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CLONING AND ANALYSIS OF T-DNA TARGETED GENE OF *Colletotrichum gloeosporioides* PATHOGENICITY-DEFECTIVE STRAIN *Cs16*

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ABSTRACT

In preliminary stage, the nucleotide sequence of *Cs16 Colletotrichum gloeosporioides* associated gene obtained from database of (NCBI), analyzed with Clustalx 1.83 in order to assess the deduced amino acid sequences. The full-length sequences of *Cs16* gene is including with specific Open Reading Frame (ORF) having 3' and 5' UTR was obtained. In order to assess the relationship of *C. gloeosporioides* associated CS16 gene with other members, a combined rooted neighbor-joining (NJ) tree was generated through MEGA 4.5 by following 1000 bootstrap replicates. The resulted alignment predicts that *Cs16* gene showed entirely diverse sequences from other species and has no any conserved domain sequences between them. In order to scrutinize the full length nucleotide sequences, the gene functional cloning of *Cs16* gene has been carried out by PCR amplification. To examine the functional analysis of *Cs16* gene in *C. gloeosporioides* and its regulatory mechanisms, the over expressing vector (pGapneoR26) was transformed into Agrobacterium strain LBA4404 that resulted successful detection of *GFP-Cs16* expression into *C. gloeosporioides* transformants and it was also substantially detected by fluorescence microscopy. The T-DNA intentional mutants of *C. gloeosporioides* mycelia illustrated phonotypic different intensification, colour, colony growth rate than wild type isolated *C. gloeosporioides* STIJ6 strain.

Keywords: *Colletotrichum gloeosporioides*, CS16 gene, pathogenicity, T-DNA.

INTRODUCTION

Colletotrichum gloeosporioides is a common plant pathogen and widely distributed throughout the world (Sutton, 1992; Cannon et al., 2000). It has a very wide host range including agro economical crops i.e. cucumber, cassava, avocado, beans, mango, cashews, eggplant, citrus, cowpea, cotton, onion, papaya, soybean, sorghum, tomato, yam, watermelon, cucurbit, wheat, cereals, spinach and legumes causing 100% rots in fruits (Shane and Sutton, 1981). There are various control measures for the management of *C. gloeosporioides*. Chemical fungicides is one of the effective control measures but due to environmental, human and animal health hazards the chemical control has been decreased which diverted the scientists to find non-chemical management strategies. Utilization of host or pathogen genes responsible for host defence or pathogenesis of the pathogen is one of the advanced and emerging trends in the control of plant pathogens. This technique is considered as safer, effective and eco-friendly management tool. Some host defence and pathogenesis genes of *C. gloeosporioides* were identified but complete genome of *C. gloeosporioides* is not yet sequenced. The

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plant cell wall degrades by *Pectate lyase* and seen easily its expression in necrotrophic phase of infection. The genetic appearance promotes host defence mechanism. During the expression of alkalinization the *Pectate lyase* was extremely affected. Naturally it was occurred at the time of fruit ripening Drori et al. (2003).

The *C. gloeosporioides* gene *CgRac1* is a main regulator of asymmetric growth and encodes protein *CgRac1* that was involved in the pathogenic germination, morphogenesis and regulation of nuclear division. The protein *CgRac1* was plentiful in hyphal tips and conidia Nesher et al. (2011). The high level of IAA in axenic culture produced by *C. gloeosporioides* during plant infection. The gene *CgOPT1* is essential for virulence and its appearance can be improved through accumulation of IAA (auxin) Chague et al. (2009). The Agrobacterium tumefaciens-mediated transformation (ATMT) is an influential technique for targeted gene interruption in some fungi on large-scale random mutagenesis, and efficient method for allocation of the T-DNA into the recipient fungal genome Soltani et al. (2009). This technique has been applicable to yeasts as well as filamentous fungi. Recently the potential of ATMT as a genetic analysis tool in functionally diverse fungi has been demonstrated Michielse et al. (2005). They successfully used green fluorescence protein (GFP) expression, homologous gene replacement and tagged mutagenesis. In the present study, we describe an ATMT protocol for the efficient transformation of *C. gloeosporioides* and GFP expression. T-DNA right flanking sequences analysis and cloning of *Cs16*, construction of over express vector, green fluorescent protein expression of gene, fungal growth analysis of T-DNA insertional gene.

MATERIAL AND METHODS

DNA extraction, amplification and purification of amplified product

The mycelium of *C. gloeosporioides* was used for DNA extraction through a standard protocol. For amplification, primers were constructed from the sequences of *C. gloeosporioides* selected from Gen Bank National Center of Biotechnology. The isolated specific gene was prepared to obtained final volume of 50 µL contained 28 µL ddH_2O, 10 µL reaction buffer (Fast Pfu Bufffer TRANS), 5 µL DNTPS, 2 µL Primer CS16F (TTTA AGCTTATGGTGCGTCTGCTCGG) and 2µL CS16R (AAATCTAGACCAGTCAC TGCTCGCCG), 2 µL Target DNA, and 1 µL polymerase Enzyme (Fast Pfu TRANS). The amplified product was imagined in 1.0% agarose gel at 3 V/cm in PBST buffer (pH 8.0) after and their molecular weights were determined using Marker C (BBI life science). The isolated gene *Cs16* purified by using Sangon Bio Tech. The amplified product was purified by using standard buffer of the purifying kit from Sangon Bio Tech.

Enzyme cut or vector digest

The enzymatic digestion was performed by mixing 66 µL ddH2O, 10 µL Buffer Tango (Thermo Scientific) 20 µL PCR purified product or 20 µL vector PGR26, 2 µL HindIII and 2 µL Xbal restriction enzyme. It was incubated at 37ºC for 5 hrs., the required fragment cut by purification.

Ligation

The ligation PGR26+*Cs16* was performed by added 6.5 µL PCR purified product after enzyme cut, 1.5 µL purified vector product after digestion, 1 µL Buffer T1(Trans) and 1 µL T4 ligase Enzyme then tube were placed into water bath (poly Science) at 16ºC for 14 hours.

Transformation to E-coli

The transformation method were performed by *Escherichia Coli*. The strain was taken from -80ºC and kept into Ice box; ligation tube was taken and added with 50 µL Ecoli. Then placed into Ice box for 30 mins. Subsequently, the tube was kept in water bath for 90 sec. at 42ºC, then again tube placed into Ice box for 5 min for cooling. The strain was taken from tube and transferred into 2 ml tube added with 100 µL liquid LB media then shacked for 1 hrs. on 180 rmp at 37ºC. After that the shacked substance was spread on 90 mm Petri plate that contained LB solid media and kanamycin 20 µL incubated at 37ºC for 24 hrs.

Colony PCR

E. coli colonies was observed and composed colony PCR product tube, each tube contained 13.2 µL ddH2O, 2 µL reaction buffer (Bufffer Easy TaqTRANS), 1.6 µL DNTPS, 1 µL Primer C6 and 1 µL C7, some amount of bacterial strain, and 0.2 µL polymerase Enzyme (Easy Taq TRANS).

Plasmid extraction

The shacked LB media 1600 µL was kept into tube and centrifuge on 8000xg for 2 minutes, then LB media discard from tubes. Subsequently, with help of pipit added 250 µl Buffer P1 and 250 µl Buffer P2 reversed for 10 times and rest for 3 minutes. Again the same tube was added with 350 µl Buffer P3 and inverted for 20 times. After that tube was placed in centrifuge at 12000 rmp for 10 minutes. When centrifuge completed sucked 740 µl substance with the help of pipit from tube and transferred into kit, then centrifuged on 8000xg for 30 sec and then reagent was discarded. The tube filled with 500 µl Buffer DW1 rest for 5 mins centrifuged at 9000xg for 30 sec, when centrifuge completed then reagent wa discarded. Subsequently, 500 µl wash Buffer was added in tube and rest for 5 mins, then centrifuged at 9000 rpm for 30 sec, and reagent was discarded, the same procedure repeated accordingly. The tube were incubated at 37ºC for 15 mins, after that 60 µl ddH_2O was added, then tube heated at 50ºC for 2 min, centrifuged at 12000 rmp for 2:30 min and plasmid extraction process was completed.

Agrobacterium-mediated transformation of *C. gloeosporioides*

The plasmid PGR26+*Cs16* product transformed into Agrobacterium LBA4404, in this method, firstly added 1 ml bacterial suspension into 2 ml tube and then centrifuged at 5800 rmp for 2:30 min. After that accumulate bacterial precipitate added with 500 µL 10% glycerin and centrifuged at 5800 for 2:30 min, the same method repeated again for five times. After completed the whole process, 100 µL ddH2O added again in the same tube and then suspended it softly and tube was kept into ice box, subsequently 1 µL PGR26+*Cs16* was added into tube and mixed gently. The mixture was transferred into electric shock cup, covered the cup with plastic bag and kept into ice box for 5 min. adjust electroporator parameter and shocked with 1800 U (volt). After that electroporator mixture transferred into 2 ml tube contained 100 µL fresh LB media shacked for 1 hour with 180 rmp at 28ºC. The substance was spread on LB solid media added with Str and Rif antibiotic and then incubated the plate for 2 days at 28ºC.

GFP fluorescence examinations

For observation of green fluorescent protein (GFP) fluorescence, culture harvested from a 8-day-old colony of each transformant growing on PDA plates. Mycelia and conidia were examined with a Leica DM5000B microscope (Germany).

Fungal transformation

IM (solid) was prepared under the following chemicals according the different rate. Dissolve 15 g of bacto agar in water to make up a total volume of 900 ml; autoclave. Add 0.8 ml of K-buffer, 1 ml of 1% (wt/vol) $CaCl_2$ $2H_2O$, 20 ml of MN buffer, 5 ml of trace elements for IM medium, 10 ml of 0.01% (wt/vol) $FeSO_4$, 2.5 ml of 20% (wt/vol) NH_4NO_3, 10 ml of 50% (vol/vol) glycerol, 40 ml of 1 M MES, pH 5.5, and 5 ml of 20% (wt/vol) glucose to make up 1 litter of solid IM. The Fresh carrying a binary vector 100 µl LBA4404 was grown on 20 ml LB medium contained 20 µl kanamycin, rif 20 µl and 20 µl Str at 28ºC overnight. The day after, take 1000 µl added in 2 ml tube centrifuged on 5800 rmp and flow was discarded. After that 1000 µl IM added solution again centrifuge 2 time by same way then transferred into flask which contained 10 ml IM 10 µL acetosyringone (AS) and shaked for 4 hours. 100 µl of agrobacterial suspension (OD600=0.2) was mixed with 100 µl of fungal conidia. The 200 µl of the mixture was spread on filter papers on IM solid media. After incubation at 21ºC for 2 days, the filter papers were transferred into PDA.

RESULTS AND DISCUSSION

Gene functional cloning of *Cs16* gene by PCR amplification

In order to scrutinize the full length nucleotide sequences, the gene functional cloning of *Cs16* gene has been carried out by PCR amplification. The PCR reaction was used by subsequent 35 cycles with initially denaturation at 95ºC for 2 min, followed by cycles second denaturation at 95ºC for 20 seconds, annealing at 50ºC for 20 seconds, extension at 72ºC for 1 min, and the final extension at 72ºC for 10 mints. The amplified Products were imagined in 1.0% agarose gel at 3V/cm in PBST buffer (pH 8.0) and their molecular weights were determined using Marker C (BBI life science). The amplified product then ligated into cloning vector (T-easy), the positive clones were amplified through colony PCR and then samples were sent to company for sequencing. Finally, about 743bp fragment of *Cs16* was obtained by blasting with ClustalW and MEGA4.0 applications, respectively (Figure 1).

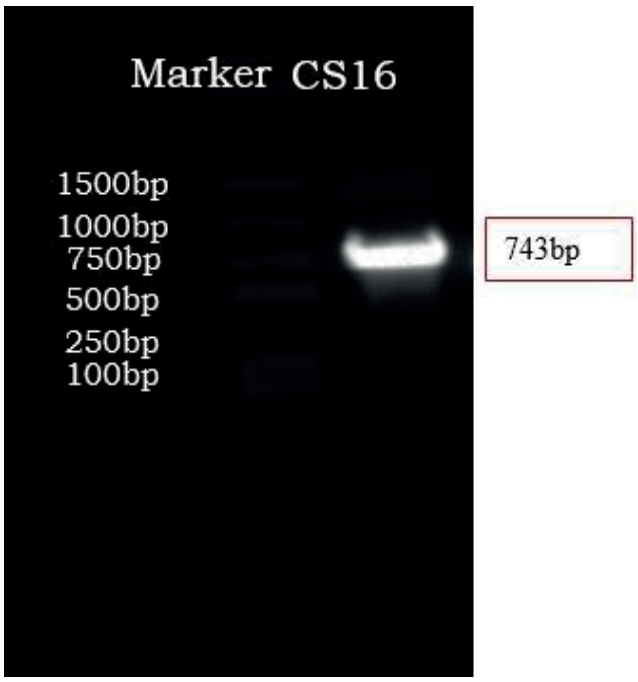


Figure 1. Cloning of right flanking sequences of *C. gloeosporioides* associated *Cs16* gene. About 743bp nucleotide sequences was obtained and amplified though the PCR. 1500bp length marker was used to quantify the amplified product.

Functional analysis of *C. gloeosporioides* associated *Cs16* gene by over-expression

In order to investigate the over expression analysis of *C. gloeosporioides* associated *Cs16* gene. The present study was examined to explore the functional analysis of *Cs16* gene in *C. gloeosporioides* and also exploring its regulatory mechanisms modulating to employing the over expressing vector (pGapneoR26). The 743bp of *Cs16* gene was ligated into pGapneoR26 expression vector (Figure 2). Positive *Cs16* + pGapneoR26 transformants were amplified and identified through colony PCR amplifications. The colony PCR confirmed *Cs16* gene clones were then double shacked and successfully transformed into a strain of E-coli and amplified. In consequence, the product was substantiated by 1% agrose gel electrophoresis. In the given results, band appearances showed on second six lanes out of eight lanes amid with marker assisted length. The Plasmid was successfully extracted according to instruction given on SanpPrep PCR Sangon Biotech D424KA5426 kit and as well as conventionally established protocols. Additionally, the overexpression construct was transformed into Agrobacterium strain LBA4404 for further transformations.

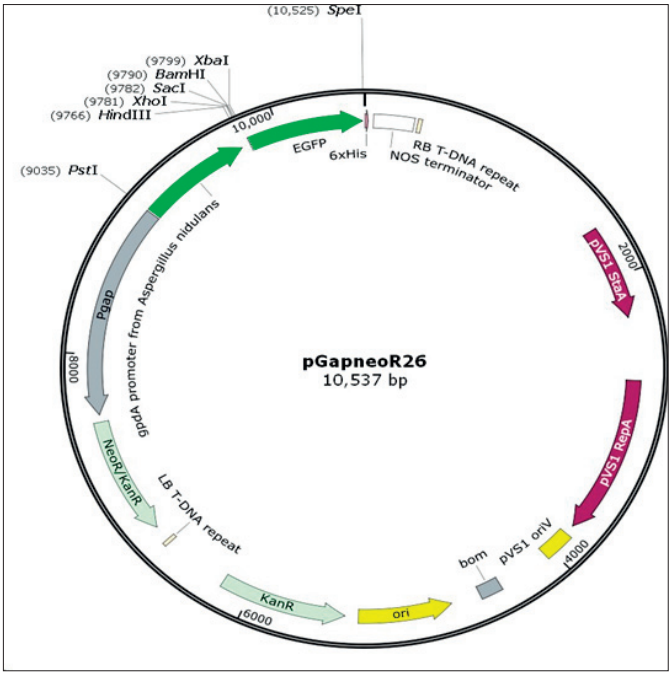


Figure 2. Diagrammatic form of PGR26 vector map used for expression analysis

Detection of GFP-*Cs16* Expression in *C. gloeosporioides* Transformants

In order to further confirm the regulatory mechanisms of *Cs16* gene functionality in *C. gloeosporioides* transformants. The GFP-*Cs16* expression was successfully detected into *C. gloeosporioides* transformants (Figure 3). The stable GFP-tagged gene *Cs16* expression inside the *C. gloeosporioides* transformants was substantially detected by fluorescence microscopy correlated with the accumulation of *Cs16* transcripts. Additionally, the fluorescence microscopic analyses were executed on energetically growing hyphae from 2 out of 15 GFP-tagged inspectional mutants. Although, premeditated mutants were randomly selected for fluorescence microscopy. At present to date, very small numbers of findings have been relied on the functionality of any reporter progression to demonstrating the regulation of pathogenicity genes.

Though in present findings, the strains of *C. gloeosporioides* articulating the GPF construct demonstrated dissimilar phenotype, that accumulating GFP seems to alter the physiology of the fungal growth behaviours. The findings articulating that identified the complexity giving signalling to pathways leading to the orientation of mortifying enzymes through pathogens.

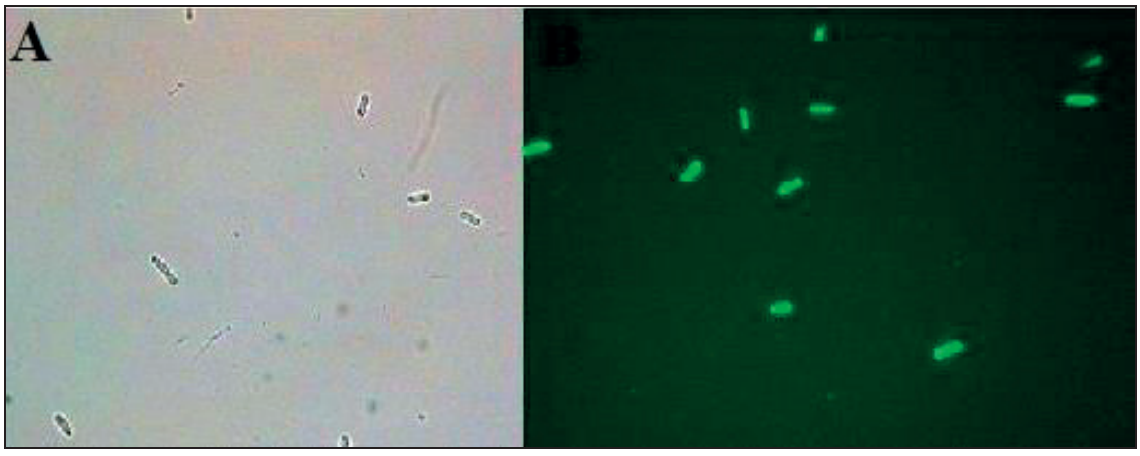


Figure 3. Expression of the gene GFP of the transformant STIJ6 in *C. gloeosporioides*.

A: Fluorescence field. B: Bright field. Scale bar corresponds to 20 μm.

Phenotypic characteristics, colony growth, spore germination of mutant strain and wild species

In order to further understand the complete mechanisms associating T-DNA intentional mutants of *C. gloeosporioides*, the study so far correlated with identifying of function of *Cs16* in *C. gloeosporioides*. The T-DNA intentional mutants of *C. gloeosporioides* mycelia illustrated phonotypic different intensification than wildtype isolated *C. gloeosporioides* STIJ6 strain.

Though, it was further amusingly to note that growth rate of T-DNA insertion of mutant transformants were considerably decreased compared to wildtype strain of *C. gloeosporioides*. Likewise, the color and the fungal colonies formation on PDA plates were also been drastically changed (Figure 4). In addition to that, the colony growth rate (8.3 cm) was observed in T-DNA insertion mutant contrast to that of wild (8.5 cm). The morphology of mutant transformants of *C. gloesporioides* compared to their parental wild type (W) on PDA plates was measure after 7 days incubation at 28ºC.

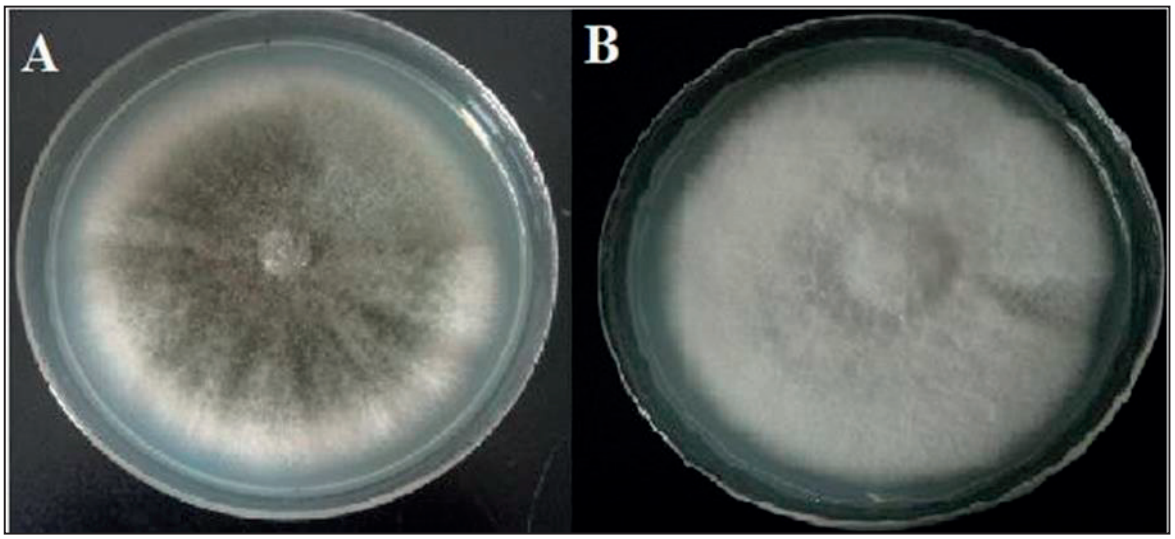


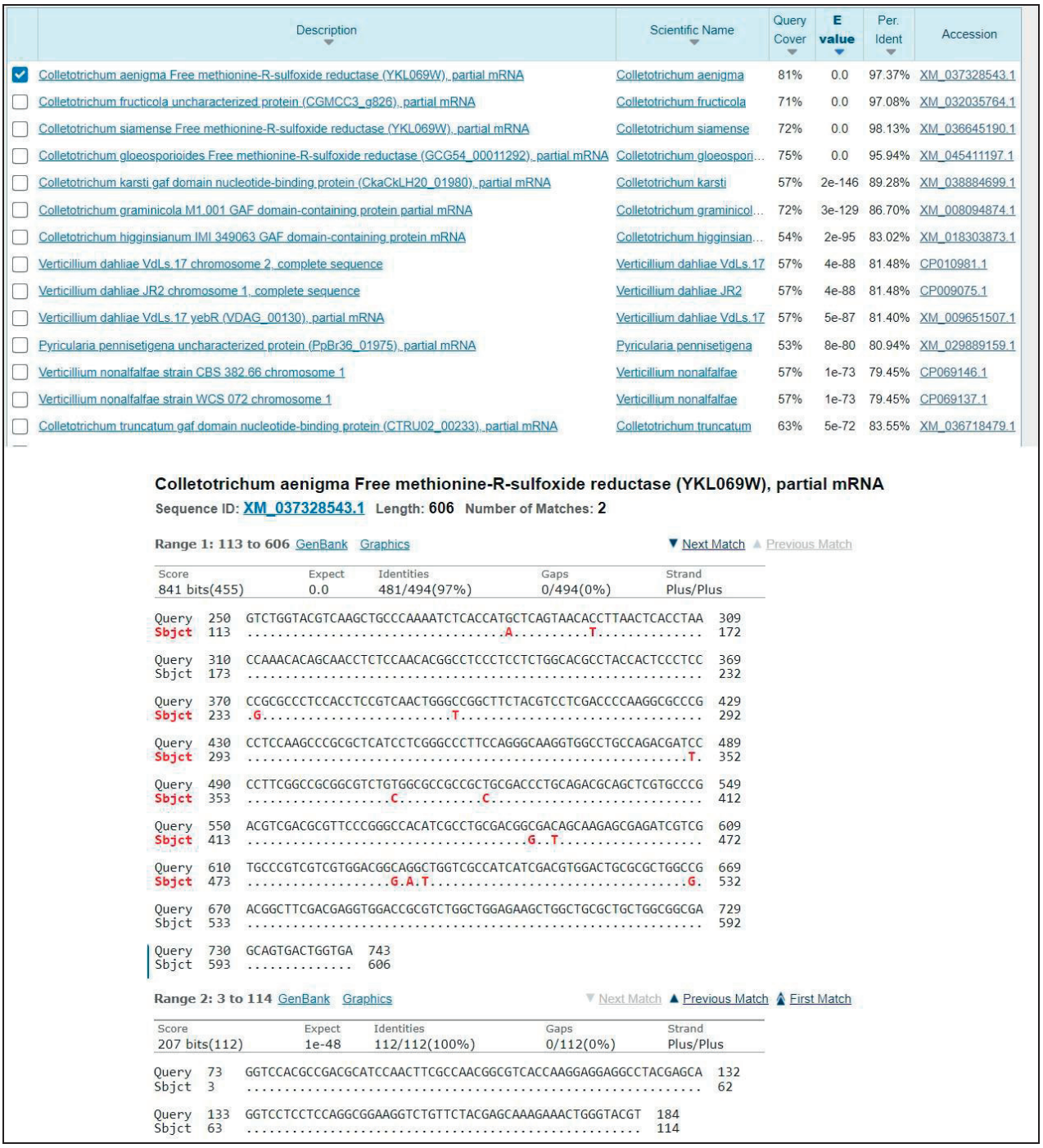
Figure 4. The colonial morphological characters of *C. gloeosporioides* STIJ6 strain and T-DNA- *Cs16* insertion mutants. Strains were inoculated onto PDA mediums and incubated at 25ºC for 8 days in the dark.
(A): Wild type of *C. gloeosporioides STIJ6* strain. (B): Mutant strain of *C. gloeosporioides* having *Cs16* gene.

***T-DNA-Cs16* Mutant Substantiation of *hph* Gene**

In relates to concluding the innovated findings, the *T-DNA-Cs16* mutant substantiations of *hph* gene has been characterized in understanding of controlled regulatory mechanisms associating *Cs16* gene of *C. gloeosporioides.* The mutant *Cs16* transformants had been proved the presence of hygromycin mutant of DNA insertion. Although, genomic DNA from the *Cs16* transformants were also been tested for the presence of *hph* gene by PCR amplification using gene specific primers *hph*-1 and *hph*-2.

Bioinformatics Analysis

The bioinformatics analysis (Figure 5) was performed in order to find the relationship of *Cs16* with other species. The targeted query showed 97.37% identity with *Colletotrichum aenigma* that is involved in free methionine R sulfoixde reductase. Fungi are heterotrophic eukaryotes that colonize all niches on Earth and play fundamental functions, in organic matter recycling, as symbionts, or as pathogens of numerous organisms.



Description	Scientific Name	Query Cover	E value	Per. Ident	Accession
Colletotrichum aenigma Free methionine-R-sulfoxide reductase (YKL069W), partial mRNA	Colletotrichum aenigma	81%	0.0	97.37%	XM_037328543.1
Colletotrichum fructicola uncharacterized protein (CGMCC3_g826), partial mRNA	Colletotrichum fructicola	71%	0.0	97.08%	XM_032035764.1
Colletotrichum siamense Free methionine-R-sulfoxide reductase (YKL069W), partial mRNA	Colletotrichum siamense	72%	0.0	98.13%	XM_036645190.1
Colletotrichum gloeosporioides Free methionine-R-sulfoxide reductase (GCG54_00011292), partial mRNA	Colletotrichum gloeospori...	75%	0.0	95.94%	XM_045411197.1
Colletotrichum karsti gaf domain nucleotide-binding protein (CkaCkLH20_01980), partial mRNA	Colletotrichum karsti	57%	2e-146	89.28%	XM_038884699.1
Colletotrichum graminicola M1.001 GAF domain-containing protein partial mRNA	Colletotrichum graminicol...	72%	3e-129	86.70%	XM_008094874.1
Colletotrichum higginsianum IMI 349063 GAF domain-containing protein mRNA	Colletotrichum higginsian...	54%	2e-95	83.02%	XM_018303873.1
Verticillium dahliae VdLs.17 chromosome 2, complete sequence	Verticillium dahliae VdLs.17	57%	4e-88	81.48%	CP010981.1
Verticillium dahliae JR2 chromosome 1, complete sequence	Verticillium dahliae JR2	57%	4e-88	81.48%	CP009075.1
Verticillium dahliae VdLs.17 yebR (VDAG_00130), partial mRNA	Verticillium dahliae VdLs.17	57%	5e-87	81.40%	XM_009651507.1
Pyricularia pennisetigena uncharacterized protein (PpBr36_01975), partial mRNA	Pyricularia pennisetigena	53%	8e-80	80.94%	XM_029889159.1
Verticillium nonalfalfae strain CBS 382.66 chromosome 1	Verticillium nonalfalfae	57%	1e-73	79.45%	CP069146.1
Verticillium nonalfalfae strain WCS 072 chromosome 1	Verticillium nonalfalfae	57%	1e-73	79.45%	CP069137.1
Colletotrichum truncatum gaf domain nucleotide-binding protein (CTRU02_00233), partial mRNA	Colletotrichum truncatum	63%	5e-72	83.55%	XM_036718479.1

Colletotrichum aenigma Free methionine-R-sulfoxide reductase (YKL069W), partial mRNA

Sequence ID: XM_037328543.1 Length: 606 Number of Matches: 2

Range 1: 113 to 606 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
841 bits(455)	0.0	481/494(97%)	0/494(0%)	Plus/Plus

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Query  250  GTCTGGTACGTCAAGCTGCCCAAAATCTCACCATGCTCAGTAACACCTTAACTCACCTAA  309
Sbjct  113  ..................................A..........T..............  172

Query  310  CCAAACACAGCAACCTCTCCAACACGGCCTCCCTCCTCTGGCACGCCTACCACTCCCTCC  369
Sbjct  173  ............................................................  232

Query  370  CCGCGCCCTCCACCTCCGTCAACTGGGCCGGCTTCTACGTCCTCGACCCCAAGGCGCCCG  429
Sbjct  233  .G.........................T...............................  292

Query  430  CCTCCAAGCCCGCGCTCATCCTCGGGCCCTTCCAGGGCAAGGTGGCCTGCCAGACGATCC  489
Sbjct  293  ............................................................T.  352

Query  490  CCTTCGGCCGCGGCGTCTGTGGCGCCGCCGCTGCGACCCTGCAGACGCAGCTCGTGCCCG  549
Sbjct  353  ................C...........C...........................  412

Query  550  ACGTCGACGCGTTCCCGGGCCACATCGCCTGCGACGGCGACAGCAAGAGCGAGATCGTCG  609
Sbjct  413  ....................................G..T...................  472

Query  610  TGCCCGTCGTCGTGGACGGCAGGCTGGTCGCCATCATCGACGTGGACTGCGCGCTGGCCG  669
Sbjct  473  ................G.A.T.............................G.  532

Query  670  ACGGCTTCGACGAGGTGGACCGCGTCTGGCTGGAGAAGCTGGCTGCGCTGCTGGCGGCGA  729
Sbjct  533  ............................................................  592

Query  730  GCAGTGACTGGTGA  743
Sbjct  593  ..............  606
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Range 2: 3 to 114 GenBank Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Identities	Gaps	Strand
207 bits(112)	1e-48	112/112(100%)	0/112(0%)	Plus/Plus

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Query  73   GGTCCACGCCGACGCATCCAACTTCGCCAACGGCGTCACCAAGGAGGAGGCCTACGAGCA  132
Sbjct  3    ............................................................  62

Query  133  GGTCCTCCTCCAGGCGGAAGGTCTGTTCTACGAGCAAAGAAACTGGGTACGT  184
Sbjct  63   ..................................................  114
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Figure 5. The bioinformatics analysis was performed using NCBI nucleotide BLAST

The nucleotide sequence of *Cs16 C. gloeosporioides* associated gene was obtained from the Information Resource or the National Center for Biotechnology Information database (NCBI). Same techniques were reported by Zhiying et al. (2013) who studied that *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *C. gloeosporioides* pathogenesis, was used for the identification of mutants of reduced in pathogenicity. An ATMT library of 4128 *C. gloeosporioides* transformants was generated. Conventional techniques to locate the putative pathogenicity gene, and then performed TAIL-PCR to isolate the target gene by using obtained genomic sequence flanking of T-DNA. In order to scrutinize the full length nucleotide sequences, the gene functional cloning of *Cs16* gene has been carried out by PCR amplification.

Primarily, competently DNA was extracted from the wild type strain of *C. gloeosporioides*. The same isolation method was adopted previously by Choi et al. (2012), who isolated genomic DNA from various fungal strain. Subsequently for PCR amplification, the gene specific primers were constructed from the sequences of *C. gloeosporioides* associated *Cs16* gene selected from GenBank National Center of Biotechnology. Finally, about 743bp fragment of *Cs16* was obtained by blasting with ClustalW and MEGA4.0 applications, respectively. The present study was examined to explore the functional analysis of *Cs16* gene in *C. gloeosporioides* and also exploring its regulatory mechanisms modulating to employing the over expressing vector (pGapneoR26). Additionally, the over-expression constructs were transformed into *Agrobacterium* strain LBA4404 for further transformations. This transformation has also conformity by adopting the procedure of transformation with the following scientists who reported that ATMT has long been used successfully as a tool for the genetic modification of a wide range of plant species. *Escherichia coli* strain XL1-blue (Stratagene) was used as a host for gene manipulations and *Agrobacterium tumefaciens* strain LBA1100 Bundock et al. (1995) as a T-DNA donor for fungal transformation. The binary vectors pTAS10 de Groot et al. (1998) and pBin-GFP-hph were transferred to this strain to yield *A. tumefaciens* pSDM2312 (Groot et al., 1998). The ATMT was applied for random insertional mutagenesis of *C. lagenarium*, constructed a binary vector pBIG2RHPH2 carrying a hygromycin-resistant gene cassette between the right and left borders of T-DNA (Tsuji et al., 2003). The *GFP-CS16* expression was successfully detected into *C. gloeosporioides* transformants. The stable GFP-tagged gene *Cs16* expression inside the *C. gloeosporioides* transformants was substantially detected by fluorescence microscopy correlated with the accumulation of *Cs16* transcripts. Our finding has the confirmation with those who previously study of Fluorescence microscopy determined the stable GFP-tagged *Hph* expression inside the *C. gloeosporioides* transformants. Seven out of 24 hygromycin-resistant isolates were randomly selected for fluorescence microscopy. Cells expressing a GFP-tagged Hph protein revealed a punctuate localization pattern of this protein throughout the cell Masha et al. (2013). According to Mansouri et al. (2009), the level of expression of *GFP* may occur differences in due to the positioning of the GFP gene in different regions of the genome of the fungus, or due the vector transformation has multiple integrations.

The mutant *Cs16* transformants had been proved the presence of hygromycin mutant of DNA insertion. Although, genomic DNA from the *Cs16* transformants were also been tested for the presence of *hph* gene by PCR amplification using gene specific primers. Our finding has the confirmation with those who previously reported that Twenty-four transformants, which had been proved to be resistant to hygromycin B at 100 µg/ml and to retain their mitotic stability, were selected and designated in MY1 to MY24. Genomic DNA from the 24 transformants was tested for the presence of the *hph* gene by PCR using specific primers *hph*-F and *hph*-R. The expected 544-bp PCR products were all detected from the 24 transformants (100%). *Hph* gene product was not detected with untransformed *C. gloeosporioides* genomic DNA (Mahsa et al., 2013). All mutants were also confirmed as true transformants so as to contain hygromycin resistant gene integration into the genome. It was confirmed by amplification of a 450bp segment of *hph* gene using primers specific for this segment. All the mutants were found to be positive with amplification of 450bp corresponding to *hph* gene into their genome. *Agrobacterium tumefaciens*-mediated transformation of several *Colletotrichum* species has been reported before (Groot et al., 1998; Tsuji et al., 2003; Takahara et al., 2004; Talhinhas et al., 2008).

CONCLUSIONS

In conclusion, we successfully extracted DNA from the *Colletotrichum gloeosporioides* wild type strain. Gene-specific primers were constructed from a 743bp fragment of *Cs16*.

The over-expressing vector (pGapneoR26). Additionally, the over-expression constructs were transformed into an *Agrobacterium* strain. The *GFP-Cs16* expression was successfully detected in *C. gloeosporioides* transformants. The color and the fungal colony formation on PDA plates have also been drastically changed. The presence of hygromycin has been demonstrated in mutant *Cs16* transformants.

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