# Functional analysis of an appressorium-spesific gene from *Colletotrichum gloeosporioides*

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**Abstract.** A novel gene (*CAS2*) specifically expressed during appressorium formation was isolated from *Colletotrichum gleosporioides* using Differential Display RT-PCR. *CAS2* comprises 368 deduced amino acid residues and is 50% identical to a hypothetical protein from *Chaetomium globosum*. ProtFun 2.2 server analysis predicted that Cas2 functions as a transport and binding protein. Based on putative transmembrane domain prediction software (HMMTOP), Cas2 protein is composed of five alpha-helical transmembrane domains with a very short external N-terminus tail and long internal C-terminus. ExPASy ScanProsite analysis showed the presence of integrin beta chain cysteine-rich domain, N-myristoylation site, EGF-like domain, 2Fe-2S ferredoxins, iron-sulfur binding region, VWFC domain, fungal hydrophobins signature, membrane lipoprotein lipid attachment site and Janus-faced atracotoxin (J-ACTX) family signature in CAS2 protein. Mutants with deleted *CAS2* were not significantly different in terms of vegetative growth, conidiation, and appressoria production compared to wild type. However, the *cas2* mutant produced multipolar germination, a feature which distinguishes it from wild type strain. Interestingly, the mutant is non-virulent to mango fruits, indicating that *CAS2* may encode proteins that function as novel virulence factors in fungal pathogens.

Keywords: appressorium formation, *Colletotrichum gloeosporioides*, spesific gene, gene disruption, pathogenicity.

## 1. Introduction

Anthracnose disease, caused by *Colletotrichum gloeosporioides* or *Glomerella cingulata*, is very common and destructive on numerous crop and ornamental plants worldwide. This fungal pathogen is one of the best-studied species among hemibiotrophic fungi for elucidating various aspects of the host-pathogen interaction with its host. The pathogenicity of *C. gloeosporioides* depends on cellular morphogenesis event. Beginning with conidial attachment onto host surfaces, appropriate physicals and chemicals from host plant induced the conidia to germinate. Subsequently, the tip of the germ tube becomes attached to the surface and begins to swell to form a dome-shaped, highly melanized infection cell, the appressorium [1]. Next, a penetration peg emerges from a small area, adhering appressorium against the host surface [2]. The fungus then uses enormous turgor pressure generated in

the appressorium to drive the penetration peg through underlying plant surface [3]. This morphogenesis is a complex process from initiation to maturation, and involves the expression of a number of genes. Identification and characterization of genes that are active during conidial-appressorium morphogenesis is important to understand the molecular mechanisms of fungal differentiation and pathogenesis, and to develop new control methods that are rationally designed with specific targets in mind.

Several genes have been identified in *C. gloeosporioides* that are specifically expressed during appressorium formation er genes involved in the process that have not been discovered [5]. The first appressorial genes identified, *in/24* and *in/26*, were isolated from the rust fungus *U. appendiculatus* by differential screening of a genomic library [6]. *in/24* is expressed when appressoria begin to mature and its expression is maintained throughout maturation. Likewise, *in/26* is upregulated during appressorial maturation, although it is constitutively expressed at low levels in non-differentiated cells. The functions of these genes are unknown. Using the same approach, two appressorium-specific genes (*Mi/23* and *Mi/29*) were identified from *M. grisea* [7] and their functions are also unknown. An additional *M. grisea* gene, *MPG1*, was isolated by differential cDNA cloning and is abundantly expressed during appressorial differentiation and early plant infection [8] during conidiation and in mycelial cultures starved for nutrient, but the importance of this gene was demonstrated by showing that *mpgl* mutants were impaired in appressorium formation. The protein encoded by *MPG1* is a hydrophobin and in addition to its role in spore and appressorium adhesion, it may also act as a developmental sensor for appressorium formation [8].

In addition to *MPG1*, a *PTH11* gene from *M. grisea* was predicted to encode an appressorial transmembrane protein. *PTH11* was identified by Restriction Enzyme Mediated Integration (REMI) mutation [9], and *pth11* mutants failed to form appressoria on inductive surfaces and have decreased pathogenicity. However, these mutants were responsive to exogenous cAMP, which helps in forming functional appressoria and restoring pathogenicity. A PTH11-GFP fusion protein was found to be localized at the cell membrane. Based on these results, it was suggested the PTH11 protein plays a role in activating appressorium signaling as a receptor for inductive surface cues.

Differential display was used to isolate a novel appressorium-specific genes (*CgCAS2*). The sequence of the gene was used to characterize and predict the features and function of the resulting protein. A gene knockout experiment was also performed to observe the gene's function in appressoria formation and pathogenesis.

## 2. Materials and methods

### *2.1. Fungal and culture conditions*

*C. gloeosporioides* PeuB was obtained from the stock culture collection of School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. The fungal cultures were grown by frequent subculturing on potato dextrose broth (PDA: Difco, USA). Conidia, germinating conidia, appressoria and mycelia were cultivated and harvested [4].

### *2.2. Genomic DNA and RNA isolation*

Total DNA of *C. gloeopsorioides* was isolated from mycelia using the method described by Hamer et al. [1]. Total RNA of conidia, germinating conidia and mycelia were extracted using TRI REAGENT® solution (Molecular Research Center, USA), while RNA from the appressoria was extracted using TRIZOL® solution in combination with mechanical cell disruption by glass beads [4] of the DNA and RNA was tested using agarose gel electrophoresis. Both DNA and RNA were stored at -20°C until further usage.

### *2.3. Clonning and sequencing of CgCAS2*

Isolation of genes active at the appressoria developmental stage (*CgCAS2*) is based on a differential display of mRNA by reverse transcription polymerase chain reaction (PCR) using arbitrary primers. A conidia suspension at $10^6$ conidia/ml was induced and incubated for 7 hours to form appressoria on a

glass petri dish (15 cm in diameter) waxed with rubber leaves and papaya fruit wax. Total RNA from appressoria and mycelia was isolated by a modified method used by Redzuan [4] trary ACP primers were used to perform independent reverse transcription PCR reactions by employing a method of GeneFishing™ (SeeGene, South Korea). After separation on 2% agarose gel, the PCR products showing differential expression in appressoria (compared to mycelia) were cloned into pCR2.1-TOPO vectors using TOPO-TA Cloning Kit (Invitrogen, USA). DNA Walking Kit (SeeGene, South Korea) was then used to obtain the full-length sequence of *CgCAS2*.

Three target specific primers (TSPs, Table 1) were designed from the newly-obtained *CgCAS2* sequence using a DNAWalking SpeedUp™ Premix Kit (Seegene, South Korea). Nested PCR was performed by using the DNA Walking Annealing Control Primers (DW-ACP) provided in the Kit and the three TSPs. Each of the DW-ACPs contained a specific ACP primer-binding site at its 3'-end (5'-AGGTC, 5'-TGGTC, 5'-GGGTC and 5'-CGGTC). The amplification contained 100 ng of *C. gloeosporioides* genomic DNA, 4 µl of 2.5 µM DW-ACP (one of DW-ACP 1, 2, 3, and 4), 1 µl of 20 µM TSP, 1, 25 µl of 2× SeeAmp™ ACP™ Master Mix II, and sufficient distilled water to make up a 50 µl reaction. In the second PCR, four PCR reactions were set up, each of which contained 3 µl of the purified PCR product, 1 µl of the 10 µM DW-ACPN provided in the kit, 1 µl of 20 µM TSP 2, 10 µl of 2× SeeAmp™ ACP™ Master Mix II, and 5 µl of distilled water to make up a 20 µl reaction. In the third PCR, four PCR reactions were prepared, each of which contained 1 µl of the second PCR products, 1 µl of the 10 µM universal primer provided in the kit, 1 µl of 10 µM TSP 3, 10 µl of 2× SeeAmp™ ACP™ Master Mix II and 7 µl of distilled water to make up a 20 µl reaction. All of the PCRs were performed on a PCR Thermal Cycler. The extracted PCR products were cloned into the pGEM®-T Easy Vector System (Promega) and sent to a commercial DNA sequencing service (First Base, Malaysia) for nucleotide sequence determination. After the upstream *CgCAS2* sequence was cloned and sequenced, two primers (Hpw-F and Hpw-R) were used to obtain the whole *CgCAS2* gene.

**Table 1.** List of oligonucleotide primers used in this study.

| Name | Sequence | Usage |
|---|---|---|
| HpTsp1 | GGTGACGACAATGATTTCT | PCR CgCAS2 ORF |
| HpTsp2 | CCCAGTCCCACTTGTTGT | PCR CgCAS2 ORF |
| HpTsp3 | TGTCACCCAGTTATTTGCT | PCR CgCAS2 ORF |
| Hpw-F | CCGAGGCATAAACCAGGGACGAG | PCR CgCAS2 ORF |
| Hpw-R | TGATCCCGTTGGTCTTTGCCTTG | PCR CgCAS2 ORF |
| TrpC-F | CCATGTCAACAAGAATAAAACGC | PCR integration gene replacement vector |

*2.4. Transformation-mediated gene replacement*

Preparation of sphaeroplasts and transformation of *C. gloeosporioides* were performed according to methods described by Rodriguez and Redman [11] gromycin transformants were selected on regeneration medium containing hygromycin B (300 µg/ml) (Sigma, USA). Before transformation, pN1389-PKAC was linearized with *Kpn*1 restriction endonuclease and precipitated with ethanol. Subsequently, 20 µg of DNA was transfected into *C. gloeosporioides* sphaeroplasts.

*2.5. Genomic DNA and RNA blot analyses*

DNA digestion, agarose gel fractionation, labeling of probes and hybridization were performed according to the kit manufacturer's instruction and standard methods [12] 2.5 kb fragment of *CgPKAC* DNA probe was labeled with [α-$^{32}$P] dCTP using Ready To Go™ DNA Labeling kit (-dCTP) (Amersham, USA). Hybridization was carried out with hybridization buffer (1 M $Na_2HPO_4.2H_2O$, 1 M $NaH_2PO_4$, 0.5 M EDTA, 0.1% [w/v] SDS) at 65°C for 4 hrs for pre-hybridization and hybridized

overnight after the labeled-probes were added. The membrane was washed at 65°C with 2× SSC for 10 min followed by 2× SSC and 0.1% SDS, 1× SSC and 0.1% SDS, and 0.5× SSC and 0.1% SDS until the radioactivity signal was low. The washed blots were exposed to Fujifilm for various times at -80°C.

*2.6. Appressorium induction on hydrophobic hard surface*

Induction of appressorium was tested on a glass slide coated with rubber wax. A total of 50 µl of wax (in chloroform) was spread on glass slide with cotton bud. Subsequently, 25 µl of conidia suspension containing $10^5$ conidia/ml were applied on the glass slides. Appressorium formation was observed every hour for 8 hours.

*2.7. Virulence assay*

Test for pathogenicity was performed as described by Kim et al. [8]. Mature green mangos were infected with conidia of *C. gloeosporioides*. Two modes of inoculation were applied in the pathogenicity test: inoculation on unwounded and wounded mango fruits. Before inoculation, fruits were surface sterilized with 70% ethanol and left to dry at room temperature. A total of 0.5 ml of conidial suspensions at $2 \times 10^4$ conidia/ml was applied to the surface of unwounded fruits by spraying the inoculum with a spray gun (Preval, USA), while wounded fruits were inoculated with 20 µl of condial suspension. Mangoes were arranged in moistened plastic trays and incubated at 30°C for two weeks to observe the disease symptoms. Number of lesions was observed daily.

## 3. Results and discussion

*3.1. Sequence analysis of the CgCAS2 gene*

A total of 2,150 bp of DNA sequence, which includes the *CgCAS2* ORF, 900 bp of promoter region, and 39 bp of 3'-end regulatory region, was obtained (Figure 1). The *CgCAS2* encodes a protein with 368 amino acids. A GTCGATGTTG sequence at nucleotide position 901 to 903, complying with the Kozak's rule, was found at the start region of the ORF (Figure 1). Comparison between the sequence of the gene and its cDNA sequence revealed a 1,214 bp ORF, which is interrupted by two introns at nucleotide positions 718 to 771 and 1,003 to 1,058, respectively. The intron/exon splice junction (GTA[Y/A]GT/[A/C]AG) of the two introns are typical of splice site sequences in other *C. gloeosporioides* genes and fit the consensus sequences found in other filamentous fungi. The second intron has the internal splicing sequence GCTAACPr necessary for lariat formation in filamentous fungi [14].

Analysis of the 900 bp upstream sequence of the coding region indicates that the 5' flanking region of the *CgCAS2* contains several potential regulatory elements (Figure 1). TATA box is absent in the *CgCAS2* promoter. However, a TATA-like sequence was detected at position -66 bp upstream of ATG. Genes from filamentous fungi often lack classical regulatory sequence of the 5' and 3' non-coding regions of other eukaryotes, and some filamentous fungi promoters do not contain any TATA boxes [14] II CCAAT signal was identified at -311 bp upstream of the start codon. Other putative regulatory elements identified at the upstream sequence are the GAGA factor binding site at -266, -466 and -668, factor NF-E1/NF-E1a/NF-E1b/NF-E1c binding site (YTATCW) at -422 and transcription factor NF-Y/CTF/CBF binding sites (ATTGG) at -261 and -758.

Within the 3'-untranslated region, a putative polyadenylation sequence (5'-AAATAA-3') is detected at the position 1,244–1,249 downstream from the ATG (Figure 1). During processing of pre-mRNA to mRNA, 5-AAAUAA-3' motif is required for proper RNA cleavage and subsequent polyadenylation. The spacing between the *CgCAS2* AAATAA element at position 1,245 and poly (A) tail is 19 bp in length, indicating that this element is most likely recognized during RNA processing [15] ORF encodes a protein with 368 amino acids.

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   -900    ggcccgacgtcgcatgctcccggccgccatggcggccgcgggaattcgatttcacagaag
   -840    tatgccaagcgaggggggggtctagacgatcgtgcagacatcttcaacgactggttttc
   -780    aataggcgtcaatgagctgcaattggaatggtgatttgggttcgtctcgtgtcaaacaga
   -720    agcttcgcctacccaagccagtgggagggaaatgtgggcaaatagatagcagagagcaag
   -660    ctagcaatacggtacagaatttcgggcgaccttcttgcgtctgctgtgcaatcaaacaaa
   -600    gcggtttgacctcaggaaacagaaggtgtcgcccaaaatgtcactgacatgtctgtccgc
   -540    agaggcacgggcttgggggaaaatgtgtcaaacccaactaccgaccgtcctgaccgaggc
   -480    ataaaccagggacgagagctgcgtcaagaaaccttcgcactcgtttacagtcgcatacta
   -420    tcatagatctgcgtggcatctgagcgatcgcatcgtcccgtttgggttagagcgccgtct
   -360    ccagccgcgcacaacgctgaatggtcccctcatttgatgtgcagcgaaccaatgcacgga
   -300    tgctgcaacttcatgctctggcacatcctcggtgagagattggccagttcgatctcgtgg
   -240    cgcggtttgggaacctcggctcagcttcccggaatatggtttgcaggggttggagttgc
   -180    ttctgctcctggttgaaacgtgatcgtttcgactacaagattgaagtagccccgatgat
   -120    acttggaacatcaatgggaccacgaaaatcatccgactattttcgtttctgtatactgat

1     ATGTTGTTGCCCAATGGGGGAGAAATCATACCCAACCCGTTTCTATCAATACCCATTGCC
       M  L  L  P  N  G  G  E  I  I  P  N  P  F  L  S  I  P  I  A
61    GCCGGGCTAGCAGTGGCCGCAGCGCAAGGTCACGGAGCAATTAGGATTTCCGGAGTTAAA
       A  G  L  A  V  A  A  A  Q  G  H  G  A  I  R  I  S  G  V  K
121   CCCGAAAATGTTCGCAGACTAGCCTACATCGCTGCGCTCGCTAGTTTTGTTCTCTCGACG
       P  E  N  V  R  R  L  A  Y  I  A  A  L  A  S  F  V  L  S  T
181   ACGGAATATCTCAACAAATGGTCAGCAAATAACTGGGTGACAGACAACAAGTGGGACTGG
      E  Y  L  N   K  W   S  A  N   N  W  V  T  D   N  K  W  D  W
241   GATCGAGAAATCATTGTCGTCACCGGCGGCAGCAGCGGCATCGGCGCAAGCATCATCAAG
      D  R  E  I  I  V  V  T  G  G   S  S  G   I  G   A  S   I  I  K
301   CACATCTTCGCAAGAAACCCCAAAGCGACCATTGTAGTGGTTGACTTGGCACCGTTATCA
       H  I  F  A  R  N  P  K  A  T  I  V  V  V  D  L  A  P  L  S
361   TGGGAACCACCCAAGGGCTCCAAGCTTCACTACTTCAAGTGTGACCTGACCGACACGGCG
       W  E  P  P  K  G  S  K  L  H  Y  F  K  C  D  L  T  D  T  A
421   GCACTGAAGACGCTTTGCACTCTCATTCGAACTCAGGTTGGGGATCCTACGGTTCTCATC
       A  L  K  T  L  C  T  L  I  R  T  Q  V  G  D  P  T  V  L  I
481   AATAATGCCGGCATTGCGCGGGGTGCAACAATTATGGAAGGCTCATATGCCGACATTGAG
       N  N  A  G  I  A  R  G  A  T  I  M  E  G  S  Y  A  D  I  E
541   CTCACCGTGAAGACAAATCTCATTGCGCCCTTCCTGTTGACGAAGGAGTTCCTGCCGTAT
       L  T  V  K  T  N  L  I  A  P  F  L  L  T  K  E  F  L  P  Y
601   ATGGTTCGCAGGAATCATGGACATATCGTCAACATCGGGTCGATGAGTTCGGTGGTCCCA
       M  V  R  R  N  H  G  H  I  V  N  I  G  S  M  S  S  V  V  P
661   CCCGTCAGAATCGCAGATTATTCTGCAACTAAAGCAGGACTAACTGCCATGCATGAGgtc
       P  V  R  I  A  D  Y  S  A  T  K  A  G  L  T  A  M  H  E
721   agtctactggtgaccacgccccaaagccgacgcttgactgacatagtacagTCTTTGCAA
                                                          S  L  Q
781   CTCGAGTTGAAGTACATCCACAAAGCACTGAAAGTTCGACAAACGCTTGGAATCTTCGGC
       L  E  L  K  Y  I  H  K  A  L  K  V  R  Q  T  L  G  I  F  G
841   TTCATCAGGACGCCTCTTGTTCCGTTCAACCCCGGACAGCCACATTTCGTTATGCCACTG
       F  I  R  T  P  L  V  P  F  N  P  G  Q  P  H  F  V  M  P  L
901   CTTCATGTCGATACTGTTGGTGAGGCAATTGTTAATGGACTTTACAGCGGATACGGCGGG
       L  H  V  D  T  V  G  E  A  I  V  N  G  L  Y  S  G  Y  G  G
961   ACCATTTACCTTCCTAGAATCATGTCTTTGGTGACTGCACTCgtaagttgtaaaattatc
       T  I  Y  L  P  R  I  M  S  L  V  T  A  L
1021  ccaaaaacaattagcatggggctaacagaatcaaacagAGGGCAGGGCCGGAATGGATA
                                            R  A  G  P  E  W  I
1081  TGGCGCCTAGCGCGAGAGACAACCGCCAGTGCAAAGGATATCCCTTACACCCCCCGCCAG
       W  R  L  A  R  E  T  T  A  S  A  K  D  I  P  Y  T  P  R  Q
1141  AAGATTAATGACTTGACGGGCACGTTTGACTTGGAAGAGGCTAGCAAGGCAAAGACCAAC
       K  I  N  D  L  T  G  T  F  D  L  E  E  A  S  K  A  K  T  N
1201  GGGATCAAGAATGAGCTTTAATCATCGAAACATTTCATCAATGTAAATAAGTATATTTTG
       G  I  K  N  E  L  -
```

**Figure 1.** Nucleotide sequence of CgCAS2 fragment showing the deduced amino acid residues and the two intron regions within the ORF. The deduced amino acids (368 residues) are indicated with abbreviations and shown below the ORF. Intron sequences are shown in lower case red letters and underlined. The potential CAAT box, TATA box, GAGA factor, factor NF-E1/NF-E1a/NF-E1b/NF-E1c binding site (YTATCW), transcription factor NF-Y/CTF/CBF binding sites (ATTGG) and polyadenylation (ATAATAA) are underlined and marked in blue letters.

The predicted CgCAS2 protein has a theoretical molecular mass of 41.7 kDa and a calculated isoelectric point of 9.4. PSORT (http://psort.nibb.ac.jp) analysis showed that there is a 65.2%

possibility that this protein is located in the cytoplasm, 17.4% in the mitochondria, 13% in the nucleus and 4.3% in the endoplasmic reticulum. Analysis of the N-terminal amino acid sequence using SignalP software predicted the presence of a signal sequence that is 24 amino acids long.

Similarity search against known proteins showed that the deduced amino acid sequence of CgCAS2 shares significant homology with some hypothetical proteins from other fungi, and the highest hits were with a hypothetical protein from *C. globosum* (CHGG09887) with 50% identity, hypothetical protein from *A. niger* (An14g01270) with 46% identity and hypothetical protein from *M. grisea* (MGG01604) with 40% identity. CgCAS2 is rich in Ala (9.8%) and Leu (10.1%).

*3.2. Disruption of CgCAS2*

Gene disruption was performed to test for the possible involvement of *CgCAS2* in appressorium morphogenesis. To construct a gene replacement vector, a 2.3 kb hygromycin resistance (*hph*) gene cassette was inserted into *Hin*dIII site of a cloned 1.8 kb *CgCAS2* fragment in pGEMCAS2 to generate the final construct, pGEMCAS2-hph (Figure 2A). Linear and circular versions of pGEMCAS2-hph were transfected into the sphaeroplasts of *C. gloeosporioides* wild type strain PeuB. Schematics of the homologous integration is shown in Figure 2B.

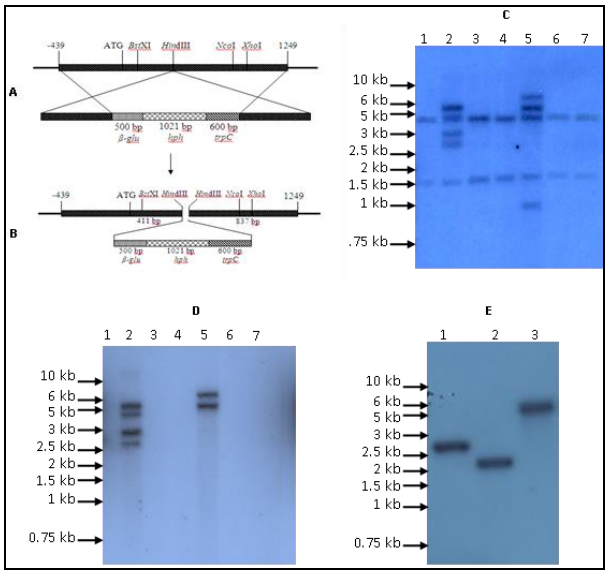


**Figure 2.** Schematic presentation of the strategy used for *CgCAS2* gene disruption in *C. gloeosporioides*. (A) Restriction map of the *CgCAS2* locus. (B) Partial map of the pGEMCAS2-hph replacement vector. DNA blot analysis of *CgCAS2* gene replacement in transformant *Cgcas2-x1* (lane 1), *Cgcas2-x2* (lane 2), *Cgcas2-x3* (lane 3), *Cgcas2-x15* (lane 4), *Cgcas2-c1* (lane 5), *Cgcas2-c2* (lane 6) and *C. gloeosporioides* wild type strain PeuB (lane 7). Genomic DNA was digested with *Xho*1 and probed with 1.8 kb of *CgCAS2*. (C) 1.1 kb of *hph* fragments. (D) The band in *Cgcas2-x2* and *Cgcas2-c1* samples showed different patterns of DNA fragments compared to the wild type strain when hybridized with 1.8 kb of *CgCAS2* probe. Probing with 1.1 kb of *hph* confirmed that both mutants carried the hygromcin resistant gene cassette in the mutant genome. To confirm that targeted integration has taken place in *Cgcas2-x2* and *Cgcas2-c1*, genomic DNA was digested with *Kpn*I and probed with 1.1 kb of *CgCAS2* gene. (E) A single band was detected when genomic DNA was digested with *Kpn*I and probed with 1.8 kb of *CgCAS2 gene*. In the *Cgcas2-c1* mutant, an increase in 2.3 kb (hygromycin cassette) was observed when compared to the wild type. Lane 1: wild type, lane2: *Cgcas2-x2*, lane3: *Cgcas2-c1*.

A total of 35 hygromycin-resistant transformants were isolated by single spore isolation and subcultured on PDA plate containing 300 g/ml hygromycin. All transformants were screened using PCR with HpF-F and HpF-R primers, which are complementary to the native *CgCAS2* DNA fragment, as well as with TrpC-F and HpF-R primers. TrpC-F primer was designed based on TrpC terminator sequence in the hygromycin resistance gene cassette. In two transformants, *Cgcas2-x2* and *Cgcas2-c1*, HpF-F and HpF-R primers did not produce the expected ~1.7 kb PCR fragment, indicating that there is an insertion of *hph* DNA fragment into the *CgCAS2* locus. TrpC-F and HpF-R primers amplified a ~1.5 kb amplicon in *Cgcas2-x2* and *Cgcas2-c1*, but not in the wild type strain that do not have *hph* gene cassette insertion (Figure 2B).

Cloning and sequencing of that fragment confirmed that homologous integration at the *CgCAS2* locus took place in the *Cgcas2-c1* mutant only. The disruption of *CgCAS2* in *Cgcas2-c1* mutant was also confirmed by Southern blot analysis (Figure 2C, 2D and 2E). In *Cgcas2-c1*, three extra bands

with the size of ~1 kb, ~6 kb and ~7 kb were observed (Figure 2C). Hybridisation with the hygromycin phosphotransferase (*hph*) gene showed that *Cgcas2-c1* produced bands with different sizes, whereas no signals were observed for the wild type (Figure 2D). To further clarify if gene replacement had occurred within *CgCAS2* locus, the genomic DNAs were digested with *Kpn*I, which has no restriction sites in wild type *CgCAS2*. When it was hybridized with the 1.7 kb fragment of *CgCAS2*, only *Cgcas2-c1* had a ~6 kb fragment, in contrast to the ~2.7 kb fragments seen in the wild type strain (Figure 2E). To test the expression of the *CgCAS2* gene by the mutant, total RNA extracted from appressoria of the wild type and *Cgcas2-c1* mutant were subjected to Northern blot analysis using the *CgCAS2* cDNA as a probe. The results confirmed the absence of *CgCAS2* transcripts in the appressoria of *Cgcas2-c1* mutants, whereas a *CgCAS2* transcript was detected in the wild type (Figure 3).

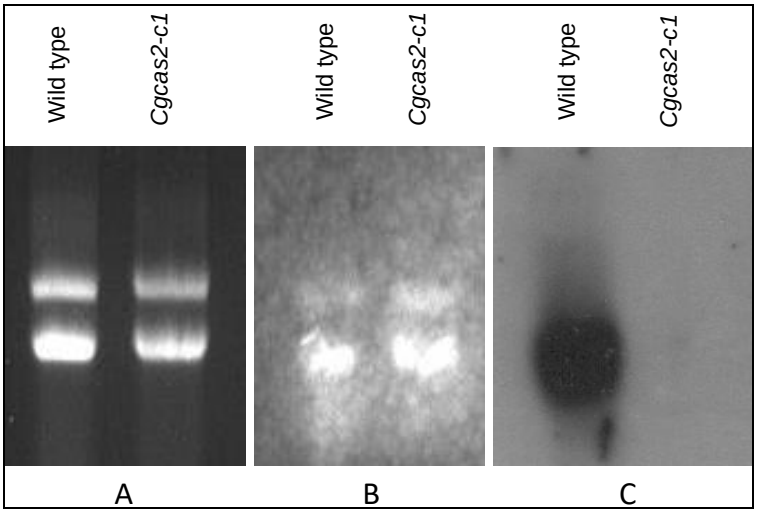


**Figure 3.** RNA blot analysis of total RNA obtained from appressoria of the wild type and the *Cgcas2* mutant of *C. gloeosporioides*. (A) The total RNA was extracted from 7-hour old appressoria induced with rubber leave wax on Petri dish. RNA was electrophoresed. (B) Blotted onto nitrocellulose membrane. (C) Hybridized with a $\alpha$-$^{32}$P-dCTP labeled 1.7 kb fragment of *CgCAS2* gene.

*3.3. The effect of CgCAS2 disruption on C. gloeosporioides morphogenesis*

The *Cgcas2* mutant strains had the typical grayish color and colony morphology similar to the wild type strain when grown on PDA. The growth rate of *Cgcas2* mutants, which was measured on PDA Petri dish cultures, is the same as that of the wild type after incubation at ambient temperature for one week. The *Cgcas2* mutant produced vegetative hyphae and abundant aerial mycelia. No obvious differences in conidial morphology was observed between the wild type and the *Cgcas2* mutant, but the amount of conidia production was slightly different (data not shown). This indicates that *CgCAS2* is essential for conidiation in *C. gloeosporioides*.

The effects of *CgCAS2* deletion mutant on germination and appressorium formation were assayed on hard surface glass slide coated with rubber leaf wax. Conidia produced by *Cgcas2* mutants were able to germinate and form appressoria. These mutant appressoria were melanised properly and had regular shapes. However, the percentage of germ tubes and appressoria formation was significantly reduced in the *Cgcas2* mutants compared to the wild type strain (Figure 4). In addition, the *Cgcas2* mutant conidia produced multipolar germination, in contrast with unipolar germination found in wild type conidia. However, appressoria differentiation only occurred at the tip of one of the germ tubes in both mutant and wild type. The remaining germ tubes in the mutant were unable to differentiate to

form appressoria. The *Cgcas2* mutant also produced longer germ tubes before forming appressoria, while the wild type conidia produced sessile appressoria (Figure 5).

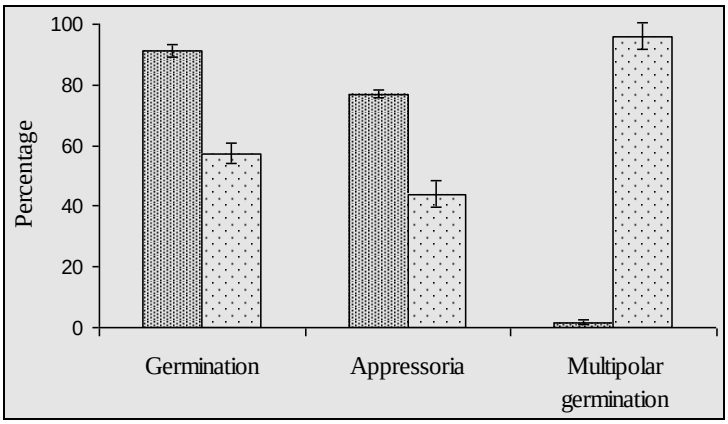


**Figure 4.** Percentage of germination, appressorium formation and multipolar germination of the wild type (▨) and the *Cgcas2* mutant (▭) conidia of *C. gloeosporioides* on hydrophobic hard surface glass slide coated with rubber leaf wax. The mean values of the same coloured bars inscribed with a common letter are not significantly different base on statistical analysis (P<0.01).

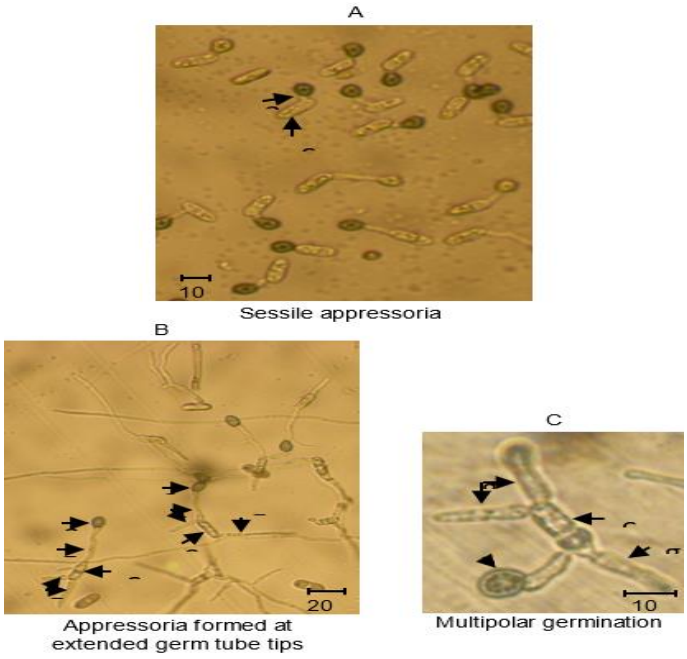


**Figure 5.** Light microscope observation of sessile appressorium formation in the wild type (A) and appressorium formation at extended germ tube tips of *Cgcas2* mutant (B) of *C. gloeosporioides*. Multipolar germination (B and C) of *Cgcas2* mutant on the hard surface of hydrophobic glass slides coated with rubber leave wax. The image was captured with an Olympus phase contrast microscope (200× magnification for A and B; 400× magnification for C) and a Nikon digital camera. a = appressorium, c = conidium, g = germ tube.

*3.4. CgCAS2 is required for C. gloeosporioides pathogenicity*

To determine the role of *CgCAS2* in pathogenesis, conidia of *Cgcas2* deletion mutants were inoculated onto mango fruits. Two methods of inoculation, i.e. direct inoculation onto wounded fruits and spray inoculation onto unwounded fruits, were employed to test for pathogenesis. In wounded fruits, the wild type strain induced typical brown lesions on fruits 3 days after inoculation (DAI) and developed typical necrotic, sunken anthracnose symptoms 7 DAI. In contrast, small brownish lesions were observed 3 DAI with the *Cgcas2* mutant, which did not develop into typical anthracnose symptoms seen in the wild type. Anthracnose disease severity was measured by lesion diameters and the *Cgcas2* mutant induced significantly smaller lesions than the wild type strain (Figure 6 & 7). When conidia were inoculated on unwounded fruits, initial symptoms by the wild type strain appeared 4 DAI and severe sunken lesion symptoms were observed 9 and 10 DAI. However, smaller brown lesions were observed on unwounded fruits sprayed with *Cgcas2* mutant conidia 6 DAI. Disease severity (based on the number of lesions) was nearly 3-fold lower in *Cgcas2* mutant compared to the wild type strain. In addition, lesions induced by *Cgcas2* mutant did not further develop into typical anthracnose symptoms. These results indicate that *CAS2* has an important role in pathogenesis of *C. gloeosporioides*.

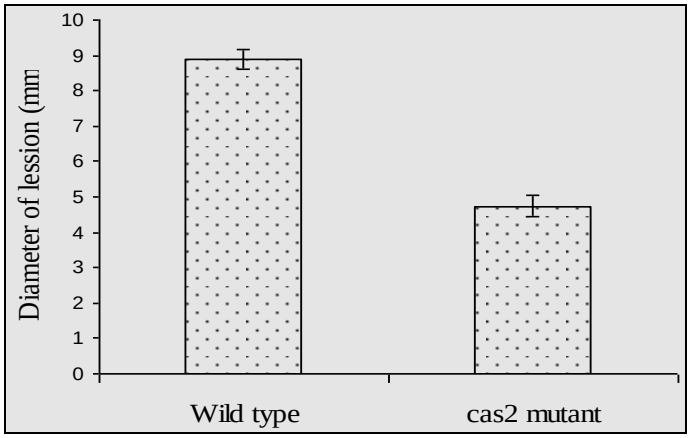


**Figure 6.** Disease severity of mango inoculated with the wild type and the *Cgcas2* mutant of *C. gloeosporioides*.

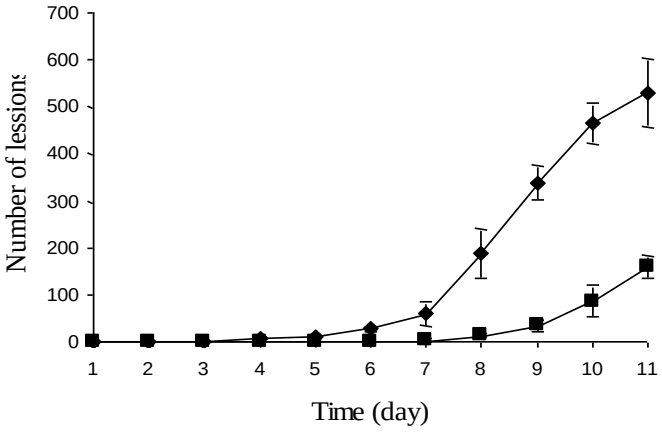


**Figure 7.** Disease severity of mango inoculated with the wild type (♦) and the *Cgcas2* mutant (■) *C. gloeosporioides*.

*CgCAS2* is present as a single copy gene in *C. gloeosporioides* genome and is uniquely expressed in the appressoria [4]. No *CgCAS2* transcripts were detected in other growth stages including conidia, germ tubes and mycelia. Comparison of CgCAS2 protein sequence with known proteins from other organisms showed that it has similarities with hypothetical proteins from several fungal species including *C. globosum, A. niger* and *M. grisea*. *CgCAS2* sequence contains putative casein kinase II phosphorylation site, glycosaminoglycan attachment site, protein kinase C phosphorylation site and short-chain dehydrogenases/reductases. The presence of the putative kinase-dependent phosphorylation motifs in CgCAS2 and the importance of kinase signaling in *C. gloeosporioides* infection pathway signify a possible role of this protein in plant infection process [16], a putative epidermal growth factor (EGF)-like domain signature, which is a receptor for soluble growth factors like ECM. Furthermore, the HMM analysis predicted that *CgCAS2* is composed of five alpha-helical transmembrane domains with the N-terminus located outside of the membrane and the C-terminus inside of the membrane. This prediction strongly suggests that *CgCAS2* could be a cell receptor in the *C. gloeosporioides* appressorium.

In higher eukaryotes, integrins use bidirectional signaling to integrate the intracellular and extracellular environments [17]. For incoming signals, ligand binding activates intracellular signaling pathways. For outgoing signals, signals received by other receptors of neuronal cells activate intracellular signaling pathways that impinge on integrin cytoplasmic domains, and make the extracellular domain competent for ligand binding on a time-scale of less than 1 second [18] family of small integral membrane proteins, shows close association with integrins [19] were first identified in mammals and play a major role in cell shape remodelling including migration, cell-to-cell contact, and motility/invasion during diverse biological processes, such as parasite entry, sperm-egg fusion, B-cell/T-cell contact, neuron out-growth, and metastasis [15] st tetraspanin gene identified was in *M. grisea* and named as *PLS1* [21] mologous to *PLS1* have been identified in other ascomycetes [16-17] are reduced in virulence [23]. In this study, *Cgcas2* mutant was also significantly reduced in virulence and did not produce typical anthracnose symptoms, but instead produced small brown lesions that are low in abundance. A simple hypothesis can be proposed, whereby mutant conidia consume more energy to produce multidirectional germination than unipolar germination, which in turn lower the mechanical force and reduce available enzymes for penetration.

Such mechanism was observed in *Cgpkac* mutant, which has reduced pathogenicity towards unwounded fruits due to the failure of appressoria to penetrate intact fruit skins, as cAMP-PKA signaling pathway is involved in the degradation of lipid bodies to generate turgor pressure. However, the effect of the deletion in the *Cgcas2* mutant with regards to the reduction in pathogenicity in wounded fruits is not well understood. It is postulated that the deletion of the *CgCAS2* resulted in high levels of phosphodiesterase and low levels of cAMP, which causes the inactivation of cAMP-PKA signaling pathway. In addition, the cAMP-PKA pathway regulates appressoria related cell wall degrading enzymes. Inactivation of cAMP-PKA pathway by deletion of *CgCAS2* may have resulted in the mutant being unable to produce cell wall degrading enzyme. More work is needed to determine whether CgCAS2 is involved in the cAMP signal cascade that regulates parasitic-saprophytic switching mode. To resolve this, it is important to detect the intracellular cAMP level and phosphodiesterase activity in *Cgcas2* mutant compared to the wild type.

## 4. Conclusions

*CgCAS2* may be a cell receptor in the *C. gloeosporioides* appressorium involved bidirectional signaling to integrate the intracellular and extracellular environments. *CgCAS2* plays an important role in the coordination of cellular processes required for pathogenic and saprophytic development in *C. gloeosporioides* appressoria.

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## 6. References

[1] Hamer J, Howard R, Chumley F and Valent B 1988 A mechanism for surface attachment in spores of a plant pathogenic fungus *Science* **239** 288–90
[2] Perfect S, Hughes H, O'Connell R and Green J 1999 *Colletotrichum*, a model genus for studies on pathology and fungal–plant interactions *Fungal Gen. Biol.* **27** 186–98
[3] Balhadère P and Talbot N 2001 *PDE1* encodes a P-Type ATPase involved in appressorium-mediated plant infection by the rice blast fungus *Magnaporthe grisea Plant Cell* **13** 1987–2004
[4] Redzuan R A 2007 *Pengenalpastian Gen yang Terlibat dalam Pembentukan Appressorium Colletotrichum gloeosporioides dan Pencirian CAS1, Gen Khusus Appressorium* (Bangi: Universiti Kebangsaan Malaysia)
[5] Hwang C S and Kolattukudy P E 1995 Isolation and characterization of genes expressed uniquely during appressorium formation by *Colletotrichum gloeosporioides* conidia induced by the host surface wax *Mol. Gen. Genetics* **247** 282–94
[6] Xuei X, Bhairi S, Staples R C and Yoder O C 1992 Characterization of *INF56*, a gene expressed during infection structure development of *Uromyces appendiculatus Gene* **110** 49–55
[7] Lee Y and Dean R 1993 cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea Plant Cell* **5** 693–700
[8] Talbot N J, Kershaw M J, Wakley G E, Vries O De, Wessels J and Hamer J E 1996 *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea Plant Cell* **8** 985–99
[9] DeZwaan T, Carroll A, Valent B and Sweigard J 1999 *Magnaporthe grisea* Pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues *Plant Cell* **11** 2013–30
[10] Pich U and Schubert I 1993 Midiprep method for isolation of DNA from plants with a high content of polyphenolics *Nucleic Acids Res.* **21** 3328
[11] Rodriguez R and Redman R 1992 Molecular transformation and genome analysis of *Colletotrichum Colletotrichum, Biology, Pathology and Control* ed J Bailey and M Jeger pp 47–66
[12] Sambrook J and Russel D 2001 *Molecular Cloning: A Loboratory Manual* (Cold Spring Harbor Lab Press)
[13] Kim Y, Lee H, Ko M, Song C, Bae C, Lee Y and Oh B 2001 Inhibition of fungal appressorium formation by pepper (*Capsicum annuum*) esterase *Mol. Plant Microbe Interact.* **14** 80–5
[14] Ballance D J 2017 Transformation systems for filamentous fungi and an overview of fungal gene structure ed S A Leong and R M Berka *Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi* 1–29
[15] Wolfgang W, Kremmer M F, Förster E, Winnacker R and Ludwig E 1997 Identification of Pirin, a novel highly conserved nuclear protein *J. Biol. Chem.* **272** 8482–9
[16] Gupta A and Chattoo B B 2007 A novel gene *MGA1* is required for appressorium formation in *Magnaporthe grisea Fungal Genet. Biol.* **44** 1157–69
[17] Takagi J, Petre B, Walz T and Springer T 2002 Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling *Cell* **110** 511–99
[18] Shimaoka M, Takagi J and Springer T A 2002 Conformational regulation of integrin structure and function *Annu. Rev. Biophys. Biomol. Struct.* **31** 485–516
[19] Hemler M E 2003 Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain *Annu. Rev. Cell Dev. Biol.* **19** 397–422

[20] Stipp C S, Kolesnikova T V and Hemler M E 2003 Functional domains in tetraspanin proteins *Trends Biochem. Sci.* **28** 106–12
[21] Clergeot P H, Gourgues M, Cots J, Laurans F, Latorse M P, Pepin R, Tharreau D, Notteghem J L and Lebrun M H 2001 *PLS1*, a gene encoding a tetraspanin-like protein is required for penetration of host leaves by the rice blast fungus, *Magnaporthe grisea Proc. Nat. Acad. Sci. U. S. A.* **98** 6963–8
[22] Veneault-Fourrey C, Lambou K and Lebrun M H 2006 Fungal Pls1 tetraspanins as key factors of penetration into host plants: a role in re-establishing polarized growth in the appressorium? *FEMS Microbiol. Lett.* **256** 179–84
[23] Barhoom S and Sharon A 2004 cAMP regulation of “pathogenic” and “saprophytic” fungal spore germination *Fungal Genet. Biol.* **41** 317–26