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Saccharomyces cerevisiae SSD1 orthologues are essential for host infection by the ascomycete plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea*

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Summary

Fungal plant pathogens have evolved diverse strategies to overcome the multilayered plant defence responses that confront them upon host invasion. Here we show that pathogenicity of the cucumber anthracnose fungus, *Colletotrichum lagenarium*, and the rice blast fungus, *Magnaporthe grisea*, requires a gene orthologous to *Saccharomyces cerevisiae SSD1*, a regulator of cell wall assembly. Screening for *C. lagenarium* insertional mutants deficient in pathogenicity led to the identification of *ClaSSD1*. Following targeted gene replacement, appressoria of *classd1* mutants retained the potential for penetration but were unable to penetrate into host epidermal cells. Transmission electron microscopy suggested that appressorial penetration by *classd1* mutants was restricted by plant cell wall-associated defence responses, which were observed less frequently with the wild-type strain. Interestingly, on non-host onion epidermis *classd1* mutants induced papilla formation faster and more abundantly than the wild type. Similarly, colonization of rice leaves by *M. grisea* was severely reduced after deletion of the orthologous *MgSSD1* gene and attempted infection by the mutants was accompanied by the accumulation of reactive oxygen species within the host cell. These results suggest that appropriate assembly of the fungal cell wall as regulated by *SSD1* allows these pathogens to establish infection by avoiding the induction of host defence responses.

Introduction

To resist attack by potential microbial pathogens, plants have evolved an enormous repertoire of defence mechanisms. These include the deposition of cell wall reinforcements (papillae), hypersensitive cell death and the synthesis of a plethora of antimicrobial secondary metabolites, which may be either preformed (phytoanticipins) or actively synthesized upon infection (phytoalexins). Many secreted plant pathogenesis-related (PR) proteins also play important roles in defence, for example cell wall proteins such as hydroxyproline-rich glycoproteins, directly toxic proteins (e.g. thaumatin, osmotin) and lytic enzymes (e.g. endoglucanases, endochitinases, proteases) that may also release fungal elicitors to activate further plant defences (Ichinose *et al*., 1989; Lamb *et al*., 1989). In addition, the local generation of reactive oxygen species (ROS) is an early plant response to attack by many pathogens and these molecules may function in oxidative cell wall cross-linking or plant defence signalling.

In the course of co-evolution with their host plants, successful (adapted) fungal pathogens have developed diverse counter defence strategies to circumvent these multilayered plant defences, for example, the detoxification of antimicrobial compounds (Papadopoulou *et al*., 1999), suppressors (Shiraishi *et al*., 1999), efflux pumps (Urban *et al*., 1999), production of ROS scavengers (Zhang *et al*., 2004; Voegele *et al*., 2005) and secretion of effectors that function to block the activity of PR proteins (Tian *et al*., 2004; Bishop *et al*., 2005).

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Colletotrichum lagenarium is the causal agent of anthracnose disease on cucumber. Following the germination of spores (conidia) on the plant surface, this ascomycete fungus invades host tissues by developing a series of specialized infection structures, including germ-tubes, appressoria, penetration pegs, biotrophic primary hyphae and necrotrophic secondary hyphae (Perfect *et al*., 1999). These specialized cells mediate the initial direct penetration of host epidermal cells by breaching the plant cuticle and cell wall layers, probably through a combination of mechanical force and localized enzymic dissolution (Tucker and Talbot, 2001). Melanization of the appressorial cell wall is crucial for appressorium function (Kubo and Furusawa, 1991), and three melanin biosynthesis genes, *PKS1*, *SCD1* and *THR1*, have been isolated and characterized (Takano *et al*., 1995; Kubo *et al*., 1996; Perpetua *et al*., 1996). The expression of these melanin biosynthesis genes was later shown to be regulated by a transcription factor encoded by the *CMR1* gene (Tsuji *et al*., 2000). A mitogen-activated protein (MAP) kinase encoded by the *CMK1* gene is indispensable for spore germination and appressorium formation (Takano *et al*., 2000). On the other hand, appressorium penetration function requires the *CST1* gene, which is homologous to the *STE12* gene of *Saccharomyces cerevisiae* and codes for a transcription factor acting downstream of *CMK1* (Tsuji *et al*., 2003a). Further studies have shown that successful penetration of cucumber epidermal cells by *C. lagenarium* appressoria also depends on the metabolic activity of peroxisomes (Kimura *et al*., 2001) and a gene involved in modifying tRNA, *APH1*, which may contribute to fungal tolerance of plant defence responses associated with penetration (Takano *et al*., 2006). Thus, all the genes so far identified as being essential for the pathogenicity of this fungus are involved in either the morphogenesis or functioning of appressoria.

In the present study, we used random insertional mutagenesis by *Agrobacterium tumefaciens*-mediated transformation (AtMT) to identify a novel mutant of *C. lagenarium* that showed attenuated pathogenicity on cucumber plants. Genetic analysis of this mutant revealed that the phenotype was caused by disruption of a gene showing significant homology to the *S. cerevisiae SSD1* gene, a regulator of cell wall biogenesis (Wheeler *et al*., 2003). Targeted gene replacement in *C. lagenarium* and the rice blast pathogen, *Magnaporthe grisea* demonstrated that orthologues of *SSD1* are essential for the initial establishment of infection by both these fungi. We present evidence that appropriate assembly of the fungal cell wall as regulated by *SSD1* is required for the pathogenicity of these fungi by avoiding the induction of basal plant defence responses.

Results

Isolation of the ClaSSD1 *gene*

A pathogenicity-deficient mutant of *C. lagenarium*, Lf2754, was identified among 10 650 hygromycin-resistant transformants obtained by random insertional mutagenesis using AtMT. In inoculation assays, the wild-type strain 104-T produced necrotic lesions on cucumber cotyledons after 5 days, whereas mutant Lf2754 produced few visible symptoms (Fig. S1A). The colony morphology of mutant Lf2754 was similar to that of the wild-type strain 104-T after 7 days growth on PDA medium, but the mutant showed a slightly slower rate of growth (Fig. S1B).

Genomic DNA adjoining the T-DNA insert was isolated from Lf2754 by the thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) and the amplified products were sequenced. In a BLASTX search (NCBI BLAST; http://www.ncbi.nlm.nih.gov/BLAST), the sequence adjacent to the T-DNA left-border showed significant homology to the *S. cerevisiae SSD1* gene. A cosmid clone p2754cos containing this region was isolated from a genomic library of *C. lagenarium* and a XhoI-digested segment containing the *SSD1* homologous region was subcloned into pBluescript SK II. The resulting construct, pBS2754Xh, was used for sequence analysis. We named the gene containing the *SSD1* homologous region *ClaSSD1* (*Colletotrichum lagenarium SSD1*) and the entire sequence of the gene was determined. The transcription initiation site was identified after 5′ rapid amplification of cDNA ends (5′-RACE). The ATG codon 43 bp downstream of the transcriptional start point is the probable translational initiation site of *ClaSSD1*. Two introns, located from nucleotides 1477 to 1526 and from 2882 to 2930, were predicted on the basis of matching with consensus 5′ splicing signals GT(A/G/T)(A/C/T)G(T/C), 3′ splicing signals (C/T)AG and internal splicing signals (G/A)CT(A/G)AC conserved in *Neurospora crassa* (Bruchez *et al*., 1993). Reverse-transcribed DNA was amplified by PCR using two pairs of primers spanning the predicted introns and comparison of the amplified cDNA with the genomic DNA sequences verified the presence of the predicted introns. Based on the likely translation initiation codon and knowledge of the introns, *ClaSSD1* is predicted to encode a protein of 956 amino acids. The information on gene structure is shown in Fig. S2. A phylogenetic tree generated on the basis of the amino acid sequences of *SSD1* homologues from *S. cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus nidulans*, *A. fumigatus*, *N. crassa*, *Fusarium graminearum*, *M. grisea*, *Ustilago maydis* and *C. lagenarium* showed that the gene is highly conserved among ascomycete filamentous fungi (Fig. 1A). The sequence alignment data are shown in Fig. S3. A search using a conserved domain database (NCBI; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) revealed that

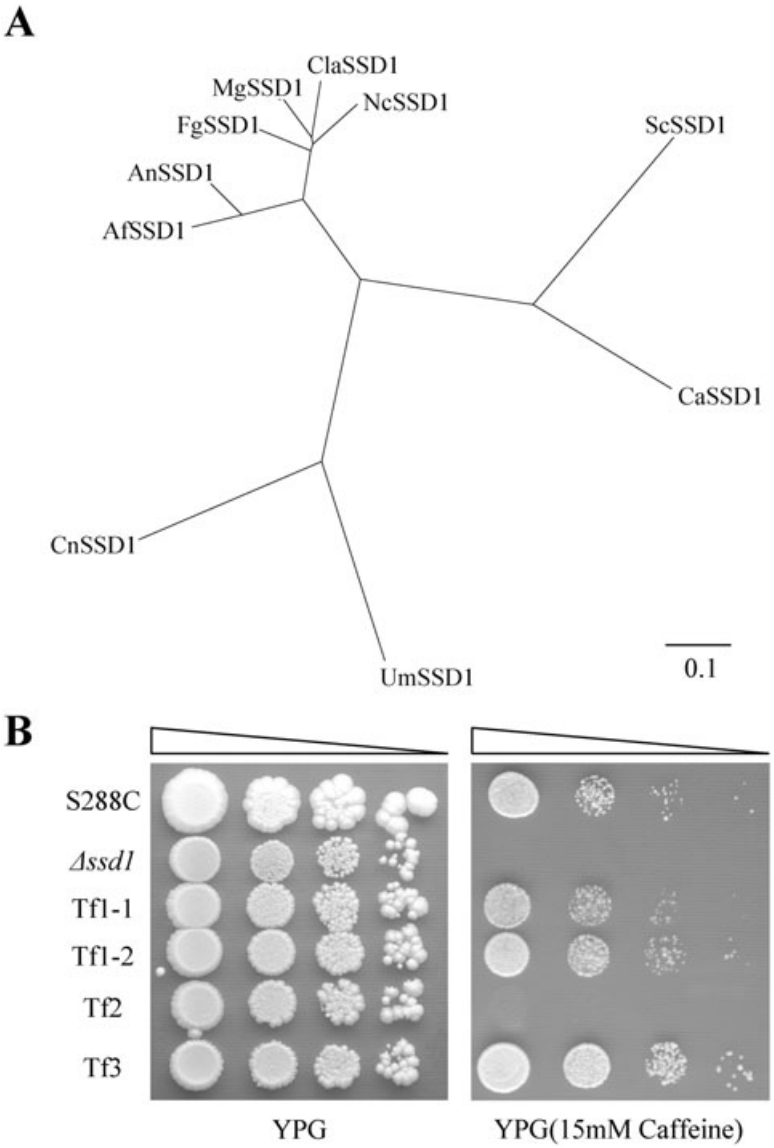

Fig. 1. Phylogenetic relationships of *ClaSSD1* and functional complementation in yeast.
A. A phylogenetic tree of *SSD1* homologues from a range of yeasts and filamentous fungi was generated on the basis of amino acid sequences. *S. cerevisiae* (ScSSD1), *C. albicans* (CaSSD1), *C. neoformans* (CnSSD1), *A. nidulans* (AnSSD1), *A. fumigatus* (AfSSD1), *N. crassa* (NcSSD1), *U. maydis* (UmSSD1), *F. graminearum* (FgSSD1), *M. grisea* (MgSSD1) and *C. lagenarium* (ClaSSD1). Scale bar indicates evolutionary distance.
B. Complementation of caffeine sensitivity in the *S. cerevisiae Δssd1* mutant by *ClaSSD1*. *ClaSSD1* was expressed in the *Δssd1* mutant (Tf1-1 and Tf1-2) using pYES2ClaSSD1. Tf2 is the *Δssd1* mutant transformed with empty vector pYES2 and Tf3 is the *Δssd1* mutant transformed with pYES2ScSSD1 to express the wild-type *S. cerevisiae SSD1* gene. Cells were cultured either on YPG medium or YPG containing 15 mM caffeine. The triangles shown above each image indicate decreasing concentrations of yeast inoculum.

Classd1p has a RNB domain. It is presumed that this domain functions as the RNA binding and catalytic domain of exoribonuclease, and is well-conserved in ribonuclease II-like proteins (Mian, 1997). To determine whether *ClaSSD1* is the functional orthologue of *S. cerevisiae SSD1*, a complementation test was performed. In *S. cerevisiae*, *ssd1* mutants show hypersensitivity to caffeine (Sutton *et al.*, 1991). We constructed the plasmids pYES2ClaSSD1 and pYES2ScSSD1 to express the cDNA of *ClaSSD1* open reading frames (ORF) and *S. cerevisiae SSD1*, respectively, in yeast under the control of the *GAL1* promoter. On YPG medium containing 15 mM caffeine, the *ssd1* transformants Tf1-1 and Tf1-2 carrying pYES2ClaSSD1 showed the normal growth phenotype indistinguishable from wild-type S288C and *ssd1* transformant Tf3 carrying pYES2ScSSD1. In contrast, the *ssd1* mutant and *ssd1* transformant Tf2 carrying the empty vector pYES2 showed the typical growth defect (Fig. 1B). Thus, *ClaSSD1* complemented the hypersensitivity of the yeast mutant to caffeine, so that *ClaSSD1* can be considered the functional orthologue of *S. cerevisiae SSD1*.

Targeted gene disruption of ClaSSD1

In order to determine whether *ClaSSD1* is involved in pathogenicity, we attempted to isolate a *ClaSSD1* disruption mutant by targeted gene replacement. The plasmid pBI2754XhBC1 was designed to replace the *ClaSSD1* gene in 104-T with the *classd1::BC1* fragment through double crossover homologous recombination (Fig. S4A). By transformation with AtMT, 108 bialaphos-resistant transformants were obtained. Four of these transformants

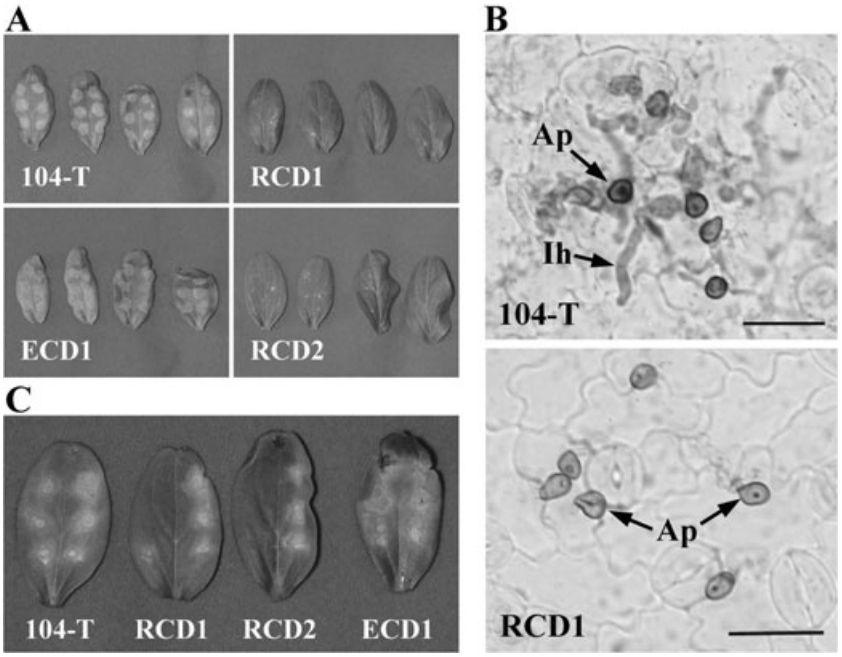

Fig. 2. Infection phenotypes of *classd1* mutants.
A. Pathogenicity assay. Conidial suspensions were spotted onto detached cucumber cotyledons. After 4 days, wild-type strain 104-T and ectopic transformant ECD1 formed anthracnose lesions, whereas *classd1* mutants RCD1 and RCD2 produced few visible symptoms.
B. Cytology of infection. Conidial suspensions were spotted onto the lower epidermis of cucumber cotyledons. After 72 h, the epidermis was removed and stained with lactophenol-Aniline blue. Appressoria (Ap) of the wild-type strain 104-T produced abundant infection hyphae (Ih) inside host epidermal cells whereas appressoria of *classd1* mutant RCD1 failed to penetrate. The percentage of appressoria forming infection hyphae is shown graphically below the microscope images. Bars = 20 μm.
C. Invasive growth ability. Detached cucumber cotyledons were scratched with a sterile pipette tip (right side of each leaf) or left intact (left side). Conidial suspensions of wild-type strain 104-T, *classd1* mutants RCD1 and RCD2 and ectopic transformant ECD1 were spotted onto the leaves. All strains formed lesions on scratched sites, indicating that *classd1* mutants retain invasive growth ability.

also showed hygromycin sensitivity, indicating they were putative disrupted mutants. Southern blot analysis was performed on these four mutants (designated RCD1, RCD2, RCD3 and RCD4) and one hygromycin-resistant transformant (designated ECD1). The wild-type 104-T and the transformant ECD1 both contained a 2.0 kb KpnI fragment (Fig. S4B; lane 1 and 6), while ECD1 contained an additional band, indicating ectopic integration. In contrast, four putative disrupted mutants (RCD1, RCD2, RCD3 and RCD4) did not contain the 2.0 kb KpnI wild-type fragment, but did contain a common 4.2 kb KpnI fragment, consistent with the length expected from a gene replacement event (Fig. S2B; Lane 2, 3, 4 and 5). These results demonstrate that the *ClaSSD1* gene was disrupted in RCD1, RCD2, RCD3 and RCD4. Reverse transcription (RT)-PCR also confirmed that *ClaSSD1* transcripts were not detected in mycelium of the RCD1 mutant (data not shown). These mutants were therefore used as *classd1* mutants in subsequent experiments. RT-PCR analysis of *ClaSSD1* expression during germination of wild-type 104-T conidia on polystyrene Petri dishes showed that RNA expression was detectable from before germination (0 h) up to 4 h, when germ-tubes started to develop appressoria (Fig. S4C). As expected, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was constitutively expressed in mycelia and conidia.

Appressoria of classd1 *mutants are defective in host penetration*

The colony morphology of *classd1* mutants grown on PDA medium for 7 days was similar to that of 104-T but, as with Lf2754, the *classd1* disruption mutants showed approximately 70% growth rate compared with the wild-type (data not shown). After inoculation of conidial suspensions onto cucumber cotyledons, *classd1* mutants RCD1 and RCD2 produced hardly any visible symptoms, whereas the wild-type 104-T and ectopic transformant ECD1 both formed clear necrotic lesions (Fig. 2A). Similarly, when *classd1* mutants were inoculated onto adult leaves, lesions were not observed (data not shown). These results indicate that *ClaSSD1* is required for the pathogenicity of *C. lagenarium* on cucumber. When we observed the behavior of *classd1* mutants on host epidermal cells with light microscopy, the mutants formed melanized appressoria on the plant surface similar to those of 104-T (Fig. 2B). However, although a basal penetration pore was visible in *classd1* appressoria, intracellular infection hyphae rarely devel-

oped inside epidermal cells, in contrast to the abundant development of intracellular hyphae by wild-type appressoria (Fig. 2B). To assess the capacity of *classd1* mutants for invasive growth inside host tissues, conidial suspension was applied to the surface of cucumber cotyledons that had been wounded by scratching with a plastic pipette tip. After incubation for 5 days, *classd1* mutants formed lesions indistinguishable from those of 104-T (Fig. 2C), indicating that the mutants retain the capacity for invasive growth. Therefore, it is likely that the attenuated pathogenicity of *classd1* mutants results from a defect in their ability to penetrate intact plant surfaces.

One possible explanation for the failure of *classd1* mutants to penetrate host epidermal cells is that appressorial function is impaired in some way. To test this, we determined the ability of *classd1* mutants to penetrate artificial cellulose membranes. After 48 h incubation, both mutant and wild-type conidia had germinated to form appressoria, which in turn penetrated the membrane surface and formed hyphae inside the membrane (Fig. 3A). These hyphae are developmentally equivalent to the intracellular infection hyphae formed *in planta* and their growth inside the membrane is typically associated with dissolution of the surrounding cellulose, visible as a clear halo after staining with $ZnCl_2$-KI-I_2 (Kubo *et al*., 1981). The frequency of penetration into cellulose membranes by both 104-T and *classd1* mutants was over 70% (Fig. 3B) and *classd1* mutants formed haloes of clearing within the membrane to the same extent as 104-T (data not shown). To assess appressorial turgor, we used a cytorrhysis assay, measuring the number of appressoria collapsed after exposure to varying concentrations of glycerol (Howard *et al*., 1991). In 4 M glycerol, 58.9% of 104-T appressoria and 59.4% of *classd1* appressoria were collapsed, indicating there was no significant decrease in the appressorial turgor of *classd1* mutants (Fig. 3C). Taken together, these results show that the ability of *classd1* appressoria to generate mechanical pressure is unaffected and that they remain functional for penetration.

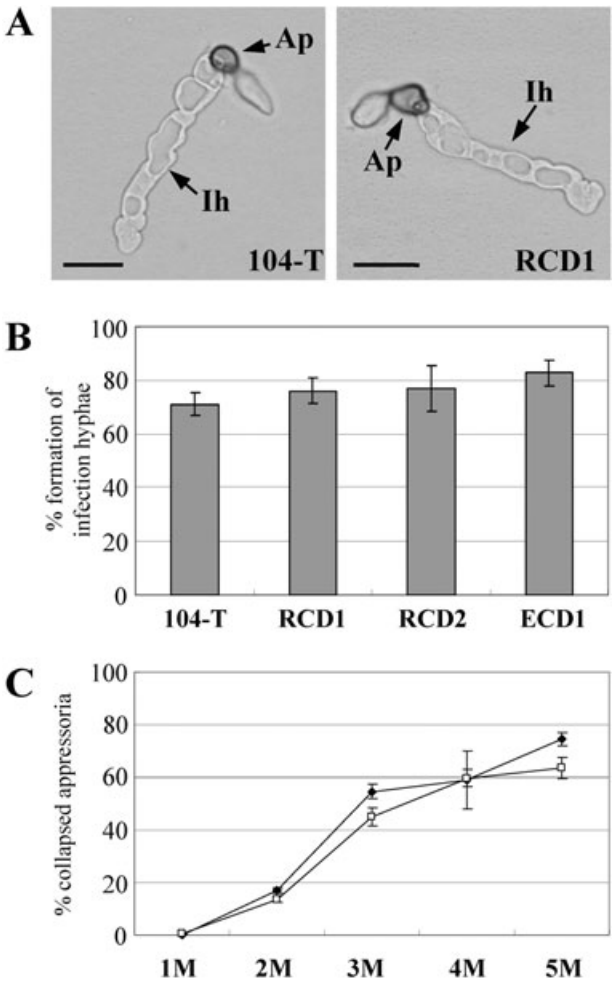

Fig. 3. Appressorial penetration ability of *classd1* mutants.
A. Cellophane penetration assay. Conidial suspensions were spotted onto cellulose dialysis membranes and incubated for 48 h. Appressoria (Ap) of both the wild-type strain 104-T and *classd1* mutant RCD1 penetrated the membrane to form infection hyphae (Ih). Bars = 10 μm.
B. Percentage penetration of cellulose membranes. Infection hyphae produced by appressoria of wild-type strain 104-T, *classd1* mutants RCD1 and RCD2 and ectopic transformant ECD1 were counted. At least 200 appressoria were counted and standard errors were calculated from three replicate experiments.
C. Appressorial cytorrhysis assay (Howard *et al*., 1991). Appressoria of the wild-type strain 104-T (solid diamonds) and *classd1* mutant (open squares) were allowed to differentiate for 48 h on a glass microscope slide, exposed to glycerol solutions (1 M to 5 M) for 15 min and the percentage of collapsed appressoria were counted. At least 200 appressoria were counted and standard errors were calculated from three replicate experiments.

Classd1p localizes to the fungal cytoplasm

The amino acid sequence of Classd1p does not contain any N-terminal secretion signal and the protein was predicted to be localized in the cytoplasm by the WoLF PSORT program (http://wolfpsort.seq.cbrc.jp). To determine the subcellular localization of Classd1p, we constructed a plasmid expressing the GFP : Classd1p fusion protein. When the GFP : Classd1p fusion protein was expressed under the control of its native promoter, GFP fluorescence was not detectable (data not shown). Therefore, the plasmid pBISCD1pGFP : ClaSSD1H was constructed in order to express the GFP : Classd1p fusion protein under control of a modified scytalone dehydrogenase (*SCD1*) promoter that confers constitutive expression. After introduction of this plasmid into the *classd1* mutant, transformants expressing GFP : Classd1p were observed using confocal microscopy. In hyphae and conidia, GFP fluorescence was uniformly distributed in the fungal cytoplasm except for vacuoles and was absent from the fungal cell wall (Fig. S5). As the transformants recov-

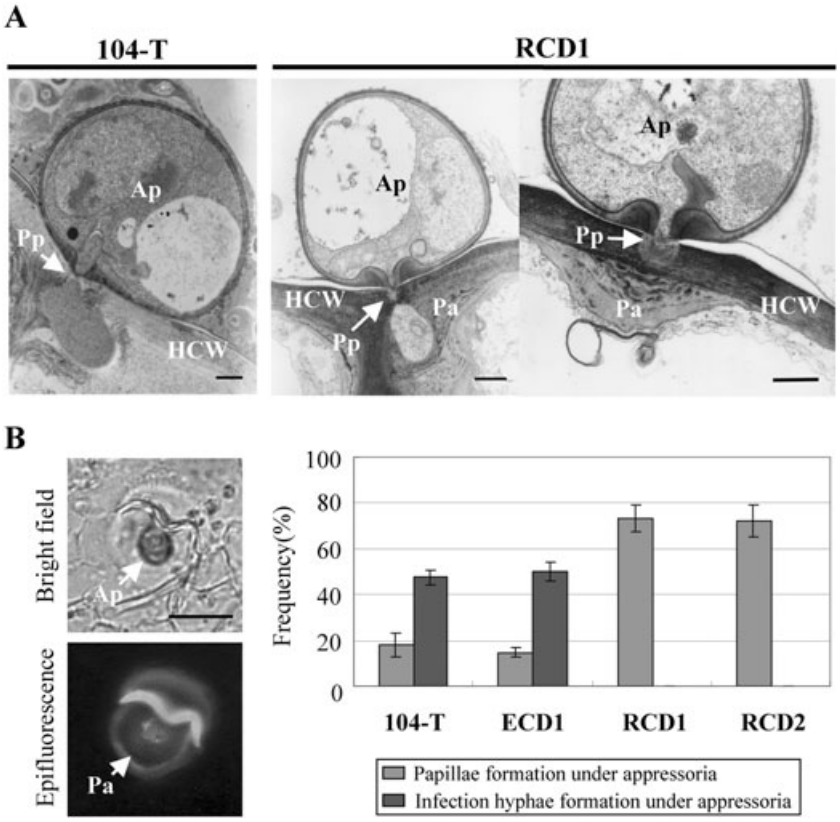

Fig. 4. Cytology of plant defence responses against fungal penetration.
A. Cucumber cotyledons were observed with transmission electron microscopy at 3 days after inoculation. Appressoria (Ap) of the *C. lagenarium* wild-type strain 104-T developed penetration pegs (Pp) which penetrated the host cuticle and cell wall to form infectious hyphae. Appressoria of *classd1* mutant RCD1 were indistinguishable from those of 104-T and their penetration pegs breached the plant cuticle. However, their further development was restricted either inside the host cell wall (Hcw) or within papillae (Pa) deposited beneath the penetration site. Bars = 500 nm.
B. Quantification of papilla formation at sites of attempted penetration by *C. lagenarium* appressoria. At 3 days, leaf epidermal strips inoculated with 104-T, RCD1, RCD2 and ECD1 were stained with Aniline blue to reveal callose and observed with epi-fluorescence or bright-field microscopy. The photos show a papilla formed beneath an attempted penetration site by appressoria of the *classd1* mutant RCD1 (bar = 10 μm). The wild-type 104-T and ectopic mutant ECD1 induced less than 20% papilla formation and the frequency of infection hyphae formation was high. In contrast, papilla formation was more than threefold greater with *classd1* mutants RCD1 and RCD2, and infection hyphae were hardly observed. At least 400 appressoria were counted for each fungal strain and standard deviations were calculated from three replicate experiments.

ered their pathogenicity (data not shown), the GFP : ClaSSD1 fusion protein appears functional. These results confirm that Classd1p has a cytoplasmic localization and does not localize to any specific subcellular structures.

Impaired host penetration by classd1 *mutants depends on plant defence responses*

Because *classd1* appressoria appeared to retain their functionality, we speculated that their failure to penetrate cucumber epidermal cells results from a defect in their ability to avoid or withstand host defence responses. To document the extent and nature of structural host defence responses associated with penetration of cucumber epidermal cells we used transmission electron microscopy (TEM). The *classd1* mutant formed appressoria that appeared identical in ultrastructure to those of 104-T (Fig. 4A), having cell walls of normal thickness and structure and a basal penetration pore that was surrounded by a funnel-shaped appressorial cone (Landes and Hoffman, 1979). Mutant appressoria were also able to form penetration pegs that penetrated through the plant cuticle and into the epidermal cell wall, which showed evidence of localized dissolution around the penetration peg (Fig. 4A). However, in all cases *classd1* appressoria induced host defence accompanied by the deposition of localized cell wall reinforcements (papillae) at sites of attempted penetration (Zeyen *et al.*, 2002) and electron-opaque material also accumulated in the epidermal cell wall adjoining the

papillae. The development of some penetration pegs became restricted inside the epidermal cell wall, while others completely penetrated the wall and grew a short distance into the lumen of the epidermal cell but these were always encased by a host papilla (Fig. 4A). In contrast, 104-T had already extensively colonized host tissues by means of intracellular primary hyphae at the same time-point (3 days after inoculation). To quantify the extent of plant defence responses against attempted appressorial penetration, percentage papilla formation was evaluated. The callose component of papillae was stained by Aniline blue and the number of papillae formed beneath appressoria was assessed at 3 days after inoculation using fluorescence microscopy. In *classd1* mutants, approximately 70% of appressoria were accompanied by callose papillae and intracellular infection hyphae did not develop from them (Fig. 4B). On the other hand, the frequency of callose papillae induced by the wild-type strain and the ectopic transformant was only 20% and intracellular infection hyphae were observed to develop from approximately 50% of their appressoria. Overall, these results strongly suggest that appressorial penetration of epidermal cells by *classd1* mutants is restricted by plant defences associated with papilla formation.

These cytological observations support the hypothesis that the impaired penetration ability of *classd1* mutants is dependent on the expression of plant defence responses. To test this hypothesis, we attempted to compromise plant defence responses by applying a transient heat-shock to host plants (Chen *et al*., 2003). Cucumber plants were heat-shocked (50°C for 30 s) and then inoculated with conidial suspensions of 104-T and the *classd1* and *cst1* mutants. Although the *cst1* mutant is defective in appressorial penetration it retains a capacity for invasive growth inside host tissues (Tsuji *et al*., 2003a). The *cst1* mutant thus provides a control to verify whether the *classd1* mutant infects host tissues through wounds caused by the transient heat-shock. After 4 days incubation, both 104-T and the *classd1* mutant formed lesions on heat-shocked cucumber cotyledons (Fig. 5A). However, the *cst1* mutant did not form any lesions, suggesting that the *classd1* mutant did not infect heat-shocked plants through wounds. Uninoculated heat-shocked plants remained healthy and developed adult leaves during the course of incubation (not illustrated), indicating that the treatment did not reduce plant viability. These data suggest that active host defence responses play a role in the failure of *classd1* mutants to penetrate host epidermal cells, while the wild-type 104-T has the ability to overcome such defences and penetrate successfully.

It has been reported that the *ssd1* mutant of *S. cerevisiae* shows sensitivity to osmotin (PR-5) (Ibeas *et al*., 2001). To examine whether the penetration defect of the *classd1* mutant is due to plant PR proteins, the expression of several *PR* genes during compatible interactions between cucumber and *C. lagenarium* was determined using RT-PCR (Fig. 5B). On the basis of the sequences deposited at GenBank, primers were designed for *Cucumis sativus PR-1a* (designated *PR-1*), *C. sativus* beta-1,3-glucanase (designated *PR-2*), *Cucumis melo* thaumatin-like protein (designated *PR-5*) and *C. melo* actin. In intact leaves before inoculation (0 h), the expression of *PR-2* and *PR-5* was not detectable, while *PR-1* expression was observed at a very low level. In leaves inoculated with either the wild-type 104-T or *classd1* mutant, as well as control leaves receiving a mock inoculation, an increased level of *PR-1* expression was observed from 12 h to 72 h after inoculation. In leaves inoculated with 104-T, expression of *PR-2* was observed from 36 h after inoculation and *PR-5* was detected at 72 h whereas in leaves inoculated with the *classd1* mutant, expression of *PR-2* and *PR-5* was not detectable. The actin gene was expressed at all time points. In neither case did the heat shock treatment significantly alter the expression of these plant defence genes except that *PR-5* was detected from 36 h in the leaves inoculated with 104-T. These results suggest that PR proteins, including PR-5, cannot be the only component of plant defence responsible for limiting penetration by the *classd1* mutant.

To investigate whether *classd1* mutants suffer defects in cell wall integrity that could increase their susceptibility to plant defence components, we tested their sensitivity to a range of abiotic stresses. The *classd1* mutants showed no greater sensitivity to osmotic or oxidative stress than the wild type when grown on PDA medium containing 0.4 M KCl or 10 mM H_2O_2, respectively (data not shown). Although the growth of *S. cerevisiae ssd1* mutants was inhibited by caffeine, neither *C. lagenarium* wild-type 104-T nor the *classd1* mutants showed any significant sensitivity to caffeine (data not shown). Thus, the *classd1* mutants do not show significant defects in the integrity of their cell walls, suggesting that impairment of appressorial penetration does not result from an increased sensitivity to environmental stress. On the other hand, *classd1* mutants did show increased sensitivity to Calcofluor White, which disrupts the assembly of fungal cell walls through binding to chitin and, to a lesser extent, glucans (Ram *et al*., 1994). Thus, growth of the mutants on PDA containing 100 $\mu g\ ml^{-1}$ Calcofluor White was reduced by over 50%, whereas growth of the 104-T strain was only reduced by 14% (Table 1). However, neither the wild-type 104-T nor *classd1* mutants showed significant sensitivity to 200 $\mu g\ ml^{-1}$ Congo Red, which inhibits cell wall construction through binding to β-1,4-glucans (Wood and Fulcher, 1983). The greater sensitivity of *classd1* mutants to Calcofluor White suggests that they may have altered cell wall composition. However, fluorescence microscopy revealed no detectable difference between wild-type 104-T and

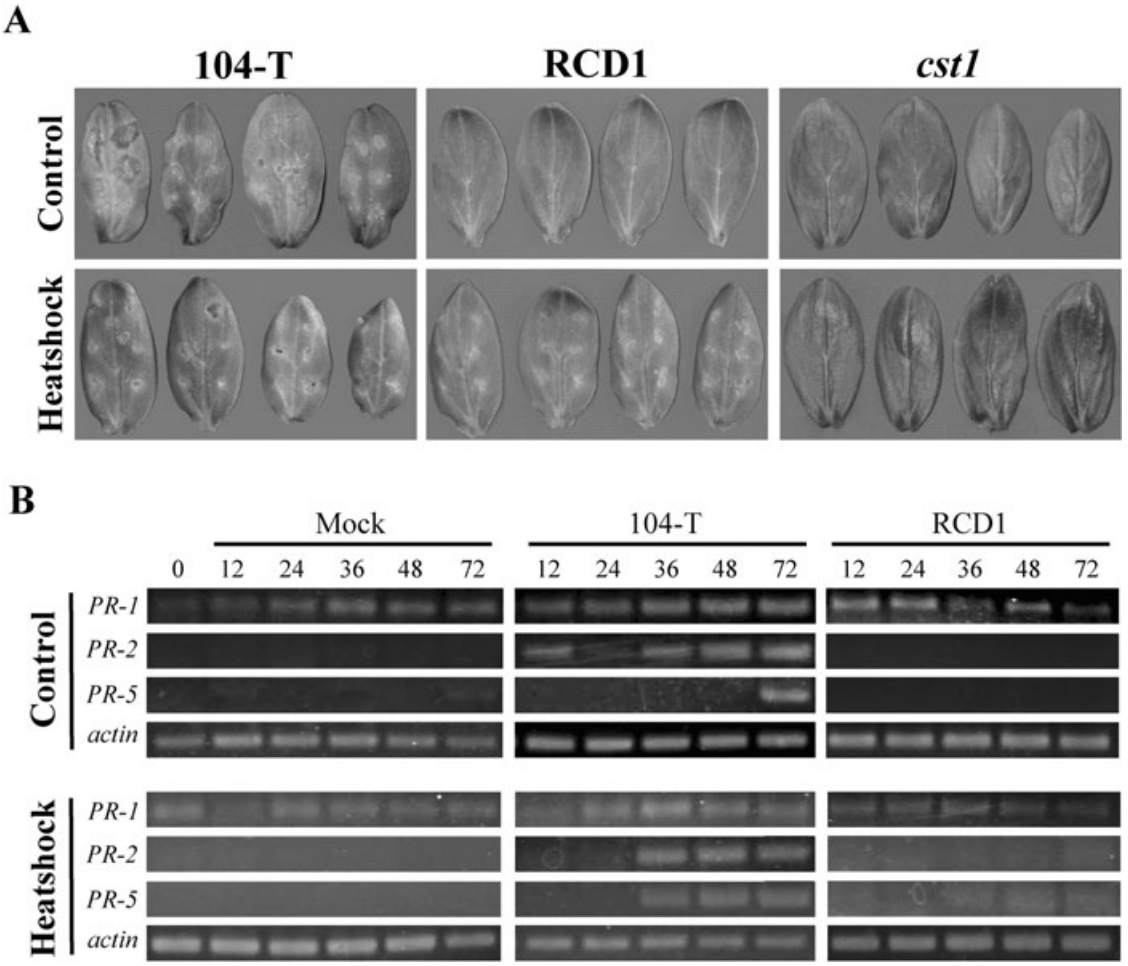

Fig. 5. Pathogenicity of *classd1* mutants on heat-shocked cotyledons and expression of cucumber *PR* genes in infected leaves.
A. Pathogenicity of *classd1* mutants on heat-shocked cotyledons. After heat treatment at 50°C for 30 s, cucumber cotyledons were spotted with droplets of conidial suspension. Control plants were not heat-shocked. After 4 days, the wild-type strain 104-T and *classd1* mutant RCD1 formed lesions on heat-shocked cotyledons while the *cst1* mutant could not form lesions.
B. Expression of cucumber *PR* genes in leaves inoculated with wild-type strain 104-T or *classd1* mutant RCD1 or control leaves receiving a mock inoculation with water. After extraction of total RNA, expression of the plant genes *PR-1*, *PR-2*, *PR-5* and actin was analysed by RT-PCR. Numbers along the top of the panel indicate time (h) after inoculation. The 0 time-point corresponds to untreated leaves.

classd1 mutants in the intensity or pattern of fluorescent labelling of hyphal and conidial cell walls by Calcofluor White, Congo Red or fluorescein-conjugated wheat germ agglutinin, a chitin-specific lectin (data not shown).

Next, we examined the capacity of the *classd1* mutants to induce papilla formation in a non-host interaction with onion epidermis. At 24 h after inoculation, papillae had developed beneath 17% of *classd1* appressoria, increasing to 20% at 48 h (Fig. 6A and B). In contrast, wild-type 104-T appressoria did not induce any papillae until 36 h, and only 7% of appresoria had induced papillae by 48 h (Fig. 6B). The penetration-deficient mutant *cst1* did not induce papillae at any time point. Thus, appressorial penetration by *classd1* mutants elicits papilla formation in onion epidermis more rapidly and with higher frequency than the wild type.

Table 1. Hyphal growth of *classd1* mutants on PDA containing Calcofluor White.

	PDA (mm)	PDA + CW (mm)	Retardation (%)
104-T	7.4	6.4	13.5
RCD1	4.1	1.9	53.7
RCD2	4.2	1.9	54.8

Colletotrichum lagenarium wild-type 104-T and *classd1* mutants RCD1, RCD2 were grown on PDA or PDA containing 100 μg ml^{-1} Calcofluor White (CW). The radius of each fungal colony was measured at 4 days after incubation and the average radius was calculated for 15 colonies. Retardation means the reduction of growth rate on PDA containing Calcofluor White compared with the growth on PDA alone.

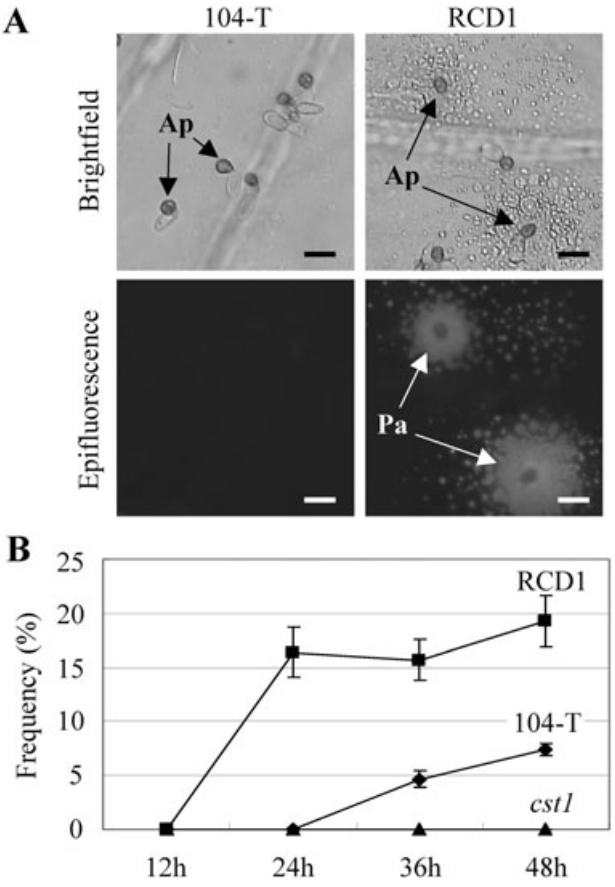

Fig. 6. Cytological assay for plant defence responses in onion epidermal cells.
A. Cellular responses of onion epidermal cells were observed at 24 h after inoculation by light and epi-fluorescence microscopy. Papilla formation (Pa) occurred at sites of attempted penetration by appressoria (Ap) of *classd1* mutant (RCD1), whereas no responses were visible in cells inoculated with wild-type strain 104-T. Bar = 10 μm.
B. Time-course of papilla formation in onion epidermis. The frequency of papillae beneath appressoria of the *classd1* mutant (closed square) and the wild-type 104-T (closed diamond) was evaluated from 12 h to 48 h after inoculation. Papilla formation beneath appressoria of the penetration deficient *cst1* mutant (closed triangle) was not observed at any time point. At least 200 appressoria were counted for each fungal strain and standard deviations were calculated from three replicate experiments.

An orthologue of ClaSSD1 *is also required for pathogenicity in* M. grisea

A gene homologous to *ClaSSD1* was identified in a database of *M. grisea* genes (Accession number MG08084.4). This gene, which we named *MgSSD1*, comprises 3339 bp interrupted by two introns and encodes a predicted protein of 1070 amino acids. To investigate whether *MgSSD1* is involved in the pathogenicity of *M. grisea*, we produced *MgSSD1* disruption mutants. The plasmid pBIG4MgSSD1AH3 was designed to replace the *MgSSD1* gene in the wild-type strain 70–15 with the *mgssd1::AH3* fragment through double crossover homologous recombination (Fig. S6A). By transformation with AtMT, 54 hygromycin-resistant transformants were obtained and of these 15 were also sensitive to bialaphos. Five of the latter transformants were randomly selected as putative *MgSSD1*-disruption mutants and designated RMD1 to 5. A hygromycin-resistant and bialaphos-resistant transformant was also selected and designated EMD1. In Southern blot analysis of genomic DNA using a *MgSSD1*-specific probe, a 2.5 kb PvuII fragment was detected in 70–15, RMD2, RMD3 and EMD1 (Fig. S6B; Lane 1, 3, 4 and 7). An additional band was also detected in RMD2, RMD3 and EMD1, indicating these transformants have an extra copy of *MgSSD1* integrated ectopically. The size of the 2.5 kb PvuII fragment detected in these transformants is consistent with that expected from a gene replacement event (Fig. S6B; lane 2, 5 and 6). These results indicate that *MgSSD1* was disrupted by homologous recombination in RMD1, RMD4 and RMD5.

The colony morphology of *mgssd1* mutants grown on PDA medium for 7 days was similar to that of 70–15 but the growth rate of *mgssd1* disruption mutants was approximately 80% of the wild type (data not shown). The pathogenicity of the *mgssd1* mutants was investigated by inoculating intact leaf sheaths of the susceptible rice line ZTS with conidial suspension (Koga *et al.*, 2004a). In leaf sheaths inoculated with the wild-type 70–15, severe lesions had developed by 6 days after inoculation. In contrast, only small pin-point lesions were observed in leaf sheaths inoculated with the *mgssd1* mutant RMD1 (Fig. 7A). This strongly suggests that *MgSSD1* is required for pathogenicity in *M. grisea*. The *mgssd1* mutants produced similar pin-point lesions on both wounded and intact leaves (data not shown), suggesting that the reduced pathogenicity of the mutants is not due to a defect in the initial penetration process.

Microscopical analysis showed that in leaf sheaths inoculated with 70–15, approximately 40% of the appressoria had produced intracellular primary hyphae inside living epidermal cells after 48 h (Fig. 7B). In contrast, in leaf sheaths inoculated with the *mgssd1* mutant RMD1, most primary hyphae were restricted within dead host cells with brown, granular contents. This cellular response resembled the 'whole plant-specific resistance' response of susceptible rice plants to *M. grisea* (Koga *et al.*, 2004a), which can be suppressed by treating plants with abscisic acid (ABA) (Koga *et al.*, 2004b). In rice plants pretreated with ABA, RMD1, produced severe lesions similar to those of 70–15 (Fig. 7A), and the resistance response was significantly suppressed (Fig. 7B). These findings suggest that the infection of *mgssd1* mutants is restricted by host defence responses that can be suppressed by ABA. The accumulation of H_2O_2 at infection sites, which has been implicated in defence responses of rice against *M. grisea* (Ono *et al.*, 2001), was investigated by staining with 3,3′-

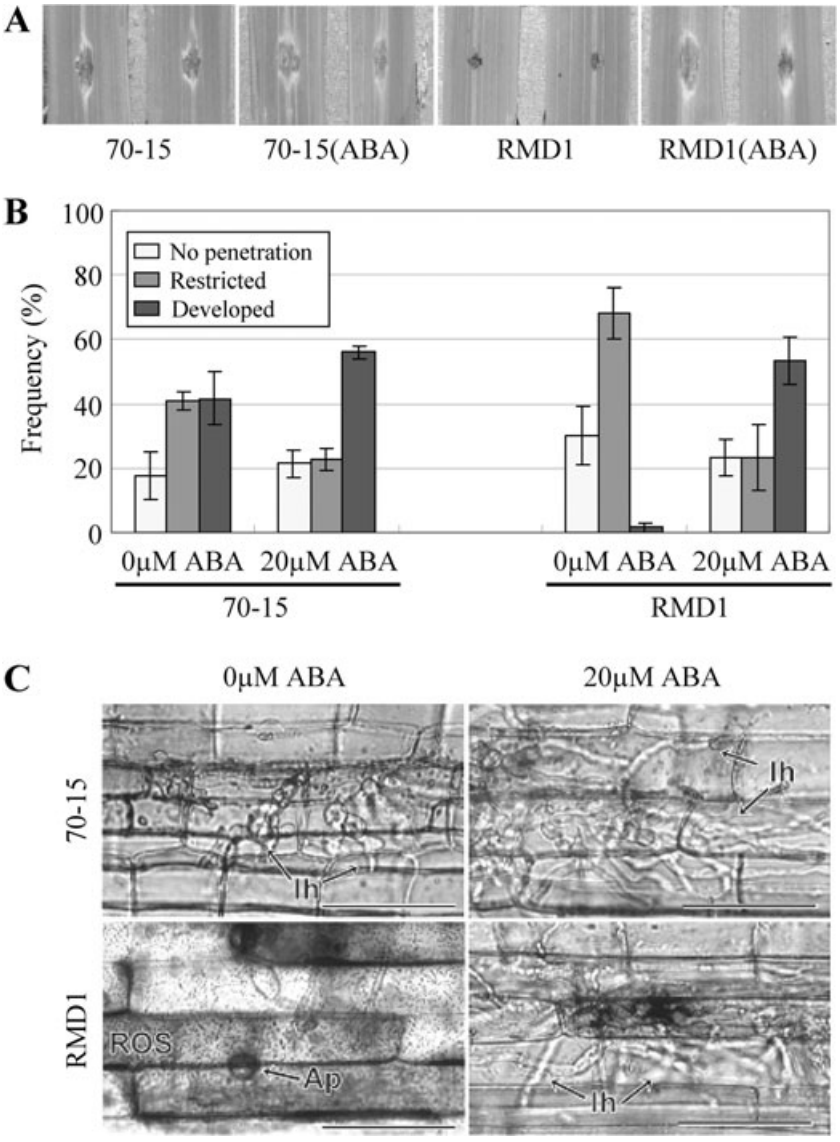

Fig. 7. Infection phenotype of *M. grisea mgssd1* mutant on rice plants. Susceptible rice plants at the 6.5 leaf stage, with or without a pretreatment with 20 μM ABA, were inoculated with either the *M. grisea* wild-type strain 70–15 or the *mgssd1* mutant RMD1.
A. Leaf blades inoculated with *M. grisea*, viewed at 6 days after inoculation.
B. Frequency of infection types in leaf sheaths inoculated with *M. grisea*. Approximately 300 penetrating appressoria were observed microscopically at 48 h after inoculation and classified into three categories as follows. (a) No penetration: appressoria formed but failed to penetrate epidermal cells. (b) Restricted: primary infection hyphae were restricted within dead host cells. (c) Developed: invading primary hyphae grew extensively in living host tissues. Values are the means ± standard deviation from three independent experiments.
C. Accumulation of reactive oxygen species (H_2O_2) in invaded host cells, as detected by DAB-staining of inoculated leaf sheaths. Dead host cells infected by hyphae of the mutant were stained reddish-brown, indicating the presence of H_2O_2, whereas living cells were unstained. Ap, appressoria. Ih, infection hyphae. Bars = 50 μm.

diaminobenzidine (DAB). As shown in Fig. 7C, living host cells containing the extensive primary hyphae of the wild-type 70–15 were not stained with DAB, whereas dead host cells infected by hyphae of the *mgssd1* mutants were stained reddish-brown with DAB, indicating the presence of H_2O_2. The frequency of DAB-stained cells corresponded to the number of dead host cells in each experiment (data not shown). These results indicate that host defence responses restricting infection of the *mgssd1* mutants include cell death and ROS accumulation.

To determine whether Mgssd1p can partially or completely replace the function of Classd1p and *vice versa*, cross-complementation experiments were performed. The complementation plasmids pBIHMgSSD1com and pBIG4ClaSSD1cp, containing full-length copies of *MgSSD1* and *ClaSSD1*, were introduced into *classd1* and *mgssd1* mutants respectively. The pathogenicity of *C. lagenarium classd1* mutants expressing Mgssd1p was indistinguishable from that of the wild-type 104-T when inoculated onto cucumber leaves (Fig. 8A). Similarly, the expression of Classd1p in *M. grisea mgssd1* mutants fully restored their pathogenicity on rice plants to wild-type levels (Fig. 8B). These results demonstrate that *ClaSSD1* and *MgSSD1* can functionally complement each other and strongly suggest that these genes are orthologous.

Discussion

The *SSD1* gene was originally identified in *S. cerevisiae* through interactions with components of the *PKC1* (protein kinase C) signalling pathway as being one of the genes required for cell integrity and proper cell wall assembly (Sutton *et al.*, 1991). However, until now the function of *SSD1* has been investigated only in yeasts. Here we show

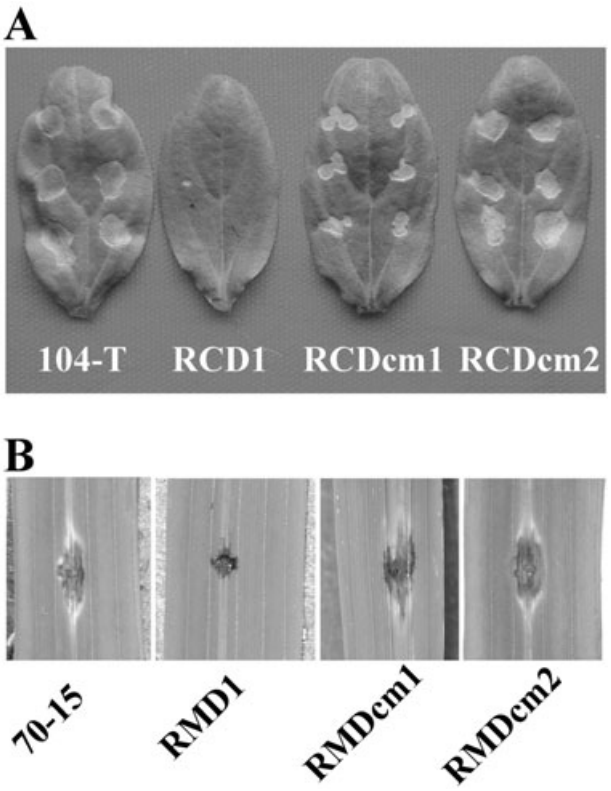

Fig. 8. Cross-complementation of *classd1* and *mgssd1* mutants.
A. Complementation of *C. lagenarium classd1* mutant by *MgSSD1*. The transformants RCDcm1 and RCDcm2 were generated by introduction of the *MgSSD1* gene into *classd1* mutant RCD1. Successful complementation was confirmed by restoration of pathogenicity: the wild-type strain 104-T and transformants RCDcm1 and RCDcm2 formed anthracnose lesions on detached cucumber cotyledons, whereas *classd1* mutant RCD1 produced no visible symptoms. Photograph taken at 6 days after inoculation.
B. Complementation of *M. grisea mgssd1* mutant by *ClaSSD1*. The transformants RMDcm1 and RMDcm2 were generated by introduction of the *ClaSSD1* gene into *mgssd1* mutant RMD1. After inoculating intact leaves of ZTS rice plants, wild-type strain 70–15 and transformants RMDcm1 and RMDcm2 formed typical blast lesions, whereas *mgssd1* mutant RMD1 produced no visible symptoms. Photograph taken at 6 days after inoculation.

for the first time that orthologues of *SSD1* play an essential role in the pathogenicity of two filamentous fungal pathogens by circumventing basal plant defence responses expressed during compatible interactions.

We isolated the homologous gene *ClaSSD1* from *C. lagenarium* by molecular analysis of a pathogenicity-deficient mutant generated by random insertional mutagenesis. Apart from a slight reduction in mycelial growth rate, the *classd1* mutants of *C. lagenarium* did not show significant alterations in their *in vitro* development (e.g. conidiation or appressorium differentiation) and TEM demonstrated that the complex structure of the appressorial cell wall and penetration pore was not affected. Furthermore, penetration assays using cellulose membranes and a cytorrhysis test for appressorial turgor all indicated that *classd1* appressoria retain the potential for penetration. As disruption of *ClaSSD1* did not appear to affect appressorial function, the failure of *classd1* mutants to penetrate host epidermal cells is probably due to plant-derived factors. This conclusion was supported by the finding that *classd1* mutants regained pathogenicity when host defence responses were compromised by a transient heat-shock.

Similarly, in *M. grisea* the deletion of *MgSSD1* reduced pathogenicity on leaf sheaths of compatible rice plants, expressed as a lower frequency of appressorial penetration into host epidermal cells and an increase in the proportion of penetrated cells undergoing hypersensitive cell death and browning accompanied by ROS production. This host cellular response closely resembles the phenomenon of whole plant-specific resistance (WPSR) previously observed in compatible blast–rice interactions and which is independent of rice *R* genes (Koga *et al*., 2004b). Remarkably, pretreatment with abscisic acid (ABA), which suppresses the expression of WPSR (Koga *et al*., 2004b), significantly reduced cellular defence responses in rice plants inoculated with *mgssd1* mutants and enabled them to successfully infect the treated plants. This again suggests that the reduced pathogenicity of the mutants is due to the expression of basal plant defence responses. Although mycelial growth rate was slightly reduced in both *classd1* and *mgssd1* mutants, a simple fitness defect is unlikely to account for their loss of pathogenicity because both mutants retain the potential for invasive growth after inoculation of wounded tissue or ABA-treated plants respectively. *ClaSSD1* and *MgSSD1* are highly homologous and can reciprocally complement the pathogenicity defects of *mgssd1* and *classd1* mutants respectively. Phylogenetic analysis also indicated that the *SSD1* gene is highly conserved among filamentous fungi. Thus, it will be interesting to determine whether genes orthologous to *SSD1* also contribute to the virulence of other plant pathogenic fungi.

Ssd1p has a conserved domain that shows weak but significant homology to RNase II-related proteins such as *N. crassa* Cyt4, *Shigella flexneri* VacB and *Escherichia coli* RNase II (Doseff and Arndt, 1995), which preferentially bind poly(A) mRNA and possibly act as post-transcriptional regulators (Uesono *et al*., 1997). However, the detailed function of *SSD1* remains unknown. In *S. cerevisiae*, several different signalling pathways have been implicated in promoting cell wall integrity and Ssd1p is a component of one such pathway (Kaeberlein and Guarente, 2002). The RAM (Regulation of Ace2p transcriptional factor and polarized morphogenesis) signalling network regulates the maintenance of polarized growth and daughter-cell-specific transcription and functions cooperatively with Ssd1p to control cell integrity (Kurischko *et al*., 2005). Similarly, deletion of a *SSD1* homologue in *C. neoformans*

indicated that the Ssd1p-containing pathway is involved in maintaining cell integrity in this basidiomycete yeast (Gerik *et al*., 2005). In the present study, a complementation assay in *S. cerevisiae* clearly showed that the *ClaSSD1* gene of *C. lagenarium* is the functional orthologue of *SSD1*. In yeast, it has been shown that Ssd1p resides in the cytoplasm (Huh *et al*., 2003). Similarly, we found that in *C. lagenarium*, a GFP : Classd1p fusion protein was restricted to the fungal cytoplasm. Thus, it is likely that Classd1p would remain inside the fungus and not be secreted into the fungal cell wall or exported into plant cells. However, it remains possible that Classd1p regulates the synthesis or assembly of other fungal proteins or polysaccharides into the cell wall or interface with host cells, which could affect plant recognition or sensitivity of the pathogen to plant defence components.

Whereas yeast *ssd1* mutants are known to be hypersensitive to a range of environmental stresses, we found that *C. lagenarium classd1* mutants were not significantly more sensitive to caffeine, KCl or H_2O_2 than the wild type. However, they did show greater sensitivity to the chitin-binding dye Calcofluor White, in common with yeast *ssd1* mutants (Kaeberlein and Guarente, 2002), suggesting that *classd1* mutants have a slightly modified cell wall composition or architecture that renders them more susceptible to this inhibitor. More detailed biochemical analysis of the cell walls of these mutants will be required to detect subtle changes in the content of chitin, glucans or other polymers. On the other hand, we found that *classd1* mutants induced papilla formation in onion epidermis more rapidly and with higher frequency than the wild-type. Therefore, the impairment of appressorial penetration observed in *classd1* mutants may result from a stronger elicitation of plant defence responses rather than a higher sensitivity to those responses.

Cell walls of the *S. cerevisiae ssd1* mutant are depleted in major structural polysaccharides such as beta-1,3-glucan and beta-1,6-glucan but are enriched with chitin and mannoproteins (Wheeler *et al*., 2003). The yeast mutant is also more virulent to mice and it was suggested by these authors that the altered cell surface composition leads to misrecognition by the innate immune system and greater induction of proinflammatory cytokine, resulting in hypervirulence. Chitin is also a major cell wall component in filamentous fungi and constitutes a pathogen-associated molecular pattern that can be recognized by the innate immune systems of both animals and plants (Nurnberger *et al*., 2004). In a recent report, the chitin receptor CEBiP was isolated from the plasma membrane of suspension cultured rice cells (Kaku *et al*., 2006). Knockdown of *CEBiP* by RNAi results in suppression of both ROS generation and changes in plant gene expression that are normally induced by the chitin elicitor, suggesting that CEBiP is required for the recognition of chitin by rice cells and subsequent induction of defence responses. It is tempting to speculate that the impaired host penetration and intracellular growth shown by *classd1* and *mgssd1* mutants might involve basal plant defence responses that are induced by recognition of fungal cell surface components such as chitin. Although no gross differences in wall composition were detectable cytochemically in *ssd1* mutants grown *in vitro*, changes in the surface composition of their infection structures produced during growth *in planta* could have a profound effect on the interaction.

The cell walls of *S. cerevisiae* and filamentous fungi contain PIR proteins (proteins with internal repeats) that protect against the cytotoxic effect of plant PR-5 proteins by blocking the binding of PR-5 to cell wall phosphomannans (Yun *et al*., 1997; Ibeas *et al*., 2001). Thus, the heterologous expression of yeast PIR2 in the plant pathogen *Fusarium oxysporum* was shown to increase both virulence and resistance to osmotin (Narasimhan *et al*., 2003). It appears that Ssd1p does not regulate the transcriptional activity of *PIR* genes but is essential for the assembly of PIR proteins into the fungal cell wall (Ibeas *et al*., 2001). It is possible that Classd1p similarly regulates assembly into the wall of components conferring resistance to plant antifungal PR proteins. However, because *PR-5* gene expression was not detectable in cucumber leaves inoculated with the *classd1* mutant, we consider that PR-5 is unlikely to be involved in inhibiting appressorial penetration by the mutant.

Reinforcement of the plant cell wall is a common response of epidermal cells to attempted fungal penetration during compatible, incompatible and non-host interactions (Heath, 1980). This response typically comprises a localized deposition of callose-rich material between the plasma membrane and cell wall beneath the penetration site (termed a papilla) and the accumulation of proteins, phenolics and other secondary metabolites within the papilla and surrounding cell wall, forming an autofluorescent halo (Zeyen *et al*., 2002). Formation of papillae and cell wall haloes involves a stereotypical polarization of the plant cytoplasm in which the host nucleus, organelles and secretory vesicles become translocated towards the site of fungal penetration (Schmelzer, 2002). In several plants, this cytoplasmic aggregation has been shown to depend upon rearrangement of the actin cytoskeleton (Gross *et al*., 1993; Kobayashi *et al*., 1994). By the analysis of actin-binding proteins in potato, an osmotin-like protein and basic chitinase were implicated in cytoplasmic aggregation (Takemoto *et al*., 1997). One possibility is that growth of the *classd1* mutant becomes inhibited by the local accumulation of antifungal compounds within papillae and the surrounding cell wall haloes. The cell wall of the fungal penetration peg is extremely thin (O'Connell *et al*., 1985) and could be particularly sensitive to plant PR proteins

such as osmotin or lytic enzymes. TEM-immunogold labelling with appropriate antibodies is necessary to determine whether such proteins do actually accumulate at *C. lagenarium* penetration sites.

The infection phenotype of the *mgssd1* mutants differed significantly from that of *classd1* mutants in that they were incapable of invasive growth following wound inoculation and a high proportion of *mgssd1* appressoria (approximately 65%) could form short primary hyphae inside epidermal cells, whereas primary hyphae formation by *classd1* mutants was almost entirely blocked by host papillae. Possibly these contrasting infection phenotypes result from qualitative differences in basal plant defence responses between cucumber and rice. In rice it appears that post-penetration responses accompanied by cell death, such as WPSR, are more significant for basal resistance to fungal invasion (Koga *et al*., 2004a,b), whereas in cucumber wall-associated defences such as papilla and halo formation may predominate.

To define more precisely the plant factors that restrict development of *Colletotrichum ssd1* mutants, we will take advantage of the fact that *C. lagenarium* can also infect *Nicotiana benthamiana* (Shen *et al*., 2001). Virus-induced gene silencing (VIGS) is a well-established method for gene knock-down in this plant host (Dean *et al*., 2005) and could be used to critically test the role of specific plant defence genes in limiting the growth of *classd1* mutants.

Experimental procedures

Fungal strains and growth conditions

Colletotrichum lagenarium (Pass.) Ellis and Halsted strain 104-T (Laboratory of Plant Pathology, Kyoto Prefectural University) and *M. grisea* (Hebert) Barr strain 70–15 (Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City) were used as the wild-type strains in this study. The *cst1* was described previously (Tsuji *et al*., 2003a). All cultures were maintained at 24°C on potato dextrose agar (PDA) medium (Difco, Detroit, MI).

Isolation of Lf2754

Agrobacterium tumefaciens-mediated transformation (AtMT) was used to generate 10 650 hygromycin-resistant transformants of *C. lagenarium* (Tsuji *et al*., 2003b). The bacterial culture of *A. tumefaciens* strain C58C1 carrying binary vector pBIG2RHPH2 (Tsuji *et al*., 2003b) and fungal conidia adjusted to 10^7–10^8 conidia ml^{-1} in induction medium (IM) (Bundock *et al*., 1995) were mixed and spread over a filter paper disc supported on IM agar. After incubation at 24°C for 2 days in the dark, the filter paper was transferred to PDA medium (containing 50 μg ml^{-1} each of hygromycin, cefotaxime and spectinomycin) and incubated at 24°C for 2 days. The growing colonies were selected as hygromycin-resistant transformants. By screening the pathogenicity of these transformants on cucumber cotyledons, 30 pathogenicity-deficient mutants were identified. Lf2754 was one of these mutants.

Cloning and sequencing

Genomic DNA adjacent to the T-DNA insert was isolated by TAIL-PCR (Tsuji *et al*., 2003b). The sequence of the amplified product was determined and used to design the primer pair 2754-S1 (5′-CCAAACAATCTTGGGCTTCT-3′) and 2754-AS1 (5′-CGAGTCAACATGTTCTCTGGTA-3′). These primers were used to screen a cosmid genomic library of *C. lagenarium*. A cosmid clone, p2754cos, containing the *ClaSSD1* gene was isolated and subjected to Southern blot analysis to obtain the genomic DNA segment containing *ClaSSD1*. p2754cos was digested with BamHI, KpnI, SmaI, XbaI and XhoI, and hybridized with the PCR product amplified using 2754-S1 and 2754-AS1. pBS2754Xh was constructed by introducing the detected XhoI segment (approximately 5 kb) into the XhoI-site of pBluescript SK II$^+$.

Using the BLASTX program in the *M. grisea* genome database (Broad Institute; http://www.broad.mit.edu/annotation/fungi/magnaporthe/), MG08084.4 was identified as a candidate orthologue of *ClaSSD1* and named *MgSSD1*. The sequence was used to design the following primer pair: MgSSD1-S1 (5′-GAAGATCTCCACTGCCTGGACAGACATA-3′), attached by the underlined BglII site, and MgSSD1-AS1 (5′-CGGAATTCTGAACATCCTTCTGCGACAC-3′), attached by the underlined EcoRI site. The PCR product amplified by MgSSD1-S1 and MgSSD1-AS1 was digested with BglII and EcoRI, and introduced into the BamHI–EcoRI site of pCB1004. The resulting construct was named pCBMgSSD1.

ClaSSD1 was sequenced following the procedure for transposon arrayed gene knockouts (TAGKO) (Hamer *et al*., 2001). The transposon EZ::TN <KAN-2> (Epicenter, Madison, WI) was modified as follows. The kanamycin phosphotransferase gene inside the transposon, which confers kanamycin resistance in *Escherichia coli*, was replaced with the bialaphos resistance gene cassette and the chloramphenicol resistance gene cassette, which confer resistance to bialaphos in *C. lagenarium* and chloramphenicol in *E. coli* respectively. The modified transposon named BC1 (2.2 kb) was inserted into pBS2754Xh. The randomly inserted clones were selected and sequenced using the transposon-specific primers KAN2-RP2 (5′-GGAAGATCTTTGTGCAATGTAACATCAGAG-3′) and KAN2-FP2 (5′-CCGAATTCTACAACAAAGCTCTCATCAACC-3′) present at the ends of the transposon. A downstream region of *ClaSSD1* that was not contained in the XhoI segment was sequenced by primer walking using p2754cos as template. The 5′ sequence of *ClaSSD1* was determined by 5′-RACE following the manufacturers' protocol (5′-Full RACE Core Set; Takara, Ohtsu, Japan). ClaSSD1rcP-AS1 (5′-(P)GCCCCGACGACGAAG-3′) was used as RT primer. First-strand cDNA was synthesized by AMV reverse transcriptase and hybrid RNA was degraded by RNase H. Synthesized cDNAs were ligated by T4 RNA ligase and the 5′ region amplified by PCR. The introns were confirmed by sequencing of cDNAs amplified by RT-PCR (SMART PCR cDNA Library Construction kit; Clontech, Mountain View, CA). Total RNA used for cDNA synthesis was extracted from germinating conidia after 3 h incubation using

the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Sequence analysis was performed with the Big-Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, UK) and an ABI PRISM 310 automated DNA sequencer (Applied Biosystems).

Phylogenetic analysis

The amino acid sequences of *SSD1* homologues were searched for in the Candida Genome Database (http://www.candidagenome.org), Broad Institute Fungal genome database (http://www.broad.mit.edu/annotation/fungi/fgi/) and TIGR database (http://www.tigr.org/tdb/fungal/index.shtml). The phylogenetic analysis was performed using ClustalW program (Thompson *et al*., 1994) based on sequences from *S. cerevisiae*, *C. albicans* (CAL0003016), *C. neoformans* (CNG02350), *A. nidulans* (AN1158.2), *A. fumigatus* (Afu1g11420), *N. crassa* (NCU01197.1), *U. maydis* (UM01220.1), *F. graminearum* (Fg07009.1), *M. grisea* (MG08084.4) and *C. lagenarium*. A phylogenetic tree was drawn using TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Construction of vectors for targeted gene disruption

For *C. lagenarium*, a binary vector pBIG4MRHrev carrying the hygromycin resistance gene cassette inside the T-DNA was used for construction of a gene replacement plasmid. pBS2754XhBC1 was selected from randomly inserted clones as described above. In pBS2754XhBC1, the transposon was inserted 862 bp from the start codon. The approximately 5 kb XhoI-fragment carrying a portion of *ClaSSD1* in pBS2754XhBC1 was introduced into the pBIG4MRHrev XhoI site. The resulting construct was named pBI2754XhBC1 and used for gene replacement.

For *M. grisea*, a binary vector pBIG4MRBrev carrying the bialaphos resistance gene cassette inside the T-DNA was used for construction of a gene replacement plasmid. pCB-MgSSD1 was digested with EcoRI and SpeI, and the segment was introduced into the EcoRI–XbaI site of pBIG4MRBrev. The resulting plasmid was named pBIG4MgSSD1. The modified transposon named AH3 (2.7 kb), carrying the hygromycin resistance gene cassette and the ampicillin resistance gene cassette, which confer resistance to hygromycin in *M. grisea* and ampicillin in *E. coli*, respectively, was inserted into pBIG4MgSSD1. A clone in which the transposon was inserted 229 bp from the start codon was named pBIG4MgSSD1AH3 and used for gene replacement.

Targeted gene disruption

Following AtMT using pBI2754XhBC1 and pBIG4MgSSD1AH3, respectively, *C. lagenarium* transformants showed bialaphos resistance while *M. grisea* transformants showed hygromycin resistance. Following a gene replacement event, the antibiotic resistance gene located at the end of the T-DNA insert was excluded, so that putative gene replacement transformants could be selected by their sensitivity to the corresponding antibiotic. For *C. lagenarium*, transformants were screened on SD medium (containing 4 μg ml^{-1} bialaphos, 50 μg ml^{-1} cefotaxime and spectinomycin respectively) and putative gene replacement transformants were screened on PDA medium (containing 50 μg ml^{-1} each of hygromycin, cefotaxime and spectinomycin). For *M. grisea*, transformants were screened on PDA medium (containing 500 μg ml^{-1} hygromycin, and 50 μg ml^{-1} each of cefotaxime and spectinomycin) and putative gene replacement transformants were screened on Czapek medium (containing 4 μg ml^{-1} bialaphos, and 50 μg ml^{-1} each of cefotaxime and spectinomycin).

Gene replacement was confirmed by Southern blot analysis. To obtain mycelia, the conidia of transformants were incubated and shaken at 70 r.p.m. in PS broth (potato starch 200 g l^{-1}, 2% sucrose) at 24°C for 5 days. Total genomic DNA was isolated from the mycelia as described previously (Takano *et al*., 1997). In *C. lagenarium*, genomic DNA was digested with KpnI and hybridized with the PCR product amplified by ClaSSD1mapb-S1 (5′-ACTTCGTCGAGAAAGCACCA-3′) and ClaSSD1mapb-AS1 (5′-ACGCGGAAGAGGACACTG-3′). In *M. grisea*, genomic DNA was digested with PvuII and hybridized with the 0.9 kb fragment obtained when the PCR product amplified by MgSSD1-S1 and MgSSD1-AS1 was digested with KpnI. Hybridization was performed following the procedure described by Takano *et al*. (1997). PCR probes were labelled with DIG-dUTP using the *Bca*BEST™ DIG Labeling Kit (Takara).

Functional complementation assays

For the yeast complementation assay, a cDNA corresponding to the *ClaSSD1* ORF was amplified by RT-PCR using the primer pair ClaSSD1orf-S1 (5′-ACGGATCCGTCAGCA TGGTGGTCAAATG-3′), attached by the underlined BamHI site, and ClaSSD1orf-AS1 (5′-GTACTAGTATCGTAGC GGCTAACCACA-3′), attached by the underlined SpeI site. The amplified PCR product was introduced into the BamHI–XbaI site of yeast shuttle vector pYES2 (Invitrogen, Carlsbad, CA) and the resulting construct was named pYES2ClaSSD1. As a positive control, a plasmid expressing *SSD1* was also constructed. The genomic DNA of *SSD1* was amplified by PCR using the primer pair ScSSD1-S1 (5′-GAAGATCT TTTGGCCCAATTATTCCATC-3′), attached by the underlined BglII site, and ScSSD1-AS1 (5′-GTCTCGAGCAAGAAA AACAGCAATGACGA-3′), attached by the underlined XhoI site. The amplified PCR product was subcloned into pGEM-T easy (Promega, Madison, WI) and the resulting construct was named pGEMScSSD1. The BglII–XhoI fragment containing *SSD1* derived from pGEMScSSD1 was introduced into the BamHI–XhoI site of pYES2 (Invitrogen) and the resulting construct was named pYES2ScSSD1. Yeast transformation was performed using the standard lithium acetate method. To confirm successful complementation, the following protocols were performed. Yeast strains were incubated overnight in YPD liquid medium at 28°C. The cells were then diluted to 10^6, 10^5, 10^4, 10^3 cells ml^{-1} and 5 μl droplets were spotted onto either YPG medium or YPG containing 15 mM caffeine, and incubated at 28°C for 7 days.

For complementation of the *C. lagenarium classd1* mutant with the *MgSSD1* gene, a genomic DNA fragment containing the full-length *MgSSD1* gene was amplified by PCR using the primer pair MgSSD1com-S1 (5′-GGAATTCTTCTTCTTT

GCCTTCACTTCTTG-3′) and MgSSD1com-AS1 (5′-GGAA TTCACTGCGTCTTGACTGTTGGA-3′), each attached by the underlined EcoRI site. The amplified PCR product was introduced into the EcoRI site of pBIG4MRHrev and the resulting construct was named pBIHMgSSD1com. For complementation of the *M. grisea mgssd1* mutant with the *ClaSSD1* gene, a genomic DNA fragment containing the full-length *ClaSSD1* gene was amplified by PCR using primer pair ClaSSD1cp-S2 (5′-GTACTAGTCCACGAGCAGCTC ACAGGATA-3′) and ClaSSD1cp-AS1 (5′-GTACTAGTCAC GGATTTTGCGTGTGTAG-3′), each attached by the underlined SpeI site. The amplified PCR product was subcloned into pGEM-T easy (Promega) and the resulting construct was named pGEMClaSSD1cp. The SpeI fragment containing *ClaSSD1* derived from pGEMClaSSD1cp was introduced into the XbaI site of pBIG4MRBrev and the resulting construct was named pBIG4ClaSSD1cp. The *classd1* mutant RCD1 and *mgssd1* mutant RMD1 were transformed by AtMT using pBIHMgSSD1com and pBIG4ClaSSD1cp respectively. The single-copy integration of each gene was confirmed by Southern blot analysis. Functional complementation was assessed in pathogenicity tests.

Analysis of Classd1p localization

For the observation of Classd1p localization by confocal microscopy, a plasmid expressing the GFP : Classd1p fusion protein was constructed as follows. A modified *SCD1* promoter, lacking its regulatory sequence in order to confer constitutive expression, was amplified by PCR using primer pair xbSCD1pS1 (5′-CGTCTAGAGTGTTTTGCGGCAGT CC-3′), attached by the underlined XbaI site, and spSCD1pAS1 (5′-GGACTAGTCTGATAGGTGGGATATTAC GTG-3′), attached by the underlined SpeI site. The binary vector pBISCD1GFPglyH was in turn constructed containing the modified *SCD1* promoter, *eGFP* and the *TEF* terminator within the T-DNA region of pBIG4MRHrev. pBISCD1GFPglyH contains a BamHI site between *eGFP* and the *TEF* terminator. *ClaSSD1* was amplified using primer pair ClaSSD1ic-S2 (5′- GGGGGATCCATGGGTGGGAACCAG CAG-3′) and ClaSSD1tc-AS2 (5′-GGGGGATCCTTACAGC GCGTAGGGATTCA-3′), attached by the underlined BamHI site. The amplified PCR product was introduced into the BamHI site of pBISCD1GFPglyH and named pBISCD1pGFP : ClaSSD1H. The in-frame ligation of *GFP-ClaSSD1* was confirmed by sequence analysis. Transformation of the *C. lagenarium classd1* mutant with this plasmid was achieved by AtMT. Confocal images were acquired using a Leica TCS SP2 confocal microscope equipped with a ×63 (1.2 N.A.) water-immersion objective. Excitation was at 488 nm and GFP fluorescence was collected between 495 and 520 nm. All images are projections of optical sections taken at 1-μm intervals and were processed using Adobe Photoshop 7.0 software.

Reverse transcription polymerase chain reaction

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and cDNA synthesis and subsequent PCR amplification were conducted using ReverTra Dash (Toyobo, Tsuruga, Japan). The OligodT 20 primer was used for cDNA synthesis. PCR was run for 35 cycles of 10 s at 98°C, 2 s at 50°C and 15 s at 74°C. For amplification of the *ClaSSD1* gene, ClaSSD1ftsq-S4 (5′-TGTTGACGCTCTCTTTGACG-3′) and ClaSSD1fl-AS1 (5′-TTCGCTCCATCTTCTGCTTT-3′) were used as PCR primers. For amplification of the *G3PDH* gene, Clg3pdh-S1 (5′-GTCGTATCGTCTTCCGCAAC-3′) and Clg3pdh-AS1 (5′-ACCTTCTTGCCGTTGATGAC-3′) were used. For the amplification of *PR* genes, we designed the following primer pairs on the basis of sequences deposited at GenBank: *PR-1* (Accession number: AF475286), CsPR1-S1 (5′-TCAGTTGTGGGTGGATGAGA-3′) and CsPR1-AS1 (5′-AATGATGAATGTGCCACCAA-3′); *PR-2* (AB009974), CsPR2-S1 (5′-GAATGGTGGAGGATCATTGG-3′) and CsPR2-AS1 (5′-GCCCTTCCTTGTCTCTTTGG-3′); *PR-5* (AY462134), CmPR5-S2 (5′-TCAACAACCGGGTTTGAG TT-3′) and CmPR5-AS1 (5′-GCTAGTGTGGCTGGTGGA AC-3′); *Actin* (AY859055), Cmact-S1 (5′-ATCGTCCTCAG TGGTGGTTC-3′) and Cmact-AS1 (5′-ACATCTGCTGGAAG GTGCTT-3′).

Pathogenicity assays and cytology

Pathogenicity tests for *C. lagenarium* were performed as described by Tsuji *et al*. (2003a). Detached cotyledons from 7 day-old cucumber (*Cucumis sativus* L. Suyo) seedlings or adult leaves from 14 day-old cucumber plants were inoculated with droplets (10 μl) of conidial suspension (5×10^5 conidia per ml) and incubated in a humid box at 24°C for 4 days. For the estimation of invasive growth, the surface of cucumber cotyledons was scratched with a sterilized plastic pipette tip and droplets of conidial suspensions were placed directly onto the wound sites. To apply a transient heat shock, cucumber cotyledons were immersed in a water bath at 50°C for 30 s (Chen *et al*., 2003). The plants were kept at room temperature until surplus water had evaporated (1 h) and then inoculated as above. For cytological analysis, the lower epidermis of cucumber cotyledons were peeled off, stained with lactophenol-Aniline blue (Takano *et al*., 1997), and observed by bright-field microscopy (Eclipse E600; Nikon, Tokyo, Japan). For the detection of callose papillae, epidermal strips were stained with 0.01% (w/v) Aniline blue in 0.15 M K_2HPO_4 and viewed by epi-fluorescence microscopy. For cytological analysis of papilla formation in onion epidermis, split pieces of onion bulb was carefully washed with distilled water and droplets of conidial suspensions were placed on the abaxial surface. After incubation in a humid plastic box, the epidermis was peeled off and observed by light microscopy.

Pathogenicity tests of *M. grisea* were carried out using the sixth leaf of susceptible rice plants (*Oryza sativa* L. ZTS) harvested at the 6.5 leaf stage. To observe lesion formation, droplets of spore suspension (5 μl) containing 500 conidia were placed onto wounds made by pricking the leaf blades with a sterilized plastic pipette tip. ABA treatment of rice leaves was carried out as described by Koga *et al*. (2004b). The inoculated plants were incubated at 24°C. For cytological observation, intact leaf sheaths were inoculated with *M. grisea* as described by Koga *et al*. (2004a). Briefly, leaf sheaths were peeled from the sixth leaves of rice plants harvested at the 6.5 leaf stage with the leaf blades and roots still attached. The leaf sheaths were then filled with conidial suspension (500

conidia ml^{-1}). The viability of host cells was assessed by plasmolysis and the extent of fungal development were observed by microscopy, as described previously (Koga *et al*., 2004a,b). To detect H_2O_2 cytochemically, pieces of the leaf sheath (10 mm) were vacuum-infiltrated with 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB)-HCl solution, pH 3.8, incubated for 8 h at room temperature in the dark and observed by light microscopy (Thordal-Christensen *et al*., 1997).

Assessment of appressorial function

To assess the ability of appressoria to penetrate artificial cellulose membranes, conidial suspension was placed onto dialysis membrane (Wako, Osaka, Japan) and incubated at 24°C for 48 h in the dark. The presence of hyphae within the membrane beneath appressoria, indicating successful penetration, was determined by light microscopy (Kubo *et al*., 1981). Cellulose dissolution around the hyphae was assessed by staining the membranes with a solution containing $ZnCl_2$ (50% w/v), KI (20% w/v) and I_2 (0.5% w/v). Appressorial turgor was determined using a cytorrhysis assay (Howard *et al*., 1991). Appressoria were allowed to form on multiwell glass microscope slides (ICN Biomedicals, Aurora, OH) and incubated in a humid box at 24°C for 48 h in the dark. Surplus water was removed and replaced with glycerol solutions varying in concentration from 1 M to 5 M. After 15 min incubation, the number of collapsed appressoria was counted. This experiment was replicated three times.

Transmission electron microscopy

Samples were prepared for TEM as described by O'Connell *et al*. (1985). Briefly, pieces of cucumber cotyledon (approximately 1×1 mm^2) were excised from beneath inoculation sites at 3 days after inoculation, fixed in glutaraldehyde (2.5% v/v) in 0.05 M cacodylate buffer (pH 7.2) for 3 h, postfixed in osmium tetroxide (1% w/v) for 2 h, dehydrated through ethanol and propylene oxide and embedded in Spurr's epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEM-1200EX II (JEOL, Tokyo, Japan).

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Pathogenicity and mycelial growth of *C. lagenarium* wild-type strain 104-T and insertional mutant Lf2754.

Fig. S2. Nucleotide and deduced amino acid sequence of *ClaSSD1*.

Fig. S3. Predicted amino acid sequence alignment of Classd1p with homologues from other filamentous fungi.

Fig. S4. Targeted gene disruption of *ClaSSD1* by AtMT.

Fig. S5. The subcellular localization of Classd1p in *C. lagenarium*.

Fig. S6. Targeted gene disruption of *MgSSD1* by AtMT.

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