Brief Report

Expression Analysis of *PKS13*, *FG08079.1* and *PKS10* Genes in *Fusarium graminearum* and *Fusarium culmorum*

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Received: October 10, 2014; **Revised:** March 14, 2015; **Accepted:** April 19, 2015

Background: Identification and quantification of mycotoxins produced by *Fusarium* species are important in controlling fungal diseases.
Objectives: Potential of zearalenone, butenolide and fusarin C production was investigated in five *Fusarium graminearum* and five *F. culmorum* isolates at molecular level.
Materials and Methods: Presence of *PKS13*, *FG08079.1* and *PKS10* genes, associated with production of zearalenone, butenolide and fusarin C, respectively, were confirmed by PCR. In addition, expression levels of them together with housekeeping gene (*β-tubulin*) were detected by real time PCR.
Results: *PKS13* and *FG08079.1* transcripts were determined in all isolates, while *PKS10* specific primers failed to amplify any product, indicative of no expression. ΔΔCT of *PKS13* was ranged between 1.79E-03-3.97E-03 and for *FG08079.1* was between 0.25E-03 and 6.02E-03. The highest *PKS13* expressions were 3.86E-03 in *F. graminearum* F9 and 3.97E-03 in *F. culmorum* F16. Maximum *FG08079.1* expressions were calculated as 6.02E-03 and 3.81E-03 in *F. graminearum* 2F and *F. culmorum* F2, respectively.
Conclusions: We revealed that ten *Fusarium* isolates produced zearalenone and butenolide under culture conditions. However, fusarin C was not generated by them in these conditions.

Keywords: *Butenolide*; *Fusarium culmorum*; *Fusarin C*; *Fusarium graminearum*; Zearalenone

1. Background

Mycotoxins are fungal secondary metabolism's products with low-molecular weight. Fungi producing mycotoxins are well-defined at genera level (1). *Fusarium* is well known genus infecting considerable plant species especially small-grain cereals (2, 3). *Fusarium graminearum* and *F. culmorum* are common mycotoxin producers. Their mycotoxins can be divided into two classes as: (i) Major consisting of trichothecenes, fumonisins, zearalenones, and (ii) minor including beauvercin and enniatins, equisetin, fusarins, butenolide (4).

Both *F. graminearum* and *F. culmorum* have the ability to produce zearalenone (ZEN), butenolide (BUT) and fusarin C (FUS C) mycotoxins (4). ZEN is a nonsteroidal estrogenic endotoxin synthesized from gene cluster that of 25 kb. Cluster consists of nine genes. *PKS4* and *PKS13* encoding two different polyketide synthases (PKS) are essential for ZEN production. Disruption of these genes resulted in loss of ZEN production (5, 6). BUT is a water-soluble endotoxin and produces by a gene cluster with eight genes on a contig, namely 1.324. Gene disruption and add-back approaches indicated that *FG08079.1* gene in the cluster is essential for BUT production. According to sequence similarity, FG08079.1 appears to be a cytochrome P450 monooxygenase, while the function of other 7 genes remained elusive (7). FUS C is one of the fusarin stereoisomers. FUS C biosynthetic pathway is controlled by cluster of nine genes reported in *F. fujikuroi*. The *fus1* is essential in production and its product is a PKS. It was shown that *PKS10* in *F. graminearum* was homologous to *fus1* of *F. fujikuroi* (8, 9).

Detection of mycotoxins within crops and their products is very important for human, animal and plant

health. Several conventional methods are effectively used for determination of mycotoxins. High-performance liquid chromatography/mass spectrometry (HPLC/MS), gas chromatography-mass spectrometry (GC-MS), liquid chromatography with tandem mass spectrometry (LC-MS/MS) and enzyme linked immunosorbent assay (ELISA) are methods regularly being used for mycotoxin determination and sometimes quantification. However, these techniques are labour intensive, time consuming and expensive. Instead of using above direct methods in identification and determination of the presence of mycotoxins, one may evaluate the presence of genes involved in biosynthesis of such substances via PCR and further investigate their expression via Real-Time PCR (10).

2. Objectives

Expression of *PKS13*, *FG08079.1* and *PKS10*, the key players of zearalenon, butenolide and fusarin C (FUS C) production, were analysed. It was aimed to determine mycotoxin production potential of *F. graminearum* and *F. culmorum* isolates and relative quantification of expression of the genes.

3. Materials and Methods

3.1. Fungal Isolation and Culture

F5, F8, F9, 1F, 2F, F2, F12, F15, F16 and F19 isolates was obtained from single-spore cultures (11). Isolates (Table 1) were kindly provided from Prof. Berna Tunali, at Samsun Ondokuz Mayis University, Plant Protection Department of Agriculture Faculty. Isolates were grown at 25°C on potato dextrose agar (PDA).

3.2. Polymerase Chain Reaction

Genomic DNA was extracted from 100 mg of 7-day-old fresh mycelium using CTAB method with minor modifications (12). Total RNA was extracted using Roche TriPure Reagent (Roche, Switzerland) that used for cDNAs synthesis using Roche cDNA synthesis kit (Roche, Switzerland) following to manufacturer's protocol.

UBC85F/R (5′-ctccggatatgttgcgtcaa-3′/5′-ggtaggtatccgacatggcaa-3′) and OPT18F/R (5′-atggtgaactcgtcgtggc-3′/5′-cccttcttacgccaatctcg-3′) primer sets were used in amplification of SCAR85 marker specific for *F. graminearum* and SCAR2-35 for *F. culmorum*, respectively (13). PCR cycling and conditions were maintained as described by Schilling *et al*. (13) in a thermocycler (Bio-Rad-T100, USA).

ZENF/R (5′-ggtagcgataacgtggagga-3′/5′-gactcaaaggtgctcggttc-3′), BUTF/R (5′-tcattgaccgagctttctga-3′/5′-ctccatcgtgtcgtctctca-3′) and FUSCF/R (5′-ttccccctgtacgattcaac-3′/5′-tcattgaccgagctttctga-3′) primers were used for amplification of *PKS13* (Acc. no: AY495638.1), *FG08079.1* (Acc. no: FG08079.1) and *PKS10* (Acc. no: AY495635.1) genes, respectively. PCR mixing were carried out as previously described by Yörük and Albayrak (14). The thermal cycling conditions were applied as 40 cycles of 94°C for 1 min, 55-57°C for 1 min and 72°C for 2 min. Prior to cycling, PCR tubes were incubated at 95°C for 5 min, and the cyclings were finished with at 72°C for 10 min Standard Taq DNA polymerase enzyme kit (Promega, USA) was used in each PCR sets.

3.3. Real-Time PCR

Real-time PCR assays were carried out in

Table 1. SCp and SDDCT values of *F. graminearum* and *F. culmorum* isolates

Isolate	Location	ΣCp			ΣΔΔCT	
		FG08079.1	***PKS13***	**β-tubulin**	***FG08079.1***	***PKS13***
F5*	Sakarya	32,18±1,14	31,42±0,05	22,93±0,34	1,64E-03	2,79E-03
F8*	Sakarya	30,99±0,07	31,52±0,59	23,28±0,034	4,78E-03	3,30E-03
F9*	Balikesir	29,86±0,22	29,80±0,07	21,78±0,20	3,68E-03	3,86E-03
1F*	Bolu	31,46±0,33	30,97±0,25	22,82±0,04	0,25E-03	3,51E-03
2F*	Cankiri	30,93±0,03	31,68±0,22	23,55±0,38	6,02E-03	3,58E-03
F2**	Marmara	31,32±0,43	31,89±0,19	23,28±0,07	3,81E-03	2,57E-03
F12**	Balikesir	31,71±0,09	31,67±0,10	22,54±0,02	1,75E-03	1,79E-03
F15**	Sinop	31,27±0,01	32,07±0,009	22,68±0,06	2,59E-03	1,49E-03
F16**	Konya	31,09±0,18	30,96±0,11	22,99±0,16	3,64E-03	3,97E-03
F19**	Konya	31,78±0,81	31,84±0,32	22,78±0,15	1,95E-03	1,87E-03

*means isolates belonging to *F. graminearum*, **means isolates belonging to *F. culmorum*

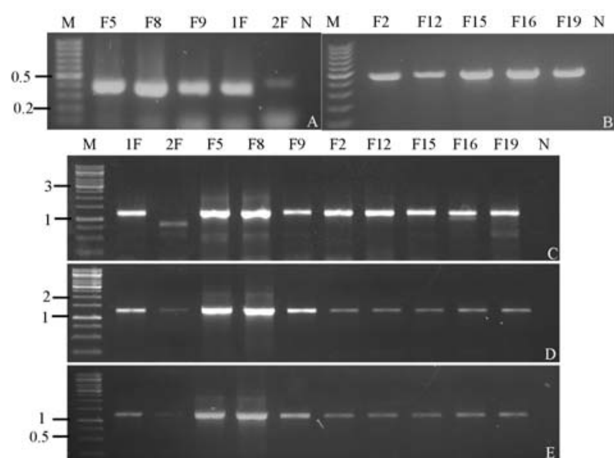

Figure 1. PCR amplification products A: of 332 bp with UBC85F/R primer in *F. graminearum* isolates B: of 472 bp with OPT18F/R primer in *F. culmorum* isolates C: of 1213 bp long with ZEAF/R primer D: of 1551 bp with BUTF/R primer and E: of 1296 bp with FUSCF/R primer in all isolates. M: 1 kb DNA size marker (Thermo, USA), N: no template control

LightCycler® 480 II (Roche, Switzerland) system using SYBR Green qPCR kit (Thermo, USA). The qPCR experiments were conducted in a reaction volume of 16 µL containing 1× SYBR Green mix, 4 pmol of each primer, cDNA amount corresponding to 500 ng RNA. *β-tubulin* gene was amplified with TUBF/R primers (5′-gaagccattgatgttgttcgt-3′/5′-tccgaccatgaagaagtgaag-3′) as internal control. Cycling conditions were followed as; 95°C for 5 min, 40 cycles of 95°C for 10 s, 55°C for 10 s, 72°C for 10 s, and final extension at 72°C for 10 min. Also, melting curve conditions were carried out to analyse the accuracy of real time PCR. Each experiment sets were replicated at least thrice. Standard curves were formed by cDNA series of 1:1/1:4/1:16/1:64 for each target genes and also for *β-tubulin*. According to the software, crossing point (ΣCp) values and target/reference ratios (ΣΔΔCT) were calculated.

4. Results

According to species specific DNA marker amplifications, five isolates (F1, F2, F5, F8 and F9) produced a common band of 332 bp with UBC85 primer pair (Figure 1 A). They were confirmed as *F. graminearum*. Gene fragment of 472 bp in length were produced with OPT18 primers in remaining isolates (Figure 1 B). It was revealed that they (F2, F12, F15, F16 and F19) belonged to *F. culmorum*.

PKS13 was amplified in all isolates as a band of 1213 bp long (Figure 1 C) except for *F. graminearum* 2F (850 bp). They yielded a common fragment of 1551 bp belonging to *FG08079.1* gene region (Figure 1 D). Amplification products of PKS10 of 1296 bp in length were obtained from all isolates (Figure 1 E). It was demonstrated that three gene sequences were carried by *F. graminearum* and *F. culmorum* isolates.

Different expression levels of *PKS13* and *FG08079.1* were detected in all isolates whereas no signal peaks were recorded for *PKS10* gene (Table 1). Also, *β-tubulin* expression levels were calculated for all isolates (Table 1). ΣCp values of *FG08079.1* and *PKS13* were found as 29.86 ± 0.22-32.18 ± 1.14 and 29.80 ± 0.07-32.07 ± 0.009, respectively. Values were between 21.78 ± 0.20-23.55 ± 0.38 for *β-tubulin*. After normalization, the highest gene expressions were recorded in 2F isolate of *F. graminearum* for *FG08079.1* and F16 isolate of *F. culmorum* for *PKS13*. While ΣΔΔCT values for *PKS13* were ranged from 1.79E-03 to 3.97E-03, the values calculated between 0.25E-03 and 6.02E-03 for *FG08079.1*.

5. Discussion

Detection of phytopathogenic fungal species and their mycotoxins is crucial in development of plant disease control strategies. Conventional and molecular marker techniques are currently used in species-specific diagnostics and determination of mycotoxin types. *Fusarium* isolates (10) was identified by SCAR marker amplification and were determined as *F. graminearum* and *F. culmorum*.

Mycotoxin types and their quantity are important in the controlling of *Fusarium* diseases. To get information about genes associated with mycotoxin production is also essential. Since genes involved in biosynthesis of ZEN, BUT and FUS C are known (4). It is possible to predict possibility of the production of these toxins via expression analysis of the genes. *PKS13*, *FG08079.1* and *PKS10* genes were investigated at genomic and transcriptomic levels. Routine tests for trichothecene and zearealenone detection in cereals have been used. There is no any kit for butenolide and FUS C detection.

ZEN production can increase by high temperature as well as plant-pathogen interaction. But, this effect may vary depending on strain. The mycotoxin generally accumulates during growth phase and stays stabile on crops and their products. Our results showed that isolates included in the study produced ZEN mycotoxin under optimal culture conditions without any plant infection and/or stress factor. Moreover, *F. graminearum* 2F isolate contained *PKS13* gene in a rather dif-

ferent amplicon size (850 bp). This may suggest the presence of other variants of this gene as seen for trichothecene (14). Slight differentiation among gene expression profiles of isolates may support this argument. Nearly the same expression patterns were obtained among isolates even when the differences were statistically significant ($p < 0.05$)

The *FG08079.1* is expressed during cell growth phase and in early stage of plant infection (7, 15). Here, *FG08079.1* was determined in all isolates under optimal culture conditions. It was noted an almost 24-fold increase in BUT production in 1F as opposed to 2F, isolates of *F. graminearum*. However, in isolates of *F. culmorum*, F2 was greater than F12-by only 2 fold. Expression levels of both *PKS13* and *FG08079.1* could provide information about aggressiveness of *Fusarium* isolates.

Despite the fact that *PKS10* present in isolates of both fungal species, no expression of the gene was detected under optimal culture conditions. It was reported that FUS C was actively synthesized during growing mycelia, under high nitrogen concentrations or acidic conditions (8, 16). Therefore, the provided condition most likely was not suitable to induce the expression of *PKS10*. Investigation other genes involved in fusarin gene cluster could be useful in FUS C production analysis. The polyketide synthase, encoded by *fusA*, contain ten domains and four are characteristics of NRPS (nonribosomal peptide synthase) domain (16). To select the NPRS instead of *PKS10* could be useful for detection of fusarin production.

Relative quantification values of *PKS13* and *FG08079.1* were under "1", which was accepted as standard gene expression value for positive calibrator. These expression values were about $\times 10^{-3}$, smaller than the reference gene. These findings are indicative of low abundance of *PKS13* and *FG08079.1* associated with production of related mycotoxins. Moreover, these results obtained from optimal culture conditions would indirectly indicate mycotoxins levels produced by pathogens in non-inducing condition. Differentiation of isolates as mycotoxin producer or non-producer is carried out in a short time by using qPCR analysis. Our data showed that molecular approaches used in determination of mycotoxin type were efficient as much as conventional approaches. At the same time, it was seen that mycotoxin amount could be sensitively determined and compared by using real-time PCR.

Occurrence of various endotoxins (aflatoxin, T-2 toxin, DON, ZEN, and fumonisin) except BUT and FUS C have been reported by using conventional methods like HPLC and ELISA in Turkey (17, 18). Chemotyping studies of *F. graminearum* and *F. culmorum* isolates are focused on class B trichothecene detection (14, 19). But, there is no studies for ZEN, BUT and FUS C chemotyping in Turkey. Number of chemotyping investigation on these three mycotoxins has been also restricted worldwide when compared to class B chemotyping. To our knowledge, this is the first report to determine the potential of *Fusarium* isolates to produce ZEN, BUT and FUS C. Finding obtained from similar studies could contribute to evaluate of infection grade and to improve of disease control strategies.

Acknowledgements

The project was partially supported by Research Found of Istanbul University by project number 23655.

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