DOI : <http://doi.org/10.22438/jeb/40/3/MRN-942>

siRNA quelling of *tri4* and *tri5* genes related to deoxynivalenol synthesis in *Fusarium graminearum* and *Fusarium culmorum*

Paper received: 06.06.2018

Revised received: 14.11.2018

Re-revised received: 07.12.2018

Accepted: 14.12.2018

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Abstract

Aim : This study was conducted to investigate the quelling of *tri4* and *tri5* genes associated with deoxynivalenol, most common class B trichothecene, via siRNA application.

Methodology : In this study, one *Fusarium graminearum* (4F) and two *F. culmorum* (9F and 20F) isolates, expressing high levels of *tri4* and *tri5* genes, were targeted for siRNA-based silencing. Full length *tri4* and *tri5* genes were amplified from all isolates, sequenced and then subjected to CLUSTALW analysis for designing siRNA molecules. Totally designed six siRNAs were separately co-transfected together with helper plasmids (pEGFP75 and pAN7-1) as 36 combinations into protoplast cultures and quelling was analysed via real time polymerase chain reaction, thin layer chromatography and spectrofluorimetric analysis.

Results : Alignment of both *tri4* and *tri5* genes' sequencing revealed high levels of similarity between isolates (94-99% and 95-100%, respectively). Transfection efficiency ranged from $50 \pm 0.00/5 \times 10^4$ to $99.33 \pm 10.06/5 \times 10^4$ and relative fluorescence unit values were calculated between 1.37 ± 0.07 and 2.89 ± 0.06 . The 0.7 kb length fragments of *hph* and *GFP* were amplified from transfectants. The real time PCR revealed that *tri4* was completely suppressed in 30 experimental sets whereas down-regulations of *tri5*, ranging from 74.5 to 99%, was detected in remaining 6 experiment groups. Thin layer chromatography assay also showed that siRNA-transfected *Fusarium* samples showed no deoxynivalenol spots.

Interpretation : This study revealed that *tri4* and *tri5* genes can be targeted in the development of less deoxynivalenol (DON)-producing strains through RNAi. Moreover, current study has great importance as it demonstrates that quelling can be used as a potential strategy in controlling diseases caused by phytopathogenic species.

Key words: *Fusarium* sp., Gene expression, Gene silencing, Transfection, Trichothecene

How to cite : Yörük E. and G. Albayrak : siRNA quelling of *tri4* and *tri5* genes related to deoxynivalenol synthesis in *Fusarium graminearum* and *Fusarium culmorum*. *J. Environ. Biol.*, **40**, 370-376 (2019).

Introduction

Quelling is the post transcriptional gene silencing mechanism in fungi (Ruvkun, 2001; Thakur, 2003). RNA interference (RNAi) based gene silencing is responsible for antiviral defence in several Ascomycota species such as *Neurospora crassa*, *Aspergillus niger* and *Fusarium culmorum*. RNAi process is elicited by noncoding double stranded short (20-25 nt) interfering RNAs (siRNA). Direct transfection of synthetic siRNA molecules to fungal cells is a powerful approach for genetic manipulations although vector-based silencing is well accepted strategy in gene silencing including quelling (Barnes *et al.*, 2008; Nakayashiki and Nguyen, 2008; Scherm *et al.*, 2011). However, application of siRNA transfection is not common in fungi.

Endotoxins produced by fungi are composed of diverse group of chemical substances. Geographic and climate differences affect the mycotoxin types and their production levels. These chemical substances occur in raw agricultural products both pre- and post-harvested (Kuiper-Goodman, 2004). Therefore, mycotoxins and food safety will continue to be of critical interest to researchers (Battilani *et al.*, 2008). *Fusarium* spp. produces several types of mycotoxins, including class-B trichothecenes (Desjardins and Proctor, 2007). Deoxynivalenol (DON) is the most accumulated class-B trichothecene on cereals, including barley (*Hordeum vulgare*), maize (*Zea mays*) and wheat (*Triticum aestivum*). Since DON is transported into dopaminergic receptors in human brain after consumption of food contaminated with mycotoxin, it is also named as vomitoxin (Sobrova *et al.*, 2010; Matny, 2015). DON is a sesquiterpenoid structured secondary metabolite.

Toxins decrease crop yield quality and quantity in agriculture. High level of DON shows acute toxic effect in humans and livestock animals. This phytotoxin has a stable structure under high temperatures and during food processing (Desjardins and Proctor, 2007; Sobrova *et al.*, 2010). Trichothecene biosynthetic pathway has been defined in *F. graminearum* and *F. sporotrichioides* strains (Brown *et al.*, 2004; Kimura *et al.*, 2007). Farnesyl pyrophosphate is the start metabolite, and various enzymes and transcription factors coded by *tri5* gene cluster are functional in the DON production (Kimura *et al.*, 2007). Several genes in *tri5* gene cluster - including *tri6*, *tri5* and *tri4* are strongly conserved in distinct trichothecene types and cluster genes expressions are essential in trichothecene production. However, some genes show variation in point of number, size and functionality. DON producers contain *tri7* and *tri13* genes as deleted and/or disrupted, therefore they are non-functional genes in the pathway (Brown *et al.*, 2004).

The *tri5* and *tri4* genes located in *tri5* gene cluster are essential in DON biosynthesis. *Tri5* is the first gene identified in trichothecene biosynthetic pathway. The gene is 1179 bp length, contains one intron and encodes trichodiene synthase (*TRI5*) which converts farnesyl pyrophosphate to trichodiene. *Tri4* is 1741 bp length including three introns, and codes a multifunctional monooxygenase (*TRI4*). The amino acid

sequence of *TRI4* is highly similar to cytochrome P450. *Tri4* protein catalysis the conversion of trichodiene to isotrichotriol (Kimura *et al.*, 2007). Alterations in media ingredients including magnesium or ferulic acid addition, H₂O₂ treatment, acidic pH supply, etc. have been used as strategy in decreasing DON production in culture conditions (Merhej *et al.*, 2010; Pinson-Gadias *et al.*, 2008). In field, fungicide treatment is the only way to reduce of *Fusarium* spp. reproduction and the amount of their related toxins (Kang *et al.*, 2001). However, since fungal strains may develop resistance to certain antimicrobials, it needs to be in development of additional and new disease control strategies (Kimura *et al.*, 2007; Leeja and Thoppil, 2007). It is also thought that the development of less mycotoxin producing strains through genetic engineering strategies may be more effective in the struggle with pathogens and their toxins.

Gene silencing technology has been reported to be a useful tool for eliminating mycotoxin contaminated food products and aggressiveness of mycotoxigenic pathogens (McDonald *et al.*, 2005; Scherm *et al.*, 2011). In view of this, this study was carried out to study silence of both *tri4* and *tri5* genes through RNAi-based strategy in order to develop less DON-producing strains.

Materials and Methods

Growth conditions and determination of hygromycin B resistance of fungal strains: Fungal spores of *F. graminearum* isolate 4F and *F. culmorum* isolate 20F were obtained from barley kernels while *F. culmorum* 9F spores were obtained from diseased wheat samples from Turkey. Fungal strains were grown on potato dextrose agar and potato dextrose broth in a light and dark cycle at 25°C for seven days. Fungal liquid cultures were incubated by shaking at 160 rpm. Fungal isolates were grown on both PDA and PDB mediums containing 25, 50, 75 and 100 µg ml⁻¹ hygromycin B for assessing antibiotic susceptibility. Minimum inhibitory concentration (MIC) and half-maximal (50%) inhibitory concentration (IC₅₀) of hygromycin B were determined through agar dilution technique and spectrophotometric analysis (at OD₆₀₀).

Nucleic acids isolation and cDNA synthesis: Genomic DNAs and total RNAs were extracted from seven-day-old fungal cultures and quelling samples. Genomic DNAs were isolated from 50 mg fresh weight mycelia through CTAB-based protocol of Doyle and Doyle (1987) with minor modifications. TriPure reagent (Roche, Switzerland) was used in total RNA extraction from 1ml liquid culture or 50 mg fresh weight mycelia according to manufacturer's recommendation. Components for cDNA synthesis were mixed in a reaction volume of 25µl containing: 1U transcript or HF reverse transcriptase, 1 µg total RNA, 60 µM random hexamer, 1X reaction buffer, 5 µM DTT, 1U protector RNase inhibitor and 1 mM dNTPs. The synthesis reaction was performed through thermal steps as follows: 65°C for 10 min, 55°C for 30 min and 85°C for 5 min. In cDNA synthesis, primer annealing was carried out at 65°C for 10 min and then RNase H activity and reverse transcription were maintained by incubation steps at 55°C for 30 min and 85°C for 5 min.

Amplification and sequencing of *tri4* and *tri5* genes: Each of *tri4* and *tri5* genes was aligned via ClustalW analysis in output deposited at GenBank with AY102584, AY102605, AY102603, AY102602, AY102571 and AY102571 accession numbers. Then, consensus regions were used for designing specific primer pairs. Full length *tri4* and *tri5* amplifications of 4F, 20F and 9F isolates were carried out by PCR in 25 µl reaction volume including 1X PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, 10 pmol of each primer, 1 U Taq DNA polymerase (Promega, USA) and 50 ng of DNA. PCR amplification was performed by pre-denaturation at 95°C for 5 min, followed by 35 cycles of amplification at 95°C for 45 sec, 55-60°C for 45 sec, 72°C for 2 min and final extension at 72°C for 5 min. PCR bands were separated on 1.5% agarose gels and visualized by gel documentation system. Amplification products belonging to *tri4* (1741 bp) and *tri5* (1187 bp) genes were purified by using a commercial kit (Roche, Switzerland) and then separately cloned into *E. coli* DH5α strain using pGEM T-Easy cloning system (Promega, U.S.A.) according to the manufacturer's instruction. Plasmids were isolated from recombinant bacterial clones using commercial plasmid isolation kit (Invitrogen, U.S.A.), and were sequenced based on Sanger dideoxy chain termination method. Nucleotide sequences were aligned via Chromas Lite Edition and then assembled by using DNA Dragon software. Total sequences of *tri4* and *tri5* genes were deposited in GenBank.

Quelling through siRNA: Multiple sequence alignment analysis was carried out in the sequences belonging to two exon regions of *tri5* and four regions of *tri4* genes. Totally 6 synthetic double stranded siRNAs were designed through PerfectIt siRNA design tool (Invitrogen, U.S.A.) according to sequence showing 100% homology with reference sequences AY102584, AY102605, AY102603, AY102602, AY102571 and AY102571 accession numbers (Table 1). The plasmid constructs pEGFP (containing *gfp* gene) and pAN7-1 (containing *hph* gene) were used in the co-transfection of *Fusarium* protoplasts with siRNAs. Protoplasts were obtained from mycelia after enzymatic digestion of cell walls by lytic enzyme (Sigma, Germany) with 20 mg ml⁻¹ final concentration. The cultures were incubated at 25°C by shaking 160 rpm for 6 hrs. Two volume of STC buffer (20% sucrose, 25 mM Tris-HCl, pH 7.5, 25mM CaCl₂) was added to filtrate and samples were centrifugated at 800xg for 10 min. Protoplasts were diluted to final concentrations of 5x10⁴. Protoplasts were transfected with plasmid vector (pAN 7-1 or pEGFP75) and siRNA molecules. 1µg plasmid DNA, 150 pmol of siRNA and 1X transfection reagent (Roche, Switzerland) were mixed in a microtube and incubated at room temperature for 15 min. Then the mixture was transferred to PDB medium immediately.

Mycelia obtained from seven-day-old transfectants were visualized in fluorescence microscopy (Leica, Germany). Spectro fluorometric measurements were carried out at 485/535 nm excitation/emission wavelengths, and relative fluorescence unit values were calculated. Co-transformants were also verified through PCR assays. GFPF/GFPR and HYGBF/HYGBR primers were used for producing of GFP (720

bp region) and hygromycin B resistance gene (707 bp length region), respectively. PCR conditions were carried out as described previously in *tri4* and *tri5* amplification.

Gene expressions and DON analysis in transfectants: *Tri4* and *tri5* primer and probe sets were designed using primer3 software and universal probe library online services (Roche, Switzerland). Real time PCR (qPCR) assays were performed in LightCycler 480 II system (Roche, Switzerland) using Taq-Man probes. qPCRs were conducted in a volume of 20 µl containing: 2 µl cDNA, 1 X probe master mix (Roche, Switzerland), 2 pmol probe and 4 pmol of primers. Cycling conditions consist of 45 cycles; followed by at 95°C for 10 sec, at 60°C for 30 sec, at 72°C for 1 sec. The slope values were calculated by standard series with a range of four dilutions. Mean crossing point (ΣCp) and ΔCp values were recorded. Target gene expression level was normalized according to *β-tubulin* and 2^{-ΔΔCT} values were determined via formula developed by Livak and Schmittgen (2001).

Reverse transcription PCR (RT-PCR) assays were performed in order to confirm qPCR findings. RT-PCRs were conducted on 25 µl volume including; 1x PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, 5 pmol primer, 1U of Taq DNA polymerase (Promega, USA) and cDNA corresponding to 1µg total RNA. The cycling conditions were performed at 94°C for 2 min for pre-denaturation, 35 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and 72°C for 2 min for final extension. RT-PCR bands were analysed as described before. The qPCR and RT-PCR analysis were replicated at least three times for each experimental set.

Thin layer chromatography (TLC) was used in order to determine DON mycotoxin both in seven-day-old control sets and co-transfectants. For this, 5 ml of fungal cultures were filtrated by double gauze. Ethyl acetate of 1/5 volume was added to filtrate and samples were mixed thoroughly. After centrifugation at 5000 xg, for 5 min (+4°C), ethyl acetate was removed. Samples were dried and then dissolved with acetonitrile. In TLC, silica gel 60 F₂₅₄ (Merck, Germany), ethyl acetate:toluene (1:1) and ethanol:aluminium chloride (99:1) were used as adsorbent, solvent.) The mycotoxin samples were co-spotted together with trichothecene standard (Sigma, Germany) on silica gels, then run for 45-60 min. Silica gels were sprayed with reactive, and Rf value was calculated via visualization at 365 nm UV light.

Results and Discussion

Fungal isolates showed no growth in *in vitro* for each concentration of hygromycin B. The MIC and IC₅₀ values of 4F, 9F and 20F isolates were determined as 25 µg ml⁻¹ and 12.5 µg ml⁻¹ hygromycin B, respectively. All fungal isolates were highly sensitive to hygromycin B. Double stranded siRNAs were designed according to *tri4* and *tri5* nucleotide sequences which belonged to 4F, 20F and 9F isolates. Amplification products of 1187 bp-length of *tri5*, were obtained from 4F and 9F, and that of 1741 bp-length of *tri4*, were obtained from 4F and 20F (Fig. 1 A).

Fig. 1: (A) *tri4* and *tri5* genes amplified from 4F and 9F isolates. (B) 0.7 kb *hph* fragment obtained from fungal isolates' genomic DNAs transfected by siRNA and pAN 7-1. M1: 100 bp DNA ladder (Thermo, U.S.A.), M2: 1 kb DNA ladder (Thermo, U.S.A.), N: No template.

Full length amplicons were cloned into pGEM-T Easy vector. The nucleotide sequences of *tri4* belonging to 4F, 9F and 20F isolates were registered in the GenBank with accession numbers KJ677970, KJ677971 and KJ677972. Similarly, KJ677973, KJ677974 and KJ677975 accession numbers were given for *tri5* gene belonging to 4F, 9F and 20F isolates, respectively, by GenBank. After sequencing, the *tri4* and *tri5* sequences were converted to amino acid data and then aligned. It was revealed in BlastP analysis that the variations found in *tri4* and *tri5* genes belonged to both reference strain (*F. graminearum* NRRL 28336) and all isolates used in this study were responsible for 25 and 16 amino acid differences (Fig. 2).

At least 10 potential regions covering exon sequences of each gene were detected for siRNA designing. After selection of DNA regions with a maximum of 100% identity, a total of 6 double-stranded siRNAs were designed, with 4 for *tri4* and 2 for *tri5* (Table 1). Protoplasts ranging from 1×10^5 to 5×10^6 were obtained from mycelia by treatment with lytic enzyme for 5-6 hrs. Putative 4F, 9F and 20F transformants were detected on selective PDB medium containing

$25 \mu\text{g ml}^{-1}$. siRNA transfection was indirectly confirmed by PCR amplification of 0.7 kb DNA fragments belonging to *hph* gene (Fig. 2B). It was observed that all isolates were successfully transfected with pEGFP75 at different siRNA combinations. Additionally, RFU values of fungal isolates transfected with siRNA and pEGFP75 plasmid were normalized as compared to no-transfected control groups. Minimum RFU value of 1.37 was recorded in 20F transfected with triple siRNA (siRNA4E1+ siRNA4E2+siRNA4E3). However, maximum RFU value was calculated as 2.89 in 4F with double siRNA transfected (siRNA4E2+siRNA4E3). In variance analysis, RFU value of each experiment set showed significant difference in comparison to control groups (Fig. 3 A-B) according to Tukey's post hoc-test ($p < 0.001$). In addition, protoplast-based transfection frequencies ranged from $50 \pm 0.0/5 \times 10^4$ to $99.33 \pm 10.06/5 \times 10^4$.

In total, 30 *tri4* specific siRNA transfected experiment sets and non-transfected fungal groups were subjected to gene expression analysis. No *tri4* expression ($\Delta\Delta\text{CT}$ values of 0.00) was recorded in all running samples in Taq-Man probe-based qPCR analysis. *Tri4* gene was totally silenced in different siRNA

Table 1 : siRNA specific to *tri4* and *tri5* genes

siRNA	Target	Exon	Sequence (5'-3')	Size(bp)
RNAi41	<i>tri4</i>	1	ACAATACGGGCGTGAGTCATGTCAA	25
sRNAi42	<i>tri4</i>	2	GAACCCTGAGAGAAGTTGGTGATGT	25
sRNAi43	<i>tri4</i>	3	ACATGCTCCTTGACTTCAAGTTCT	25
sRNAi44	<i>tri4</i>	4	CATCTTGGAGATCTCCCAGAGAT	23
siRNAi51	<i>tri5</i>	1	AAGCGACTACAGGCTTCCCTCCAAA	25
sRNAi52	<i>tri5</i>	2	CAGGATCTGATGACTACCCTCAATT	25

Fig.2 : BlastP analysis of trichodiene synthase protein encoded by partial region of *tri5* gene. '**' indicates conserved amino acids while '.' and ':' mean mutations in amino acids sequences.

agarose gels, siRNA-transfected sets showed no bands on gels. Quelling was verified by TLC. No spots corresponding to DON was yielded from *tri4* and *tri5* silenced isolates in silica gels. However, control sets showed spots with Rf value of 0.28 (data not shown). *Fusarium* spp. including *F. graminearum* and *F. culmorum* cause devastating diseases worldwide and epidemics leading to significant yield and economic losses and mycotoxin contamination in foods (Matny, 2015). Additionally, stable (in food processing phase) mycotoxin molecules produced by *F. graminearum* and *F. culmorum* are also causative agents for animals and humans (Desjardins and Proctor, 2007). siRNA based quelling strategy was performed for inhibition of trichothecene production. For this purpose, *tri4* and *tri5* genes encoded proteins which are essential in trichothecene biosynthesis

Isolate	siRNA	$\Sigma\Delta CT(\text{treated})$	$\Sigma\Delta CT(\text{untreated})$	$\Sigma\Delta\Delta CT$	$2^{-\Delta\Delta CT}$ / Downregulation
4F	sRNAi51	2.68±0.79	0.71±0.14	1.97±0.65	0.255
4F	siRNA52	4.89±0.85	0.71±0.14	4.18±0.71	0.055
4F	siRNA51+52	5.23±0.15	0.71±0.14	4.52±0.14	0.043
9F	sRNAi51	5.64±0.57	-1.82±0.13	7.46±0.7	0.056
9F	siRNA52	5.72±0.03	-1.82±0.13	7.54±0.8	0.05
9F	siRNA51+52	3.23±0.9	-1.82±0.13	5.05±0.52	0.03

Fig. 3: One-way ANOVA profiles of spectro fluorimetric values belonging to transfectants of (A) *F. graminearum* 4F; (B) *F. culmorum* 20F and 9F isolates.

were targeted to silence of DON. Both genes have not been used before in trichothecene quelling. At first, high level of DON producing isolates, *in vitro* culture conditions among 38 isolates, were selected without plant-microbe interaction by using qPCR analysis. Amplification based chemotyping confidently provided data about mycotoxin profiling in a short time. At the same time, DON production was verified with TLC. PCR applications are effectively used for quantitation of endotoxins commonly detected with sophisticated analytic techniques because microarray is not commercially available (Güldener *et al.*, 2006; Pinson-Gadais *et al.*, 2008; Merhej *et al.*, 2010). *Tri4* and *tri5* specific siRNA mediated quelling was developed in this study.

The *tri6* gene, which encodes transcription factor for *tri5* gene cluster, have been subjected to vector based genetic manipulation studies to inhibit trichothecenes biosynthesis (McDonald *et al.*, 2005; Scherm *et al.*, 2011). As in this study, *gfp* and/ or *hph* based verification systems were used in *tri6* quelling studies. However, *tri6* does not include intronic regions whereas both genes used in this study contain at least one intron. The *tri6* silencing studies resulted in down regulation of *tri6* and decrease in trichothecene production. Product encoded by *tri6* is a zinc finger transcription factor, and other general transcription factors replace it in the absence of TRI6 protein in the cell. However, Scherm *et al.* (2011) reported that *tri6* quelling in some experimental sets dramatically led to up regulation and/or increase in toxin production. Even if vector-based quelling provides development of stable silenced strains for generations, positive selection of these strains or isolates in population will be

time-consuming in disease management. Therefore, novel strategies have to be developed for quelling of *Fusarium* mycotoxins. Since siRNA-based quelling offers an alternative approach, Barnes *et al.* (2008) used siRNA delivery to protoplasts of *Aspergillus niger*. They successfully obtained down-regulated specific genes (*uidA* and *GUS*) in the species. But, they determined that RNA interference mechanism resulted in transient quelling instead of stable gene silencing. The siRNA transfection targeted to genes associated with mycotoxin production provided a powerful approach, in this study. *Tri4* expression was totally quelled in siRNA delivered *Fusarium* isolates. Similarly, *tri5* gene was also silenced up to 99% with siRNA. Both real-time PCR assay and TLC analysis showed absence of DON production.

Lysosome based quelling may provide a powerful tool in struggling with diseases. This study revealed that *tri4* and *tri5* genes can be targeted in the development of less DON-producing strains through RNAi in *F. graminearum* and *F. culmorum*. Although siRNA transfection lead to transient quelling, it was shown that the highest level of down-regulation could be obtained from this study.

Acknowledgments

The study was supported by Research Fund of Istanbul University by project number 23489. Authors are grateful to Dr. B. Tunalı for providing fungal material and Dr. P.J. Punt and Dr. H. Nakayashiki for plasmids.

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