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Note

Expression in Cereal Plants of Genes That Inactivate *Fusarium* Mycotoxins

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Trichothecene 3-*O*-acetyltransferase (encoded by *Tri101*) inactivates the virulence factor of the cereal pathogen *Fusarium graminearum*. Zearalenone hydrolase (encoded by *zhd101*) detoxifies the oestrogenic mycotoxin produced by the same pathogen. These genes were introduced into a model monocotyledon rice plant to evaluate their usefulness for decontamination of mycotoxins. The strong and constitutive rice *Act1* promoter did not cause accumulation of TRI101 protein in transgenic rice plants. In contrast, the same promoter was suitable for transgenic production of ZHD101 protein; so far, five promising T_0 plants have been generated. Low transgenic expression of *Tri101* was suggested to be increased by addition of an Ω enhancer sequence upstream of the start codon.

Key words: Fusarium head blight; mycotoxin and phytotoxin; transgenic cereals; trichothecene resistance; zearalenone detoxification

Trichothecenes are a group of sesquiterpenoid secondary metabolites that inhibit protein synthesis in eukaryotes. They are produced by phytopathogenic *Fusarium* species (*e.g.*, *F. graminearum*) that cause head blight (FHB) of wheat, barley, and maize. The fungus also produces another mycotoxin, zearalenone, that shows potent oestrogenicity in humans and livestock. Since the mold-infected cereal plants accumulate much trichothecenes and zearalenone in grains, FHB poses threats of mycotoxin contamination of agricultural products.

In addition to their toxic effects on animals, trichothecenes are also known as a virulence factor in host-parasite interactions.[1,2] It is expected that transgenic expression of *Tri101* (*i.e.*, the 3-*O*-acetyltransferase gene responsible for self-protection of the trichothecene-producing fungi) reduces the disease severity of susceptible cereals by inactivation of this virulence factor.[3–6] In a model dicotyledonous tobacco plant, *Tri101* was expressed at a high level and consequently protected the transgenic lines from the phytotoxic effects of trichothecene 4,15-diacetoxyscirpenol (DAS).[7] In addition to *Tri101*, we have recently isolated the zearalenone detoxifying gene *zhd101* from a fungus, *Clonostachys rosea*.[8] As a first step towards FHB resistance and mycotoxin decontamination in susceptible cereals, we attempted to evaluate the usefulness of these mycotoxin inactivating genes.

For expression of the trichothecene resistance gene, two vectors were constructed (Fig. 1A: top and middle): (1) pAct1-*Tri101*, in which the resistance gene is placed under constitutive and powerful rice *Act1* promoter-intron[9] and (2) pAct1-*spTri101*, which contains an N-terminal signal peptide sequence of a wheat acidic β-1,3-glucanase gene (*Glb3*; AF112967)[10] fused to 5′ end of *Tri101*. Recombinant TRI101 was purified from *Escherichia coli* DE3 (BL21) carrying pET-19b*Tri101*[3] using a Hi Trap Chelating HP Column (Amersham Biosciences). Purified His-tagged TRI101 protein was used to prepare the rabbit polyclonal antibodies. For expression of the zearalenone detoxfying gene, we constructed pAct1-*egfp::zhd101* to facilitate monitoring of the ZHD101 fusion protein production by green fluorescence (Fig. 1A: bottom). The fusion gene expressed

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Abbreviations: 3-ADON, 3-*O*-acetyldeoxynivalenol; DAS, diacetoxyscirpenol; DIG, digoxigenin; DON, deoxynivalenol; FHB, Fusarium head blight; RT, reverse transcription

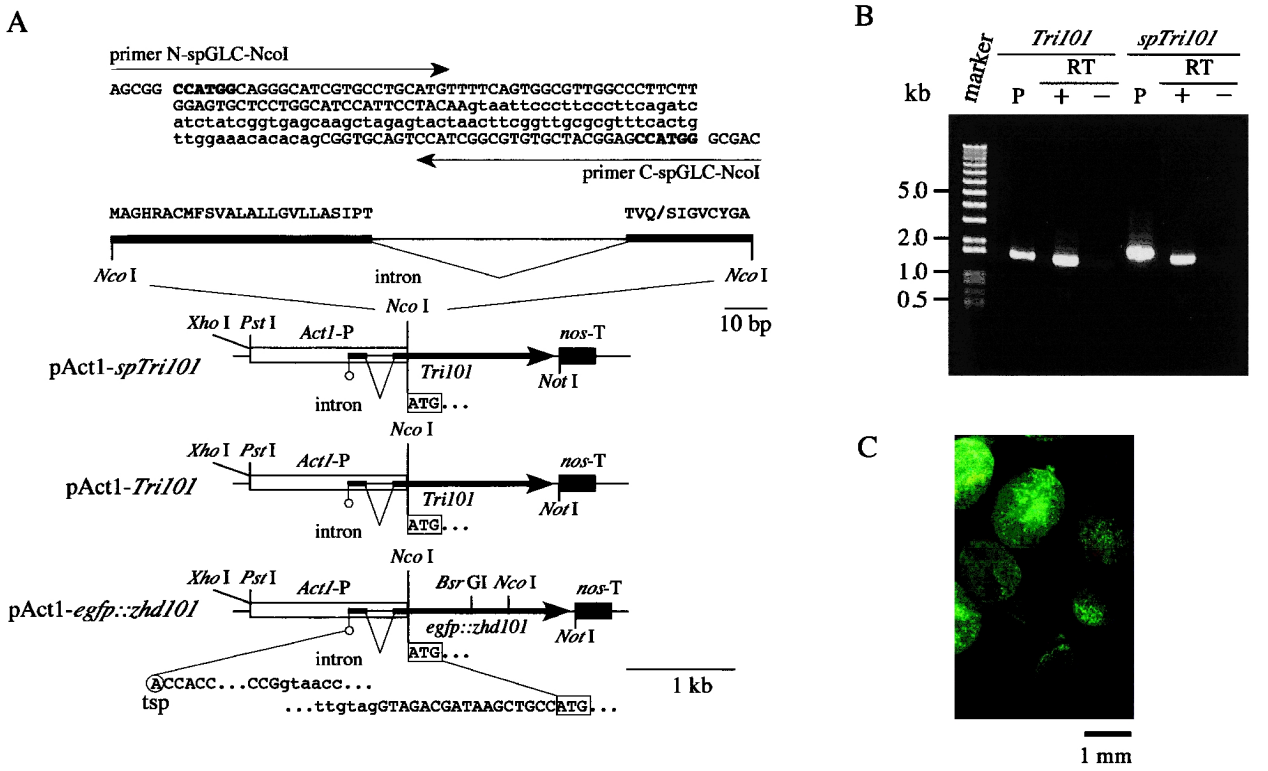

Fig. 1. Vectors Used for the Mycotoxin Inactivation and Their Transient Expression in Wheat Calli.

(A) Trichothecene resistance and zearalenone detoxifying vectors. Rice *Act1* promoter-first intron segments (*empty box*) are labeled as "*Act1*-P" and nopaline synthase terminator (*closed box*) as "*nos*-T". The *Act1* non-coding first exon/intron sequence enhances transcription of the fused downstream gene in monocotyledonous systems. Open circles indicate the reported transcription start point (*tsp*) of the *Act1* gene.[9] Translation start codons are boxed. Top: Structure of pAct1-*spTri101*. Genomic DNA sequence of a signal peptide from wheat acidic β-glucanase (*Glb3*)[10] is shown above the vector map. Large and small letters (which corresponds to thick and thin lines) represent exons and introns, respectively. The translated signal peptide sequence is shown above the thick lines and the processing site is indicated by a slash. Primers N-spGLC-NcoI and C-spGLC-NcoI (marked by arrows) were used for amplification of the *Glb3* signal sequence from wheat genomic DNA (*i.e.*, the signal peptide portion contains an intron derived from *Glb3*). *Nco* I recognition sequences (shown in *bold letters*) were created on both sides of the amplified fragment. This PCR product was inserted at the *Nco* I site of pAct1-*Tri101*. Middle: Structure of pAct1-*Tri101*. The internal *Nco* I site of *Tri101* was eliminated by a synonymous A to T mutation at position 296 (AB000874) by site directed mutagenesis. Bottom: Structure of pAct1-*egfp::zhd101*. The *Bsr* GI-*Not* I segment of pEGFP-1 (Clontech) downstream of *egfp* was replaced with a synthetic *Srf* I polylinker comprising of 5′-GTACAGGCCCGGGCCGC-3′ and 5′-GGCCGCGGCCCGGGCCT-3′ to generate pEGFP-SrfI. Vent DNA polymerase (New England Biolabs) was used for amplification of *zhd101* with primers GFP-zhd-5′ (5′-ATGCGCACTCGCAGCACAATCTCG-3′) and GFP-zhd-3′ (5′-TGTACCGTTCAAAGATGCTTCTGC-3′), and the amplified fragment was inserted at the *Srf* I site of pEGFP-SrfI in the correct orientation to generate pEGFP-*zhd101*. *Tri101* in pAct1-*Tri101* was replaced with the *Nco* I-*Not* I fragment of *egfp::zhd101* fusion in pEGFP-*zhd101*. (B) Transient expression of *Tri101* and *spTri101* in wheat callus cells as assessed by RT-PCR. Two μg of vector was precipitated on 0.6 mg of gold particles and used for each bombardment. Total RNA was isolated two days after the bombardment and the synthesized cDNA was used for RT-PCR. "P" indicates amplification directly from the bombarded vector. DNA size markers (1kb Plus DNA Ladder, Invitrogen) are indicated on the left. (C) Transient expression of *egfp::zhd101* in the bombarded calli monitored by fluorescence stereomicroscope (Leica MZ12). Filter sets GFP-Plus (460–500 nm excitation, 505 nm emission, and > 510 nm emission barrier) were used.

in *E. coli* retained the ability to cleave the lactone ring of zearalenone as assessed by an enzyme assay *in vitro* (data not shown).

To check the performance of these vectors, expression of the genes was evaluated by transient assays before the generation of transgenic cereals. Using the PDS-1000/He particle delivery system (Bio-Rad Laboratories), the immature-embryo-scutellum-derived calli of wheat (*Triticum aestivum* cv 'Norin 61') were bombarded with the vectors. Two days after the bombardment, total RNA was isolated with the RNeasy Plant Mini kit (Qiagen). Oligo(dT) primed cDNA was synthesized with the Superscript First-strand Synthesis System (Invitrogen) and used for reverse transcription (RT)-PCR. Primer Tri101RT (5′-CTTCAATCGGTCCATATCCTCATCCCTCAGAG-3′) was used in pair with primer Tri101FT (5′-ATGGCTTTCAAGATACAGCTCGACACCCTCGGC-3′) and N-spGLC-NcoI (5′-AGCGGCCATGGCAGGGCATCGTGCCTGCATG-3′) for amplifications of *Tri101* and *spTri101*, respectively. Although TRI101 protein was not detected on a Western blot (data not shown), the corresponding cDNA was amplified by RT-PCR (Fig. 1B). Proper

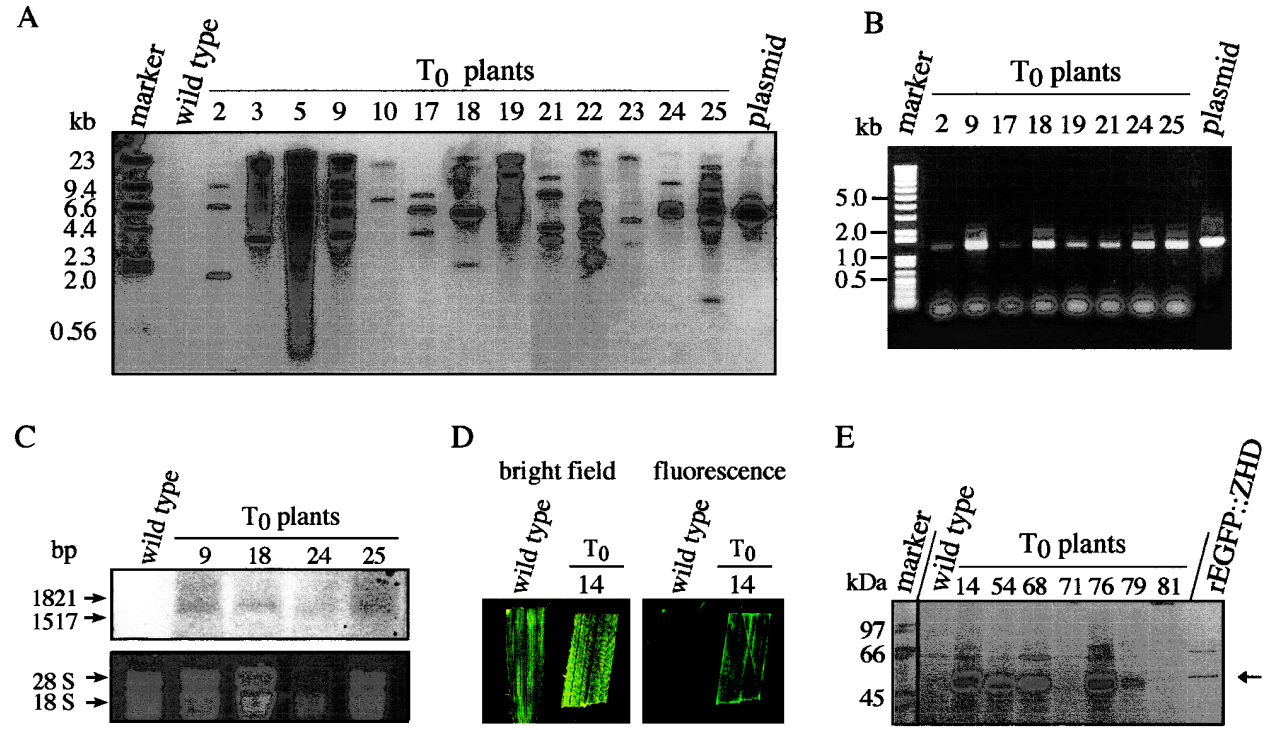

Fig. 2. Analyses of Transgenic Rice Plants Transformed with Mycotoxin Inactivation Genes.

(A) Southern blot analysis of thirteen T0 lines with *spTri101*. The probe was prepared by PCR using the DIG PCR-DNA labeling kit (Roche) with primers Tri101FT and Tri101RT. Each lane contains ten μg of DNA digested with *Pst* I. (B) RT-PCR analysis of eight T0 lines with *spTri101*. The amplified cDNA products (2, 9, 17, 18, 19, 21, 24, and 25) were smaller in size than a control product (plasmid) amplified directly from pAct1-*spTri101*. Control reactions without RT did not provide amplified PCR products (data not shown). DNA size markers are shown on the left. (C) Northern blot analysis of four T0 lines with *Tri101*. Ten-μg portions of total RNA were put on each lane. (D) Stereomicroscopic observation of detached leaves from non-transformed (wild type) and transgenic *egfp::zhd101* (No. 14) T0 plants (more than 5 months after the bombardment) under bright field (left) and fluorescence (right) conditions. Filter sets GFP-Plant (450–490 nm excitation, 495 nm emission, and 500–550 nm emission barrier) were used. (E) Western blot analysis of transgenic T0 plants expressing *egfp::zhd101* (line numbers shown) and recombinant EGFP::ZHD101 protein (rEGFP::ZHD). The leaf extracts of five transgenic plants (No. 14, 54, 68, 76, and 79) revealed expected size of bands (marked by an arrow) on the blot.

processing of the genes (*i.e.*, the absence of cryptic splicing) was confirmed by sequencing of the amplified cDNA products. The results suggest that the *Tri101* sequence does not have undesirable features, at least for transcription in wheat and other cereal plants. Similar conclusions were obtained for *zhd101* by this transient assay (data not shown). In addition to the detection of the mRNA by RT-PCR, production of EGFP::ZHD101 fusion protein was directly confirmed by visual monitoring of the green fluorescence in the bombarded calli (Fig. 1C).

Based on the above results, we next introduced each vector into a model monocotyledon rice plant (*Oryza sativa* L. *japonica* cv 'Nipponbare') by co-transformation with a bialaphos resistant vector pDM302.[11] Using the biolistic method,[11] we have generated 300 candidate *Tri101*/*spTri101* T0 plants resulting from fifteen independent transformation events and seven *egfp::zhd101* T0 plants resulting from six events. The expression levels of the fungal transgenes in cereals were subsequently analyzed.

To facilitate screening of trichothecene-resistant lines, we assayed the leaf protein extracts by Western blot analysis in search for TRI101 protein. However, the screening failed to provide evidence of TRI101 protein production with all these transgenic plants. To gain insight into the molecular basis of such inefficient gene expression in the T0 plants, we analyzed thirteen independent *spTri101* lines. Genomic DNA was isolated from the leaves using Nucleon PhytoPure (Amersham Biosciences), digested with *Pst* I that cut once upstream of the *Act1* promoter, and hybridized with the digoxigenin (DIG)-labeled *Tri101* probe on a Southern blot. As shown in Fig. 2A, *Tri101* proved to occur with different integration patterns in the genome of these transgenic lines. Among these thirteen independent lines, eight (lines 2, 9, 17, 18, 19, 21, 24, and 25) accumulated *spTri101* mRNA to a level detectable by RT-PCR; fragments of expected size (*i.e.*, 1.5 kb) were amplified with primers N-spGLC-NcoI and Tri101RT (Fig. 2B). DIG-labeled *Tri101* riboprobe was prepared by using the DIG RNA labeling kit (Roche) and the transcript level was further examined. As shown in Fig. 2C, four of the eight lines (lines 9, 18, 24, and 25) had signals of weak to moderate intensity

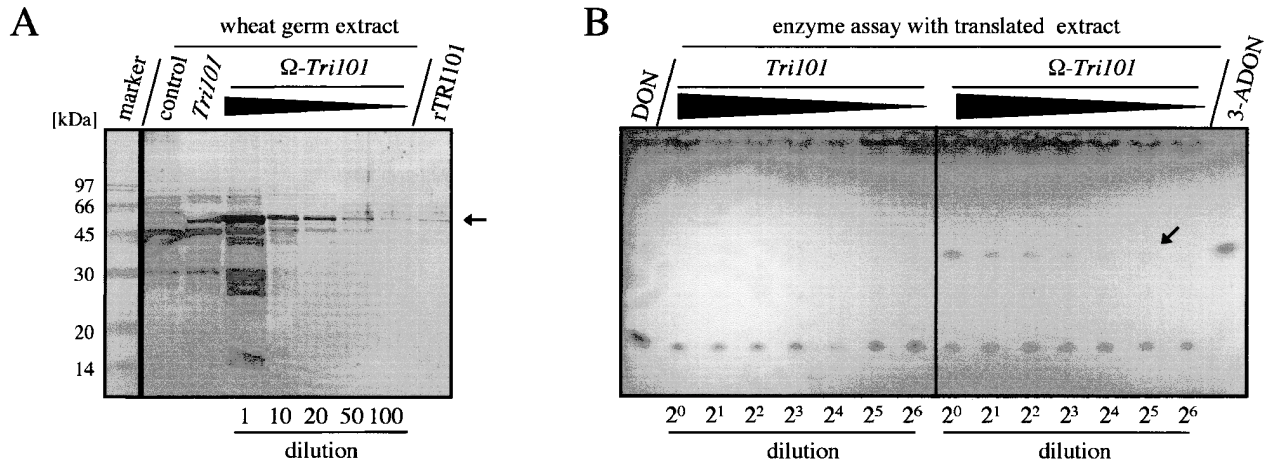

Fig. 3. *In vitro* Translation of *Tri101*.

RNA of *Tri101* was prepared from pET12a-*Tri101* (*Tri101* coding region cloned in the *Nde* I-*Bam* HI sites of pET-12a; Novagen) and pEU3-NII-*Tri101* (*Tri101* coding region inserted into the *Eco* RV site of the Ω vector pEU3-NII; TOYOBO) using the Riboprobe *in vitro* Transcription Systems (Promega). (A) Western blot analysis of the *in vitro* translated product and recombinant TRI101 (rTRI101). A series of dilutions (10, 20, 50, and 100-fold) of the reaction mixture containing Ω-*Tri101* RNA was put on (10 μl each lane) to compare the amount of TRI101 protein produced by the *in vitro* system. An arrow indicates TRI101 protein. (B) 3-*O*-acetyltransferase activity of TRI101 protein translated either in the presence or absence of Ω. The reaction mixture with the Ω vector was diluted 2^1, 2^2, 2^3, 2^4, 2^5, and 2^6-fold, assayed for the enzyme activity using DON as a substrate, and developed on a TLC plate under the conditions as described previously.[3] An arrow indicates the product 3-ADON.

on a northern blot. However, these northern-positive lines did not show TRI101 enzyme activity as assessed by an acetylation assay *in vitro*. Besides, portions of transgenic calli (cultured on a medium with 5 mg/l bialaphos) corresponding to these lines did not show DAS resistance phenotype (data not shown). These results are consistent with the negative screening results on Western blots.

In contrast to the low expression level of *Tri101*, five out of seven T0 plants transformed with *egfp::zhd101* accumulated sufficient amounts of the fusion protein as demonstrated by GFP fluorescence (one example is shown in Fig. 2D). Production of EGFP::ZHD101 was further confirmed by Western blot analysis. Leaf proteins were extracted and electrophoresed as described previously.[12] Proteins (20 μg each lane) were transferred to a PVDF membrane and incubated with the 5000-fold diluted primary antibodies against EGFP (Clontech). The Amplified Alkaline Phosphatase Goat Anti-rabbit Immun-blot Assay kit (Bio-Rad) was used for the detection. As shown in Fig. 2E, full-length EGFP::ZHD101 fusion protein (marked by an arrow) was detected on a Western blot using the GFP antibody. These promising *egfp::zhd101* lines are now growing in the greenhouse and will be used for *in planta* detoxification experiments (*i.e.*, detoxification at developing endosperms) at anthesis in the near future.

Recently, Okubara *et al.* have observed similarly low expression of *Tri101* in cereal plants; *i.e.*, none of the *Tri101* wheat lines showed significant increases in FHB resistance due to the very small amount (or lack) of TRI101 enzyme in the transgenic plants.[13] Inefficient translation is often associated with biased codon usages of a transgene. However, the *Tri101* sequence generally has unbiased use of codons (*i.e.*, "unbiased" here implies that the frequency of each codon use is less than twice *and* within 2% excess compared to that of the host organisms) for the rice plant (see Codon Usage Database; http://www.kazusa.or.jp/codon). Possible causes may be attributed to contiguous arrangement of some specific codons or instability of the *Tri101* mRNA. However, extensive gene engineering and long plant regeneration periods are necessary before the effect can actually be evaluated in the whole plant system.

To improve the low expression of the transgene, we examined the effects of the tobacco mosaic virus Ω translation enhancer sequence[14] placed just upstream of *Tri101* using an *in vitro* translation system. Ten μg each of RNA with or without Ω was added to the wheat germ extract (Proteios Wheat Germ Cell-free Protein Synthesis kit; TOYOBO) supplemented with creatine kinase and RNase inhibitor in a final volume of 50 μl. Translation was initiated by placing the 50 μl reaction mix under 250 μl of the buffer mix as described in the manufacturer's protocol. After incubation at 26°C for 20 h, efficiencies of translation were evaluated. As shown in Fig. 3A, TRI101 (marked by an arrow) was detected from each vector on a Western blot. The amount of TRI101 protein from the Ω vector was at least 20-fold greater than that from the original vector lacking Ω. A similar result was obtained from the *in vitro* acety-

lation assay using deoxynivalenol (DON) as a substrate; although the acetyltransferase activity was not detected from the original vector, use of the Ω vector resulted in detection of the product 3-*O*-acetyldeoxynivalenol (3-ADON) even after 2^5-fold dilution of the reaction mixture (Fig. 3B; marked by an arrow). This is also the first confirmative evidence that TRI101 protein is properly folded to an active conformation in the wheat system. It may be possible that simple addition of the enhancer to the vector supports transgenic expression of *Tri101* in cereals.

In conclusion, use of a strong and constitutive rice *Act1* promoter was not appropriate to achieve accumulation of TRI101 protein in transgenic rice plants while the same promoter was suitable for production of EGFP::ZHD101 fusion protein. New Ω transformation vectors with various types of promoters are now being constructed for generation of transgenic wheat resistant to trichothecenes.

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