**Tri13 and Tri7 Determine Deoxynivalenol- and Nivalenol-Producing Chemotypes of Gibberella zeae**

Theresa Lee,¹ You-Kyoung Han,¹ Kook-Hyung Kim,¹ Sung-Hwan Yun,² and Yin-Won Lee¹*

School of Agricultural Biotechnology and Research Center for New Bio-materials in Agriculture, Seoul National University, Suwon 441-744,¹ and Division of Life Sciences, Soonchunhyang University, Asan 336-745,² Korea

Received 10 December 2001/Accepted 11 February 2002

**Gibberella zeae**, a major cause of cereal scab, can be divided into two chemotypes based on production of the 8-ketotrichothecenes deoxynivalenol (DON) and nivalenol (NIV). We cloned and sequenced a Tri13 homolog from each chemotype. The Tri13 from a NIV chemotype strain (88-1) is located in the trichothecene gene cluster and carries an open reading frame similar to that of Fusarium sporotrichioides, whereas the Tri13 from a DON chemotype strain (H-11) carries several mutations. To confirm the roles of the Tri13 and Tri7 genes in trichothecene production by G. zeae, we genetically altered toxin production in 88-1 and H-11. In transgenic strains, the targeted deletion of Tri13 from the genome of 88-1 caused production of DON rather than NIV. Heterologous expression of the 88-1 Tri13 gene alone or in combination with the 88-1 Tri7 gene conferred on H-11 the ability to synthesize NIV; in the latter case, 4-acetylnivalenol (4-ANIV) also was produced. These results suggest that Tri13 and Tri7 are required for oxygenation and acetylation of the oxygen at C-4 during synthesis of NIV and 4-ANIV in G. zeae. These functional analyses of the Tri13 and Tri7 genes provide the first clear evidence for the genetic basis of the DON and NIV chemotypes in G. zeae.

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**Materials and Methods**

Strains, media, and culture conditions. *G. zeae* strains H-11 (a DON producer) and 88-1 (a NIV producer) were described previously (22) and were used...
Plasmid construction. For insertion of Tri7 from 88-1 into the genome of H-11, a plasmid containing an intact copy of Tri7 was prepared. Tri7 was amplified from 88-1 genomic DNA by using primers Ntri7/p1 and Ntri7/p2 and cloned into pCR2.1TOPO as described above. The Tri7 insertion vector pTri7/p1 (9.4 kb) was then created by subcloning a 2.1-kb hygromycin B resistance gene (HygB) taken from pBcATH into the XbaI site of the pCR2.1TOPO-Tri7 vector described above. For deletion of Tri7 from the genome of 88-1 via double crossover, a plasmid harboring a 3.4-kb fragment carrying the 5' and 3' flanking sequences of the 88-1 Tri7 ORF was constructed. This fragment, which carried 0.9 and 2.5 kb of the 5' and 3' flanking sequences, was obtained by inverse PCR with primers Tri7/deln/delp1 and Tri7/deln/delp2, using NheI-digested and self-ligated 88-1 genomic DNA as a template. The inverse PCR product was cloned into pCR2.1TOPO along with the HygB gene, creating a 9.4-kb plasmid designated pdelN7H.

Plasmids used for insertion or deletion of the 88-1 Tri13 gene were prepared by the same strategies described above for Tri7. For an insertion plasmid, primers NWTri13/p1 and NWTri13/p2 were used to amplify the entire Tri13 ORF from 88-1 genomic DNA. The 3.6-kb amplified product was cloned into pCR2.1TOPO and then removed from the cloning vector by digestion with EcoRI. This EcoRI fragment was subcloned into an EcoRI site of pBCGT, which contains a gene conferring resistance to Geneticin (GenR), to create the 9.8-kb plasmid pNtri13G. To create a deletion plasmid, 88-1 genomic DNA was digested with EcoRI, self-ligated, and used as a template for inverse PCR with primers delTri13/p1 and delTri13/p2, each including a BgII recognition sequence. The PCR amplified a 2.0-kb fragment carrying the 5' and 3' flanking sequences of the 88-1 Tri13 gene. The amplified product was cleaned by phenol extraction, digested with BgII, and ligated into BgII-digested pNfri1-1 containing GenR and no EcoRI site. The resulting 7.3-kb plasmid was designated pdelN13G (Table 1).

Fungal transformation. For sporation, mycelial plugs of each strain were inoculated into CMC liquid medium (15 g of carboxymethyl cellulose, 1 g of yeast extract, 0.5 g of MgSO4, 1 g of NH4NO3, and 1 g of KH2PO4 per liter) at 25°C with shaking (100 rpm) for 3 days. Fungal conidia produced in CMC culture were inoculated into 100 ml of YPG liquid medium (3 g of yeast extract, 10 g of peptone, and 20 g of glucose per liter) at 30°C and grown for 12 h with shaking at 25°C. Mycelia were harvested by filtration through sterile Whatman no. 2 filter paper and incubated in 80 ml of 1 M NH4Cl containing Driselase (10 mg/ml) (Interspec Products, Inc., San Mateo, Calif.) to generate protoplasts. Further steps in transformation were as previously described (45). Each transformant was transferred to fresh potato dextrose agar medium amended with the desired antibiotics and purified by single-conidium isolation. For insertion of the 88-1 Tri7 or Tri13 gene, circular pNTri7H or pNtri13G was transformed into protoplasts of H-11. For gene deletions, plasmids pdelN7H and pdelN13G were linearized by digestion with NheI and EcoRI, respectively, prior to transformation.

Trichothecene analysis. Transgenic and wild-type strains of G. zeae were screened for trichothecene production on rice medium. Rice cultures were harvested after 3 weeks of incubation at 25°C and extracted as previously described (36). A portion of each extract was reacted with trimethylsilylating reagent and analyzed with a JEOL JMS-AX 505 gas chromatograph-mass spectrometer in full-scan mode using a DB-5 fused silica column (0.25 mm [inside diameter] by 30 m; 0.25-µm film) (J & W Scientific, Folsom, Calif.). The column temperature was maintained at 120°C for 5 min and then increased to 270°C at 5°C per min.

## TABLE 1. PCR primers and plasmids used in this study

<table>
<thead>
<tr>
<th>Primer or plasmid</th>
<th>GenBank accession no. (bp)</th>
</tr>
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<tbody>
<tr>
<td>Ntri7/p1</td>
<td>AF336365 (3362–3385)</td>
</tr>
<tr>
<td>Ntri7/p2</td>
<td>AF336365 (6734–6707)</td>
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</tr>
<tr>
<td>Tri13/p1</td>
<td>AY064209 (853–8530)</td>
</tr>
<tr>
<td>Tri13/p2</td>
<td>AY064209 (2505–2528)</td>
</tr>
</tbody>
</table>

**a** AmpR, resistant to ampicillin; KanR, resistant to kanamycin; Chra, resistant to chloramphenicol.

**b** The number in parentheses indicates the primer position in the deposited sequence.

**c** A BgII recognition site (AGACT) was added to each 5' end.
The injector, ion source, and interface temperatures were 280, 200, and 250°C, respectively. The ionizing voltage was 70 eV. The injector, ion source, and interface temperatures were 280, 200, and 250°C, respectively. The ionizing voltage was 70 eV.

Nucleotide sequence accession numbers. The sequences of the \textit{Tri13} genes obtained from \textit{G. zeae} 88-1 and H-11 have been deposited in GenBank under accession numbers AF330109 and AF330108, respectively.

\section*{RESULTS}

\section*{Comparative sequence analysis of \textit{Tri13}.} The putative ORF of a \textit{Tri13} homolog from \textit{G. zeae} strain 88-1 was identified by sequence comparison with the cDNA sequence of \textit{Tri13} from \textit{F. sporotrichioides} (GenBank accession number AF330109). The \textit{Tri13} ORF is located in the trichothecene gene cluster of \textit{G. zeae} 88-1 immediately upstream of \textit{Tri12}, as it is in \textit{F. sporotrichioides}. This 1,853-bp ORF is interrupted once by a putative intron of 62 bp. The \textit{Tri13} genes from 88-1 and \textit{F. sporotrichioides} are 78 and 80\% identical at the nucleotide and amino acid levels, respectively; both exhibit similarities to a putative cytochrome P450 monooxygenase. Seventeen amino acids at the N terminus of the \textit{F. sporotrichioides} \textit{Tri13} protein are missing in the corresponding region of the putative \textit{Tri13} protein of 88-1 (data not shown).

\textit{G. zeae} strain H-11 carries a \textit{Tri13} homolog that is strikingly different from the \textit{Tri13} homologs of 88-1 and \textit{F. sporotrichioides}. The H-11 \textit{Tri13} gene is only 65 and 61\% identical to the \textit{Tri13} genes from \textit{G. zeae} 88-1 and \textit{F. sporotrichioides}, respectively. In addition, alignment of these nucleotide sequences reveals many alterations present only in the H-11 gene. This gene appears to have incurred several substitutions, insertions, and deletions, causing a deficient translation start and frameshifts in the putative \textit{TRI13} amino acid sequence. Nucleotide alignment of \textit{Tri13} fragments amplified from genomic DNAs of other \textit{G. zeae} isolates revealed that these features found in H-11 \textit{Tri13} are highly conserved among DON-producing isolates of the \textit{G. zeae} strains tested from Korea and the United States (data not shown).

\textbf{Molecular manipulations of \textit{Tri7} and \textit{Tri13}.} (i) Deletion of either the \textit{Tri7} or \textit{Tri13} ORF from the \textit{G. zeae} genome. The region containing the entire \textit{Tri7} ORF or an internal portion of the \textit{Tri13} ORF in the genome of strain 88-1 was deleted by targeted gene replacement using linearized pdelN7H and pdelN13G, respectively, via double crossover between homologous regions (Fig. 1A and B). The desired transfectants sustaining a deletion of either \textit{Tri7} or \textit{Tri13} were identified by gel blot analysis (Fig. 1C and D). In transformants \textit{Tri13} ORF but carried a 520-bp deletion of the \textit{Tri13} ORF but carried a 520-bp deletion of the \textit{Tri13} ORF. In transformants \textit{Tri13} ORF and \textit{Tri13} ORF, which was replaced with the vector pdelN13G (Fig. 1B and D).
(ii) Heterologous expression of intact Tri7 and/or Tri13 ORFs. The DON-producing H-11 strain of G. zeae was transformed with the circular vectors pNTri7H and pNTri13G, either singly or in sequential combination (Table 1). Vector pNTri7H carries the Tri7 ORF from 88-1 and the HygBR gene; vector pNTri13G carries the Tri13 ORF from 88-1 and the GenR gene. The resulting HygBR GenR, HygBR GenN, or HygBR GenR transformants were purified by single-conidium isolation, and integration events were examined by gel blot analysis.

The probe, a 3.6-kb EcoRI fragment from pNTri13G carrying the 88-1 Tri13 ORF, hybridized with genomic DNAs from all HygBR GenR transformants tested. In EcoRI-digested genomic DNAs of transformants TxN13-2 and TxN13-3, the 3.6-kb fragment of the heterologous Tri13 ORF hybridized along with a 2.5-kb fragment of the native Tri13, indicating that the intact 88-1 Tri13 ORF integrated at an ectopic site of the recipient genome (Fig. 2A). In EcoRI-digested genomic DNAs from all HygBR GenN transformants tested, a 3.4-kb probe carrying the 88-1 Tri7 ORF hybridized with a single 3.6-kb fragment of the heterologous Tri7 but not with a native Tri7, probably because the probe shared only 80% nucleotide identity with the native Tri7 (data not shown). The same probe identified HygBR GenN transformants carrying both intact 88-1 Tri7 and Tri13 ORFs at ectopic sites that were created by retransformation of transformant TxN13-2 with pNTri7H (Fig. 2B).

Trichothecene production by transgenic strains. (i) Transgenic G. zeae 88-1 strains with either Tri7 or Tri13 deleted. G. zeae 88-1 produced NIV and 4-ANIV in rice cultures (Fig. 3A), whereas all of the transgenic 88-1 strains tested that had sustained deletions of the Tri7 ORF produced NIV but no 4-ANIV (Fig. 3C). In contrast, other 88-1 strains resulting from ectopic integration of the transforming vector (pdelN7H) produced NIV and 4-ANIV similarly to the wild-type strain (88-1) (Fig. 3D).

Transgenic strains of 88-1 with targeted deletions of the Tri13 ORF from the 88-1 genome exhibited more dramatic changes in trichothecene production. All of the transgenic strains tested that had sustained deletions of the Tri13 ORF produced neither NIV nor 4-ANIV. Instead, they produced DON (Fig. 3E). Other strains resulting from ectopic integrations of pdelN13G produced NIV and 4-ANIV similarly to wild-type 88-1 (Fig. 3F).

(ii) Transgenic G. zeae H-11 strains carrying heterologous Tri7 and/or Tri13 ORFs. G. zeae H-11 produced DON and 15-ADON in rice cultures (Fig. 3B). Transgenic G. zeae strains of H-11 carrying intact copies of the 88-1 Tri7 ORF produced DON and 15-ADON similarly to wild-type H-11 (data not shown). However, heterologous expression of the 88-1 Tri13 ORF in the genetic background of H-11 caused changes in trichothecene production. GC-MS analysis showed that transgenic H-11 strains carrying the 88-1 Tri13 ORF produced only NIV; neither 4-ANIV nor DON was produced (Fig. 3G). Furthermore, transgenic H-11 strains carrying intact copies of both the 88-1 Tri7 and Tri13 ORFs produced both NIV and 4-ANIV (Fig. 3H).

DISCUSSION

We located the Tri13 gene in the trichothecene gene cluster and found that the gene differs drastically between two chemotypes of G. zeae. In G. zeae strain H-11, several mutations are present in the nucleotide sequence of Tri13. These mutations were found in all DON-producing G. zeae field isolates tested, suggesting that the lack of a viable Tri13 gene is specific to the DON chemotype. Taken together with previous results concerning Tri7 (22), these results suggest that both the Tri7 and Tri13 genes are nonfunctional in all DON chemotypes. In a previous experiment, disruption of Tri7 in F. sporotrichioides caused accumulation of HT-2 toxin rather than T-2 toxin in fungal liquid culture, suggesting that Tri7 is required for acet-
FIG. 3. Total ion chromatograms of extracts of *G. zeae* cultures. (A) Wild-type 88-1; (B) wild-type H-11; (C) transgenic 88-1 with *Tri7* deleted (TxNΔ7-1); (D) transgenic 88-1 carrying an ectopic integration of pdelN7H (TxNΔ7-6); (E) transgenic 88-1 with *Tri13* deleted (TxNΔ13-1); (F) transgenic 88-1 carrying an ectopic integration of pdelN13G (TxNΔ13-4); (G) transgenic H-11 carrying *Tri13* from 88-1 (TxN13-2); (H) transgenic H-11 carrying both *Tri13* and *Tri7* from 88-1 (TxN713-1).

The functions of *Tri7* and *Tri13* in trichothecene production by *G. zeae* have not yet been conclusively determined. To confirm their functions, we employed molecular manipulations, including gene deletion and insertions, as described in this study. When the *Tri13* gene in the NIV-producing *G. zeae* strain 88-1 was deleted, DON, instead of NIV, was detected in rice cultures. This result indicates that the *Tri13* protein is responsible for the oxygenation at C-4 during synthesis of NIV. In addition, heterologous expression of the functional copy of *Tri13* in the genetic background of the DON chemotype H-11 showed that *Tri13* is sufficient for conversion from DON production to NIV production in transgenic H-11 strains.

The same molecular strategies were used to confirm that the *G. zeae* *TRI7* protein is involved in acetylation of the oxygen at C-4 of NIV, as in *F. sporotrichioides* (5). Heterologous expression of both the *Tri13* and *Tri7* genes in H-11 caused production of both NIV and 4-ANIV. Therefore, these functional analyses have confirmed that the *Tri13* gene is the determinant for the DON-NIV switching in *G. zeae* and that the *Tri7* gene is responsible for further modification of NIV. Further confirmation of the enzymatic activities of these proteins and their roles in trichothecene biosynthesis awaits detailed biochemical studies.

Transgenic strains produced toxins in quantities similar to those produced by recipient strains, although the kinds of trichothecenes were switched with respect to each other. H-11 produced approximately 5 times more trichothecenes than did 88-1. Transgenic H-11 carrying the 88-1 *Tri13* gene produced more NIV than did wild-type 88-1. The amount of DON produced by transgenic 88-1 with *Tri13* deleted was much less than that produced by the wild-type H-11. Studies of field isolates also revealed higher levels of DON production by DON chemotypes than of NIV production by NIV chemotypes (36). This difference may be attributed to differences in regulatory factors such as the *Tri6* and *Tri10* genes (34, 39) and/or in another quantitative genetic element(s).

In addition to functional studies, the transgenic strains created in this study will be useful in evaluating the relative contributions of the two types of trichothecenes in *G. zeae* pathogenesis toward cereals. Previous studies using a DON-deficient *G. zeae* mutant showed that DON was responsible for reduced virulence by *G. zeae* toward wheat (29, 33). However, the role of NIV in the virulence of *G. zeae* has not been quantitatively analyzed, although NIV is known to be less phytotoxic than DON (11). Combinations of isogenic strains differing only in trichothecene production, such as 88-1 and a transgenic 88-1 *Tri13* deletion strain or H-11 and a transgenic H-11 strain carrying the 88-1 *Tri13* gene, would be appropriate for these studies.

All DON chemotype isolates of *G. zeae* examined thus far carry defective sequences for both *Tri17* and *Tri13*, which raises two questions regarding the population of *G. zeae* in Korea. First, is the presence of both defective genes in the trichothecene gene clusters common to all DON-producing isolates? Based on our functional studies, which suggest that *Tri7* is not directly involved in DON-NIV switching, we can expect to find NIV- and DON-producing isolates of *G. zeae* that carry functional *Tri13* but not *Tri7* genes and functional *Tri7* but not *Tri13* genes, respectively. In the former case, the isolate should not be able to produce 4-ANIV because it has a defective *Tri7* gene. The latter case may be rare, because a functional *Tri7* gene may be dispensable, and thus mutations could accumulate. A PCR assay using primers derived from *Tri7* (22) and *Tri13* designed to reveal polymorphisms between the two che-
motypes would be useful in testing these possibilities. This PCR assay would also provide a more reliable method to determine the chemotypes of G. zeae field isolates.

The second question is whether polymorphisms in Tri7 and Tri13 between DON and NIV chemotypes reflect the genetic diversity of Korean G. zeae populations. No polymorphisms were found in the gene clusters including Tri7 and Tri13 among the Korean NIV-producing isolates tested (unpublished data). In contrast, in the DON-producing isolates, the gene clusters differed at the two Tri genes. Significant conservation of this structural difference between the Tri gene clusters in the two chemotypes would suggest the presence of chemotype-specific lineages in Korea. According to the description by O’Donnell et al. (32), a preliminary study showed that Korean G. zeae populations from barley were dominated by a single lineage (lineage 6) and that those from maize were dominated by lineage 7, but lineage 3 was a relatively common component (K. A. Zeller, J. L. Vargas, Y.-W. Lee, R. L. Bowden, and J. F. Leslie, Abstr. National Fusarium Head Blight Forum, abstr. 163, 2001). It is likely that at least lineages 6 and 7 in Korean populations are specific to the NIV and DON chemotypes, respectively. Confirmation of this hypothesis will require a phylogenetic study of Korean G. zeae populations.

ACKNOWLEDGMENTS

This study was supported by a grant (M1-01-KG-0001-01-K07-01-028-1-0) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Science and Technology and by a grant (2000-2-22100-004-3) from the Korean Science and Engineering Foundation. T. L. and Y. K. H. were supported by postdoctoral and graduate fellowships, respectively, from the Korean Science and Engineering Foundation. T. L. and Y. K. H. were supported by postdoctoral and graduate fellowships, respectively, from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Science and Technology and by a grant (2000-2-22100-004-3) from the Korean Science and Engineering Foundation. T. L. and Y. K. H. were supported by postdoctoral and graduate fellowships, respectively, from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Science and Technology and by a grant (2000-2-22100-004-3) from the Korean Science and Engineering Foundation.