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Cloning and Functional Analysis of *tri14* in *Trichoderma brevicompactum*

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Abstract: Trichodermin, one of the trichothecenes, is a potential antifungal antibiotic and a protein synthesis inhibitor. Trichodermin is synthesized mainly by *Trichoderma brevicompactum*, but the biosynthetic pathway has yet been completely elucidated. Our earlier transcriptome assay on the trichodermin synthesis in *T. brevicompactum* 0248 identified a unique *tri14* transcript, designated *Tbtri14*, that was co-regulated with other *tri* cluster genes. However, the function of this gene remains unknown. In this study, the full cDNA and DNA sequence of *Tbtri14* was isolated from *T. brevicompactum* 0248. It was found the *Tbtri14* gene encodes 364 amino acids with a 59 bp intron. For gene function study, the *Tbtri14* was deleted in the 0248 strain. The silencing of *Tbtri14* dramatically increased the transcription of *Tbtri11*, it had little influence on the expression of *Tbtri4* and *Tbtri5* as well as the production of trichodermin in *Δtri14* deletion mutants. This implied that *Tbtri14* may not be directly involved in trichodermin biosynthesis. Instead, it may act as a gene regulator, such as a repressor for *Tbtri11*.

Keywords: *Trichoderma brevicompactum*, *tri14*, Functional Analysis, Trichodermin

Introduction

Trichothecenes are a large group of sesquiterpenoid-derived secondary metabolites and have mainly been synthesized by *Fusarium* and certain other fungal genera like *Stachybotrys*, *Myrothecium*, *Trichoderma* and *Trichothecium* (Shentu *et al*., 2014; Wilkins *et al*., 2003). The trichothecene biosynthetic pathway in *Fusarium* has been studied in detail and about 18 genes were found to be involved (Kimura *et al*., 2007). Most of the *Fusarium* genes directly involved in trichothecene biosynthesis are positioned at a locus designated the core trichothecene biosynthetic gene cluster (*tri* cluster) (Brown *et al*., 2004). *Trichoderma* species produce trichothecenes, most notably trichodermin and harzianum A (HA) that are synthesized mainly by *T. brevicompactum* and *T. arundinaceum*, respectively. Trichodermin and HA both have strong antifungal activities against phytopathogens such as *Rhizoctonia solani* and *Botrytis cinerea* (Moradi *et al*., 2012; Tijerino *et al*., 2011; Malmierca *et al*., 2012). Furthermore, HA shows extremely similar chemical structure to trichodermin. The biosynthetic pathway of trichothecenes by *Trichoderma* has yet been completely elucidated and the function of many genes in the *Trichoderma* is little known.

It was revealed that *Trichoderma* species also have a trichothecene biosynthetic gene cluster with orthologues of seven genes (*tri3*, *tri4, tri6, tri10, tri11, tri12* and *tri14*) in the *Fusarium tri* cluster and *tri4*, *tri11* are directly involved in trichothecenes biosynthesis (Cardoza *et al*., 2011). The *tri5* gene (encoding the trichodiene synthase), located outside the cluster, is responsible for the first committed step in HA biosynthesis (Cardoza *et al*., 2011). It keeps unkown that other genes in *tri* cluster involve in trichothecenes biosynthesis or not.

The *tri14* gene is located adjacent to the *tri* cluster in *Trichoderma*, it was considered as a part of the cluster because its transcription was co-regulated with the other cluster genes (Dyer *et al*., 2005; Brown *et al*., 2002). However, the function of *tri14* remains unknown so far. In this study, the full sequence of *Tbtri14* cDNA was cloned from *T. brevicompactum* 0248 and analyzed. For study of the gene function, *Tbtri14* in strain 0248 was

deleted. The influence on the trichodermin production as well as the expression levels of three genes directly involved in trichothecene biosynthesis *Tbtri4*, *Tbtri5* and *Tbtri11* were investigated.

Materials and Methods

Strains, Plasmids and Culture Conditions

Escherichia coli JM109 was used for plasmid construction and maintenance. The *E. coli* was cultivated at 37°C in Luria–Bertani (LB) medium containing 100 μg mL^{-1} ampicillin (Brayner *et al*., 2006).

T. brevicompactum 0248, isolated in our previous study and deposited in China General Microbiological Culture Collection Center (CGMCC No. 6985), was used as the original strain and transformation host. The cultivation of strain 0248 was carried out as described in the literature (Shentu *et al*., 2014).

Agrobacterium tumefaciens AGL-1, obtained from Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, was grown in YEB medium (Lacorte *et al*., 1991) with 100 μg mL^{-1} rifampicin at 28°C before used for *Agrobacterium Tumefaciens*-Mediated Transformation (ATMT). Induction Medium (IM) and M-100 medium were used in the transformation experiment (Bundock *et al*., 1995).

The plasmid pSilent-1, kindly provided by Fungal Genetics Stock Center (FGSC) in USA, carries a hygromycin-resistant gene (*hph*) cassette. The plasmid pCAMBIA0380, obtained from Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, was used for ATMT.

All the primers used in this study were synthesized by Shanghai Sunny Biotechnology Co. Ltd and listed in Table 1.

Cloning and Sequencing of the tri14 Gene

Total RNA was extracted from fresh mycelia by using Spin Column Fungal Total RNA Purification Kit (Sangon, Shanghai, China). Genomic DNA of the strain 0248 was extracted by using Ezup Column Fungi Genomic DNA Purification Kit (Sangon, Shanghai, China). The *Tri14* cDNA was amplified from the total cDNA by Polymerase Chain Reaction (PCR) with the primer 14-F/14-R and subcloned into the pMD18-T vector for sequence verification.

Construction of Recombinant Vector

A 1-kb upstream DNA fragment before the *tri14* gene start codon was amplified using total DNA as template and following primers pf1 and pr2, the forward primer pf1 containing *BstXI* site and the reverse primer pr2 containing a 22 bp reverse complementary sequence of *hph* cassette. To obtain the *hph* expression cassette, hygromycin resistance gene was amplified by PCR using plasmid pSilent-1 as the template with the primers pf3 and pr4. Similarly, another DNA fragment downstream of the *tri14* gene stop codon was amplified using total DNA as template and following primers pf5 and pr6, the forward primer pf5 containing a 22 bp overlapping sequence of *hph* gene and the reverse primer pr6 containing a *BglII* site (Fig. 1). Then, the three linearised fragments were equimolarly mixed and cyclised in a fusion PCR to generate a gene knockout fragment (Cao *et al*., 2014). The gene knockout fragment was cloned into the *Bst*XI/*Xma*I sites of pCAMBIA0380 to generate a vector designated pCAMBIA0380-kt.

Transformation of T. brevicompactum 0248

The expression vector pCAMBIA0380-kt was introduced into *A. tumefaciens* AGL-1 by heat shocking at 37° for 5 min after pretreatment with liquid nitrogen for 3 min. The primer H-F/H-R was used to screen the positive cloning that was named as *A. tumefaciens* AP03HS. The AP03HS-mediated transformation of *T. brevicompactum* 0248 was then performed as described earlier (Almeida *et al*., 2007; dos Reis *et al*., 2004).

Genetic Stability Analysis of Transformants

The transformants were purified by single-spore isolation and analyzed by PCR and qRT-PCR to confirm the deletion in the *Tbtri14* gene. The selected transformants were cultured in PDA plates without hygromycin B for 72 h. The fresh mycelia was picked up and inoculated into new PDA plates without hygromycin B and incubated for another 60 h. After this procedure was repeated for five times, mycelia from each transformants were inoculated in PDA plates with 100 μg mL^{-1} hygromycin B and their growth was observed.

Analysis of Gene Expression by using qRT-PCR

Total cDNA was extracted as described above and diluted 10 times before used. qRT-PCRs were performed in a Applied Biosystems StepOnePlus Real-Time PCR System using a SYBR® *Premix Ex Taq*™ (Tli RHaseH Plus) reagent (TaKaRa, Dalian, China). The primers used in qRT-PCRs were given in Table 1. All qRT-PCRs for each gene were carried out in triplicate within a plate and the *β-tubulin* gene of *T. brevicompactum* 0248 was used as the reference gene (Zhan *et al.*, 2013). The quantification of the *Tbtri4*, *Tbtri5*, *Tbtri11 and Tbtri14* gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Analysis of Trichodermin by Using GC

The fermentation broth was separated from the mycelia by filtration using a Buchner funnel and extracted exhaustively with ethyl acetate (v/v, 1:2). The organic fractions were combined and evaporated to dryness in a vacuum at 50°C. The recovered residues were re-suspended in methanol and subjected to Gas Chromatographic (GC) analysis for the quantification of trichodermin (Shentu *et al*., 2013; 2008).

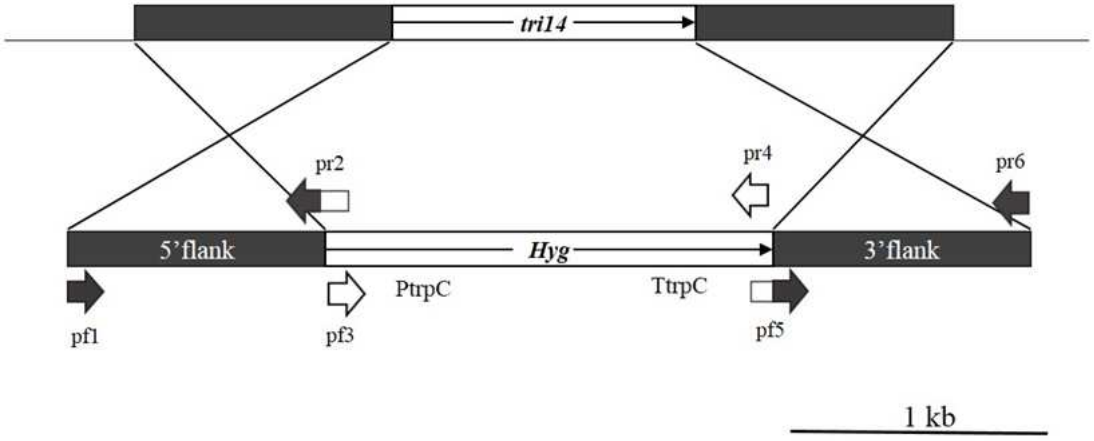

Fig. 1. Strategy for the deletion of the *Tbtri14* coding region

Table 1. Primer pairs used in this study

Name	Sequence (5'-3')	Notes
pf1	GTCCCAGGGCGGTGGAGAGATCGTGATGCAAAGC	For *Tbtri14* gene verification
pr2	TCCTTCAATATCAGTTAACGTCCAACCACAGGTGTTACTGAG	
pf3	GACGTTAACTGATATTGAAGGA	For vector construction
pr4	TTCCGTCACCAGCCCCTGGGTT	
pf5	AACCCAGGGGCTGGTGACGGAAGTTATGGAGTAGGCTGCGAC	
pr6	GACAGATCTCAACTGATCTAAGTCAAGCAC	
H-F	GAAGAGGTAAACCCGAAACG	For *hph* gene detection
H-R	GGCAAACTGTGATGGACGAC	
Tri4-F	TTCGGTCCCATTAGTC	
Tri4-R	CCATCGCTCAGGTTTA	
Tri5-F	TTCTAAATGCCCGACCAC	
Tri5-R	GAGCCACGGAAACCCT	
Tri11-F	CTGATTGGAAAGCAGAGC	
Tri11-R	AGCCCGAGATAGGAAGGA	
Tri14-F	AGGAATGAATCCCAAAT	
Tri14-R	GACATCCGAGACGAAA	
Tub-F	ATGCCACCCTGTCTATGC	
Tub-R	CGAAGGTCGGAGTTGAGC	

Results

Sequence Analysis of Tbtri14 from T. brevicompactum 0248

The full cDNA sequence (1,092 bp) and DNA sequence (1,151 bp) of *Tbtri14* from *T. brevicompactum* 0248 were obtained in this study. The nucleotide sequence of *tri14* cDNA (Supplementary 1) and its predicated protein has been submitted to GenBank under accession no. KJ957748. It was predicted that the molecular mass of *Tb*TRI14 is 40.11 kDa and the isoelectric point is 4.62 by using ProtParam of ExPASy. The analysis of the *Tb*TRI14 sequence by using InterPro of EMBL-EBI indicated that there was a non-cytoplasmic domain, a signal peptide and a polypeptide belonging to the calcium-dependent phosphotriesterase superfamily. A tertiary structure analysis of *Tb*TRI14 by using CPHmodels of CBS predicted that it may be a protein with a barrel structure. The deduced protein sequence of *Tb*TRI14 was compared with those of the Tri14 from *T. arundinaceum*, *S. chlorohalonata* and *F. sporotrichioides*, the identities were 99, 67 and 64%, respectively. However, the function of Tri14 proteins in these three strains was also unknown (Cardoza *et al*., 2011; Brown *et al*., 2002).

Genetic Stability Analysis of Transformants

With ATMT, a total of 58 hygromycin-resistant transformants were obtained. Screened by PCR, three were identified which had the hygromycin cassette replacing the ORF of *Tbtri14* (data not show), designated kt26, kt31, kt52. The genetic stability analysis of the three transformants showed that the loss-of-function mutations could be passed down steadily to the next generation. The mutants were identical to each other with respect to the phenotypes observed.

Effect of Tbtri14 Deleting on the Expression Levels of Tbtri4, *Tbtri5*, *Tbtri11*

The relative expression levels of *Tbtri14* and other three key genes involved in trichothecenes biosynthesis *Tbtri4*, *Tbtri5* and *Tbtri11* in *Δtri14* knockout mutant (kt26) and wild strain 0248 were detected. As shown in Fig. 2A, the expression of the *Tbtri14* gene in the wild strain changed with time (40, 52, 64, 76 and 88 h), with the highest expression level observed at 40 h. At each time point, the *Tbtri14* expression level was not detected in *Δtri14* knockout mutants. This indicated that the *Tbtri14* gene was completely silenced in *Δtri14* deletion mutants.

Surprisingly, there was a significant increase in the *Tbtri11* expression in *Δtri14* deletion mutants than that in wild strain at 40, 52, 64, 76 and 88 h (Fig. 2B). In contrast to *Tbtri11,* the expressions of *Tbtri4* (Fig. 2C) and *Tbtri5* (Fig. 2D) were hardly affected by the deleting of the *Tbtri14* gene; no significant difference in the expression levels of these three genes between the transformant and wild strain was detected.

Effect of tri14 Deleting on the Production of Trichodermin

Fermentation broths from transformant kt26 and wild strain 0248 were assayed for trichodermin production. As shown in Fig. 3, the trichodermin yields produced by kt26 transformant was similar to those produced by wild strain at different time. For both transformant and wild strain, the trichodermin was rapidly synthesized from 40 to 64 h. After 64 h, no increase in the trichodermin concentrations was found.

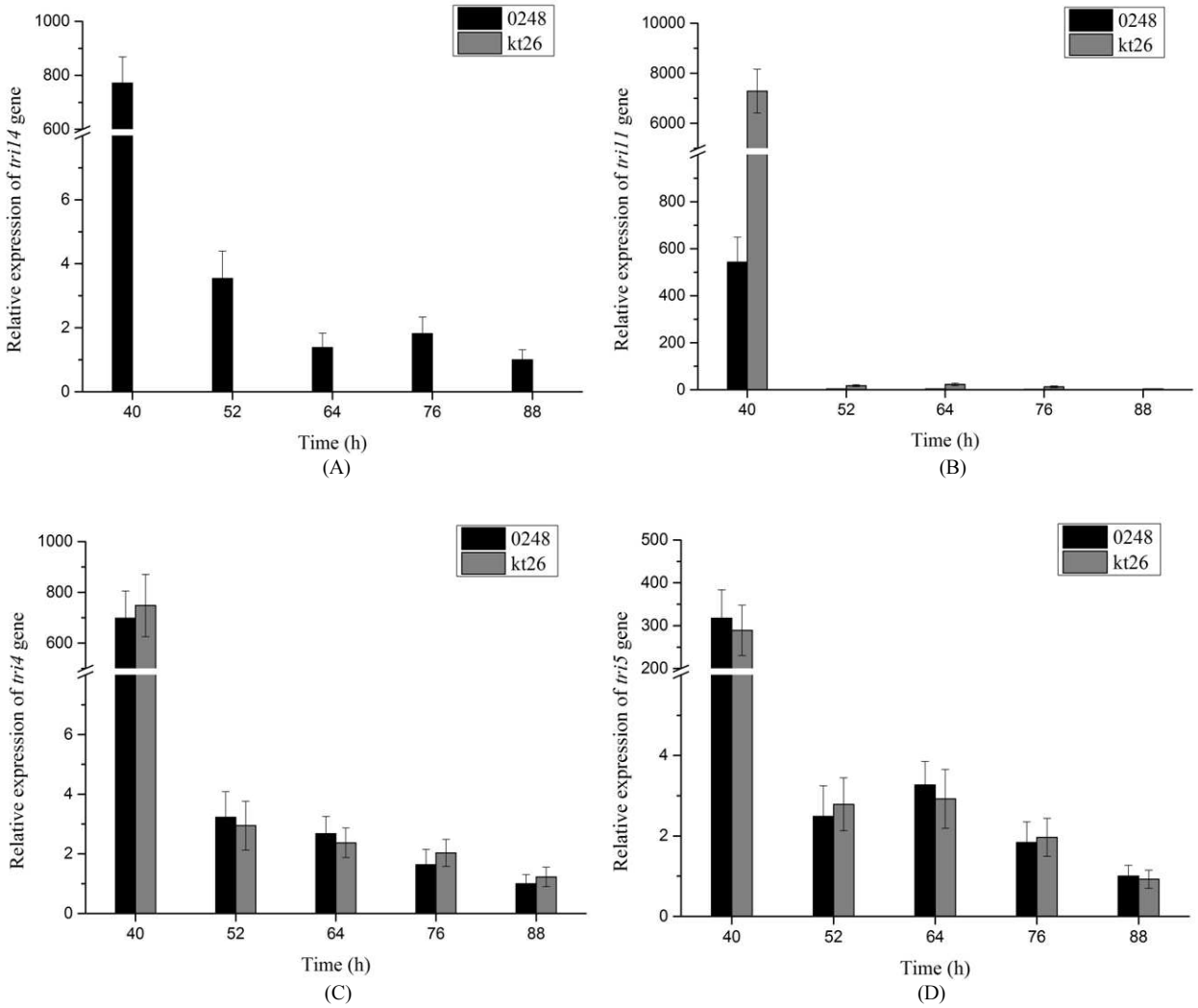

Fig. 2. Relative expression levels of *tri* cluster genes from wild strain 0248 and transformant kt26. The relative expression levels of *Tbtri14* (A) *Tbtri11* (B) *Tbtri4* (C) and *Tbtri5* (D) were detected by using qRT-PCR. The strain 0248 cultured for 88 h was used as control and β-tubulin as reference gene

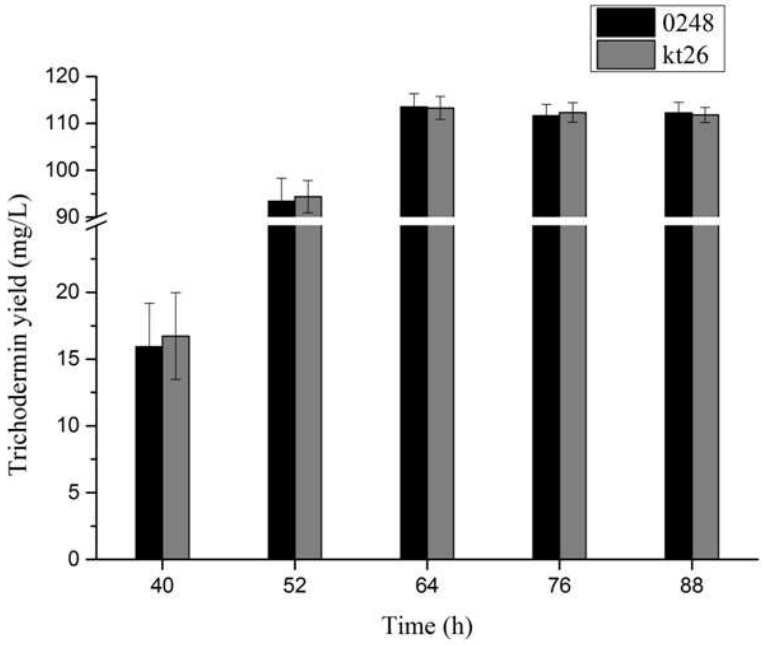

Fig. 3. Trichodermin production by wild strain 0248 and transformant kt26

Discussion

Transcriptome sequencing and gene expression analysis of the 0248 strain identified a unique *Tbtri14* transcript that is highly similar to the *Fusarium tri14* gene. Furthermore, *Tbtri14* was shown to have a significant differential expression under the trichodermin-producing condition. The cDNA and DNA sequence of *Tbtri14* was cloned and analyzed in this study. Subsequently, the function of the *Tbtri14* gene was also researched in the 0248 strain.

It was found that while the expression levels of *Tbtri14* in the three *Δtri14* deletion mutant was completely silenced, the yields of trichodermin were comparable between the mutant and the wild strain (Fig. 3). This indicated that *Tbtri14* may be indirectly involved in the trichodermin synthesis in *T. brevicompactum*. Similar to this finding, deletion of the *Fstri14* gene in *F. sporotrichioides* was found not to change the synthesis of T-2 toxin that also belongs to the trichothecenes family, which implied that *Fstri14* may not be required for the T-2 toxin synthesis in *F. sporotrichioides* (Brown *et al*., 2002). The study of the *tri14* function in *F. graminearum* showed that *Fgtri14* was required for the wild-type levels of virulence on wheat, but not for the synthesis of deoxynivalenol (another trichothecene mycotoxin) in cracked corn culture.

With the deleting of *Tbtri14*, the expression levels of *Tbtri11* were significantly increased in the transformant. This implied that the expression of *Tbtri14* may repress the transcription of *Tbtri11* in *T. brevicompactum* 0248. This result was consistent with the earlier assumption that the *Fgtri14* gene in *F. graminearum* may act as a regulator of the deoxynivalenol synthesis by affecting the transcription of other relevant genes (Dyer *et al*., 2005).

The *tri11* gene in *T. arundinaceum* (*Tatri11*) has been shown to encode a requisite enzyme, *Ta*TRI11, in the HA biosynthetic pathway; it catalyzes the C-4 hydroxylation of trichothecene (Cardoza *et al.*, 2011). As we know that both trichodermin and HA can be synthesized by the *Trichoderma* species and their chemical structure are extremely similar (Degenkolb *et al*., 2008). The only difference is the side group at the C-4 position; trichodermin has an acetyl group while HA has an octa-2,4,6-trienedioic acid (Malmierca *et al*., 2012). Thus, it is very likely that the trichodermin in *T. brevicompactum* shares the similar biosynthetic pathway with the HA in *T. arundinaceum*. In other words, the *Tbtri11*-encoded *Tb*TRI11 may also catalyze the C-4 hydroxylation of trichothecene in the biosynthetic pathway of trichodermin, as does the *Ta*TRI11 in HA biosynthesis. However, with the significant enhancement of the *Tbtri11* expression in the *T. brevicompactum* transformant, the yield of trichodermin was not improved. This implied that the expression of *Tbtri11* is probably not a rate-limiting step in trichodermin biosynthesis. Other *tri* cluster genes (e.g., *Tbtri3*, *Tbtri4* or *Tbtri5*), of which the expression was not affected by the deleting of *Tbtri14*, may play critical roles in trichodermin biosynthesis. However, it was recently reported that overexpression of *Tatri4* in *T. arundinaceum* did not enhance the HA biosynthesis

either (Malmierca *et al*., 2012). In contrast, overexpression of *Tbtri5* in *T. brevicompactum* IBT 40841 resulted in an increased production of trichodermin. These results indicated that *Tbtri5* is most likely a critical gene that limits the trichodermin synthesis in *Trichoderma* (Tijerino *et al*., 2011).

Conclusion

Tbtri14 may not be required for trichodermin biosynthesis in *T. brevicompactum* 0248. Instead, it may act as a regulator of other relevant genes, such as a repressor for *Tbtri11*. The results obtained from this research provide new information for future study on the biosynthesis pathway of trichodermin or other trichothecenes.

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Author's Contributions

Xu-Ping Shentu: Conceived of the research, designed the study, drafted and revised the paper.

Xiao-Feng Yuan: Involved in sample preparation and performed most of the laboratory work.

Wei-Ping Liu: Performed most of the laboratory work.

Jian-Feng Xu: Involved in manuscript writing and scientific discussion.

Xiao-Ping Yu: Conceived of the research, designed the study.

Ethics

This article is original containing unpublished materials. All authors have read and approved the manuscript and no ethical issues involved.

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Supplementary data

The DNA Sequence of tri14

The Sequence Marked with Red is Introne

ATGCAACCACAGGTGTTACTGAGCTCACTGCTCCCCTTCGGTGACTACATTTCAGGATGGTCTTGG
AGCAGTCTGTTGGGAAATCAACCATGTCCACCTCTGCCGGCAGGTGATCTTATCATGCGGAAATATCA
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TCTCCTCACAATGGATCTGAGAACCAAGAAAGTAATTTCTCAGATTCGACTCAACGATATAAGCAATG
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CCATATCACGAATTCACCACCTCCAATGTAATGAATTTGCCTGAAAAATACAATAACACAATTCTCCTT
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TATACAATGAGGTCGAGTATTTGGGATTCATTCCTAGTCGGTTAACCAATGCCTTAGCGACAGCCGCTC
GGCAAATGTCGGATCGAATATACGTAGTTTCAGTCTACACAGACGGCGCAAATATCACTGTCGCCGGA
TATAGCAGCGAGTTCGTGCTTCAAGATATCACGGCCGAGGTGGATGCTCTCGTAGCATGA

The Deduced Amino Acid Sequence of TbTri14

MQPQVLLSSLLPFGDYISGWSWSSLLGNQPCPPLPAGDLIMRKYQMYPENFMWDKKRCVAYVSNLY
NATLSIYDPYKSEVVDTISFPGLSHPGDSATPNPLHTSGLILRPDAATADLLEIVIDNGDCFFSNGNNVSGPD
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LQDITAEVDALVA-