Restoration of wild-type virulence to *Tri5* disruption mutants of *Gibberella zeae* via gene reversion and mutant complementation

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***Gibberella zeae* is a pathogen of small grain crops and produces trichothecene mycotoxins in infected host tissue. The role of trichothecenes in the virulence of *G. zeae* was previously investigated using trichothecene-non-producing mutants that were generated via transformation-mediated disruption of a gene (*Tri5*) that encodes the first enzyme in the trichothecene biosynthetic pathway. The mutants were less virulent on some hosts than the wild-type strain from which they were derived. Here, we used two approaches to determine whether the reduced virulence of mutants was due specifically to *Tri5* disruption or to non-target effects caused by the transformation process. First, we generated a revertant from a *Tri5* disruption mutant by allowing the mutant to pass through the sexual phase of its life cycle. In approximately 2% of the resulting progeny the disrupted *Tri5* had reverted to wild-type; however, only one of three revertant progeny also regained the ability to produce trichothecenes. In the second approach, we complemented the *Tri5* mutation in a disruption mutant by transforming the mutant with a plasmid carrying a functional copy of *Tri5*. In all transformants examined, the ability to produce trichothecenes was restored. The restoration of trichothecene production in the revertant progeny and in the complemented mutant was accompanied by restoration of wild-type or near wild-type levels of virulence on wheat seedlings (cultivar Wheaton). The results indicate that the reduced virulence of the mutants was caused by disruption of *Tri5* rather than non-target effects resulting from the transformation process. The results also provide further evidence that trichothecenes contribute to the virulence of plant-pathogenic fungi.**

Keywords: *Gibberella zeae*, trichothecene, *Tri5*, gene disruption

INTRODUCTION

Gibberella zeae (Schwein.) Petch (anamorph *Fusarium graminearum*) is a filamentous ascomycete that causes severe and economically important diseases on a variety of plants, including maize, barley, wheat and carnation (Nelson *et al.*, 1975; Cook, 1981; Kommedahl & Windels, 1981). On small grains, *G. zeae* can cause seedling blight, root rot and head blight (or scab). In addition, *G. zeae* produces the trichothecene mycotoxins deoxynivalenol and acetylated derivatives of deoxynivalenol in infected grain (Cook, 1981; Atanassov *et al.*, 1994). Trichothecenes are sesquiterpenoids produced by several fungal genera, including species of *Fusarium*, *Myrothecium*, *Stachybotrys* and *Trichothecium*, and by two species of the plant genus *Baccharis*

(Sharma & Kim, 1991; Jarvis *et al.*, 1991). These toxins are harmful to both plants and animals and their toxicity is thought to occur as a result of their ability to inhibit protein biosynthesis (Sharma & Kim, 1991; Wang & Miller, 1988; Jarvis, 1991). The phytotoxicity of trichothecenes and the fact that many trichothecene-producing fungi are plant pathogens has led to the hypothesis that these toxins are virulence factors. Indeed, mutants of *F. sporotrichioides*, *G. pulicaris* and *G. zeae* that are unable to produce trichothecenes exhibit reduced virulence on some hosts (Desjardins *et al.*, 1989, 1992; Proctor *et al.*, 1995). The trichothecene-deficient mutants of *G. pulicaris* and *G. zeae* were generated via transformation-mediated disruption of a gene, *Tri5*, that encodes the enzyme trichodiene synthase. This enzyme catalyses the cyclization of the isoprenoid intermediate farnesyl

pyrophosphate to trichodiene, which serves as the parent compound for all trichothecenes (Hohn & VanMiddlesworth, 1986). The formation of trichodiene marks the biochemical step at which trichothecene biosynthesis branches from general isoprenoid biosynthesis. Disruption of *Tri5* prevents the formation of functional trichodiene synthase and as a result precludes the formation of trichothecenes or intermediates in trichothecene biosynthesis. However, disruption of *Tri5* should not prevent the formation of isoprenoids for which trichodiene is not an intermediate.

Transformation-mediated gene disruption is a powerful tool for examining the role of genes in the pathogenicity of fungi on plants (Desjardins *et al.*, 1992; Herrmann *et al.*, 1996; Bowyer *et al.*, 1995; Panaccione *et al.*, 1992; Rogers *et al.*, 1994; Stahl *et al.*, 1994; VanEtten *et al.*, 1995). Gene disruption has the advantage over chemical and UV light-induced mutagenesis in that it allows for the generation of defined mutations in targeted genes. However, the transformation process itself can cause alterations at loci in addition to the targeted gene (Keller *et al.*, 1990; Oliver & Osbourn, 1995). Thus, a change in virulence that accompanies gene disruption is not always sufficient proof that the target gene is involved in pathogenicity. Several approaches have been employed to determine whether observed reductions in virulence following gene disruption are caused by the disruption or by non-target effects of transformation. These include (i) analysis of more than one disruption mutant (Desjardins *et al.*, 1992; Herrmann *et al.*, 1996; Talbot *et al.*, 1996), (ii) analysis of transformants in which the disruption vector integrated ectopically (Desjardins *et al.*, 1992; Herrmann *et al.*, 1996; Talbot *et al.*, 1996), (iii) complementation of the disruption mutant with a wild-type copy of the disrupted gene via a second transformation (Talbot *et al.*, 1996) and (iv) outcrossing a disruption mutant with a wild-type strain and analysing the resulting meiotic progeny for cosegregation of the disrupted gene and reduced virulence (Desjardins *et al.*, 1992; Talbot *et al.*, 1996). In the initial characterization of trichothecene-non-producing mutants of *G. zeae*, the virulence of two *Tri5* disruption mutants and a transformant in which the disruption vector had integrated at an ectopic site was examined (Proctor *et al.*, 1995). The other approaches were not employed because a suitable second selectable marker was not available and although *G. zeae* can outcross, analysis of the resulting meiotic progeny is cumbersome because the fungus is homothallic.

In this study, we used two approaches to determine whether the reduced virulence of *Tri5* mutants of *G. zeae* was due specifically to disruption of this gene or to non-target effects resulting from the transformation process. The first approach was based on the observation that in filamentous fungi closely linked duplicated sequences can recombine when the fungi pass through the sexual phase of their life cycle and as a result of this recombination one copy of the duplicated sequence can be deleted in meiotic progeny (Desjardins *et al.*, 1992; Miller *et al.*, 1986; Selker *et al.*, 1987). We

exploited this phenomenon to generate a trichothecene-producing, meiotic progeny with a wild-type *Tri5* from a disruption mutant that had two closely linked, mutated copies of the *Tri5* coding region. In the second approach, we complemented the *Tri5* mutation in a disruption mutant by introducing a functional copy of *Tri5* into the mutant via a second transformation.

METHODS

Fungal strains and manipulations. The trichothecene-producing, wild-type strain, GZ3639 (previously Z3639) and the trichothecene-deficient, *Tri5* disruption mutants GZT33 and GZT40 have been described previously (Proctor *et al.*, 1995). These strains were routinely cultured on V8 juice agar (Tuite, 1969) at 25 °C under white and blue fluorescent lights for 12 h d⁻¹. GZT33 was selfed following a method similar to that described to cross *G. fujikuroi* (Klittich & Leslie, 1988). GZT33 was grown on carrot agar at 25 °C under fluorescent light as described above for 5–7 d. At this time, 2 ml sterile water was added to the culture and spread with a glass rod to flatten the mycelium. Excess water was then removed and the cultures were incubated for an additional 1–2 weeks until cirrhi were observed oozing from developing perithecia. To obtain single ascospore cultures, a single cirrhus was picked and vortexed vigorously in 1 ml water. The resulting suspension was then spread over 3% (w/v) water agar and incubated for 8–10 h. With the aid of a stereomicroscope, small blocks of agar around individual germinating ascospores were cut from the water agar and transferred to V8 juice agar. Care was taken to avoid clumps of two or more ascospores.

Nucleic acid manipulations. Except when indicated otherwise, fungal genomic DNA was isolated as described previously (Hohn & Desjardins, 1992) except that after the chloroform extraction, the aqueous phase was incubated with RNase for 30 min at room temperature, combined with an equal volume of 5 M LiCl, incubated for 15 min on ice and then centrifuged at 14000 g for 10 min. DNA was precipitated from the supernatant with 2.5 vols 95% (v/v) ethanol and resuspended in Tris/EDTA buffer, pH 8 (Sambrook *et al.*, 1989). Southern blotting and PCR experiments were as described previously except for the modifications noted below (Hohn & Desjardins, 1992; Proctor *et al.*, 1995).

In PCR experiments designed to distinguish between the disrupted and wild-type *Tri5*, DNA templates were obtained by growing individual strains on V8 juice agar in 100 × 15 mm Petri plates until the mycelium covered the entire agar surface. At this time, the mycelium was scraped from the agar and dried under vacuum overnight. The mycelium was then ground, mixed with extraction buffer and extracted with phenol/chloroform (1:1). DNA was purified from the aqueous phase using GeneClean (Bio 101) as described by the manufacturer except that samples were rinsed only once with NEW solution and DNA was eluted with 20 µl of Tris/EDTA buffer (Sambrook *et al.*, 1989). Two to five microlitres of the resulting solution was used as a template in PCR. The sequences of the PCR primers were as follows: 65, 5' GAAACAGCTATGACCATG 3'; 170, 5' GGCATGGTTGTATACAGC 3'; 246, 5' CTCCTTCACATCGTCCTTGG 3'; 248, 5' CTATGCCTACAGCATCCAGG 3'.

Construction of *Tri5* complementation plasmid. A plasmid containing a functional *Tri5* gene, consisting of the 5' flanking and coding regions of the *G. zeae Tri5* and the 3' flanking region of the *F. sporotrichioides Tri5*, was constructed to

Fig. 1. Maps of *Tri5* alleles and PCR strategy for distinguishing between them in progeny from disruption mutant GZT33. (a) *Tri5-1* allele in wild-type strain GZ3639; (b) *Tri5-2* (disrupted) allele in GZT33; (c) *Tri5-3* (disrupted) allele in GZT40. Open boxes represent the *Tri5* coding region, jagged ends of open boxes represent truncated ends of the coding region, hatched boxes represent the *HygB* marker and the wavy line represents the bacterial cloning vector sequence. The numbered arrows indicate positions of sequences complementary to PCR primers 65, 170, 246 and 248 and the direction of the arrows indicate the direction in which DNA synthesis is primed. Bars indicate sizes of possible PCR products.

facilitate the complementation of *Tri5* disruption mutant GZT40. The 5' flanking sequences were isolated by rescuing a portion of plasmid pGZTS4-1 from transformant GZT33 (Proctor *et al.*, 1995). Plasmid rescue was accomplished by digesting genomic DNA from GZT33 with *Sall*. Following *Sall* digestion, the DNA fragments were isolated using GeneClean and then ligated to themselves. Transformation of *Escherichia coli* with the resulting ligation products yielded eight colonies. Analysis of six of these revealed that three carried the expected rescued plasmid (pRESC1-4) while the other three carried the intact disruption plasmid pGZTS4-1 (Proctor *et al.*, 1995). The plasmid pRESC1-4 was found to contain 776 bp of sequence upstream of the *Tri5* coding region. Construction of the complementation plasmid began with a 281 bp fragment amplified from the 3' flanking sequence of the *F. sporotrichioides Tri5* gene (nt 3–284 downstream of the stop codon). The primers used to amplify this fragment contained *Bst*XI and *Not*I sites and the resulting product was cloned into pBluescriptII KS(-) to yield pADD1-3. The *Sall*–*Hind*III fragment from pRESC1-4 containing the 776 bp promoter region and nt 1–829 of the *G. zeae Tri5* coding region was then cloned into pADD1-3 to generate pADD2B. Next, an amplified portion of the *G. zeae Tri5* coding region was cloned into *Xho*I/*Not*I-cut pADD2B to yield pADD3. This was accomplished using the existing *Xho*I site at nt 49 of the *G. zeae Tri5* and a *Not*I site introduced via the PCR primers immediately 3' of the *Tri5* stop codon. The entire chimeric *Tri5* gene in pADD3 was amplified and cloned in the TA cloning vector pCRII (Invitrogen) to yield pADD4. The complementation plasmid pADD6-1 was then constructed by transferring the chimeric *Tri5* gene from pADD4 as an *Apal*–*Kpn*I fragment into the fungal transformation vector

Fig. 2. (a) PCR analysis of selected GZT33 progeny for wild-type *Tri5*. Bands are the amplification products from reactions with genomic DNA templates isolated from wild-type strain GZ3639 (lane 1), *Tri5* mutant parent GZT33 (lane 2) and progeny GZP1-1 (lane 3), GZP3-1 (lane 4), GZP1-6 (lane 5), GZP1-15 (lane 6) and GZP3-9 (lane 7). (b) Southern blot analysis of GZT33 progeny. Genomic DNA was digested with *Xmn*I and probed with 32 P-labelled wild-type *Tri5* coding region (nt 2–1187). Lanes: 1, GZ3639; 2, GZT33; 3, GZP1-1; 4, GZP3-1; 5, GZP1-6; 6, GZP1-15; 7, GZP3-9; 8, GZP5-15.

Table 1. Trichothecene production and virulence of progeny of *Tri5* disruption mutant GZT33

Strain	Genotype*	Trichothecene production†	Emergence (%)‡	Seedling height (cm)‡
Control (water)	—	—	92.7 a	26.2 a
GZT33	<i>Tri5-2</i>	0	53.3 c	27.7 ab
GZP1-1	<i>Tri5-2</i>	0	56.7 bc	27.0 ab
GZP1-4	<i>Tri5-2</i>	0	67.3 b	28.4 b
GZP3-1	<i>Tri5-2</i>	0	58.7 bc	28.0 ab
GZP1-6	<i>Tri5-1</i>	0	62.0 bc	27.3 ab
GZP1-15	<i>Tri5-1</i>	0	62.7 bc	26.8 ab
GZP3-9	<i>Tri5-1</i>	238	25.3 d	8.2 c
GZ3639	<i>Tri5-1</i>	209	29.0 d	8.9 c

* Genotypes were determined by PCR and Southern blot analysis (Fig. 2). *Tri5-1* is the wild-type *Tri5* allele. *Tri5-2* is the disrupted *Tri5* allele present in GZT33.

† Data are expressed as μg triacetyldeoxynivalenol (g dry wt corn meal culture)⁻¹ and are the means from two or more assays.

‡ Data are means from three experiments. In each experiment there were five pots per treatment (strain) and 10 seeds inoculated with *G. zeae* or water were sown in each pot. Emergence is the percentage of seedlings that emerged from the 10 seeds that were sown. Seedling height was determined by measuring the length of seedlings from the seed to the tip of the tallest leaf. Seedlings that did not emerge were not included in analysis of seedling height data. The overall *F*-tests for strains were significant ($P < 0.01$). For percentage emergence, the mean square error (variance for the entire data set) was 20.3 with 117 degrees of freedom. For seedling height, the mean square error was 56.3 with 694 degrees of freedom. Within a column, means not followed by the same letter are significantly different ($P < 0.05$) according to a paired *t*-test of least squares means.

pAMDS4-1 (J. Jones, T. Leathers & T. Hohn, unpublished) which contains the *Aspergillus nidulans amdS* gene.

Transformation of GZT40. Protoplasting and transformation procedures were carried out at room temperature unless otherwise indicated and were similar to those described previously (Hohn & Desjardins, 1992; Salch & Beremand, 1993). To prepare protoplasts, conidia of GZT40 were harvested from 7–10-d-old V8 juice agar cultures and used to inoculate 250 ml Erlenmeyer flasks containing 100 ml YEPD medium (w/v, 1% peptone, 0.3% yeast extract, 2% glucose). Conidia were inoculated at a concentration of between 1×10^6 and 5×10^6 conidia ml⁻¹ and incubated at 28 °C for 8–9 h at 200 r.p.m. Germlings were harvested by centrifugation in a table top centrifuge at 3000 *g* for 10 min and washed twice with 50 ml 0.7 M NaCl. After the second wash, the pellet was resuspended in 20 ml of a solution containing 100 mg Novozyme (Novo Nordisk), 500 mg driselase (Sigma) and 1 mg chitinase (Sigma). Incubation at 30 °C resulted in 100% protoplast formation within 30–90 min. Protoplasts were pelleted at 3000 *g* for 5 min and resuspended in 10 ml 0.7 M NaCl. Protoplasts were again pelleted at 3000 *g* for 5 min and washed twice in 10 ml STC (0.8 M sorbitol, 0.05 M Tris, pH 8.0, 50 mM CaCl₂). After the second wash, the protoplast pellet was resuspended in STC to 1×10^8 protoplasts ml⁻¹ and then further diluted with SPTC (STC with 40%, w/v, PEG 6000) and DMSO (protoplasts/SPTC/DMSO, 8:2:0:1). The resulting solution was frozen as 100 μl aliquots at -80 °C.

Transformation was performed by thawing a frozen 100 μl protoplast aliquot on ice and diluting with 100 μl STC containing 1–10 μg DNA and 50 μl 30% PEG. Following a 20 min incubation at room temperature, an additional 2 ml of

30% PEG was carefully added and incubation was continued at room temperature for 5 min. The transformation mixture was diluted further with 4 ml STC and then 600 μl aliquots were mixed with 6.5 ml molten SL medium containing 15 mM CsCl and 1% agarose and spread on SL medium. SL medium consisted of a previously described basal medium (Correll *et al.*, 1987) supplemented with 10 mM acetamide (Sigma). Transformation plates were incubated at 28 °C and transformants were initially detected after 2 or 3 d. Putative transformants were transferred to SL agarose slants prior to picking single spore isolates.

Trichothecene analysis. The ability of *G. zeae* to produce trichothecenes was analysed by culturing the fungus on sterile corn meal, extracting the cultures with ethyl acetate and analysing the extract via GC and GC-MS as described previously (Proctor *et al.*, 1995).

Virulence assays. Virulence assays were carried out, as described previously (Proctor *et al.*, 1995), by soaking wheat seeds of cultivar Wheaton in a suspension of 5×10^5 macroconidia ml⁻¹ and then sowing the seeds into moistened vermiculite. Disease was assessed 2 weeks after sowing.

RESULTS

Reversion of disrupted *Tri5*

The *Tri5* locus (allele *Tri5-2*) in disruption mutant GZT33 contains two copies of the *Tri5* coding region (nt 2–1098) separated from one another by about 3 kb of bacterial vector sequences (Fig. 1b) (Proctor *et al.*, 1995). In addition, the downstream copy of the *Tri5* coding

region in GZT33 is interrupted by the *HygB* gene. This *Tri5* duplication made GZT33 a good candidate for gene reversion experiments because recombination between the duplicated sequences could restore the wild-type *Tri5* allele (*Tri5*-1). We speculated that this sort of recombination might be induced by passing GZT33 through the sexual phase of its life cycle. Because *G. zeae* is homothallic, it was possible to self-fertilize GZT33 and obtain viable ascospore progeny. One hundred and forty of these progeny were isolated (20 from each of seven perithecia) and screened for the presence of the wild-type *Tri5* using two PCR protocols. In the first protocol, a combination of three oligonucleotides (170, 246 and 248) were used as primers (Fig. 1). When these three oligonucleotides are combined in one reaction, oligonucleotides 170 and 246 amplify a 931 bp product from a wild-type *Tri5* DNA template. However, if a disrupted *Tri5* serves as the template, oligonucleotides 246 and 248 amplify a 688 bp product (Fig. 1). Oligonucleotides 170 and 246 can also amplify a 3135 bp product from a disrupted *Tri5* template, but this product was generally not detected under our PCR conditions. In the second PCR protocol, oligonucleotides 65 and 170 were employed. These primers amplify a 996 bp product from a *Tri5*-2 template but fail to amplify a product from the wild-type *Tri5* template (Fig. 1). Genomic DNA of selected progeny was also analysed by Southern blot analysis using *Xmn*I-digested genomic DNA and the wild-type *Tri5* coding region as a hybridization probe.

PCR analysis of the 140 progeny of GZT33 indicated that three progeny, GZP1-6, GZP1-15 and GZP3-9, carried a wild-type *Tri5*. In the PCR analysis in which oligonucleotides 170, 246 and 248 were employed, the 931 bp fragment indicative of the wild-type *Tri5* was amplified from these three progeny (Fig. 2a). No fragment was amplified from these progeny in PCR analysis employing oligonucleotides 65 and 170, which is also consistent with the presence of a wild-type *Tri5* in these three progeny. In addition, Southern analysis of *Xmn*I-digested DNA from progeny GZP1-6, GZP1-15 and GZP3-9 yielded a hybridization pattern identical to the pattern obtained with wild-type DNA (Fig. 2b). A fourth progeny, GZP5-15, appeared to have a disrupted *Tri5* gene as indicated by the amplification of a 688 bp fragment in the PCR analysis employing oligonucleotides 170, 246 and 248. However, in PCR with oligonucleotides 65 and 170, no fragment was amplified, suggesting that the structure of the disrupted *Tri5* in GZP5-15 was different from the *Tri5*-2 allele present in GZT33. Southern analysis of *Xmn*I-digested DNA from GZP5-15 revealed a hybridization pattern different from that obtained with both GZT33 and GZ3639 (wild-type) DNA (Fig. 2b). Comparison of the hybridization pattern with previously published hybridization patterns (Proctor *et al.*, 1995) indicated that the structure of *Tri5* in GZP5-15 is similar to that obtained with the *Tri5* disruption mutant GZT40. PCR analyses of the remaining 136 progeny from GZT33 were consistent with the presence of the *Tri5*-2 (disrupted) allele in these

Fig. 3. Map of plasmid pADD6-1. Abbreviations: Gz *Tri5* 5', 5' flanking sequence of *G. zeae Tri5*; Gz *Tri5* CDS, coding region of *G. zeae Tri5*; Fs *Tri5* 3', 3' flanking sequence of *F. sporotrichioides Tri5*; *amdS*, coding region and 3' flanking sequence of *amdS* gene from *A. nidulans*; Promoter 1, Promoter 1 sequence from *Cochliobolus heterostrophus*. Only those restriction sites employed for cloning or Southern blot analysis are shown.

progeny. The PCR and Southern results from two representative progeny, GZP1-1 and GZP3-1, of this type are shown in Fig. 2.

Strains GZ3639 and GZT33 and selected progeny were analysed for their ability to produce trichothecenes. Parent strain GZT33 and progeny GZP1-1, GZP1-4 and GZP3-1, all of which carry a disrupted *Tri5*-2 allele, did not produce trichothecenes (Table 1). Surprisingly, putative revertant progeny GZP1-6 and GZP1-15 also failed to produce trichothecenes. The only progeny that produced trichothecenes was the *Tri5* revertant GZP3-9 and it did so at levels that were comparable to those produced by the wild-type progenitor, GZ3639 (Table 1).

Complementation of *Tri5* disruption mutant

The *Tri5* disruption mutant GZT40 carries a single, non-functional copy of the *Tri5* gene (allele *Tri5*-3) that is interrupted by the *HygB* gene (Fig. 1) (Proctor *et al.*, 1995). To complement the disrupted *Tri5* gene in GZT40 it was necessary to first construct complementation vector, pADD6-1 (Fig. 3), carrying a functional copy of *Tri5* (allele *Tri5*-4). This functional *Tri5* consisted of the 776 bp promoter region and 1187 bp coding region of the wild-type *G. zeae Tri5* and a 281 bp fragment containing the transcription termination sequence from the wild-type *F. sporotrichioides Tri5*. The vector pADD6-1 also carried the *A. nidulans amdS* gene (Corrick & Hynes, 1987) fused to Promoter 1 from *Cochliobolus heterostrophus* (Turgeon *et al.*, 1987). This chimeric Promoter 1/*amdS* gene facilitated the

Table 2. Trichothecene production and virulence of the complemented *Tri5* disruption mutant of *G. zeae*

Strain	Genotype*	Trichothecene production†	Emergence (%)‡	Seedling height (cm)‡
Control (water)	—	—	93.1 a	19.4 b
GZT40	<i>Tri5-3</i>	0	68.7 b	21.1 a
GZT201-1	<i>Tri5-3/Tri5-4</i>	670	45.0 c	18.7 b
GZT201-5	<i>Tri5-3/Tri5-4</i>	434	31.3 cd	19.8 ab
GZ3639	<i>Tri5-1</i>	224	22.5 d	19.5 ab

* Genotypes were determined by PCR and Southern blot analysis (Fig. 4). *Tri5-1* is the wild-type *Tri5* allele. *Tri5-3* is the disrupted allele present in GZT40. *Tri5-4* is a functional copy of *Tri5* derived from *G. zeae* and *F. sporotrichioides* sequences (see Methods).

† Data are expressed as µg triacetyldeoxynivalenol (g dry wt corn meal culture)⁻¹ and are the means from two assays.

‡ Data are means from two experiments. Virulence assays were designed and the data analysed as described for Table 1 except that there were eight pots per treatment in each experiment. The overall *F*-tests for strains were significant ($P < 0.01$). For percentage emergence, the mean square error (variance for the entire data set) was 20.7 with 70 degrees of freedom. For seedling height, the mean square error was 16.8 with 409 degrees of freedom. Within a column, means not followed by the same letter are significantly different ($P < 0.01$) according to a paired *t*-test of least squares means.

selection of pADD6-1 transformants by their ability to grow on media in which acetamide is the sole source of nitrogen.

Transformation of GZT40 with pADD6-1 resulted in the isolation of four transformants. Two transformants, GZT201-1 and GZT201-5, were selected for further analysis. Single spore isolates of these transformants were found to be restored to trichothecene production when grown on corn meal (Table 2). Southern blot analysis revealed that the integration of pADD6-1 had occurred ectopically in GZT201-1 but at the *Tri5* locus in GZT201-5 (Fig. 4).

Virulence of *Tri5* revertant

Virulence of *G. zeae* strains was evaluated via a seedling blight assay (Proctor *et al.*, 1995). In the assay, disease severity was assessed by measuring the ability of the fungus to inhibit seedling emergence and the growth of seedlings once they had emerged. In virulence assays for the reversion study, the results obtained by measuring emergence and growth (i.e. seedling height) showed the same trend. The *Tri5* disruption mutant GZT33 caused significantly less ($P < 0.05$) seedling blight than its wild-type progenitor, GZ3639 (Table 1). Five, trichothecene-deficient progeny of GZT33, including three with a disrupted *Tri5* (GZP1-1, GZP1-4 and GZP3-1) and the two with a revertant *Tri5*, (GZP1-6 and GZP1-15), also caused less disease than GZ3639. With the exception of GZP1-4, these progeny did not differ significantly from GZT33 in their ability to cause seedling blight (Table 1). In marked contrast to its siblings, the trichothecene-producing, *Tri5* revertant progeny, GZP3-9, caused significantly more seedling blight than GZT33 (Table 1). In addition, the severity of seedling blight caused by progeny GZP3-9 did not differ significantly from that

caused by the wild-type progenitor, GZ3639 (Table 1). Thus, the trichothecene-deficient strains caused consistently less disease than the trichothecene-producing strains.

Virulence of complemented *Tri5* disruption mutant

We examined the virulence of transformants GZT201-1 and GZT201-5 in the wheat seedling blight assay. In contrast to previous experiments (Proctor *et al.*, 1995), seedling height was not a reliable indicator of differences in the virulence of the *Tri5* disruption mutant GZT40 and the wild-type progenitor, GZ3639. In the first virulence assay carried out for this study, the height of seedlings from water- or GZT40-inoculated seeds was significantly greater ($P < 0.05$) than that of seedlings derived from seeds inoculated with GZ3639, GZT201-1 or GZT201-5. However, in the second assay, there were no significant differences in the height of seedlings from any *G. zeae* inoculation treatments. On the other hand, emergence data were consistent in both experiments. The wild-type progenitor caused significantly greater ($P < 0.01$) inhibition of seedling emergence than the *Tri5* mutant GZT40 in both assays. In addition, the two complemented *Tri5* mutants, GZT201-1 and GZT201-5, caused greater inhibition of seedling emergence than GZT40. Statistical analysis of emergence data also indicated that GZT201-1 caused significantly less ($P < 0.01$) inhibition of emergence than the wild-type progenitor strain GZ3639 (Table 2).

DISCUSSION

Transformation-mediated gene disruption has been used increasingly in recent years to evaluate the role of fungal genes in pathogenicity (Herrmann *et al.*, 1996; Bowyer

Fig. 4. Southern blot analysis of *Stul*-digested genomic DNA from pADD6-1 transformants. Blots were probed with 32 P-labelled *G. zeae* *Tri5* coding region. Lanes: 1, GZT201-1; 2, GZT201-2; 3, GZT201-3; 4, GZT201-5; 5, GZT201-6; 6, GZT40; 7, GZ3639 (wild-type). Only transformants GZT201-1, GZT201-2, GZT201-3 and GZT201-5 were restored to trichothecene production. Isolate GZT201-6 (lane 5) does not contain a copy of the transformation vector.

et al., 1995; VanEtten *et al.*, 1995; Talbot *et al.*, 1993). Although gene disruption is a powerful approach, it has the disadvantage that the transformation process itself can cause alterations in the fungal genome apart from the gene targeted for disruption (Keller *et al.*, 1990; Oliver & Osbourn, 1995). This phenomenon of non-target effects of transformation may be particularly problematic when analysing the roles of genes involved in complex phenotypes, such as virulence, where changes at any one of a number of loci could significantly alter the phenotype. In this study, we used gene reversion and mutant complementation to determine whether the reduced virulence of disruption mutants of *G. zeae* was caused by disruption of *Tri5* or by non-target effects of transformation. The gene reversion approach used in this study was based on observations that, during meiosis in other filamentous fungi (i.e. *A. nidulans*, *G. pulicaris* and *Neurospora crassa*) homologous recombination between closely linked copies of a duplicated sequence can lead to the loss of one copy of the sequence as well as the sequence between the two copies (Desjardins *et al.*, 1992; Miller *et al.*, 1986; Selker *et al.*, 1987). Disruption mutant GZT33 has two copies of a 1.1 kb region of the *Tri5* coding region separated from each other by approximately 3 kb of sequence corresponding to a bacterial cloning vector (Fig. 1) (Proctor

et al., 1995). We speculated that if this mutant passed through the sexual phase of its life cycle, the duplicated regions might recombine in such a way as to restore the wild-type *Tri5* coding region in some progeny. Because *G. zeae* is homothallic, a single strain of the fungus can complete the sexual phase. We supposed that progeny that resulted from selfing and that had recombined at *Tri5* would be genetically identical to their parent except for the structure of *Tri5*. Thus, if the reduced virulence of the *Tri5* disruption mutant were due to non-target effects of transformation (i.e. an alteration at a locus other than *Tri5*), revertant progeny should also exhibit reduced virulence. Alternatively, if the reduced virulence were caused by *Tri5* disruption, the revertant should exhibit wild-type levels of virulence. The latter alternative appears to be the case, since the trichothecene-producing, *Tri5* revertant progeny, GZP3-9, exhibited wild-type levels of virulence on wheat seedlings. These results are consistent with those from an experiment in which genetically marked (via transformation) derivatives of GZP3-9 exhibited wild-type levels of head blight on wheat in the field (Desjardins *et al.*, 1996).

The interpretation of the *Tri5* revertant phenotypes was complicated by progeny GZP1-6 and GZP1-15, in which reversion of the disrupted *Tri5* to wild-type was not accompanied by restoration of trichothecene production. The cause of this inability to produce trichothecenes is not known. Initially, we speculated that a nucleotide sequence error may have been introduced into the *Tri5* coding region of progeny GZP1-6 and GZP1-15 via the disruption vector, pGZTS4-1 (Proctor *et al.*, 1995). The two rounds of *Taq* PCR to which the truncated *Tri5* coding region in the disruption vector was subjected could have introduced an error in this DNA. The error could have been incorporated into the *Tri5* of GZP1-6 and GZP1-15 through recombination events leading to the initial disruption and subsequent reversion of *Tri5*. That such an error existed was ruled out by nucleotide sequence analysis which revealed that the *Tri5* coding regions in the disruption vector, progeny GZP1-6, and strain GZ3639 (wild-type) were identical, except for the expected truncation and *HygB* insertion in the disruption vector. We also speculated that the inability of GZP1-6 and GZP1-15 to produce trichothecenes may have been caused by repeated-induced point mutations (RIP) and methylation induced premeiotically (MIP). RIP and/or MIP occur in *N. crassa* and *Ascobolus immersus* when strains carrying two or more copies of a gene pass through the sexual cycle and result in the inactivation of all copies of the repeated gene (Cambareri *et al.*, 1989; Rhounim *et al.*, 1992). Nucleotide sequence analysis of the GZP1-6 *Tri5* coding region revealed no differences from that of the wild-type (GZ3639) coding region (data not shown). This indicates that a RIP-like phenomenon had not occurred in the trichothecene-deficient revertant progeny. Likewise, Southern blot analysis employing methylation-sensitive and -insensitive enzymes (*Sau3AI* and *MboI*) and the *Tri5* coding region as a hybridization probe, revealed no evidence for a MIP-like phenomenon

in the trichothecene-deficient revertant progeny (data not shown). The inability of progeny GZP1-6 and GZP1-15 to produce trichothecenes reveals the limitations of the reversion approach, namely revertants may not be isogenic with their parent and revertants may acquire other genetic alterations that affect the phenotype of interest. However, the fact that progeny GZP1-6 and GZP1-15 were unable to produce trichothecenes and exhibited reduced virulence on wheat seedlings strengthens the correlation between trichothecene production and virulence.

That the structure of *Tri5* in progeny GZP5-15 is the same as the *Tri5*-3 allele in disruption mutant GZT40 (Proctor *et al.*, 1995) suggests that the two predicted types of intrachromatid homologous recombination events may have occurred as GZT33 passed through its sexual phase. Intrachromatid recombination between the 5' ends of the duplicated *Tri5* coding regions (i.e. upstream of the *HygB* gene insert, Fig. 1) should yield the *Tri5*-3 allele, while intrachromatid recombination between the 3' ends of the *Tri5* coding regions should yield the *Tri5*-1 (wild-type) allele. However, the *Tri5*-1 and *Tri5*-3 alleles and other constructs could also be generated via crossing over.

The rationale behind the complementation approach was that if the disrupted *Tri5* in GZT40 was responsible for the reduced virulence of this mutant, introduction of a functional *Tri5* should restore high levels of virulence. However, if the reduced virulence of GZT40 were caused by non-target effects of transformation, introduction of a functional *Tri5* would not restore wild-type levels of virulence to the mutant. The fact that the two complemented mutants examined, GZT201-1 and GZT201-5, exhibited relatively high levels of virulence on wheat seedlings indicates that the reduced virulence of GZT40 was due primarily to disruption of *Tri5* rather than non-target effects of transformation.

The disadvantage of the complementation approach is that it employs a second transformation step to determine whether an earlier transformation altered the phenotype of interest. This problem was not evident in the current study since the complemented strains were more virulent than the mutant (GZT40) from which they were derived. However, the second transformation, or perhaps the combination of two transformations, may have caused the slightly reduced virulence of strain GZT201-1 as compared to the wild-type progenitor.

Data from both the revertant and complementation approaches provide further evidence that the ability to produce trichothecenes enhances the ability of *G. zeae* to cause seedling blight. These results combined with results from recent field studies with *Tri5* disruption mutants and revertants (Desjardins *et al.*, 1996) support the hypothesis that trichothecenes are virulence factors of *G. zeae* on both seedlings and adult plants of wheat.

ACKNOWLEDGEMENTS

We thank A. E. Desjardins for helpful comments throughout this study, K. M. MacDonald and M. L. Moore for excellent

technical assistance, L. W. Tjarks for preparing oligonucleotides and running sequencing gels, R. F. Sylvester for preparing figures and T. C. Nelsen for statistical analysis of virulence data.

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Received 24 December 1996; revised 7 April 1997; accepted 14 April 1997.