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Sequence analysis and expression pattern of *MGTA1* gene in rice blast pathogen *Magnaporthe grisea**

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Abstract: *MGTA1*, a putative fungal $Zn(II)_2Cys_6$ transcriptional activator-encoding gene, was isolated from rice blast pathogen *Magnaporthe grisea*, which is homologous to *CLTA1* from *Colletotrichum lindemuthianum* with 51% identity at protein level. *MGTA1* cassette contains a 2370 bp open reading frame, consisting of 6 exons, coding a 790 amino acid peptide. *MGTA1* gene exists as a single copy in genomes of 7 strains of *M. grisea*, and is expressed in tip hyphae, conidia, and mature appressoria of strain Guy11.

Key words: *Magnaporthe grisea*, *MGTA1* gene, $Zn(II)_2Cys_6$
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INTRODUCTION

Fungi cause most plant diseases of all groups of microbes. During their infection cycles, fungal pathogens must undergo two key processes: first, penetration through cuticles into host plant cells; second, colonization in host cells utilizing nutrients from their hosts. To penetrate host cells, fungi develop a series of specialized infection structures such as appressorium, penetration peg, and infection hypha. The appressorium-mediated penetration is a process typical of some filamentous fungal pathogens, e.g., *Magnaporthe grisea* (Talbot, 1995), *Colletotrichum lindemuthianum* (Bailey and Jeger, 1992; O'Connell *et al*., 1985), and *Erysiphe graminis* (Kunoh *et al.*, 1979). During colonization in plant hosts, fungal pathogens exhibit two main modes to obtain nutrients: biotrophy, where nutrients are obtained from living host cells, e.g., *E. graminis* (Aist and Bushnell, 1991); and necrotrophy, where nutrients are obtained from dead host cells killed by the fungi.

C. lindemuthianum has an intermediary nutrition strategy called hemibiotrophy. After penetrating its host cuticle and epidermal cell wall, it initially grows as a biotroph with primary intracellular hyphae for one or a few days. Subsequently, secondary narrow hyphae are formed, killing its host cells and proliferating by necrotrophic growth (Perfect *et al.*, 1999). *CLTA1* gene, a putative fungal $Zn(II)_2Cys_6$ family transcriptional activator is involved in the switch between these two phases. A null mutant of *CLTA1* gene stops at biotrophic phase and loses its pathogenicity on common bean. In infected cells, mutants can form primary intracellular hyphae but cannot differentiate infectious secondary hyphae (Dufresne *et al.*, 2000).

In *M. grisea*, different from *C. lindemuthianum*, no distinct switch between biotrophic and necrotrophic phases in the post-penetration processes was

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found, although *M. grisea* does not induce visible necrotic damages on the infected rice leaves until 4~5 d after infection. Firstly, the penetration peg of *M. grisea* differentiates into bulbous primary infection hyphae in the plant epidermal cells, and then primary hyphae differentiate narrow secondary hyphae and spread in the leaf (Heath *et al.*, 1990a; 1990b). The mechanism involved in colonization of *M. grisea* in rice leaf leaves much to be understood.

In view of the similarity in penetration of *C. lindemuthianum* as *M. grisea* and the importance of *CLTA1* in the colonization of *C. lindemuthianum*, *CLTA1* homologous genes in *M. grisea* probably play an important role in the colonization of *M. grisea* and account for the differences of nutrition between these two fungi. A homologous gene (*MGTA1*) to *CLTA1* with 51% identity to *CLTA1* at the protein level in *M. grisea* was identified in this study.

MATERIALS AND METHODS

Strains, media, and growth of *M. grisea*

M. grisea strains Guy11, Y91-11, Y90-1, 84-7-3, 2000-034E3, 2001-068F1 and 2001-060G1 were used in this study. The fungi were grown routinely on complete medium (Talbot *et al.*, 1993). Long-term storage of *M. grisea* was carried out by growing the fungus through sterile filter paper discs, desiccating for 48 h and storing them at −20 °C. Mycelia collected from 2-day-old complete medium cultures shaken at 150 r/min at 27 °C were used for the isolation of fungal genomic DNA.

Nucleic acid manipulation and analysis

Genomic DNA was extracted from fungal mycelium using a CTAB (hexadecyltrimethylammonium bromide) procedure described by Talbot *et al.*(1993). Routine PCR, gel electrophoresis, restriction enzyme digestion and cloning in *Escherichia coli* were carried out using standard procedures (Sambrook *et al.*, 1989). Elongase Amplification System (Invitrogen, USA) was used to amplify relatively long DNA molecules (>5 kb) form genomic DNA, and the PCR products were cloned into PCR-XL-TOPO vector (Invitrogen, USA). DIG high prime DNA labelling and detection starter kit I (Roche, Germany) was used in the DNA gel blot hybridization.

DNA Sequencing and Sequence Analysis

The DNA clones and cDNA clones were sequenced using AB3730 autosequencer (ABI, USA). BLAST program (Altschul *et al.*, 1997) was used to search for homologues against GenBank database (http://www.ncbi.nlm.nih.gov/blast/) and fungal genome database (Broad Institute, http://www.broad.mit.edu/annotation/). Multiple sequence alignments were determined using CLUSTAL V software (Higgins *et al.*, 1992).

Construction of *MGTA1(p)::eGFP::HPH* fusion vector

pEGFP (Clontech, USA) was digested with *Pvu*II and *Hpa*I to remove lacZ promoter, and self-ligated to generate pEGFP11. A 1.5 kb promoter fragment of *MGTA1* was amplified using the primer MGTA150 (5′-GCATTCCTTGGGCCCCGCATAAC-3′) and MGTA130n (5′-GTGCCATGGTGGTCGAAGTGCTGAAGCCAC-3′), and cloned into easy vector pGEM-T (Promega, USA) to generate pMP4. Then this promoter fragment was cut with *Nco*I and cloned into pEGFP11. A plasmid with *MGTA1* promoter and *eGFP* gene in the same orientation was selected and named pLM7. A 2.3 kb *Apa*I *MGTA1(p)::eGFP* fragment from pLM7 was cloned into the *Apa*I site of pCB1004 (Caroll *et al.*, 1994) to generate two plasmids with different insertion orientation: pELM3 and pELM6. A 1.4 kb *Kpn*I-*Bam*HI fragment covering *HPH* gene under control of trpC was cut from pCSN43 (Staben *et al*., 1989) and cloned into pLM7 to generate pLMH. The *MGTA1(p)::eGFP::HPH* vectors pELM3, pELM6 and pLMH were verified by restriction digestion and used to transform protoplast after linearization.

Fungal transformations

Protoplast preparation and transformation were done as described previously (Talbot *et al.*, 1993). DNA was transformed into the *M. grisea* strain Guy11 and transformants selected for hygromycin resistance in complete media with 200 μg/ml hygromycin.

Examination of *eGFP* fluorescence

Fluorescence of *MGTA1(p)::eGFP::HPH* fusion

transformed strains were detected using an excitation wavelength of 488 nm and an emission between 500 and 530 nm. Transformants were viewed using an Olympus-BX51 microscope with UV epifluorescence and appropriate filters.

RESULTS

Isolation of *MGTA1* gene

The 746 amino acid sequence of *CLTA1* was used to search for homologous genes against *M. grisea* genome database (Broad Institute, http://www.broad.mit.edu/annotation/fungi/magnaporthe/) with tblastN and blastP program (Altschul *et al.*, 1997). Seven genome contigs and 14 hypothetical genes with *E*-values less than 8e-04 were found in the *M. grisea* database (Table 1 and Table 2).

Table 1 Result of tblastN with Clta1p against *M. grisea* genome database

Contig noes	Score (bits)	*E*-value
M. grisea contig 2.2025	223	4e-65
M. grisea contig 2.275	71	4e-12
M. grisea contig 2.1096	68	4e-11
M. grisea contig 2.274	51	3e-06
M. grisea contig 2.1386	48	4e-05
M. grisea contig 2.474	46	1e-04
M. grisea contig 2.488	44	7e-04

Table 2 Result of blastP with Clta1p against *M. grisea* genome database

Hypothetical gene	Score (bits)	*E*-value
MG10529.4	540	e-154
MG10528.4	80	3e-15
MG01486.4	71	1e-12
MG05829.4	68	1e-11
MG10212.4	55	9e-08
MG03848.4	54	2e-07
MG07450.4	51	1e-06
MG09443.4	47	1e-05
MG08418.4	47	1e-05
MG02377.4	46	3e-05
MG09118.4	45	6e-05
MG02408.4	44	2e-04
MG06086.4	42	6e-04
MG00318.4	42	8e-04

Among those hypothetical genes, the gene most identical (Score=540, *E*-value=e-154) to *CLTA1* was *MG10529.4* (GenBank accession No. EAA46835.1) located in *M. grisea* contig 2.2025 (Broad Institute). So, the hypothetical gene *MG10529.4*, namely *MGTA1* in this paper, was the potential homologous gene to *CLTA1*.

A 5.2 kb DNA fragment covering *MGTA1* gene full length was amplified from genomic DNA of *M. grisea* strain Guy11 using forward primer MGTA150 and reverse primer MGTA130 (5′-GCTGCGTGG GATGGGGGTGTG-3′) (Fig.1a). The fragment was cloned into PCR-XL-TOPO vector (Invitrogen, USA). Three independent clones (pTL12, pTL20, pTL22) were selected and verified exactly as recombination clones by restriction digestion (Fig.1b).

A 3.0 kb *MGTA1* cDNA fragment was amplified from a cDNA library constructed with RNA extracted from 24 h-old appressoria of *M. grisea* strain Guy11 (Lu *et al.*, 2005) using forward primer Tl20.w2f (5′-AAACCATAATAGCCGCAAGG-3′) and reverse primer Tl20.w2r (5′-GTCATTCCCAAGATC TAGTC-3′). The fragment was cloned into easy vector pGEM-T (Promega, USA). Three independent clones (pW2FW2R4, pW2FW2R7, pW2FW2R8) were selected and verified as recombination clones by restriction digestion (Fig.1c).

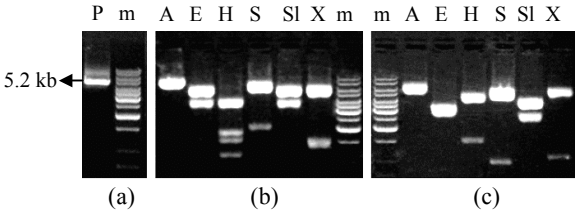


Fig.1 5.2 kb PCR product and digestion of pTL20 and pW2FW2R4 (a) 5.2 kb fragment containing *MGTA1* gene amplified from *M. grisea* strain Guy11 genomic DNA; (b) pTL20 digestion with restriction enzymes; (c) pW2FW2R4 digestion with restriction enzymes. P, 5.2 kb PCR product; m, 1 kb DNA ladder; A, *Apa*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; Sl, *Sal*I; X, *Xho*I

Sequencing analysis of *MGTA1* gene

Three DNA clones (pTL12, pTL20 and pTL22) and 3 cDNA clones (pW2FW2R4, pW2FW2R7 and pW2FW2R8) were sequenced on both strands. The genomic DNA sequence, cDNA sequence and deduced protein sequence of *MGTA1* were submitted to GenBank (accession No. AY786158). Six nt differing

was identified among 5.2 kb DNA sequence between our result (strain Guy11) and *M. grisea* database (strain 70-15, Broad Institute).

Analysis of these 3 cDNA sequences revealed an open reading frame of 2370 bp, coding a 790 amino acid peptide (GenBank accession No. AY786158). Compared with *MGTA1* cDNA sequence, *MGTA1* genomic DNA cassette contains 6 introns and 6 exons. Of these 6 introns, a 100 bp long 5′-intron locates at the 405 bp upstream of the *MGTA1* initiation codon. *MG10529.4* not having exactly the same location as that of the second and the third intron of *MGTA1* leads to a difference in a stretch of 28-amino acids between the two proteins (Fig.2). Since intron locations of *MGTA1* were determined experimentally using a cDNA sequence, the automatic annotation of these two introns in *MG10529.4* was erroneously estimated.

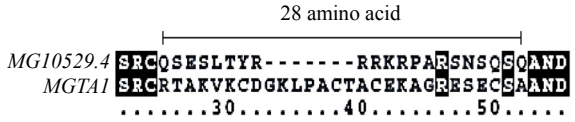


Fig.2 28-amino acid divergence between deduced protein sequences of *MG10529.4* and *MGTA1*

The 790 amino acid polypeptide of *MGTA1* gene has 51% identity to *CLTA1* gene (*E*-value=e-173). Three conserved functional domains, characteristic of transcription factors from the zinc binuclear cluster family ($Zn(II)_2Cys_6$ DNA binding domain, middle homology region and activation domain), appear at the regions from aa 22 to 51, 276 to 315 and 628 to 738 respectively in Mgta1p (Fig.3). These 3 domains are identical to the homologous domains of Clta1p with 93%, 75%, 22% identity respectively. The features of Mgta1p sequence indicate that this protein is a potential transcriptional activator from the fungal zinc-binuclear cluster family.

Copies of *MGTA1* gene in 7 *M. grisea* strains

In *M. grisea* genome database (strain 70-15, Broad Institute), only one copy of *MGTA1* was found. The number of copies of *MGTA1* in 7 *M. grisea* strains isolated from 5 host plants in different geographic locations (Y91-11, Y90-1, 84-7-3, 2000-034E3, 2001-068F1, 2001-060G1 and Guy11) was assessed by Southern hybridization. Firstly, genomic DNA from strain Guy11 was digested with six restriction enzymes (*Apa*I, *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I, and *Xho*I), separated by agarose gel electrophoresis, blotted on Nylon membrane and hybridized with a 1 kb probe of *MGTA1* internal fragment. Only one band emerged in each lane of digested DNA (Fig.4a). Then, genomic DNA of other 6 *M. grisea* strains and strain Guy11 were digested by *Sac* I and blotted by the same probe as above. Only one band emerged in each lane of digested DNA for each strain (Fig.4b). These facts implied that only one copy of *MGTA1* gene exists in each genome of these 7 strains.

***MGTA1* expression in conidia, mycelia and appressoria**

To investigate the expression pattern of *MGTA1* gene, an *MGTA1(p)::eGFP::HPH* fusion vector was constructed and introduced into *M. grisea* strain Guy11. Eight transformants containing single insertion were selected from 30 hygromycin-resistant transformants by Southern analysis (data not showed). Mycelia and conidia at different stages of development of these 8 transformants were examined for *eGFP* fluorescence under control of *MGTA1* promoter. Green *eGFP* fluorescence appeared faintly in hyphal tips from mycelia cultivated on complete media and increased lightly in conidia (Fig.5a and Fig.5b). The conidia of 8 transformants were induced to germinate and form appressoria on terylene membranes and sterile onion epidermis. During the germinating process, the *eGFP* fluorescence of conidia decreased rapidly. In germ tubes and young appressoria, no *eGFP* fluorescence was detected. While in mature appressoria, *eGFP* fluorescence was observed again (Fig.5c). On onion epidermis, infection hyphae were observed clearly in the infected cells after incubation of 28 h, but no *eGFP* fluorescence was detected.

DISCUSSION

M. grisea is a well-known ascomycete that causes rice blast (Ou, 1985). As a model fungal pathogen, its pre-penetration processes, including conidium dissemination, adhesion (Hamer *et al.*, 1988) and germination, appressorium differentiation (Howard *et al.*, 1991) and maturation (Howard and Ferrari, 1989), penetration peg differentiation (Bourett and Howard, 1990; 1992), had been reported. Several

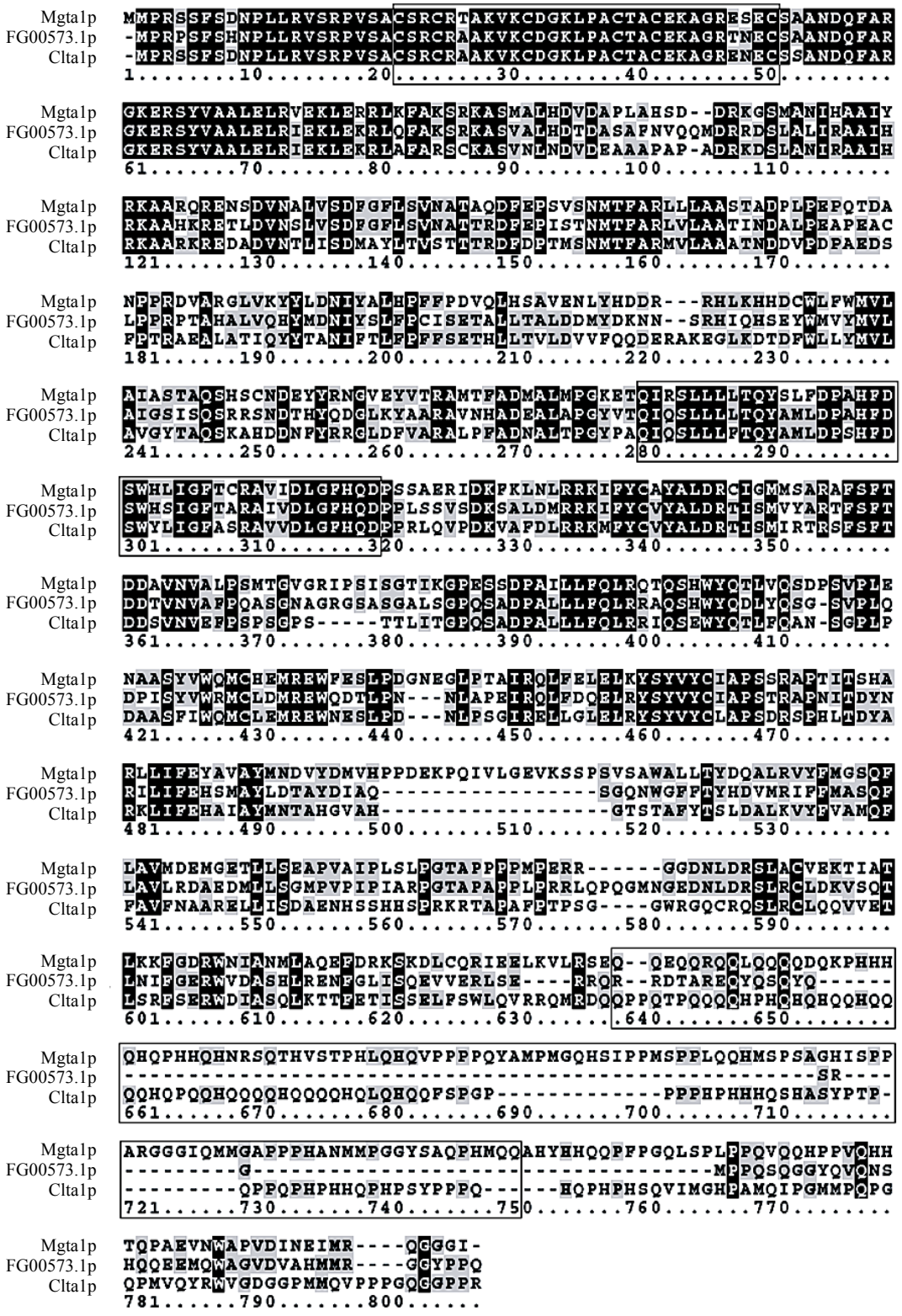
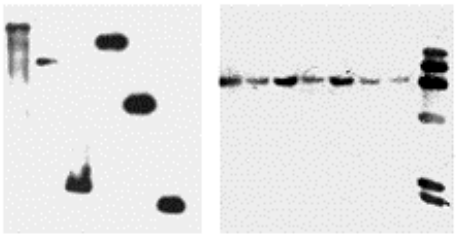


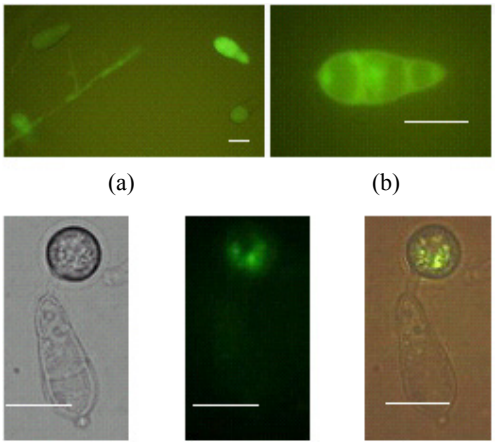
Fig.3 Sequence alignment of Mgta1p*, FG00573.1p and Clta1p. The 3 conserved functional domains were indicated in boxes

*The sequence of Mgta1p deduced from cDNA sequence, not the MG10529.4p automatic estimated from *M. grisea* genome database (Broad Institute)



(a) (b)

Fig.4 Copies of *MGTA1* gene in *M. grisea* strains (a) Southern blot of genomic DNA from *M. grisea* strain Guy11 strain with 1 kb fragment of *MGTA1* gene. Genomic DNA was digested with different enzymes (left to right: *Apa*I, *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I, *Xho*I); (b) Southern blot of genomic DNAs of 7 *M. grisea* strains by 1 kb fragment of *MGTA1* gene (The strains and hosts (from left to right): Y91-11, summer grass; Y90-1, goose grass; 84-7-3, millet; 2000-034E3, rice; 2001-068F1, rice; 2001-060G1, rice; Guy11). Genomic DNA was digested with *Sac*I



(a) (b)

c_1 c_2 c_3

(c)

Fig.5 Fluorescence in *M. grisea MGTA1(p)::eGFP* transformants (Bars=10 μm) (a) Fluorescence of conidia and tip hyphae; (b) Zoom of a conidium's fluorescence; (c) Fluorescence of appressorium. c_1, c_2 and c_3 show the image under bright-field illumination, UV light and bright-UV compound light, respectively

chemical and physical factors activating these processes have been found and many genes related to these processes have been cloned and characterized (Balhadere and Talbot, 2001; Talbot *et al*., 1993; Xu and Hamer, 1996). The post-penetration processes of *M. grisea*, including infection hyphae differentiation and colonization, are far less known (Balhadere and Talbot, 2001). Our work focuses on genes involved in such processes. In this study, we isolated *MGTA1*, a novel gene homologous to *CLTA1* involved in the post-penetration processes of *C. lindemuthianum* (Dufresne *et al*., 2000). Clarification of *MGTA1* functions, especially in pathogenicity, is helpful for us to understand the infection process of *M. grisea* and the difference of nutrition in post-penetration processes between *M. grisea* and *C. lindemuthianum*. Work on *MGTA1* gene replacement, mutant analysis, functional recovery, and cross-complement of *MGTA1* and *CLTA1* is being carried out now in our lab. The *MGTA1* gene replacement vector was constructed successfully (data not shown).

MGTA1 gene expression in *M. grisea* strain Guy11 was demonstrated by *MGTA1(p)::eGFP::HPH* fusion expression experiment. Unlike the *CLTA1* gene expressed in later phase of the *C. lindemuthianum* infection cycle (Dufresne *et al*., 2000), *MGTA1* gene is expressed in several phases of the *M. grisea* infection cycle: tip hypha, conidium and mature appressorium. While in infectious hypha, *eGFP* fluorescence cannot be detected. It is possible that the fluorescence is too weak to be detected under the shades of host cell cuticles. The different expression patterns indicate that some potential differences probably exist between *MGTA1* and *CLTA1* gene functions.

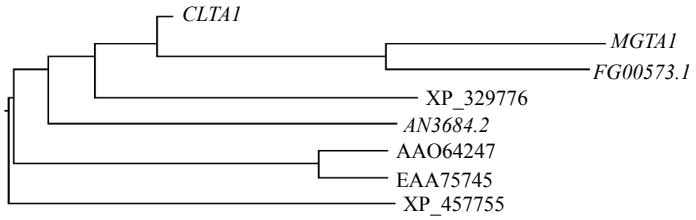
Several homologous genes of *MGTA1* were found in some fungal genomes by searching against GenBank database with Mgta1p (Fig.6). In these homologues, a predicted protein FG00573.1 from *Gibberella zeae* had 54% identity to the protein Mgta1p and 56% identity to Clta1p (Fig.3). *G. zeae* does not form appressorium for plant penetration, and the *CLTA1* null mutants of *C. lindemuthianum* can form appressoria normally (Dufresne *et al*., 2000), so *MGTA1* is possibly involved in other processes. It will be interesting to determine the function of *MGTA1*, *FG00573.1* and their homologous genes fungal pathogens.

MGTA1 encodes a putative transcription factor of the $Zn(II)_2Cys_6$ family. $Zn(II)_2Cys_6$ DNA binding proteins are unique fungal transcriptional activators involved in a wide range of processes, including primary and secondary metabolism, drug resistance, and meiotic development (Todd and Andrianopoulos, 1997). Fifty-four hypothetical proteins with $Zn(II)_2Cys_6$ domain were found in the *M. grisea* genome database (Broad Institute). Up to now, these genes have not been researched well yet.

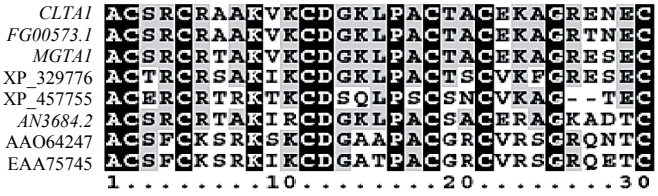
Sequences producing significant alignments

GenBenk accession No.	Gene name	Organisms	Score (bits)	*E*-value
AY78615	*MGTA1*	*Magnaporthe grisea*		
EAA69234	*FG00573.1* (hypothetical)	*Gibberella zeae*	650	0.0
AAG25917	*CLTA1*	*Colletotrichum lindemuthianum*	612	e-173
XP_329776	Hypothetical	*Neurospora crassa*	404	e-111
EAA59892	*AN3684.2* (hypothetical)	*Aspergillus nidulans*	208	4e-52
AAO64247	Unknown	*Fusarium sporotrichioides*	163	1e-38
EAA75745	Hypothetical	*Gibberella zeae*	157	1e-36
XP_457755	Unnamed	*Debaryomyces hansenii*	113	2e-23

(a)



(b)



(c)

Fig.6 Scores of 8 top hits of blast to GenBank using Mgta1p (a) GenBank accession No., gene name, organisms, and scores of 8 top hits; (b) Phylogeny of the 8 top hit protein sequences (marked with gene name or GenBank accession No.); (c) Sequence alignment of $Zn(II)_2Cys_6$ domains of the 8 top hits

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