

# The Role of Cutinase and its Impact on Pathogenicity of *Colletotrichum truncatum*

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## Abstract

The phytopathogenic fungus, *Colletotrichum truncatum* infects and colonises chili fruit through direct penetration of the cuticle. The cutinase gene of *C. truncatum* (*CtCut1*), a cutin degrading enzyme was identified, cloned and shown to be essential in breaching the cuticle of chili fruit. The expression of *CtCut1* gene was studied through RNA-mediated gene silencing and its impact on fungal pathogenicity was demonstrated. The vector, pAA1 encoding a hairpin RNA of GFP and *CtCut1* was constructed and transformed into *C. truncatum* pathotype F8-3B (virulent strain). F8-3B-pAA1 transformants exhibited reduced patterns of infection with one isolate having a 45.8% reduction in cutinase activity (reduction in *CtCut1* transcript) in comparison to the wild type. Importantly, *CtCut1*-deficient strains were unable to infect detached chili and soybean hosts as efficiently as the wild type. There was a delay in the infection period by the transformants. Nevertheless, artificial wounding of the cuticle enabled these F8-3B-pAA1 transformants to infect and colonise host tissues, resulting in typical anthracnose disease lesions. Coupled with microscopy, these data suggested that the defect in pathogenicity was likely due to a failure in penetration of the host cutin. Knowledge of the plant-fungal interactions arose from the development of a fungal transformation system for *C. truncatum* and implementation of RNAi technology. This technology thus provides an alternative genetic tool for studies of gene function, particularly of essential pathogenicity genes.

**Keywords:** Cutinase, Pathogenicity, *Colletotrichum truncatum*, Anthracnose

## Introduction

For successful pathogenicity, fungi employ various infection strategies to breach the host cuticle. While some pathogens use mechanical means through the formation of specialised infection structures [1], others enter by direct penetration of the cuticle through the assistance of extracellular cutinase activity [2,3].

*Colletotrichum truncatum*, a pathogen of legumes, cereals, grasses and vegetable crops such as *Capsicum* species is an ascomycete that infects its host by direct penetration through the host cuticle [4]. The process of infection/penetration is thought to be triggered by cutin degrading enzymes such as cutinase. Cutinases from many fungi have been isolated and characterised [5-8]. Cutinases have been suggested to participate in carbon acquisition for saprophytic growth [9]. They were also presumed to have a role in fungal penetration [10,11] and in surface adhesion [12].

However, the importance of cutinase in pathogenesis of fungi remains controversial since disruption of the cutinase gene in various fungi resulted in contradictory results. The overexpression of *MfCut1* in *Monilinia fructicola* increased pathogenicity on *Prunus* spp. and targeted gene replacement of *Magnaporthe grisea* and *Pyrenopeziza brassicae* cutinases dramatically reduced pathogenicity on rice, barley and oilseed rape hosts, respectively [11,13,14]. On the contrary, disruption of cutinase from other fungi such as *Nectria haematococca* [15] and *Botrytis cinerea* [16] resulted in conflicting evidence of the role of cutinase in pathogenicity. Pathogenicity of transformants in both fungi was unaltered compared to their wild type and control counterparts. The relevance of cutinase gene in fungal pathogenicity of *C. truncatum* however, has not yet been identified.

Cutinase has been proposed to be the hydrolyzing agent of the plant cuticle and/or elicitor of cutin monomers to trigger signaling pathways

within fungal pathogens [17,18]. Despite the identification of cutinase in a range of *Colletotrichum* species such as in *C. gloeosporioides* [3,6], *C. kahawae* [6], *C. trifolii* [19], *C. lagenarium* [20] and *C. capsici* [5], characterisation of the gene and its function in the *C. truncatum*-chili interaction remains unelucidated.

Analysis of gene function has been traditionally performed by conventional methods such as examining for phenotypic or biochemical changes in response to mutation of the gene of interest [20,21]. The process however, can be laborious and time consuming. Furthermore, the abundant wealth of information provided by the ever increasing sequencing of fungal species genomes requires an efficient method to link the genetic information to its biological function with much more accuracy. This has led to the development of alternative methods such as RNA-mediated gene silencing.

RNA-mediated gene silencing is a powerful tool for functional analysis of genes [22]. Initially discovered in *Caenorhabditis elegans* [23], the strategy has now been applied to a range of organisms across different kingdoms [24]. Termed as Post Transcriptional Gene Silencing (PTGS) in plants, RNA interference (RNAi) in animals and quelling in fungi [25], the phenomenon exploits the endogenous gene regulatory mechanism of eukaryotic cells. Double stranded RNA

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(dsRNA) is cleaved by a nuclease (Dicer) of the RNase III family into Smaller Interfering RNAs (siRNAs) of 21 to 28 nucleotides in length, that are then incorporated into a ribonucleoprotein complex forming the RNA-Induced Silencing Complex (RISC). The RISC recognises homologous mRNAs by complementary base pairing and triggers sequence-specific degradation of the homologous mRNA [26].

Studies of gene function through specific inhibition of pathogenicity or plant-fungal interaction gene expression using RNA-mediated gene silencing have been reported in many ascomycete fungi [27] including *Colletotrichum gloeosporioides* [28], *Magnaporthe oryzae* and *Colletotrichum lagenarium* [29]. However, such silencing mechanism has not yet been employed in *C. truncatum* to understand pathogenicity.

The aim of this paper was to identify functional cutinase genes in *C. truncatum* and then determine if the cutinase gene, *CtCut1* of *C. truncatum* plays a role in pathogenicity. Utilising a short (515 bp) cDNA fragment of *CtCut1* as the silencing target for gene silencing by RNAi, the efficacy of the pAA1 plasmid harboring the hairpin RNA cassette as a mediator for gene silencing was examined. Coupled with an established *Agrobacterium tumefaciens*-Mediated Transformation (ATMT) system for *C. truncatum* [30], the RNA-mediated gene silencing approach was successfully applied to study the function of *C. truncatum* cutinase gene in response to contact with its hosts.

## Materials and Methods

### Strains, culture conditions and plant materials

*Colletotrichum truncatum* pathotype F8-3B [31,32] and its transformant expressing GFP [30] that caused anthracnose disease of chili (*Capsicum* spp.) were grown and maintained on Potato Dextrose Agar (PDA; Difco Laboratories, France) at 24°C with 12 h photoperiod. Medium for the GFP-tagged F8-3B transformant was supplemented with 250 µg/ml of hygromycin B antibiotic. Conidiospores obtained from 14-day-old cultures were adjusted to 10<sup>6</sup> conidiospores/ml and used as the recipient in all transformation experiments. Genomic DNA/RNA extraction was carried out on fungal mycelium grown in 50 ml Czapek-Dox broth (CDB; Difco Laboratories, France) for 7 days under the same temperature and photoperiod described above until a mycelial mat was formed.

*Agrobacterium tumefaciens* strain AGL1, used as the donor for fungal transformation was grown and maintained in Luria Bertani (LB) broth containing 5 mM glucose (LBG; Sigma Aldrich, USA) at 28°C. One Shot<sup>®</sup> TOP10 Chemically Competent *Escherichia coli* (Invitrogen, Australia) used as the host for gene manipulation was maintained in LB broth at 37°C.

For pathogenicity assays, *Capsicum annuum* genotype Bangchang plants were established in pots in a glasshouse and grown under controlled conditions of light (18 h photoperiod), temperature (28°C) and humidity (60-70%). One-month-old *Glycine max* genotype Bragg soybean plants were also established in pots and maintained in the glasshouse.

### Nucleic acid manipulations

Total RNA was extracted from saprophytic stage (growing in CDB) and pathogenic stage (2-, 6- and 24-hours post-inoculated) mycelium using RNeasy Plant Mini kit (Qiagen, Australia). The RNA extraction was performed following the manufacturer's instructions. Contaminating genomic DNA in all RNA samples were removed by DNase I (Qiagen, Australia) treatment at room temperature for 30 min.

The integrity and quantity of the total RNA was confirmed by 1.5% agarose gel electrophoresis and 1 kb Plus DNA Ladder (Invitrogen, Australia). First strand cDNA was performed using the Omniscript RT-PCR Kit (Qiagen, Australia) and the oligo-dT primer (Qiagen, Australia) according to the manufacturer's protocol. Gene transcript was evaluated by reverse transcription polymerase chain reaction (RT-PCR) using Cut1F and Cut1R primers (Table 1), designed based on orthologues of fungal cutinase. The *GAPDH* gene was used to normalise the expression levels of the studied gene. A total of three replications were conducted for the experiment.

Genomic DNA was extracted from fungal mycelium using DNeasy Plant Mini kit (Qiagen, Australia) following the manufacturer's protocol. PCR was conducted to amplify cutinase gene in *C. truncatum* F8-3B using gCUT-F and gCUT-R primers (Table 1). The PCR reaction components consisted of 20 ng genomic DNA, 250 µM dNTPs, 0.5 µM of each primer, 1.5 mM MgCl<sub>2</sub>, PCR buffer and 0.5 U *Taq* DNA Polymerase (Scientifix, Australia) in a total volume of 25 µl. Conditions for amplification were an initial stage of denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final elongation step at 72°C for 5 min. to ensure that any remaining single stranded DNA was fully extended. The PCR reaction was done in PTC-100™ Programmable Thermal Controller (MJ Research, Inc, USA). Both PCR products derived from the cDNA and genomic DNA were cloned and sequenced (AGRF, Australia).

Alignment of the sequence was generated using CLUSTALW (1.81) Multiple Sequence Alignments program [33]. Sequence analysis was carried out using BLAST (Basic Local Alignment Search Tool), accessed through the Internet (<http://www.ncbi.nlm.nih.gov/>) and the *C. graminicola* Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>). Six frame translations on the nucleotide sequence were performed using SDSC Biology Workbench 3.2 (<http://workbench.sdsc.edu/>) [34]. A homology search was done at the nucleotide and protein level using BLASTN and

Name	Sequence (5' → 3') <sup>a</sup>	Applications
Cut1F	CTTGTGCGTGCCGCTCCTGT	Reverse transcription PCR
Cut1R	AGAGTCGCTTAGCCTCGTTGAT	Reverse transcription PCR
gCUT-F	AATCCCTTACAACCTTCTCTGACA	Cutinase gene amplification
gCUT-R	ATCAACCGCCACGATACAGACAA	Cutinase gene amplification
SmaI-CutS-F	TATATA <b>accggg</b> TCATCTCTCTTGCCGTTTC	Cassette construction, Southern analysis
XbaI-CutS-R	TCAGAT <b>tctaga</b> GTCCAAGGTTCTGCAGGTTTC	Cassette construction, Southern analysis
BamHI-CutAS-F	TAGTAT <b>ggatcc</b> GTCCAAGGTTCTGCAGGTTTC	Cassette construction
XhoI-CutAS-R	ATTCG <b>actcag</b> TCATCTCTCTTGCCGTTTC	Cassette construction
S-GFP-R	TCGCCGATGGGGGTGTTCTGCT	PCR, Southern analysis
5NQ-CUTF	TCTGTTGCGCTACACCAAGAA	Quantitative real-time PCR
5NQ-CUTR	CAAGAAATGCGCCGGCAGGAT	Quantitative real-time PCR
hphF	GCTGCGCCGATGGTTTCTACA	pAA1 analysis
hphR	GCGCGTCTGCTGCTCCAT	pAA1 analysis

<sup>a</sup>Restriction sites are bolded lower case letters

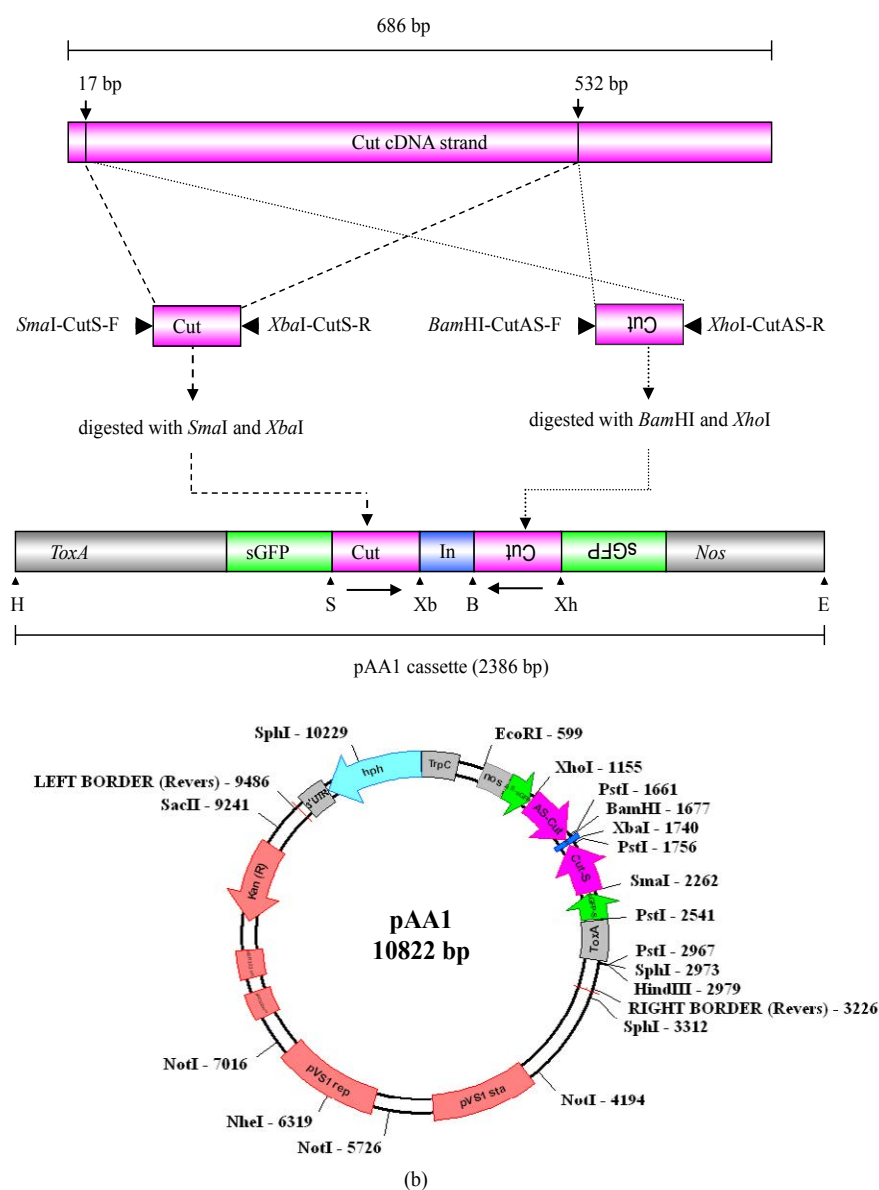
**Table 1:** List of primers used in the study.

BLASTX [35]. Bioinformatics tools were used to analyse and confirm the cloned gene, which was later designated as *CtCut1*. The protein prediction/manipulation tools in the Gene Infinity program (<http://www.geneinfinity.org/>) were also used to predict the cutinase protein molecular weight and protein isoelectric point.

### Vector construction and fungal transformation

Two binary vectors, designated as pJF1\_ShhGFP and pAA1 were generated for RNA-mediated gene silencing. For this, the sGFP cassette driven by the *ToxA* promoter from the pJF1 [36] plasmid was removed by digestion at the *HindIII-EcoRI* site, forming an open plasmid for cloning of a desired fragment. A 1354-bp DNA fragment containing the regulatory sequences and the sense and antisense of GFP

interrupted by *C. truncatum* cutinase (Accession number HQ406775) intron to form hairpin structure was synthesised and cloned into the *HindIII-EcoRI* digested site of pJF1, resulting in pJF1\_ShhGFP fungal silencing plasmid (DNA2.0, USA). The second fungal silencing vector, pAA1 was constructed by cloning the sense and antisense strand of the cutinase cDNA into *SmaI-XhoI* and *BamHI-XbaI* sites of the pJF1\_ShhGFP plasmid. The internal cutinase fragment (17 bp to 532 bp) was amplified with primers *SmaI-CutS-F* and *XbaI-CutS-R* to generate the sense (540 bp) and with primers *BamHI-CutAS-F* and *XhoI-CutAS-R* to generate the antisense (541 bp) (Figures 1a and b). Amplifications were performed in two separate reactions with 20 ng/μl *C. truncatum* cutinase DNA previously cloned into pCR4-TOPO vector (Invitrogen, Australia) as template. The amplified antisense fragment was digested



**Figure 1:** a) Schematic representation map of pAA1 cassette expressing *Cut* hairpin RNA, conferring the cutinase gene in *Colletotrichum truncatum*. The pAA1 recombinant silencing plasmid was derived from pJF1\_ShhGFP. pJF1\_ShhGFP was a derivative of plasmid pJF1. Arrows indicate the direction of the *Cut* gene fragment. In represents the 57-bp intron from *CtCut1*. *ToxA* represents the *Pyrenophora tritici-repentis* *ToxA* promoter and *Nos* represents *Nos* terminator. Restriction enzymes site: H, *HindIII*; S, *SmaI*; Xb, *XbaI*; B, *BamHI* and E, *EcoRI*. The nucleotide sequence of the *cutinase* gene is available from the NCBI database (Accession no. HQ406775). b) Plasmid map of pAA1.

with *Bam*HI and *Xba*I enzymes (New England Biolabs, UK), cloned into *Bam*HI-*Xba*I sites of the pJF1\_ShHGFP plasmid and the vector was named pAA1AS. Next, the amplified sense fragment was digested with *Sma*I (New England Biolabs, UK) at 25°C for 16 h and followed by digestion with *Xho*I (New England Biolabs, UK) at 37°C for another 16 h. The fragment was cloned into the *Sma*I-*Xho*I site of the pAA\_CutAS plasmid, resulting in the pAA\_CutASS plasmid. pAA\_CutASS plasmid was later named pAA1.

Fourteen-day-old germinated conidiospores-transformants expressing GFP were transformed with pJF1\_ShHGFP and its wild type was transformed with pAA1, respectively by the ATMT method as described [30]. Both pJF1\_ShHGFP and pAA1 transformed strains were selected on PDA containing 250 µg/ml hygromycin B antibiotic. Since the transformant-expressing-GFP carried the same selection marker (hygromycin B antibiotic) as the current pJF1\_ShHGFP transformant, the double-transformed individuals were unable to be selected on the selective medium. Subsequent analyses were performed only on single-spored putative transformants of pAA1, or also known as F8-3B-pAA1 transformants; with pJF1-ShhGFP transformants used as the control for the experiment.

### Microscopy

Mycelium and conidiospores of putative F8-3B-pAA1 transformants containing the inverted repeat cassette within the T-DNA insert were observed using a Leica DMRBE microscope (Leica Microsystems, Germany), for the lack of GFP expression which confirmed the success of the inverted repeat (hairpin) structure formation in the pAA1 plasmid. Similarly, the mycelium and conidiospores derived from infected fruits and leaves in the pathogenicity assays were observed for the lack of GFP expression. Successful transformants were those with no GFP expression. For this, all specimens were placed on a glass slide in a water droplet, covered with a cover slip and observed under the microscope. The images were captured by a Leica DC300F digital camera using Leica IM50 v.4 software. Wild type F8-3B and transformants expressing GFP [30] were used as the controls.

### PCR and Southern blot analysis

PCR was used to confirm the presence of T-DNA in ten putative F8-3B-pAA1 (F1, F2.1, F2.2, F4, F5.1, F5.2, F7, F8, F9 and F10) transformants by amplifying an internal 544 bp region of the *hph* gene [37] using *hph*F and *hph*R primers (Table 1). Putative transformants of F2.1 and F2.2, and F5.1 and F5.2 were derived from separate single spores of the same transformation event for each isolate respectively. Another PCR was performed with the 207 bp region of the *sgfp* gene using the primers; *Sma*I-CutS-F and S-GFP-R (Table 1).

Southern blot analysis was performed to confirm the copy number of the endogenous *cutinase* gene in the wild type and the integration of T-DNA containing the hairpin cassette in the transformed fungal genome. Transformed fungal genomic DNA was extracted using a DNA extraction method that utilizes high SDS concentration [38]. A total of 8 µg genomic DNA was digested with 20 U *Hind*III (New England Biolabs, UK) at 37°C for 8 h. pAA1 plasmid was prepared using *Hind*III and *Eco*RI restriction enzymes. Fragments were separated on 1.0% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics Cooperation, Indianapolis, USA). A 540-bp cutinase probe was generated by PCR using the *Sma*I-CutS-F and *Xba*I-CutS-R primers (Table 1), labeled with digoxigenin (DIG) according to the DIG High Prime DNA Labelling and Detection Starter Kit II protocol (Roche Diagnostics GmbH, Germany). Pre-

hybridization and hybridization of bound fragment(s) was carried out at 42°C for 1.5 h and overnight, respectively. The blot was washed and processed further following the DIG High Prime DNA Labelling and Detection Starter Kit II protocol. The number of bands appeared on the blot indicated the number of copies of the 540 bp cutinase probe that also represented the number of copies of the cutinase genes in the genome. Wild type *C. truncatum* genomic DNA and pAA1 plasmid were used as controls.

### Quantitative real-time PCR (qRT-PCR) analysis

The mycelium of 7-day-old F8-3B-pAA1 transformants (F2, F4, F5, F7 and F8) were inoculated onto the surface of 4-week-old young chili leaves of *C. annuum* genotype Bangchang following the procedure by Chen et al. [39]. After 6 h, the inoculated fungal mycelium were collected, freeze-dried and subjected to RNA extraction and cDNA conversion. Wild type F8-3B was used as the control. The transcript levels of *CtCut1* that encoded for the *cutinase* gene of *C. truncatum* in five transformants in response to gene knockdown were quantified. The reactions were performed with ABI qPCR universal master mix (Applied Biosystems, USA) in ABI-Applied Biosystems 7900 HT Thermal Cycler Real Time PCR machine by MCLAB, USA. Triplicate qRT-PCR reactions were performed for all biological replicates. Elongation Factor 1 (EF1) from wild type *C. truncatum* pathotype F8-3B was used as a reference gene for the qRT-PCR experiment because of its stability in diverse developmental stages [40]. Analysis of the qRT-PCR data was performed using the comparative delta Ct ( $\Delta$ Ct) method [41]. Two independent experiments were performed with three technical replicates each. Results from the experiments were statistically analysed using ANOVA.

### Pathogenicity bioassays

Fruit of *C. annuum* genotype Bangchang which is susceptible to *C. truncatum* pathotype F8-3B [31] was used in the pathogenicity bioassays. Leaves of *G. max* genotype Bragg, the susceptible leguminous host of *C. truncatum* [42] was alternately used to verify the infection characteristics of the fungus. F8-3B-pAA1 transformants that displayed a single copy gene T-DNA insertion in their genome were randomly selected for use in the pathogenicity assay. Detached fruits and leaves for pathogenicity assays were surface sterilised with 1.5% sodium hypochlorite (NaOCl) for 5 min, followed by several rinses with sterile distilled water before fungal inoculation.

### Chili fruit inoculation

Preliminary non-wound chili fruit bioassay was carried out on detached *C. annuum* genotype Bangchang fruit. Agar plugs from 8-day-old cultures consisting of active growing mycelium of transformed strains; F1, F2, F4, F5, F7, F8, F9, F10 and a wild type were excised with a cork-borer and placed onto the surface sterilised fruit. The wild type F8-3B was used as the control and was designated as FW.

Subsequent pathogenicity bioassay using a wild type and two or three F8-3B-pAA1 transformants (F2, F4 and F8) was carried out comparing inoculation method (wound versus non-wound inoculation). Wound inoculation of the detached chili fruit consisted of pricking the surface of the fruit to penetrate the cuticle to a depth of 1 to 2 mm with a sterile needle, then placing agar plugs of actively growing mycelium from 14 to 21-day-old cultures of transformed strains and a wild type over the wound site. Fruits were placed in a plastic box on a tray, containing 900 ml water and sealed to provide humidity and incubated at 28°C in 12 h photoperiod. Three fruit replicates for each treatment was used and the experiment was replicated twice.



Symptoms of the typical anthracnose lesions were evaluated daily and the disease severity on chili fruit caused by F8-3B-pAA1 transformants and wild type F8-3B was scored on a 0-9 rating scale according to the method by Montri et al. [32]. The anthracnose scores were recorded as soon as the first symptom of the disease was observed on the fruit surface between 1 and 9 days after inoculation (DAI) for wound-inoculated fruit and between 1 and 13 DAI for non-wound inoculated fruit. Pathogenicity of the transformed strain was considered retained if the lesion score was similar to the wild type. A consensus rating score was given for each strain based on the predominant score for the three replicates. Ratings were based on comparison to a set of key scale diagrams developed for this disease with each rating representing a range of lesion sizes adjusted for fruit size [32].

### Soybean leaf inoculation

Soybean is a very susceptible host to *C. truncatum* and inoculated leaves showed infection within 3 days (unpublished). Hence, soybean leaves of *G. max* genotype Bragg were also used to assess the degree of infection caused by F8-3B-pAA1 transformants (F1, F2, F4, F5, F7, F8, F9 and F10). Surface sterilised detached leaves from 1-month-old soybean plants were placed on moist filter paper disc in Petri dishes. Agar plugs containing actively growing mycelium of F8-3B-pAA1 transformants were inoculated onto the left corner of the abaxial surface of the same detached leaves. Wild type F8-3B, designated as FW was inoculated onto the right corner of the abaxial surface of the detached leaves as the control. Inoculated leaves were incubated at 28°C with 95% RH in 12 h photoperiod. Anthracnose symptoms consisting of sunken and water soaked tissue which developed around the fungal plug on the leaves were evaluated at 5 DAI. Disease severity caused by the transformed strains on the leaves was measured based on the percentage of the diameter lesion produced relative to the wild type. Pathogenicity of the transformants was considered retained if the diameter of the lesion was similar to the wild type. The experiment was conducted with two leaves with each leaf consisting of two replicates of the transformants and a wild type. The experiment was repeated twice and the results obtained were statistically analysed using ANOVA.

### Chili leaf inoculation for screening of germ tube and appressoria development

The sterilised abaxial surface of detached young leaves from *C. annum* genotype Bangchang placed on moist filter paper discs in Petri dishes were inoculated with 10 µl of  $2 \times 10^6$  conidiospores/ml of F8-3B-pAA1 transformants (F2, F4, F5, F7 and F8). At least two leaves were used per fungal sample and three positions on the abaxial leaf surface (basal end, mid rib and tip end) were inoculated. Wild type F8-3B was used as a control and was designated as FW. The Petri dishes were sealed with parafilm and incubated at 28°C with 12 h photoperiod. Two time points, 6 hours after inoculation (HAI) and 24 HAI were selected to determine the appropriate time for counting germ tube and appressoria structures.

Inoculated tissue samples were excised into 1 cm<sup>2</sup> leaf pieces, and then placed in acetic alcohol solution (absolute ethanol: glacial acetic acid, 1:2) for 24 h to 48 h according to the method by Khan and Hsiang [43] to remove the chlorophyll. The acetic alcohol solution containing the leaf tissues was replaced when the acetic alcohol solution turned dark green. Cleared tissues were removed and rinsed with sterile distilled water, placed on glass slide with the abaxial surface facing up in drops of 0.05% trypan blue (w/v) and examined under the Leica DMRBE microscope (Leica Microsystems, Germany). The images

were captured by a Leica DC300F digital camera using Leica IM50 v.4 software.

Each leaf tissue was considered a separate replicate. A random three out of the six replicates were chosen per fungal sample. A total of 100 conidiospores per tissue were observed for conidiospore germination and appressoria formation. Conidiospores were considered germinated when germ tubes were present, regardless of germ tube length. The occurrence of a globular structure either at the conidiospore or at the end of the germ tube with a diameter larger than the germ tube was considered to be an appressorium and these were counted [44]. Results from the experiments were statistically analysed using analysis of variance (ANOVA).

## Results

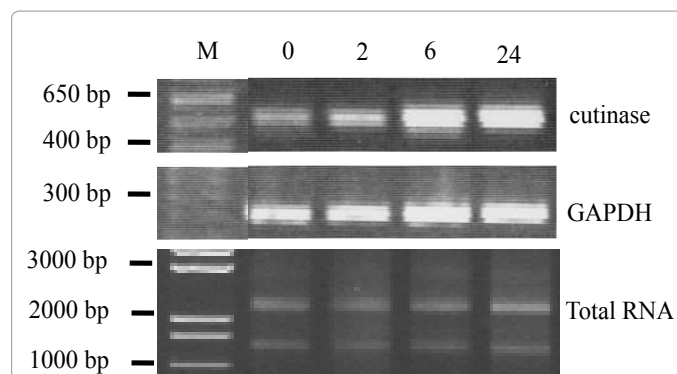
### Expression analysis of *C. truncatum* cutinase gene transcript on chili host

Expression analysis of cutinase gene transcript using equal amounts of RNA as the template revealed that the transcript was upregulated during *C. truncatum* infection (Figure 2). Similar RT-PCR results were obtained in all three independent biological replicates.

### *C. truncatum* cutinase 1 (CtCut1)

A 1936-bp cutinase genomic DNA fragment from *C. truncatum* pathotype F8-3B was obtained and was designated as CtCut1 (Figure 3). The primer binding sites of the sequence were identified. The predicted molecular weight for CtCut1 DNA was 1196.35 kDa. The nucleotide sequence was found to contain sequence identical to the partial gene obtained previously from the RT-PCR results (data not shown). Sequence analysis of the gene revealed an open reading frame (ORF) of 684 nucleotides and a stop codon (TGA), interrupted by a single 57-bp intron (Figure 3). The predicted ORF encoded a polypeptide of 228 amino acids with a predicted molecular weight of 23.81 kDa and a predicted protein isoelectric point (pI) of 7.82. The nucleotide and predicted amino acid sequences showed significant 99% identity to the cutinase of *C. capsici* isolated from papaya, 85% and 84% to *G. cingulata* and *C. gloeosporioides*.

Following the bioinformatics assessment, CtCut1 DNA was submitted to the NCBI GenBank database and was attributed the accession number, HQ406775.



**Figure 2:** The pattern of *C. truncatum* cutinase gene transcript expressed during the early stages of chili infection. The number on top of the agarose lane denotes the hours after inoculation (HAI) of the fungal mycelium on the host under laboratory conditions. The *GAPDH* gene was used to normalise the expression levels and the total RNA was used to demonstrate the equal loading for cDNA conversion. M, 1 kb Plus DNA marker.

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1      atcccttacaactttcctctgacatccaccctgagaaagacgtcgcgaaactcgtagtgg 60
2      ttgaccatctctctagatcacccgtcgaatcgagaaagtcataggccgcattttcccctca 120
121    ctccaaagtcagtcttctcctcctggcgcagaaggcggagggctggccccttttctc 180
181    cagccaaagagatggaagagtcttctcctcagaccattcccattttgatgttctgtcgcgaaa 240
241    agccgatcctcatgtgatctcgcagccttgaacatctaccttgcggactgtgggatcta 300
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421    atcatggaatggatgttaagccggataaacgagggcaatcatggccatagacgctccatggc 480
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541    agattcaatctctcactcagaccccaaacactctctccttcgctcgaagctcagccagcg 600
601    cgctcactccaagatcattctcttctcctcggcaagtcgtcgcctctgaagaccagtccca 660
      M K F L S I I S L A V S L V A A A
661    atacacaaaatgaagttcctcagcatcatctctcttgcggtttcccttgtcgtgcccgt 720
      P V E V G L D T G V A N L E A R Q S S T
721    cctgtcgaagtccggttggacaaccggtgttgccaaccttgaggcccgccagctcctcgacc 780
      R N E L E S G S S N C P K V I Y I F A
781    cgcaacgagcttgagtctggcagcagctccaactgccccaaagtcattctacatctttgct 840
      R A S T E P G N M
841    cgtgctctactgagccggtaacatggttaagtgacagttaagcgtcctcccttccacag 900
      * G I S A G P I V A D A L
901    gatgataactgactcctataaacagggcatcagggcagggcccattgtgcgcgagcgtctc 960
      E S R Y G A S Q V W V Q G V G G P Y S A
961    gaaaacggctacgggcctcagaggtctgggtccaggcgttggcggcccttactctgcc 1020
      D L A S N F I I P E G T S R V A I N E A
1021    gaccggcctccaacttcaatcaatcaccgagggcaccctccggcgtcggcctcaacgaggt 1080
      K R L F T L A N T K C P N S A V V A G G
1081    aagcgactcttccagctcgcacaaacaccaagtgcaccaactccgcgctcgtgcagggcga 1140
      Y S Q G T A V M A S S I S E L S S T I Q
1141    tacagcagggcagcagcgtgatggcgtcctccatctccgagttgagctccaagatccag 1200
      N Q I K G V V L F G Y T K N L Q N L G R
1201    aaccagatcaagggcgtgttctgttccgctacaccaagaacctgcagaaccttggagcg 1260
      I P N F S T S K T E V Y C A L A D A V C
1261    atcccgacttctcagacatccaagaccgaggtgtactgtgccctcgccgatgctgtgtgc 1320
      Y G T L F I L P A H F L Y Q A D A A T S
1321    tacggcagctgtttatcctgcggcgcatttctgtaccagggcagatgctgctactctc 1380
      A P R F L A A R I G *
1381    gcccagagattccttgctgctcgcattggctgagctccacgcgagggaaatatttccacg 1440
1441    gacctaggctgaggggagaacgggtctgaacaggctcgaaggacgggctccgtggaaa 1500
1501    agatactgagtatctggttggctgttgatcggctcagcttggggaggaggtcgaagtg 1560
1561    gatcgggttttctggttcttatgcttatgcttgctttaaactccttttttggctcactct 1620
1621    ttcattgctgatagggttttgatgattgtagcggggaagtaaaacgaaatgcaacgtgct 1680
1681    ttattgctaaggggtatcattgactcattacttctcaagtggtatcatgataatgcctc 1740
1741    acacggcgtctccgacaaaaatctgactgcattctcagggctcctcgggtctgccccgcaa 1800
1801    gtacggcgtatgcctctgagaatggtaactgacatcagggggatgcagcgggtgtgacac 1860
1861    tatacgacatgtcttgcatcctcgaatgtaggttttgatgagatgttgatagtgtctg 1920
1921    tatcgtggcggctcgaa 1936
    
```

**Figure 3:** Nucleotide sequence of a cloned genomic DNA for *Colletotrichum truncatum* isolate F8-3B *CtCut1* gene (accession no. HQ406775). The deduced amino acid sequence is shown above the open reading frame. The untranslated sequence gap beginning at nucleotide 868 is a 57-bp intron. The CAAT and TAAATA boxes for transcription as well as the potential polyadenylation site, TAAA are underlined. The three potential AATs are italicized. The "\*" indicates the stop codon of the ORF (represented by the amino acid sequences). "" represents the false stop codon in the intron region. Translation was carried out using SIXFRAME (Biology Workbench 3.2, University of California San Diego, 1999). Primer sites used to amplify the gene are shaded in grey. Bolded nucleotide sequence was used as probe in Southern analysis. The cutinase cDNA fragment was also used to generate the hairpin transcriptional unit for RNAi silencing.

### Phenotypic analysis of *CtCut1* silenced transformants and the integration of pAA1 T-DNA with hairpin structure in *C. truncatum* genome

All *C. truncatum* transformed strains were able to grow on the hygromycin selective medium. Phenotypes of the F8-3B-pAA1 transformants and its wild type (FW) were indistinguishable with respect to colony morphology, growth rate and conidiospore production. The unique feature of the fungus with the formation of concentric rings during

sporulation, was exhibited in all individuals. The conidiospores and hyphae of F8-3B-pAA1 transformants and wild type samples did not fluoresce under the GFP filter indicating that the silencing construct have been integrated into the genome and was functioning efficiently.

Single-spore-derived F8-3B-pAA1 transformed strains were subjected to PCR analysis. The hph and sGFP regions of T-DNA insertions were detected through amplification of the expected size fragments. All of the transformants amplified a 544-bp and a 207-bp fragment (Figure 4a). Neither fragment was amplified from the wild type.

Southern blot analysis indicated that 85.7% of the fungal genomes contained the T-DNA insertion (Figure 4b). Restricted genomic DNA of seven F8-3B-pAA1 transformants probed with a DIG-labelled 540 bp cutinase sense fragment confirmed the integration of the hairpin structure into the genome of F8-3B-pAA1 transformants.

*Hind*III was expected to cut the T-DNA sequence once only. All the transformants tested contained one insert of the T-DNA, with the exception of transformant F5, which contained three copies and transformants F4 and F9, which contained two copies of the T-DNA (Figure 4b). A higher molecular endogenous cutinase band was observed in transformant F4, suggesting the integration of T-DNA adjacent to the endogenous *cutinase* gene or the occurrence of partial T-DNA integration into the genome. Transformants F10 however, did not exhibit any additional bands apart from the endogenous cutinase band (Figure 4b). Incidentally, this transformant was able to grow in the selective medium, suggesting that the integration of nicked T-DNA strand containing the hygromycin B resistant fragment into the fungal genome.

The wild type copy of the endogenous *cutinase* gene, *CtCut1* was visible in all genomes as a hybridising band at ~8 kb (Figure 4b). As *Hind*III and *Eco*RI cut within the T-DNA insert and outside of the exogenous *cutinase* silencing transcriptional unit, a band of 2386 bp which corresponded to the intact pAA1 cassette, was expected and observed (Figure 4b). Undigested and/or partially digested pAA1 plasmid was also seen hybridising at ~10 kb (Figure 4b).

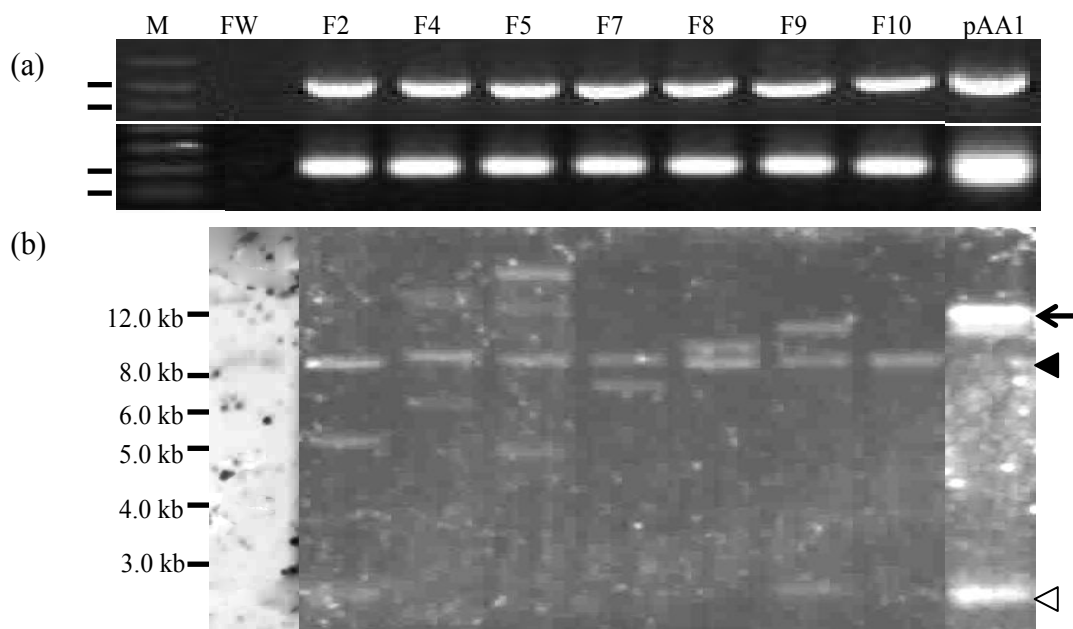
#### Expression analysis of CtCut1 silenced transformants of *C. truncatum*

Comparative *CtCut1* transcription of 6 HAI *C. truncatum* mycelium of five F8-3B-pAA1 transformants and one wild type were

determined using qRT-PCR. Differential expression of *CtCut1* that encodes for the *cutinase* gene in *C. truncatum* was detected from these transformants at 6 HAI (Figure 5). In the wild type (FW) mycelium, the amount of *CtCut1* transcript when normalized to EF1 transcript gave a relative expression value of  $2.45 \pm 0.11SE$  compared with  $1.33 \pm 0.29SE$  from an average of five transformants. These data indicated that almost one-fold of decrease in the relative expression of *CtCut1* in F8-3B-pAA1 transformants during infection. The highest significant decrease occurred in transformant F2 followed by F5, F8 and F4 with decrease of *CtCut1* transcript of 72.0%, 52.7%, 45.1%, and 44.3%, respectively in comparison to the expression in the wild type. In contrast, transformant F7 displayed no significant difference to the wild type although a slight reduction occurred in *CtCut1* transcript (14.9%). Overall, F8-3B-pAA1 transformants exhibited 54.2% of cutinase activity in comparison to the wild type.

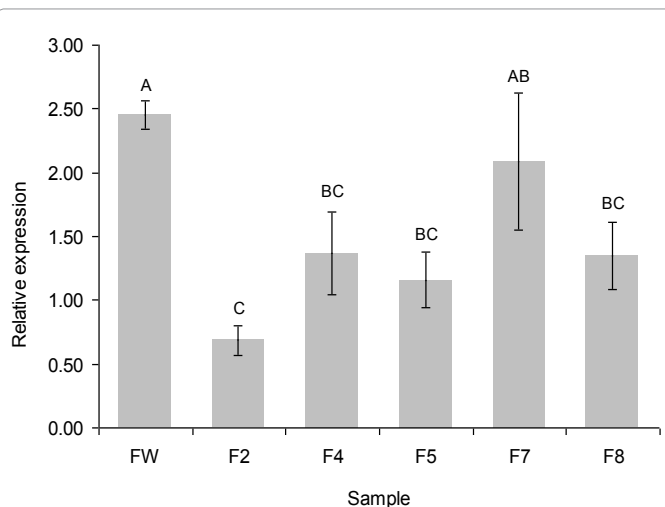
#### Infection of chili fruits

In the preliminary pathogenicity bioassay with non-wound detached chili fruit, all F8-3B-pAA1 transformants generally showed reduced or no infection compared to the wild type at 13 DAI (data not shown). In a subsequent detailed pathogenicity assay on wounded and non-wounded fruit, the transformed strains were found less aggressive on non-wounded fruit compared to the wild type at both 9 and 13 DAI (Table 2). Very little or no infection occurred in fruit up to 9 DAI for the transformed strains however, by 13 DAI symptom of the disease were displayed at the site of the transformed inoculum (Figure 6), suggesting that there was a delay in the transformants to invade the host tissues. On wounded fruit there was no difference in severity of infection between wild type and transformed strains at 2 or 4 DAI (Table 2).



**Figure 4:** Evidence of pAA1 cassette integration in several independent hygromycin resistant *C. truncatum* pathotype F8-3B fungal genome. (a) PCR analysis of genomic DNA with primers specific for the amplification of an internal 544-bp fragment of the *hph* marker gene (top panel) and the 207 bp *sgfp* gene (lower panel). Wild type, indicated as FW was used as negative control and pAA1 plasmid was used as positive control. M, 1 kb Plus DNA Ladder (bars from top to bottom: 650 bp, 500 bp, 300 bp and 200 bp). (b) Southern analysis of the hygromycin-resistant transformants. Genomic DNA (8 µg) was digested with *Hind*III and probed with DIG-labelled 540 bp cutinase sense fragment. Closed triangle indicates the single copy endogenous *cutinase* gene (hybridised at ~8.0 kb) in the fungal genome. Open triangle represents the exogenous *cutinase* silencing transcriptional unit (2386 bp) within the T-DNA fragment. Non-transformed *C. truncatum* (FW) was used as negative control. *Hind*III+*Eco*RI-digested pAA1 plasmid was used as positive control. Arrow corresponds to the undigested plasmid.



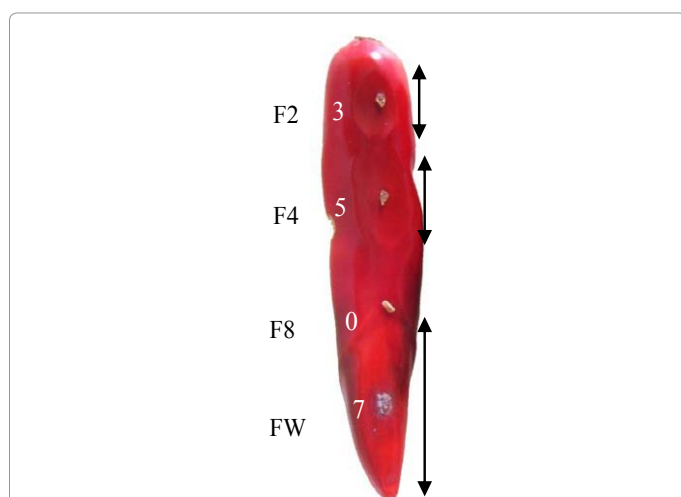


**Figure 5:** Quantitative gene expression of *CtCut1* transcript in F8-3B wild type (FW) and F8 3B-pAA1 transformants at 6 hours after inoculation (HAI) with *Capsicum annuum* genotype Bangchang. The Y-axis represents the relative expression in fold change of *CtCut1* gene relative to Elongation Factor 1 (EF1) reference gene expression. Bars on graph represent standard error based on mean of two independent experiments. Different letters over the bars indicate significant differences conducted using analysis of variance (ANOVA). LSD (1.034) was calculated at  $p < 0.05$  ( $n=2$ ). All data derived from three technical replicates per sample per experiment.

Method	Wounded fruit		Non-wounded fruit	
	2	4	9	13
Isolate / DAI				
F2	3	5	0	3
F4	nt	nt	1	5
F8	1	5	0	0
FW	3	5	3	7

Note: not tested (nt)

**Table 2:** Consensus ratings of disease severity based on Montri et al [32] disease scales for pAA1-transformed and wild type *Colletotrichum truncatum* infection of *Capsicum annuum* genotype Bangchang.



**Figure 6:** A representational image of the pathogenic development of wild type and transformed *Colletotrichum truncatum* pathotype F8-3B on *Capsicum annuum* genotype Bangchang. Fruit surface of non-wounded chili were inoculated with 14-day-old *C. truncatum* transformants F2, F4 and F8. Wild type (FW) was used as the control. The sample name of the transformants is indicated on the left side of panel. Arrow and the numerical score next to the infected fruit indicate the area of anthracnose sunken lesion.

GFP fluorescence was not detected in all conidiospores isolated from the infected fruits; neither from the wild type nor the transformants (data not shown).

### Germ tubes and appressoria development on chili leaves

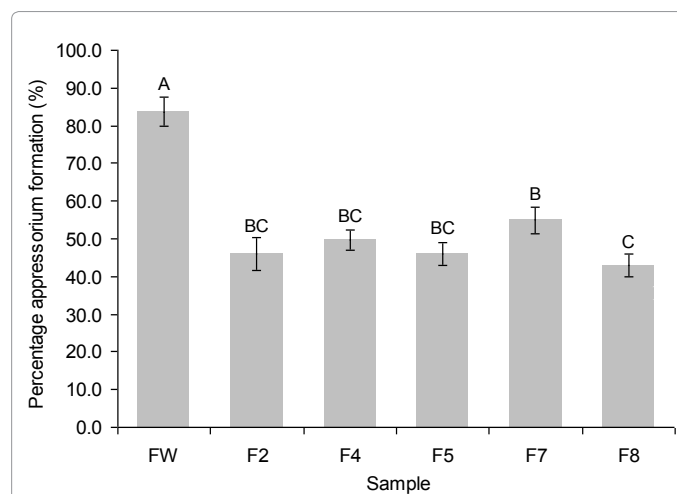
Germinated conidia formed appressoria by 6 HAI on the leaf of *C. annuum* genotype Bangchang. At 6 HAI, the highest number of appressoria formation occurred in the wild type as opposed to the F8-3B-pAA1 transformants (Figure 7). However, no significant difference in germ tube length was found between the wild type and the transformants, except that the length of germ tubes with appressoria in the transformants were generally longer compared to the wild type (data not shown).

### Soybean leaf bioassay

Differences in severity of infection were observed between the F8-3B-pAA1 transformants 5 DAI on soybean leaves compared to the wild type. The transformed strains selected can be categorised into two levels of pathogenicity with high (transformants F2, F5 and F8) and low (transformants F1, F4, F7, F9 and F10) reduction in infection efficiency (Figure 8). These were clearly observed by the different degrees of anthracnose symptom produced in the *inplanta* soybean bioassay (data not shown). Lesion region in soybean leaves demonstrated that transformants with reduced expression of the *cutinase* gene (F2, F5 and F8) displayed a lower degree of pathogenicity compared to the wild type.

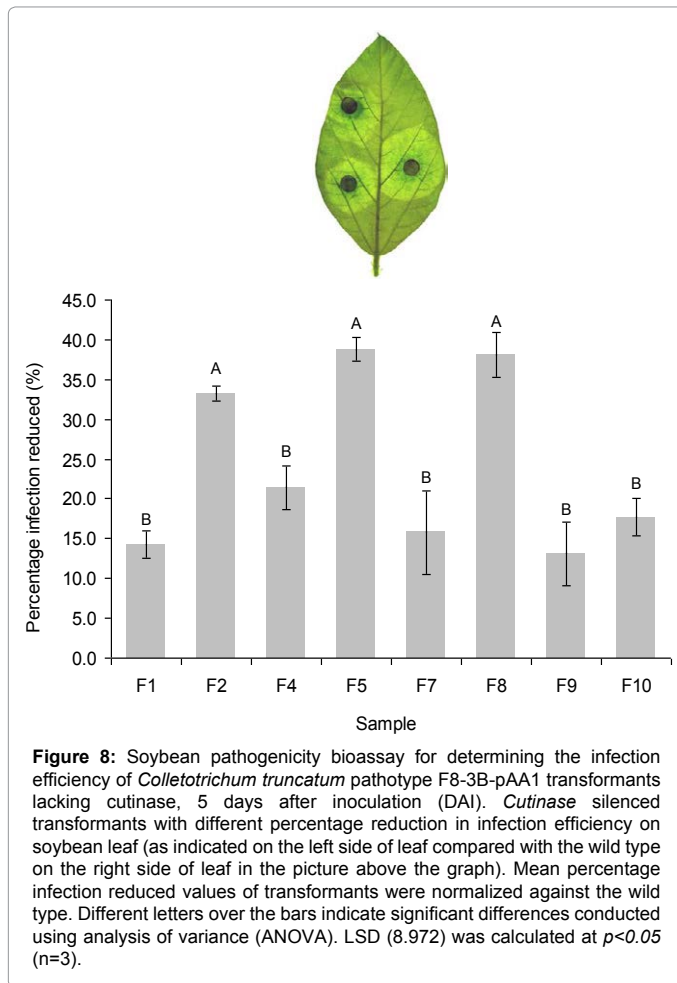
### Discussion

A full length gene encoding for cutinase, *CtCut1* of *C. truncatum* was successfully isolated and characterised. The amino acid sequence predicted from the nucleotide sequence had a relatively high degree of homology to the cutinases of other fungi in the *Colletotrichum* genus [5,45] as well as of other ascomycetes [46,47]. The similarity of the conserved consensus of *CtCut1* sequence with the other cutinases also suggested that the cloned DNA was cutinase of *C. truncatum*. The GYSQG motif, known to be the signature pattern for cutinase gene [48] was also present in *CtCut1*. The presence of the classical catalytic



**Figure 7:** Quantification of appressoria formation in *Colletotrichum truncatum* pathotype F8-3B wild type and transformants on *Capsicum annuum* genotype Bangchang leaf, 6 hours after inoculation (HAI). Different letters over the bars indicate significant differences conducted using analysis of variance (ANOVA). LSD (10.616) was calculated at  $p < 0.05$  ( $n=3$ ).





triad of Asp, Ser and His [49] as well as the essential carboxyl group within the cloned *CtCut1* also resembled those cutinases from other fungi [5,45,48].

RNA-mediated gene silencing in *C. truncatum* was successfully achieved following the development of a hairpin plasmid, pAA1 using a relatively short fragment of sGFP and *CtCut1* as the silencing target. Variable but efficient knock-down of the *cutinase* gene of *C. truncatum* was achieved. The pAA1 plasmid was stably transformed into *C. truncatum* through *Agrobacterium tumefaciens*-mediated transformation with pAA1-dsRNA synthesised *in vitro*. Conidiospores and hyphae of F8-3B-pAA1 transformants were unable to fluoresce under the GFP filter compared to the pJF1 transformants expressing GFP [30]. This was the first indication that the hairpin cassette was incorporated into the fungal genome, and thus had activated the endogenous RNAi machinery within the pathogen.

In addition, the transformed strains were able to survive many rounds of subculture on selective medium, suggesting that the integration was stable. Southern analysis confirmed that the pAA1 cassette had been integrated ectopically into the genome of all transformed strains of *C. truncatum*. Interestingly, the pAA1 hairpin that induced the gene silencing process of *CtCut1* did not produce significant off-target effects in *C. truncatum*; albeit the number of T-DNA copies harbouring the pAA1 cassette in the genome. All the transformed strains exhibited no significant changes in their morphology, growth rate and conidiospore production. Therefore,

it was likely that the only gene affected by the pAA1 hairpin was the *CtCut1*.

The pAA1 plasmid was versatile as it contained restriction enzyme sites that allowed the creation of hairpin cassette of any gene to be silenced as long as the primers used to amplify the gene of interest were tailored with the compatible restriction enzyme sites. The success of the silencing was predicted to be partially enhanced by the relatively short 57-bp intron sequence in the pAA1 plasmid. Short intron-enhanced gene silencing has been reported to provide stable and high silencing frequencies in *M. oryzae* [29]; which in some cases even led to complete silenced phenotypes [50]. In addition, the intron sequence used to form the hairpin in the pAA1 plasmid derived from the *cutinase* gene of *C. truncatum* was likely to reduce the possibility of organism rejection. Thus, proper regulation of RNA-silencing machinery that enables high levels of gene silencing was predicted to occur. In contrast, Praveen et al. [51] reported that the use of intron sequences from non-related organisms were likely to yield a lower gene silencing efficacy.

Considering the limited detailed genome information of *C. truncatum* and despite the occurrence of partial integration of T-DNA such as observed in transformant F10, the RNAi approach still produced silenced transformants of some degree; which is usually sufficient for practical use [29]. Basically, there were less challenges or effort in the inactivation of the gene such as the need to identify the number of copies or members of the gene in the genome. Nevertheless, the flexibility of RNA-mediated gene silencing to simultaneously silence homologous genes is achievable in *C. truncatum* because the targeted *CtCut1* gene suppression occurred in a sequence specific and not locus specific manner [29]. This would mean that silencing of *cutinase* gene using this approach was easily performed without the necessity to tailor knockdown per each gene member.

Although further work is needed to identify the presence of cutinase members in *C. truncatum*, Southern analysis revealed that *C. truncatum* had a single copy of the *cutinase* gene in the genome. This may have likely corresponded to the presence of one *cutinase* gene member in the pathogen. Further, there have been reports indicating the divergence of cutinase in other *Colletotrichum* species genomes [52]. In contrast, Skamnioti et al. [53] reported the presence of the multimembered cutinase families in *Magnaportha grisea*, *Fusarium graminearum*, *Botrytis cinerea*, *Aspergillus nidulans* and *Neurospora crassa*. In such event, the RNA-mediated gene silencing of *cutinase* in *C. truncatum* appeared to have simultaneously suppressed the entire gene family (homologous genes) and consequently avoided gene compensation. Baulcombe [54] demonstrated that the co-suppression of gene family occurred during the disruption of *quelling defective (qde-1)* gene mutation which resulted in the silencing of a tomato RNA-dependent RNA polymerase homologue in *N. crassa*. Simultaneous silencing of homologues was also observed in the silencing of *OsRac* gene. Three members of the *OsRac* gene family in *Oryza sativa* were suppressed when inverted repeat sequences derived from highly conserved regions of the gene family were used [55]. Not surprisingly, the RNA-mediated gene silencing approach has been adopted in many fungal gene manipulation studies (as reviewed thereafter by Salame et al. [27]).

More importantly, the expression of *CtCut1* transcript in the F8-3B-pAA1 transformants was lower than the wild type *C. truncatum*. A wide range of reduced mRNA level of *CtCut1* (14.9% to 72%) was observed among the transformed strains. Such variation among transformants using this technique has been observed in other fungal

pathogens as well, including *Phanerochaete chrysosporium* [56] and *Botrytis cinerea* [57]. As indicated earlier, the differences could also have been due to the ectopic integration of the T-DNA within the genome of the pathogen. Additionally, the genome complexity of different microorganisms and the environmental changes may perhaps have contributed to the variability [56].

Likewise, the infection and colonisation by F8-3B-pAA1 transformants on detached chili and soybean hosts were inefficient compared to the wild type F8-3B, further suggesting the involvement of the gene during pathogenesis. Transformed strains were unable to infect normally and there was a delay in infection (as indicated by the extremely slow development of anthracnose symptoms on chili fruits). This suggested that the direct penetration through host cuticle during the infection process of *C. truncatum* [4] was largely governed by the presence of cutinase in the fungus.

In contrast, there was no difference in infection and colonisation by F8-3B-pAA1 transformants and wild type on wounded fruit, suggesting that cutinase appeared to no longer have an important role in cell infection after penetration of the cuticle. Hence, there was no disadvantage to the F8-3B-pAA1 transformants having reduced expression of cutinase once they had infected the pericarp tissue. The results were compatible to previously reported work where it was demonstrated that *C. capsici* pathotype F8-3B was able to infect chili fruits if wound inoculation was applied to the fruit [31]. If the hypothesis that cutinase assisted with the penetration of the fungal pathogen, then obviously, the wound inoculation method is unlikely to demonstrate the function of the gene since the host first layer of defence (cuticle and epidermis) has been disrupted.

The reduced level of cutinase expression in the transformants may have interrupted or delayed pathogen recognition of the host surface thus, resulting in the formation of lower number of appressoria. Cutinase may be required to stimulate a host reaction, which in turn is recognised by the pathogen to initiate appressoria formation and penetration. Therefore, the inability of the pathogen to express the effector (cutinase) may have led to poor infection rates. Deising et al. [12] reported that cutinase was involved during the adhesion of *Uromyces viciae* uredospores to the host cuticle. In another report, Woloshuk and Kolattukudy [17] indicated that the expression of cutinase from spores of *F. solani* was triggered upon contact with the plant cuticle. Indeed, Li et al. [58] reported that low levels of cutinase present in the saprophytic phase of *F. pisi* f. sp. *psii* was essential to induce higher levels of cutinase expression during infection of the fungus to penetrate its host. Since the endogenous cutinase in these F8-3B-pAA1 transformants were suppressed, the regulation of normal cutinase activity was affected. Whether this abnormal/improper regulation of cutinase activity happened due to its incapability to secrete sufficient levels of cutinase at the beginning to induce cutin monomers from the host or from the failure of the pathogen to detect the host cutin monomers is unknown.

The soybean pathogenicity bioassay revealed different levels of soybean leaf infection when inoculated with various selected F8-3B-pAA1 transformants. These observations confirmed that the disturbance of *CtCut1* gene via RNA-mediated gene silencing (knockdown) altered pathogenicity of the transformants. Indeed, the relative expression of the five chosen F8-3B-pAA1 transformants showed different levels of *CtCut1* transcription as compared to the control wild type in the qRT-PCR assay. Lower levels of *CtCut1* transcripts translated to reduced infection. Similar observations were shown in the *Pyrenopeziza brassicae*-*Brassica napus* system where mutants lacking cutinase transcript expression failed to penetrate the

cuticle of *Brassica napus* cotyledon [14]. In another report, Lee et al. [13] showed that the pathogenicity of *Monilinia fructicola* on peach petal was more aggressive when the gene was overexpressed, thus indicating the involvement of the gene during infection.

Taken together, these results demonstrated that pAA1 plasmid was efficient and could be applicable in most genes/*Colletotrichum* species to induce gene silencing. The observation indicated that the silencing event of cutinase had possibly brought an alteration in pathogenicity within the transformants. More durable and novel forms of disease control can be practiced when knowledge of pathogen pathogenicity genes that trigger resistance is achievable/available through this alternative genetic tool approach.

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