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Isolation and Gene Disruption of the *Tox5* Gene Encoding Trichodiene Synthase in *Gibberella pulicaris*

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The trichodiene synthase gene (*Tox5*) was isolated from *Gibberella pulicaris*, and its nucleotide sequence was determined. *Tox5* was disrupted through transformation with a plasmid carrying a doubly truncated copy of the coding region and a selectable marker for resistance to hygromycin B (Hyg^r). Analysis of 82 transformants for their ability to produce the trichothecene, 4,15-diacetoxyscirpenol (DAS), resulted in the identification of five DAS$^-$ strains. Southern hybridization analysis of DAS$^-$ Hyg^r transformants indicated that the plasmid integrated at the *Tox5* locus. The disrupted *Tox5* gene was shown to be mitotically stable. Analysis of nine tetrads revealed either the cosegregation of the disrupter plasmid and the DAS$^-$ phenotype or the loss of the disrupter plasmid. These results demonstrate the feasibility of using gene disruption in *G. pulicaris* and suggest a general method for obtaining *Tox5*$^-$ mutants in other trichothecene-producing fungi.

Additional keywords: genetic analysis, genetic transformation.

Gibberella pulicaris (Fr.:Fr.) Sacc. (anamorph: *Fusarium sambucinum* Fuckel) is an ascomycete that causes an economically important dry rot in potato tubers worldwide (Boyd 1972). It also produces trichothecenes, a family of sesquiterpenoid toxins that are potent inhibitors of protein synthesis (McLaughlin *et al.* 1977). Trichothecenes are produced by many species of *Fusarium* and closely related genera (Ueno 1980). Problems resulting from the accumulation of these toxic metabolites in agricultural products have stimulated the study of their biosynthesis and function. Although considerable progress has been made in studies of trichothecene biosynthesis (McCormick *et al.* 1990), including the isolation of a biosynthetic enzyme (Hohn and VanMiddlesworth 1986), the function of trichothecenes remains unknown. Because trichothecenes are phytotoxic (Cutler and Jarvis 1985) and many trichothecene-producing fungi are plant pathogens, it has been proposed that trichothecenes play a role in plant disease (Bean *et al.* 1984). Studies with mutants blocked at different steps in the trichothecene biosynthetic pathway have provided evidence that the production of trichothecenes can greatly enhance the virulence of *Fusarium sporotrichioides* Sherb. on parsnip root slices (Desjardins *et al.* 1989).

Previous investigations of trichothecene production in *G. pulicaris* have demonstrated the advantages of this organism as an experimental system for biochemical and genetic studies (Desjardins and Beremand 1987; Hohn and Beremand 1989a). More than 70 field strains of *G. pulicaris* have been shown to produce trichothecenes, and evidence linking high levels of trichothecene production with increased levels of virulence has been reported (Beremand *et al.* 1991). To further investigate the involvement of trichothecenes in plant diseases caused by *G. pulicaris*, we obtained mutants deficient in trichothecene production. Identification of such mutants is difficult because trichothecenes are not essential for growth or development under most conditions, and screening methods for trichothecenes are time-consuming. An immunoassay screening method has been successfully employed to isolate *Tox*$^-$ mutants from *F. sporotrichioides* (Beremand 1987); however, the development of a suitable immunoassay for the major trichothecene produced by *G. pulicaris*, 4,15-diacetoxyscirpenol (DAS), has proven difficult, and no DAS$^-$ mutants are yet available.

Trichodiene synthase catalyzes the first committed step in the biosynthesis of the trichothecenes. Therefore, mutants without a functional trichodiene synthase gene (*Tox5*) are particularly valuable for studies of trichothecene function. Gene disruption has been shown to be a useful method for constructing defined mutations in filamentous fungi (Fincham 1989). The development of a genetic transformation system (Salch and Beremand 1988) has enabled us to employ this technique in *G. pulicaris*. In this paper, we describe the isolation of the *Tox5* gene from *G. pulicaris* and the construction of *Tox5*$^-$ mutants through gene disruption. Details concerning the effects of *Tox5* gene disruption on virulence are described elsewhere (Desjardins *et al.* 1992).

MATERIALS AND METHODS

Strains and genetic crosses. A strain of *G. pulicaris* from potato in Germany, R-6380 (Fusarium Research Center, Pennsylvania State University), was the recipient strain for

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fungal transformations. Strain R-6380 produced DAS in liquid culture, was male- and female-fertile, and was mating type Mat1-1 (Desjardins and Beremand 1987). Strain 1815-1-5, which was used as a Mat1-2 parent in genetic crosses with transformants, is a fifth generation progeny produced by a successive series of backcrosses to strain R-6380 with selection for Mat1-2, high male and female fertility, and DAS production (Beremand 1989). Techniques for crossing strains, strain storage, and tetrad isolations have been described (Desjardins and Beremand 1987). *Escherichia coli* (Migula) Castellani and Chalmers XL1-Blue competent cells from Stratagene Cloning Systems (La Jolla, CA) were used in all *E. coli* transformations.

Isolation of the trichodiene synthase gene. A λgt11 library was constructed from *G. pulicaris* R-6380 total DNA as previously described (Hohn and Beremand 1989b). The *Bam*HI fragment of pTS37-4 (470-531) containing the entire coding sequence for the trichodiene synthase gene of *F. sporotrichioides* was used as a hybridization probe. The labeled probe, plaque transfer, and hybridization conditions were prepared as described (Hohn and Beremand 1989b).

Nucleotide sequence analysis. A series of nested deletions, differing in size by approximately 250 bp, was generated with the Erase-a-Base system (Promega, Madison, WI) from a DNA fragment cloned into the plasmid pTZ19U (pGP58-1, see below). Template DNA and sequencing reactions were prepared as described (Hohn and Beremand 1989b). Both commercially available and specifically synthesized primers were employed. All reactions were performed with [α-^{35}S]dATP.

Disrupter plasmid construction. Molecular biology techniques followed the protocols of Maniatis *et al.* (1982). The disrupter plasmid pGP73-2 was constructed by cloning the 889-bp *Sma*I-*Kpn*I fragment of pGP58-1 (see below) into pUCH1 (Turgeon *et al.* 1987). Besides having functions required for replication and selection in *E. coli*, pGP73-2 also carries a hybrid hygromycin B phosphotransferase gene. This hybrid gene consists of *Cochliobolus heterostrophus* (Drechs.) Drechs. DNA (promoter 1) fused in-frame with the hygromycin B phosphotransferase coding region.

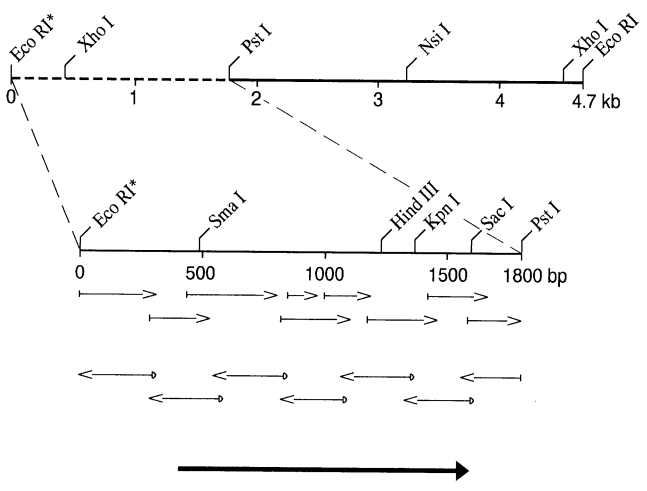

Fig. 1. Restriction map of the 4.7-kb *Eco*RI fragment and sequencing strategy for the *Tox5* gene. The small arrows below the map indicate individual sequencing reactions and the direction of sequencing. Open semicircles at the end of the arrows represent sequencing reactions primed with specifically synthesized oligonucleotides. The large heavy arrow represents the open reading frame of the *Tox5* gene and the direction of transcription. The asterisk by the *Eco*RI site at the 5′ end of the fragment (*Eco*RI*) indicates that this restriction site was introduced during the construction of the λgt11 library.

Transformation of *G. pulicaris*. Strain R-6380 was transformed by the method of Salch and Beremand (1988). Conidia (2×10^7) from this strain grown on V8 agar medium were used to inoculate 100 ml of YPG (2% glucose, 1.0% peptone, and 0.3% yeast extract) medium in 300-ml flasks. The flasks were incubated at 250 rpm for 13–15 hr at 28° C. Mycelium was collected and washed three times with 1.2 M KCl by centrifugation. Protoplasts were prepared by resuspending mycelium from 100 ml of culture in 50 ml of 1.2 M KCl with a mixture of cell wall degrading enzymes. The mixture of cell wall degrading enzymes included: Novozyme 234, 20 mg/ml (Novolabs, Wilton, CT); driselase, 20 mg/ml; and chitinase, 50 μg/ml (Sigma Chemical Co., St. Louis, MO). Isolated protoplasts (2.5×10^8) were transformed in 625 μl of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) with 125 μg of plasmid DNA. Protoplasts were regenerated by plating in molten (50° C), nonselective medium (0.1% yeast extract, 0.1% casein-enzyme hydrolysate, 1.6% agar, and 1.0 M sucrose). After 24 hr, plates were overlaid with 1% agar containing 50 μg/ml of hygromycin B (Sigma Chem. Co.). Colonies were transferred to fresh medium containing hygromycin B (50 μg/ml). Individual transformants isolated from a single spore were retested for hygromycin B resistance before further study.

Southern blotting analysis. DNA was isolated by using a modification of the procedure described by Raeder and Broda (1985). Cultures were grown in 25 ml of YPG medium for 1–2 days at 28° C on a gyratory shaker (200 rpm). Mycelium was harvested by filtration, washed with H_2O, and lyophilized overnight. The lyophilized mycelium (0.15–0.20 g) was broken up with a pipet tip and resuspended in 1.5 ml of extraction buffer (200 mM Tris, pH 8.5, 250 mM NaCl, 25 mM EDTA, pH 8.0, and 0.5% sodium dodecyl sulfate [SDS]). After phenol-chloroform and chloroform extractions, the aqueous layer was recovered (400 μl), and the DNA was precipitated by the addition of 0.54 vol of isopropanol. Samples were immediately centrifuged for 30 s at 13,000 × *g*. The pellet was washed with 70% ethanol, allowed to dry for several minutes in a vacuum desiccator, and then dissolved in 100 μl of TE (10 mM Tris, pH 8.0, 1.0 mM EDTA). After samples were incubated overnight at 50° C, they were digested with the indicated restriction enzymes. DNA fragments were separated by agarose gel electrophoresis (0.8 or 1.0% agarose) and transferred by diffusion to a nylon membrane as described by Maniatis *et al.* (1982). DNA was crosslinked to the membrane by exposure to UV light. Prehybridization was performed in a solution of 7.0% SDS, 50% formamide, 125 mM $NaPO_4$, pH 7.2, and 10 mM EDTA. After a 1- to 3-hr incubation at 42° C, the [^{32}P] labeled hybridization probe was added, and incubation continued for 16 hr. The blot was washed twice with each of the following solutions: 2× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M

sodium citrate, pH 7.0), 0.1% SDS for 15 min at room temperature; 0.1× SSC, 0.5% SDS for 20 min at 65° C; and 0.1× SSC, 0.1% SDS for 20 min at room temperature. Blots were exposed to Kodak XAR X-ray film (Eastman Kodak Co., Rochester, NY).

Trichothecene toxin assays. Liquid cultures were analyzed for trichothecene toxins by gas-liquid chromatography (GLC) as described by McCormick *et al.* (1990). An aliquot (2.5 ml) from a 7-day-old, 25-ml liquid culture in YEPD (0.3% yeast extract, 0.3% peptone, and 5.0% glucose) medium (Ueno *et al.* 1975) was transferred to a test tube and mixed with 5 ml of ethyl acetate by vortexing (90 s). The layers were separated by centrifugation. The organic layer was removed and dried under a stream of N_2 and then resuspended in 1 ml of ethyl acetate. A 2-μl aliquot was analyzed by GLC. The detection limit for DAS was equivalent to approximately 4 μM in the original liquid culture.

RESULTS

Cloning and sequencing of the *Tox5* gene. A single positive recombinant phage was isolated from a λgt11 library of *G. pulicaris* DNA; its 4.7-kb insert was cloned into pTZ18U to yield pGP53-1. Plasmid pGP58-1 was constructed to facilitate characterization of the *Tox5* gene by cloning the 1.8-kb *Eco*RI-*Pst*I fragment of pGP53-1 into pTZ19U. Restriction maps for the inserts cloned in plasmids pGP53-1 and pGP58-1, along with the sequencing strategy for the *Tox5* gene, are shown in Figure 1.

The nucleotide sequence of the pGP58-1 insert is shown in Figure 2. Comparison of this sequence with the *Tox5* gene of *F. sporotrichioides* (Hohn and Beremand 1989b) revealed a high degree of homology (89%) and permitted identification of the coding region for the *G. pulicaris* gene. Alignment of the deduced amino acid sequences of trichodiene synthase from *G. pulicaris* and *F. sporotrichioides* is presented in Figure 3. The aligned sequences are 96% identical; however, the *G. pulicaris* sequence contains nine additional amino acids near the C-terminus to give it a total of 383 amino acids and a calculated molecular weight of M_r 44,700. Of the 18 amino acid differences that occur between the two sequences, 14 can be considered conservative changes. A 60-nucleotide intron sequence that interrupts amino acid 157 is also present in the same position in the *F. sporotrichioides* sequence. The 5′ noncoding region contains two possible TATA-box sequences in approximately the same positions as these sequences occur in the *Tox5* gene of *F. sporotrichioides*. In addition, a perfect 42-nucleotide direct repeat is present starting at nucleotide 37.

Gene disruption of *Tox5*. The *Tox5* gene was disrupted after *G. pulicaris* was transformed with a plasmid containing a doubly truncated portion of the *Tox5* coding region and a selectable marker for resistance to hygromycin B. The plasmid, pGP73-2 (Fig. 4), was constructed for this purpose by insertion of a gene fragment coding for amino acids 29–303 into the fungal cloning vector pUCH1 (Turgeon *et al.* 1987). Homologous integration of pGP73-2 should produce two copies of the *Tox5* gene with each copy truncated at one end. Because substantial portions of the *Tox5* coding region were deleted at both the 5′ and 3′ ends, the truncated copies of the gene would be expected to make nonfunctional products.

After *G. pulicaris* was transformed with pGP73-2, hygromycin B resistant transformants were isolated. Transformants were grown under nonselective conditions, and culture extracts were analyzed for the major *G. pulicaris* trichothecene, DAS. Of the 82 transformants analyzed, 77 produced levels ranging from 15 to 100 mg/L, whereas five transformants did not produce detectable levels of DAS.

Southern hybridization analysis. Those transformants that did not produce trichothecenes were further characterized by Southern blotting to determine if plasmid DNA had integrated at the *Tox5* locus. Total DNA was extracted after growth on a nonselective medium and was submitted to agarose gel electrophoresis after digestion with either *Xho*I or *Eco*RI. The resulting blots were probed with radiolabeled pGP73-2.

Samples digested with *Xho*I, which does not cut within pGP73-2, yielded hybridization patterns that were consistent with the integration of one or more copies of pGP73-2 at the *Tox5* locus (Fig. 5B). A 4.1-kb *Xho*I band corresponding to the progenitor *Tox5* locus was replaced by a band of approximately 10 kb in transformants BC51, 96, and 97 and by bands greater than 10 kb in transformants BC81 and 93. The *Eco*RI hybridization pattern was also consistent with the integration of the transforming plasmid at the *Tox5* locus (Fig. 5A). There are two *Eco*RI sites within pGP73-2, and the progenitor *Tox5* locus resides on a 9.8-kb *Eco*RI fragment. Loss of the 9.8-kb *Eco*RI band carrying *Tox5* was observed in all five DAS^- transformants. Hybridization patterns for *Eco*RI-digested DNA from transformants BC51, 96, and 97 contained the three bands expected for the integration of a single copy of pGP73-2 at the *Tox5* locus. However, *Eco*RI-digested DNA from transformants BC81 and 93 produced an additional 1.9-kb band consistent with the tandem integration of two or more copies of the plasmid pGP73-2 at the *Tox5* locus.

Stability of the *Tox5* disruption. To determine mitotic stability, we retested DAS^- transformants for the production of trichothecenes three or more times over a period of several months. The fact that DAS^- transformants did not produce detectable levels of trichothecenes under these conditions suggests that the *Tox5* disruption is mitotically stable. To test the meiotic stability of the *Tox5* disruption, we crossed transformant 51 (Mat1-1, *Tox5*$^-$, and Hyg^r) and strain 1810-1-5 (Mat1-2, *Tox5*$^+$, and Hyg^s), a strain which is isogenic with the R-6380 progenitor strain (Beremand 1989).

Ascospores picked from individual tetrads were analyzed for trichothecene production and hygromycin B resistance. In a total of nine complete tetrads analyzed, two different segregation patterns were observed. DAS^- and Hyg^r (1:1) cosegregated as expected for a single genetic locus in seven tetrads. In the other two tetrads, all of the progeny were DAS^+ and Hyg^s (8:0), a result consistent with the loss of the transforming plasmid.

Southern analysis of progeny from tetrads representing both segregation patterns (data not shown) gave the expected hybridization patterns for the observed phenotypes.

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         10          20          30          40          50          60          70
TGTACCTATC GCTTGCGTAG CTCTTTACTA CATGTGCCGA GCTAAAGATA AAATCGGACT AAAGATTCGT
                                    ***
         80          90         100         110         120         130         140
CCCGGGAGCC GAGCTAAAGA TAAAATCGGA CTAAAGATTC GTCCCGGGAG CCGAATGCTA TCTCAAGCTC
         **  *
        150         160         170         180         190         200         210
GTCGTGTTGC AGGGGATGGA AGACCTCCAG TGTACGTCAC GGTCTCTATC ACTACGAATT TACTGGGAAG

        220         230         240         250         260         270         280
GCTATTTGCA TTAACGTCAA GTTAATCATT AGGCCTAACA ACACAAGCAC AACTAAAGAT TGTGGATGGT

        290         300         310         320         330         340         350
TGACATTTAC CATATGCTGA TATATAGTTG ATAGCAACAG CACTTTGCAA TAGAACAATA ATAGCGATTT

        360         370         380         390         400
GACTTGAAAA CTCACCAAGA ATCGTTACCA ATTATTATAC CATTATCATC

          409         418         427         436         445         454
ATG GAG AAC TTT CCC ACT GAG TAT TTT CTC AAC ACT TCT GTG CGC CTT CTC GAG
Met Glu Asn Phe Pro Thr Glu Tyr Phe Leu Asn Thr Ser Val Arg Leu Leu Glu

          463         472         481      SmaI 490         499         508
TAC ATT CGA TAC CGA GAT AGC AAT TAT ACC CGG GAA GAG CGC ATC GAG AAT TTG
Tyr Ile Arg Tyr Arg Asp Ser Asn Tyr Thr Arg Glu Glu Arg Ile Glu Asn Leu

          517         526         535         544         553         562
CAC TAT GCT TAC AAC AAG GCT GCT CAT CAC TTT GCT CAG CCA CGA CAA CAG CAG
His Tyr Ala Tyr Asn Lys Ala Ala His His Phe Ala Gln Pro Arg Gln Gln Gln

          571         580         589         598         607         616
CTG CTC AAG GTA GAC CCT AAG CGA CTA CAG GCT TCC CTC CAA ACC ATT GTT GGC
Leu Leu Lys Val Asp Pro Lys Arg Leu Gln Ala Ser Leu Gln Thr Ile Val Gly

          625         634         643         652         661         670
ATG GTG GTA TAC AGT TGG GCA AAG GTC TCC AAA GAG TGT ATG GCG GAT CTA TCA
Met Val Val Tyr Ser Trp Ala Lys Val Ser Lys Glu Cys Met Ala Asp Leu Ser

          679         688         697         706         715         724
ATT CAT TAC ACG TAC ACA CTT GTT TTG GAT GAC AGC AGC GAT GAT CCG TAT CCG
Ile His Tyr Thr Tyr Thr Leu Val Leu Asp Asp Ser Ser Asp Asp Pro Tyr Pro

          733         742         751         760         769         778
GCC ATG ATG AAC TAT TTC AAC GAT CTT CAG GCT GGA CGA GAA CAG GCA CAC CCC
Ala Met Met Asn Tyr Phe Asn Asp Leu Gln Ala Gly Arg Glu Gln Ala His Pro

          787         796         805         814         823         832
TGG TGG GCG CTT GTC AAT GAG CAC TTT CCC AAT GTC CTT CGA CAT TTT GGT CCC
Trp Trp Ala Leu Val Asn Glu His Phe Pro Asn Val Leu Arg His Phe Gly Pro

          841         850         859         868
TTC TGC TCA TTG AAC CTT ATC CGC AGC ACT CTT GAC T  GTAAGTACCCTGGCTCTAT
Phe Cys Ser Leu Asn Leu Ile Arg Ser Thr Leu Asp P

                                                  931         940         949
TATTTCACCACCCCAATAAGCTAACAGTGATGGAATTGCAG  TT  TTT GAG GGA TGC TGG ATC
                                               he  Phe Glu Gly Cys Trp Ile
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(**Fig. 2** *continued on next page*)

Fig. 2. Nucleotide sequence of the *Tox5* gene and predicted amino sequence. The untranslated region beginning at nucleotide 869 is a 60-nucleotide intron within which a postulated internal consensus sequence is double-underlined. The 42-nucleotide repeat sequence in the 5′-noncoding region is indicated by asterisks under the first three nucleotides of each repeat.

(**Fig. 2** *continued from previous page*)

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          958          967          976          985          994         1003
GAG CAG TAC AAC TTT GGA GGA TTT CCA GGA TCT CAT GAT TAT CCT CAG TTT CTT
Glu Gln Tyr Asn Phe Gly Gly Phe Pro Gly Ser His Asp Tyr Pro Gln Phe Leu

         1012         1021         1030         1039         1048         1057
CGA CGC ATG AAT GGC TTG GGC CAC TGC GTC GGG GCG TCT TTG TGG CCC AAG GAG
Arg Arg Met Asn Gly Leu Gly His Cys Val Gly Ala Ser Leu Trp Pro Lys Glu

         1066         1075         1084         1093         1102         1111
CAG TTT GAC GAG CGA GGT CTA TTC CTT GAA ATC ACA TCA GCC ATT GCT CAG ATG
Gln Phe Asp Glu Arg Gly Leu Phe Leu Glu Ile Thr Ser Ala Ile Ala Gln Met

         1120         1129         1138         1147         1156         1165
GAG AAC TGG ATG GTC TGG GTA AAT GAT CTT ATG TCA TTC TAC AAG GAG TTC GAT
Glu Asn Trp Met Val Trp Val Asn Asp Leu Met Ser Phe Tyr Lys Glu Phe Asp

         1174         1183         1192         1201         1210         1219
GAT GAG CGT GAC CAG ATC AGT CTC GTC AAG AAC TAC GTC GTC TCT GAT GAG ATC
Asp Glu Arg Asp Gln Ile Ser Leu Val Lys Asn Tyr Val Val Ser Asp Glu Ile

         1228         1237         1246         1255         1264         1273
ACT CTC CAT GAA GCT TTA GAG AAG CTC ACC CAG GAC ACT CTA CAC TCG TCC AAG
Thr Leu His Glu Ala Leu Glu Lys Leu Thr Gln Asp Thr Leu His Ser Ser Lys

         1282         1291         1300         1309         1318         1327
CAG ATG GTA GCT GTC TTC TCT GAG AAG GAC CCC CAG GTG ATG GAC ACG ATT GAG
Gln Met Val Ala Val Phe Ser Glu Lys Asp Pro Gln Val Met Asp Thr Ile Glu

         1336         1345         1354         1363         1372  KpnI   1381
TGC TTC ATG CAC GGC TAT GTC ACG TGG CAC TTG TGC GAT CAC AGG TAC CGC CTT
Cys Phe Met His Gly Tyr Val Thr Trp His Leu Cys Asp His Arg Tyr Arg Leu

         1390         1399         1408         1417         1426         1435
AAT GAG ATC TAC GAA AAG GTC AAA GGA CAA AAG ACC GAG GAC GCT GAG AAG TTT
Asn Glu Ile Tyr Glu Lys Val Lys Gly Gln Lys Thr Glu Asp Ala Glu Lys Phe

         1444         1453         1462         1471         1480         1489
TGC AAG TTC TAT GAG CAG GCT GCT AAT GTC GGA GCC GTT TCG CCT TCG GAG TGG
Cys Lys Phe Tyr Glu Gln Ala Ala Asn Val Gly Ala Val Ser Pro Ser Glu Trp

         1498         1507         1516         1525         1534         1543
GCT TAT CCA CCT ATT GCG CAA TTG GCA AAC ATT CGG ACC AAG GAT GTG AAG GAT
Ala Tyr Pro Pro Ile Ala Gln Leu Ala Asn Ile Arg Thr Lys Asp Val Lys Asp

         1552         1561         1570         1579         1588         1597
TTG AAG GAT GTG AAG GAT CTG AAG GAG ATT CAG AAG CCT CTT CTG AGC TCA ATT
Leu Lys Asp Val Lys Asp Leu Lys Glu Ile Gln Lys Pro Leu Leu Ser Ser Ile

         1606                 1622         1632         1642         1652         1662
GAG CTA GTG GAA TGA     CCGACGGTGA GATGGAAGTA TGTTTTGCGG GTACTCGCTA GGAGAATACT
Glu Leu Val Glu End

        1672         1682         1692         1702         1712         1722         1732
GGTCGTTTAT CATGATTACA AATAGCTTGG TTGTGTTTTT ATTAGCATTT ACAGTTGAAC AAGGATAATT

        1742         1752         1762         1772         1782         1792      1799
CCTACTGAAT AGGCAGCTGA AACTGATGTC TGTAACTCCA GCCTGTTCGT TATCCGCTTG CCTGCAG
                                                                      PstI
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Fs MENFPTEYFL NTTVRLLEYI RYRDSNYTRE ERIENLHYAY NKAAHHFAQP RQQQLLKVDP 60
Gp MENFPTEYFL NTSVRLLEYI RYRDSNYTRE ERIENLHYAY NKAAHHFAQP RQQQLLKVDP

Fs KRLQASLQTI VGMVVYSWAK VSKECMADLS IHYTYTLVLD DSKDDPYPTM VNYFDDLQAG 120
Gp KRLQASLQTI VGMVVYSWAK VSKECMADLS IHYTYTLVLD DSSDDPYPAM MNYFNDLQAG

Fs REQAHPWWAL VNEHFPNVLR HFGPFCSLNL IRSTLDFFEG CWIEQYNFGG FPGSHDYPQF 180
Gp REQAHPWWAL VNEHFPNVLR HFGPFCSLNL IRSTLDFFEG CWIEQYNFGG FPGSHDYPQF

Fs LRRMNGLGHC VGASLWPKEQ FNERSLFLEI TSAIAQMENW MVWVNDLMSF YKEFDDERDQ 240
Gp LRRMNGLGHC VGASLWPKEQ FNERGLFLEI TSAIAQMENW MVWVNDLMSF YKEFDDERDQ

Fs ISLVKNYVVS DEISLHEALE KLTQDTLHSS KQMVAVFSDK DPQVMDTIEC FMHGYVTWHL 300
Gp ISLVKNYVVS DEITLHEALE KLTQDTLHSS KQMVAVFSEK DPQVMDTIEC FMHGYVTWHL

Fs CDRRYRLSEI YEKVKEEKTE DAQKFCKFYE QAANVGAVSP SEWAYPPVAQ LANVRSKDV• 360
Gp CDHRYRLNEI YEKVKGQKTE DAEKFCKFYE QAANVGAVSP SEWAYPPIAQ LANIRTKDVK

Fs ••••••••KE VQKPFLSSIE LVE 374
Gp DLKDVKDLKE IQKPLLSSIE LVE 383
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Fig. 3. Alignment of deduced amino acid sequences for the *Tox5* genes from *Gibberella pulicaris* (Gp) and *Fusarium sporotrichioides* (Fs). Sequence differences at a particular position are indicated with boxes.

DISCUSSION

We have cloned and sequenced the *G. pulicaris* gene coding for trichodiene synthase. Comparison of its deduced amino acid sequence with the trichodiene synthase of *F. sporotrichioides* revealed that these sequences differ primarily in the presence of nine additional amino acids near the C-terminus of the *G. pulicaris* sequence. The *G. pulicaris* trichodiene synthase gene is referred to as *Tox5* to indicate its similarity to the *F. sporotrichioides* gene. A *G. pulicaris* gene involved in trichothecene biosynthesis was previously designated *Tox1* (Beremand and Desjardins 1988).

The native *Tox5* gene was disrupted by a one-step procedure first described in *Saccharomyces cerevisiae* Hansen (Shortle *et al.* 1982). After being transformed with a plasmid containing a portion of the cloned *Tox5* coding region truncated at both ends, five DAS$^-$ transformants were isolated. These transformants were shown by Southern analysis to have integrated one or more copies of the disrupter plasmid at the *Tox5* locus. This gene disruption strategy was chosen because one-step gene disruption involving gene replacement and gene disruption occurs at low frequencies in some filamentous ascomycetes (Miller *et al.* 1985; Paietta and Marzluf 1985). Efficient transformation protocols are not presently available for *G. pulicaris* or other trichothecene-producing fungi. One disadvantage of this approach is its potential reversibility through loss of the disrupter plasmid. Excision of the disrupter plasmid and the subsequent restoration of gene function can occur as a result of recombination between the two nonfunctional copies of the target gene. For this reason, the mitotic and meiotic stabilities of the disrupted *Tox5* gene were determined. We

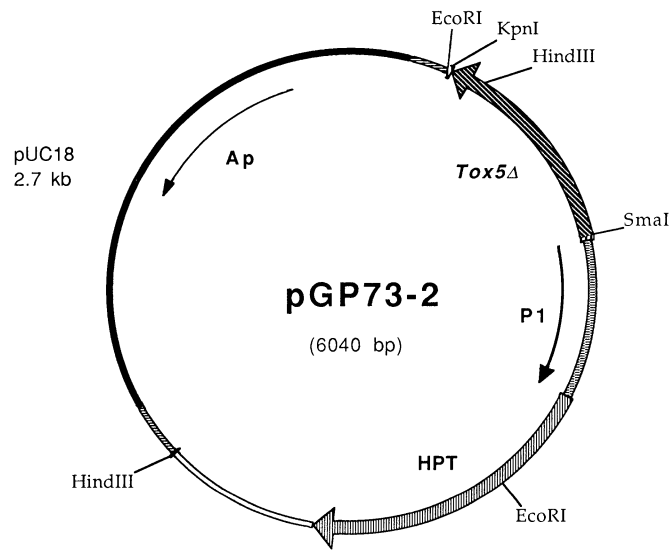

Fig. 4. Map of plasmid pGP73-2. Abbreviations: Ap, β-lactamase; HPT, hygromycin B phosphotransferase; P1, promoter 1 from *Cochliobolus heterostrophus*; *Tox5*Δ, truncated *Tox5* coding region.

concluded that the *Tox5* disruption was mitotically stable after multiple analyses failed to detect trichothecenes in cultures of DAS$^-$ transformants grown under a variety of conditions. Genetic analysis revealed that the disrupted *Tox5* gene was meiotically unstable.

Genetic crosses between transformant BC51 and a strain isogenic with the BC51 progenitor revealed that the DAS$^-$ phenotype was stably inherited in seven of nine tetrads. Cosegregation of the DAS$^-$ Hyg^r phenotypes in all seven

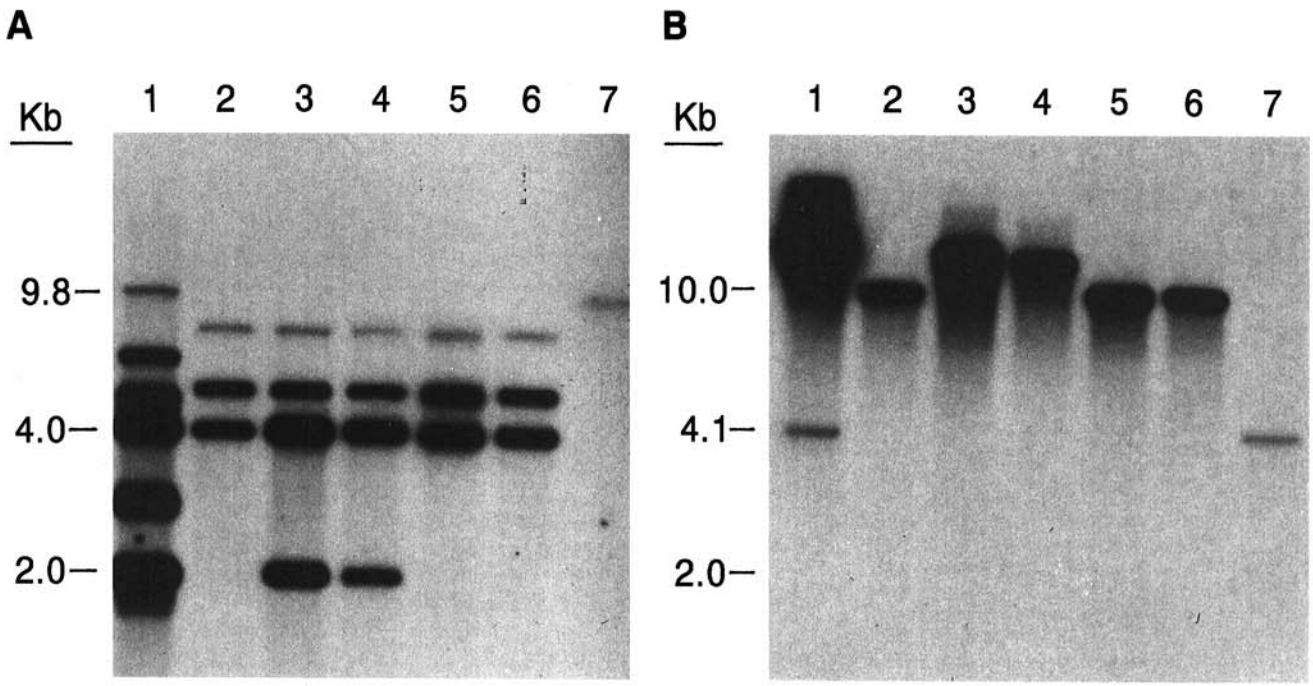

Fig. 5. Southern blot analysis of digested genomic DNA from DAS$^-$ transformants. Blots were probed with ^{32}P-labeled pGP73-2. **A,** *Eco*RI-digested samples. **B,** *Xho*I-digested samples. Lanes: 1, DAS$^+$ transformant; 2–6, DAS$^-$ transformants BC51, 81, 93, 96, and 97; 7, progenitor strain R-6380.

tetrads demonstrates that the integrated disrupter plasmid and the DAS$^-$ phenotype are closely linked. The absence of the DAS$^-$ Hyg^r phenotypes in the progeny from two of nine tetrads indicates that the disrupter plasmid was excised. The relatively high frequency of disrupter plasmid excision during meiosis suggests that a two-step gene disruption procedure may be feasible in *G. pulicaris*. A two-step gene disruption procedure involving gene replacement and gene disruption has been demonstrated in *Aspergillus nidulans* (Eidam) G. Wint. (Miller *et al.* 1985).

Construction of a defined *Tox5*$^-$ mutant has provided additional evidence that trichodiene or trichodiene metabolites do not function in primary metabolism or development. The growth rate and morphology of *Tox5*$^-$ strains were indistinguishable from those of the progenitor strain. Similar results were previously obtained with mutants of *F. sporotrichioides* blocked at steps in the pathway after trichodiene (Beremand 1987). Precursor feeding studies have demonstrated that the trichothecene pathway is functional in *Tox5*$^-$ mutants. Trichothecene precursors such as trichodiene and isotrichotriol were efficiently converted to DAS by *Tox5*$^-$ mutants (S. P. McCormick, T. M. Hohn, and M. N. Beremand, unpublished). Based on a correlation between increased fertility and high trichothecene production levels observed in a survey of 70 strains of *G. pulicaris*, it has been suggested that trichothecenes may play a role in sexual reproduction (Beremand *et al.* 1991). The fact that *Tox5*$^-$ strains are fertile when crossed either as a male or a female parent with isogenic host strains argues against this.

Trichothecene production by *G. pulicaris* appears to be a virulence factor in some plant diseases. All five *Tox5*$^-$ mutants were less virulent than the wild-type on parsnip root slices but had wild-type virulence on potato tuber slices (Desjardins *et al.* 1992). Greatly reduced virulence was also observed on parsnip slices for trichothecene-deficient mutants of *F. sporotrichioides* (Desjardins *et al.* 1989).

We have demonstrated the feasibility of gene disruption in *G. pulicaris*. The same molecular disruption strategy has also been used to disrupt the *Tox5* gene in *F. sporotrichioides* (T. M. Hohn and M. N. Beremand, unpublished). Preliminary results indicate that the trichodiene synthase coding region can be amplified by polymerase chain reaction from *G. zeae* (Schwein.) Petch and closely related *Fusarium* species; this suggests it may be possible to apply the approach described here to other trichothecene-producing fungi.

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LITERATURE CITED

Bean, G. A., Fernado, T., Jarvis, B. B., and Burton, B. 1984. The isolation and identification of trichothecene metabolites from a plant pathogenic strain of *Myrothecium roridum*. J. Nat. Prod. 47:727-729.

Beremand, M. N. 1987. Isolation and characterization of mutants blocked in T-2 toxin biosynthesis. Appl. Environ. Microbiol. 53:1855-1859.

Beremand, M. N. 1989. Genetic and mutational tools for investigating the genetics and molecular biology of trichothecene production in *Gibberella pulicaris* (*Fusarium sambucinum*). Mycopathologia 107:67-74.

Beremand M. N., and Desjardins A. E. 1988. Trichothecene biosynthesis in *Gibberella pulicaris*: Inheritance of C-8 hydroxylation. J. Indus.

Microbiol. 3:167-174.
Beremand, M. N., Desjardins, A. E., Hohn, T. M., and VanMiddlesworth, F. 1991. A survey of *Fusarium sambucinum* (*Gibberella pulicaris*) for mating type, trichothecene production, and other selected traits. Phytopathology 81:1452-1458.
Boyd, A. E. W. 1972. Potato storage diseases. Rev. Plant Pathol. 51:297-321.
Cutler, H. G., and Jarvis, B. B. 1985. Preliminary observations on the effects of macrocyclic trichothecenes on plant growth. Environ. Exp. Bot. 25:115-128.
Desjardins, A. E., and Beremand, M. N. 1987. A genetic system for trichothecene toxin production in *Gibberella pulicaris* (*Fusarium sambucinum*). Phytopathology 77:678-683.
Desjardins, A. E., Hohn, T. M., and McCormick, S. P. 1992. Effect of gene disruption of trichodiene synthase on the virulence of *Gibberella pulicaris*. Mol. Plant-Microbe Interact. 5:214-222.
Desjardins, A. E., Spencer, G. F., Plattner, R. D., and Beremand, M. N. 1989. Furanocoumarin phytoalexins, trichothecene toxins and infection of *Pastinaca sativa* by *Fusarium sporotrichioides*. Phytopathology 79:170-175.
Fincham, J. R. S. 1989. Transformation in fungi. Microbiol. Rev. 53:148-170.
Hohn, T. M., and Beremand, M. N. 1989a. Regulation of trichodiene synthase in *Fusarium sporotrichioides* and *Gibberella pulicaris* (*Fusarium sambucinum*). Appl. Environ. Microbiol. 55:1500-1503.
Hohn, T. M., and Beremand, P. D. 1989b. Isolation and nucleotide sequence of a sesquiterpene cylase gene from the trichothecene-producing fungus *Fusarium sporotrichioides*. Gene 79:131-138.
Hohn, T. M., and VanMiddlesworth, F. 1986. Purification and characterization of the sesquiterpene cyclase trichodiene synthetase from *Fusarium sporotrichioides*. Arch. Biochem. Biophys. 251:756-761.
Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McCormick, S. P., Taylor, S. L., Plattner, R. D., and Beremand, M. N. 1990. Bioconversion of possible T-2 toxin precursors by a mutant strain of *Fusarium sporotrichioides*. Appl. Environ. Microbiol. 56:702-706.
McLaughlin, C. S., Vaughn, M. H., Campbell, J. M., Wei, C. M., Stafford, M. E., and Hansin, B. S. 1977. Inhibition of protein synthesis by trichothecenes. Pages 263-273 in: Mycotoxins in Human and Animal Health. H. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman, eds. Pathotox Publishers, Park Forest South, IL.
Miller, B. L., Miller, K. Y., and Timberlake, W. E. 1985. Direct and indirect gene replacements in *Aspergillus nidulans*. Mol. Cell. Biol. 5:1714-1721.
Paietta, J. V., and Marzluf, G. A. 1985. Gene disruption by transformation in *Neurospora crassa*. Mol. Cell. Biol. 5:1554-1559.
Raeder, V., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1:17-20.
Salch, Y. P., and Beremand, M. N. 1988. Development of a transformation system for *Fusarium sambucinum*. J. Cell Biochem. Suppl. 12C:290.
Shortle, D. P., Haber, J. E., and Botstein, D. 1982. Lethal disruption of the yeast actin gene by integrative DNA transformation. Science 217:371-373.
Turgeon, B. G., Garber, R. C., and Yoder, O. C. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. 17:3297-3305.
Ueno, Y. 1980. Trichothecene mycotoxins: Mycology, chemistry and toxicology. Adv. Nutr. Sci. 3:301-353.
Ueno, Y., Sawono, M., and Ishii, K. 1975. Production of trichothecene mycotoxins by *Fusarium* species in shake culture. Appl. Microbiol. 30:4-9.