The *Colletotrichum lagenarium* Ste12-Like Gene *CST1* Is Essential for Appressorium Penetration

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Submitted 23 September 2002. Accepted 16 December 2002.

Colletotrichum lagenarium is the causal agent of anthracnose of cucumber. This fungus produces a darkly melanized infection structure, appressoria, to penetrate the host leaves. The *C. lagenarium* *CMK1* gene, a homologue of the *Saccharomyces cerevisiae* *FUS3/KSS1* mitogen-activated protein (MAP) kinase genes, was shown to regulate conidial germination, appressorium formation, and invasive growth. In *S. cerevisiae*, Ste12p is known to be a transcriptional factor downstream of Fus3p/Kss1p MAP kinases. To evaluate the CMK1 MAP kinase pathway, we isolated the Ste12 homologue *CST1* gene from *C. lagenarium* and characterized. The *cst1Δ* strains were nonpathogenic on intact host leaves, but could form lesions when inoculated on wounded leaves. Conidia of the *cst1Δ* strains could germinate and form melanized appressoria on both host leaf surface and artificial cellulose membrane, but could not produce infectious hyphae from appressoria, suggesting that *CST1* is essential for appressorium penetration in *C. lagenarium*. In addition, matured appressoria of the *cst1Δ* strains contained an extremely low level of lipid droplets compared with that of the wild-type strain. Lipid droplets were abundant in conidia of the *cst1Δ* strains, but rapidly disappeared during appressorium formation. This mischeduled lipid degradation might be related to the failure of appressorium penetration in the *cst1Δ* strain.

Additional keywords: Cys2His2, homeodomain, zinc fingers.

Colletotrichum spp. cause anthracnose diseases that typically appear as small or large dark-colored spots or slightly sunken lesions on the foliage, stems, or fruit (Agrios 1988). These fungi have had a substantial impact on world agricultural and horticultural sciences, through both their capacity to cause economic crop losses and their utility as model systems for fundamental studies in physiological, genetic, and molecular plant pathology.

Colletotrichum lagenarium is the causal agent of cucumber anthracnose disease. The infection process is composed of several key steps; conidial attachment on host plants; germination; and appressorium formation, penetration, and subsequent invasive growth in host plants. This fungus produces darkly melan-

ized appressoria. The melanin is essential for appressorium-mediated penetration (Kubo and Furusawa 1991), and three melanin biosynthesis structural genes (*PKS1*, *THR1*, and *SCD1*) and one regulatory gene (*CMR1*) were isolated and characterized from *C. lagenarium* (Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995; Tsuji et al. 2000). The causal agent of rice blast, *Magnaporthe grisea*, is known to produce melanized appressoria, and follows similar infection process with *C. lagenarium*. Melanized appressoria of *M. grisea* generate a high turgor pressure to penetrate into the intact host plant surface (Howard et al. 1991). Glycerol is considered to be an osmolyte for generation of enormous turgor pressure in appressoria, and the melanin layer of appressorium is required for preventing the efflux of intracellular glycerol (de Jong et al. 1997; Howard and Ferrari 1989; Money 1997). Also, recent biochemical and cytological studies suggested that glycogen and lipid reserves stored in conidia could contribute to glycerol production in appressoria (Thines et al. 2000; Weber et al. 2001).

Recent molecular genetic studies have demonstrated that the mitogen-activated protein (MAP) kinase signal transduction pathways play pivotal roles for fungal pathogenesis (Xu 2000). In *M. grisea*, two MAP kinase genes *PMK1* and *MPS1*, homologues of the yeast MAP kinase gene *FUS3/KSS1* and *SLT2*, were isolated and characterized for pathogenicity, respectively (Elion et al. 1993; Lee et al. 1993). Analysis of knockout mutants of these genes in *M. grisea* indicated that the PMK1 MAP kinase pathway is involved in appressorium formation and invasive growth in host plants (Xu and Hamer 1996), and the MPS1 MAP kinase pathway is in appressorium penetration (Xu et al. 1998). In *C. lagenarium*, the MAP kinase gene *CMK1* encoding the PMK1 MAP kinase homologue has been shown to regulate diverse steps of fungal pathogenesis, including conidial germination, appressorium formation, and invasive growth in host plants (Takano et al. 2000). PMK1 homologues were shown to be necessary for appressorium formation and invasive growth in other foliar fungal pathogens, *Cochliobolus heterostrophus* and *Pyrenophora teres* (Lev et al. 1999; Ruiz-Roldan et al. 2001). The pathway also was essential for pathogenicity in the necrotrophic pathogen *Botrytis cinerea* and the vascular wilt pathogen *Fusarium oxysporum* (Di Pietro et al. 2001; Zheng et al. 2000). Thus, recent studies have indicated that the MAP kinase pathways are conserved among many plant-pathogenic fungi for plant infection, and advanced studies have been directed toward the characterization of downstream components responsible for these pathogen-related signal pathways.

The MAP kinase pathways are best characterized in *Saccharomyces cerevisiae*, and shown to regulate diverse cell responses such as mating, filamentous growth, cell integrity, and

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Nucleotide and amino acid sequence data are available in the DDBJ/EMBL/GenBank databases under accession no. AB090340.

high osmolarity growth (Gustin et al. 1998). *S. cerevisiae* Ste12p is a downstream transcription factor regulated by MAP kinases Fus3p and Kss1p in a cell-type-specific manner (Cook et al. 1997; Madhani and Fink 1997, 1998). The FUS3/KSS1 MAP kinase pathways share the MAP kinase kinase Ste7p, and MAP kinase kinase Ste11p, but diverge into two MAP kinases, Fus3p and Kss1p (Gustin et al. 1998). Ste12p is a direct target of Fus3p for mating in haploid cells, and Kss1p for filamentous growth in diploid (Cook et al. 1997; Madhani and Fink 1997, 1998). *STE12* homologues have been identified

from other fungi, and some play an important role for mating. In *Candida lusitanae*, *STE12* homologue *CLS12* has been shown to regulate a specific mating response during its life cycle (Young et al. 2000). *STE12* homologues *SteA* and *StlA* also were identified from filamentous fungi *Aspergillus nidulans* and *Penicillium marneffeii*, respectively (Borneman et al. 2001; Vallim et al. 2000). In *A. nidulans*, the *steA* mutant impaired complete sexual reproduction, but showed no defect in vegetative growth and asexual reproduction (Vallim et al. 2000). In asexual ascomycete *P. marneffeii*, *stlA* was dispensable for vegetative growth and asexual reproduction, but able to complement the mating defect of an *A. nidulans steA* mutant (Borneman et al. 2001). Some *STE12* homologues were important not only for mating but also other cellular responses. *Filobasidiella neoformans* is the species that has a mating-type-specific *STE12* homologue in each mating type, and both *CnSte12 α* and *CnSte12 β* have been shown to be important not only for mating but also for survival in the host tissue (Chang et al. 2000, 2001). In *Candida albicans*, the *STE12* homologue *CPH1* was shown to be partially required for filamentous growth, but the role for mating remains to be determined (Liu et al. 1994). In plant-pathogenic fungus *M. grisea*, *MST12* has been shown to be essential for appressorium penetration and invasive growth in plants, but dispensable for vegetative growth, conidiation, and mating (Park et al. 2002).

In this report, we isolated *CST1* gene coding for yeast *STE12* homologue from *C. lagenarium*. Analysis of *cst1* knockout mutants demonstrated that *CST1* plays an essential role for appressorium penetration.

RESULTS

Isolation of the *CST1* gene.

A polymerase chain reaction (PCR)-based strategy was used to isolate the *STE12* homologue from *C. lagenarium*. A 157-bp fragment was amplified by PCR using two degenerate oligonucleotide primers, CST-S1 primer and CST-AS1 primer. The nucleotide sequence of the fragment showed a significant homology with the corresponding regions of *S. cerevisiae STE12* gene (Dolan et al. 1989; Errede and Ammerer 1989). The fragment was used to screen a genomic cosmid library of *C. lagenarium* 104-T, and two cosmid clones pCSTCOS1 and pCSTCOS2 were isolated. Three restriction fragments from pCSTCOS1 that hybridized to the PCR probe, a 6-kb *Apa*I, a 4-kb *Bam*HI, and a 4-kb *Kpn*I fragment, were subcloned into pBluescript II SK⁺. The resultant plasmids were defined as pBSCSA, pBSCSB, and pBSCSK, respectively (Fig. 1A). The sequence of these fragments revealed an open reading frame composed of 697 codons, and was designated *CST1* (*Colletotrichum* Ste12 homologue). Three introns of 55, 76, and 203 nucleotides were predicted on the basis of matching with consensus 5' splicing signals GTDHGY and 3' splicing signals YAG and internal splicing signals RCTRAC conserved in *Neurospora crassa* (Bruchez et al. 1993). Reverse-transcribed DNA was amplified by PCR using two pairs of primers spanning predicted introns, and the nucleotide sequence of the amplified cDNA was compared with that of genomic DNA to verify the predicted introns.

Cst1p has a homeodomain and Cys2His2 zinc finger domain.

The deduced amino acid sequence of the *CST1* contained two putative DNA-binding motifs, a homeodomain at the N-terminal region (amino acids 59 to 203) and Cys2His2 zinc fingers at the C-terminal region (amino acids 572 to 617) (Fig. 2). The homeodomain is present in all Ste12-family proteins ever identified. The second putative DNA binding motif, the Cys2His2 zinc fingers, is absent from *S. cerevisiae*, *Candida*

Fig. 1. A, Cloning of the *CST1* gene of *Colletotrichum lagenarium*. A cosmid clone pCSTCOS1 was selected from a wild-type cosmid library of *C. lagenarium* probed with the amplified DNA. Hybridizing fragments were subcloned into pBluescriptII(SK⁺) and sequenced. The shaded arrow indicates the open reading frame. Open boxes within the shaded arrow indicate introns. cDNA clones also were sequenced for identification of introns. **B,** Isolation of the *CST1* knock-out mutants. Predicted restriction map of *CST1* gene disrupted by homologous recombination with pCBCSTRRep2. Through double crossing over, a 9.0-kb *Eco*RI fragment of wild-type 104-T is predicted to be replaced by a 7.7-kb *Eco*RI fragment containing a hygromycin-resistant *hph* gene. **C,** DNA blot analysis of transformants. Genomic DNA was digested with *Eco*RI and probed with *Apa*I/*Sal*I fragment upstream of *CST1*. In the wild type, a single 9.0-kb band was recognized. In transformants RCS2-1 (R2-1), 2-2 (R2-2), and 2-3 (R2-3), the 9.0-kb band disappeared and the expected 7.7-kb band was recognized. In transformant RCS2-4 (R2-4), the 9.0-kb band containing *CST1* was intact, showing that disruption had not occurred. Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; X, *Xho*I.

albicans, and *Kluyveromyces marxianus* Ste12 proteins. Cst1p showed significant homology with Ste12-family proteins from filamentous fungi, *M. grisea* Mst12p (74.2%), *A. nidulans* SteAp (58.1%), and *P. marneffei* StlAp (57.9%). On the other hand, the lesser extent of homology with that was recognized from yeast, *S. cerevisiae* Ste12p (23.0%), *C. albicans* Ste12p (23.2%), *K. marxianus* Ste12p (23.4%), *F. neoformans* Ste12ap (26.4%), and *F. neoformans* Ste12ap (28.1%). However, in the homeodomain, Cst1p shared over 60% identity with these yeast Ste12 proteins. Thus, the homology between Cst1p and the yeast Ste12 proteins tends to be restricted to putative DNA binding motifs. In *S. cerevisiae*, Ste12p binds to pheromone response elements with HD-helix III (amino acids 150 to 160 in Ste12p) of homeodomain (Dolan et al. 1989; Kirkman-Correia et al. 1993; Yuan and Fields 1991). This helix was highly conserved among identified Ste12-family proteins, including Cst1p. In addition, tryptophan and phenylalanine residues of the HD-Helix III (amino acids 153 and 154 in Ste12p) are considered to be essential amino acid residues for DNA binding (Yuan and Fields 1991). The tryptophan residue was uniformly conserved among identified Ste12-family proteins, but the phenylalanine residue was replaced to another aromatic amino acid, tyrosine, in Cst1p as well as Ste12-family proteins from other filamentous fungi.

Cst1p is a putative transcription factor; therefore, transcriptional activation domains were also of interest. Several putative phosphorylation sites are predicted in Cst1p for cAMP-

and cGMP-dependent protein kinases (3 sites), protein kinase C (5 sites), and casein kinase II (12 sites) by PROSITE computer program. Moreover, acidic regions (amino acids 626 to 659) with a net charge of -12 and the glutamine-rich region (amino acids 431 to 446) also were recognized in Cst1p. The region outside the homeodomain of *S. cerevisiae* Ste12p is known to interact with other proteins such as a MAP kinase Kss1p, or a repressor Dig1p, or coactivators Mcm1p and Tec1p (Bardwell et al. 1998a; Errede and Ammerer 1989; Madhani and Fink 1997; Olson et al. 2000). However, Cst1p shared little homology in the interaction region with yeast Ste12p. Cst1p completely lacked the domain (amino acids 303 to 313 in Ste12p) which were conserved commonly in the ascomycetous yeast proteins and is necessary for the response to pheromone stimulation in *S. cerevisiae* (Pi et al. 1997).

CST1 is required for fungal pathogenicity.

We isolated *CST1* knockout mutants to define the function of Cst1p. Plasmid pCBCSTrep2 was constructed to replace the *CST1* gene with a hygromycin-resistant gene in the wild-type strain through double crossover homologous recombination (Fig. 1B). The occurrence of gene replacement was confirmed by genomic DNA blot analysis (Fig. 1C). A single 9.0-kb band was present in wild type, whereas a 7.7-kb band was present in RCS2-1, 2-2, and 2-3, as expected for a proper gene replacement event. In transformant RCS2-4, the 9.0-kb band containing *CST1* was intact, showing that disruption had not occurred.

Fig. 3. A, Five-day-old culture of the wild-type strain and the *cst1Δ* strains. White-orange conidial masses were observed in the center of each colony. B, Fourteen-day-old cultures showing the different appearance of conidial masses. Conidial masses of the *cst1Δ* strain RCS2-1 started getting dark from the center of the colony. Bar = 2 mm. C, Pathogenicity assays on cucumber leaves. The *cst1Δ* strains RCS2-1 and RCS2-2 and ectopic strain RCS2-4 were assayed. Detached cucumber leaves were drop inoculated and incubated at 24°C for 7 days. The right half leaves were wounded by scratching with a sterile toothpick. Wild-type strain 104-T (WT) and the *pks1* mutant 79215 (ALB) were used as controls.

Deletion of *CST1* did not inhibit vegetative hyphal growth and conidiation in *Colletotrichum lagenarium*. Growth rate of the *cst1Δ* strains was similar to that of the wild-type strain 104-T on potato dextrose agar (PDA) or Czapek agar medium (data not shown). Both the wild-type strain and the *cst1Δ* strains produced an average of 10^7 conidia/plate (data not shown). High osmotic stress also produced no difference in growth rate between the wild-type strain and the *cst1Δ* strains. The *cst1Δ* strains retained the wild-type level of colonial growth on PDA medium containing either 0.5 M sorbitol, 0.5 M NaCl, or 0.5 M KCl (data not shown).

Colonial appearance of the *cst1Δ* strains were indistinguishable from those of the wild type in the early stage of mycelial culture, but became slightly different in the later stage. Both the wild-type and *cst1Δ* strains produced white-orange conidial masses in the central area of the colony by 5 days after inoculation (Fig. 3A). The wild-type conidial masses retained the light orange color for long-time incubation over 2 weeks. In contrast, the color of conidial masses of the *cst1Δ* strains gradually turned to dark brown (Fig. 3B), and some conidia germinated and produced melanized appressorium-like structures on 14-day-old mycelial cultures grown on PDA medium (data not shown). These results suggest that *CST1* is dispensable for the growth and conidiation of *C. lagenarium*, but required for the maintenance of conidial dormancy.

To assess whether *CST1* is required for pathogenicity, the inoculation assays were performed using host cucumber leaves. Drops of conidial suspension were spotted onto the intact leaves and incubated at 24°C. The wild-type strain 104-T formed yellow-brown lesions by 5 days after inoculation. In contrast, the *cst1Δ* strains produced no lesion even at 9 days after inoculation (Fig. 3C). This result indicates that *CST1* is indispensable for pathogenicity in *C. lagenarium*. Consequently, we assessed whether the *cst1Δ* strains are able to grow invasively in host leaves. As a control strain retaining infectious growth ability, we used the albino mutant 79215 which carries a mutation of the melanin biosynthesis gene *PKS1* and cannot penetrate the host leaf. Drops of conidial suspension were spotted at the wounded sites of leaves. The *cst1Δ* strains formed yellow-brown lesions around the wound sites of leaves by 5 days after inoculation (Fig. 3C), although the lesions that produced the *cst1Δ* strains were slightly smaller than the *pks1* mutant 79215. This result suggests that involvement of *CST1* in invasive growth is partial, and that the complete loss of

pathogenicity of the *cst1Δ* strains is due to a defect in a step before host invasion.

Appressoria produced by *CST1* knockout mutants impaired in penetration.

To determine the steps leading to loss of pathogenicity, infection-related morphogenesis of the *cst1Δ* strain was investigated on glass slides and cellulose membrane. When incubated in water on a glass slide, the wild-type conidia started to germinate by 3 h and to produce appressoria by 6 h after the start of conidial incubation. Approximately 80% of germinating conidia produced appressoria by 12 h. Kinetics of conidial germination was normal on the *cst1Δ* strains. Appressorium formation of the *cst1Δ* strains was slightly delayed, but over 70% of conidia were able to produce darkly melanized appressoria by 12 hr, as well as the wild-type strain (Table 1). There is no significant difference between the *cst1Δ* strains and the wild-type 104-T on the size of appressoria produced at 24 h after the start of conidial incubation (data not shown). This result indicates that *CST1* is dispensable for conidial germination but might be involved partially in appressorium formation in *C. lagenarium*.

Previous studies indicated that yeast extract can restore the conidial germination of the *cmk1Δ* strain, but the germinating conidia still failed to form appressoria (Takano et al. 2000). We investigated the effect of yeast extract on appressorium formation of the *cst1Δ* strains. When incubated in 0.1% yeast extract solution on a glass slide, conidia of the wild-type strain generally formed normal appressoria, although some conidia produced secondary germ tubes and appressoria (Fig. 4A). However, interestingly, germinating conidia of the *cst1Δ* strains tended to produce imperfect appressoria, resulting in aberrant infection structures (Fig. 4B). The frequency is over 70% of germinating

Table 1. Time course of conidial germination and appressorium formation on a glass surface

Time, strains (genotype)	Conidial germination ^a	Appressorium formation
6 h		
104-T (Wild-type)	56.9 ± 1.7	45.3 ± 1.2
RCS2-1 (104- <i>Tcst1Δ</i> , Hyg ^r)	65.7 ± 3.8	24.3 ± 1.6
RCS2-2 (104- <i>Tcst1Δ</i> , Hyg ^r)	70.6 ± 4.9	21.1 ± 3.0
RCS2-4 (104-T, Hyg ^r)	60.7 ± 5.7	37.9 ± 10.2
12 h		
104-T (Wild-type)	81.4 ± 8.2	73.8 ± 7.6
RCS2-1 (104- <i>Tcst1Δ</i> , Hyg ^r)	92.5 ± 1.2	79.2 ± 9.9
RCS2-2 (104- <i>Tcst1Δ</i> , Hyg ^r)	90.4 ± 2.8	75.7 ± 2.3
RCS2-4 (104-T, Hyg ^r)	79.3 ± 8.1	73.1 ± 5.8
24 h		
104-T (Wild-type)	90.0 ± 3.4	87.5 ± 3.4
RCS2-1 (104- <i>Tcst1Δ</i> , Hyg ^r)	90.4 ± 2.8	82.9 ± 3.1
RCS2-2 (104- <i>Tcst1Δ</i> , Hyg ^r)	92.6 ± 1.5	81.7 ± 1.4
RCS2-4 (104-T, Hyg ^r)	88.6 ± 0.6	86.0 ± 1.4

^a Percentage of conidial germination or appressorium formation was counted at each time course after the start of conidial incubation at 24°C. Approximately 200 conidia were observed in each sample and each experiment was repeated at least three times.

Fig. 4. Germinating conidia of the *cst1Δ* strain on glass slide. Conidia were incubated in 0.1% yeast extract solution at 24°C for 24 hr. **A**, Wild-type strain 104-T; **B**, *cst1Δ* strain RCS2-1. Abbreviations: Ap, appressorium; Co, conidium; Sg, secondary germ-tube; Sa, secondary appressoria; Aa, aberrant appressoria. Bar = 10 μm.

conidia, which was significantly higher compared with the wild-type strain (Table 2). Other complex nutrients, peptone and tryptone, also induced the aberrant structures of the *cst1Δ* strains (data not shown).

To access penetration ability of appressoria in the *cst1Δ* strains, conidia were incubated on cellulose membrane. Approximately 90% of the wild-type appressoria penetrated cellulose membrane and produced infectious hyphae by 48 h after the start of conidial incubation (Fig. 5A). A low frequency of infectious hyphae also was observed even in the *pks1* mutant 79215 that cannot penetrate the host leaf (Fig. 5C). In contrast, appressoria of the *cst1Δ* strains produced no infectious hyphae in cellulose membrane by 48 hr, even at 96 h after the incubation (Fig. 5B). Moreover, we scraped appressoria from cellulose membrane with a brush, and observed the membrane with a light microscope. Holes produced by penetration pegs were observed abundantly on the membrane on which the wild-type strain 104-T had been incubated (Fig. 5D); whereas, no hole was observed in membrane of the *cst1Δ* strains (data not shown). These results suggest that the *cst1Δ* strains are defective in penetration peg formation from appressorium and, thus, lack the ability for appressorium penetration. To confirm further that the loss of pathogenicity is due to the failure of appressorium penetration, we observed infectious morphogenesis of the *cst1Δ* strains on the host leaf. The wild-type strain also produced infectious hyphae on the host leaf by 4 days after inoculation (Fig. 5E). Conidia of the *cst1Δ* strains produced melanized appressoria, but no infectious hyphae were observed from the appressoria (Fig. 5F). These results suggest that the loss of pathogenicity of the *cst1Δ* strain is due to a defect in appressorium penetration.

Appressoria produced by *CST1* knockout mutants lack lipid droplets.

The wild-type appressoria produced on a glass slide contained a lot of lipid droplets confirmed by Nile-Red staining (Fig. 6E). Appressoria of the *pks1* mutant 79215 also contained the same level of droplets as the wild-type strain (Fig. 6G). The positional distribution of orange fluorescence signal by Nile-Red was consistent with that of the droplets in appressoria of the *pks1* mutant 79215 (Fig. 6G and H), although the fluorescent signals could not be detected in matured appressoria of the wild-type strain by an interference of melanin layer.

Through observation of multiple samples, we recognized that matured appressoria produced by the *cst1Δ* strains contained an extremely low level of lipid droplets compared with that of the wild-type strain (Fig. 6E and F). Therefore, we traced the distribution of lipid droplets during the development of infection-related morphogenesis. Lipid droplets were abundant in conidia both of the wild-type strain and of the *cst1Δ* strains (Fig. 6A through D). Both droplets uniformly translocated to appressoria during the development, and over 90% of formed appressoria contained a large number of lipid droplets at 8 h after the start of conidial incubation. In the wild-type

strain, the lipid droplets in appressoria persisted for over 48 h after the incubation. In contrast, the lipid droplets in appressoria of the *cst1Δ* strains rapidly disappeared during the maturation, and could hardly be observed at 24 h after the incubation (Fig. 7). This finding indicates the possibility that *CST1* regulates the genes involved in the lipid metabolism during the development of infectious morphogenesis.

DISCUSSION

Recent studies have revealed that several plant-pathogenic fungi require components of extensively conserved signal transduction pathways for pathogenicity. In *C. lagenarium*, the yeast *FUS3/KSS1* MAP kinase gene homologue *CMK1* has been shown to be essential for pathogenicity (Takano et al. 2000). Here, we identified and characterized a *CST1* gene homologous to the yeast *STE12* gene coding for a downstream transcriptional factor of *FUS3/KSS1* MAP kinase. Analysis of the *cst1* knockout mutant demonstrated that *CST1* is also essential for pathogenicity in *C. lagenarium*.

Cst1p shows a feature characteristic of *STE12* homologues from filamentous fungi.

CST1 was isolated on the basis of homology to the conserved homeodomains found in previously identified Ste12-family proteins. In *S. cerevisiae*, the region downstream from the homeodomain is known to interact with several regulatory proteins, Kss1p, Dig1p, Mcm1p, or Tec1p in *S. cerevisiae* (Bardwell et al. 1998a, 1998b; Errede and Ammerer 1989; Madhani and Fink 1997; Olson et al. 2000). However, the deduced amino acid sequence of *CST1* showed little homology with the yeast Ste12-family proteins in the region. On the other hand, Cst1p had a feature of *STE12* homologues from filamentous fungi, showing marked significant homology with the entire sequences of *M. grisea* Mst12p, *A. nidulans* SteAp, and *P. marneffei* StlAp. Cst1p contained the Cys2His2 zinc finger domain that is absent from the ascomycetous yeast Ste12-family proteins. The region between the homeodomain and the Cys2His2 zinc finger domain is conserved well among the Ste12-family proteins from filamentous fungi, and contains several identical stretches of amino acid residues (amino acids 354 to 370 and 407 to 425 in Mst12p) (Park et al. 2002). Cst1p showed identity with those highly conserved areas (amino acids 348 to 364 and 402 to 420), together with the putative cAMP- and cGMP-dependent protein kinase phosphorylation site (amino acids 413 to 416). This suggests the possibility that these Ste12-family proteins, including Cst1p, have a unique regulatory mechanism with a different set of partner proteins or interacting regions, which is distinct from the yeast Ste12-family proteins.

Roles of *CST1* for appressorium function.

The *cst1Δ* strain produced darkly melanized appressoria, but failed to penetrate not only plant epidermal surface but also artificial cellulose membrane. This suggests that *CST1* is required for normal appressorium function in *C. lagenarium*. The failure of appressorium penetration is considered to be caused by a defect in either penetration peg formation or appressorium turgor generation, or both.

In *M. grisea*, high turgor pressure in melanized appressorium enables mechanical penetration, and the melanin layer contributes to preventing the leakage of intracellular solute for turgor generation (de Jong et al. 1997; Howard et al. 1991). Therefore, the melanin-deficient mutants of *M. grisea* fail to penetrate the host leaves (Chumley and Valent 1990; de Jong et al. 1997). *C. lagenarium* is predicted to have a similar turgor-driven infection process with *M. grisea*. The

Table 2. Aberrant infection structure formation by yeast extract treatment in the *cst1Δ* strains

Strains (genotype)	Distilled water ^a	0.1% Yeast extract
104-T (Wild-type)	3.0 ± 1.4	5.7 ± 1.2
RCS2-1 (104-T <i>cst1Δ</i> , Hyg ^r)	11.3 ± 4.5	70.6 ± 2.8
RCS2-2 (104-T <i>cst1Δ</i> , Hyg ^r)	13.5 ± 4.4	79.3 ± 1.2
RCS2-4 (104-T, Hyg ^r)	4.2 ± 3.0	10.6 ± 3.1

^a Percentage of germinating conidia that produced aberrant infection structure was counted at 24 h after the start of conidial incubation at 24°C. Approximately 200 germinating conidia were observed in each sample and each experiment was repeated at least three times.

melanin-deficient strains of *C. lagenarium* also were unable to penetrate the host leaves, raising the possibility of the failure of normal turgor generation by a similar mechanism with *M. grisea*. However, the melaninless appressoria produced by the *pks1* mutant 79215 of *C. lagenarium* still retain a little ability to produce penetration peg and infectious hyphae on the artificial cellulose membrane, whereas the darkly melanized appressoria produced by the *cst1Δ* strains completely lacks the ability. This result suggests that the *cst1Δ* strains fail to produce penetration peg regardless of turgor generation, and that *CST1* regulates genes involved in penetration peg formation in *C. lagenarium*. However we cannot exclude

the possibility of complex involvement of turgor generation in the *cst1Δ* strains.

In *M. grisea*, lipid droplets observed in conidia translocated to appressoria and were rapidly degraded at the onset of turgor generation, suggesting that lipid degradation might contribute to appressorium turgor generation (Thines et al. 2000). Numerous lipid droplets also were observed in conidia of *C. lagenarium* and mobilized to appressoria during the conidial germination and appressorium formation process. Appressoria produced by the wild-type strain were filled with numerous lipid droplets. However, interestingly, the *cst1Δ* strains lacked the lipid droplets in appressoria. The wild-type level of lipid droplets were observed

Fig. 5. Appressorium formation and penetration of the *cst1Δ* strains **A–D**, on the cellulose membrane 2 days after inoculation, **E–F**, on the epidermal layer of cucumber cotyledon 4 days after inoculation. **A** and **E**, Wild-type strain 104-T; **B** and **F**, the *cst1Δ* strain RCS2-1; **C**, the *pks1* mutant 79215; **D**, the cellulose membrane after the removal of the wild type appressoria. Abbreviations: Ap, appressorium; Co, conidium; Ih, infectious hyphae; Ho, penetration hole. Bar = 10 μm.

in conidia of the *cst1Δ* strains, but rapidly disappeared during the development of appressorium formation. Based on the interpretation in *M. grisea*, we expect that lipid droplets contribute to increasing appressorium turgor in *C. lagenarium*, and misscheduled lipid degradation causes a defect in appressorium function in the *cst1Δ* strains. However, in *C. lagenarium*, the relationship between degradation of lipid reserves and appressorium turgor generation is still unclear. The behavior of

lipid droplets in the wild-type strain of *C. lagenarium* was slightly different from that in *M. grisea*. Lipid droplets in appressoria of *M. grisea* produced on glass surface were completely degraded during maturation (Weber et al. 2001), whereas those were still observed in fully melanized appressoria of *C. lagenarium* incubated for 48 h. Thus, at present, we cannot exclude the possibility that this fungus does not utilize the substance stored in lipid droplets to support full function of

Fig. 6. Cellular distribution of lipid droplets in conidium and appressorium of the *cst1Δ* strain RCS2-1. Conidia and appressoria produced on the glass surface were stained by Nile-Red solution. **A–D**, Ungerminating conidia before incubation; **E–H**, appressoria at 24 h after incubation. **A**, **C**, **E**, **F**, and **G** are differential-interference contrast images. **B**, **D**, and **H** are epifluorescence images of Nile-Red stained material of **A**, **C**, and **G**, respectively. Ap, appressorium; Co, conidium. Bar = 5 μ m.

appressoria. In *M. grisea*, the Pmk1p MAP kinase is shown to regulate the transport of storage carbohydrate and lipid reserves to the appressorium, whereas the CPKA/SUM1-encoded PKA is the degradation of those storage reserves for appressorium turgor generation (Thines et al. 2000). Although several signal transduction pathways required for appressorium formation and function are identified in *C. lagenarium*, the role of intracellular materials remains to be investigated (Takano et al. 2000, 2001).

Signal transduction pathways regulating fungal infectious morphogenesis.

CMK1 is shown to regulate diverse aspects of infectious morphogenesis, including conidial germination, appressorium formation, and infectious growth (Takano et al. 2000). On the other hand, *CST1* mainly is required for appressorium penetration that is a subsequent event to appressorium formation. This suggests the possibility that the CMK1 MAP kinase pathway may be involved in appressorium penetration through Cst1p function, although we cannot estimate the role of *CMK1* on appressorium penetration due to the failure of appressorium formation in the *cmk1Δ* strain. The relationship between *CMK1* and *CST1* in *C. lagenarium* is similar to that between *PMK1* and *MST12* in *M. grisea*. The *pmk1* mutant of *M. grisea* is defective in appressorium formation, whereas the *mst12* mutant is defective in appressorium penetration (Park et al. 2002; Xu and Hamer 1996). Preliminary results from yeast two-hybrid assays also suggested the possibility that Mst12p is physically associated with Pmk1p (Park et al. 2002). At present, the physical interaction between Cmk1p and Cst1p remains to be investigated.

CST1 seemed dispensable for primary functions of *CMK1* in fungal infectious morphogenesis, suggesting that another redundant transcriptional factor or factors is associated with Cmk1p. However, the involvement of *CST1* could not be excluded absolutely. Especially, it is possible to postulate that *CST1* is involved partially in appressorium formation and infectious growth. When incubated in the nutrient solution, conidia of the *cst1Δ* strains tended to produce appressoria imperfectly, showing an intermediate phenotypic appearance between the wild-type strain 104-T and the *cmk1Δ* strain. In addition, the *cst1Δ* strain retained invasive growth ability in host leaves, but the lesions formed on wounded leaves were relatively smaller than those formed by the control strain. Thus, *CST1* may regulate diverse aspects of fungal infectious morphogenesis by means of coregulation with different factors at different stages of cellular differentiation. In *S. cerevisiae*, Ste12p is known to form heterodimer with Mcm1p to activate pheromone response element-regulated genes in mating, and with Tec1p to regulate filamentation response-element (FRE) genes during filamentous growth (Lengeler et al. 2000; Madhani and Fink 1997).

In *M. grisea*, the *PMK1* MAP kinase pathway is proposed to act cooperatively with a cAMP-PKA signaling pathway for appressorium formation and function (Thines et al. 2000; Xu and Hamer 1996). In *C. lagenarium*, the mutant of *CMK1* MAP kinase gene showed lack of conidial germination, appressorium formation, and invasive growth, whereas the mutant *SGC1* impairing PKA activity showed a reduced germination of conidia and a lack of appressorium penetration (Takano et al. 2000, 2001). Therefore, the Cmk1p MAP kinase was speculated to regulate cooperatively with a cAMP-PKA pathway at least for conidial germination in *C. lagenarium* (Takano et al. 2001). However, the *cst1Δ* strain showed no effect on conidial germination, implying that another transcriptional factor or factors associated with these pathways must be present in this process. On the other hand, the involvement of *CST1* in the cAMP-PKA signaling pathway is predicted by a defect of appressorium

penetration in the *cst1Δ* strain, and also supported by the mis-scheduled germination of conidia. This phenotypic appearance is similar to that of the *rpkl* mutant of *C. lagenarium* that lacks the PKA regulatory subunit. The improperly high PKA activity of the *rpkl* mutant resulted in mis-scheduled germination of the conidia on mycelial colonies (Takano et al. 2001). This might suggest that Cst1p function is repressed by high PKA activity. This finding is supported by the result that the high PKA activity inhibits FRE-dependent transcription depending on Ste12p and Tec1p in diploid cells of *S. cerevisiae* (Lorenz and Heitman 1997). In *C. lagenarium*, the PKA activity must be regulated properly during appressorium formation and penetration process for the production of functional appressoria (Takano et al. 2001). This might suggest that *CST1* also must be highly regulated during these processes for appressorium penetration.

We present evidence that *CST1* is required for appressorium penetration and partially involved in appressorium formation and infectious growth in host plants, implying the possibility that *CST1* may function under cooperative control of the MAP kinase and cAMP-PKA signaling pathways. We speculate that several transcriptional factors, including Cst1p, regulated by these signaling pathways may form a transcriptional complex and cooperatively regulate different sets of genes at different stages of fungal infectious morphogenesis. Further experiments will be needed to identify the components interacted with Cst1p and the genes regulated. These studies will enable us to understand how the fungal pathogen utilizes these signal pathways for infection.

MATERIALS AND METHODS

Fungal strains and culture conditions.

Colletotrichum lagenarium (Pass.) Ellis and Halsted strain 104-T (stock culture of the Laboratory of Plant Pathology, Kyoto Prefectural University) was used as the wild-type strain. The *pks1* mutant 79215 previously was isolated by UV irradiation (Kubo and Furusawa 1991; Takano et al. 1995). All *C. lagenarium* cultures were maintained on PDA medium (Difco Laboratories, Detroit) or Czapek agar medium at 24°C.

Fig. 7. Quantitative analysis of lipid distribution. Conidia were allowed to germinate on the glass surface and form appressoria. Percentage of appressorium formation was indicated as bars. Black, white, and gray bars represent the wild-type strain 104-T and the *cst1Δ* strains RCS2-1 and RCS2-2, respectively. Percentage of appressoria containing lipid droplets were counted for the wild-type strain 104-T (closed circle) and the *cst1Δ* strains RCS2-1 (open square) and RCS2-2 (open triangle). Approximately 200 conidia were observed in each sample and each experiment was repeated at least three times.

CSTI cloning.

Two degenerate oligonucleotide primers, CST-S1 primer (5'-CGGGATCCAARAARTTYGARGARGG-3') and CST-AS1 primer (5'-CGGAATTCACYYTGYTTYTGYTG-3'), were designed on the basis of the conserved amino acid sequences among Ste12-family proteins from *S. cerevisiae*, *Candida albicans*, *A. nidulans*, and *F. neoformans* (Chang et al. 2000; Errede and Ammerer 1989; Liu et al. 1994; Vallim et al. 2000). The underlined regions are added restriction sites *Bam*HI and *Eco*RI, respectively. These primers were used to amplify *CSTI* partial fragment by PCR using KOD DASH DNA polymerase (TOYOBO, Osaka, Japan). PCR conditions were as follows: 1 cycle (1 min at 94°C, 1 min at 37°C, and 1 min at 72°C), followed by 30 cycles (1 min at 94°C, 1 min at 50°C, and 2 min at 72°C). PCR products were digested with *Bam*HI and *Eco*RI, cloned into pBluescript II KS⁺ vector, and sequenced. The cloned fragment showed significant homology with *S. cerevisiae* *STE12*. Two cosmid clones were isolated from a *Colletotrichum lagenarium* cosmid library probed with the cloned PCR product. The cosmid clones obtained were digested with various restriction enzymes and applied to Southern blotting. The fragments shown to hybridize to the PCR probe were selected and subcloned into pBluescript II SK⁺. The nucleotide sequence of a representative clone was analyzed with a 373A Perkin-Elmer auto-sequencer (Applied Biosystems, Tokyo). Homologues in GenBank were searched with the BLAST program (Altschul et al. 1997). Amino acid sequence alignment was performed with the CLUSTAL W program (Thompson et al. 1994).

Plasmid construction for *CSTI* replacement.

Plasmid pCBCSTrep2 was designed to replace *CSTI* with *hph* by homologous recombination. Fungal transformation vectors pCB1531 and pCB1636 (Sweigard et al. 1997) were used to construct the *CSTI* replacement plasmid pCBCSTrep2. Plasmid pCB1531 was double-digested with *Sal*I and *Xho*I, and self-ligated. The resultant plasmid was defined as pCB1531SX. A 5-kb *Apa*I fragment of pBSCSA was introduced to the *Apa*I site of pCB1531SX, and the resultant plasmid was named pCBCST1. Subsequently, a hygromycin-resistant gene cassette derived from pCB1636 was introduced into *Sal*I-*Xho*I site of pCBCST1 to make pCBCSTrep2.

Fungal transformation.

Fungal transformation experiments were performed by the standard polyethylene glycol method as described by Kubo and associates (1991b). Hygromycin-resistant transformants were selected on PDA medium containing 1 M glucose and hygromycin B (50 µg/ml).

Pathogenicity test.

The inoculation assay was performed as described by Tsuji and associates (1997). Ten-day-old seedlings of cucumber (*Cucumis sativus* L. 'suyo') were used. Excised cotyledons were placed in a humid plastic box. Conidia of *C. lagenarium* were obtained from the 7-day-old culture. Drops (10 µl) of conidial suspension (5 × 10⁵ conidia/ml) were spotted on the surface of these leaves. For estimation of infectious hyphal growth, 5-µl drops were spotted on wound sites prepared by scratching with a sterile toothpick. After inoculation, the humid plastic box was maintained with a 12-h light condition at 24°C for 5 days. For microscopic observation, 10-µl drops were spotted on the underside of cotyledon. After the incubation at 24°C for 4 days, the epidermal layers were peeled off and stained by lactophenol aniline blue (Takano et al. 1997).

Cytological assays.

For appressorium formation and penetration assays in vitro, conidia were harvested from 7-day-old PDA cultures and suspended in distilled water. The conidial suspension, adjusted to 1 × 10⁵ conidia per ml, was poured onto a multiwell glass slide (Glass 8-well multitest slide; ICN Biomedicals, Aurora, OH, U.S.A.) or a cellulose membrane (Seamless Cellulose Tubing; Wako Pure Chemicals, Osaka, Japan), and incubated at 24°C. Germlings were observed with a Nikon ECLIPSE E600 microscope with differential-interference contrast optics. Lipid droplets in conidia and appressoria were visualized by staining with a Nile Red solution (Greenspan et al. 1985; Weber et al. 1999) consisting of polyvinylpyrrolidone at 20 mg/ml and Nile Red Oxazone (Sigma-Aldrich, St. Louis) at 2.5 µg/ml in 50 mM Tris-HCl (pH 7.5). Nile Red fluorescence was viewed on a fluorescent microscope with the B-2A filter (450- to 490-nm excitation wavelength, 505-nm wavelength dichroic mirror, and 520-nm wavelength barrier filter).

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Ministry of Education, Science, Sports and Culture of Japan (No. 12460024 and 12052219), and Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science (No. 05878).

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