ORIGINAL ARTICLE



## Genome-wide identification, classification and expression analysis in fungal–plant interactions of cutinase gene family and functional analysis of a putative *ClCUT7* in *Curvularia lunata*

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**Abstract** Cutinase is described as playing various roles in fungal-plant pathogen interactions, such as eliciting host-derived signals, fungal spore attachment and carbon acquisition during saprophytic growth. However, the characteristics of the cutinase genes, their expression in compatible interactions and their roles in pathogenesis have not been reported in Curvularia lunata, an important leaf spot pathogen of maize in China. Therefore, a cutinase gene family analysis could have profound significance. In this study, we identified 13 cutinase genes (ClCUT1 to ClCUT13) in the C. lunata genome. Multiple sequence alignment showed that most fungal cutinase proteins had one highly conserved GYSQG motif and a similar DxVCxG[ST]-[LIVMF](3)-x(3)H motif. Gene structure analyses of the cutinases revealed a complex intron-exon pattern with differences in the position and number of introns and exons. Based on phylogenetic relationship analysis, C. lunata cutinases and 78 known cutinase proteins

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from other fungi were classified into four groups with subgroups, but the C. lunata cutinases clustered in only three of the four groups. Motif analyses showed that each group of cutinases from C. lunata had a common motif. Real-time PCR indicated that transcript levels of the cutinase genes in a compatible interaction between pathogen and host had varied expression patterns. Interestingly, the transcript levels of ClCUT7 gradually increased during early pathogenesis with the most significant up-regulation at 3 h post-inoculation. When ClCUT7 was deleted, pathogenicity of the mutant decreased on unwounded maize (Zea mays) leaves. On wounded maize leaves, however, the mutant caused symptoms similar to the wild-type strain. Moreover, the ClCUT7 mutant had an approximately 10 % reduction in growth rate when cutin was the sole carbon source. In conclusion, we identified and characterized the cutinase family genes of C. lunata, analyzed their expression patterns in a compatible host-pathogen interaction, and explored the role of ClCUT7 in pathogenicity. This work will increase our understanding of cutinase genes in other fungal-plant pathogens.

**Keywords** Cutinase · Compatible interaction · Expression patterns · Pathogenicity

#### Introduction

The cuticle is the outermost layer of the aerial part of all plants and forms the first obstacle to invasion by plant pathogens. However, most plant pathogens produce cutinases that catalyze the degradation of the cuticle and promote penetration by the pathogen. Degradation of the host plant cuticle at the inoculation site leads to successful penetration and infection by various pathogens (Maddock 1979; Shaykh et al. 1977). Moreover, if the cutinase activity of a fungal–plant pathogen is deleted, its pathogenicity is often weakened (Davies et al. 2000; Zhang et al. 2005) or eliminated (Dickman and Patil 1986; Stahl and Schafer 1992; Stahl et al. 1994).

Curvularia leaf spot of maize (Zea mays) caused by Curvularia lunata (Wakker) Boed was one of the most severe diseases in China's maize (corn) production zone during the 1990s and is still prevalent nationwide (Dai et al. 1995, 1998; Gao et al. 2014a, b; Ito et al.1979; Li et al. 2006; Macri and Lenna 1974). The fungus causes water-soaked or necrotic spots with yellow halos on leaves (Hou et al. 2013). It is presumed that this pathogen produces a series of degradation enzymes that soften the cell wall and breach the host's defenses. Although some degradation enzymes such as polygalacturonase, polymethylgalacturonase, cellulases and laccases also have important roles in colonization by the pathogen (Feng et al. 2002; Zhou et al. 2010), little is known about the characteristics of the cutinase genes, their expression, or their roles in the pathogenesis of C. lunata. Therefore, an investigation of the cutinase genes in C. lunata is important and timely.

The recent completion of a draft genome of *C. lunata* provided information to help identify the cutinase genes of this fungus (Gao et al. 2014a, b). In this study, we identified 13 cutinase genes in *C. lunata*, and analyzed the structures of their encoded proteins, gene intron–exon distributions, phylogenetic relationships, motif distributions, and their expression patterns in a compatible host–pathogen interaction. We also characterized the function of an up-regulated cutinase gene, *ClCUT7*, in the penetration process. The results of these analyses suggested that the cutinases of *C. lunata* are involved in its pathogenesis on maize. These results will lead to future systematic analyses of the functions of the cutinase genes in this plant pathogen.

#### Materials and methods

#### Fungal strains and plant material

The highly virulent strain CX-3 of *C. lunata*, provided by Prof. Jie Chen (Shanghai Jiaotong University, Shanghai, China) was used as the wild-type strain. The maize inbred line 'Huangzao 4' was used as the susceptible host plant. Maize seeds were surface sterilized in 10 % sodium hypochlorite for 5 min, rinsed with sterile water, and germinated on wet filter paper for 2 days at 25 °C. Germinated seeds were transferred to  $14 \times 18$ -cm pots and kept in a growth chamber for 4 weeks at 25 °C with a 14 h photoperiod and 60 % relative humidity.

#### Cutinase sequence search

Data for the *C. lunata* genome were downloaded from http://www.ncbi.nlm.nih.gov/nuccore/JFHG00000000. Pedicted protein databases were constructed using BioEdit<sup>®</sup> software. The keyword "cutinase" was used to search the National Center for Biotechnology Information (NCBI) protein database. The known cutinase proteins from plant pathogens were downloaded (Additional file 1) as queries against the predicted protein databases from *C. lunata*. Proteins with a significant *E*-value (<1.0<sup>-10</sup>) were extracted from the predicted protein database of *C. lunata* and redundant protein sequences were discarded. The candidate proteins were analyzed using the conserved domain database (CDD) server at NCBI. The proteins containing cutinase domains were used for further analyses.

#### Sequence analysis

The molecular weights (kDa) and isoelectric points (PI) of each predicted cutinase protein were calculated using online ExPASy programs (http://www.expasy.org/tools/). Multiple alignments were made with the Clustal W2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and GENEDOC v. 2.7000<sup>©</sup> software. Amino acid sequences used for conserved residues analysis were as indicated (Additional file 2). All predicted cutinase protein sequences from C. lunata, plus 78 other known cutinase amino acid sequences (Additional file 3) downloaded from the NCBI database, were aligned for phylogenetic analyses with MEGA6.0<sup>©</sup> (http://megasoftware.net) using the neighborjoining method (minimum evolution criterion; bootstrap values performed on 1000 replicates) (Staitou and Nei 1987). To reveal the exon-intron organization of the cutinase genes in C. lunata, we aligned the genomic and cDNA sequences using Clustal W2 (http://www.ebi.ac.uk/Tools/ msa/clustalw2/). Structural analyses of the cutinase genes were performed using the online server GSDS (http://gsds. cbi.pku.edu.cn/). Conserved motifs were identified using the online MEME analysis tool (http://meme.nbcr.net/ meme/cgi-bin/meme.cgi) with the following parameters: optimum motif width  $\geq 6$  and  $\leq 200$ ; maximum number of motifs = 25.

#### Sample preparation, RNA isolation and cDNA synthesis

To analyze the transcript levels of cutinase genes during host–pathogen interactions, maize plants at the seven-leaf stage were inoculated using an air sprayer with  $1 \times 10^6$  conidia/ml suspensions of *C. lunata* CX-3 in 2 % sucrose and 0.02 % Tween 20 as described (Huang et al. 2008). Control plants were sprayed with sucrose and Tween 20 only. Maize leaves were collected at 0, 0.5, 1.5, 3 and 24 h

post-inoculation (hpi). All samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Total RNAs were extracted using a QIAGEN RNeasy Plant<sup>®</sup> Kit (Beijing, China) following the manufacturer's recommendations. RNA concentrations were measured on a NanoDrop<sup>®</sup> spectrophotometer (ND-1000) and quality was checked by agarose gel electrophoresis (28SrRNA/18Sr RNA ratios). We synthesized cDNA from the total RNAs (0.5–1 mg) using an MMLV reverse transcriptase cDNA Synthesis Kit<sup>®</sup> following the manufacturer's instructions (Cat#D6130, TaKaRa, Tokyo, Japan).

#### **Quantitative PCR**

Quantitative RT-PCR (qRT-PCR) was performed in 20-ml reaction volumes using Super-Real PreMixPlus<sup>®</sup> (SYBR-Green; Cat. # FP205, Tiangen Biotech, Beijing, China) on an ABI PRISM7500 sequence-detection system according to manufacturer's instructions. *GAPDH* was used as the internal control. We used the comparative  $C_t$  method ( $\Delta\Delta C_t$ ) to calculate relative expression levels. Statistical analysis of gene expression was conducted using ANOVA (SAS 8.2). The primer pairs used for qRT-PCR are listed in Additional file 4. The experiments were repeated at least twice.

#### Deletion of the ClCUT7 gene

Nucleotide sequences from 3000 bp upstream and 2500 bp downstream of the ClCUT7 gene were identified from the C. lunata genome. We constructed a gene deletion cassette using a double-joint PCR technique (DJ-PCR) (Yu et al. 2004). A deletion cassette containing flanking sequences of the ClCUT7 gene and a selectable marker hygromycin phosphotransferase (*hph*) gene was amplified by three sequential cycles of PCR. In the first cycle, the two flanking fragments of ClCUT7 and hph with the TrpC promoter of Aspergillus nidulans (HPH), were amplified with primers cu1 s/cu1a, cu2 s/cu2a and hphs/hpha (Additional file 5), respectively. In the second cycle, two fusion fragments were amplified using the first-round PCR products as templates. In the third PCR cycle, the deletion cassette was developed with primers cu1s and cu2a using 100-fold dilutions of the two fusion fragments as templates from the second round of PCR. The deletion cassette products were introduced into protoplasts of wild-type strain CX-3 as described (Liu et al. 2010). Hygromycin-resistant transformants were isolated and screened for durable resistance by growing them for six consecutive transfers on potato dextrose agar (PDA) containing 250  $\mu$ g ml<sup>-1</sup> hygromycin. DNA from the hygromycin-resistant transformants was extracted as described previously (Liu et al. 2011) and used to detect the gene deletion mutants. The resulting mutants were confirmed by Southern blot analysis and RT-PCR. The probe used for Southern blotting was amplified with primers cus and cua, and labeled with biotin. Southern blotting was performed as described (Liu et al. 2011). Primers used for construction of the deletion cassette, the initial detection of the gene deletion mutant and RT-PCR are listed in Additional file 5, Table S5.

#### Colony characteristics and pathogenicity assay

Mycelium from C. lunata (wild-type strain CX-3) and the ClCUT7 deletion mutant were inoculated onto plates containing PDA and incubated for 7 days at 25 °C. Colony morphology was documented with a Nikon D3200 DSLR camera. To measure vegetative growth, a 4-mm mycelial plug from each strain was transferred to fresh PDA plates and incubated in the dark for 7 days at 25 °C. Colony diameters were measured and recorded daily. Vegetative growth was based on the change in colony diameter after 7 days. Each experiment was performed three times. Conidia production was determined by inoculating both wild-type and mutant strains onto PDA plates and incubating them for 12 days at 25 °C in darkness. Then 10 ml of sterile distilled H<sub>2</sub>O was added to each plate, the conidia dislodged with a sterile swab, and then counted using a hemocytometer. Conidial morphology was observed and photographed using an Olympus BX51 microscope (Olympus, Center Valley, PA, USA). To observe appressorium formation, conidia were inoculated onto cellophane disks placed on PDA, examined microscopically after 6, 9, and 12 h, and photographed using Olympus cellSens® standard software. For pathogenicity assays, we removed the fourth leaf of maize cultivar Huang Zao 4 at the seventh leaf stage, cut it into 5-cm fragments, and placed the fragments on moist filter paper in humid petri dishes at 25 °C. Wounded and unwounded leaves were inoculated with a suspension of  $1 \times 10^6$  conidia/ml in 0.02 % Tween 20 as previously described (Liu et al. 2010). Lesion development was examined and recorded as described by Gao et al. (2014a, b), and then photographed using a Nikon D3200 DSLR camera. Each experiment was conducted at least three times.

### Results

#### Identification of cutinase proteins

To identify putative cutinase proteins in the *C. lunata* genome, 34 cutinase proteins from the necrotrophic pathogens *Rhizoctonia solani*, *Phytophthora capsici*, *Fusarium solani*, and *Phaeosphaeria nodorum* SN 15 were used as queries in BLASTP searches for possible homologous cutinase proteins from *C. lunata* using BioEdit<sup>®</sup> software. As a result,

Designated gene name	ORF length (bp)	Amino acid (aa)	Molecular weight (kD)	Isoelectric point (pI)	Location	
CICUTI	651	216	21.6	9.16	Scaffold20	
ClCUT2	747	248	25.9	5.54	Scaffold1	
ClCUT3	1326	441	40.8	7.61	Scaffold19	
ClCUT4	750	249	25.8	8.96	Scaffold4	
ClCUT5	714	237	25.1	5.73	Scaffold30	
ClCUT6	708	235	23.9	8.31	Scaffold10	
ClCUT7	675	224	23.8	7.55	Scaffold30	
ClCUT8	1272	423	43.6	5.10	Scaffold15	
ClCUT9	996	331	34.1	5.42	Scaffold8	
ClCUT10	1071	356	37.1	5.75	Scaffold20	
ClCUT11	999	332	33.9	5.48	Scaffold3	
ClCUT12	957	318	34.4	6.06	Scaffold1	
ClCUT13	402	133	14.7	8.59	Scaffold14	

Table 1 Characteristics of 13 cutinase genes (ClCUT) in Curvularia lunata

31 candidate proteins from *C. lunata* were obtained that had probability E-value thresholds of  $1.0^{-10}$ . Subsequently, all candidate protein sequences were surveyed using a CDD search in NCBI to further verify whether they contained cutinase domains. Eighteen candidate protein sequences lacked cutinase domains and were discarded. Consequently, 13 nonredundant cutinase proteins were identified and designated as ClCUT1 to ClCUT13. Using ExPASy<sup>®</sup> tools, we found that the shortest cutinase protein consisted of 133 amino acid residues and the longest had 411 amino acid residues. The respective ORF lengths ranged from 402 to 1326 nucleotides. The predicted molecular weights/isoelectric points of all cutinase proteins ranged from 14.7/9.16 to 40.8/5.10, respectively. All cutinase sequences were distributed on 10 different scaffolds in the *C. lunata* genome (Table 1).

# Multiple sequence alignment and intron–exon organization

We conducted a protein sequence alignment among all cutinases from *C. lunata*, 10 cutinase proteins from other plant pathogens, and analyzed some conserved amino acid sites. These data showed that 12 cutinase proteins had highly conserved GYSQG motifs and a DxVCxG[ST]-[LIVMF] (3)-x(3)H motif similar to cutinase proteins from other phytopathogens. We also noted that ClCUT13 lacked the conserved GYSQG motif, but contained the DxVCxG[ST]-[LIVMF](3)-x(3)H motif (Fig. 1).

All predicted cutinase genes had different intronexon patterns in relation to both the position and number of introns, which ranged from 0 to 4 per gene. Based on the number of introns, the cutinases were segregated into four patterns. Pattern1 (*ClCUT3* and *ClCUT8*), pattern 2 (*ClCUT1*, *ClCUT2*, *ClCUT4*, *ClCUT9*, *ClCUT10* and *ClCUT11*), pattern 3 (*ClCUT5*, *ClCUT6* and *ClCUT12*), and pattern 4 (*ClCUT7* and *ClCUT13*) contained 1, 2, 3, and 4 introns, respectively. Furthermore, significant differences in size (41-1326 bp) between the exons were observed. These results showed the different cutinases had variable, complex intron–exon structures (Fig. 2).

### Phylogenetic and motif analyses

To examine the relationship between the cutinase genes of C. lunata and those from other fungal organisms, we constructed a phylogenetic tree based on the multiple sequence alignment of the 13 CICUT protein sequences and 78 known cutinases from other fungi. The phylogenetic tree divided these cutinase genes into four groups (Groups 1, 2, 3, 4). Group 1 and Group 3 were further divided into two subgroups (1a and 1b, 3a and 3b), respectively. In our analysis, ClCUT1, ClCUT2 and CICUT3 were clustered in Group 1a, but CICUT7 was in Group 1b. CICUT4, CICUT5 and CICUT6 were clustered in Group 2. CICUT8, CICUT9, CICUT11, CICUT12 and CICUT13 constituted Group 3a. CICUT10 was placed in Group 3b as a separate clade. None of the *ClCUT* genes from C. lunata were clustered in Group 4. The motif distribution generated by MEME analysis was consistent with the phylogenetic analysis (Fig. 3). Members of the same class shared several of the conserved motifs, which indicated that genes containing these motifs were probably produced from gene expansion within the same class or subclass. For instance, members of Group 1a contained motifs 1, 2, 3 and 10, whereas Group 1b had different motifs. All members of Group 2 contained motifs 1, 2, 3, and 8, whereas Group 3 members contained motifs 2, 4 and 7. It was noted that all ClCUT genes had motifs 1, 2 and 3, whereas ClCUT13 lacked motifs 1 and 3 (Fig. 4). It is likely that ClCUT13 lost motifs 1 and 3 during evolution. All motifs with characteristic sequences identified by MEME tools are listed in Additional file 6.

		* 26	•	200	* 400		400	* 440	* 160		
CERMD01175		MECOURAR		COUNTRADAT	~ 400	DDCAR	420	NCONCETAN	~ 480		157
Cleum1	:	MICOVRAK	CPDITLYMAGISQC	COLUMNAANILSA		DDSAF VSSF		NCKDVCKISAA		:	157
M035164994	:	DENBACT KALAAD	CPUIKLVMAGISQC	ACUMUNA ADOL DR.			WARGE DI	NCOCUTCUDTS		:	134
M035163931	:		CPNTCTVTSCVSOC	AMUMHNAADQUDF		FTASHOVAZ				:	126
BC1177242	:	MUTSTLSS	CPDTRIVISCVSOC	COLVHNAAKLLDA		FTTAKISSI		NCDPVOCVSAD		:	159
BC34783153	:	Mitmemt.co	CPD TRIVISCYSOC	COLVENAAKTTDA		FUTAKISSE		NGDPVQGVSAD		:	159
CICUT2	:	INTTASE	CROTHINICOC	ACT VHKALORITA		ACAARWNAU				:	207
M035164870	:	LATEAGOK	CECTRVVISCUSOC	GOLVHNAARTLGP		OGISVNSAR	WTFC PY	NPAPVPGLPSD		:	199
CICUT3	:	IMKOAKSO	CPDTRVTVSCVSOC	AMUVHNA ENKC		LSASDASCA	WMEC DI	KRAATTGLSAD		:	395
CICUT4	:		CPDSWTWSSCVSOC	AAUNHRATEELDA		GVKDO AGV	WINC TOKLO	DRDOIDKEDKD		:	174
CICUTS	:		CRSATTWCCCVSOC	AAMNHRATEDLSD		AVKNOTAGY	WTECHTOABA	DNOOTDREPKD		:	184
CICUTE	:	I.FOOANOK	CPDTPVVSCGVSOC	TANTAGATPKLSE		AVRACHKGT	VT FOYTKNKO	NNGGTPSYPAF		:	191
C5FMD90612	:		CENOTEALVEVSOC	ASIMHRAAFKLDV		SLEDRIKAU	WMEC POORLGA	LODKEPIGLEP		:	182
CICUT7	:		CENOTESLVGVSOC	ASIMHKAADOLPV		SLEPKTKSV	WMEGN PNT.RLGR	LODKEPAALOS		:	180
C5EMD94256	-		CPOTHITTNGYSOC	AW MGDILAGPG-	GCSOFTT-GL	DETTAPONATAAA	TTWG VIHEANE	PYNVLDGAGLO-K	SGRPEPSLSRLNRYSS		194
CICUT11			CPOTHT.TTN GESOG	AW TGDILGGPG-	GCSOTTT-GL	DNTSAPGNATAAA	TTWO VMHEANO	PYNVLDGSDLO-K	SGRSADSLARLNRYSS		192
CICUT9			CPOSHTVVNGVSFG	AHVMGDVLGGPG-	GCTKVSN-GL	DSTSSAGKATAAA	TTWG VMHTANO	PYNVLDGASKO-K	NPRSASDLALLNRYAP	-	193
C5EMD93730			CPDTT TVTN GYSOG	AMIGSATLSGGGE	-DACDVDPOTT-GL	DPNSKAGOATKAV	TING VKHTANO	SYNVLDGADKO-V	WPRTGANLERMNRESS		202
C1CUT8			CPDTLIVINGYSOC	AMMAGALLSGGGE	-DACNVDALTT-GL	DPASOAGOALKA	TING VKHTANO	PYNALDGADKA-V	WPRTGVNLDRLNRYSS		202
CICUT12		MRNYTAR	CPDSHITTYNGESOC	GSUML DVLGGGGG	PIWGCEOKSNPSM	NTTSPPGSKTAAA	TVFCPTRRSANO	SYTHEGEOISDEG	APRSAFELAGLOPYADAG		210
CICUT13	-		FFFS	BILGGGGG	DAGLGCSSRTNPGL	NASTSPONK	TIEG VRHTANO	VYNTASGANSDGI	NPRRGTOLISLNRFSN		90
CICUT10		NOAYSKK	CPDSKLIVI GESOG	GSTAODMLGGGGG	-PTFKCEOGDNPAL	DASVAPGSNVVAA	WTECAVSRSRGO	NFTVGGGKDYDGT	RARTPEOLOGLNOYAD		217
RS47189543	:	AAOAIS	CPNOHEVI SGYSKC	ALVLHGLSLSSN-		LRSK ASI	IVEG PARNINSPW	PINNPSVDLSPKD	GSSSS0		162
			cp q5S q			6	6 5Gd		-		
			-r 9 9								
		*	480	* 500	*	520	* 540	*	560 *		
C5EMD91175	:	NTKIICATGDLI	AGQAIILPPH	SYGANANEAAR	TSKM				AGKAAARLVKARSFTA	:	214
CICUT1	:	NTKVFCAACCLI	AGQAVILPPH	SYGANANQAAK	WMSKAGA				GGAAAARAVSARTFTA	:	216
M035164994	:	RVLVICHDRDNI	EGGAQIRQAH	TYGQDVSKAAS	MQAAAA			GQG	AGQGGVEAPGAGAAAGAN	:	198
M035163931	:	RTLVVCHAGENV	QGGGMILPAH	TYGQDVGKAAA	WMEAAAAVVASMG	AAPATEAAPGTEA	VPGAGAVLGAGAVP	GTGGAPGTEAAPGTG	ASPGTEAAPGTGAAPGTE	:	235
BC1177242	:	RTDIICHAGDNI	QGGSLILLAH	TYGMDTTABAA	WKKAAGL					:	202
BC34783153	:	RTDIICHAGDNI	QGGSLILLAH	TYGMDTTABAA	WKKAAGL					:	202
C1CUT2	:	NTLIICHEGELI	ENTAILTPENS	NYEQDAPTEAA	BOAARV						248
M035164870	:	DSMIFCHTCDU	KCCFOLFDD		and the second se					-	262
C1CUT3		and the second	enders prore	TYNRDAGA∉AD	EIVRITKMN			G	PPGGGNMAPLPFPPPTPS	:	
	:	KIKQFCGTADQI	CGGGGGDGGATGSH	TYNRDAGAAAD SYGSSAEAAAT	AIQAAGLA			G	PPGGGNMAPLPFPPPTPS	:	441
CICUT4	:	KIKQFCGTADQI KVKIICQPGDAV	GGGGGDGGATGSH LGTLAVLPAH	TYNRDAGAJAD SYGSSAEAJAT TYGLRADEGVD	UVRITKMN AIQAAGLA UVQQVKAAQAKIK	ARNKKREAEK		G	PPGGGNMAPLPFPPPTPS 		249
CICUT4 CICUT5	:	KIKQFCGTADQI KVKIICQPGDAV KLKIFCGGAVRITV	CGGGGDGGATGSHI CLGTLAVLPAH CDGNLSAAVLLPH	TYNRDAGAAAD SYGSSAEAAAT TYGLRADEGVD SYGRDAAEAGR	FIVRITKMN FAIQAAGLA FIVQQVKAAQAKIK FIIGKINAVRG	ARNKKREAEK		G	PPGGGNMAPLPFPPPTPS 		249 237
CICUT4 CICUT5 CICUT6	: : :	KIKQFCGTAPQI KVKIICQPGPAV KLKIFCGGAVRT NLKVYCAAGPLV	GGGGDGGATGSH LGTLAVLPAH CDGNLSAAVLLPH SGTLTVTAAH	TYNRDAGAPAD SYGSSAEAPAT TYGLRADEGVD SYGRDAAPAGR TYNDDAMG-PAPQ	FIVRITKMN FAIQAAGLA FIVQQVKAAQAKIK FIIGKINAVRG FIESKIGA	ARNKKREAEK		G	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA-		249 237 235
ClCUT4 ClCUT5 ClCUT6 C5EMD90612		KIKQFCGTALQI KVKIICQPGDAV KLKIFCGGAVRDTV NLKVYCAACILV KVLQVCAKCIPV	GGGGDGGATGSH LGTLAVLPAH DGNLSAAVLLPH SGTLTVTAAH CDSGSCQYYH	TYNRDAGADAD SYGSSAEADAT TYGLRADEGVD SYGRDAADOGR TYNDDAMG-POPQ TYVLPKLMDPOVA	FIVRITKMN FAIQAAGLA FIVQQVKAAQAKIK FIIGKINAVRG FIESKIGA FIVRAFQGSPRL	ARNKKREAEK SRS		G	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA-		441 249 237 235 230
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7		KIKQFCGTADQI KVKIICQPGDAV KIKIFCGGAVRDTV NIKVYCAAGILV KVLQVCAKGIPV KVLEVCAKGIPV	GGGGDGDGATGSH LGTLAVLPAH DGNLSAAVLLPH SGTLTVTAAH DSGSCQYYH DSGTCQYYH	TYNRDAGABAD SYGSSAEABAT TYGLRADBCAT SYGRDAABAGR TYNDDAMG-PAPQ TYVLPKLMDPAVA TYVLPKLMDPAVA	FIVRITKMN FAIQAAGLA FIVQQVKAAQAKIK FIIGKINAVRG FIESKIGA FIVRAFQGSPRL YIVKAFKS	ARNKKREAEK SRS		G	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA-		441 249 237 235 230 224
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7 C5EMD94256		KIKQFGG-TADQI KVKIICQ-PGIAV KLKIFCGGAVRITV NIKVYGA-ACILV KVLQVCA-KCIPV KVLEVCA-KCIPV VLRSYGA-KCIPI	GGGGDGGATGSH LGTLAVLPAH DGNLSAAVLLPH SGTLTVTAAH DSGSCQYH DSGTCQYH ANGDDLSKH	TYNRDAGAAAD SYGSSAEAAAT TYGLRADEGVD SYGRDAAEAGR TYNDDAMG-PAPQ TYVLPRLMDPAVA TYVLPRLMDPAVA SYFELYTG-DAST	PIVRITKMN PAIQAAGLA PIVQQVKAAQAKIKI PIIGKINAVRG FIESKIGA PIVRAFQGSPRL YIVKAFKS YAVNKINA	ARNKKREAEK SRS		G	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- 		441 249 237 235 230 224 268
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7 C5EMD94256 ClCUT11		KIKQFGG-TAPQI KVKIIGQ-PGTAV KLKIFGGAVRT NLKVYGA-AGTIV KVLQVGA-KGIPV KVLEVGA-KGIPV VLESYGA-KGIPI VLRSYGA-KGIPI	GGGDGGATGSH LGTLAVLPAH DGNLSAAVLLPH SGTLTVTAAH DSGSCQYYH DSGTCQYYH DSGTCQYH ANGDDLSKH ANGDDLSKH	TYNRDAGA-AD SYGSSAEA-AT TYGLRADEGVD SYGRDAAE-GR TYNDDAMG-P-PC TYVLPRLMDP-VA TYVLPRLMDP-VA SYFELYTG-D-ST SYFELYTD-D-ST	PIVRITKMN PATQAGLA PIVQQVKAAQAKIK PITGKINAVRG PIESKIGA PIVRAFQGSPRL VVKAFKS AVNKIND AVGKVND	ARNKKREAEK SRS VAPLCDAP-TI AAPLCAAP-TI		G 	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- 		441 249 237 235 230 224 268 268
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7 C5EMD94256 ClCUT11 ClCUT9		KIKQFGG-TATQI KVKIICQ-PGTAV KLKIFGGAVRTV NLKVGA-ACTLV KVLQVGA-KGIPV KVLEVGA-KGIPV VLRSVGA-KGIPI VLRSVGA-KGIPI VLRSVGA-ACTPV	GGGDGGATCSH CG-TLAVIPA DGNLSAAVLLH SG-TLTVTAAH DSGSCQYY DSGTCQYY ANGDDISK ANGDDISK ANGDDVER ASCGNVVAD	TYNRDAGAAAD SYGSSAEAAT TYGLRADEGVD SYGRDAAEAGR TYNDDAMG-PAPC TYVLPKLMDPAVA TYVLPKLMDPAVA SYFELYTC-DAST SYFELYTC-DAST	F VRITKMN A CAAGLA V QQVAAQAXIX FI IGKINAVRG	ARNKKREAEK	PSPSAS	G 	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- 		441 249 237 235 230 224 268 268 268 272
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7 C5EMD94256 ClCUT11 ClCUT9 C5EMD93730		KIKUGG-G-TAQ KVKIIGQ-PGIAV KIKIGGAVRGTV NIKVIGA-AGIV KVLQVGA-KGIV VLSVGA-KGIV VLSVGA-KGIV VLSVGA-KGIV VLSVGA-AGIV VLSSVGA-AGIV	GGGDGGATCSH LGTLAVIPAH DGNLSAAVILH SGTLTVTAAH DSGSCQYH DSGTCQYH ANGDDISKH ANGDDVEK AGGNVVAH AGGTNVAH	TYNRDAGA AD SYGSAE-A AT YGLRAD-EGVD SYGRDAA-P GR TYNDDAMG-P PC TYNLPKLMDP VA SYFELYTG-D ST SYFELYTG-D ST SYFELYTD-D SS NYFELYTD-D SS NYFELYTD-ESS	PIVRITKMN PIVQVKAAQARIKI PIVQVKAAQARIKI PIVRAFQGSPRI PIVRAFQGSPRI	ARNKKREAEK SRS VAPLCDAP-TH AAPLCAAP-TI VAPLPSCGSSSS SSSVLSSSATPTH	SPSAS TPSAS VSPTP TPTPS-PEPTPTPT		PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- 		441 249 237 235 230 224 268 268 272 305
ClCUT4 ClCUT5 ClCUT6 CSEMD90612 ClCUT7 CSEMD94256 ClCUT11 ClCUT9 CSEMD93730 ClCUT8		KIKQFGC-TAQ KVKIICQ-PGIAV KLKIGGGAVRTV NLKVICA-AGIU KVLQVCA-KGIPV VLRSVCA-KGIPV VLRSVCA-KGIPI VLRSVCA-AGIPV VLRSVCA-AGIPU VLRSVCA-AGIPI VLRSVCA-AGIPI	GGGCDGGATGS   LG TLAVIPA   DGNLSAAVLP   SG TLIVTAA   DSGSC QYY   AN GDDLSR   AN GDDVER   AG GNVAD   AG GSNVAD   AG GSNVAD	TYNRDAGA AD SYGSAEAL TYGLRADECVD SYGRDAA-DCK TYNLDRAMC-P.PC TYVLPKLMDP VA TYVLPKLMDP VA TYVLPKLMDP VA SYFELYTC-D.ST SYFELYTD-D SS NYFELYTD-ESS NYFELYTD-ESS	PURITKMN FLQAAGLA	ARNKKREAEK SRS VAPLCDAP-TI AAPLCAAP-TI VAPLPSCGASSSI SSSVLSSSATPTI SSSVLSSSVAPPTS	SPSAS TPSAS VPTPT TPTSAS TPTPS-PEPTPT TPTASSAEVSPTEI	G SS SS AS PTSAASTSEVSS PSSTVIPASSTPVSS	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- 		441 249 237 235 230 224 268 272 305 311
ClCUT4 ClCUT5 ClCUT6 CSEMD90612 ClCUT7 CSEMD94256 ClCUT11 ClCUT9 CSEMD93730 ClCUT8 ClCUT12		KINGFGG-TALCO KVKIIG Q-FG AX KKIFGGGAVR TV NLKVYGA-ACIP KVL2VGA-KCIP KVL2VGA-KCIP VLSYGA-KCIP VLSYGA-KCIP VLSYGA-ACIP VLSYGA-ACIP ULSYGA-ACIP LLRSYGQ-FGCIP	GGGGDGGATGS LG TLAVLPA DSGSC QYH DSGSC QYH AN GDDLSR AN GDDLSR AG GNVAD AG GNVAD AG GTNVAD AG GTNVAD AG GTNVAD AFHTDNKDSR	TYNRDAGA AD SYGSAE-A AT TYGLRAD-PCGVD SYGRDAA-PCGVD SYGRDAA-PCVT TYNLPKIMDP VA TYVLPKIMDP VA SYFELYTG-D ST NYFELYTD-D ST NYFELYTD-D SS DYFLYTD-PS SYFELYTD-PS SYFELYTD-PS SYFELYTD-PS	IVRITKMN FLQAAGLA FLQCVKAAQAKIK, FLIGKINAVRG FLSKIGA FLVRAFQGSPRL MVKAFKS MVKINN MVKINN MVKINN MVKINN MVKINN MVKINN MVKIN	ARNKKREAEK	SPSAS	G S S S S S S S S S S S S S S S S S S S	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- AVASSVTIAAVASS- VVASSGVILATAVAS- VSATSVINTPTASVSA- TESSSTVIVPPSIPG- DIKSSIVLPPTMVSSIL FILIFVLFVIYFAIRKFA		441 249 237 235 230 224 268 272 305 311 299
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 C5EMD94256 ClCUT11 ClCUT9 C5EMD93730 ClCUT8 ClCUT12 ClCUT12 ClCUT13		KINGFGG-TALCO KVKIIGG-PGAV KKIFGGGAVR M NLKVYGA-ACT KVL2VGA-ACT VLSYGA-KCT VLSYGA-KCT VLSYGA-KCT VLSYGA-ACT VL	GGGCDGGATGS LG TLAVLPA CGNLSAAVLP SG TLTVTAA DSGSC QYY DSGTC QYY AN GDDLSR AN GDDVER AG GTVVAD AG GTVVAD AG GSVVAD AG GSVVAD AFHTDNKDSR ARGFDPN A	TINRDAGA AD SIGSAEAAT TIGLRADEGVD SIGRDAA-ED GR TINDDAG-P PC TIVLPKLMDP VA SIFELTG-D ST SIFELYTD-D ST NIFELYTD-D SS NIFELYTD-ESS NIFELYTD-ESS SIFELYTD-ESS SIFELYTD-ESS SIFELYTD-ESS SIFELYTD-ESS	IVRITKMN	ARNKKREAEK- SRS	SPSAS	G SS SS ST ST ST ST SST SST SST SST SST	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- AVASSVTIAAVASS- VVASSTGVTLATAVAS- VSATSUTNTTAVAS- TESSSTVIVPPTSVSA- TESSSTVIVPPSIPG- DIKSSIVLTPTTMVSSYL FILIFVLFVIYFAIRKFA		441 249 237 235 230 224 268 268 272 305 311 299 133
ClCUT4 ClCUT5 ClCUT6 C5EMD90612 ClCUT7 C5EMD94256 ClCUT11 ClCUT9 C5EMD93730 ClCUT8 ClCUT12 ClCUT13 ClCUT13 ClCUT13		KINGFGG-TALGO KVKIIGQ-FGGAVR NLKVFGGAVR KVLUQGA-AGCIU NLKVFGA-AGCIU VLLVGA-KCIP VLRSYGA-KCIP VLRSYGA-ACIP VLRSYGA-AGIP VLRSYGA-AGIP VLRSYGA-AGIP VLRSYGA-AGIP VLRSYGA-AGIP	GGGCDGGATGS   LG-TLAVDPA   DGNLSAAVLP   SG-TLIVTAA   DSGSCQYY   ANGDDLSR   ANGDDLSR   AGGNVAD   AGGNVAD   AGGNVAD   AGGNVAD   AGGNVAD   AGGNVAD   AGGNVAD   AGGNVAD   AGGPDNAST   ANGFDPNAST   ANGSEPQNAST	TINRDAGAAD SIGSAEAAT TIGLRADEGVD SIGRDAA-DCR TINDDAMG-PPC TIVLPKLMDPVA TIVLPKLMDPVA SIFELYTG-DST SIFELYTD-DSS NIFELYTD-ESS NIFELYTD-ESS DIFDIVGD-DAS SIFILYTD-FSS NIFELYTD-FSS NIFFSS NIFELYTD-FSS NIFELYTD-FSS NIFELYTD-FSS NIFFSSS NIFS	IVRITKMN	ARNKKREAEK SRS	PSPSAS	G ST ST ST ST STS STVIPASTPVSS SSTVIPASSTPVSS SSTVI	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- AVASSVTIAAVASS- VVASSTGVLATAVASS- VSATSVINTPTASVSA- ESSSTVIVPEPSIPG- DIKSSIVLTPTTMVSSYL FILIFVLFVIYFAIRKFA APASATSSIVAKPAKPGS		441 249 237 235 230 224 268 272 305 311 299 133 306
ClCUT4 ClCUT5 ClCUT6 CSEMD90612 ClCUT7 CSEMD94256 ClCUT11 ClCUT9 CSEMD93730 ClCUT8 ClCUT12 ClCUT13 ClCUT13 ClCUT10 RS47189543		KINGFGG-TALCO KVKIIG Q-FG AX KKIFGGAVR TV NLKVYGA-KCIP KVLEVGA-KCIP VLRSYGA-KCIP VLRSYGA-KCIP VLRSYGA-KCIP VLRSYGA-ACIP VLRSYGA-ACIP ILRPYGQ-FCIP ULRSYGA-ACIP ILRPYGQ-FCIP VLRSYGA-ACIP NLASFGN-TGCIP	GGGGDGGATGS   LGTLAVLPA   DSGSCQYH   SGTLTVTAA   DSGSCQYH   SGGUDISR   ANGDDISR   AGGNVAD   AGGNVAD   AGGUVAD   AG	TYNRDAGA AD SYGSAE-A AT TYGLRADECVD SYGRDAA-P CCV TYNLDRIMDP VA SYFELYTG-D ST TYVLPKIMDP VA SYFELYTD-D ST NYFFLYTD-D SS DYFDLYTD-FSS DYFDLYCD-F AS SYFNIFST-F GS NYFLYTD-F SS DYFNIFST-F GS NYFLYTDSIAV AA	IVRITKMN AIQAAGLA IVQQVKAAQAKIK IGKINAVRG IVRAFQGSPRL IVRAFQGSPRL	ARNKKREAEK	SPSAS	G SS SS ST PTSAASTSEVSS PSSTVIPASSTPVS V SSTVIPASSTPVS	PPGGGNMAPLPFPPPTPS -VAATESAKLVAKAILA- AVASSVTIAAVASS- VVASSGVILATAVAS- VSATSVINTPTASVSA- TESSSTVIVPPSIPG- DIKSSIVLTPTTMVSSYL FILIFVLFVIYFAIRKFA APASATSSIVAKPAKPGS		441 249 237 235 230 224 268 272 305 311 299 133 306 205

Fig. 1 Multiple sequence alignment of ClCUT proteins in *C. lunata* and 10 known cutinase proteins from different plant pathogens. *Marked with degree of colors* representing for different levels of con-

servation of each column in the alignment. Two sites with conserved residues are outlined in *red* 



Fig. 2 Intron-exon structures of *ClCUT* genes from *Curvularia lunata*. Exons and introns are indicated by *open boxes* and *lines*, respectively. Different intron types are represented by 0, 1, and 2.

The 5'-3' scale indicates the DNA sequence size. The names of the *ClCUT* genes and intron–exon structures are indicated at the *left* and *right* sides, respectively



Fig. 3 Unrooted phylogenetic tree representing relationships among CICUT proteins and cutinase proteins from other plant pathogens. Amino acid sequences of all CICUT and 78 cutinase proteins were aligned by the Clustal W2 program. The phylogenetic tree was con-

structed using the neighbor-joining method in MEGA 6.0. The number for each interior branch was the percentage of bootstrap values (1000 replicates). *Black dots* denoted the 13 CICUT proteins. The *red arcs* indicate different groups or subgroups of cutinase proteins

#### Expression analysis of cutinase genes

To reveal expression patterns of the cutinase genes during the infection process in maize, we collected leaves at five different times post-inoculation. We then used quantitative-PCR to analyze the cutinase gene expression patterns. Distinct patterns of expression were noted during the compatible interaction between *C. lunata* and maize (Fig. 5). Transcripts of *ClCUT7*, *ClCUT8*, and *ClCUT13* were significantly up-regulated during conidial germination (1.5 hpi) and penetration (3 hpi). The transcript level of *ClCUT7* increased gradually during early infection (0–3 hpi) and reached an eightfold higher expression at 3 hpi compared to conidia at 0 hpi. It then decreased to its lowest level during establishment and colonization at 24 hpi. It is possible that these changes reflect appressorium adhesion and penetration by the pathogen. The transcript levels of *ClCUT8* and *ClCUT13* had a threefold increase in expression during conidial germination (1.5 hpi) and colonization (24 hpi) compared to their levels at 0 hpi. We suggest that *ClCUT8* and *ClCUT13* are not only associated with pathogen penetration, but also colonization. In this study, we also found that the transcript level of *ClCUT6* showed significant upregulation at 24 hpi compared with other time points. This suggests that *ClCUT6* might have a role in establishment and colonization. In addition, the transcript levels of six cutinase genes (*ClCUT2*, *ClCUT4*, *ClCUT9*, *ClCUT10*, *ClCUT11* and *ClCUT12*) showed little differential expression (<threefold change) at every time point. We therefore considered these genes to be constitutively expressed.





Fig. 4 Schematic diagram of amino acid motifs for all ClCUT proteins from different groups or subgroups. The ClCUT name is listed on the *left*. The *black solid line* represents the corresponding ClCUT protein and its length. The *different-colored boxes* indicate different

Deletion and functional analysis of *ClCUT7* 

To verify the function of *ClCUT7*, we generated a *ClCUT7* deletion mutant. A 3057-bp deletion cassette containing an 847-bp upstream sequence of ClCUT7, 1343 bp of the hygromycin phosphotransferase (hph) gene with the TrpC promoter, and 867 bp of the sequence downstream of ClCUT7 were obtained by double-joint PCR (Fig. 6a). The ClCUT7 gene was deleted from wild-type C. lunata by transformation of C. lunata protoplasts with the deletion cassette. Four hygromycin-resistant transformants were selected, and the hph gene and ClCUT7 gene were detected by PCR. The results showed that one transformant had the hph gene, but lacked the ClCUT7 gene, suggesting that this transformant might be a ClCUT7 gene-disruption mutant. The gene-deletion mutant was confirmed by Southern blotting and RT-PCR and designated  $\triangle$ ClCUT7 (Fig. 6b). The absence of ClCUT7 mRNA in the  $\Delta$ ClCUT7 transformant was confirmed by RT-PCR (Fig. 6c).

 $\Delta$ ClCUT7 had no obvious changes in phenotype, including colony morphology (Fig. 6d), vegetative

motifs and their positions in each ClCUT amino acid sequence. A detailed motif sequence characteristic of all ClCUT proteins is shown in the supporting information Fig S1

growth, conidia production (Table 2), or morphology of the conidia and appressoria (Additional file 7). Three days after inoculation of wounded maize leaves with a conidial suspension, small black lesions were formed at the inoculation sites by both the wild-type strain and  $\Delta$ ClCUT7. The deletion of *ClCUT7* apparently had no effect on the mutant's pathogenicity on wounded leaves compared to the wild-type strain (Fig. 6e; Table 2). However, when unwounded leaves were inoculated with these strains,  $\Delta$ ClCUT7 produced smaller lesions than the wild-type strain, suggesting that the ClCUT7 deletion had some effect on fungal penetration and resulted in decreased pathogenicity (Fig. 6f; Table 2). To further determine if ClCUT7 could degrade the cuticle, we cultured the  $\Delta$ ClCUT7 and wild-type strains on media containing either glucose or cutin as a carbon source. Mycelial growth rates for both strains on the medium containing glucose as a carbon source were similar. In contrast, growth of the ClCUT7 mutant was approximately 10 % slower than the wild-type strain on the medium containing cutin as a carbon source.

**Fig. 5** Expression patterns produced by qRT-PCR of 10 *ClCUT* genes from *C. lunata* during a compatible interaction with maize (*Zea mays*). The relative expression level of mRNA (y-axis) was calculated by the comparative  $C_t$  method. The *C. lunata GADPH* gene was used as an internal control to normalize the data. Sample time is in hours post-inoculation (x-axis). *Error bars* were calculated based on three replicates





Fig. 6 Construction and confirmation of the *ClCUT7* deletion mutant and functional analysis of the *ClCUT7* gene. **a** Construction of the deletion cassette for *ClCUT7*. *Arrows* indicate orientations of the *ClCUT7* gene (*red*) and hygromycin phosphotransferase (*hph*) gene (*blue*). The deletion cassettes of *ClCUT7* contained homologous sequences flanking the *hph* gene to replace the *ClCUT7* gene ORF. Primer hphs and hpha (Table S5) using amplification of *hph* gene and primer cus and cua (Table S5) using amplification of *ClCUT7* genes were employed to screen the *ClCUT7* deletion mutant. Total of genomic DNA samples isolated from wild-type strain CX-3 and  $\Delta$ ClCUT7 were digested with Page I (**b**) and *Hin*dIII (**c**), then subjected to Southern blot analysis. A hybrid probe, a 610-bp PCR fragment amplified from plasmid pBHt1 with primer hphts (5'-AACTCACCGCGACGTCTGTC-3') and hphta (5'-TTGTC-

CGTCAGGAATTGTT-3') (b); A hybrid probe, a 510-bp PCR fragment amplified from the DNA of wild type strain CX-3 with primer JZts (5'-ACCAGCGAGCCCAGTTTC-3') and JZta (5'-CCTCAC-CGTTCAAGCATTT-3') (c); d total RNA samples from wild-type strain CX-3 and  $\Delta$ ClCUT7 were subjected to RT-PCR using ClCUT7 gene-specific primers cus and cua (Table S5). The RT-PCR product is a 510-bp fragment in the wild-type CX-3 strain as predicted, but is missing in the  $\Delta$ ClCUT7 mutant; e colony morphology of the wildtype strain CX-3 and  $\Delta$ ClCUT7 on PDA; f disease symptoms on wounded leaves of maize inoculated with conidial suspensions from the wild-type CX-3 strain and the  $\Delta$ ClCUT7 mutant. g Unwounded leaves inoculated with the same conidial suspensions. The leaves were photographed 3 days after inoculation

Table 2 Phenotypic analysis of a Curvularia lunata CICUT7 gene disruption mutant compared with wild-type strain CX-3

Strain	Growth rate (mm/24 h)	Conidiation (log <sub>10</sub> CFU/ml)	Necrotic area on wound leaf (mm <sup>2</sup> )	Necrotic area on unwound leaf (mm <sup>2</sup> )
CX-3	$7.55 \pm 0.04a$	$6.47 \pm 0.10a$	$76.13 \pm 2.23a$	$70.72 \pm 0.86a$
$\Delta$ ClCUT7	$7.45\pm0.03a$	$6.26\pm0.19a$	$74.99\pm0.99a$	$49.05\pm1.76\mathrm{b}$

Data in all columns are the means of three independent experiments with standard deviation. The statistical analysis was performed using the SAS statistical package. Statistically significant analysis of variance was further analyzed using least significant difference tests. Different letters in each column indicate significant differences at P = 0.05

#### Discussion

Previous publications have reported 3 cutinase genes in *Neurospora crassa*, 4 in *Aspergillus nidulans*, 11 in *Botrytis cinerea*, 12 in *Fusarium graminearum*, and 17 in *Magnaporthe grisea* (Skamnioti et al. 2008). In this study, 13 non-redundant cutinase proteins were identified in *C. lunata*. The large number of cutinase genes in *C. lunata* 

indicated that this fungus produces cutinases that may be able to degrade different types of plant cuticles from various host plants. This may explain the broad host range of *C. lunata*, which includes: sorghum (Akram et al. 2014), lotus (Cui and Sun 2012), rice (Liu et al. 2014), strawberry (Verma and Gupta 2010), spinach (Pandey et al. 2011), zoysia (Roberts and Tredway 2008), and some varieties of turfgrass (Goldring et al. 2007). This pathogen is similar to *M. grisea*, which produces numerous cutinases able to breach the cuticles of various grasses. Additionally, it has been suggested that a large number of cutinases reflects the need for this enzyme during colonization of the host (Skamnioti et al. 2008).

In this study, 12 of 13 cutinase proteins of *C. lunata* possessed the same amino acid sites (GYSQG). It has been reported that cutinase protein sequences contain a highly conserved motif (GYSQG) surrounding a cutinase-active serine, and a less precise motif (DxVCxG[ST]-[LIVMF] (3)-x(3)H) that carries aspartate and histidine residues at the active site (Skamnioti et al. 2008). The amino acid motif GYSQG of the cutinase protein is thought to be crucial for its function. However, when we analyzed intron–exon structures of all 13 cutinase proteins, different and complex intron–exon structures were obtained, suggesting the cutinase genes have undergone ancient and extensive diversification.

C. lunata has an infection process similar to most necrotrophic fungal-plant pathogens. This process includes: host recognition and conidial adhesion; germ tube emergence, elongation, and appressorium formation; host penetration; and establishment in and colonization of plant tissues (Xue et al. 2010). To understand the role of cutinase in the C. lunata-maize interaction, we investigated the expression of the pathogen's 13 cutinase genes. Transcript levels of the cutinases quantified by q-PCR indicated that CICUT7 was up-regulated early in infection. This suggested that it is associated with cuticle degradation because the cuticle is the first structural obstacle to plant pathogens. Therefore, we examined the role of *ClCUT7* in pathogenicity by knocking out this gene. Additionally, we found that six of the cutinase genes (ClCUT2, ClCUT4, ClCUT9, ClCUT10, ClCUT11 and ClCUT12) appeared to be constitutively expressed. Previous research indicated that most of the cutinase genes in M. oryzae belonged to constitutively expressed gene groups (Skamnioti et al. 2008). Hence, these cutinase genes may operate synergistically in the interaction between the pathogen and its host plant. The transcript levels of three other cutinase genes (ClCUT1, ClCUT3, and ClCUT5) were not detected in leaf tissue samples, but were detected by quantitative PCR when we used cDNA from pathogen hyphae as templates. It is possible that the expression of ClCUT1, ClCUT3, and ClCUT5 was inhibited by the host plant during pathogenesis. Recently, several studies demonstrated that host-induced gene silencing could inhibit the expression of genes in some plant pathogens (Govindarajulu et al. 2014; Ghag et al. 2014). The expression of ClCUT8 and ClCUT13 also increased significantly during conidial germination (1.5 hpi) and colonization (24 hpi), respectively. We suggest that both genes may be involved in pathogen penetration and colonization. The specific role of these two genes is being investigated.

Fungal cutinase genes reportedly have different roles in pathogenicity (Dantzing et al. 1986; Kolattukudy 1985; Köller 1991; Sweigard et al. 1992). For example, in Fusarium solani f. sp. pisi, the presence of cutinases at the site of infection was detected serologically, and the application of chemical cutinase inhibitors prevented fungal penetration (Kolattukudy 1985; Köller 1991). The pathogenicity of cutinase-deficient mutants was reduced but could be restored by adding exogenous cutinases or complementation with the native cutinase gene (Dantzing et al. 1986; Köller et al. 1982). Other studies, however, have provided contradicting evidence about the role of cutinase in pathogenicity (Stahl and Schafer 1992; Stahl et al. 1994). The deletion of the cutinase gene CUT1 of M. grisea did not alter the pathogenicity of this fungus on rice (Sweigard et al. 1992). Therefore, the role of cutinase in the plant infection process remains controversial.

*C. lunata* directly penetrates the cuticle of maize leaves (Mandokhot et al. 1979), suggesting that cutinase played a role in penetration by degrading the cuticle. In our study, deletion of *ClCUT7* had some effect on cuticle degradation, resulting in decreased pathogenicity. This result suggested the cutinases are involved in the pathogenicity of *C. lunata*. Overall, our study characterized the cutinase genes in *C. lunata*, improved our understanding of their expression patterns during compatible interactions with maize, and explored the role of *ClCUT7* in pathogenicity. These findings are a preliminary step in future systematic analyses of cutinase gene functions in *C. lunata*.

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#### Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

**Ethical statement** This article does not contain any studies with human participants or animals performed by any of the authors.

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