Cgl-SLT2 is required for appressorium formation, sporulation and pathogenicity in Colletotrichum gloeosporioides

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Abstract

The mitogen-activated protein (MAP) kinase pathways has been implicated in the pathogenicity of various pathogenic fungi and plays important roles in regulating pathogenicity-related morphogenesis. This work describes the isolation and characterization of MAP kinase gene, Cgl-SLT2, from Colletotrichum gloeosporioides. A DNA sequence, including 1,633 bp of Cgl-SLT2 open-reading frame and its promoter and terminator regions, was isolated via DNA walking and cloned. To analyze gene function, a gene disruption cassette containing hygromycin-resistant gene was constructed, and Cgl-SLT2 was inactivated via gene deletion. Analysis on Cgl-slt2 mutant revealed a defect in vegetative growth and sporulation as compared to the wild-type strain. When grown under nutrient-limiting conditions, hyperbranched hyphal morphology was observed in the mutant. Conidia induction for germination on rubber wax-coated hard surfaces revealed no differences in the percentage of conidial germination between the wild-type and Cgl-slt2 mutant. However, the percentage of appressorium formation in the mutant was greatly reduced. Bipolar germination in the mutant was higher than in the wild-type at 8-h post-induction. A pathogenicity assay revealed that the mutant was unable to infect either wounded or unwounded mangoes. These results suggest that the Cgl-SLT2 MAP kinase is required for C. gloeosporioides conidiation, polarized growth, appressorium formation and pathogenicity.

Key words: Colletotrichum gloeosporioides, MAP kinase, pathogenicity, appressorium.

Introduction

Phytopathogenic fungi employ various techniques to overcome plant barriers to successfully colonize hosts. Some fungi penetrate intact host surfaces, whereas others enter the host via a wound or natural opening (Prusky and Lichter, 2008). To breach host surfaces, a vital fungal sensing mechanism relays information regarding the surrounding environment. A family serine/threonine protein kinases, known as mitogen-activated protein (MAP) kinases, is involved in transducing signals from plant surfaces in response to physical and chemical stimuli, inducing differentiation processes in fungi (Xu, 2000).

Colletotrichum gloeosporioides has been implicated in vast host losses due to the ability of the species to form latent infections in the host, in which symptoms are not visible until after the climacteric period of the fruit begins (Prusky and Lichter, 2008). C. gloeosporioides infects an extensive range of tropical and subtropical crops, such as papaya (Carica papaya), mango (Mangifera indica), avocado (Persea americana) and rubber (Hevea brasiliensis). The disease severity is attributed to the ability of C. gloeosporioides to infect during different stages of plant growth, including flowering stages, mature plants and fruiting stages.

When conidia encounter plant surfaces, Colletotrichum sp. will attach and germinate, forming a
germ tube that subsequently forms an appressorium at the terminal end. The appressorium is an infective structure that produces a penetration peg, which can penetrate the plant epidermis, leading to the formation of primary and secondary hyphae (Prusky and Lichter, 2008). Subsequently, an acervulus is formed at the infected tissue, characterized by orange to pink conidia masses formed in lesions (Arauz, 2000). Appressorium development is a complex process involving various signals, including physical and chemical stimuli. In the plant fungal pathogen model Magnaporthe oryzae (formerly known as M. grisea), appressorium formation requires hydrophobic surface induction (Ebbole, 2007). In C. gloeosporioides, appressorium differentiation is triggered by hard surface contact, host wax, ammonium accumulation and a fruit ripening hormone, such as ethylene (Kim et al., 2002; Miyata et al., 2010; Priyatno et al., 2012). In the rust fungus Uromyces appendiculatus, surface topography, such as ridges, has been reported to induce appressorium formation (Kumamoto, 2008). In M. oryzae and Colletotrichum sp., appressorium melanization is required for function (Wang et al., 2005). The process of melanization inside the appressorium acts as a semi-permeable barrier, allowing water molecules but not ions and small molecules to diffuse through (Ebbole, 2007). Carbohydrate and lipid breakdown results in glycerol accumulation, and to support the high glycerol content, water from the surrounding environment moves into the appressorium, generating a high turgor pressure (Wang et al., 2005).

The MAP kinase cascade consists of three conserved kinases: MAP kinase kinase (MEKK), MAP kinase kinase (MEK) and MAP kinase (MAPK) (Xu, 2000). In the model yeast Saccharomyces cerevisiae, the MAP kinase cascade regulates mating (Fus3), filamentation (Kss1), high-osmolarity growth (Hog1), cell integrity (Slt2) and spore wall assembly (Smk1) (Xu, 2000). In M. oryzae, the inactivation of PMK1, which is functionally related to S. cerevisiae FUS3, yields defects in both appressorium formation and invasive growth in plants (Xu and Hamer, 1996). Subsequent studies have shown that Colletotrichum orbiculare (formerly known as C. lagenarium) CMK1, which is related to the PMK1 gene, also regulates appressorium morphogenesis and pathogenicity. The conserved role of both MAP kinases Pmk1 and Cmk1 suggests that a general signaling pathway regulates appressorium morphogenesis (Takano et al., 2000). Other well-characterized FUS3 MAP kinase homologs include Claviceps purpurea CPMK1, Cochliobolus heterostrophus CHK1 and Fusarium oxysporum FMK1, which are all responsible for pathogenesis in their respective host plants (Lev et al., 1999; Di Pietro et al., 2001; Mey et al., 2002b). Besides that functional characterization of the SLT2-related MAP kinase has been carried out for several phytopathogenic fungi such as M. oryzae MPS1, C. orbiculare MAF1, Mycosphaerella graminicola MgSLT2 and C. purpurea CPMK2 (Xu et al., 1998; Kojima et al., 2002; Mey et al., 2002a; Mehrabi et al., 2006). Generally, Slt2 homologs in these phytopathogenic fungi are required for invasive growth in the host and conidiation. Some phytopathogenic fungi with mutations in this gene display a heightened sensitivity towards a cell wall-degrading enzyme, indicating that these genes participate in cell wall integrity maintenance (Mey et al., 2002a; Mehrabi et al., 2006).

In this report, we isolated Cgl-SLT2, a homolog of S. cerevisiae SLT2, and investigated its function by generating a knockout mutant of C. gloeosporioides.

Materials and Methods

Strains and culture conditions

C. gloeosporioides strain PeuB was obtained from the stock culture collection of the Molecular Mycology Laboratory, Universiti Kebangsaan Malaysia and used as the wild-type strain throughout this work. The wild-type and mutant strains were grown on potato dextrose agar (PDA) (Difco, USA) at 30 °C for all initial work. Wild-type and mutant strains were maintained on PDA supplemented with 1 M sorbitol (Sigma, USA) for all assays performed, unless otherwise stated. Hygromycin B-resistant mutants were selected on PDA supplemented with 300 µg/mL hygromycin B (Bio Basic, Canada). Fungal mycelia were harvested from 3-day-old cultures grown in PDYE potato dextrose broth supplemented with 3% yeast extract and used for genomic DNA extraction.

Genomic DNA isolation

Genomic DNA was isolated using two different methods. For general molecular biology manipulation, total DNA of C. gloeosporioides was isolated using polyvinylpyrrolidone (PVP) as described by Oh et al. (2009). Genomic DNA for screening fungal transformants via PCR was isolated as follows. Fungal mycelia (0.1 g) grown in PDYE were transferred into 2 mL microcentrifuge tubes containing 450 µL of extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 1% SDS), 200 µL glass beads and 50 µL of 10% SDS. Mycelial cells were vortexed vigorously for 10 min and placed on ice for 15 min. This step was repeated once, and 400 µL phenol:chloroform was then added. After centrifugation at 13,000 rpm, the aqueous phase was transferred to a new microcentrifuge tube, and 1/10 volume of 3 M sodium acetate was added. The mixture was placed at -80 °C for 30 min before centrifugation at 13,000 rpm. The pellet was washed with 70% ethanol, dissolved in 20 µL of dH2O and stored at -20 °C.

Isolation of the Cgl-SLT2 gene

Cgl-SLT2 was isolated using the parMAFF and parMAFR primers (Table 1), which were designed to flank
the conserved region (subdomain IX to subdomain XI) of the gene based on the MAF1 sequence from C. orbiculare. From the obtained partial sequence, three target-specific primers (TSPs), designated TSP1-1, TSP2-1 and TSP3-1 (Table 1), were designed for the first round of DNA walking (DNA Walking SpeedUp™ Premix Kit, Seegene, Korea). After amplification, three additional target-specific primers were designed (TSP 1-2, TSP 2-2 and TSP 3-2; Table 1) based on the new sequence obtained. Three additional sets of target-specific primers (TSP1-3, TSP2-3 and TSP3-3; Table 1) were designed for downstream DNA walking from the partial Cgl-SLT2 sequence to amplify the downstream region. Cgl-Slt2 amino acids were aligned with amino acids of C. orbiculare Maf1 (AAC63682) and S. cerevisiae Slt2 (AAB68912) using the CLUSTALW program (Thompson et al., 1994).

Isolation of RNA and Cgl-SLT2 cDNA synthesis

Total RNA was extracted from C. gloeosporioides conidia using TRIzol® (Invitrogen, USA) according to the manufacturer’s instructions. A total of 5 μg total RNA were used as the template for cDNA synthesis with oligo(dT) primers using the SuperScript first-strand synthesis system (Invitrogen, USA) according to the manufacturer’s instructions. The cDNA synthesis was performed with reaction conditions consisting of 1 cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 15 min.

Construction of the gene disruption cassette

The gene disruption cassette was constructed using the plasmid pN1389, which contains the hygromycin resistance cassette driven by the Aspergillus niger glucoamylase promoter (Priyatno et al., 2012). Approximately 660 bp at the 5’ region of the gene was PCR-amplified using the primers 5F-MAFkpn and 5R-MAFbam (Table 1), which contained KpnI and BamHI sites, respectively. The sequence at the 3’ region of the gene was PCR-amplified using the primers 3F-MAFsd1 and 3R-MAFsph (Table 1), which contained SdaI and SphI restriction enzyme sites, respectively. PCR-amplified fragments were ligated into the pGEMT-Easy vector and transformed into E. coli DH5α. The 3’ fragments were digested and ligated into the SdaI and SphI sites of pN1389 followed by the 5’ fragments at the KpnI and BamHI sites to generate pGDMAF1.

Table 1 - Oligonucleotide primers used in this study.

| Name       | Description                              | Sequence                  |
|------------|------------------------------------------|---------------------------|
| parMAFF    | Forward primer for partial Cgl-SLT2 amplification | 5’ CGTGGATGATCATCTTGGC 3’ |
| parMAFR    | Reverse primer for partial Cgl-SLT2 amplification | 5’ GGCAACGAGCGGAAGCGGTAG 3’ |
| TSP1-1     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ CAAGTTTGAGAGCTACTC 3’ |
| TSP2-1     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ GAGGATGGAGGATCTGGTTC 3’ |
| TSP3-1     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ GATCTGGTTCAGCTGGTCAGTAG 3’ |
| TSP1-2     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ AGGATGAGGGGAAGAGAG 3’ |
| TSP2-2     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ AATGCCGTAAGCTCCCTGG 3’ |
| TSP3-2     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ CTGCTGCCGAGTAAGCTCCTG 3’ |
| TSP1-3     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ CCCCTCAAGCGGTATCA 3’ |
| TSP2-3     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ GAGCACCCCTACCTTCATCT 3’ |
| TSP3-3     | Target-specific primer used in DNA walking for Cgl-SLT2 isolation | 5’ GGTGTTCGAGGATGTCGGTAGATG 3’ |
| 5F-MAFkpn  | Forward primer for amplification of 5’ flanking region | 5’ CGTGATCATCTTGCCATCCATC 3’ |
| 5R-MAFbam  | Reverse primer for amplification of 5’ flanking region | 5’ GGTACGATATTGGCCGCTTTTG 3’ |
| 3F-MAFsd1  | Forward primer for amplification of 3’ flanking region | 5’ TACCTGCGAGATTCTCCTGG 3’ |
| 3R-MAFsph  | Reverse primer for amplification of 3’ flanking region | 5’ TCGATCGACGGCGAAAGGG 3’ |
| ItgF       | Forward primer for PCR screening of transformants | 5’ GCGGCGTCTATCTAACTTAGTTACTG 3’ |
| fMAFR      | Reverse primer for PCR screening of transformants | 5’ TCTGCTGCGATGAAAGCTCCTG 3’ |
| cDNAF      | Forward primer for reverse transcription PCR of Cgl-SLT2 | 5’ CAAGGGAGCGAAAGGGTCTTACAG 3’ |
| cDNAR      | Reverse primer for reverse transcription PCR of Cgl-SLT2 | 5’ TTACTCTTCTCGCCATCATAGTCCG 3’ |
| GPDF       | Forward primer for reverse transcription PCR of GPD | 5’ ATGCTCCACAGAGTCCG 3’ |
| GPDR       | Reverse primer for reverse transcription PCR of GPD | 5’ TTACTTGGAGGCACTGACCTTG 3’ |
Fungal transformation and screening

Fungal spheroplasts were generated using the method described previously (Rodriguez and Redman, 1992). Fungal protoplasts were transformed using 20 μg of linearized plasmid pGDMAF1 via PEG-mediated spheroplasts transformation. Transformants were grown on PDA supplemented with 300 μg/mL hygromycin B, and putative positive transformants were screened via PCR. A total of 1 μL of genomic DNA were used as the template in PCR to screen for positive transformants using the primers ItgF and flMAFR (Table 1). PCR was performed in a 20 μL reaction containing 1 μL of genomic DNA (300 ng), 1 μL of 2.5 mM dNTPs, 2 μL of 10X PCR buffer, 1 μL of 20 μM of each primer, 1 unit of Taq polymerase and 1.5 μL of 25 mM MgCl₂, and the conditions consisted of one cycle of denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 90 s, 60 °C for 2 min and 72 °C for 30 s and a final elongation step at 72 °C for 15 min.

Southern blot analysis

A total of 20 μg genomic DNA was digested and gel-fractionated according to standard molecular methods. For copy number determination, 20 μg of genomic DNA was digested using the restriction enzymes EcoRI, HindIII, XbaI and XhoI. For verifying transformants, genomic DNA was digested with XhoI only. A 1 kb span of the ORF sequence was used as a DNA probe and labeled using [α-32P]dCTP with the Ready-To-Go DNA Labeling Beads kit (-dCTP) according to the manufacturer’s instructions (Amersham, USA). Hybridization was performed as described previously (Kamaruddin et al., 2008).

Wild-type and mutant Cgl-slt2 expression analysis

The presence of a Cgl-SLT2 transcript in the wild-type and the Cgl-slt2 mutant was assessed via reverse transcription PCR using the primer pairs CDNAF and cDNAAR (Table 1). As a control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GPD) was amplified from both wild-type and the mutant C. gloeosporioides transcript in the wild-type and the Cgl-slt2 mutant RNA using the primer pairs GPDF and GPDR (Table 1). The reverse transcription PCR conditions were similar to those described for the amplification of Cgl-SLT2 cDNA.

Vegetative growth, conidiation, appressorium formation and hyphal morphology observation

Vegetative growth was measured on PDA after 6 days in triplicate on petri dish. Conidiation potential was measured by counting conidia from 7-day-old plate cultures grown on PDA alone or PDA with 1 M sorbitol. Conidia were harvested in 10 mL of sterile distilled water and quantified using a hemacytometer under a light microscope. Appressorium formation induction from conidia harvested from PDA and PDA with 1 M sorbitol was performed using wax that was extracted from rubber leaves and coated onto glass slides (Priyatno et al., 2012). A spore suspension (10⁵ spores/mL) was dropped onto glass slides and incubated in a humid environment for 8 h to induce appressorium formation. After 8 h, the percentage of appressoria was quantified. Hyphal morphology was observed by transferring agar plugs containing mycelia from PDA to 1.5% (w/v) bacteriological agar, incubating at 30 °C for 3 days and observing them under light microscope.

Pathogenicity assay

Pathogenicity assays were performed as previously described (Priyatno et al., 2012). Prior to the pathogenicity assay, healthy mangoes were surface-sterilized with 70% ethanol. Conidia were harvested from 7-day-old cultures grown on PDA with 1 M sorbitol using sterile distilled water. Conidial suspensions were adjusted to a final concentration of 5 x 10⁴ spores/mL and spray-inoculated onto mangoes. Inoculated mangoes were stored in a humid chamber and incubated at 30 °C for up to 10 days. To inoculate fungal conidia into wounded fruits, sterile needles were used to puncture the fruit surface. Subsequently, 25 μL of a 10⁵ spores/mL spore suspension was dropped onto the wounded site. The same amount of spore suspension was dropped onto an adjacent unwounded site of the same fruit as a control. Five replicates were used in pathogenicity assays.

Results

Identification of the Cgl-SLT2-encoding gene

Cgl-SLT2 including the promoter and terminator region was successfully isolated via PCR amplification and DNA walking. In addition, total RNA isolated from C. gloeosporioides conidia was used as a template for reverse transcription PCR to clone Cgl-SLT2 cDNA. Alignment between the cDNA and the gene revealed a 1,633 bp Cgl-SLT2 ORF spanning four introns that encodes a putative 420 amino acid protein. The sequence was submitted to GenBank under the accession number JQ322774. Cgl-Slt2 harbored all of the 11 conserved protein kinase subdomains and the TEY sequence, a site for threonine and tyrosine phosphorylation required for kinase activation (Figure 1). The deduced amino acid sequence exhibited 96% identity to C. orbiculare Maf1 and 68% identity to S. cerevisiae Slt2.

Cgl-SLT2 gene disruption

Before constructing the gene disruption cassette, the Cgl-SLT2 copy number was determined. Southern blotting showed that the gene was present as a single copy in the genome (Figure 2A). A gene replacement vector pGDMAF1 was constructed and transformed into C. gloeosporioides PeuB spheroplasts. Nine positive transformants were obtained on PDA supplemented with 300 μg/mL hygromycin
B. PCR-based screening was employed to detect transformants containing the hygromycin-resistant gene cassette integrated at the targeted site in the *C. gloeosporioides* genome. Transformants that yielded the appropriate product during PCR screening were designated *Cgl-slt2* mutants. Genomic DNA was isolated from the wild-type and the *Cgl-slt2* mutant, and a single copy with no ectopic integration was observed using genomic DNA blot analysis using a 1.1 kb probe from the ORF of the gene (Figure 2B). An increase of approximately 1.9 kb corresponded to the hygromycin cassette that was integrated into the ORF was observed (Figure 2C). Reverse transcription PCR analysis revealed the absence of *Cgl-SLT2* transcripts in the mutant, indicating a successful inactivation of the gene’s coding region (Figure 2D). This mutant colony was distinct from the wild-type strain and showed less dense aerial mycelial growth.

### Cgl-Slt2 Hyphal Morphology and Conidiation

The *Cgl-slt2* mutant grown on minimal medium (1.5% w/v bacteriological agar) displayed hyperbranched hyphal morphology (Figure 3). Under nutritional stress, the mutant strain was unable to form radial colonies, indicating a defect in polarized growth. When conidia were harvested by scraping the mycelial mat of the *Cgl-slt2* mutant from PDA plates, a high hyphal fragmentation incidence was observed, which is similar to the autolysis phenotype in fungi. However, this phenotype was ameliorated with the addition of 1 M sorbitol to the culture plate (data not shown). Hyphal fragmentation indicated a low tensile strength of the *Cgl-slt2* mutant cell wall. Conidiation of the *Cgl-slt2* mutant

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**Figure 1** - The amino acid sequence alignment of Cgl-Slt2 of *C. gloeosporioides* (JQ322774), Maf1 of *C. orbiculare* (AAL50116), Mps1 of *M. oryzae* (AAC63682) and Slt2 of *S. cerevisiae* (AA86912). Sequence alignment was performed using CLUSTALW. Identical amino acids are indicated by white letters on a black background. Similar residues are indicated by a gray background. Gaps introduced for alignment are indicated by hyphens. The protein kinase subdomains are indicated by the Roman numerals above the sequences. The protein kinase activation sequence TEY is indicated by asterisks.
grown on PDA after 7 days of culture was significantly reduced at $1.48 \pm 0.27 \times 10^4$ spores/mL compared to the wild-type at $2.98 \pm 0.21 \times 10^7$ spores/mL (Table 2). Conidiation for the Cgl-slt2 mutant was increased to $4 \pm 0.28 \times 10^6$ spores/mL by the addition of 1 M sorbitol to the culture medium.

**Cgl-SLT2 and appressorium**

The conidia of Cgl-slt2 mutants exhibited normal morphology and germination frequency when harvested from PDA plates supplemented with 1 M sorbitol. However, the Cgl-slt2 mutant exhibited a different style of conidial germination on a hard surface coated with plant wax compared to the wild-type conidia. On an inductive surface, approximately 89% of the wild-type conidia underwent unipolar germination, whereas 72% of the Cgl-slt2 conidia underwent bipolar germination at 8-h post-induction. In addition, mature appressorium formation was absent in the Cgl-slt2 strain (Figure 4B) compared to the wild-type strain (Figure 4A). The germ tube swelled to form an appressorium, but the size of the appressorium was much smaller than the wild-type. In addition, melanization was greatly reduced in Cgl-slt2 appressoria (Figure 4B). After 8 h, approximately 46% of the mutant germlings...
formed an appressorium-like swelling at the terminal end of the germ tube, whereas approximately 90% of the wild-type germ tubes produced mature melanized appressoria. For conidia harvested from PDA plates without 1 M sorbitol, the wild-type formed normal mature appressoria at 8-h post-induction (Figure 4C), whereas the mutant was unable to produce appressoria but formed the germ tube at both conidial polar ends (Figure 4D).

**CgL-SLT2 and fungal pathogenicity**

To investigate the pathogenicity of the CgL-slt2 strains, mutant and wild-type conidia were harvested from PDA supplemented with 1 M sorbitol and analyzed using

**Figure 4 - Appressorium formation in wild-type and CgL-slt2 strains.** Appressorium formation induced from conidia harvested from *C. gloeosporioides* grown on PDA supplemented with 1 M sorbitol in wild-type (a) and CgL-slt2 strains (b) Appressorium formation induced from conidia harvested from *C. gloeosporioides* grown on PDA alone in wild-type (c) and CgL-slt2 strains (d). The CgL-slt2 mutant showed an impaired ability to form mature appressoria, and the melanization of appressoria was not observed. The image was captured using an Olympus light microscope under 200X magnification. (a: appressorium; gt: germ tube; c: conidium; sg: second germ tube). The scale bar indicates 10 μm.

**Table 2 - Comparison of wild-type *C. gloeosporioides* and CgL-slt2 mutant characteristics.**

| Strain      | Vegetative growth (cm)³ | Conidiation (per mL)⁴ | Germination (%)² | Appressorium formation (%)² | Bipolar germination (%)² |
|-------------|--------------------------|------------------------|-----------------|------------------------------|--------------------------|
| Wild-type   | 8.5 ± 0.1                | 2.98 ± 0.21 x10⁷       | 93.3 ± 2.3      | 90.6 ± 1.15                  | 11.3 ± 5.5               |
| CgL-slt2    | 6.7 ± 0.08               | 1.48 ± 0.27 x10⁴       | 92.3 ± 5.0      | 46 ± 1                       | 72.3 ± 4.5               |

³Diameter of colonies grown on potato dextrose agar at 30 °C for 6 days. ⁴Conidia harvested from 7-day-old cultures. ²Percentage of conidia germinated after induction on glass slides coated with rubber wax at 30 °C for 8 h. The mean and standard deviation were obtained from three independent experiments. ⁴Percentage of appressorium formation after induction on a glass surface coated with rubber wax at 30 °C for 8 h. The mean and standard deviation were obtained from three independent experiments. ²Percentage of bipolar germination 8-h post-induction on a glass surface coated with rubber wax at 30 °C. The mean and standard deviation were obtained from three independent experiments.

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**Figure 3 - Light microscopy images of CgL-slt2 (a) and wild-type strains (b) on minimal media agar.** The CgL-slt2 mutant exhibited increased hyphal branching compared to the wild-type. The image was captured using an Olympus light microscope under 100X magnification. The scale bar indicates 50 μm.
an infection assay. The wild-type strain induced extensive lesion formation on the host, indicating that the strain is virulent towards its host, mangoes. However, the mutant strain failed to generate lesions on the host (Figure 5A). When 25 μL of spore suspension was spotted onto the surface of intact mangoes, lesions were also absent with the mutant. To investigate whether the inability to infect intact fruits was due to abnormal appressorium formation thus, prevent a breach in the fruit surface, drops of spore suspension were spotted onto the surface of wounded mangoes, which allowed for direct entry into the fruits. However, the Cgl-slt2 mutant remained unable to infect wounded fruits, whereas the wild-type strain formed lesions typical of anthracnose symptoms (Figure 5B).

**Discussion**

In this study, *C. gloeosporioides* Cgl-SLT2, a homolog of SLT2 MAP kinase, which regulates cell wall integrity and polarized growth in *S. cerevisiae*, was isolated and characterized. In *C. orbiculare*, MAF1 is predominantly required for an early appressorium differentiation phase, and a maf1 mutant fails to form appressoria on glass slides (Kojima et al., 2002). In contrast, MPS1, a homolog of SLT2 in *M. oryzae*, is not required for appressorium formation but plays a pivotal role in the penetration of plant surfaces (Xu et al., 1998). Interestingly, *M. graminicola* MgSLT2, a homolog of SLT2, is dispensable for penetration but essential for invasive growth in the host (Mehrabi et al., 2006). In this study, Cgl-SLT2 was shown to regulate normal appressorium formation and invasive growth in the host. These results indicate that although homologs in *C. orbiculare*, *M. oryzae*, *M. graminicola* and *C. gloeosporioides* regulate pathogenicity, the group of genes that they regulate could vary between different species.

The vegetative Cgl-slt2 mutant growth rate on plates was lower than wild-type. In contrast, the *C. orbiculare* maf1 mutant has been shown to exhibit a slightly higher vegetative growth rate compared to wild-type (Kojima et al., 2002), and the *M. oryzae* mps1 mutant has been reported to exhibit an identical vegetative growth rate as wild-type (Xu et al., 1998).

Conidia are an etiological agent of anthracnose disease in the field caused by *Colletotrichum* sp. Thus, conidiation ability is vital for these phytopathogenic fungi. The Cgl-slt2 mutant produced significantly less conidia compared to wild-type, which has also been observed in *M. oryzae*, *C. orbiculare* and *C. purpurea* (Xu et al., 1998; Kojima et al., 2002; Mey et al., 2002a). In the *M. oryzae* mps1 mutant, the sporulation defect was shown to be due to the limited ability to form normal aerial hyphae, and this anomaly was also observed in the Cgl-slt2 mutant. Sporulation was enhanced in the Cgl-slt2 mutant with the addition of 1 M sorbitol, which has also been reported for the *M. oryzae* mps1 mutant. Interestingly, the mps1 mutant underwent progressive autolysis radiating from the central part of the colony, which was rescued through the addition of 1 M sorbitol (Xu et al., 1998). A detailed investigation of the vegetative properties of the *M. graminicola* IPO323AMgSlt2 mutant also revealed that it underwent autolysis 11-days post-inoculation (Mehrabi et al., 2006).

In this study, autolysis-like phenotypes, including a high incidence of hyphal fragmentation, were also observed in the Cgl-slt2 mutant and were suppressed in the presence of 1 M sorbitol. A similar lytic phenotype was observed in *S. cerevisiae* slt2 mutants, in which a temperature-dependent lytic phenotype was observed at a restrictive temperature of 37 °C and suppressed with the addition of 1 M sorbitol (Martyn et al., 2000). These data indicate that the regulation of this gene’s morphogenetic pathway is similar in the presence of an osmostabilant, and this regulation may be conserved between yeasts and filamentous fungi.

Under nutrient-limiting conditions, Cgl-slt2 mutants displayed hyperbranched hyphal morphology, which was not observed in the wild-type strain. *Neurospora crassa* mutants with hyperbranched hyphal morphology have been suggested to lack the ability to establish polarized growth

![Figure 5](image)

*Figure 5* - A pathogenic assay with wild-type and Cgl-slt2 strains on mangoes. (a) Mangoes were spray-inoculated with a 10⁵ conidia/mL suspension. Images were obtained 10-days post-inoculation. The picture in the left panel is the wild-type strain, the middle panel is the mutant strain (Cgl-slt2) and the right panel is the control (sterile dH₂O). (b) Wounded mangoes were inoculated with 25 μL of a 10⁵ conidia/mL suspension. The picture in the left panel is the wild-type strain, the middle panel is the mutant strain (Cgl-slt2) and the right panel is the control (sterile dH₂O). Cgl-slt2 mutants were unable to infect wounded or unwounded fruits compared to the wild-type.
Cell growth polarity is an important process in filamentous fungi, allowing the efficient colonization and exploitation of new substrata (Harris and Momany, 2004). A study by Mazzoni et al. (1993) showed that Slt2 was also required for polarized growth in S. cerevisiae, and these mutants exhibited defects in actin localization. Additionally, the C. purpurea Cpmk2 mutant, homologous to SLT2, exhibited some hyperbranched hyphae (Mey et al., 2002a). Phenotypic analysis of the M. oryzae mps1 mutant suggested that polarized growth played an important role in penetrating hyphae formation. Since the Cgl-slt2 mutant displayed conditional defects in polarized growth and was unable to infect wounded fruits, we conclude that the Cgl-slt2 mutant is defective in polarized growth, leading to the inability to develop penetrating hyphae.

Interestingly, the Cgl-slt2 mutant also formed abnormal appressoria. Two different MAP kinases have been characterized in C. orbiculare that are involved in different stages of appressorium formation. C. orbiculare Cmk1 participates in appressorium maturation, whereas Maf1 participates in appressorium formation. The Cgl-slt2 mutant was unable to form appressoria when induced on a glass surface coated with rubber wax. However, when the culture medium was supplemented with sorbitol, spores harvested from Cgl-slt2 mutants formed swollen appressorium-like structures, which were smaller and unmelanized, similar to the C. orbiculare cmk1 mutant (Takano et al., 2000). Precise melanization and sizes of the appressorium are crucial during turgor generation within the appressorium structure (Tsuji et al., 2003). These observations suggest that the Cgl-SLT2 MAP kinase participates in the regulation of normal appressorium formation, which is triggered by the presence of an osmostabilant. Based on the analogy of the phenotypic traits observed in C. orbiculare maf1 and cmk1 mutants, the addition of sorbitol to the Cgl-slt2 mutant culture medium could trigger the activation of MAP kinase, which is homologous to the C. gloeosporioides CMK1.

When the pathogenicity assay was performed, the Cgl-slt2 mutant was unable to infect unwounded mangoes. This is likely to be due to the inability of the Cgl-slt2 mutant to produce melanized mature appressoria, which are required to breach the host’s thick cuticle barrier. When a puncture wound was introduced onto the mango’s surface prior to inoculation, Cgl-slt2 was still unable to infect the host, unlike the wild-type, indicating the inability to establish invasive growth in the host. Conversely, the C. orbiculare maf1 mutant is able to invasively grow on wounded cucumber leaves. The high percentage of Cgl-slt2 conidia undergoing bipolar germination may account for this observation. Bipolar germination was termed saprophytic germination by Barhoom and Sharon (2004) when only unidirectional germination resulted in plant infection by C. gloeosporioides. Spores induced to germinate in a bipolar manner and then transferred to plant surfaces developed mycelia on the surface without penetration, indicating that they were not pathogenic. The inability to infect plant hosts coincided with the high incidence of bipolar germination in the Cgl-slt2 strain, consistent with the saprophytic germination pattern.

Alternatively, the inability of Cgl-slt2 to produce anthracnose symptoms on wounded fruits may be due to the hypersensitivity of the mutant to reactive oxygen species produced by the plant upon wounding. Plants produce maximum levels of superoxide within several minutes of wounding and hydrogen peroxide at 4-6 h after wounding (León et al., 2001). A study performed by Alic et al. (2003) showed that the S. cerevisiae slt2 mutant was sensitive to oxidative stress, and this gene was upregulated when the mutant was exposed to linoleic acid hydroperoxide for 5 min, indicating that Slt2 regulates a subset of oxidative stress-responsive genes downstream. Similar roles may be performed by Cgl-Slt2, which may regulate a subset of genes required to protect the fungus against host reactive oxygen species. This hypothesis remains to be tested and verified with well-designed experiments.

Conclusions

The results in this study suggest that MAPK may be involved in the conidiation, appressorium formation, polarized growth and pathogenicity of C. gloeosporioides and that these processes are regulated by nutrients, osmos tabilants and host signals.

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References

Alic N, Higgins VJ, Pichova A, Breitenbach M., Dawes IW (2003) Lipid hydroperoxides activate the mitogen-activated protein kinase Mpk1p in Saccharomyces cerevisiae. J Biol Chem 278(43):41849-41855.

Arauz LF (2000) Mango anthracnose: economic impact and current options for integrated management. Plant Dis 84(6):600-611.

Barhoom S, Sharon A (2004) cAMP regulation of “pathogenic” and “saprophytic” fungal spore germination. Fungal Genet Biol 41(3):317-326.

Di Pietro A, Garcia-Maceira FI, Méglecz E, Ronceiro MIG (2001) A MAP kinase of the vascular wilt fungus Fusarium oxysporum is essential for root penetration and pathogenesis. Mol Microbiol 39(5):1140-1152.
Ebbole DJ (2007) Magnaporthe as a model for understanding host-pathogen interactions. Annu Rev Phytopathol 45(1):437-456.

Harris SD, Momany M (2004) Polarity in filamentous fungi: moving beyond the yeast paradigm. Fungal Genet Biol 41(4):391-400.

Kamaruddin N, Bakar FDA, Mahadi NM, Murad AMA (2008) Isolation and characterisation of a gene encoding the Colletotrichum gloeosporioides regulatory subunit of protein kinase A. J Biol Sci 8:730-737.

Lev S, Sharon A, Hadar R, Ma H, Horwitz BA (1999) A mitogen-activated protein kinase homolog is involved in conidiation, appressorium formation, and pathogenicity: Diverse roles for mitogen-activated protein kinase homologs in fungal pathogens. Proc Natl Acad Sci USA 96(23):13542-13547.

Maztoni, C, Zarov P, Rambourg A, Mann C (1993) The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in Saccharomyces cerevisiae. J Cell Biol 123(6):1821-1833.

Mey G, Held K, Scheffer J, Tenberge K, Tudyynski P (2002a) CPMK2, an SLT2-homologous mitogen-activated-protein (MAP) kinase, is essential for pathogenesis of Claviceps purpurea on rye: evidence for a second conserved pathogenesis-related MAP kinase cascade in phytopathogenic fungi. Mol Microbiol 46:305-318.

Mey G, Oesaer B, Lebrun MH, Tudyynski P (2002b) The biotrophic, non-appressorium-forming grass pathogen Claviceps purpurea needs a Fus3/Pmk1 homologous mitogen-activated protein kinase for colonization of rye ovarian tissue. Mol Plant-Microbe Interact 15(4):303-312.

Miyara I, Shafran H, Davidzon M, Sherman A, Prusky D (2010) pH Regulation of ammonia secretion by Colletotrichum gloeosporioides and its effect on appressorium formation and pathogenicity. Mol Plant-Microbe Interact 23(3):304-316.

Oh SSL, Bakar FDA, Adnan AM, Mahadi NM, Hassan O, Murad AMA (2009) Isolation and Characterisation of glyceroldehyde-3-phosphate dehydrogenase gene of Trichoderma virens UKM1. Biotechnology 8:194-203.

Priyatno T, Bakar FDA, Kamaruddin N, Mahadi NM, Murad AMA (2012) Inactivation of the catalytic subunit of cAMP-dependent protein kinase A causes delayed appressorium formation and reduced pathogenicity of Colletotrichum gloeosporioides. ScientificWorldJournal article ID 545784.

Prusky D, Lichter A (2008) Mechanisms modulating fungal attack in post-harvest pathogen interactions and their control. Eur J Plant Pathol 121: 281-289.

Rodriguez R, Redman R (1992) Molecular transformation and genome analysis of Colletotrichum. In: Bailey, J., Jeger, M. (eds). Colletotrichum: Biology, Pathology and Control. CAB International, Wallingford, UK, 47-66.

Takano Y, Kikuchi T, Kubo Y, Hamer JE, Mise K, Furusawa I (2000) The Colletotrichum lagenarium MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. Mol Plant-Microbe Interact 13(4):374-383.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673-4680.

Tsug S, Tsuge S, Shiraishi T, Kubo Y (2003) Expression pattern of melanin biosynthesis enzymes during infectious morphogenesis of Colletotrichum lagenarium. J Gen Plant Pathol 69(3):169-175.

Vogt N, Seiler S (2008) The RH01-specific GTPase-activating protein LRG1 regulates polar tip growth in parallel to Ndr kinase signaling in Neurospora. Mol Biol Cell 19(11):4554-4569.

Wang Z, Jenkinson J, Holcombe I, Soanes D, Veneault-Forrey C, Bhambra G, Talbot N (2005) The molecular biology of appressorium turgor generation by the rice blast fungus Magnaporthe grisea. Biochem Soc Trans 33:384-388.

Xu JR (2000) MAP kinases in fungal pathogens. Fungal Genet Biol 31(3):137-152.

Xu JR, Hamer JE (1996) MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus Magnaporthe grisea. Genes Dev 10(21):2696-2706.

Xu JR, Staijer CJ, Hamer JE (1998) Inactivation of the mitogen-activated protein kinase Msps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. Proc Natl Acad Sci USA 95(21):12713-12718.

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