

Identification of Deoxynivalenol- and Nivalenol-Producing Chemotypes of *Gibberella zeae* by Using PCR

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Gibberella zeae, a major cause of cereal scab, may be divided into two chemotypes based on production of the trichothecenes deoxynivalenol (DON) and nivalenol (NIV). We cloned and sequenced the gene cluster for trichothecene biosynthesis from each chemotype. *G. zeae* H-11 is a DON producer isolated from corn, and *G. zeae* 88-1 is a NIV producer from barley. We sequenced a 23-kb gene cluster from H-11 and a 26-kb cluster from 88-1, along with the unlinked *Tri101* genes. Each gene cluster contained 10 *Tri* gene homologues in the same order and transcriptional directions as those of *Fusarium sporotrichioides*. Between H-11 and 88-1 all of the *Tri* homologues except *Tri7* were conserved, with identities ranging from 88 to 98% and 82 to 99% at the nucleotide and amino acid levels, respectively. The *Tri7* sequences were only 80% identical at the nucleotide level. We aligned the *Tri7* genes and found that the *Tri7* open reading frame of H-11 carried several mutations and an insertion containing 10 copies of an 11-bp tandem repeat. The *Tri7* gene from 88-1 carried neither the repeat nor the mutations. We assayed 100 *G. zeae* isolates of both chemotypes by PCR amplification with a primer pair derived from the *Tri7* gene and could differentiate the chemotypes by polyacrylamide gel electrophoresis. The PCR-based method developed in this study should provide a simple and reliable diagnostic tool for differentiating the two chemotypes of *G. zeae*.

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is an important pathogen of cereal crops in many areas of the world. The fungus causes head and seedling blight of small grains, such as wheat and barley; ear and stalk rot of corn; and stem rot of carnation (7, 20, 28). Head blight and ear rot reduce the yield of grain, and harvested grain is often contaminated with mycotoxins, such as trichothecenes and zearalenone (23). The fungus produces 8-ketotrichothecenes, including deoxynivalenol (DON), 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol (NIV), and 4-acetylnivalenol (4-ANIV), as well as an estrogenic mycotoxin, zearalenone (27, 35). Of these 8-ketotrichothecenes, DON and NIV are frequently found in cereals harvested in Korea and Japan (17, 38, 40). Compared to DON, NIV is present at higher levels in cereals and exhibits greater toxicity (33). Therefore, NIV is of greater concern in these countries.

Based on 8-ketotrichothecene production, Ichinoe et al. (15) divided *G. zeae* into two chemotypes groups. The DON chemotype produces DON and acetyl-DON, and the NIV chemotype produces NIV and 4-ANIV. These chemotypes appear to differ in geographic distribution. The NIV chemotype has been reported in several countries of Africa, Asia, and Europe (10, 14, 22, 35, 37), but it has not been reported in North America (1, 27). Since the NIV chemotype is of great concern in the countries where it has appeared, an efficient system for differentiating DON and NIV chemotypes is desired. Recently O'Donnell et al. (30) divided global population of *G. zeae* into seven biogeographically structured lineages based on phyloge-

netic analysis using six gene genealogies. The two chemotypes, however, do not appear to correlate with the global phylogenetic structure of *G. zeae*.

Although the geographic distribution of DON and NIV chemotypes has been well studied, little is known about the genetic basis of 8-ketotrichothecene production by these chemotypes. However, the molecular genetics of the production of T-2 toxin by *Fusarium sporotrichioides* has been studied intensively, using various mutant strains blocked at specific steps in the trichothecene pathway (9, 13). Many trichothecene biosynthesis genes are localized in a gene cluster comprising at least 10 genes. These genes include those encoding trichodiene synthetase (*Tri5*) (11), P450 oxygenases (*Tri4* and *Tri11*) (2, 12), acetyltransferase (*Tri3*) (25), a transcription factor (*Tri6*) (32), a toxin efflux pump (*Tri12*) (3), and several unidentified hypothetical proteins (*Tri7*, *Tri8*, *Tri9*, and *Tri10*) (13, 26). Another acetyltransferase (*Tri101*) (19) is unlinked to the cluster. Homologues of *Tri5*, *Tri6*, *Tri11*, *Tri12*, and *Tri101* have been previously found in *G. zeae* (18, 24, 31, 39), and all of the genes exhibited high degrees of sequence homology to those of *F. sporotrichioides*. A homologue of the *F. sporotrichioides* *Tri12* was designated *Tri102* in *G. zeae* (39).

In this study we built on the *F. sporotrichioides* results to analyze the trichothecene biosynthesis gene clusters in the DON and NIV chemotypes of *G. zeae*. The objectives of the study were to identify genetic differences between the trichothecene biosynthetic pathways of the two chemotypes, and to develop a rapid method for *G. zeae* chemotype identification based on PCR analysis.

MATERIALS AND METHODS

Strains, media, and culture conditions. *G. zeae* strains H-11 (a DON producer) and 88-1 (a NIV producer) were isolated from Korean corn and barley,

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respectively, and used for sequence analysis. For PCR assays, 100 field isolates of *G. zea* were used. Of these isolates, 25 DON producers were from the United States. The remaining 25 DON and 50 NIV producers were from a previous study of Korean strains (35). Fungi were cultured on potato dextrose agar (Difco Laboratories, Detroit, Mich.) and preserved as 25% glycerol stock cultures frozen at -80°C . To isolate genomic DNA, fungal conidia were inoculated into 100 ml of complete liquid medium (8) at 10^6 per ml. Cultures were incubated in 250-ml Erlenmeyer flasks at 25°C for 48 h on a rotary shaker (200 rpm), after which mycelia were harvested and lyophilized. *Escherichia coli* strains were grown on Luria-Bertani agar or liquid medium supplemented with ampicillin (75 $\mu\text{g}/\text{ml}$).

DNA manipulations and PCR conditions. Fungal genomic DNA was prepared as previously described (16). *E. coli* colonies carrying recombinant plasmids were screened by a single-tube mini-prep method (21). For sequencing, plasmids were purified from 5-ml *E. coli* cultures using a Qiagen kit (Qiagen Inc., Valencia, Calif.). Standard procedures were used for restriction endonuclease digestions, ligations, and agarose or polyacrylamide gel electrophoresis (34).

PCR primers used for *Tri* gene amplification in this study are listed in Table 1. They were synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chungwon, Korea), dissolved at 100 μM in sterilized water, and stored at -20°C . For PCRs, about 50 ng of genomic DNA was used as a template in a 50- μl reaction mixture containing Ex *Taq* PCR buffer, which contains 2 mM MgCl_2 (TaKaRa Biomedicals, Shiga, Japan), deoxynucleoside triphosphate mixture (0.2 mM each), a 2 μM concentration of each primer, and 1.25 U of Ex *Taq* (TaKaRa Biomedicals). PCRs were performed in a thermal cycler (MJ Research, Inc., Waltham, Mass.) set to the following: denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min 30 s; and a final extension step at 72°C for 10 min. After PCR was completed, PCR mixtures were stored at 4°C .

Amplification of *Tri* gene clusters. We amplified *Tri* genes from both strains (Fig. 1) by using several sets of degenerate primers that were based on the known sequences of *Tri* genes from *G. zea* and *F. sporotrichioides*. Most of the degenerate primer pairs successfully amplified fragments of the expected sizes from the two strains. When necessary, primers were redesigned according to the consensus sequences of already-acquired *Tri* genes (e.g., primer pairs 5-6 and 5'-6' in Table 1 and Fig. 1). The PCR product sequences were used to design specific primers for amplifying regions flanking the amplified *Tri* genes. A PCR was performed using two primers, each derived from different *Tri* genes based on the assumption that the order of the *Tri* genes in the *G. zea* and *F. sporotrichioides* clusters is the same. For example, PCR using primers 31 and 43 successfully amplified the region between *Tri6* and *Tri5* from H-11 (Fig. 1). When this strategy failed, inverse PCR using primers derived from the known sequences was performed as previously described (29, 41). These PCR strategies yielded various sizes of DNA fragments sufficient for construction of contigs for both strains.

Cloning and sequencing. The amplified PCR products were analyzed by agarose gel electrophoresis. PCR products of the expected sizes were cloned into pCR2.1TOPO using the TOPO TA cloning kit (Invitrogen, San Diego, Calif.). Sequencing of the inserts in pCR2.1 TOPO was initiated with M13 reverse and forward primers and then extended using specific primers corresponding to the newly sequenced regions. DNA sequencing was done at the National Instrumentation Center for Environmental Management, Seoul National University, Suwon, Korea, using an ABI377 automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). Primers for sequencing were designed using the PrimerSelect program (DNASTAR, Inc., Madison, Wis.). Sequences were assembled using the SeqMan program (DNASTAR, Inc.) and analyzed with the MegAlign and MapDraw programs (DNASTAR, Inc.). BLAST (4) searches were done against the NCBI and GenBank databases.

PCR assays. To amplify the inserted region of *Tri7* from 50 DON- and 50 NIV-producing isolates, we designed a specific set of primers. The sequence of primer GzTri7/f1 (forward) is 5'-GGCTTTACGACTCCTCAACAATGG-3', and the sequence of primer GzTri7/r1 (reverse) is 5'-AGAGCCCTGCGAAAG (C/T)ACTGGTGC-3'. PCRs were performed in a thermal cycler set to the following: denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. After PCR was completed, PCR mixtures were stored at 4°C . PCR products were electrophoresed on 5% polyacrylamide gels; the products of the expected size (~ 160 bp) were purified using a QIAquick PCR purification kit (Qiagen Inc.) and directly sequenced using primers GzTri7/f1 and GzTri7/r1.

Nucleotide sequence accession numbers. The sequences of the trichothecene biosynthesis gene clusters we obtained from *G. zea* 88-1 and H-11 have been deposited in GenBank under accession numbers AF336365 and AF336366, respectively.

RESULTS

Structural organization of trichothecene biosynthesis gene clusters from DON and NIV chemotypes. Construction of contigs using the sequenced PCR fragments yielded 26- and 23-kb gene clusters from *G. zea* 88-1 and H-11, respectively. Each cluster carried 8 open reading frames (ORFs), all of which were readily identified by sequence comparisons with current databases. Two additional ORFs (designated *Tri9* and *Tri10*) were found between *Tri5* and *Tri11* as those in the trichothecene gene cluster of *F. sporotrichioides* (Fig. 1). The order and transcription directions of the ORFs are identical for the two *G. zea* chemotypes and *F. sporotrichioides*.

Comparative sequence analysis. We compared the sizes of the ORFs, putative introns, and adjacent noncoding DNA regions (Table 2) and the percent identities of the ORFs and their flanking sequences (Table 3) in the two *G. zea* *Tri* clusters and other strains of *G. zea* and *F. sporotrichioides*. All of the *Tri* genes except *Tri7* exhibited significant conservation between the two strains, ranging from 88% (*Tri8*) to 98% (*Tri10*) identity at the nucleotide level and from 82% (*Tri8*) to 99% (*Tri10*) at the amino acid level. The flanking regions exhibited less nucleotide sequence identity than the ORFs, ranging from 57% (region between *Tri3* and *Tri4*) to 89% (region between *Tri5* and *Tri11*). In particular, strain H-11 lacked a 226-bp stretch in the noncoding region between *Tri3* and *Tri4* that was present in strain 88-1 (data not shown), giving this region the lowest percent identity overall.

The gene with the highest identity for the two chemotypes was *Tri10*, which is not part of the *Tri* gene cluster. The nucleotide and amino acid sequence identities were 98 and 99%, respectively. In addition, several *Tri* genes from other *G. zea* strains, including *Tri5*, *Tri6*, *Tri11*, *Tri102*, and *Tri101* (18, 24, 31, 39), were compared to the corresponding *Tri* ORFs of H-11 and 88-1 (Table 3). These genes were also highly similar to those of both chemotypes at both the nucleotide and amino acid levels. In contrast, the *Tri* genes of *F. sporotrichioides* were less similar to those of the *G. zea* chemotypes, ranging from 76 to 86% identity for nucleotide sequences and from 74 to 91% identity for amino acid sequences.

Alignment of *Tri7* ORFs from *G. zea* DON and NIV chemotypes and *F. sporotrichioides*. Unlike the other *Tri* ORFs, the *Tri7* ORFs from the two *G. zea* chemotypes and from *F. sporotrichioides* exhibited striking differences. First, the nucleotide sequence of the H-11 *Tri7* ORF exhibited only 80% identity to that of 88-1 and 64% identity to that of *F. sporotrichioides* (Table 3). This level of conservation was significantly lower than that found for the other *Tri* ORFs. Second, alignment of the nucleotide sequences of these three *Tri7* ORFs revealed several alterations present only in H-11 (data not shown). For example, a substitution in a potential start codon in H-11 appeared to have occurred, causing a deficient translation start signal (by comparison with *F. sporotrichioides*). The H-11 *Tri7* nucleotide sequence also appeared to have had several deletions, an addition, or other substitutions, resulting in frame shifts that created an internal stop in the putative *Tri7* amino acid sequence. Due to these features, comparisons of the H-11 *Tri7* amino acid sequence with those of 88-1 and *F. sporotrichioides* could not be made. Finally, the most substantial differences between the H-11 *Tri7* gene and the other

TABLE 1. Primers used for PCR-based cloning

Primer no.	Primer designation	Sequence (5' to 3') ^b	Position (bp) ^c
1	Tri5/p1	AGATCGTACAGCTAAATTCAGAGC	15500–15523
2	Tri5/p2	CCACTAGCTCAATTGAACTTAGGA	17393–17370
3	Tri6/p1	TGGAGNGCNTTNCNCTNTTTGA	12663–12685
4	Tri6/p2	TTGTGTATNCGNCTATAGTGATC	13276–13254
5	Tri4/p2	TCNGTYITNCCNGDATNGC	9757–9776
5'	Tri4/2p2	GTCGATGTCGGCTTTAGTGGTGTC	9810–9833
6	Tri4/p1	GGNCCNTAYYTNNGARTTYTA	11293–11274
6'	Tri4/2p1	AGCCTTAGTCAATATCCCCATCAG	11431–11409
7	Tri3/p1	CAGCGCTATACAGATGGAAGTC	6823–6844
8	Tri3/p2	TCGGTATAGTTTGCATCATTGTAG	8487–8464
9	Tri7/p1	CGCATCGAAAGTGAAGGTT	4618–4637
9'	Tri7/p5	TGCCCTCCTGCCATCGTC	4750–4768
10	Tri7/p2	ATGAACCAAAATGATACCCAGATG	5823–5800
11	Tri11/p2	ACATTNAGNGGGCTTGTTC	21419–21440
12	Tri11/p1	ATGTTTCAATATTCNTTNTGGCC	23140–23118
13	Tri102/p2	TGGTGNGNACATGATCNGTNAGG	23890–23913
14	Tri102/p1	GACCTNGAATCNCAGCCNGACGAC	25661–25638
15	Itri5n/p1	ACTTGACTAGGTAGCCACGGTGTTTTATTC	15723–15694
16	Itri5n/p2	GAAAAGGTCAAGCATCAGGATACAGAGGA	17204–17232
17	Itri65/p1	GAAGTCAAAGTCAAGCAAACAAGTGG	12878–12852
18	Itri65/p2	GCCAAGCAAATGCCGTATCCCAGTT	12971–12996
19	Itri7n/p1	GAAACCGCGATAGGTAAGTACTGCTAAG	4837–4808
20	Itri7n/p2	GCCACAAAGCAAGACGGAGACGAAGGAG	5744–5771
21	Itri11n/p2	TAGCCGCAATTGGTACCAGGATAGCAG	21481–21455
22	Itri11n/p1	CCGCAGGGGATGAAAAAGAGGTTGTA	22979–23005
23	I2tri5/p2	GCGATGCTGCCAGGCTAAGGTTAAAG	14905–14880
24	I2tri5/p1	AACTAATTGTCTGTAACCTGAGCCTGTAACCA	17553–17584
25	Itri4nd/p2	CYGATTGGAGGAGAACACTTGATAACATTT	9936–9907
26	Itri4n/p1	GCTTTGGTCCCGGATCTTTTCGAGTG	11305–11331
26'	Itri4d/p1	GGACGATGGGGCTTGAGGATGTGATG	8425–8451 ^d
27	Itri8/p1	TTAATTTAGGCGAACAAAGAGACGGAAGACAGT	1191–1160
28	Itri8/p2	AGTATCGGTATCGCGTTTCATTCTGG	4817–4844
29	Itri102n/p5	ATTTTGGCCGGCTTTGTCTGCATCCTTCTC	24716–24688
30	Itri102nd/p1	TGAAGAACAAGTAGGATAGYGCAGACATT	25510–25538
31	Tri6nd/p2	CACGACCAGGAAGGACAACAATG	13197–13219
32	Tri6nd/p1	CTTGGCGGGATCGGGAGAC	12713–12695
33	Tri5n/p1	GGGGAGTTGTTAGGCTTGGATTG	14358–14335
34	Itri3nd/p1	TCCGAAGAGAAGTAGCGATGAARAGAT	6857–6931
35	Itri3d/p2	TCCGTTGAGAGCATAGACTTTGTTGTGAA	8322–8350
36	Itri53/p3	TGATCCTGATGCTTGACCTTTTC	17227–17204
37	Itri53/p2	CTTGCAAACTGAACAGGCGACGGTATG	18282–18309
38	Tri8/delp2	ACAGCGTGGACACAGCAACCCTTACTT	4203–4229
39	I53n/np3	TCGGCAACATGGCAACGGACTT	20553–20574
40	I11n/np4	GCTTTTCGCGCTGTTGCCGTAGG	20764–20742
41	8n/p2	AGATGAATTACCAGGCAGATGTCCAGAAGTCG	2567–2598
42	7d/p1	AGGCCGTGAAAATGACCCGCTTGATGTA	1963–1936 ^d
43	5d/np1	CACTACGAAAAGTCCGGCCATCACATA	12866–12840 ^d
44	7d/np1	TGTGCCGTGAGCCAGAAATGAAGC	2271–2247 ^d
45	I7d/p1	ACGGTGTACGAGTGGTTTGTGTCTATTCA	2363–2392 ^d
46	Itri102d/p4	TATCCCTCTTTCCTTGGTGACTACTGG	21988–22016 ^d
47	Tri5d/p4	TGGTTTGGGTCATTGTGTTGGGGCTTCT	14063–14090 ^d
48	I11d/np4	TGCTTTCATTAATTTTCGCTTTTC	17990–17967 ^d
48'	I11d/np2	CTTGCAACTTTAACGTGTCCAGAT	20533–20556 ^d
49	I4d/np3	ATGGCAAGTTATGGGTCAGC	9187–9207 ^d
50	Itri102nd/p2	GCTAATGTCTACTACTCGCTATGGCACTGG	21122–21092 ^d

^a Shown in Fig. 1.^b R = A or G; Y = C or T; D = A or G or T; N = A or C or G or T.^c According to the deposited sequence (GenBank accession no. AF336365; from *G. zeae* 88-1).^d According to the deposited sequence (GenBank accession no. AF336366; from *G. zeae* H-11).

two *Tri7* genes were found within a putative intron sequence. The *Tri7* ORFs of 88-1 and *F. sporotrichioides* were interrupted by a putative intron at the same position, identified from the consensus signals for intron splicing (5'-GTAAT~TAG-3'). The corresponding region of H-11 was, however, much longer (151 bp) than those of 88-1 and *F. sporotrichioides* (51 bp each).

This size difference was due to the presence of 10 tandem repeats of an 11-bp nucleotide stretch (CACAATATTAG) within that region of the H-11 *Tri7* sequence. These repeats were not present in either 88-1 or *F. sporotrichioides*.

Amplification of the inserted regions of *Tri7* genes from field isolates. Selected regions spanning the *Tri7* insertion from *G.*

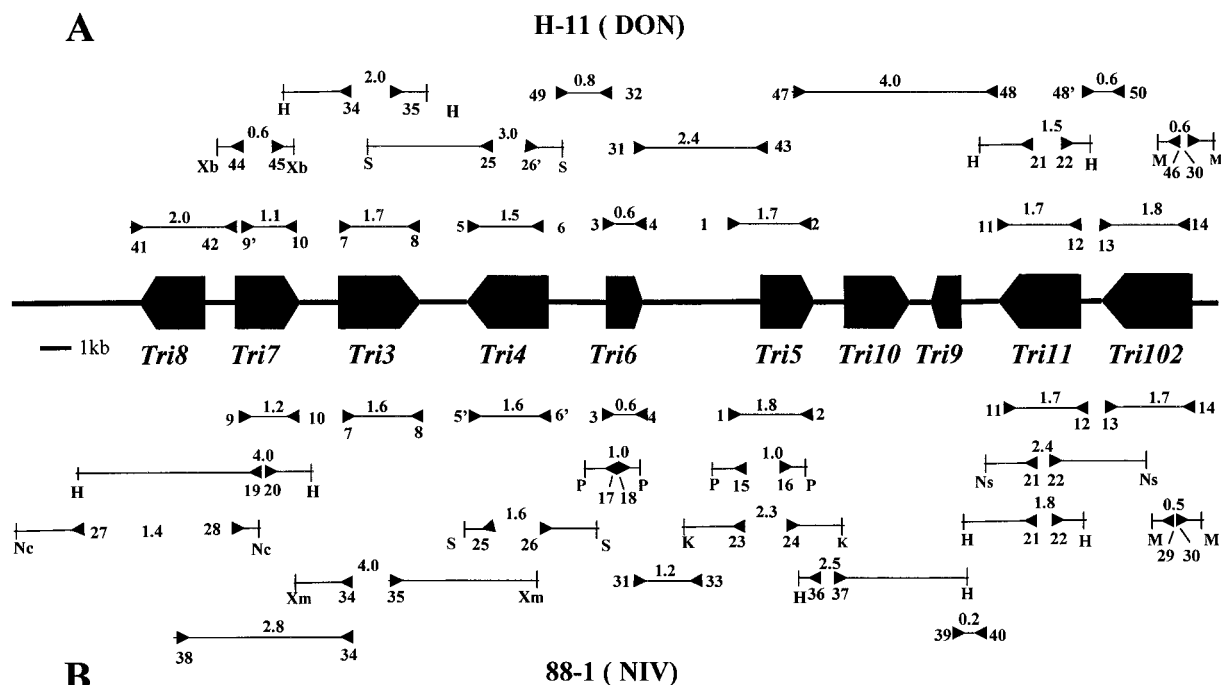


FIG. 1. PCR strategies for amplification of trichothecene biosynthesis gene clusters from *G. zeae* strains H-11 (A) and 88-1 (B). Block arrows indicate the locations of *Tri* ORFs and their transcription directions within the gene cluster. The connecting thick lines represent noncoding DNA regions. The inverted arrowheads indicate PCR primer locations and orientations. Numbers above the thin lines bounded by inverted arrowheads indicate the approximate sizes (in kilobases) of amplified PCR products. Numbers next to or below the inverted arrowheads indicate the primers used (see Table 1 for primer names and sequences), and the letters indicate restriction enzyme sites: H, *Hind*III; K, *Kpn*I; M, *Mse*I; Nc, *Nco*I; Ns, *Nsi*I; P, *Pst*I; S, *Spe*I; Xa, *Xba*I; Xm, *Xmn*I. Note that *Tri101* is not part of the cluster and is not shown here.

zeae field isolates were PCR amplified using primers GzTri7/f1 and GzTri7/r1. Amplification yielded products of various sizes from DON chemotypes but yielded products of only one size (~160 bp) from all NIV chemotypes tested (Fig. 2). The amplified DON chemotype products were of distinctly lower mobility than the NIV chemotype products on 5% polyacrylamide

gels. Direct sequencing of the PCR products revealed that the actual sizes of the products from DON chemotypes varied, ranging from 173 to 327 bp, depending on the number of 11-bp repeats within each sequence, whereas the products from NIV chemotypes were identical in size (161 bp) due to a lack of the repeat. The inserted repeats in the *Tri7* sequences varied from

TABLE 2. Sizes of *Tri* ORFs, introns, and noncoding regions in trichothecene biosynthesis gene clusters of *G. zeae* and *F. sporotrichoides*

Region(s)	No. of nucleotides (bp)/amino acids				Putative intron(s) (bp) ^a			
	88-1	H-11	<i>G. zeae</i> ^b	<i>F. sporotrichoides</i> ^b	88-1	H-11	<i>G. zeae</i> ^b	<i>F. sporotrichoides</i> ^b
8	1,335/445	1,338/446	NA ^c	1,344/447	0	0	NA	0
8-7	701/— ^d	681/—						
7	1,377/442	1,444/431	NA	1,250/398	51	151	NA	53
7-3	799/—	812/—						
3	1,763/512	1,768/512	NA	1,783/513	55, 49, 58, 65	56, 49, 58, 69	NA	56, 61, 59, 65 ^e
3-4	1,165/—	946/—						
4	1,742/521	1,742/521	NA	1,743/520	58, 52, 69	58, 52, 69	NA	58, 52, 70 ^e
4-6	1,171/—	1,168/—						
6	657/219	657/219	657/218	654/217	0	0	0	0
6-5	2,935/—	2,886/—						
5	1,180/376	1,187/376	1,187/375	1,185/374	52	59	59	60
11	1,741/493	1,741/493	1,741/492	1,722/492	57, 54, 78, 73	57, 54, 78, 73	57, 55, 82, 68	49, 54, 76, 64
11-102	649/—	632/—						
102	1,905/591	1,902/590	1,906/598	1,902/598	56, 76	56, 76	60, 76	56, 49 ^e
101	1,202/400 ^f	1,356/452	1,356/451	1,380/459	0	0	0	0

^a The order of introns is 5' to 3' in each ORF.

^b Sequences of noncoding DNA regions are not compared here. Refer to Table 3 for accession numbers of each sequence.

^c NA, not available.

^d —, no translation product from noncoding DNA regions.

^e Confirmed by cDNA sequencing.

^f Putative translation product from an incomplete ORF.

TABLE 3. Sequence homology of *G. zeae* and *F. sporotrichioides* *Tri* ORFs and noncoding regions^a

Region(s)	% Nucleotide (% amino acid) identity between ^b :				
	88-1 and H-11	88-1 and <i>F. sporotrichioides</i>	H-11 and <i>F. sporotrichioides</i>	88-1 and <i>G. zeae</i>	H-11 and <i>G. zeae</i>
8	88 (82)	81 (77)	81 (75)	— ^c	—
8-7	68	—	—	—	—
7	80 ^d	73 (72)	64 ^d	—	—
7-3	86	—	—	—	—
3	93 (96)	83 (85)	82 (84)	—	—
3-4	57	—	—	—	—
4	96 (96)	83 (85)	84 (88)	—	—
4-6	88	—	—	—	—
6	94 (95)	86 (86)	85 (85)	95 (95)	96 (96)
6-5	82	—	—	—	—
5	94 (97)	84 (90)	85 (91)	94 (97)	100 (100)
5-11	89	—	—	—	—
11	94 (97)	84 (91)	84 (91)	91 (94)	92 (94)
11-102	81	—	—	—	—
102	92 (89)	79 (75)	76 (74)	90 (85)	90 (85)
101	98 (99)	80 (78)	80 (77)	99 (100)	99 (99)

^a Homology is presented as percent identity at both the nucleotide and amino acid (in parentheses) levels for *Tri* genes and at the nucleotide level only for noncoding DNA regions. Percent identities were calculated from pairwise comparison of the sequences using the Martinez/Needleman-Wunsch method (for nucleotides) and the Lipman-Pearson alignment (for amino acids) in the DNASTAR program.

^b 88-1 is the NIV chemotype, and H-11 is the DON chemotype. *G. zeae* represents the sequences of other *G. zeae* strains from previous studies; GenBank accession numbers are AB017495 (*Tri6*), U22464 (*Tri5*), AB024617 (*Tri11* and *102*), and AB011417 (*Tri101*). The sequence of *Tri5* in *G. zeae* is from a North American strain, and the other sequences are from a Japanese strain. *F. sporotrichioides* GenBank accession numbers are U22463 (*Tri8*, *Tri7*, and *Tri3*), U22462 (*Tri4*), U22150 (*Tri6*), M27246 (*Tri5*), AF011355 (*Tri11* and *Tri12*), and AF127176 (*Tri101*).

^c —, not determined.

^d Homology at the amino acid level was not determined because the *Tri7* gene of H-11 was defective.

2 to 16 copies for the 50 DON-producing isolates (data not shown). This variation was not, however, related to their geographical origins.

DISCUSSION

We determined the sequence of the trichothecene biosynthesis gene cluster from DON and NIV-producing *G. zeae* isolates. We used these sequences to identify potential changes that might be responsible for differences in 8-ketotrichothecene biosynthesis.

Based on sequence differences, we think that the *Tri7* gene may be one of the elements responsible for the difference in trichothecene production between the two chemotypes, as either no *Tri7* protein or a truncated version is synthesized in H-11. These features are conserved in all of the DON isolates tested (data not shown), suggesting that the *Tri7* gene is non-functional in all DON chemotypes. To confirm the role of *Tri7* protein in *G. zeae* trichothecene biosynthesis, functional studies are needed. Previous experiments suggest that *Tri7* protein is required for acetylation of the hydroxyl group at C-4 of T-2 toxin produced by *F. sporotrichioides* (13, 26). If the function of *Tri7* in *G. zeae* is the same as in *F. sporotrichioides*, *Tri7* protein is not required for DON production but is required for acetylation of the hydroxyl group at C-4 of NIV to convert to 4-ANIV; NIV chemotypes usually produce both NIV and 4-ANIV. Recently, an additional homologue of oxygenase (*TriD*) was found immediately upstream of *Tri102* in both *F. sporotrichioides* and a DON-producing strain of *G. zeae*. However, sequence comparison showed that the *TriD* gene of the DON chemotype was defective (6). If *TriD* is functional in the NIV chemotypes as in *F. sporotrichioides*, both *TriD* and *Tri7* would be determinants for biosynthesis of NIV and 4-ANIV in *G. zeae*.

The presence of different numbers of repeats within the *Tri7* gene sequences of DON isolates suggests certain possibilities. The defective *Tri7* gene in DON chemotypes of *G. zeae* may no longer be under selection pressure. This lack of selection would allow mutations to accumulate. Another possible mechanism for the variable number of repeats among the DON isolates is unequal crossing over during sexual recombination (5). To test this possibility, it is necessary to evaluate ascospore progeny from *G. zeae* DON chemotypes and see if the number of repeats present in the progeny is the same as in the parents. The *Tri7* gene sequence could also be used as a



FIG. 2. PCR amplification patterns of the inserted regions of *Tri7* from DON- or NIV-producing isolates of *G. zeae* on a 5% polyacrylamide gel. Lanes 1 and 20, 100-bp DNA ladder; lane 2, 88-1 (NIV chemotype); lanes 3 to 7, Korean NIV chemotypes. Lanes 8 to 19 show DON chemotypes with various numbers of repeats (in parentheses): lane 8, Korean (2); lane 9, United States (3); lane 10, Korean (4); lane 11, United States (5); lane 12, Korean (6); lane 13, Korean (7); lane 14, United States (8); lane 15, United States (9); lane 16, Korean (10); lane 17, United States (11); lane 18, United States (13); lane 19, United States (16)

marker to assess genetic diversity among DON isolates as well as to distinguish the chemotypes of *G. zeae*.

O'Donnell et al. (30) estimated global genetic diversity of *G. zeae* using a molecular phylogenetic analysis with multiple-gene genealogies. They discovered seven lineages within 37 isolates of *G. zeae* from a worldwide collection and demonstrated that the trichothecene chemotypes were not lineage specific. However, no *Tri7* polymorphism was found in the Korean NIV-producing isolates in this study. These results are consistent with the hypothesis that there is a NIV chemotype-specific lineage in Korea, most likely lineage 6. In addition, the two Korean *G. zeae* chemotypes appear to be different lineages; 88-1 and H-11 were identified as lineages 6 and 7, respectively, based on sequence comparisons of *Tri101*. It is unclear whether the other Korean DON-producing isolates are lineage 7, as found for North American isolates. To confirm these results further studies are needed to determine the lineage(s) of Korean *G. zeae* isolates.

Differentiating chemotypes of *G. zeae* isolates is of great practical importance in several Asian countries, including Korea and Japan, where both chemotypes are common (15, 35, 36). Such a technique would also be useful in plant quarantine for North American countries, including the United States and Canada, where no NIV chemotypes have been detected (1, 27). The use of a PCR assay with *Tri7* primers would avoid the difficulties that arise from time-consuming and laborious chemical analyses of toxins. In this study, we demonstrated that a PCR assay with *Tri7* primers provides a rapid and reliable method for differentiating the two chemotypes of *G. zeae* from Korea and North America. If this PCR assay works on isolates from other locations, then it may be globally applicable. This assay also could be applicable for evaluating contamination of cereal samples with either DON- or NIV-producing isolates of *G. zeae*. This method, which is based on the size variation of PCR products, should be more reliable than other PCR-based detection systems that are based on the absence or presence of a PCR band and which may yield false-negative results. In addition, our studies of the nucleotide sequences of the *Tri* genes from the two chemotypes will allow development of a DNA probe hybridizing to different restriction fragment(s) of genomic DNA from each chemotype.

The sequence similarity in other *Tri* genes and noncoding regions between the two chemotypes of *G. zeae* most likely reflects their evolutionary divergence. However, the conservation of mutations, except for the number of repeats, within the *Tri7* genes of DON isolates from both Korea and the United States leads us to question whether all DON isolates in different lineages have a common ancestry. Future studies are needed to determine if the *Tri7* polymorphisms found in this study also are found in the other lineages.

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