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Research Paper

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

H.Y. Yong[1], F.D.A. Bakar[1], R.M. Illias[2], N.M. Mahadi[3], A.M.A. Murad[1]

[1]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia.
[2]Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, Skudai, Johor, Malaysia.
[3]Malaysia Genome Institute, Jalan Bangi, Kajang, Selangor, Malaysia.

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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

Send correspondence to A.M.A. Murad. School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia. E-mail: munir@ukm.my.

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; Miyara *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 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 dH_2O 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 (AAL50116), *M. oryzae* Mps1 (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 cDNAF and cDNAR primer pair (Table 1) was used to amplify *Cgl-SLT2* cDNA. PCR was performed in a 20 μL reaction mixture containing 1 μL of cDNA from each sample, 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 $MgCl_2$. PCR conditions consisted 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 were PCR-amplified using the primers 5F-MAFkpn and 5R-MAFbam (Table 1), which contained *Kpn*I and *Bam*HI sites, respectively. The sequence at the 3' region of the gene was PCR-amplified using the primers 3F-MAFsda and 3R-MAFsph (Table 1), which contained *Sda*I and *Sph*I 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 *Sda*1 and *Sph*1 sites of pN1389 followed by the 5' fragments at the *Kpn*1 and *Bam*HI sites to generate pGDMAF1.

Table 1 - Oligonucleotide primers used in this study.

Name	Description	Sequence
parMAFF	Forward primer for partial *Cgl-SLT2* amplification	5' CGTTGGATGCATCTTGGC 3'
parMAFR	Reverse primer for partial *Cgl-SLT2* amplification	5' GCGAACGAGCGGAAGCGGTAG 3'
TSP1-1	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' CAGGTTGCGGACGTACTC 3'
TSP2-1	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' GAGGATGTGGAGGATCTGGTTC 3'
TSP3-1	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' GATCTGGTTCAGCTGGTCGACGTAG 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' CTCGTCGGCGATGAAGTCCTG 3'
TSP1-3	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' CCCCTCAAGCCGTATCA 3'
TSP2-3	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' GAGCACCCCTACCTTCACATCT 3'
TSP3-3	Target-specific primer used in DNA walking for *Cgl-SLT2* isolation	5' GGTTGTCGAGGATGTCGGTGAGATG 3'
5F-MAFkpn	Forward primer for amplification of 5' flanking region	5' CGGTACCATCCTGCCCATCCCAT 3'
5R-MAFbam	Reverse primer for amplification of 5' flanking region	5' GGATCCCATGATTGCGGCTTTTTG 3'
3F-MAFsda	Forward primer for amplification of 3' flanking region	5' TACCTGCAGGATATTCCCCGACCC 3'
3R-MAFsph	Reverse primer for amplification of 3' flanking region	5' TGCATGCACAGGCTGGGGAAAGG 3'
ItgF	Forward primer for PCR screening of transformants	5' GCGGCCGCTGATCCACTTAACGTTACTG 3'
flMAFR	Reverse primer for PCR screening of transformants	5' CTCGTCGGCGATGAAGTCCTG 3'
cDNAF	Forward primer for reverse transcription PCR of *Cgl-SLT2*	5' CAGGGACGCAAGGTTTTCAAG 3'
cDNAR	Reverse primer for reverse transcription PCR of *Cgl-SLT2*	5' TTACCTTCTGCCATCAAGTCCG 3'
GPDF	Forward primer for reverse transcription PCR of *GPD*	5' ATGGCTCCCATCAAGGTCGG 3'
GPDR	Reverse primer for reverse transcription PCR of *GPD*	5' TTACTTGGAGGCATCGACCTTGG 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 $MgCl_2$, 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 *Eco*RI, *Hin*dIII, *Xba*I and *Xho*I. For verifying transformants, genomic DNA was digested with *Xho*I only. A 1 kb span of the ORF sequence was used as a DNA probe and labeled using [α-^{32}P] 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 cDNAR (Table 1). As a control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GPD*) was amplified from both wild-type *C. gloeosporioides* and *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 (10^5 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 10^4 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 10^5 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

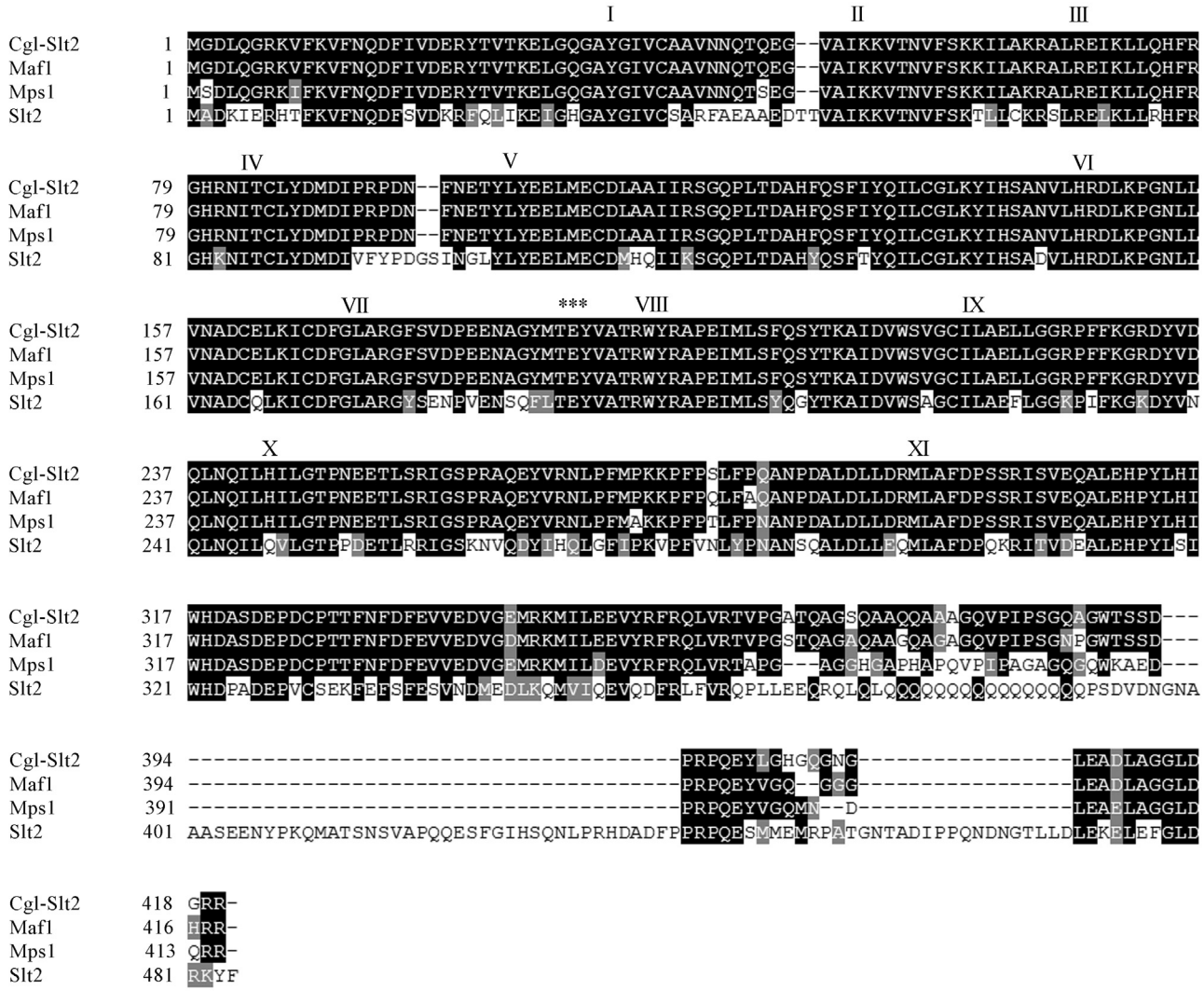

Figure 1 - The amino acid sequence alignment of Cgl-Slt2 of *C. gloeosporioides* (JQ322774), Maf1 of *C. orbiculare* (AAL50116), Mps1 of *M. oryzae* (AAC63682) and Slt2 of *S. cerevisiae* (AAB68912). Sequence alignment was performed using CLUSTALW. Identical amino acids are indicated by white letters 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.

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

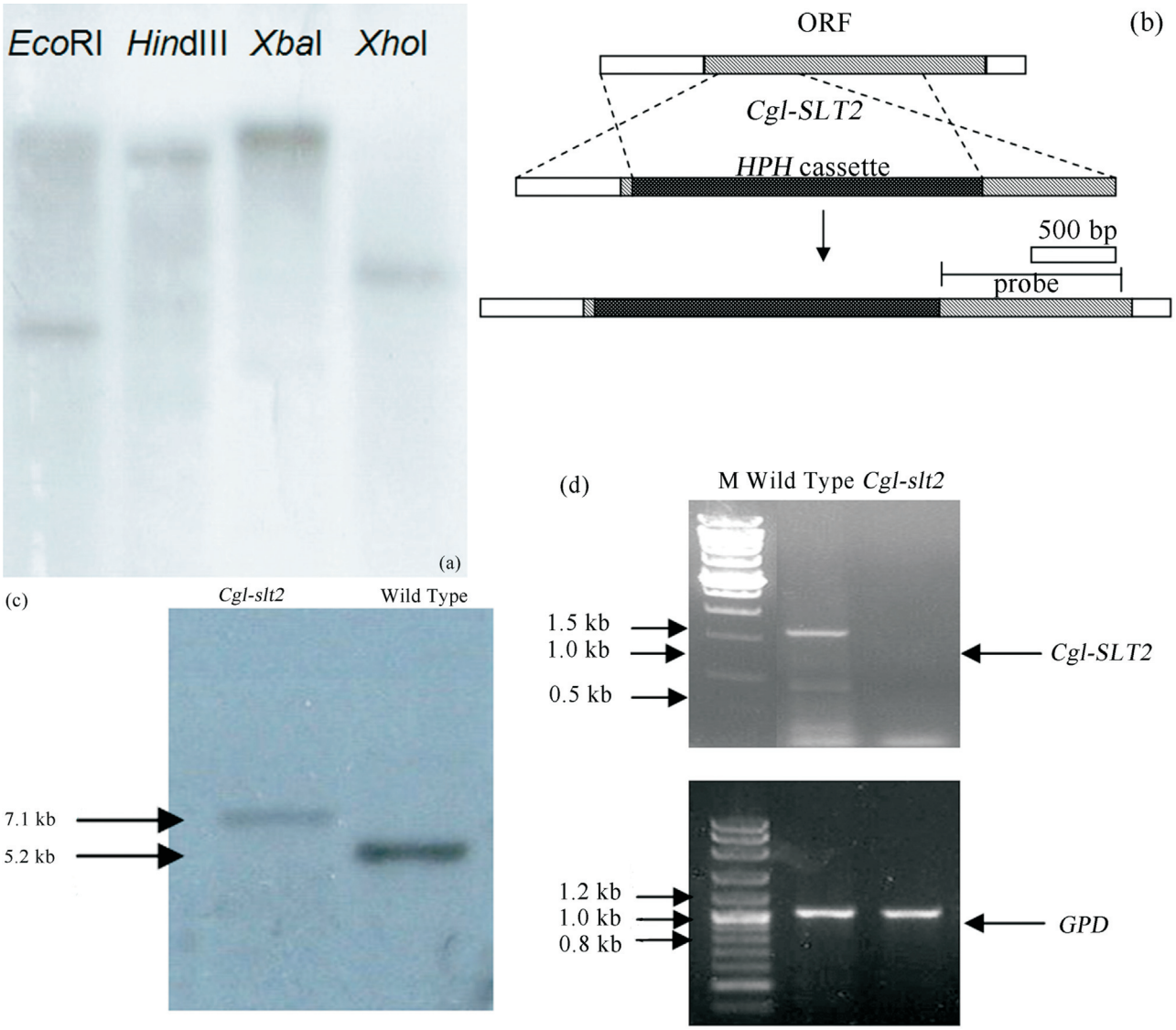

Figure 2 - Copy number determination and the targeted gene disruption of *Cgl-SLT2*. (a) A Southern blot analysis shows the *Cgl-SLT2* copy number in the *C. gloeosporioides Peu*B genome. Genomic DNA was digested with different restriction enzymes, including *Eco*RI, *Hin*dIII, *Xba*I and *Xho*I, and probed with the *Cgl-SLT2* ORF. (b) A schematic representation of the targeted gene disruption of *C. gloeosporioides Cgl-SLT2*. A linearized plasmid harboring the disruption construct was transformed into the *C. gloeosporioides* spheroplasts. A portion of the *Cgl-SLT2* ORF was replaced with an *hph* cassette via homologous recombination. A 1 kb fragment was used as a probe for Southern blot analysis. (c) A Southern blot analysis of the strain with disrupted *Cgl-SLT2* and wild-type. Genomic DNA was digested with *Xho*I and probed with *Cgl-SLT2.* (d) *Cgl-SLT2* expression analysis in wild-type and *Cgl-slt2* strains via reverse transcription PCR using *GPD* as a control. Gene disruption resulted in the loss of *Cgl-SLT2* transcripts. M: Molecular marker.

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

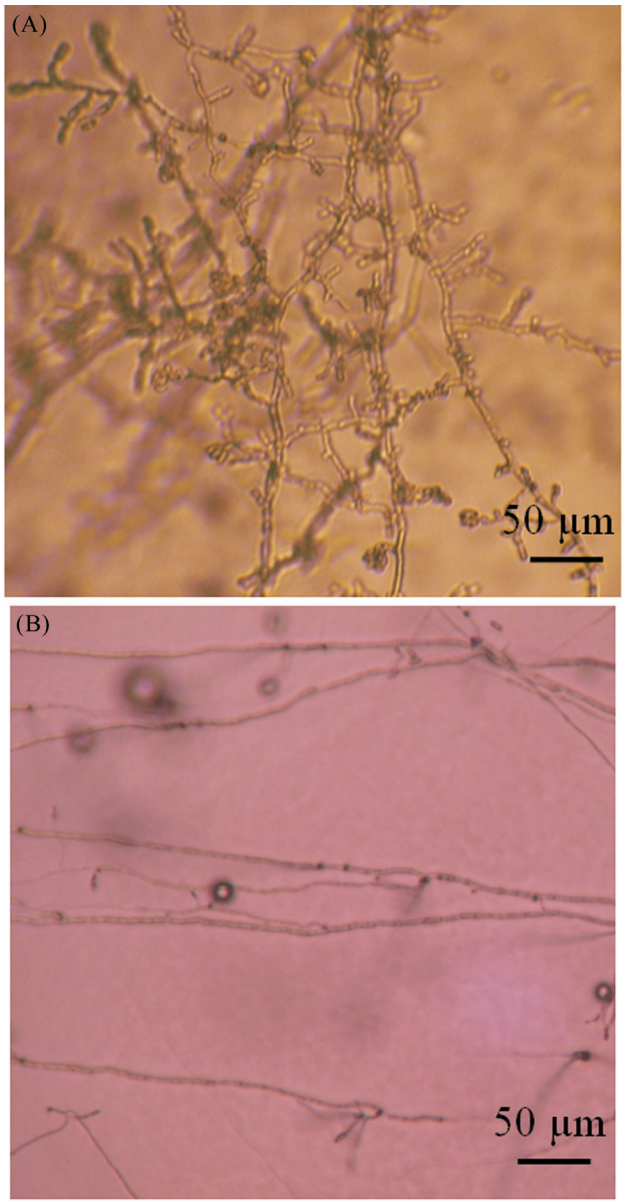

Figure 3 - Light microscopy images of *Cgl-slt2* (a) and wild-type strains (b) on minimal media agar. The *Cgl-slt2* mutant exhibited increased hyphal branching compared to the wild-type. The image was captured using an Olympus light microscope under 100X magnification. The scale bar indicates 50 µm.

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

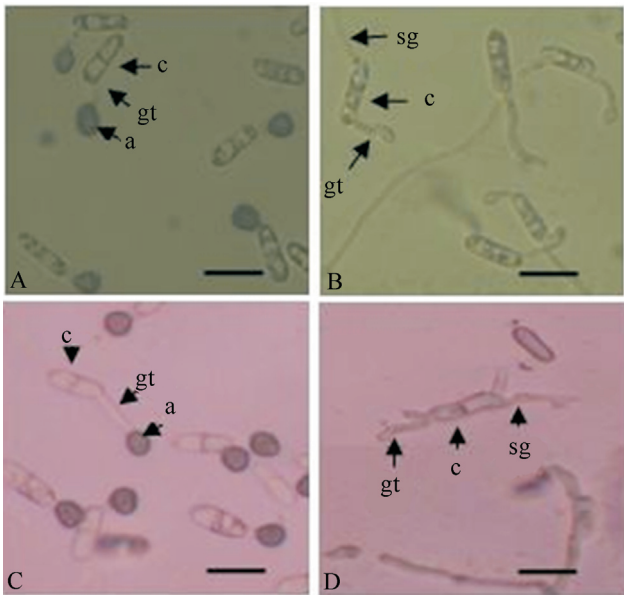

Figure 4 - Appressorium formation in wild-type and *Cgl-slt2* strains. Appressorium formation induced from conidia harvested from *C. gloeosporioides* grown on PDA supplemented with 1 M sorbitol in wild-type (a) and *Cgl-slt2* strains (b) Appressorium formation induced from conidia harvested from *C. 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)[a]	Conidiation (per mL)[b]	Germination (%)[c]	Appressorium formation (%)[d]	Bipolar germination (%)[e]
Wild-type	8.5 ± 0.1	2.98 ± 0.21 x10^7	93.3 ± 2.3	90.6 ± 1.15	11.3 ± 5.5
Cgl-slt2	6.7 ± 0.08	1.48 ± 0.27 x10^4	92.3 ± 5.0	46 ± 1	72.3 ± 4.5

[a]Diameter of colonies grown on potato dextrose agar at 30 °C for 6 days. [b]Conidia harvested from 7-day-old cultures. [c]Percentage 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. [d]Percentage 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. [e]Percentage 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.

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, preventing 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.

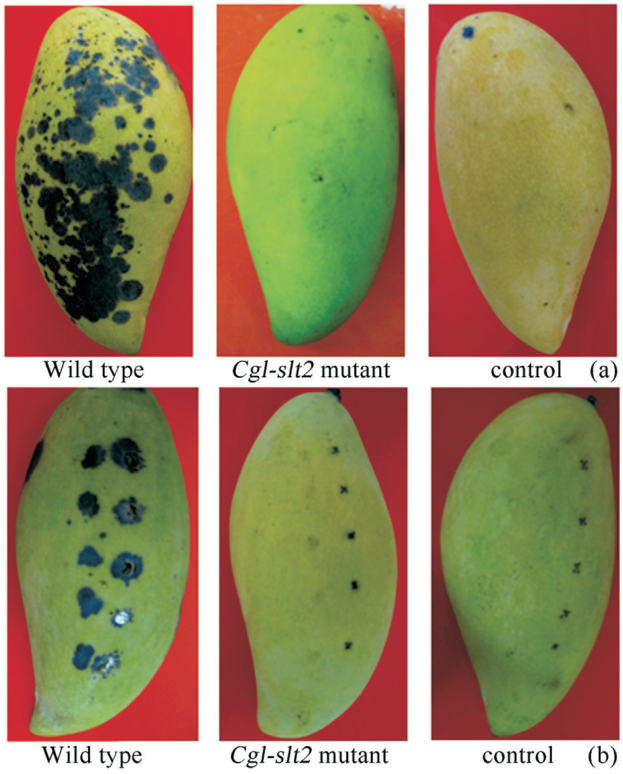

Figure 5 - A pathogenicity assay with wild-type and *Cgl-slt2* strains on mangoes. (a) Mangoes were spray-inoculated with a 10^5 conidia/mL suspension. Images were obtained 10-days post-inoculation. The picture in the left panel is the wild-type strain, the middle panel is the mutant strain (*Cgl-slt2*) and the right panel is the control (sterile dH_2O). (b) Wounded mangoes were inoculated with 25 µL of a 10^5 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 dH_2O). *Cgl-slt2* mutants were unable to infect wounded or unwounded fruits compared to the wild-type.

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* IPO323ΔMgSlt2 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* s*lt2* 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

(Vogt and Seiler, 2008). 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, osmostabilants and host signals.

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