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Isolation and Characterization Of Novel Egyptian Regulatory Gene Tri 10 From *Fusarium fujikuroi* (MG211161)

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Abstract

The Egyptian isolate *Fusarium fujikuroi* (MG211161) has been previously isolated and showed high accumulation of T-2 toxin. In the present study, Tri10 gene fragment was isolated and characterized from *Fusarium fujikuroi.* A regulatory gene that control the pathway of cluster genes (Tri 3, Tri 5, Tri 4, Tri 8, Tri 6 and Tri 7) to produce T-2 toxin. The gene fragment was PCR amplified using nested primer. Nucleotide sequence analysis indicated 522 base pair fragment, that showed 98% similarity with the gene isolated from *F. fujikuroi* (HF679024.1). Amplified sequence was submitted to the gene bank and registered with the accession number (MG674224).

Keywords: Fusarium, Tri 10 gene, Nested PCR

Introduction

Nested polymerase chain reaction (Nested PCR) is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites [**1].** Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product. This allows amplification for a low number of runs in the first round, limiting non-specific products. The second nested primer set should only amplify the intended product from the first round of amplification and not non-specific product. This allows running more total cycles while minimizing non-specific products, which is useful for very rare templates or PCR with high background [**2]**.

[**3]** summarized the current understanding of the pathway of biosynthesis, the function of cloned tri gene and evolution of Tri gene, focusing on *Fusarium* species. Following the isolation of Tri5 gene encoding the first enzyme trichodine synthase, 10 biosynthetic gene (two regulatory gene, seven pathway gene and one transporter gene were functionally identified in the Tri 5 gene cluster. At least three pathway gene, Tri 101 separated alone, tri 1 and tri 16 (located in Tri 1 and tTri 16 two gene cluster), were found outside of Tri 5 gene cluster.

[**4]** studied some fungal genes for trichothecene biosynthesis (Tri genes) are known to be under control of transcription factors encoded by Tri 6 and Tri 10.

[**5]** reported the characterization of Tri10, a novel regulatory gene within the trichothecene gene cluster. Comparison of Tri 10 genomic and mRNA sequences revealed that removal of a single 77-bp intron provided a 1,260-bp open reading frame, encoding a 420-amino-acid protein. Disruption of Tri 10 in *Fusarium sporotrichioides* abolished T-2 toxin production and dramatically decreased the transcript accumulation for four trichothecene genes (Tri 4, Tri 5, Tri 6, and Tri 101).

[**5]** suggested a model in which Tri 10 acts upstream of the cluster encoded transcription factor Tri 6 and is necessary for full expression of both the other trichothecene genes and the genes for the primary metabolic pathway that precedes the trichothecene biosynthetic pathway, as well as for wild-type levels of trichothecene self-protection.

[**6]** investigated Tri 10, a regulatory gene in trichothecene mycotoxin-producing *Fusarium* species, is required for trichothecene biosynthesis and the coordinated expression of four trichothecene pathway-specific genes (Tri 4, Tri 5, Tri 6, and Tri 101) and the isoprenoid biosynthetic gene for farnesyl pyrophosphate synthetase (FPPS). They showed that Tri 10 regulates six more trichothecene genes (Tri 3, Tri 7, Tri 8, Tri 9, Tri 11, and Tri 12).

2. Materials and methods

2.1 RNA extraction and purification

Pure isolates of *Fusarium fujikuroi* (MG211161) were grown on broth slant PDA medium for 3 days in an incubator at 25°C, then RNA were extracted according to protocol of Thermo Scientific kit. Purified RNA was stored at - 70°C until further use. The RNA were quantified by Nanodrop Spectrophotometer to assess the concentration and purity of nucleic acids. cDNA was constructed from RNA using Biolab Reverse Transcriptase kit.

2.2 Construction of primers

A set of new species-specific primers was designed using PRIMER3 tools (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) based on GenBank sequence isolated of *Gibberella pulicaris* (AF386074.1) type B (Tri 10) gene, complete cds, *Fusarium sporotrichioides* (AF359360.3) trichothecene gene and *Fusarium sp.* (AF364179.1) FRC R-06979 regulatory protein (Tri 10). The specificity of the primers was also validated by BLAST software for searching the primer sequences (http: //www.ncbi.nlm.nih. gov/BLAST). The list of nested primers *Fusarium fujikuroi* (MG211161).

The primers for the first PCR round were: Forward Primer Tri 10: (AAGCTTGTACTACCTGGACGTCGTGTTTC), Reverse Primer Tri 10: (GATATCAGCTGAATGTCGCTTCATAAGAC), Forward Primer Tri 10 (inner): (GCCGTGGGGAAAAGAGAGTGGTTG) and Reverse Primer Tri 10 (inner): (GCCGTTCTTGCACCCCAGTACCTC).The designed primers were synthesized at LGC biosearch technology, USA and obtained commercially.

2.3 PCR conditions

The two rounds nested PCR amplification using primers (F-Tri10 /R-Tri10) for first round and two set of Tri10 inner primer based on *Fusarium* species-specific primers [F-Tri10 (inner)/ R-Tri10(inner)] was used for second round. Thus, The PCR mastermix consisted of 1µl forward primer (F-Tri10), 1 µl reverse primer (R-Tri10), promgea tag 0.25ul, $MgCl_2$ 2.5ul, buffer 2.5ul , buffer green 2.5ul , dNTPs 1ul and

DNA 2ul for 25 ul PCR mixture. PCR was performed on Thermal Mastercycler, Multi Gene, Gradienta. The PCR program was as follow: first denaturation step at 95°C for 2 min, 35 cycles 95°C for 30 min (denaturation), 53°C for 1.30 min (annealing), and 72°C for 1 min (extension), while the final extension step was at 72°C for 5 min. Second PCR round master mix was performed in 25 µl of PCR mixture, including 1 µl of PCR product of first PCR round, two different set of primers 1µl F-Tri10–(inner) and 1ul of R-Tri 10 (inner), a 1µl mixture containing of each dNTPs and 0.25 U *Taq* DNA polymerase (Promgea). The PCR program was as follow: 95°C for 2 min (first denaturation step), 35 cycles of 95°C for 30 min (denaturation), 59°C for 1 min (annealing), and 72°C for 1 min (extension), while the final extension at 72°C for 5 min.

2.4 Agarose Gel Electrophoresis

PCR products obtained from the first PCR round were separated on 1% agarose gels in 0.5 M Tris Borate EDTA (TBE) buffer (pH 8) at room temperature at a constant voltage of 80 V for 60 minutes. While, PCR products obtained from the second round were separated on 1.5% agarose gels. This standard technique was used to separate, and identify purified DNA fragments via electrophoresis through an ethidium bromide (0.5 µl ml^{-1}) stained agarose. The gels were analyzed and photographed using gel documentation system (Alpha-chem. Imager, USA).

2.5 PCR product purification

PCR product purification was carried out according to PCR clean up system, promgea, wizard R SV gel protocol.

2.6 Sequencing of nested Tri 10 gene fragment

DNA sequencing was carried out by Macrogen Inc., Korea. The nested gene sequences were blasted for comparison to NCBI Gene Bank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and registrated in gene bank.

2.7 Tri 10 gene data analysis

The Tri 10 nested gene nucleotide sequences were then compared to those in the public domain databases NCBI (National Centre for Biotechnology information; www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Alignment of Tri 10 nested gene sequences was carried out using Clustal-W program.

3. Results

Agarose gel electrophoresis of PCR products obtained after the first round of amplification revealed different DNA bands with different size range from 100 to 700 bp. While, second round PCR amplification indicated only a single DNA band with molecular weight of 522 bp.

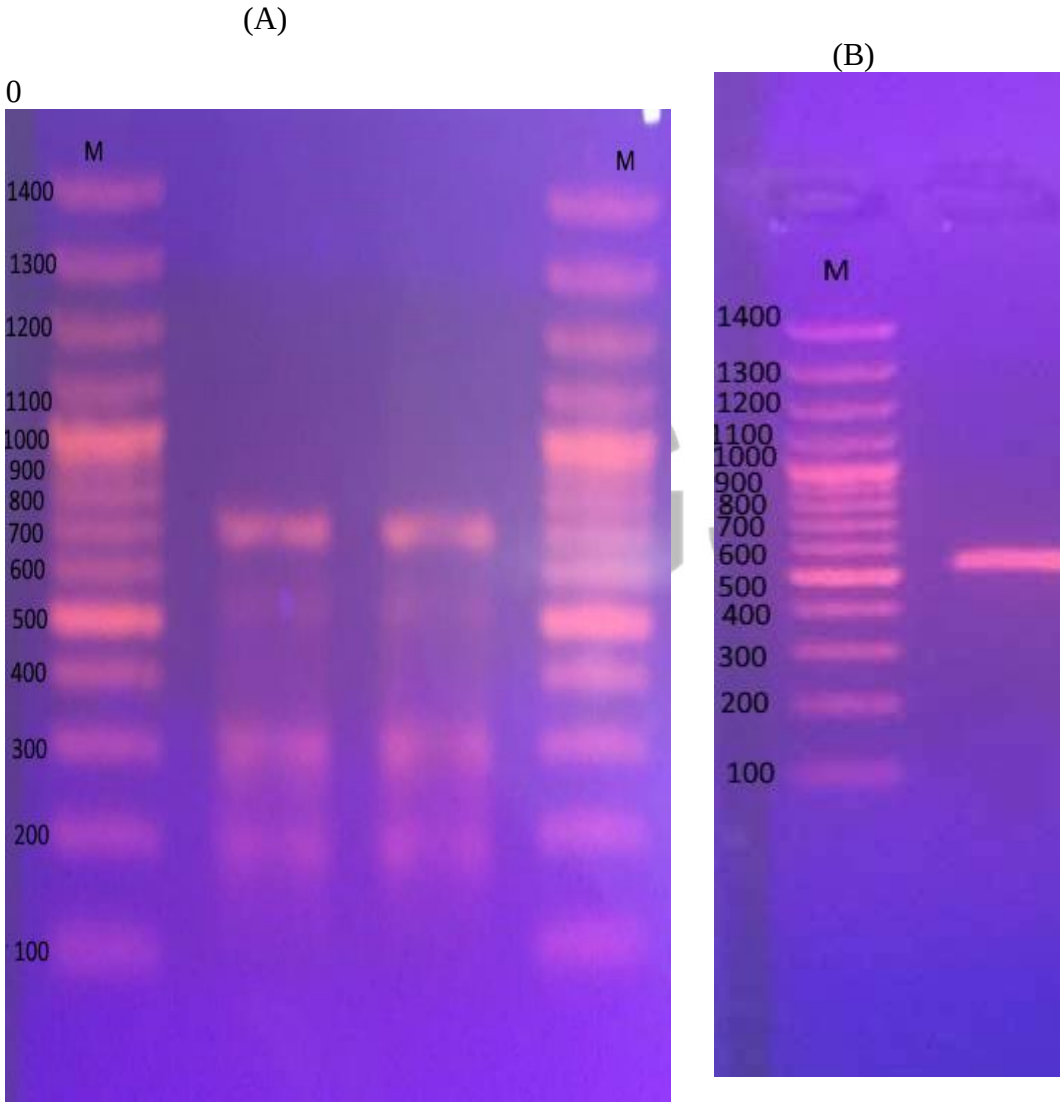


Figure 1: Agarose gels of first round PCR amplified products with tri 10–F and tri 10-R primers (A) and re-amplified nPCR products with inner primers tri 10-F and tri 10-R (B) together with cDNA template from of *Fusarium fujikuroi* (MG211161), M: DNA ladder 100-10.000 bp.

The PCR amplified Tri 10 gene fragment was subjected to nucleotide sequencing as mentioned in the methods. Nucleotide sequence indicated a truncated gene fragment of 522 bp. The PCR amplification of cDNA isolated from *Fusarium fujikuroi* (MG211161) using inner primers F-Tri10 and R-Tri 10 resulted in a specific amplification of a single amplicon of 522 bp.

3.1 Alignment of isolated nested tri 10 gene (MG674224)

Nucleotide sequence of the amplified nested tri 10 gene (MG674224) was aligned with that of the gene isolated from *Fusarium fujikuroi* (HF679024.1) and showed 98% identity (Figure 2).

Query 15 GTA-TCTTAGGCATGGGGGATGTTGGCGTCGAAGTCTTCTTGACCCATCGTGC--TACTA 71

Sbjct 2135002 GTATTCTTAGGCATGGGGGATGTTGGCGTCGAAGTCTTCTTGACCCATCGTGCCGTACTA 2134943

Query 72 ATTCGTCCTCGCTCGTCGACTCCAATGAACTCATATGAGCCATTGGCCATCAGACTTGAC 131

Sbjct 2134942 ATTCGTCCTCGCTCGTCGACTCCAATGAACTCATATGAGCCATTGGCCATCAGACTTGAC 2134883

Query 132 TCCCAAGTCGATCCATCTTCCATCACGATTTCGGCGGACTCGTCACCCATTTGGGGAAGC 191

Sbjct 2134882 TCCCAGGTCGATCCATCTTCCATCACGATTTCGGCGGACTCGTCACCCATTTGGGGAAGT 2134823

Query 192 GGGCTAATAACTGCAACGACGTCCCTTTGGTCAAGCGAGTCTTCGGTATCGAGAGATCCT 251

Sbjct 2134822
GGGCTAATAACTGCAACGACGTCCCTTTGGTCAAGCGAGTCTTCGGTATCGAGAGAT
CCT 2134763

Query 252
GGAGATGCTGGCGTGTCATAGTCCTCACTGCGAACAATAAGAAGATCGTTCATGCCA
AGG 311
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2134762
GGAGATGCTGGCGTGTCATAGTCCTCACTGCGAACAATAAGAAGATCGTTCATGCCA
AGG 2134703

Query 312
GTTGGCTTCTTATGGAACAGGTGAGGGCAACGCTTGGCCATACGAGGTACAATTTGA
GAG 371
| ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2134702
GCTGGCTTCGTATGGAACAGGTGAGGGCAACGCTTGGCCATACGAGGTACAATTTG
AGAG 2134643

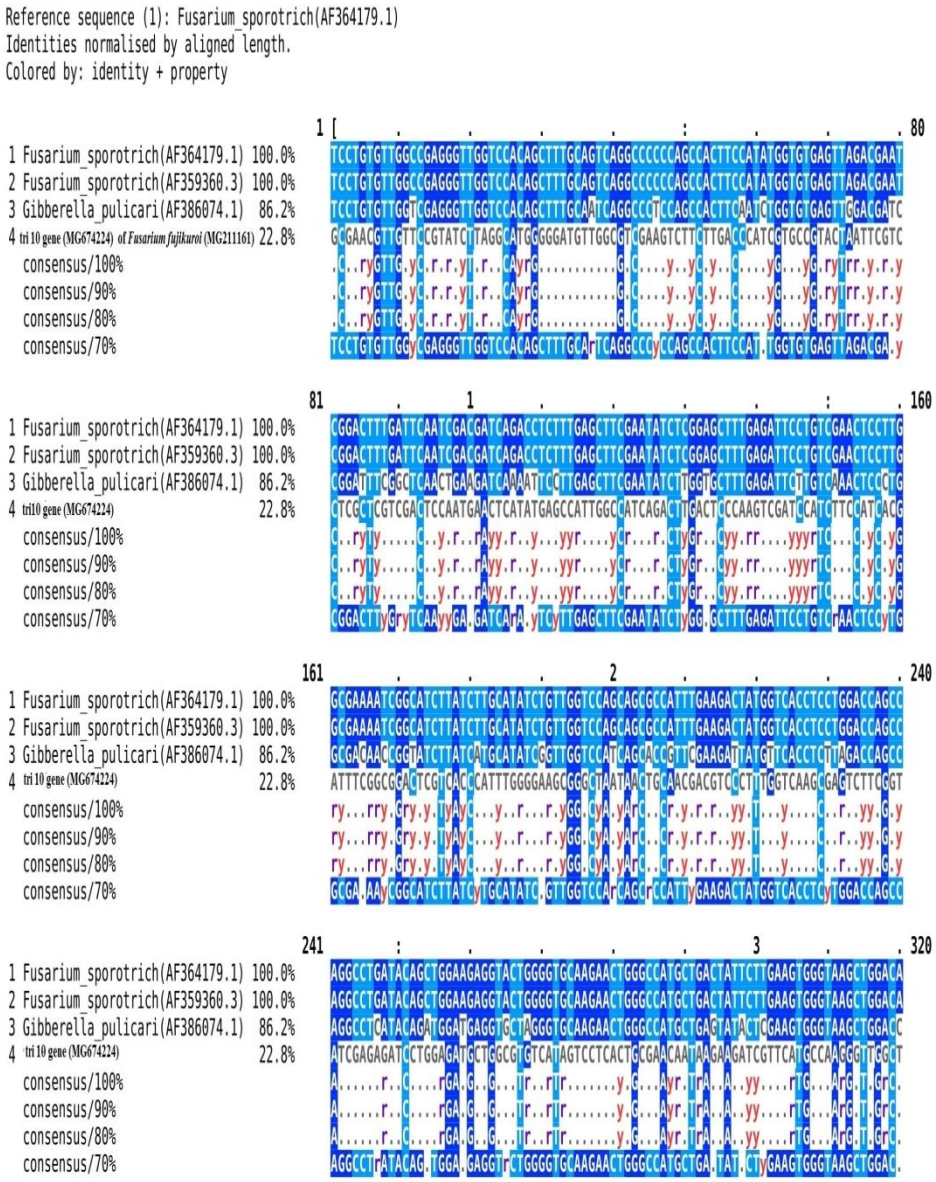
Query 372
CCCGTGATGAGGCTGGACGGTACTACATCAAATGCGGGTATTGGGCGCTTCTCGCC
GACT 431
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2134642
CCCGTGATGAGGCTGGACGGTACTACATCAAATGCGGGTATTGGGCGCTTCTCGCC
GACT 2134583

Query 432
TCTTGTAATTGAAGGAGTAGTCGTGGGCGATGAACCAAGCGGCGTTGTCTGGCTCCT
GGT 491
|||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct 2134582
TCTTGTAATTGAAGGAGTAGTCGTGGGCGATGGACCAAGCGGCGTTGTCTGGCTCCT
GGT 2134523

Query 492 CGAGGTACTGGG-GTGCAAGAA 512
|||||| ||||| |||||||||
Sbjct 2134522 CGAGGTGCTGGGTGTGCAAGAA 2134501

Figure 2. Nucleotide sequence alignment between the PCR amplified 522 bp gene fragments and the gene isolated from *Fusarium fujikuroi* (MG674224) and the gene isolated from *Fusarium fujikuroi* (HF679024.1), showing 98% identity.

Alignment of nucleotide sequence of nested Tri 10 gene (MG674224) type (A+B) isolated from F. *fujikuroi* with other Tri 10 genes isolated from trichothecnces producing *Fusarium* species is indicated in Figure 3. Alignment was carried out between our isolated gene fragment and the trichothecene genes from *Fusarium sporotrichioides* (AF364179.1) type A, *Fusarium sporotrichioides* (AF359360.3) type A and *Gibberella pulicaris* (AF386074.1) type B. An overall similarity (70%) among consensus sequences of the four selected genes was indicated as shown in Figure 3.



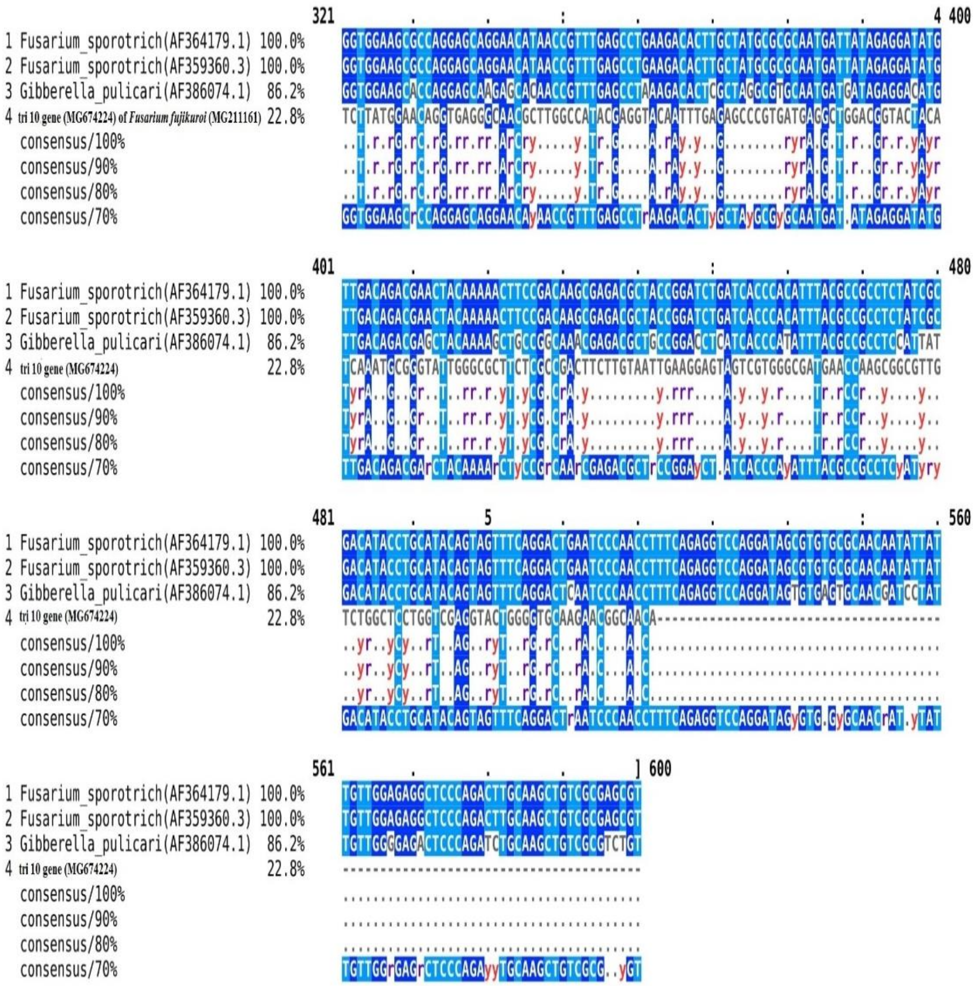


Figure 3. Sequence alignment of isolated nested Tri10 gene (MG674224) from *Fusarium fujikuroi* (MG211161) with *Fusarium sporotrichioides* gene (AF364179.1), *Fusarium sporotrichioides* trichothecene gene (AF359360.3) and *Gibberella pulicaris* type B (Tri10) gene (AF386074.1) using Multiple Sequence Alignment (www.ebi.ac.uk/Tools/msa/). Blue colour indicates conserved bases, dots indicate gaps between sequences. (y) indicates A and T and (r) indicates G and C.

4. Discussions

In the present study, we isolated and characterized regulatory gene Tri 10 from *Fusarium fujikuroi* which T-2 toxin producer([**7**], [**8**]). This sequence was with the accession number (MG674224). submitted to the gene bank and registered Isolated gene fragment indicated 98% similarity with the gene isolated from *Fusarium fujikuroi* (HF679024.1), sequence which control the pathway of cluster gene (Tri 3, Tri 5, Tri 4, Tri 8, Tri 6, Tri 7) to produce T-2 toxin 522 base pair. Our result was in accordance with [**5**] and [**6**] who characterized Tri 10 gene, a regulatory gene in trichothecene mycotoxin-producing

Fusarium species, is required for trichothecene biosynthesis and the co-ordinated expression of four trichothecene pathway-specific genes (Tri 4, Tri 5, Tri 6, and Tri 101) and the isoprenoid biosynthetic gene for farnesyl pyrophosphate synthetase (FPPS) and showed that six more trichothecene genes (Tri 3, Tri 7, Tri 8, Tri 9, Tri 11, and Tri 12) are regulated by Tri 10 gene**.**

Of the first time Egyptian Tri 10 gene (MG674224) was isolated and characterized from *Fusarium fujikuroi* species which was registered in gene bank (Tri 10 gene and isolate). Moreover **[3]** summarized the current understanding of the pathways of biosynthesis, function of cloned Tri 10 gene ,where tri10 encoding a novel type of regulatory protein which encode 420 amino acid residues doesn't show any similarity to protein of known function. Alignment of nested Tri 10 gene (MG674224) with of *Gibberella pulicaris* (AF386074.1) type B (Tri 10) gene, complete cds, *Fusarium sporotrichioides* (AF359360.3) trichothecene gene and *Fusarium* sp (AF364179.1) FRC R-06979 regulatory protein (Tri 10) indicated that our gene sharing 70 % identity of the consensus sequences common for all trichothecene coding genes. Results obtained in this study indicated that the used nested primers could be used as molecular markers for *Fusarium* trichothecnce producing species.

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