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Tri4 and *tri5* Gene Expression Analysis in *Fusarium graminearum* and *F. culmorum* Isolates by qPCR

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Abstract: The *tri5* gene cluster is associated with production of trichothecene B type mycotoxins in *Fusarium graminearum* and *F. culmorum*. Multifunctional oxygenase and trichodiene synthase encoded by *tri4* and *tri5* genes, respectively, belonging to this cluster are responsible for production of trichothecenes. In this study, *tri4* and *tri5* gene expression levels were determined in 14 *F. graminearum* and 24 *F. culmorum* isolates causing head blight in wheat, maize and barley collected from different regions of Turkey. Isolates producing oxygenase and trichodiene synthase in high levels were distinguished from low-producers using two-step reverse-transcription comparative quantitative real-time PCR. Slope values were -3.63, -3.4 and -3.4 for amplification of *tri4*, *tri5* and *β-tubulin* genes, respectively. Cp values were ranged from 17.08±0.0 to 38.82±1.66. The highest *tri4* and *tri5* expression levels were determined in 4F isolate of *F. graminearum*. $\Delta\Delta$CT ratios were calculated as 0.1371 for *tri4* gene and 0.865 for *tri5* gene. However, maximum *tri4* and *tri5* expressions were detected in 20F ($\Delta\Delta$CT = 0.2196) and 9F ($\Delta\Delta$CT = 0.3761) isolates in *F. culmorum*, respectively. Quantification of definite toxin content can contribute to determination of pathogen infection grade and to gaining the information about fungal biomass on field crops and their products.

Key words: *Fusarium graminearum*, *Fusarium culmorum*, class B trichothecenes, *tri5* gene cluster, quantitative gene expression

INTRODUCTION

Mycotoxins are secondary metabolites produced by fungal organisms. *Fusarium* species produce several exotoxin types such as zearalenone, fumonisins, butenolide, trichothecenes etc. (Desjardins and Proctor, 2007). Class B trichothecenes include deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives. Cereals which were infected by *Fusarium* sp. are also contaminated with them. Moreover, their acute toxicity is harmful to health of human and animal consuming the contaminated foods (Goswami and Kistler, 2004; Foroud and Eudes, 2009; Asran and Amal, 2011).

Currently, techniques based on amplification of eleven genes located in *tri5* gene cluster are effectively used for chemotyping of *Fusarium* sp. producing trichothecene B (Chandler *et al*., 2003; Haratian *et al*., 2008; Yoruk and Albayrak, 2012). The eleven genes are separately responsible for catalysis of each step of the trichothecene B biosynthesis. Among them, *tri5* and *tri4* genes code trichodiene synthase and multifunctional oxygenase responsible for catalysing the first and second steps of biosynthetic pathway, respectively. Therefore, both of main genes are required for synthesis of trichothecenes. Determination of their expression levels is also essential for discrimination and identification of isolates (Doohan *et al*., 1999; Marin *et al*., 2010). Moreover, findings obtained from related studies contribute to understanding the importance of these genes in biochemical pathway. In addition, microbiological determination approaches such as HPLC (high performance liquid chromatography) or MS (mass spectrometry) are used to determine mycotoxins. Although results obtained from these approaches are reliable, cost of them is extremely higher than amplification based ones. Moreover, their throughputs are limited. Commercial ELISA (enzyme linked immunosorbent assay) based test systems are also used to determine toxin contents despite their low accuracy. Also, preparation of samples used in it is still time consuming (Ward *et al*., 2004; Gherbawy and Voigt, 2010).

Toxin biosynthetic key genes in *tri5* cluster are used in amplification by polymerase chain reaction (PCR) for the quantification of trichothecene B. To determine their gene expression levels is more reliable, inexpensive, laborious and precise than microbiological determination approaches. Besides, it is possible to detect the mycotoxins even if pathogen produces only a little

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Table 1:Geographic origin, hosts and gene expression values of *F. graminearum* and *F. culmorum* isolates used in this study

				ΣCp			ΣΔΔCT	
Isolate	Species	Host	Origin	*tri4*	*tri5*	β-*tubulin*	*tri4*	*tri5*
F5	Fg	Wheat	Sakarya	25.82±0.2	25.14±0.08	22.21±0.06	8.20E-02	0.131
F6	Fg	Wheat	Sakarya	25.67±0.17	24.94±0.03	22.28±0.38	9.54E-02	0.161
F7	Fg	Wheat	Sakarya	25.83±0.06	24.86±0.04	21.96±0.14	6.84E-02	0.133
F8	Fg	Wheat	Sakarya	26.17±0.29	25.88±0.1	22.07±0.48	5.54E-02	0.169
F9	Fg	Wheat	Balikesir	25.94±0.03	25.19±0.21	22.8±0.23	0.1135	0.195
1F	Fg	Wheat	Bolu	25.85±0.17	25.13±0.05	22.76±0.16	0.117	0.194
2F	Fg	Wheat	Çankiri	25.85±0.07	23.84±0.29	21.86±0.71	6.27E-02	0.295
3F	Fg	Maize	Samsun	28.15±0.23	27.38±0.22	25.14±0.03	0.1243	0.214
4F	Fg	Barley	Bolu	26.25±0.61	25.54±0.24	23.3±0.22	0.1371*	0.865*
5F	Fg	Maize	Samsun	25.59±0.57	24.69±0.13	21.84±0.0	7.43E-02	0.138
6F	Fg	Maize	Samsun	27.49±2.54	38.82±1.66	21.89±0.14	2.07E-02	1.14E-02
7F	Fg	Maize	Samsun	26.36±0.24	24.96±0.03	22.79±0.19	8.41E-02	0.226
14F	Fg	Wheat	Kastamonu	25.95±0.95	27.6±4.26	22.48±0.00	8.41E-02	1.78E-03
15F	Fg	Wheat	Sakarya	25.44±0.41	25.13±0.2	24.8±0.00	7.73E-02	8.10E-02
F1	Fc	Wheat	Marmara	24.4±0.59	22.12±0.04	20.81±0.15	8.25E-02	0.339
F2	Fc	Wheat	Marmara	21.14±1.46	22.03±0.0	17.52±0.43	8.11E-02	0.329
F3	Fc	Wheat	Konya	23.56±0.0	22.05±0.0	20.89±0.13	0.1568	0.3222
F4	Fc	Wheat	Marmara	23.91±0.12	21.79±0.25	21.06±0.17	0.1387	0.3479
F10	Fc	Wheat	Bilecik	23.58±0.67	22.14±0.07	20.54±0.2	0.1219	0.3076
F12	Fc	Wheat	Balikesir	23.76±0.19	22.41±0.1	20.61±0.16	0.1122	0.2916
F14	Fc	Wheat	Bilecik	24.17±0.15	22.26±0.15	20.88±0.2	0.1025	0.2621
F15	Fc	Wheat	Sinop	23.42±0.06	22.42±0.2	20.71±0.0	0.1527	0.2941
F16	Fc	Wheat	Konya	23.69±0.08	22.3±0.36	20.76±0.08	0.1312	0.2657
F17	Fc	Wheat	Konya	23.36±0.33	22.17±0.17	20.68±0.09	0.1561	0.2924
F19	Fc	Wheat	Konya	23.92±0.2	22.29±0.19	20.5±0.04	9.37E-02	0.2853
F20	Fc	Wheat	Bilecik	23.32±0.29	22.24±0.06	20.37±0.08	0.1289	0.3105
F21	Fc	Wheat	Usak	23.99±0.41	22.75±0.28	20.99±0.44	0.1251	0.336
F24	Fc	Wheat	Konya	23.69±0.13	22.32±0.08	20.74±0.0	0.1297	0.3449
8F	Fc	Wheat	Ankara	35.87±0.52	33.03±0.28	24.49±0.16	3.74E-04	2.38E-03
9F	Fc	Wheat	Isparta	28.1±0.11	18.48±0.45	22.49±0.11	2.05E-02	0.3761*
10F	Fc	Wheat	Samsun	19.32±0.28	27.05±0.17	17.08±0.0	0.212	2.59E-04
11F	Fc	Wheat	Çorum	33.67±0.45	32.11±0.19	22.7±0.0	4.99E-04	1.89E-03
12F	Fc	Wheat	Amasya	33.09±0.68	31.28±0.95	23.59±0.02	1.38E-03	1.04E-02
13F	Fc	Wheat	Konya	32.16±0.28	28.0±0.38	22.14±0.03	9.63E-04	1.92E-02
17F	Fc	Wheat	Ankara	31.06±0.07	29.99±0.36	23.11±0.17	4.04E-03	8.77E-03
18F	Fc	Wheat	Eskisehir	31.06±0.11	29.65±0.04	23.55±0.22	5.49E-03	1.98E-02
19F	Fc	Wheat	Eskisehir	31.44±0.3	30.45±0.55	23.14±0.09	3.16E-03	2.09E-02
20F	Fc	Barley	Dinar	23.19±0.06	18.98±0.15	21.0±0.38	0.2196*	0.287

*Maximum gene expression, Fg: *Fusarium graminearum*, Fc: *Fusarium culmorum*

Table 2: Primer and prob sets used in this study

Code	Forward primer (5'-3')	Reverse primer (5'-3')	Prob (5'-3')	Product size
Tri4	atggatgaaaggctcgaggt	actgtcggtgcttttgacg	-	139
Tox5*	gctgctcatcactttgctcag	ctgatctggtcacgctcatc	-	658
FusTbl	gaagccattgatgttgttcgt	tccgaccatgaagaagtgaag	-	465
Tri4P	gcgagaggatactggtcgtc	aagaagctccgagaggagttg	ggaggctg	63
Tri5P	caatcgttggcatggttgta	atagatccgccatgcactct	ctgggcaa	62
FusTbl	ggtttccaaatcacccactc	tcaacagggtacccataccg	ggtggtgg	61

*Primers designed by Hue *et al.* (1999)

amount of them. Therefore, quantification of trichothecene B type mycotoxins is a basic and powerful approach for getting knowledge about activity of genes located in that cluster (Kimura *et al.*, 2003; Alexander *et al.*, 2011).

In this study, *tri4* and *tri5* gene expression analysis were carried out in 14 *F. graminearum* and 24 *F. culmorum* isolates causing head blight in wheat, maize and barley collected from different regions of Turkey by using a two-step reverse-transcription comparative real-time PCR.

MATERIALS AND METHODS

14 *F. graminearum* and 24 *F. culmorum* monosporic isolates (Leslie *et al.*, 2006) were kindly provided from Dr. Berna Tunali, Department of Plant Protection, Agricultural Faculty, Samsun On Dokuz Mayis University. Collection region, hosts and gene expression values are listed in Table 1.

Fungal isolates were grown in potato dextrose agar (PDA) plates at 25°C for 7 days. Total RNA extracted from 7-day-old mycelium by using TriPure isolation reagent

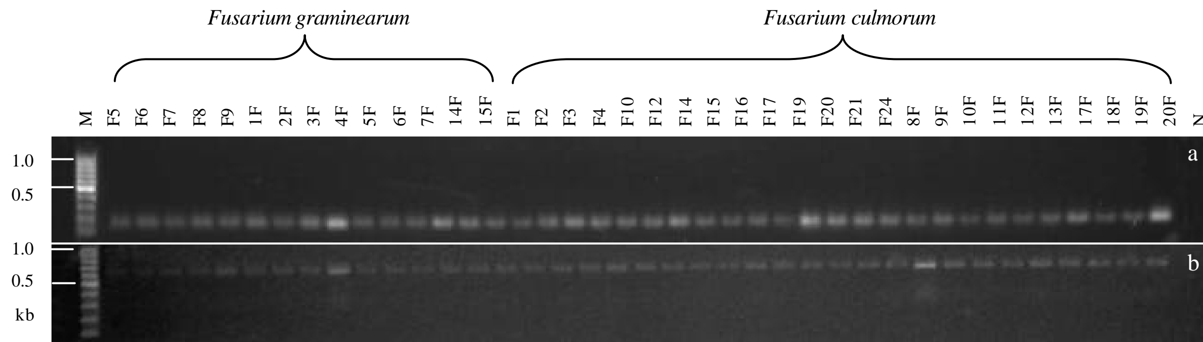

Fig. 1(a-b): Tri4 gene amplicons by (a) tri4 primers and (b) tri5 gene bands using Tox5 primers in all isolates by RT-PCR. M: 100 bp DNA ladder (Thermo), N: Negative control (no template)

s(Roche, Switzerland) including phenol and guanidine thiocyanate. Quantity of total RNA was assessed by Nanodrop 2000C. Complementary DNA (cDNA) synthesis was carried out in a reaction volume of 25 μL containing: 1 μg total RNA, 60 μM random hexamer, 1X reaction buffer, 5 μM DTT, 1U protector RNase inhibitor, 1 mM dNTPs and 1U transcriptor HF reverse transcriptase. cDNA synthesis was performed in thermal cycler (Bio-Rad, France) at 65°C for 10 min, 55°C for 30 min and 85°C for 5 min.

For gene expression analysis, primers and probe sets (Table 2) were designed using primer3 (Rozen and Skaletsky, 2000) and universal probe library online services (www.rocheappliedscience.com) to amplify *tri4*, *tri5* and β-*tubulin* genes. qPCR assays were performed in Light Cycler 480 II system (Roche, Switzerland) using Taq-Man probes and conducted in a reaction volume of 20 μL containing: cDNA amount corresponding to 1 μg total RNA, 1X probe master mix, 2pmol probe and 4pmol of each primers. β-*tubulin* gene was used as house keeping gene. Cycling conditions consisted of pre-denaturation at 95°C for 10 min; followed by 45 cycles at 95°C for 10 s, at 58°C for 30 s, at 72°C for 1 s. For each experiment set, standard series with a range of three dilutions were prepared in the determination of slope values. Crossing point values (Cp) were determined for three genes in all isolates. The *tri4* and *tri5* gene expression levels were normalized according to the β-*tubulin* gene and target/reference ratio results. Each experiment sets were replicated at least three times.

For confirmation of quantitative gene expression results, three times replicated RT-PCR assays were performed using cDNA molecules of 38 isolates to amplify each of three genes. PCR was carried out in a volume of 25 μL comprised of cDNA amount corresponding to 1 μg total RNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM of dNTPs, 10 pmol of each primer and 1U of Taq DNA polymerase (Promega, USA). Tox5 primer set designed by Hue *et al*. (1999) and Tri4 and FusTb1 primers designed (by us for the first time) in this study were used in the amplification of *tri5*, *tri4* and β-*tubulin* genes, respectively (Table 2). PCR conditions were performed at 94°C for 5 min; 35 cycles at 94°C for 45 s, at 58°C for 45 s, at 72°C for 45 s and at 72°C for 5 min.

Furthermore, amplification of *tri5, tri4* and β-*tubulin* genes in the genomic DNA's was performed using Tox5, Tri4 and FusTb1 primers, respectively, in a volume of total 25 μL. The procedure was followed RT-PCR described previously, except for using genomic DNA as template (50 ng). RT-PCR and PCR amplification products were separated by electrophoresis in 1.5% agarose gels and visualized using gel visualization system (Avegene, X-lite 200).

RESULTS

Total RNA molecules with high quality (A_{260}/A_{280} = 1.9-2) and quantity (1.5-2 μg μL^{-1}) were isolated from 38 fungal samples. Expressions of *tri4* and *tri5* genes together with housekeeping gene (β-*tubulin*) in all isolates were detected by RT-PCR and qPCR techniques.

A 139 bp *tri4* amplicon (Fig. 1a) and 658 bp fragment (Fig. 1b) belonging to *tri5* gene were yielded in all isolates by RT-PCR analysis. The most strong amplification products were obtained from 4F and 20 F isolates for *tri4* and also from 4F and 9F for *tri 5* genes (Fig. 1a and 1b). In addition, 465 bp long common bands of β-*tubulin* were observed in 38 isolates with a similar banding pattern (data not shown).

In qPCR analysis, Cp and slope values and target/reference ratios were calculated according to signal peaks (Table 1). Slope values were -3.63, -3.4 and -3.4 for amplification of *tri4*, *tri5* and β-*tubulin* genes, respectively. That the slope values reach to -3.3 indicated expression levels of three genes as valuable. ΣCp values for *tri4* were calculated ranged from 19.32±0.28 to 35.87±0.52. ΣCp values for *tri5* gene were found as 18.48±0.45-38.82±1.66 whereas values for β-*tubulin* were between 17.08±0.0 and 25.14±0.03. As shown in Table 1, the highest *tri4* and *tri5* expression levels were obtained from 4F isolate of *F. graminearum*. Σ△△CT ratios were calculated 0.1371 for *tri4* and 0.865 for *tri5* gene amplifications. However, maximum *tri4* and *tri5* levels were detected in 20F (Σ△△CT=0.2196) and 9F (Σ△△CT=0.3761) isolates of *F. culmorum*, respectively. It is clear that RT-PCR and qPCR findings verify each other.

Presence of the *tri4*, *tri5* and β-*tubulin* genes were confirmed with standard PCR assay. Tri4 primer pair amplified 139 bp amplicons in all isolates (data not shown). Tox5 primer pairs produced 658 bp DNA fragment while FusTb1 yielded 465 bp amplicons (data not shown). Thus, the expression profile obtained in this study is reliable.

DISCUSSION

There is not any definite struggle strategy to be used in the *Fusarium* infections of field crops. Therefore, it seems to be difficult to overcome the *Fusarium* problems in agriculture in the near future. So, detection of mycotoxins via chemotyping has become an important strategy to avoid them in the food chain (Wilbert and Kemmelmeier, 2003; Gherbawy and Voigt, 2010). Activation of eleven genes shows differences from one strain to another even when they located in *tri5* cluster. Expression of some *tri* genes is definitely required for production of mycotoxins. However, a number of strains contain them as pseudogene or completely deleted ones (Chandler *et al*., 2003; Kim *et al*., 2003; Kimura *et al*., 2003; Alexander *et al*., 2011). In that situation, mycotoxin can still continue to be produced. Scherm *et al*. (2011) determined an increase of trichothecene production in silenced *tri6* gene samples when they are compared to control groups. The results of this study indicate that some genes found in that cluster are not essential in the production of mycotoxins. For trichothecene B production, determining the gene expression and expression levels of each gene is very important.

Fungal microorganisms producing high levels of definite mycotoxins can be discriminated from non-producers and also low-producers. For this purpose, quantification of target genes encoding those mycotoxins and their expression levels can now be carried out by qPCR assay. For the first time, Schnerr *et al*. (2001) applied this assay for quantification of trichodiene synthase in 30 wheat samples. They showed PCR based method was as useful as microbiological determination approaches.

In Turkey, genotyping of *F. graminearum* and *F. culmorum* isolates was done by different research groups (Arici and Koc, 2010; Gurel *et al*., 2010; Yoruk *et al*., 2011, 2012). Chemotypes for trichothecene B in both of two species, which are associated with especially head blight, were studied with HPLC and PCR analysis (Tunali *et al*., 2006; Yoruk and Albayrak, 2012; Mert-Turk and Gencer, 2013).

In this study, expression of *tri4* and *tri5* genes, encoding oxygenase and trichodiene synthase respectively, were analyzed from 38 local *Fusarium* isolates using two-step transcription comparative quantitative real-time PCR. The results indicated that different amount of mycotoxins were produced by different isolates, although they may contain the same gene(s). Also, isolates have maximum gene expression profiles were distinguished from low-producers. In some countries, proposed mycotoxin limits are determined in food and feed products. Our results could be useful for that purpose if there were legislative limitations for trichothecenes in Turkey. Quantification of definite toxin content can be also useful in the evaluation of infection grade. Mycotoxin quantification can inform us about fungal biomass and their products on field crops. Moreover, results obtained from similar studies can be used in routine monitoring programs associated with disease controlling.

Expression analysis of genes responsible for class B trichothecene production in phytopathogenic *Fusarium* species was carried out in this study. Trichothecene producer isolates were easily distinguished from low-producers by qPCR assays in a short time. This kind of studies allow pre-selection of isolates which produce high level of trichothecenes in order to use further investigation such as gene disruption, gene silencing etc. Although all isolates possess the *tri4* and *tri5* genes, significant differences in gene expression profiles were shown. Isolate selection by distinguishing producers and also high-producers from low- and non-producers is so important in plant pathology. Therefore, selection of trichothecene producers and determination of gene expression levels are efficient strategies especially in quelling. This paper report will useful for wide range of researchers in plant pathology.

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