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CglCUT1 gene required for cutinase activity and pathogenicity of *Colletotrichum gloeosporioides* causing anthracnose of *Camellia oleifera*

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Abstract *Colletotrichum gloeosporioides* is the causal agent of *Camellia oleifera* anthracnose, mainly infecting fruits and leaves. The fungus secretes degrading enzymes to destroy the cuticle of aerial plant parts and help infect the host successfully. To validate whether a cutinase gene (*CglCUT1*) was required for cutinase activity and pathogenicity of *C. gloeosporioides*, the *CglCUT1* gene was cloned and analyzed. The characterization of *CglCUT1* predicted protein suggests that the cloned DNA encoded a cutinase in *C. gloeosporioides* affecting *C. oleifera*. The *CglCUT1* showed a high homology to those from *C. gloeosporioides* causing papaya anthracnose and *C. capsici* causing pepper anthracnose, as well as those of other ascomycetes. The whole *CglCUT1* gene was knocked-out and the knockout mutant (Δ*CglCUT*39) was subsequently complemented using *Agrobacterium tumefaciens* mediated transformation. The knockout transformants exhibited significant decreases in cutinase activity and virulence compared with the wild-type strain. The complemented transformants of the disrupted transformant Δ*CglCUT*39 showed a significant increase in cutinase activity and virulence compared with the disrupted transformant Δ*CglCUT*39. This study suggests that the *CglCUT1* gene has a positive effect on fungal virulence of the hemibiotrophic *C. gloeosporioides* on *C. oleifera*.

Keywords *Camellia oleifera* · Anthracnose · Gene-knockout · Cutinase activity · Pathogenicity

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Introduction

Tea oil camellia (*Camellia oleifera* Abel.) is an important edible oil woody plant native to southern China and comprises over 3,500,000 ha mostly south of the Yangtze River in China (Deng and Xie 2008; Zhuang 2008). Among the woody plant species grown for edible oil production, *C. oleifera* is valued as equivalent to palm, olive and coconut throughout the world (Zhuang 2008). Camellia oil, extracted from *C. oleifera* seeds, is regarded as a top quality edible oil rich in vitamins and unsaturated fatty acids, and also used for medical purposes for enhancing human immunity, reducing low density lipoprotein cholesterol and preventing cardiovascular disease (Wang et al. 2007). However, anthracnose is a serious disease of *C. oleifera* in most areas south of the Yangtze River. The disease generally causes 20 % to 40 % fruit drop and 10 % to 30 % camellia seed loss, and also leads to death of the branches and sometimes entire plants (Wu 1985; Jin et al. 2009). The disease can spread very quickly and is difficult to

control; thus, it results in severe economic losses and poses a huge threat to the *C. oleifera* industry in China (Zhou et al. 2007; Liu et al. 2009).

The pathogen causing anthracnose of *C. oleifera* was described as *Colletotrichum gloeosporioides* (Penzig) Penzig et Sacc. (telemorph: *Glomerella cingulata*) (Li et al. 2014). A hemibiotrophic ascomycete, *C. gloeosporioides* causes anthracnose diseases on a wide range of subtropical and tropical plants (Bernstein et al. 1995; Freeman et al. 1996). During the infection process, conidia attach to the plant surfaces, germinate to develop appressoria and penetrate host surfaces using a combination of mechanical force and enzymatic degradation (Bechinger et al. 1999). Cutinase is an important enzyme that could help the fungus directly penetrate the cuticle of aerial plant parts, which is the first physical defensive barrier (Kolattukudy et al. 1995). Cutinase activity in fungal cultures grown on purified cutin as the sole carbon source has been reported for more than 20 plant pathogens (Trail and Köller 1990).

Although more studies on cutinases have been carried out in the past decades, the role of cutinases in fungal pathogenicity still remains controversial. Cutinase-deficient mutants of *Alternaria alternata* lost pathogenicity to Japanese pear (Tanabe et al. 1988). The cutinase gene disruption in *Fusarium solani* f. sp. *pisi* isolate 77–2-3 resulted in a significant decrease in virulence on pea (Rogers et al. 1994). The cutinase *Pbc1* disruption mutants of *Pyrenopeziza brassicae* failed to penetrate the cuticular layer and were unable to develop disease symptoms (Li et al. 2003). Also, the insertion of a *F. solani* f. sp. *pisi* cutinase gene into *Mycosphaerella* spp., a cutinase-deficient wound pathogen, enabled it to infect intact papaya fruits (Dickman et al. 1989). However, disruption of the cutinase gene did not alter infection of gerbera flowers or tomato fruits by *Botrytis cinerea* (van Kan et al. 1997). The disruption of the *cutA* gene of a race of *F. solani* f. sp. *cucurbitae* had no effect on its virulence to *Cucurbita maxima* and *C. moschata* (Crowhurst et al. 1997). Interestingly, disruption of the *cut1* gene in *Magnaporthe grisea* did not affect the fungal pathogenicity, but the *cut2* gene in *M. grisea* was required for full virulence on host rice (Sweigard et al. 1992; Skamnioti and Gurr 2007). Collectively, studies on cutinase-deficient mutants generated in a range of phytopathogenic fungi have not provided consistent evidence to support a definitive conserved role of cutinase in fungal pathogenicity.

The involvement of cutinases in the pathogenicity of *Colletotrichum* spp. had been rarely studied. Cutinase activity-deficient mutants of *C. gloeosporioides* generated by UV radiation did not affect fungal pathogenicity to papaya (Dickman and Patil 1986). Only one cutinase gene had been sequenced from genomic libraries of *C. gloeosporioides* and *C. capsici*, respectively, but the role of the cutinase gene in pathogenicity had not been studied in depth (Ettinger et al. 1987). The cutinase mutants of *C. lagenarium* supported poor correlations between cutinase production and penetration and disease expression (Bonnen and Hammerschmidt 1989). At present, the characterization of the *CglCUT1* gene remains to be performed and the role of the *CglCUT1* gene in pathogenicity of *C. gloeosporioides* has not been evaluated. This lack of information provides the basis for this study to clone and analyze the *CglCUT1* gene and elucidate whether *CglCUT1* is required for cutinase activity and pathogenicity in the anthracnose fungus *C. gloeosporioides* of *C. oleifera* using a gene knockout approach.

Materials and methods

Strains, plasmids and culture conditions

The wild-type strain MC171 of *C. gloeosporioides* is a single spore isolate collected from a diseased fruit of *C. oleifera* in Macheng (115.03°E, 31.17°N), Hubei province, China. The wild-type strain and its derivatives were cultured on potato dextrose agar (PDA) at 25 °C and stored in PDA slants at 4 °C.

Seven Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth (PDB) were inoculated with 1 ml of a 1×10^6 conidia/ml suspension of the wild-type strain, the disrupted transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182) and the complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63), respectively. The liquid cultures were incubated on a shaker at 160 rpm, 25 °C for 5 days. The mycelia were harvested by filtering the cultures through two layers of sterile cheesecloth and used to extract genomic DNA.

Cloning and analysis of the CglCUT1 gene

Genomic DNA was extracted with the hexadecyl trimethyl ammonium bromide (CTAB) method

following Sambrook et al. (1989). The DNA sample was stored at −20 °C before using.

To obtain the full coding region of the *CglCUT1* gene, a pair of specific primers cutF and cutR (Table 1) were designed with PRIMER (version 5.0) based on the cutinase gene sequence of the anthracnose fungus *C. gloeosporioides* on papaya (Ettinger et al. 1987). Polymerase chain reaction (PCR) conditions were described as follows: 1 cycle at 95 °C for 5 min, 35 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 90 s, and 1 final extension cycle at 72 °C for 10 min. PCR reactions were run on a PTC-200 Thermal Cycler (BIO-RAD, Hercules, CA, USA). Amplified production was analyzed on a 1.0 % agarose gel in TBE buffer and purified using a Nucleic Acid Purification kit (Axygen, Hangzhou, China). The target DNA was ligated into the vector pMD18-T, transformed into competent cells of *Escherichia coli* DH5α (Takara, Dalian, China) and sequenced by Sunny Biotechnology Co., Ltd. (Shanghai, China).

The *CglCUT1* nucleotide sequence was compared to the GenBank nr database using BLAST and analyzed with the DNAman program (version 5.2). The molecular mass and isolatric points of the predicted *CglCUT1* protein was analyzed with the PeptideMass program (http://web.expasy.org/peptide_mass). The domains of the *CglCUT1* protein were analyzed with the SMART data base (http://smart.embl.de). The signal of the *CglCUT1* protein was predicted using the SignalP (version 4.1) program (http://www.cbs.dtu.dk/services/SignalP/). The structure of the *CglCUT1* protein was predicted with the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/) and the Swiss-model program (http://swissmodel.expasy.org/). A neighbor-joining (NJ) tree was constructed using ClustalX (version 2.0) and MEGA (version 5.1) program.

Targeted gene disruption and complementation

Vectors KS1004 and pneoP3300III were used for gene replacement vector construction (Liu et al. 2013; Qin et al. 2011). To assess the function of the *CglCUT1* gene, a vector was constructed for the targeted disruption of the whole *CglCUT1* gene by homologous replacement (Fig. 2). A pair of gene-specific primers F1-SP-*Hind*III and F1-AP-*Sal*I (Table 1) were used to amplify a 0.93 kb fragment upstream of the 5′ coding region. Another pair of gene-specific primers F2-SP-*Xba*I and F2-AP-*Kpn*I (Table 1) were used to amplify a 1.27 kb fragment downstream of the 3′ coding region. The 0.93 kb *Hind*III/*Sal*I-fragment, and 1.27 kb *Xba*I/*Kpn*I-fragment were cloned into the corresponding restricted sites of the vector KS1004, resulting in the preliminary vector KS1004-*CglCUT1*. The *Hyg-CglCUT1* cassette (with the 0.93 kb *Hind*III/*Sal*I-fragment, the 1.9 kb Hyg-fragment and the 1.27 kb *Xba*I/*Kpn*I-fragment) was cloned into the corresponding sites of vector pneoP3300III, resulting in the gene

Table 1 Primers used in the study

Name	Sequence (5'- 3')	Product Length	Annealing Temp
cutF cutR	TTGAGGGAACCTTGTTGACG AAGCGAATGACGAAGTGAGTG	1357 bp	56 °C
F1-SP-*Hind*III F1-AP-*Sal*I	AAGCTT CTGGGTTTTGTTGGTTTCTGGGCGA GTCGAC CATTTTAGATGTTTGGGGACGGGGCG	928 bp	60 °C
F2-SP-*Xba*I F2-AP-*Kpn*I	TCTAGA CTTTGTCATTCGCTTTTGTCACTCGCT GGTACC TGTCCCTGTTTATTGCTTCTGTGTTGG	1270 bp	59 °C
hphF hphR	TTCTGCGGGCGATTTGTG AGCGTCTCCGACCTGATG	887 bp	58 °C
C-SP-*Hind*III C-AP-*BamH*I	AAGCTT TGGCACCCTTCAGACACATTCGC GGATCC GACACGCCCCCTTCATCACAACC	3820 bp	61 °C
ComF ComR	GTTCCTCAGCATCGTGTCCC GTCCGCAATGTCGCAGTAGA	620 bp	55 °C

[a] Restriction enzyme recognition sites are indicated by the underline

[b] All above primers were designed using Primer program (version 5.0), based on the related sequence from GenBank

replacement vector p3300neo*CglCUT1* (Liu et al. 2013; Qin et al. 2011). The vector p3300neo*CglCUT1* was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation, and the T-DNA within the plasmid transformed into the conidia of the wild-type stain with the *A. tumefaciens* mediated transformation (ATMT) method as described by Li et al. (2005).

A pair of primers, Com-SP-*Hind*III and Com-AP-*BamH*I (Table 1) were used to amplify a 3.82 kb fragment, including the cutinase gene ORF and its promoter, from the genomic DNA of the wild-type stain. The 3.82 kb fragment was cloned into the corresponding sites of vector pneoP3300III, resulting in the complemented vector p3300neoCom*CglCUT1*. Then vector p3300neoCom*CglCUT1* was also transformed into *A. tumefaciens* EHA105 by electroporation. The T-DNA was transformed into the conidia of the disrupted transformant Δ*CglCUT*39 with the ATMT method.

Identification of transformants

The hygromycin resistance gene (*hph*) in the vector KS1004 was used as the first selectable marker and the neomycin resistance gene (*neo*) in vector pneoP3300III was used as the second selectable marker for screening of transformants. The disrupted transformants and the complement transformants were screened on PDA supplemented with 120 μg/ml of hygromycin B (Roche, Germany) and sub-cultured on PDA supplemented with 300 μg/ml neomycin/G418 (Ameresco, USA). The disrupted transformants and the complement transformants were confirmed for the presence of insertions by amplifying an 887-bp internal region of the hygromycin resistance gene with the primers hphF/hphR and by amplifying a 620-bp internal region of *CglCUT1* with the primers ComF/ComR (Table 1). The genomic DNAs of the wild-type strain and its derivatives were digested by the restriction enzyme *Hind*III and analyzed by Southern blot to confirm the presence of 887-bp *hph* and 620-bp *CglCUT1* insertions, using gene image alkphos direct labelling and detection system kit (GE Healthcare, UK).

Phenotyping of transformants

The wild-type strain, the disrupted stains (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182) and the complemented strains (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63) were cultured on PDA plates in the dark at 25 °C. Then their colonial morphologies were observed and their growth rates of mycelia were measured on the 7th day post incubation. Erlenmeyer flasks (100 ml) containing 50 ml of PDB were inoculated with 1 ml of a 1×10^6 conidia/ml suspension of the aforementioned strains and were incubated on a shaker at 160 rpm, 25 °C for 7 days. The mycelia were harvested by filtering the cultures through two layers of sterile cheesecloth and dried at 105 °C with an electric drying oven (Jinghong DHG-9146 A, China) for 10 h before measuring their dry weights. Each treatment was carried out with three replicates. Conidial suspension droplets (10 μl/drop) were also spotted on plastic microscope coverslips placed in 9-cm-diameter Petri dishes, and conidial germination and appressorial formation were examined by compound microscopy (Olympus, Japan) at 24 h after incubation (Liu et al. 2013).

Enzyme production and activity assay

The wild-type strain and its transformants were cultured on PDA plates in the dark for 7 to 10 days at 25 °C. A conidial suspension of 1.2×10^6 conidia/ml was obtained. For production of cutinase, Erlenmeyer flasks (250 ml) containing 100 ml of Czapek-Dox broth (CDB), a basal mineral salt medium, and 0.2 g cutin as the sole carbon source were inoculated with 1 ml of a conidial suspension and incubated on a shaker at 160 rpm, 25 °C (Chen et al. 2007). Cutin was obtained from Fuji apples purchased from a local supermarket and purified as previously described by Kolattukudy et al. (1981).

Culture filtrate was obtained from cutin-induced culture after 7 days of growth. Cutinase activity was measured with ρ-nitrophenyl butyrate (Sigma, USA) as a substrate (PNBase; Purdy and Kolattukudy 1975). Each reaction mixture consisted of 200 μl of culture filtrate, 1380 μl of 50 mM sodium phosphate buffer (pH 7.0), 200 μl of 0.4 % Triton X-100 equilibrated to 37 °C and 20 μl of 1.76 % (*v*/v) PNB in acetonitrile. The reactions were carried out at 37 °C for 10 min (Davies et al. 2000). The production of ρ-nitrophenol was measured at 405 nm using a digital recording spectrophotometer (Mapuda, China). All assays were performed in triplicate. The molar extinction

coefficient for PNB of 6.83×10^3 at pH 7.0 was used to calculate enzyme activity (Koller and Parker 1989).

Pathogenicity test

Conidia were obtained as described above. The conidial suspension was adjusted to 1×10^6 conidia/ml with sterile distilled water. Detached healthy fruits (E'you 151) were placed on a layer of wet filter paper in an enamel basin and each fruit was inoculated with a 10 μl droplet of the conidial suspension. Inoculated fruits were incubated in a growth chamber at 25 °C and the symptoms were observed daily. The diameters of lesions on inoculated fruits were measured on the 10th day post-inoculation (dpi). The pathogenicity test was carried out twice.

Statistical analysis

The data from all quantitative assays were analyzed with Data Processing System (DPS) statistical analysis software (Version 3.01, China Agric. Press, Beijing, China), using analysis of variance (ANOVA). When significant treatment effects were found ($P < 0.05$), multiple comparison of means was analyzed using the test of least significant difference (LSD).

Results

Analysis of the CglCUT1 gene sequence

A 1357 bp PCR-fragment (GenBank No. KP331429) was obtained with the primers cutF and cutR, including the full open reading frame of *CglCUT1* gene. The ORF of *CglCUT1* was 727 -bp -long with a predicted 52 -bp intron. The protein sequence deduced from the ORF (GenBank No. AKH80819) encodes 224 amino acids, with a theoretical molecular mass of 23.4 kDa and a deduced isoelectric point of 6.58. The *CglCUT1* enzymatic domain was found extending from amino acid 44 to 223 of the protein. A protein signal appeared significantly and the cleavage site was between 16 and 17 amino acids in the *CglCUT1* protein. The predicted structure of the protein contains several alpha-helix and beta-strand regions. The *CglCUT1* showed high sequence similarities with cutinase proteins from several fungal species, such as *C. truncatum* (83.8 %), *C. capsici* (83.7 %), *Magnaporthe grisea* (60.3 %), *Alternaria brassicicola* (57.5 %), and *Aspergillus oryzae* (49.1 %).

A neighbor-joining (NJ) tree (Fig. 1) was developed with another 19 protein sequences retrieved from GenBank, representing cutinase sequences from 13 ascomycetes, two basidiomycetes, two oomycetes and two bacteria. The phylogenetic tree revealed that the bacterial cutinases from *Rhodococcus rhodochrous* and *Streptomyces pratensis* were located outside the main group, which clustered ascomycetous, oomycetous and basidiomycetous cutinases in separate subgroups. The cutinases from Basidiomycota and Oomycota were located in the main group, but formed separate and distinct branches, respectively. The cutinases from ascomycete fell into two main branches and the *CglCUT1* sequence formed a subclade with the homologous protein of the papaya anthracnose pathogen. The protein sequences of the *Colletotrichum* genus were closely grouped with *M. grisea* (Fig. 2).

Targeted gene disruption and complementation

Three *CglCUT1*-disrupted transformants, Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182, were randomly selected from 248 transformants resistant to hygromycin B. One of the characterized *CglCUT1*-disrupted mutants (Δ*CglCUT*39) was subsequently chosen for complementation. The complement transformants, CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CgCUT*39–63, were obtained with resistance to neomycin/G418 and hygromycin B.

The disrupted transformants lacked a 620-bp *CglCUT1* fragment, but had an 887-bp fragment carrying a gene for resistance to hygromycin (Fig. 3A). The complement transformants had a 620-bp *CglCUT1* fragment and an 887-bp fragment for hygromycin resistance cassette (Fig. 3A). However, the wild-type MC171 had only a 620-bp *CglCUT1* fragment, but lacked an 887-bp fragment (Fig. 3A). In addition, Southern blot analysis showed similar results as revealed by PCR analysis (Fig. 3B). All aforementioned results indicated that the *CglCUT1* gene was disrupted in Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182 and was successfully complemented in the CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63 transformants.

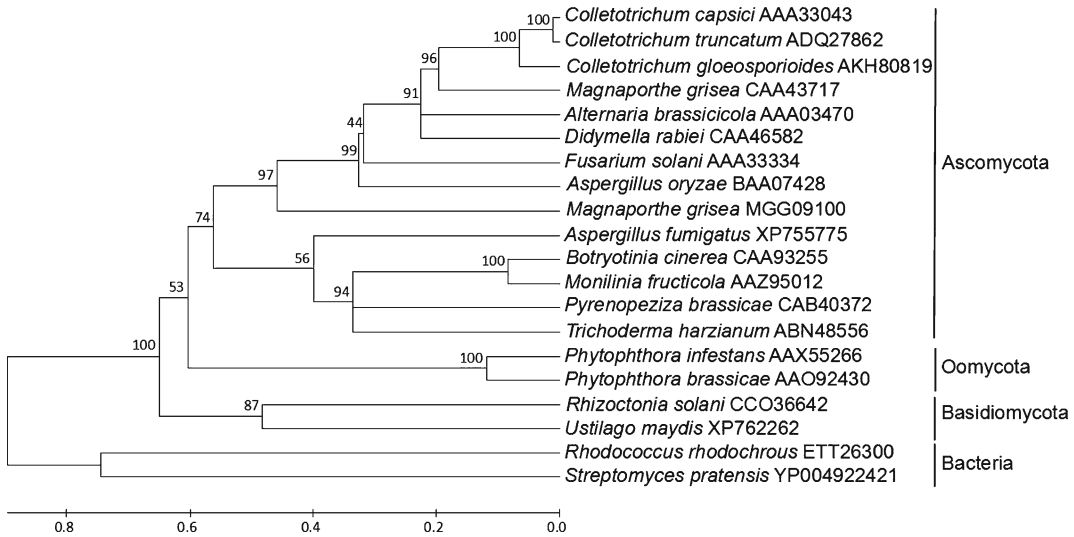

Fig. 1 The phylogenetic tree was generated using the Neighbor-Joining algorithm in the MEGA program. The phylogenetic tree showed the relation between the *CglCUT1* protein sequence and 19 other cutinase sequences from Ascomycota, Basidiomycota, Oomycota and bacteria. *Magnaporthe grisea* CAA43717 stands for *cut*1 protein sequence, *Magnaporthe grisea* MGG09100 stands for *cut*2 protein sequence

Phenotypic analysis of transformants

The colony morphology of the disrupted transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182) was similar to the wild type MC171. The cultures of the disrupted transformants initially developed into a white and floccose colony, and gradually turned into olive green and gray-black on PDA medium at 25 °C (Fig 4). The growth rate of Δ*CglCUT*39 had no significant differences in comparison with that of the wild type MC171, but growth rates of Δ*CglCUT*90 and Δ*CglCUT*182 were significantly reduced (Table 2). The colony morphology of the complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63) was similar to the wild type MC171. (Fig. 4). The colony of CΔ*CglCUT*39–36 had no significant differences in comparison with

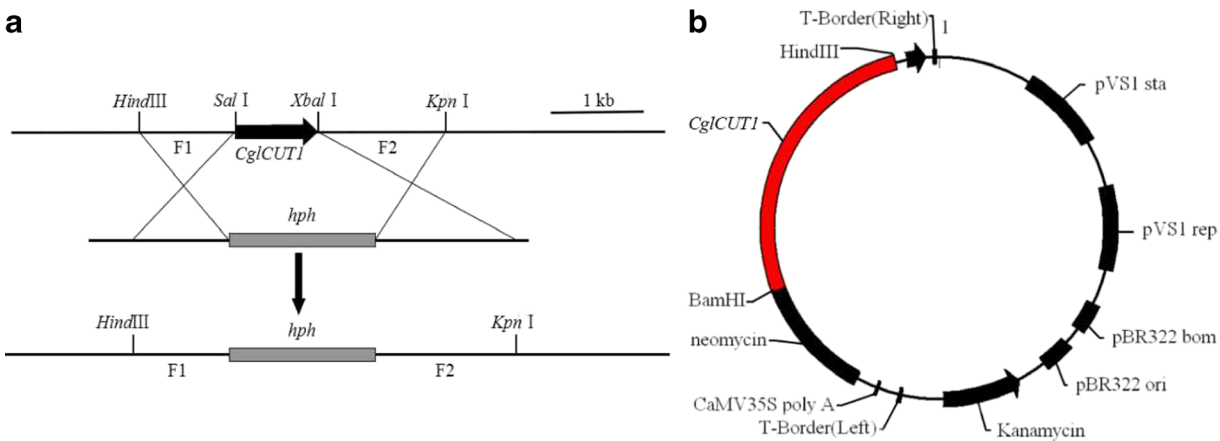

Fig. 2 Targeted disruption strategy and the map of the complementation vector p3300neoCom*CglCUT1*. **a**: Targeted disruption of *CglCUT1* gene using homologous recombination method. A map shows the gene disruption vector construction and a gene replacement event. The vector p3300neo*CglCUT1* contained a 0.9 kb *Hind*III/*Sal*I fragment, a 1.2 kb *Xba*I/*Kpn*I fragment, and a hygromycin resistance cassette (*hph*). B: The map shows the complementation vector construction p3300neoCom*CglCUT1* that contains a 3.8 kb *Hind*III/B*amH*I fragment including the *CglCUT1* (in red color), and G418 resistance gene (neomycin)

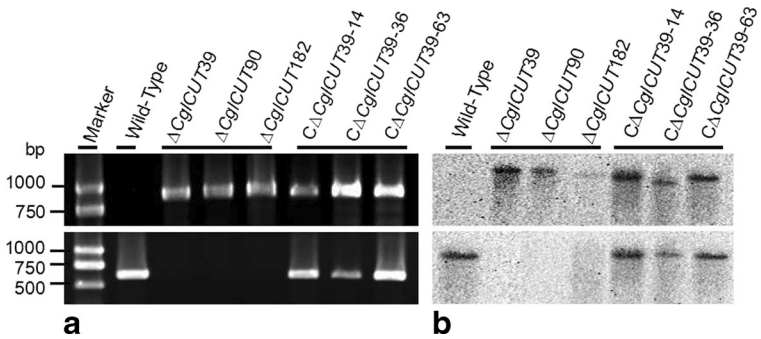

Fig. 3 PCR amplification and Southern blot analysis of transformants. Genomic DNAs were extracted from three knockout transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182), the complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63), and the wild-type strain. **a**: An 887-bp fragment of the *hph* gene that confers resistance to hygromycin was amplified by PCR using primers hphF and hphR (up). A 620-bp fragment of CglCUT1 gene was amplified using primers ComF and ComR (down). **b**: Genomic DNAs of the transformants were digested with *Hind*III and then blotted and hybridized using the 887-bp *hph* fragment as probe. The result showed the presence of the *hph* gene in the knockout transformants and the complemented transformants (up). The blot was hybridized using the 620-bp *CglCUT1* fragment as probe. The result showed the presence or absence of the *CglCUT1* gene in the strains (down)

that of Δ*CglCUT*39 and the wild type MC171. However, the colony color of CΔ*CglCUT*39–14 and CΔ*CglCUT*39–63 became lighter in comparison with that of Δ*CglCUT*39 and the wild type MC171. The growth rates of the complemented transformants were slower than those of Δ*CglCUT*39 and the wild type MC171 (Table 2).

The dry weight of mycelium after 7 days post incubation was not significantly different between the wild type MC171 and all transformants (Table 2). All selected transformants and the wild type MC171 produced conidia normally, and the conidia germinated and formed appressoria on plastic microscope coverslips normally.

Cutinase activity

In initial experiments, cutinase activity was continuously measured from 1 to 8 days after inoculation with 1 ml conidial suspension of the wild-type MC171 at 2.5×10^6 conidia/ml in 100 ml medium. Cutinase activity increased slowly from 2.16 to 10.67 U/ml in 3 days or less, and then increased rapidly from 43.72 to 88.01 U/ml after 4 days or longer, and reached a peak at 7 days post incubation (Fig. 5A). Thus, the cutinase activities among the transformants were measured at 7 days after incubation.

The cutinase activity of the disrupted transformants was significantly lower than wild-type (Fig. 5B).

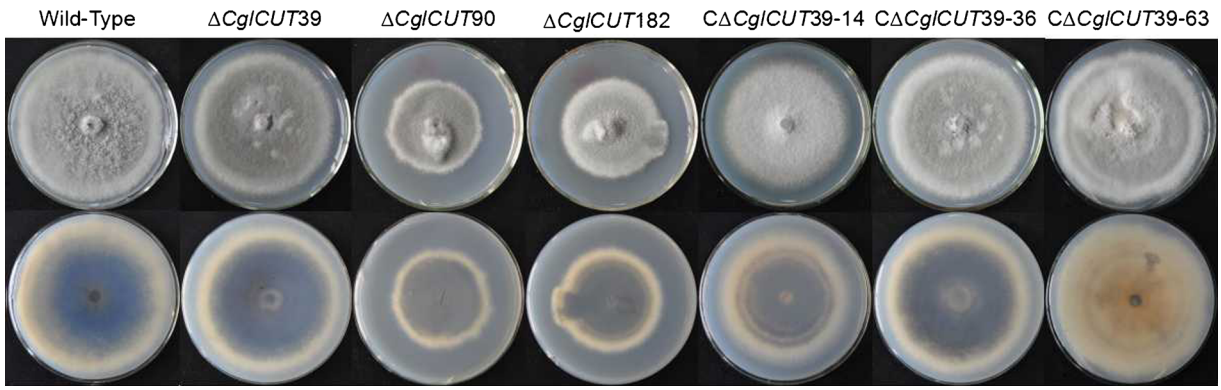

Fig. 4 Colonial morphology of *CglCUT1* disrupted and complemented transformants. The disrupted transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182), complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63), and the wild-type strain were cultured on PDA plates in dark at 25 °C for 7 days. The photographs were taken from the top (upper row) and bottom (lower row) sides of the culture plates

Table 2 Growth rate and dry weight (mean ± SD) of mycelium of transformants

Strains	Growth rate of mycelium (mm/d)	Dry weight of mycelium(g)
Wild type	12.286 ± 0.128 a	0.438 ± 0.023 b
Δ*CglCUT*39	12.000 ± 0.090 b	0.460 ± 0.046 ab
Δ*CglCUT*90	7.168 ± 0.074 f	0.451 ± 0.039 ab
Δ*CglCUT*182	8.024 ± 0.108 e	0.448 ± 0.014 b
CΔ*CglCUT*39–14	10.690 ± 0.058 d	0.449 ± 0.050 b
CΔ*CglCUT*39–36	11.857 ± 0.090 c	0.500 ± 0.022 a
CΔ*CglCUT*39–63	11.786 ± 0.149 c	0.456 ± 0.031 ab

The letters, a to f, mean the significant difference (significant treatment effects $P < 0.05$)

Cutinase activity of Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182 decreased to 6.76 U/ml, 6.25 U/ml, and 4.23 U/ml, respectively. Compared with that of the wild-type, the cutinase activity of Δ*CgCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182 decreased by 87.1 %, 88.0 % and 91.9 %, respectively. The cutinase activity of the complemented transformants was restored to almost wild type levels. Cutinase activity of CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63 increased to 44.12 U/ml, 42.42 U/ml and 46.67 U/ml, respectively. Compared with the knockout mutant Δ*CglCUT*39, the cutinase activity of CΔ*CgCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63 increased by 84.7 %, 84.1 % and 85.6 %, respectively. However, cutinase activities of all three complement transformants were slightly lower than that of the wild type.

Pathogenicity test

Lesion diameters were measured after inoculation with conidial suspensions of the wild-type MC171, the knockout transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182) and the complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63) at 10 days post inoculation (Table 3). The virulence of the disrupted transformants decreased significantly. The lesion diameters on camellia fruits caused by the knockout transformants were less than 3.00 ± 0.99 mm (mean ± SD) and were 78.9 % smaller compared with that (14.20 ± 1.56 mm) caused by the wild-type MC171. Restored virulence was observed for complemented transformants. The lesion diameters on camellia fruits caused by the complemented

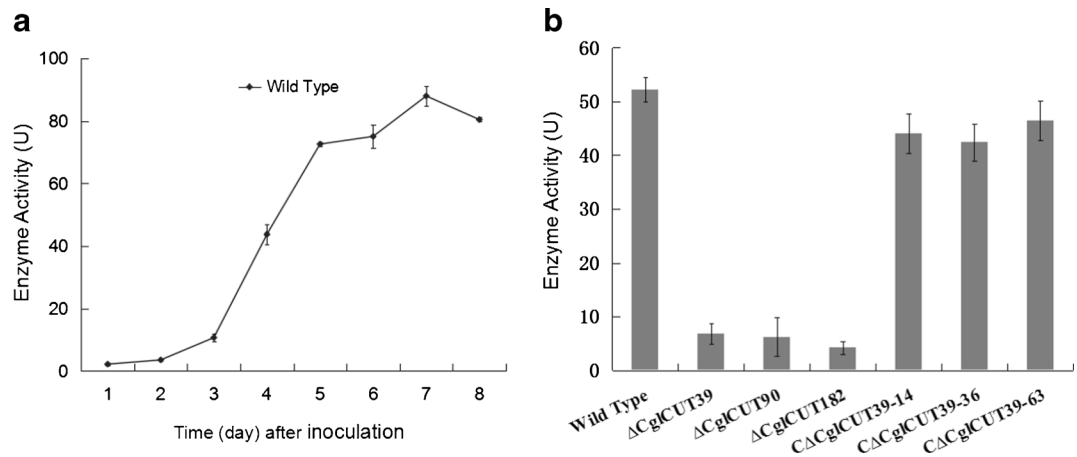

Fig. 5 Enzyme production and activity assay. **a**: Cutinase activity was continuously measured during an incubation from 1 to 8 days post inoculation (dpi) with conidial suspension of the wild-type MC171 at 2.5×10^6 conidia/ml. Cutinase activity reached a peak at 7th dpi. **b**: The cutinase activities of disrupted transformants, complemented transformants and the wild-type. Conidial suspensions (1.2×10^6 conidia/ml) of disrupted transformants (Δ*CgCUT*39, Δ*CgCUT*90 and Δ*CgCUT*182), complemented transformants (CΔ*CgCUT*39–14, CΔ*CgCUT*39–36 and CΔ*CgCUT*39–63), and the wild-type strain were used for inoculation. At 7 dpi, enzyme activities were measured from culture filtrates. The error bars stand for standard deviation (SD)

Table 3 The lesion diameter (mean ± SD) on the fruits measured at 10 days post-inoculation

Strain	Lesion Diameters(mm)	Virulence
Wild Type MC171	14.20 ± 1.56 a	+++++
△*CglCUT*39	2.61 ± 0.72 d	+
△*CglCUT*90	3.00 ± 0.99 d	+
△*CglCUT*182	2.83 ± 0.49 d	+
C△*CglCUT*39–14	9.93 ± 1.51 bc	++++
C△*CglCUT*39–36	8.96 ± 1.81 c	++++
C△*CglCUT*39–63	10.14 ± 1.49 b	++++

The letters, a to d, mean the significant difference (significant treatment effects, $P < 0.05$). "+"stands for low pathogenicity, "++++" stands for high pathogenicity

transformants were more than 8.96 ± 1.81 mm, increasing by 70.9 % compared with that caused by disrupted transformant Δ*CglCUT*39, but were slightly less than that caused by the wild type.

When the fruits were sectioned longitudinally, the inner fruit tissues inoculated with the wild-type and the complemented transformants were dark brown to black with decay, whereas none or slight decays were observed in the inner tissues of the fruits inoculated with the disrupted transformants (Fig 6). These results suggest that the *CglCUT1* plays a critical role in virulence of *C. gloeosporioides* during pathogenesis on Camellia.

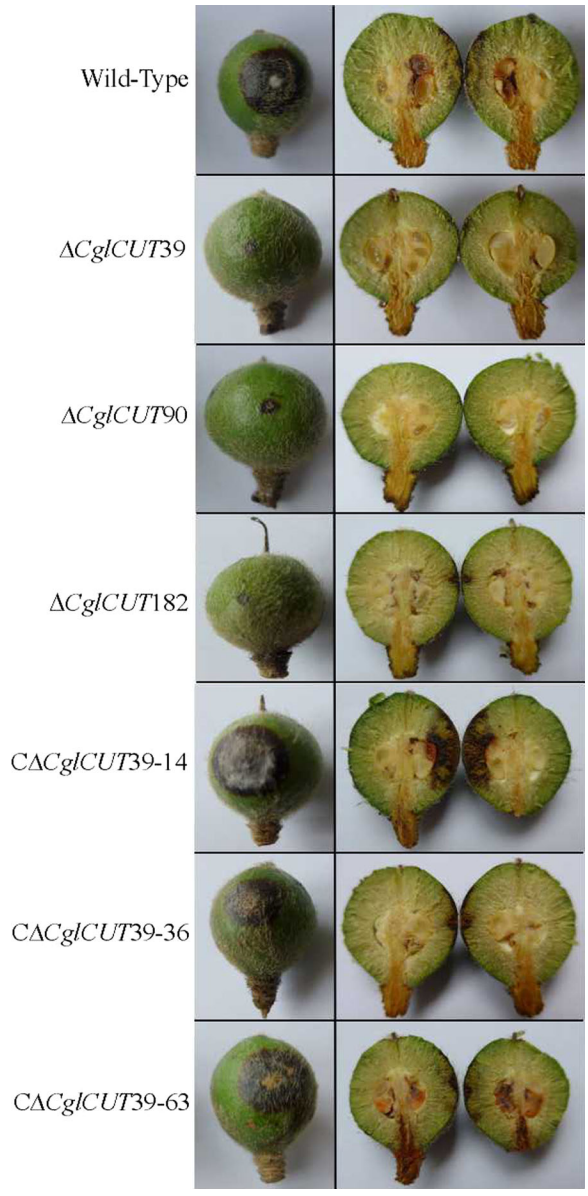

Fig. 6 Pathogenicity test of *C. gloeosporioides* strains. Detached fruit of camellia were inoculated with conidial suspensions (1.0×10^6 conidia/ml) of CglCUT1 disrupted transformants (Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182), complemented transformants (CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63), and the wild-type strain. Photographs were taken at 10 days post inoculation, showing disease lesion development on the inoculated fruit surface, and in the inner tissues displayed by longitudinally cutting across the inoculation sites

Discussion

As an important cutin degrading enzyme, cutinase has been purified or sequenced from a number of plant pathogenic fungi, including *Botrytis cinerea*, *Monilinia fructicola*, *Alternaria brassicicola*, *Fusarium solani* f. sp. *pisi* and *C. kahawae* (Chen et al. 2007, Purdy et al. 1975, Trail and Köller 1993, van der Vlugt-Bermans et al. 1997, Wang et al. 2000). However, the *CglCUT1* gene in the anthracnose fungus *C. gloeosporioides* of *C. oleifera* had not been characterized. Thus, the full length *CglCUT1* gene in the anthracnose fungus *C. gloeosporioides* of *C. oleifera* was cloned and characterized in this study. The GYSQG motif, the signature pattern for cutinases, was present in the *CglCUT1* predicted protein sequence (Belbahri et al. 2008). The predicted molecular weight of the *CglCUT1* protein was 23.4 kDa and was similar to several reported fungal cutinases, such as the 21 kDa protein from *C. kahawae*, 20.8 kDa from *M. fructicola*, and 24 kDa from *Alternaria brassicicola*. The structure of the *CglCUT1* protein had alpha-helix and beta-strand regions, which belong to the class of serine esterases and to the

superfamily of the α/β hydrolases (Longhi et al. 1997). The characterization of *CglCUT1* sequence suggests that the cloned DNA was the cutinase of this *C. gloeosporioides* that causes disease on *C. oleifera.*

The deduced amino acids sequences of the *CglCUT1* have the highest homology to the cutinases sequence of other fungi in the *Colletotrichum* genus (Ettinger et al. 1987; Chen et al. 2007), as well as those of other ascomycetes. The *CglCUT1* protein formed a subgroup with the cutinases of the *Colletotrichum* genus in the NJ tree. Furthermore, the cutinases of *Colletotrichum* genus were grouped very closely with the orthalogue of *Magnaporthe grisea***.** All the fungal cutinases were clustered in a single clade in the NJ tree, with the bacterial cutinases forming a separate cluster outside the fungal cutinase group. Within the fungal main group, the cutinases of Ascomycota, Oomycota and Basidiomycota were clustered in separate subgroups.

To elucidate whether the *CglCUT1* gene was required for cutinase activity and thus for fungal virulence, knockout transformants were obtained using homologous recombination by ATMT method. The knockout transformants were able to survive many rounds of subculture on selective medium, suggesting that the integration was stable. PCR amplification and Southern analysis further confirmed that the *CglCUT1* gene was successfully knock-out from genome. The strains with the *CglCUT1* gene knocked out did not exhibit significant changes in their morphology, conidiophore development and conidia germination.

The reduced level of cutinase in the knockout transformants may have interrupted pathogen recognition of the host cuticle and led to poor infection (Deising et al. 1992; Woloshuk and Kolattukudy 1986). In this study, cutinase activities of knockout mutants Δ*CglCUT*39, Δ*CglCUT*90 and Δ*CglCUT*182 decreased by more than 87.1 % and lesion diameters on host camellia fruits inoculated with knockout transformants decreased by more than 78.9 % compared with that caused by the wild-type. Cutinase activities of the complemented transformants CΔ*CglCUT*39–14, CΔ*CglCUT*39–36 and CΔ*CglCUT*39–63 increased by more than 84.1 % and lesion diameters increased by more than 70.9 % compared with that caused by the knockout transformant Δ*CgCUT*39. All aforementioned results suggest that the *CglCUT1* gene contributes to the fungal cutinase activity and is required for virulence of *C. gloeosporioides* causing anthracnose of *C. oleifera*.

Previous studies have demonstrated that pathogenic fungi could produce extra-cellular enzymes, such as pectinase and chitosanase to degrade the cell wall components of plants (Kolattukudy et al. 1995). The degradation products of host cell walls could act as damage associated molecular patterns that trigger the host plant immunity. As a defense strategy, plants can produce phytoalexins and a number of pathogenesis-related (PR) proteins, such as chitinase and peroxidase to inhibit fungal growth (Kikot et al. 2009). The importance of *CglCUT1* cutinase in *C. gloeosporioides* virulence revealed in this study may give us an insight into the applications of cutin and pathogenesis-related proteins of *C. oleifera*, such as facilitating molecular approaches for breeding disease resistance in *C. oleifera*. Some broad-spectrum fungicides, such as chlorothalonil and carmazine, are not ideal for practical disease management of this important edible oil producing woody plant (Wang et al. 2014). The importance of *CglCUT1* cutinase in *C. gloeosporioides* virulence revealed in this study may also provide a direction towards the identification of fungicides that could inhibit production and activities of degrading enzymes secreted by pathogens during infection processes (Dikaman et al. 1983).

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