PROGRESS IN BIOLOGICAL SCIENCES

# Enzymatic detoxification of Don in transgenic plants via expression of *Fusarium graminearum Tri101* gene

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**Abstract**

*Fusarium graminearum* is causal agent of economically catastrophic disease of cereal Fusarium Head Blight (FHB) around the world. In addition to causing a loss of yield, this fungus causes serious threats to humans and animals due to the contamination of grain with the trichothecene mycotoxin. *TRI101* gene, a *Fusarium* spp. gene, encodes an enzyme that transfers an acetyl group to the C3 hydroxyl of trichothecenes. We introduced *TRI101* gene from *F. graminearum* to tobacco to test its usefulness for decontamination of mycotoxins. The acetyltransferase activity of this gene was detected in transgenic plants. The growth pattern of T1 seedling was examined at present of deoxynivalenol (DON) and crude extract of mycotoxin. A significant difference in growth rate was seen in plants expressing TRI101 as compared to wild type in DON assay. There was no significant difference in growth rate of the wild type and transgenic plants in presence of a mixture of mycotoxins. According to the results, it seems although FgTRI101 enzyme is suitable for detoxification of DON, more studies are required to evaluate its performance in detoxification of mixture of Fusarium toxins.

**Keywords:** Detoxification, DON, Fusarium Head Blight, *F.gTRI101*, Mycotoxin

## Introduction

*Fusarium graminearum* is the causal agent of Fusarium Head Blight, the most destructive disease of wheat worldwide (1-3). It is in forth place of 'Top 10' of fungal plant pathogen based on their perceived importance, scientifically or economically (4). *Fusarium* epidemics led to 70% grain quality losses annually in wheat-growing areas in Caspian littoral in Northern Iran because of warm and humid condition during flowering and early stages of wheat kernel development (5). This disease mainly reduces grain quality rather than lowering grain yield and results in trichothecene contaminated grain. Trichothecenes, such as deoxynivalenol, can accumulate in infected grain and their end used products. Consumption of the contaminated products can cause health problems in livestock and humans (1, 6, 7). Since trichothecenes are antibiotics, the producing organisms need to have special adaptations for self-protection. Trichothecene 3-O-acetyltransferase (*Tri101*) is an enzyme which catalyzes the conversion of toxic trichothecenes to less-toxic 3A-products; therefore, has been proposed as a metabolic self-protection mechanism in *F. graminearum* (8, 9). Acetylation by *Tri101* is the main defense mechanism against 3-hydroxylated trichothecenes in *Fusarium* rather than other mechanisms and Mutants of *F. sporotrichioides* produced by disruption of *Tri101* were altered in their abilities to synthesize mycotoxins (10).

To identify whether the *Tri101* expression in transgenic tobacco, as a model plants, can reduce the effects of mycotoxins in plants and detoxifying them, we generated transgenic tobacco plants stably expressing *Tri101* from *F. graminearum* and the DON acetylase activity of the transgenic plants was studied.

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## Materials and methods

### Strain, media and DNA extraction of *F. Graminearum*

The single spore isolate of *F. graminearum* which was previously isolated from naturally infected plants was used throughout this study. According to HPLC analysis, this isolate was a DON producing isolate (Fig. 1). DNA extraction was done using the modified CTAB method (11). Mycelia were lifted from the Potato Dextrose Agar (PDA) plates, and washed twice with sterile water. It was then dried with sterilized filter paper and ground with a tissue lizer [UTRA-TURRAX T8 (IKA$^{TM}$)]. The powder was mixed with 1.4 ml of CTAB buffer (12). The resulting mixture was incubated at 42ºC for 10 min followed by 65ºC for 10 min. after cooling in room temperature mixture of phenol-chloroform and isoamyl alcohol (25:24:1) was added to the solution and centrifuged at 10000g at 4ºC for 5 minfor precipitation of DNA 500 µl of isopropanol was added to the solution and was incubated at -20°C for 20 min. DNA was washed with 70% ethanol and re-suspended in distilled water. The quantity of DNA was measured by optical absorption at 260 nm (A260) using spectrophotometer.

### Isolation of *TRI101* gene

Primers were designed for *TRI101* gene of *F. graminearum* using the *Fusarium Comparative Database Site* (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html). Primer sequences were as follows: TriFw 5'-GGATCCACCAAAAT GGCTTTCAA GATACAGCTCG-3', TriRe 5'-CCCGGGGCTAA TCTAACCAACGTACTGCGC-3' with an expected amplified fragment length of 1356 bp. Extracted DNA (50 ng) was used as template in PCR for amplification of *Tri101*. The reaction mixture contained (50 µl totals): Expand PCR buffer, 2 mM $MgCl_2$, dNTP mixture (0.2 mM each), and 1.25 U of Expand enzyme (Roche). Amplification consisted of an initial denaturing cycle of 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension cycle of 72°C for 10 min. Amplified PCR products were analyzed by Agarose gel electrophoresis. PCR products of the expected size were cloned into pGEM-T Easy according to recommended protocol by Company (Promega, Madison, WI). Right cloning was ensured by DNA sequencing (Shinegene Molecular Biotech, China). Sequences were assembled using the SeqMan program (DNASTAR, Inc.) and analyzed with the MegAlign and Map Draw programs (DNASTAR, Inc.).

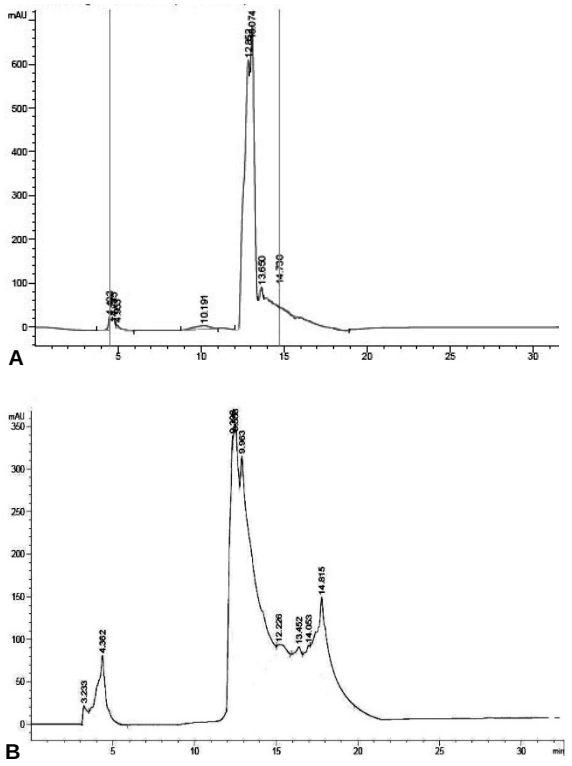


**Figure 1.** HPLC separation of **(A)** standard DON, **(B)** mycotoxins extract from *F. graminearum*.

### Leaf-Disk transformation of tobacco plants

As *Tri101* gene has restriction site for *Sac* I enzyme, *Bam*HI (Fermentas, Ukraine)/*Sma* I (Fermentas, Ukraine) double digestion was used to excision from pGEM T Easy vector. Then the gene was sub-cloned into pBI121 binary vector under the control of 35S promoter and Nos terminator through digestion with *Bam*HI (Fermentas, Ukraine) and *ECI*136 II (Fermentas, Ukraine) in order to construct pATRI plasmid (Fig. 2). Restriction digestions were performed following the recommended protocol by the company. The insertion of *Tri101* gene was confirmed by PCR using TriFw/TriRe primer pairs (the same protocol described above). The recombinant vector pATRI

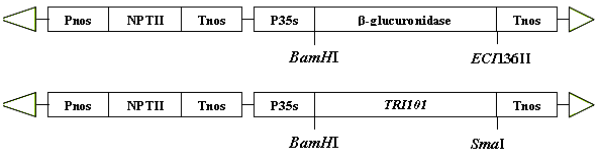


**Figure 2.** Schematic map of pATRI construct. *GUS* (ß-glucuronidase) gene was replaced with *Tri101* gene in pBI121 vector.

was transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* LBA4404 by freeze and thaw method (13) which was subsequently used to transform *Nicotiana tabacum* cv. Xanthi (14). Transformed lines of *N. tabacum* with pATRI were selected in MS medium containing 0.1 $mgl^{-1}$ NAA, 2 $mgl^{-1}$ BA and 100 $mgl^{-1}$ kanamycin as regeneration medium. Antibiotic resistant plantlets were separated and transferred to MS basal medium supplement with 100 $mgl^{-1}$ kanamycin (15). Rooting of shoots was done on rooting medium (MS medium containing 100 $mgl^{-1}$ kanamycin, 200 $mgl^{-1}$ kanamycin cefotoxime, 2% sucrose, 0.7% agar; PH 5.7). The regenerate plants were grown in a greenhouse under 16 h light/8 h dark condition at 25-28°C. For collection self seeds flowers were covered during the flowering period.

### Molecular analysis of transformed plants

Genomic DNA extraction from Tobacco leaves was carried out using CTAB method (12). The resulting DNA was used as a template in PCR and the presence of the trans-genes was tested by PCR using primers TriFw/TriRe. RNA was extracted from putative transformants using RNX plus kit (Cinagen, Iran). Total RNA was converted to single-strand cDNA using OligodT primer. Transcription of the trans-genes in positive lines in PCR analysis was tested by reverse transcriptase PCR using the same primers (TriFw/TriRe primers) and amplified under the same condition as described for *Tri101* gene.

### Extraction of mycotoxins

Mycotoxins were extracted following the protocol described by Shahbazi et al. (16). DON was detected by HPLC. The HPLC equipment consisted of Agilent 1200 Series Preparative HPLC System with using $C_{18}$ HPLC column (USA) and setting on 218 nm.

### DON tolerance assay of T1 seedling

Stability and co-integration of transgenic plants was checked by germination T1 seeds on MS medium. Selfed seeds from transgenic lines and wild type tobacco were germinated on mesh in Petripaltes (approximately 60 seeds/mesh) containing kanamycin incase of transgenic lines (100 $mgl^{-1}$). When two leaves were appeared, agar carefully removed and seedlings along with mesh were transferred to 100 ml liquid MS medium containing 4 ppm DON (Biopure, Astria) or 8 µl of extracted mycotoxin and were kept on shaker. After one week, the seedlings were removed and transferred to fresh flasks having 100 ml of fresh liquid MS medium containing 10 ppm DON or 20 µl of mycotoxins mixture. Media were changed one week interval with fresh medium. MS medium without DON or mycotoxin was used for growing negative controls. After 3 weeks, wet weight of plants was determined to obtain the biomass increase. Data were analyzed using MSTAT-C software in $p \leq 0.05$ level.

### Detection of *in vitro* trichothecene 3-oacetyltransferase activity in transgenic lines

Protein was extracted from 100 mg of tobacco plant leaves. The supernatant containing soluble proteins was used to analyze the expression of the recombinant protein. For detection of acetyltransferase activity 100 µg of total leaf protein extract was used. DON (40 µg) was mixed with protein and 1 mM acetyl-CoA was used as coenzyme. Total volume was adjusted to 250 $\mu$l with 50 mM Tris-HCl pH 7.5 and remained at 37°C for 12 h. Then reaction mixture was extracted with ethyl acetate (9) and separated on TLC papers (Ridel-deHaen™, Cat. No.37368) and developed by solvent ethyl acetate/toluene (3:1). The papers were visualized with fluorescent indicator at 254 nm (9).

## Results

For studying the ability of *TRI101* gene in acetylation of DON, this gene was isolated from *F. graminearum*. PCR amplification was

performed on the genomic DNA of *F. graminearum* using designed primer pair and the specific band of approximately 1400 bp was amplified (Fig. 3).

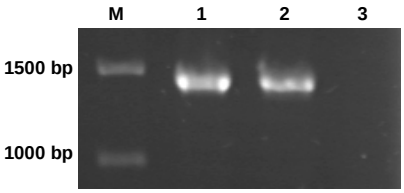


**Figure 3.** PCR amplification of *FgTRI101* gene using TriFw/TriRe primers M: 1Kb DNA Ladder (Fermentas), 1, 2: *TRI101 gene* amplified fragment (1356 bp). 3: negative control.

PCR products then were cloned into pGEM-TA vector. The presence of insertion gene (*TRI101*) and absence of the undesired spontaneous mutations were checked by sequencing. The integrity of (*Tri101*) cloning in pBI121, resulted in pATR1, confirmed by observation of a 1356bp fragment in PCR, (Fig. 4). The pATRI mobilized into the *A. tumefaciens* LBA4404 and subsequently used for transformation of leaf-disk of tobacco.

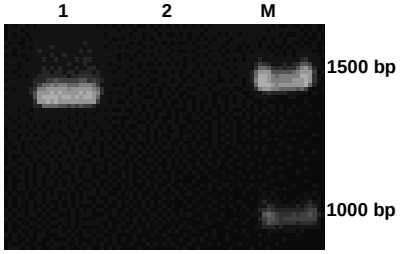


**Figure 4.** Confirmation of pATRI containing *FgTRI101* gene. 1: PCR products of amplification of pATRI using TriFw/TriRe primers, 2: negative control, M: 1Kb DNA Ladder (Fermentas).

Two weeks after leaf-dick transformation shoot generation occurred, especially from the mid-vein. The callus phase was totally omitted in this protocol. Several shoots which obtained from independent transformation were rooted and grew in seedlet by self crossing. To test the presence of stable integration of transgene, genomic PCR was done. The PCR profile of the transformed plants exhibited the presence of a sharp 1356bp fragment of the *TRI101* gene (Fig. 5) which confirmed the integration of interested gene in the genome. RT-PCR analysis was further used to confirm transcription of transformed gene. This reaction

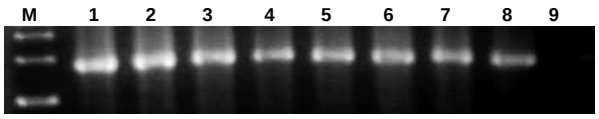


**Figure 5.** PCR analysis of putative transgeni c tobaccos. M: 1kb DNA Ladder (from above 1500bp and 1000bp), 1-8: *FgTRI101* Transformed tobaccos 9: non-transformed tobacco.

resulted 1356bp fragment in transgenic plant while the negative control without reverse transcriptase was negative showing indicated there was no contamination of genomic DNA. No band was amplified by using RNA from non-transformed plant (Fig. 6).

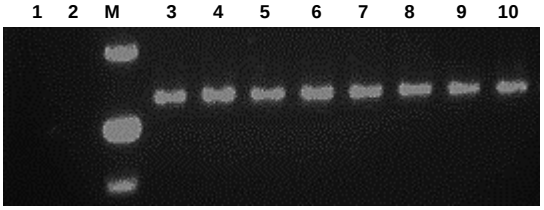


**Figure 6.** RT-PCR of *FgTRI101* transcript in transgenic tobacco plants. 1: non-transformed plant, 2: negative control without the reverse transcriptase enzyme, 3-10: transgenic plants amplifying 1356bp fragment, M: 1Kb DNA Ladder (Fermentas).

To test the ability to tolerate pure DON or crude mycotoxins in FgTRI101 transformed tobaccos, seeds from transformed and non-transformed plants were germinated in liquid MS medium. At 2 leaf stage seedling were transformed to medium containing graduate increasing DON or crude mycotoxins. There was no significant difference in growth rate in transformed and non transformed plants in control medium. In DON containing medium transgenic plants grew significantly more than non-transformed although the growth of both of them was inhibited compared with MS medium (Table 1). FgTRI101 transformed plants also showed better resistance to crude mycotoxins as compared with wild type, but it was not significant (Table 2).

In enzymatic reaction using protein extract from transgenic and non-transformed plants, 3ADON was detected only in transgenic line extracts, not in control ones which confirmed the C-3 acetylation function of Tri101 in transgenic plants (Fig. 7).

**Table 1.** Effect of DON on growth of seedlings (T0 stage) in liquid medium. Increase of biomass (mg) is detected.

| | MS | MS +DON |
|---|---|---|
| **Wild type plant** | 38216.67[a] | 32497[c] |
| **Transformed plant** | 37480[a] | 34896[b] |

Each value represents the mean of three independent experiments, with average standard deviation p≤0.05.

**Table 2.** Effect of extracted mycotoxins on growth of seedlings (T0 stage) in liquid medium. Increase of biomass (mg) is detected.

| | MS | MS + crude mycotoxin |
|---|---|---|
| **Wild type plant** | 38216.67[a] | 30366.67[b] |
| **Transformed plant** | 37480[a] | 30840[b] |

Each value represents the mean of three independent experiments, with average standard deviation p≤0.05.

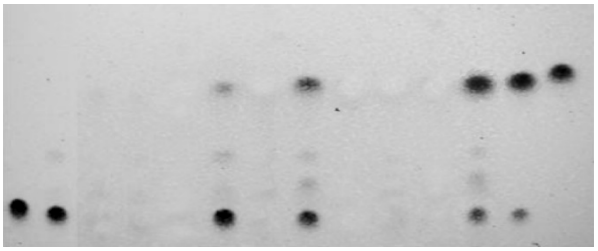

**Figure 7.** Acetyltransferase assay of synthesis AYT1 in Tobacco. 1: DON Standard sample, 2: $C^-$ protein extract from non-transformed tobacco+ DON, 3-6: protein extract from transformed tobacco+ DON, Lane 7: 3ADON Standard sample.

## Discussion

It has been proven that the relationship between trichothecene accumulation in the host plant and the ability of *Fusarium* spp. to cause disease is linear and positive. Fungi without the ability of DON production are unable to spread rapidly throughout the plant and induction PR metabolites (17). Therefore, removal or degradation of the toxin by the host plant can reduce aggressiveness of pathogen. In the other hand, the mechanisms of resistance to trichothecene are well known and therefore, manipulation of such mechanisms would be an attractive alternative for biotechnologists. Detoxification of DON through converting to a low-risk component, 3ADON, by the enzymatic reaction highly focused on recent projects. Acetyltranferases from *Fusarium* spp. frequently have used to produce transgenic plants. We introduced trichothecene-3-O-acetyltansferase from *F. graminearum* (FgTRI101) to tobacco and the results showed that transgenic plants are able to convert DON to 3-A-DON. As it was expected, FgTRI101 plants represented more tolerance than the wild type in the present of DON and the effect of the toxin on their growth was less observed. Previous studies indicated that the seeds of transgenic tobacco plants transformed with ortholoug gene from *Fusarium sporotrichoioides* (FsTRI101) displayed a more germination ability compared with the wild type (18). FgTRI101 has been also introduced to rice (19) and after enhancing expression with Ω enhancer, transgenic lines generate longer roots compared with the wild type in DON containing medium (9). There are a few reports on introduction of TRI101 gene into important crops such as wheat, rice and barley (9, 20, 21); in which resistance to DON has been tested (9, 21, 22). In present study, transgenic plants were treated with crude extract of mycotoxins and unexpectedly, they could not combat significantly in comparison with the wild types. Greenhouse experiments performed to test in FsTRI101 transgenic wheat to infection, showed moderate tolerance (20). However it should be mentioned that *F. graminearum* isolate which had been used in the above study was a DON producing isolate (23).

The kinetic analysis properties of FgTRI101, reported in our study, indicated that this enzyme has been much more effective in converting DON to 3ADON compared with ortholog from *F. sporotrichioides* (24). The average concentrations of deoxynivalenol in infected wheat are generally high in epidemic years (between 336 and 678 µg/kg) (25) whereas the screening of DON tolerance is usually performed in lower concentration. Hence, selection of the candidate genes for adverse effects of the disease should be performed conservatively. There are some reports indicating a substantial increase in lipase 3 h after DON treatment in the FHB susceptible cultivar; in the resistance cultivar this increase has been observed with a slight shift, 6 h after treatment (26). Lipases are reported to be able on deacetylation of their substrates. What is the role of these enzymes in DON tolerance and how they can

influence the action of trichothecene-3-O-acetyltansferase enzymes in transgenic plant, is not well understood and should be investigated more precisely.

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