:1). The acetylation mixture was added to two volumes of ice water and then extracted with ethyl acetate by vortexing. The extract was dried under a stream of nitrogen, redissolved in 1 ml of ethyl acetate and analyzed by gas-liquid chromatography. Concentrations of TriAc-DON were determined from a 25 to 800 µg/ml standard curve.

To assess whether trichothecenes were produced in inoculated seedlings, shoots of all seedlings from a treatment were pooled and extracted with 100 ml of ethyl acetate over night. The extract was concentrated under vacuum and then dried under a stream of nitrogen. The residue was redissolved in 1 ml of ethyl acetate and then acetylated and analyzed for the

presence of TriAc-DON as described above.

Samples were analyzed by flame ionization detection on an Hewlett-Packard (Palo Alto, CA) 5890 Gas Chromatograph fitted with a 30 m, DB1, 0.25 mm, fused silica capillary column. The column was held at 120°C at the time of injection, then heated to 210°C at 15°C/min and held for 1 min; then heated to 260°C at 5°C/min and held for 8 min.

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