A Kelch Repeat Protein, Cokel1p, Associates with Microtubules and Is Involved in Appressorium Development in *Colletotrichum orbiculare*

Ayumu Sakaguchi, Toshihiko Miyaji, Gento Tsuji, and Yasuyuki Kubo

Laboratory of Plant Pathology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522, Japan

Submitted 9 June 2009. Accepted 10 August 2009.

Kelch repeat proteins are conserved in diverse organisms and some are known to mediate fundamental cellular functions. We isolated the gene *CoKEL1*, encoding a novel kelch repeat protein, from *Colletotrichum orbiculare*. Analysis of a *cokel1* mutant indicated that *CoKEL1* is involved in proper appressorium development and cell wall synthesis. Appressoria produced by *cokel1* disruption mutants showed irregular shape and impairment of turgor generation and the mutant appressoria rarely penetrated to form infection hyphae in host epidermal cells. Accordingly, *cokel1* mutants had reduced pathogenicity on host leaves compared with the wild type. Furthermore, the *cokel1* mutant was more sensitive to cell-wall-degrading enzymes and showed altered labeling with the cell wall stain Calcofluor white. Cokel1p was localized on cortical and spindle microtubules in vegetative hyphae. These results suggest that Cokel1p is a microtubule-associated protein involved in infection-related morphogenesis and pathogenicity. This is the first report that a kelch repeat protein is required for the pathogenicity of a fungal plant pathogen.

Colletotrichum orbiculare (syn. *C. lagenarium*) is the causal agent of cucumber anthracnose disease. A series of changes in fungal morphology, including formation of a specialized infection structure called the appressorium, is important for successful infection (Kubo and Furusawa 1991). A number of metabolic functions and signal transduction pathways required for appressorium development have been characterized. *CMK1*, a homolog of the *Saccharomyces cerevisiae* *FUS3/KSS1* mitogen-activated protein (MAP) kinase, and *MAF1*, a homolog of the *S. cerevisiae* *SLT2* MAP kinase, are both involved in appressorium development and pathogenicity of *C. orbiculare* (Kojima et al. 2002; Takano et al. 2000). *Cst1*, homologous to the *STE12* transcription factor, is required for penetration peg formation by appressoria (Tsuji et al. 2003b). The cyclic AMP (cAMP) signaling pathway is also essential for pathogenicity in this fungus. The adenylate cyclase gene, *CAC1*, and the protein kinase A (PKA) gene, *CPK1*, were shown to be involved in conidial germination, appressorial penetration, and invasive growth into host tissue (Yamauchi et al. 2004). Metabolic pathways essential for appressorium function include melanin

synthesis (Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995; Tsuji et al. 2000) and peroxisome metabolism (Asakura et al. 2009; Kimura et al. 2001).

Previously, we identified a gene, *CoKEL2* (previously named *ClakEL2*), encoding a kelch repeat protein, which is homologous to *Schizosaccharomyces pombe* *tea1p*. Cokel2p localizes to the apices of growing hyphae and germ tubes in a microtubule-dependent manner and is involved in normal appressorium development on artificial substrata (Sakaguchi et al. 2008). Although *CoKEL2* is involved in appressorium development, the *cokel2* mutant could form normal appressoria with full virulence on the host plant when supported by calcium-dependent bypass pathways which transduce plant-derived signals (Sakaguchi et al. 2008). Kelch repeat proteins are composed of multiple kelch motifs, each consisting of a segment of 44 to 56 amino acids. Several kelch repeat proteins have been functionally characterized in diverse organisms, including viruses, plants, fungi, and mammals (Adams et al. 2000). In yeast, *S. pombe* *tea1p* localizes to cell ends in a microtubule-dependent manner and functions as part of the “polarisome” complex in establishing cell polarity (Feierbach et al. 2004; Mata and Nurse 1997). The mechanism by which *tea1p* is transferred to the cell apex requires binding of *tea1p* to the partner protein *tip1p* at the growing microtubule plus end. In addition, the protein *tea3p* functions as a cell-end marker dependent on microtubules and *tea1p* (Arellano et al. 2002). In *Aspergillus nidulans*, the *tea1* homologue *TeaA* was shown to be involved in polarized growth (Takeshita et al. 2008).

The relationship between kelch repeat proteins and signal transduction pathways has also been analyzed. In *S. pombe*, a mutant of the kelch repeat encoding gene *RAL2* lacks mating activity and the ability to respond to mating pheromone (Fukui et al. 1989). In *Saccharomyces cerevisiae*, overexpression of the *KEL1* gene suppresses the cell fusion defect caused by hyperactive *Pkc1p*, indicating that *Kel1p* acts upstream of or parallel to the PKC1 pathway (Philips and Herskowitz 1998). More direct interactions between kelch repeat proteins and the signal transduction pathway were indicated by the report that *S. cerevisiae* *Krh1p* and *Krh2p* (also called *Gpb1p* and *Gpb2p*) are binding partners of the Gα protein *Gpa2p*, which functions in glucose- and sucrose-induced cAMP signaling (Harashima and Heitman 2002). The two kelch repeat proteins *Krh1p* and *Krh2p* act as downstream effectors of *Gpa2p* and negatively regulate the PKA pathway (Peeters et al. 2007).

In this study, we identified the gene *CoKEL1* encoding a novel kelch repeat protein in *C. orbiculare*. Targeted gene disruption indicated that *CoKEL1* is required for normal appressorium development and cell wall synthesis and, in con-

Corresponding author: Yasuyuki Kubo; E-mail: y_kubo@kpu.ac.jp.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplemental figures are published online. Also, Figures 1 through 5 and 7 appear in color online.

trast to *CoKEL2*, is essential for invasion of host tissue. Moreover, we show that Cokel1p is localized on cortical and spindle microtubules, indicating that Cokel1p is a microtubule-associated protein. Our study is the first to show that a kelch repeat protein is required for the pathogenicity of a plant-pathogenic fungus.

RESULTS

Isolation of the *CoKEL1* gene encoding a kelch repeat protein.

CoKEL1 was identified as part of a screen for restriction enzyme-mediated integration (REMI) mutants of *C. orbiculare* with impaired pathogenicity. Mutant P24 was found to produce less severe lesions on cucumber cotyledons than the wild-type strain 104-T (Fig. 1A). DNA segments adjacent to the inserted plasmid were isolated from mutant P24 by thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) (Fig. 1B). The TAIL-PCR product was sequenced and, on this basis, a primer pair was designed to isolate a genomic clone from a cosmid library of *C. orbiculare*. Sequence analysis revealed that the TAIL-PCR product encoded a reading frame with significant homology to proteins containing the kelch motif. The *CoKEL1* locus is shown schematically in Figure 1B. The transcriptional initiation site was found by 5' rapid amplification

of cDNA ends (RACE). The ATG codon 265 bp downstream of the transcriptional initiation site is the probable translational initiation site of *CoKEL1*. The upstream region of the predicted *CoKEL1* open reading frame (ORF) contained upstream open reading frames (uORF) of 30 bp. Based on the preferred translation initiation codon, *CoKEL1* was predicted to encode a protein of 555 amino acids, and six kelch motifs were found in the carboxyl-terminal region of Cokel1p (Fig. 1C).

To investigate the distribution of *CoKEL1* homologs in other fungal genomes, the National Center for Biotechnology Information nonredundant protein database was searched using blastp. The deduced amino acid sequence of *CoKEL1* showed high similarity to a number of fungal hypothetical proteins. A phylogenetic tree based on the amino acid sequences of *CoKEL1* and *CoKEL2* homologs from other fungi was constructed using MEGA version 4.0 software (Tamura et al. 2007) (Supplementary Fig. S1). Genes homologous to *CoKEL1* are well conserved among ascomycetes fungi and *CoKEL1* and *CoKEL2* were found to belong to different clades. Cokel2p showed high homology to *S. pombe* tealp (*E* value = 2e-77) but Cokel1p was less similar to tealp (*E* value = 2e-21).

CoKEL1 is involved in appressorium development.

To further define the function of *CoKEL1*, we used *Agrobacterium tumefaciens*-mediated transformation (AtMT) to generate *cokel1* targeted disruption mutants by homologous recombination. The *A. tumefaciens* binary gene replacement cosmid vector p24cosKEL1Tn was introduced into the wild-type strain 104-T by AtMT. Gene replacement of *CoKEL1* were verified by DNA gel blot analysis (Supplementary Fig. S2). Impairment of *CoKEL1* transcription was confirmed by reverse-transcription PCR (RT-PCR). The expected fragment could be amplified from the wild-type strain and ectopic transformant but no transcript was detected in the *cokel1* mutants.

The *cokel1* mutants did not differ from the wild type in their growth rate on potato dextrose agar (PDA) medium when incubated at 24°C but showed reduced conidiation (Table 1). The wild type usually produced orange conidial masses in the central region of the colony on PDA. In contrast, *cokel1* mutants produced black conidial masses on PDA. Conidia of *cokel1* mutants germinated normally but formed morphologically aberrant appressoria on glass microscope slides. Approximately 90% of the appressoria produced by the wild type had a spherical form whereas approximately 50% of the appressoria produced by *cokel1* mutants were irregular or elliptical in shape, sometimes with a swollen neck region adjoining the spore. Many mutant appressoria also showed germination from an appressorium which was not observed for the wild type

Fig. 1. Identification of the *CoKEL1* gene in *Colletotrichum orbiculare*. **A**, Pathogenicity assays of restriction enzyme-mediated integration (REMI) mutant P24. Detached cucumber cotyledons were droplet inoculated with conidial suspension and incubated at 24°C for 5 days. 104-T, the wild type; P24, REMI mutant; Mock, distilled water control. **B**, Schematic representation of the *CoKEL1* locus in the *C. orbiculare* wild-type strain and mutant P24. Exons are indicated as black boxes. The predicted kelch-repeat-encoding regions are presented as open boxes. The transcription initiation site is shown by a bent arrow. The open reading frame of *CoKEL1* is shown with the predicted ATG start codon designated as +1, and ending with a TGA termination codon at position 1668. **C**, Alignment of the predicted kelch repeats in Cokel1p. Identical or similar residues are shown on shaded backgrounds. The lefthand numbers show amino acid positions of the kelch motifs in Cokel1p. Each amino acid sequence were examined manually for the presence of eight key conserved residues, including four hydrophobic residues (h) followed by a double glycine element (GG), and separated by two characteristically spaced aromatic residues (a) (Adams et al. 2000).

Table 1. Growth and conidiation of *Colletotrichum orbiculare cokel1* mutants grown on potato dextrose agar (PDA) medium

Strain ^a	Vegetative growth (mm/3 days) ^b	Conidia (×10 ⁷ /colony) ^c
104-T	7.77 ± 0.26	3.68 ± 0.44
RK1-1	7.77 ± 0.17	0.99 ± 0.35
RK1-2	7.88 ± 0.11	0.98 ± 0.29
EK1-1	7.79 ± 0.19	3.08 ± 0.80
RK1C-1	7.81 ± 0.10	3.71 ± 0.53

^a Strains: 104-T, wild type; RK1-1 and RK1-2, *cokel1* mutant strains; EK1-1, *CoKEL1* ectopic transformant; RK1C-1, *GFP::CoKEL1* complemented transformant of RK1-1.

^b Each strain was grown on PDA at 24°C, and radial growth was estimated by comparing colony diameters at 3 and 6 days. Radial growth was measured for five colonies of each strain, and the means and standard deviations were calculated.

^c Production of conidia was measured in 7-day-old cultures. Conidia produced by five colonies of each strain were counted, and the means and standard deviations were calculated.

(Fig. 2A and B). The generation of appressorial turgor pressure is required for mechanical penetration of the plant surface (Bechinger et al. 1999). Therefore, we evaluated the appressorial turgor of the *cokell* mutant using a cytorrhysis assay (Howard et al. 1991). The proportion of collapsed appressoria at glycerol concentrations above 2 M was higher in the *cokell* mutant than the wild type (Fig. 2C), indicating that the mutant was unable to produce wild-type levels of turgor pressure.

***CoKEL1* is involved in appressorium-mediated plant invasion.**

To assess the requirement of *CoKEL1* for fungal pathogenicity, conidial suspensions were inoculated onto cucumber cotyledons. On intact cotyledons, *cokell* mutants produced smaller lesions than those produced by the 104-T wild-type strain (Fig. 3A). In contrast, when conidial suspensions were placed onto wound sites produced by scratching with a toothpick, *cokell*

mutants formed lesions indistinguishable from those of the wild type (Fig. 3B).

These results suggested that the *cokell* mutant has a defect in appressorium-mediated penetration of host tissues. To determine whether appressoria of the *cokell* mutant retained the ability to penetrate and form infection hyphae on artificial substrata, we incubated conidia of the mutant on cellophane membranes. Both the *cokell* mutant and the wild-type formed melanized appressoria which penetrated the cellophane membranes, producing infection hyphae inside the membrane (Fig. 4A). When the membranes were stained with $\text{ZnCl}_2/\text{KI}/\text{I}_2$ solution, haloes were observed around appressoria, indicating that the *cokell* mutant secretes cellulose-degrading enzymes during infection (Fig. 4B). Appressoria of the *cokell* mutants also penetrated cellophane with frequency similar to that of the wild type (Fig. 4C). To verify whether appressoria of *cokell* mutants penetrated cucumber epidermis, we inoculated conidial suspensions onto the abaxial surface of cucumber cotyledons, and infection sites were observed with microscopy after 3 days. In contrast to the penetration assay on cellophane membranes, appressoria of the *cokell* mutants rarely penetrated cucumber epidermal cells to form intracellular infection hyphae (Fig. 4A). In the wild type, 27% of appressoria successfully penetrated to form infection hyphae on cucumber cotyledon, whereas only 3% of the *cokell* appressoria formed infection hyphae (Fig. 4C). On cucumber cotyledons where host resistance had been impaired by heat treatment, 58% of wild-type appressoria formed infection hyphae compared with only 7% of *cokell* appressoria (Fig. 4C).

Cell wall synthesis is affected in the *cokell* mutant.

Vegetative growth of the *cokell* mutant was affected at 28°C. The colony morphology of the mutant was similar to the wild-type when incubated at 24°C but, at 28°C, *cokell* colonies showed irregular margins (Supplementary Fig. S3). Radial growth of the wild type was also faster than the *cokell* mutant at 28°C. However, the colony morphology and growth rate of the *cokell* mutant at 28°C were restored when incubated on PDA containing 0.5 M sorbitol. Thus, hyphal growth of the *cokell* mutant was impaired by high temperature and restored

Fig. 2. Appressorium formation by *cokell* mutants. Conidial suspensions of each strain prepared in distilled water were incubated on multi-well glass slides at 24°C for 24 h. **A**, Appressorium formation of *cokell* mutant RK1-1 and the wild-type strain 104-T. Ap, Appressoria. Co, Conidia. Scale bars = 10 μm . **B**, Percentage of conidial germination (closed bars), appressorium formation (shaded bars), and abnormal appressorium formation (open bars). 104-T, the wild type; RK1-1 and RK1-2, *cokell* mutants; EK1-1, ectopic transformant; RK1C-1, *GFP::CoKEL1-1*-complemented transformant of RK1-1. Approximately 200 conidia were observed per well, and three replicates were examined. Means and standard errors were calculated from three independent experiments. **C**, Appressorial cytorrhysis assay. Appressoria formed on multi-well glass slides were exposed to glycerol solutions ranging from 0 to 4 M and the percentage of collapsed spherical appressoria was counted. Approximately 200 conidia were observed per well and three replicates were examined. Means and standard errors were calculated from four independent experiments. Solid line, the wild-type 104-T; dotted line, *cokell* mutant.

Fig. 3. Pathogenicity of *cokell* mutants on cucumber. **A**, Pathogenicity assay of *cokell* mutants on intact cucumber cotyledons. Conidial suspensions were droplet inoculated onto detached cucumber cotyledon and incubated at 24°C for 6 days. WT, wild-type 104-T; RK1-1 and RK1-2, *cokell* mutants; EK1-1, ectopic transformant; RK1C-1, *GFP::CoKEL1-1*-complemented transformant of RK1-1; Mock, distilled water control. **B**, Pathogenicity assay of *cokell* mutants on wounded cucumber cotyledons. Conidial suspensions were droplet inoculated onto wound sites (prepared by scratching the leaf surface with a sterile toothpick) and incubated at 24°C for 6 days. WT, wild type 104-T; RK1-1, *cokell* mutant; Mock, distilled water control.

by high osmotic conditions. *S. cerevisiae* mutants of the Pkc1 and Slr2 MAP kinases, which are involved in cell wall integrity, show osmoremedial cell lysis at 37°C (Lee et al. 1993). Similarly, in *Magnaporthe oryzae*, the mycelial autolysis phenotype of mutants defective in *MPS1*, a MAP kinase gene closely related to Slr2, could be complemented by 1 M sorbitol (Xu et al. 1998). Therefore, we postulated that cell wall synthesis might be impaired in the *cokell* mutant. To determine

whether *cokell* mutants have weakened cell walls, we counted the number of protoplasts released after digestion of mycelia with lysing enzymes for 2 or 3 h. The mean number of protoplasts released from mycelia of the *cokell* mutant was three times higher than from wild-type mycelia (Fig. 5A). Furthermore, the *cokell* mutant was more sensitive to caffeine, a chemical that affects cell wall integrity. Hyphal growth rates of the *cokell* mutant were approximately 85% of those of the wild type and *CoKEL1*-reintroduced transformant on the PDA containing 5 mM caffeine, while remaining the same as those on PDA without caffeine (Supplementary Fig. S4). Moreover, the abundance of cell wall polysaccharides labeled by Calcofluor white was also affected in the *cokell* mutants. Thus, appressorial cell walls of the wild type stained strongly with Calcofluor white, whereas fluorescent labeling was not detectable in appressoria of the *cokell* mutant (Fig. 5B).

Cokellp is a microtubule-associated protein.

To further elucidate the cellular role of Cokellp, the green fluorescent protein (GFP) gene was fused to the C-terminus of *CoKEL1* and expressed under the native *CoKEL1* promoter. Introduction of the *GFP-CoKEL1* fusion gene into the *cokell* mutant fully restored wild-type conidiation, appressorium formation, and pathogenicity (Figs. 2 and 3), showing that the *GFP-CoKEL1* fusion protein was functional. However, GFP fluorescence could not be detected in the complemented transformants. Therefore, we constructed a *GFP-CoKEL1* fusion gene expressed under a constitutive promoter. This construct was introduced into the *cokell* mutant and one transformant was named TFK1G. The GFP signals in some TFK1G hyphae were detected as filamentous structures similar to a cortical microtubule (data not shown). Therefore, to visualize microtubules, we introduced an *mRFP1- α TUB1* fusion gene into the TFK1G transformant, named TFK1GR. In most TFK1GR hyphae, GFP-Cokellp distributed uniformly through the cytoplasm and did not show localization at specific intracellular sites whereas mRFP1- α TUB1 detected filamentous structures (Fig. 6A).

On the other hand, GFP-Cokellp signal also corresponded to a microtubule labeled by mRFP1- α TUB1. In some TFK1GR hyphae, cytoplasm and filamentous structures were labeled by GFP-Cokellp and these filamentous structures co-localized with mRFP1- α TUB1 on cortical microtubules (Fig. 6B a through c). Furthermore, punctuate GFP signals were observed when the hyphae of this strain were treated with the microtubule depolymerizing drug benomyl (Fig. 6B d through f). These results indicate that Cokellp is only partly localized on microtubules.

Furthermore, the localization of GFP-Cokellp was cell cycle dependent. Thus, in cells undergoing mitosis, the long, filamentous microtubules labeled by mRFP1- α TUB1 disappeared and thick bundles were observed corresponding to mitotic spindles.

Fig. 4. Penetration ability of the *cokell* mutant on cellophane and cucumber cotyledons. **A**, Microscopic observation of the infection process. Conidial suspensions of the wild-type 104-T and *cokell* mutant were incubated on cellophane membranes at 24°C for 48 h or on the abaxial surface of cucumber cotyledons at 24°C for 72 h. Ce., Cellulose membrane; Cu., cucumber cotyledons; H.s.Cu., cucumber cotyledons subjected to heat treatment at 50°C for 30 s. Scale bars = 10 μ m. Ap, Appressoria; Ih, Infection hyphae stained with aniline blue. **B**, Conidial suspensions of the wild-type 104-T and *cokell* mutant were incubated on cellophane membranes at 24°C for 48 h and the membranes were stained with $ZnCl_2/KI/I_2$ solution. Ap, Appressoria; Ha, Halo. Scale bar = 10 μ m. **C**, Percentage of appressoria forming infection hyphae. In total, at least 200 appressoria were observed for each sample, based on examination of three replicates. Means and standard errors were calculated from three independent experiments.

In these cells, GFP-Cokel1p signals were detected on the spindle microtubules labeled by mRFP1- α TUB1 (Fig. 6B g through i). On the other hand, the localization of GFP-Cokel1p at specific intracellular sites was not observed during appressorium formation (data not shown).

cokel1-cokel2 double mutants show an additive phenotype.

The above results indicated that *CoKEL1* is involved in appressorium morphogenesis. Recently, we isolated another kelch repeat encoding gene, *CoKEL2*, in *C. orbiculare*. *CoKEL2* was also involved in appressorium morphogenesis but, unlike *CoKEL1*, was not essential for fungal pathogenicity (Sakaguchi et al. 2008). To further investigate the roles of *CoKEL1* and *CoKEL2* in appressorium development, *cokel1-cokel2* double mutants were isolated. The gene replacement vector pBIG3CKEL2Tn was introduced into the *cokel1* mutant RK1-

1 by AtMT, and the replacement of *CoKEL2* was confirmed by DNA gel blot analysis (data not shown). The *cokel1-cokel2* double mutants showed an additive phenotype for both appressorium morphogenesis and pathogenicity compared with the *cokel1* and *cokel2* single mutants. The *cokel1-cokel2* mutants formed abnormal appressorium similar to those of *cokel2* on glass slides (Fig. 7A). Approximately 90% of *cokel2* appressoria showed lateral germination, as reported previously (Sakaguchi et al. 2008), and appressoria of the *cokel1-cokel2* double mutant underwent lateral germination to a similar extent (Fig. 7B). On the other hand, pathogenicity of the *cokel1-cokel2* mutants on cucumber cotyledons was reduced to the same extent as the *cokel1* single mutant, whereas the *cokel2* single mutant formed much larger necrotic lesions, similar to those of the wild type (Fig. 7C).

Fig. 5. Cell wall synthesis is altered in the *cokel1* mutant. **A**, Sensitivity of the *cokel1* mutant to cell-wall-degrading enzymes. Bars represent the average number of protoplasts released from wild-type or *cokel1* mutant mycelia after incubation with Lysing enzyme for 2 or 3 h. Means and standard deviations were calculated from five independent experiments. **B**, Appressoria of the *cokel1* mutant stained with Calcofluor white to detect cell wall polysaccharides. Conidia germinated for 6 h were stained with Calcofluor white (40 ng ml⁻¹). Ap, Appressoria. Scale bars = 10 μ m.

Fig. 6. Cokel1p is associated with microtubules. **A**, *GFP-CoKEL1* and *mRFP1- α TUB1* expression in vegetative hyphae grown on potato dextrose agar. Green fluorescent protein (GFP) fluorescence was distributed uniformly through the cytoplasm with no preferential labeling of specific cell components while mRFP1 fluorescence was detected as filamentous microtubule structures. Scale bars = 10 μ m. **B**, Localization of Cokel1p-GFP signals to microtubules was demonstrated by coexpression of mRFP1- α TUB1. **a** through **c**, Filamentous structure of Cokel1p-GFP corresponded to cortical microtubule labeling mRFP1- α TUB1. **d** through **f**, Vegetative hyphae were treated with a microtubule depolymerizing drug, benomyl (10 μ g ml⁻¹). Cokel1p-GFP and mRFP1- α TUB1 fluorescence colocalized in punctate structures within the cytoplasm. **g** through **i**, Hyphal cells undergoing mitosis, showing that Cokel1p-GFP signals localize on spindle microtubules. Cm, cortical microtubule; Sp, spindle microtubule; DIC, differential-interference contrast images; GFP-Cokel1p, epifluorescence images of GFP-Cokel1p; mRFP1- α TUB1, epifluorescence images of mRFP1- α TUB1; merge, overlay of green and red fluorescence. Scale bars = 5 μ m.

DISCUSSION

Cokel1p of *C. orbiculare* is a microtubule-associated protein.

From a forward genetic screen for pathogenicity mutants in *C. orbiculare*, we have identified the gene *CoKEL1*, which encodes a protein with a C-terminal region containing multiple repeats of the kelch motif. The kelch repeat forms β -propeller structure that is considered to function as a protein–protein interaction module mediating association with diverse partners (Adams et al. 2000; Prag and Adams 2003). For example, the *Drosophila* kelch protein is a component of ring canals which are the intercellular bridges connecting the nurse cells and the oocyte in an egg chamber and is involved in the transport of nurse cell cytoplasm to the oocyte. The kelch repeat is necessary and sufficient for ring canal localization (Prag and Adams 1993; Robinson and Cooley 1997). Keap1, a mammalian actin-binding protein, sequesters the transcription factor Nrf1 and protects it against electrophilic and oxidative stress (Kang et al. 2002). *Arabidopsis thaliana* ACBP4 and ACBP5, acyl-CoA-binding proteins, are involved in maintaining an oleoyl-CoA pool in the cytosol and transfer of oleoyl-CoA esters from

the chloroplast to the endoplasmic reticulum (Leung et al. 2004). The crystal structure of a fungal galactose oxidase from *Dactylium dendroides* has been determined and the propeller structure shown to be the catalytic domain of the enzyme (Ito et al. 1994).

Some kelch repeat proteins are associated with the actin or microtubule cytoskeleton (Adams et al. 2000). For example, the kelch repeat in tea1p of *Schizosaccharomyces pombe* mediates interaction with CLIP170 tip1p, which is essential for appropriate localization at microtubule plus ends and cell apices (Brunner and Nurse 2000; Feierbach et al. 2004; Martin et al. 2005). Similarly, tea1p homologues such as Cokel2p of *C. orbiculare* and TeaA of *Aspergillus nidulans* associate with microtubule plus ends in growing cells (Mata and Nurse 1997; Sakaguchi et al. 2008; Takeshita et al. 2008). In the present study, we observed the localization of GFP-Cokel1p expressed from a strong constitutive promoter, because the expression level provided by the *CoKEL1* native promoter was low. Significantly, GFP-Cokel1p localized at cytoplasmic and spindle microtubules, indicating that Cokel1p is also a microtubule-associated protein. However, the cellular localization differed from that of Cokel2p in that Cokel1p was distributed along the length of microtubule filaments rather than at microtubule ends and cell apices. This finding suggests that Cokel1p is not conveyed along microtubules like tea1p or Cokel2p but, rather, resides at microtubules. Phylogenetic analysis indicated that *CoKEL1* and *CoKEL2* belong to different clades, which supports the idea that *CoKEL1* and *CoKEL2* are functionally distinct proteins.

The association of GFP-Cokel1p to microtubules was not visible in all hyphal cells and appeared to be restricted to a subset of cells. This localization pattern suggests that the association of Cokel1p with microtubules may be dependent on cell-autonomous factors, such as cell-cycle stage, and may be regulated by Cokel1p-associated proteins or signaling pathways that activate Cokel1p under specific cellular conditions or developmental stages.

The two kelch repeat proteins Cokel1p and Cokel2p are both involved in appressorium development and are microtubule-associating proteins. Therefore, to investigate the functional relationship between Cokel1p and Cokel2p during appressorium development, we generated double mutants. The *cokel1-cokel2* mutants showed an additive phenotype compared with that of the single mutants, suggesting that these two kelch repeat proteins do not function in the same cellular processes.

CoKEL1 is required for full virulence in *C. orbiculare*.

In the human pathogen *Candida albicans*, a kelch repeat protein was shown to be involved in the host–fungus interaction. Thus, the *MDS3* gene was required for the expression of filamentation-associated genes in alkaline media and contributed to full virulence (Davis et al. 2002). In contrast, kelch repeat proteins were not previously reported to play a role in the virulence of plant-pathogenic fungi.

Targeted disruption of *CoKEL1* strongly reduced appressorium-mediated infection of host epidermal cells. Accordingly, the *cokel1* mutants produced smaller lesions than the wild type on intact cucumber leaves. However, appressoria of *cokel1* mutants could penetrate and form hyphae inside cellophane membranes with efficiency similar to that of the wild-type strain, indicating that *CoKEL1* is not essential for differentiation of the appressorial penetration peg and infection hyphae. On the other hand, the appressoria of *cokel1* mutants formed very few infection hyphae on intact host leaves. In *C. orbiculare*, the ability of some pathogenicity mutants to penetrate cucumber cotyledons can be restored by impairing host resistance with a heat-shock treatment (Asakura et al. 2006; Takano

Fig. 7. Phenotype of *cokel1-cokel2* double mutants. **A**, Appressorium formation by the *cokel1-cokel2* double mutant (DK-1) compared with that of *cokel2* single mutant (RK2-1). Conidial suspensions of each strain in distilled water were incubated on multi-well glass slides at 24°C for 24 h. Ap, appressoria; Co, conidia. Scale bars = 10 μ m. **B**, Percentage of lateral germination from appressoria formed on slide glass. WT, wild-type 104-T; RK1-1, *cokel1* mutant; RK2-1, *cokel2* mutant; DK-1 and DK-2, *cokel1-cokel2* mutants. In total, at least 200 appressoria were observed for each sample, based on examination of three replicates. Means and standard errors were calculated from three independent experiments. **C**, Pathogenicity of *cokel1-cokel2* double mutants on intact leaves. Conidial suspensions were droplet-inoculated onto detached cucumber cotyledons and incubated at 24°C for 6 days. WT, wild-type 104-T; RK1-1, *cokel1* mutant; RK2-1, *cokel2* mutant; DK-1 and DK-2, *cokel1-cokel2* mutants; Mock, distilled water control.

et al. 2006; Tanaka et al. 2007). However, heat treatment did not restore the penetration efficiency of *cokell* mutants on cucumber cotyledons, suggesting that the reduced pathogenicity of *cokell* mutants results from impaired appressorium function.

***CoKEL1* is involved in infection-related morphogenesis.**

Although the frequency of appressorium formation and melanization were not affected by disruption of *CoKEL1*, appressorium morphogenesis was perturbed, and approximately half the conidia of *cokell* mutants formed irregularly shaped appressoria. Moreover, vegetative hyphae of the *cokell* mutant were more sensitive to cell-wall-degrading enzymes than the wild type, and appressorial cell walls of the *cokell* mutant showed reduced staining by the chitin-specific dye Calcofluor white. These results indicate that the disruption of *CoKEL1* may affect development of the appressorial cell wall. Furthermore, cytorrhysis assays showed that, even in *cokell* appressoria retaining normal (spherical) shape, turgor generation was significantly lower than in wild-type appressoria. These results indicate that *CoKEL1* plays a role not only in appressorium morphogenesis but also in the generation of physical force by appressoria.

The generation of high turgor pressure allows the melanized appressoria of pathogens such as *Colletotrichum* and *Magnaporthe* spp. to breach the host cuticle and epidermal cell wall by means of mechanical force (Wang et al. 2005). There is evidence that melanization is critical for turgor generation by reducing the porosity of appressorial cell walls and, thus, trapping osmotically active solutes such as glycerol within the appressorial cytoplasm (Howard et al. 1991). We propose that abnormal cell wall construction in the *cokell* mutant allows solutes to escape from the appressorium, resulting in the generation of insufficient turgor pressure for successful penetration of host epidermal cells.

MATERIALS AND METHODS

Strains and culture conditions.

Strain 104-T (MAFF240422) of *C. orbiculare* (Berk. & Mont.) Arx (syn. *C. lagenarium* (Pass.) Ellis & Halst.) was used as the wild-type strain. All *C. orbiculare* strains were maintained at 24°C in the dark on potato dextrose media (3.9% [wt/vol] PDA; Difco Laboratories, Detroit) or SD medium (0.67% [wt/vol] yeast nitrogen base without amino acids, 2% [wt/vol] glucose, 2% [wt/vol] agar). *Escherichia coli* DH5- α was used as a host for gene manipulation and was maintained on Luria-Bertani medium (Sambrook et al. 1989) at 37°C. *Agrobacterium tumefaciens* C58C1 was used as a T-DNA donor for fungal transformation and was maintained on AB-minimal medium (Lichtenstein and Draper 1986) at 28°C.

Fungal transformation.

Protoplast preparation and transformation of *C. orbiculare* were performed according to the method described previously (Kubo et al. 1991). In REMI mutagenesis, the vector pCB1490 containing the hygromycin resistance gene was introduced into fungal protoplasts with restriction enzyme *Pst*I. Transformants with the hygromycin resistance gene were selected on regeneration medium containing hygromycin B (Wako Chemicals, Osaka, Japan) at 50 μ g/ml. Strain P24 was isolated after screening the REMI transformants. *C. orbiculare* was transformed by AtMT as described previously (Tsuiji et al. 2003a). Hygromycin-resistant transformants were selected on PDA medium containing hygromycin B (Wako Chemicals) at 100 μ g/ml, cefotaxim (Wako Chemicals) at 100 μ g/ml, and spectinomycin (Wako Chemicals) at 100 μ g/ml. Bialaphos-resistant transformants were selected on SD medium containing bialaphos at 4 μ g/ml

(Meiji Seika Kaisha, Ltd., Tokyo), cefotaxim at 100 μ g/ml, and spectinomycin at 100 μ g/ml. Sulfonyleurea-resistant transformants were selected on SD medium containing chlorimuron ethyl (Chem Service, West Chester, PA, U.S.A.) at 4 μ g/ml, cefotaxim at 100 μ g/ml, and spectinomycin at 100 μ g/ml.

Cloning and sequencing.

To identify the disrupted gene in mutant P24, a TAIL-PCR protocol was applied to genomic DNA (Liu et al. 1995). Fungal genomic DNA flanking the pCB1490 plasmid insert was analyzed as described previously (Tsuiji et al. 2003a). Amplified PCR products were sequenced with the Big-Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Japan, Ltd., Tokyo) and an ABI PRISM 310 automated DNA sequencer (Applied Biosystems Japan, Ltd.). Genomic clones of *CoKEL1* were selected from a cosmid library of *C. orbiculare* 104-T by PCR using primer pair P24S1 (5'-TAGACCCA CACGTCGTTGAA-3') and P24AS1 (5'-ACGTGAGCGACA TGAACAAG-3') designed from the obtained DNA sequence. To obtain the entire *CoKEL1* ORF, the EZ::TN <KAN-2> Insertion Kit (Epicenter, Madison, WI, U.S.A.) was used to insert a transposon containing a selection marker into the target DNA. Clones containing the <KAN-2> transposon was sequenced using primers KAN2-RP2 (5'-GGAAGATCTTTG TGCAATGTAACATCAGAG-3') and KAN2-FP3 (5'-GCAG GCGATGCAAGCTTCAGGGTTG-3'). RACE analysis was performed using the 5' Full RACE Core Set (Takara Bio, Ohtsu, Japan) to determine the transcription initiation site of *CoKEL1*. The *CoKEL1* sequence is available in the DDBJ database under accession no. AB259752.

Plasmid constructs.

To construct the *CoKEL1* gene-replacement vector p24cosKEL1Tn, the 2.7-kb transposon EZ::TN <KAN-2>, in which the kanamycin resistance gene was replaced by the ampicillin resistance gene and the hygromycin resistance gene, was inserted into *A. tumefaciens* binary cosmid vector p24cosKEL1 containing the *CoKEL1* gene. The vector p24cosKEL1Tn was selected from randomly inserted clones and contained the transposon at 320 bp downstream from the start codon. Disruption of the *CoKEL1* gene was confirmed by genomic DNA blot analysis of selected transformants.

For construction of the *EGFP::CoKEL1* fusion gene regulated by a constitutive promoter, *A. tumefaciens* binary vector pBI-TEFpGFPB was used as starting construct. The plasmid pBI-TEFpGFPB contains the bialaphos resistance gene and the enhanced GFP (EGFP) ORF linked by replacement of the stop codon with a spacer encoding five glycine residues. The expression of *EGFP* was controlled by the translation elongation factor from *Aureobasidium pullulans* derived from pTEFEGFP (van den Wymelenberg et al. 1997). The full region of the *CoKEL1* ORF was amplified by PCR with primers KEL1S2 (5'-CGGAATTCATGGCTGATTCGCCAGGT-3') and KEL1AS3 (5'-CGGGATCCTAGACCTCGATGGTGAAGT-3') and was digested with *Eco*RI and *Bam*HI, introduced into the *Eco*RI and *Bam*HI site of pBI-TEFpGFPB, and designated as pBI-TEFpGFP-KEL1.

For construction of the *EGFP::CoKEL1* fusion gene regulated by the native *CoKEL1* promoter, *Agrobacterium tumefaciens* binary vector pBI-SCD1pGFPB was used as starting construct. The plasmid pBI-SCD1pGFPB contains the bialaphos resistance gene and EGFP ORF linked by replacement of the stop codon with a spacer encoding five glycine residues. The *EGFP* was controlled by a 221-bp 5' upstream region of the *C. orbiculare* *SCD1* gene (Takano et al. 2001). The last codon of EGFP was fused to 15 nucleotides encoding a glycine linker followed by the *Eco*RI, *Bam*HI site and the termi-

nator of the glucoamylase gene of *Aspergillus awamori*. To construct the *EGFP::CoKEL1* fusion vector, the full region of the *CoKEL1* ORF was amplified by PCR with primers KEL1S2 and KEL1AS3 and digested with *EcoRI* and *BamHI*, introduced into the *EcoRI* and *BamHI* site of pBI-SCD1pGFPB, and designated as pBI-SCD1pGFP-KEL1. The *SCD1* promoter was then exchanged for the native *CoKEL1* promoter. However, use of the *CoKEL1* promoter containing its uORF would have introduced a premature stop codon upstream of the start ATG of *EGFP*. To avoid this, the *SCD1* promoter and *EGFP* containing a Kozack sequence was exchanged for *EGFP* without a Kozack sequence upstream of the ATG codon, which was amplified by PCR using primers GFPATG (5'-GATTCTAGAATGGTGAGCAAGGGCGAG-3') and eGFPASgly (5'-CGGAATTCACCACCACCACCACCCTTGACAGCTCGTCCAT-3'). The GFPATG primer contains a terminal *XbaI* site, whereas eGFPASgly contains a terminal *EcoRI* site. The amplified product was digested with *XbaI* and *BamHI* and introduced into the *XbaI* and *BamHI* site of pBI-SCD1pGFP::KEL1, which contained the *SCD1* promoter and *EGFP*. The resulting plasmid was designated pBI-ATGGFP::KEL1. The 1,083-bp 5' upstream region of the *CoKEL1* gene was amplified by PCR using primers KEL1plusAS (5'-GGACTAGTATCAGCCATGGTTCGGCG-3'), containing uORF sequence overlapping *CoKEL1* (ATGGCTGAT) and a terminal *SpeI*, and T7 (5'-TAATACGACTCACTATAGGG-3'). The plasmid pBSKEL1, containing a 1,083-bp 5' upstream region of the *CoKEL1* gene and the *CoKEL1* ORF, was used as a template for PCR. The amplified product was digested with *SpeI* and introduced into the *XbaI* site of pBI-ATGGFP::KEL1. The resulting clone was named pBI-GFP::KEL1.

For labeling of the microtubule, pBI-SCD1pmRFP1:: α -TUB1S was used as previously described (Sakaguchi et al. 2008).

Genomic DNA blot analysis.

Genomic DNA of *C. orbiculare* was isolated from mycelia and DNA blot analysis was performed as described previously (Takano et al. 1997). DNA digestion, gel electrophoresis, labeling of probes, and hybridization were performed according to the manufacturer's instructions and standard methods (Sambrook et al. 1989). DNA probes were labeled with digoxigenin (DIG)-dUTP using the *BcaBEST* DIG labeling kit (Takara Bio). Hybridized DNA was detected by Anti-Digoxigenin-AP Fab fragments (Roche Diagnostics, Tokyo) and light emission generated by enzymatic dephosphorylation of CDP-Star Detection Reagent (GE Healthcare, Tokyo) by alkaline phosphatase was detected using FUJIFILM LAS1000 plus.

Pathogenicity tests.

Inoculation assays on cucumber cotyledons (*Cucumis sativus* L. 'Suyo') were performed as described previously (Tsuji et al. 2003b). For estimation of invasive growth ability, 3- μ l drops of conidial suspension (5×10^5 conidia ml^{-1}) were spotted onto wound sites prepared by scratching the leaf surface with a sterile toothpick. After inoculation, the cotyledons were incubated in a humid box at 24°C with a 16-h photoperiod for 5 days.

Microscopy.

For observation of appressorium formation and penetration in vitro, conidia were harvested from 7-day-old PDA cultures and suspended in distilled water. The conidial suspension was adjusted to 10^5 conidia ml^{-1} and 20- μ l droplets were placed onto eight-well multitest glass slides (ICN Biomedicals, Aurora, OH, U.S.A.) or cellophane membranes (Wako Chemi-

cals) and incubated at 24°C in the dark. Cellulose dissolution was assessed by staining the membranes with a solution containing ZnCl_2 (50%, wt/vol), KI (20%, wt/vol), and I_2 (0.5%, wt/vol). Germlings were observed with a Nikon ECLIPSE E600 microscope using differential-interference contrast optics (Nikon, Tokyo). For observation of infectious hypha in cucumber leaves, the conidial suspension of each strain was spotted onto the abaxial surface of cucumber cotyledons and incubated at 24°C for 3 days. Then, the inoculation site was peeled off and stained with 0.1% lactophenol-anilin blue. For observation of GFP and mRFP1 fluorescence, cells were viewed with an epifluorescence microscope equipped with a GFP(R)-BP filter (460- to 500-nm excitation filter, 505-nm dichroic mirror, and 510- to 560-nm barrier filter) and G-2A filter (510- to 560-nm excitation filter, 575-nm dichroic mirror, and >590-nm barrier filter), respectively. For staining cell wall polysaccharides, Fluorescent Brightener 28 (Calcofluor white, MP Biomedicals, Inc., Burlingame, CA, U.S.A.) (40 ng ml^{-1}) was applied to germlings grown for 6 h on multitest glass slides. After 10 min, the stained cells were washed with distilled water and observed by epifluorescence microscopy with a UV-1A filter (365/10-nm excitation filter, 400-nm dichroic mirror, and 400 nm-barrier filter).

For protoplast preparation, conidia of each strain were incubated in potato-sucrose liquid medium at 24°C for 3 days with shaking at 90 rpm. Mycelia were collected by filtration, washed in a solution of 1.2 M MgSO_4 and 10 mM Na_2HPO_4 , and then transferred to the same solution supplemented with Lysing enzymes (5 mg/ml) (Sigma), and incubated at 24°C. Protoplasts released from mycelia after treatment with enzymes for 2 and 3 h were then counted on a hemacytometer slide using a microscope.

ACKNOWLEDGMENTS

We thank R. O'Connell for carefully reading the manuscript and giving useful suggestions. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (nos.19380029 and 21380031).

LITERATURE CITED

- Adams, J., Kelso, R., and Cooley, L. 2000. The kelch repeat superfamily of proteins: Propellers of cell function. *Trends Cell Biol.* 10:17-24.
- Arellano, M., Niccoli, T., and Nurse, P. 2002. Tea3p is a cell end marker activating polarized growth in *Schizosaccharomyces pombe*. *Curr. Biol.* 12:751-756.
- Asakura, M., Okuno, T., and Takano, Y. 2006. Multiple contributions of peroxisomal metabolic function to fungal pathogenicity in *Colletotrichum lagenarium*. *Appl. Environ. Microbiol.* 72:6345-6354.
- Asakura, M., Ninomiya, S., Sugimoto, M., Oku, M., Yamashita, S., Okuno, T., Sakai, Y., and Takano, Y. 2009. Atg26-mediated pexophagy is required for host invasion by the pla1291-nt pathogenic fungus *Colletotrichum orbiculare*. *Plant Cell* 1291-1304.
- Bechinger, C., Giebel, K. F., Schnell, M., Leiderer, P., Deising, H. B., and Bastmeyer, M. 1999. Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. *Science* 285:1896-1899.
- Brunner, D., and Nurse, P. 2000. CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell* 102:695-704.
- Davis, D. A., Bruno, V. M., Loza, L., Filler, S. G., and Mitchell, A. P. 2002. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* 162:1573-1581.
- Feierbach, B., Verde, F., and Chang, F. 2004. Regulation of a formin complex by the microtubule plus end protein tea1p. *J. Cell Biol.* 165:697-707.
- Fukui, Y., Miyake, S., Satoh, M., and Yamamoto, M. 1989. Characterization of the *Schizosaccharomyces pombe* *ral2* gene implicated in activation of the *ras1* gene product. *Mol. Cell. Biol.* 9:5617-5622.
- Harashima, T., and Heitman, J. 2002. The Galpa protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gbeta subunits. *Mol. Cell* 10:163-173.

- Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. U.S.A.* 88:11281-11284.
- Ito, N., Phillips, S. E., Yadav, K. D., and Knowles, P. F. 1994. Crystal structure of a free radical enzyme, galactose oxidase. *J. Mol. Biol.* 238:794-814.
- Kang, M. I., Kobayashi, A., Wakabayashi, N., Kim, S. G., and Yamamoto, M. 2002. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proc. Natl. Acad. Sci. U.S.A.* 101:2046-2051.
- Kimura, A., Takano, Y., Furusawa, I., and Okuno, T. 2001. Peroxisomal metabolic function is required for appressorium-mediated plant infection by *Colletotrichum lagenarium*. *Plant Cell* 13:1945-1957.
- Kojima, K., Kikuchi, T., Takano, Y., Oshiro, T., and Okuno, T. 2002. The mitogen-activated protein kinase gene MAF1 is essential for the early differentiation phase of appressorium formation in *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* 15:1268-1276.
- Kubo, Y., and Furusawa, I. 1991. Melanin biosynthesis: Prerequisite for successful invasion of the plant host by appressoria of *Colletotrichum* and *Pyricularia*. Pages 205-217 in: *The Fungal Spore and Disease Initiation in Plants and Animals*. G. T. Cole and H. C. Hoch, eds. Plenum Publishing, New York.
- Kubo, Y., Nakamura, H., Kobayashi, K., Okuno, T., and Furusawa, I. 1991. Cloning of a melanin biosynthetic gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* 4:440-445.
- Kubo, Y., Takano, Y., Endo, N., Yasuda, N., Tajima, S., and Furusawa, I. 1996. Cloning and structural analysis of the melanin biosynthesis gene SCD1 encoding scytalone dehydratase in *Colletotrichum lagenarium*. *Appl. Environ. Microbiol.* 62:4340-4344.
- Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin D. E. 1993. A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. *Mol. Cell. Biol.* 13:3067-3075.
- Leung K. C., Li, H. Y., Mishra, G., and Chye, M. L. 2004. ACBP4 and ACBP5, novel *Arabidopsis* acyl-CoA-binding proteins with kelch motifs that bind oleoyl-CoA. *Plant Mol. Biol.* 55:297-309.
- Lichtenstein, C., and Draper, J. 1986. Genetic engineering of plants. Pages 67-119 in: *DNA Cloning: A Practical Approach*, Vol. 2. D. M. Glover, ed. IRL Press, Oxford.
- Liu, Y. G., Mitsuoka, N., Teruo, O., and Whittier, R. F. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* 8:457-463.
- Martin, S. G., McDonald, W. H., Yates, J. R., and Chang, F. 2005. Tea4p links microtubule plus ends with the formin for3p in the establishment of cell polarity. *Dev. Cell.* 8:479-491.
- Mata, J., and Nurse, P. 1997. tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell. *Cell* 89:939-949.
- Peeters, T., Versele, M., and Thevelein, J. M. 2007. Directly from Galpha to protein kinase A: The kelch repeat protein bypass of adenylate cyclase. *Trends Biochem. Sci.* 32:547-554.
- Perpetua, N. S., Kubo, Y., Yasuda, N., Takano, Y., and Furusawa, I. 1996. Cloning and characterization of a melanin biosynthetic THR1 reductase gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* 9:323-329.
- Philips, J., and Herskowitz, I. 1998. Identification of Kel1p, a kelch domain-containing protein involved in cell fusion and morphology in *Saccharomyces cerevisiae*. *J. Cell Biol.* 143:375-389.
- Prag, S., and Adams, J. C. 1993. kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell* 72:681-693.
- Prag, S., and Adams, J. C. 2003. Molecular phylogeny of the kelch-repeat superfamily reveals an expansion of BTB/kelch proteins in animals. *BMC Bioinf.* 17:42.
- Robinson, D. N., and Cooley, L. 1997. *Drosophila* kelch is an oligomeric ring canal actin organizer. *J. Cell Biol.* 138:799-810.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sakaguchi, A., Miyaji, T., Tsuji, G., and Kubo, Y. 2008. Kelch repeat protein Clakel2p and calcium signaling control appressorium development in *Colletotrichum lagenarium*. *Eukaryot. Cell.* 7:102-111.
- Takano, Y., Kubo, Y., Shimizu, K., Mise, K., Okuno, T., and Furusawa, I. 1995. Structural analysis of PKS1, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. *Mol. Gen. Genet.* 249:162-167.
- Takano, Y., Kubo, Y., Kawamura, C., Tsuge, T., and Furusawa, I. 1997. The *Alternaria alternata* melanin biosynthesis gene restores appressorial melanization and penetration of cellulose membranes in the melanin-deficient albino mutant of *Colletotrichum lagenarium*. *Fungal Genet. Biol.* 21:131-140.
- Takano, Y., Kikuchi, T., Kubo, Y., Hamer, J. E., Mise, K., and Furusawa, I. 2000. The *Colletotrichum lagenarium* MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. *Mol. Plant-Microbe Interact.* 13:374-383.
- Takano, Y., Oshiro, E., and Okuno, T. 2001. Microtubule dynamics during infection-related morphogenesis of *Colletotrichum lagenarium*. *Fungal Genet. Biol.* 34:107-121.
- Takano, Y., Takayanagi, N., Hori, H., Ikeuchi, Y., Suzuki, T., Kimura, A., and Okuno, T. 2006. A gene involved in modifying transfer RNA is required for fungal pathogenicity and stress tolerance of *Colletotrichum lagenarium*. *Mol. Microbiol.* 60:81-92.
- Takeshita, N., Higashitsuji, Y., Konzack, S., and Fischer, R. 2008. Apical sterol-rich membranes are essential for localizing cell end markers that determine growth directionality in the filamentous fungus *Aspergillus nidulans*. *Mol. Biol. Cell* 19:339-3351.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
- Tanaka, S., Yamada, K., Yabumoto, K., Fujii, S., Huser, A., Tsuji, G., Koga, H., Dohi, K., Mori, M., Shiraishi, T., O'Connell, R., and Kubo Y. 2007. *Saccharomyces cerevisiae* SSD1 orthologues are essential for host infection by the ascomycete plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea*. *Mol. Microbiol.* 64:1332-1349.
- Tsuji, G., Kenmochi, Y., Takano, Y., Sweigard, J., Farrall, L., Furusawa, I., Horino, O., and Kubo, Y. 2000. Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and Pig1p of *Magnaporthe grisea*, contain Cys2His2 zinc finger and Zn (II)2Cys6 binuclear cluster DNA binding motifs, and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. *Mol. Microbiol.* 38:940-954.
- Tsuji, G., Fujii, S., Fujihara, N., Hirose, C., Tsuge, S., Shiraishi, T., and Kubo, Y. 2003a. *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. *J. Gen. Plant Pathol.* 69:230-239.
- Tsuji, G., Fujii, S., Tsuge, S., Shiraishi, T., and Kubo, Y. 2003b. The *Colletotrichum lagenarium* Ste12-like gene CST1 is essential for appressorium penetration. *Mol. Plant-Microbe Interact.* 16:315-325.
- van den Wymelenberg A. J., Cullen, D., Spear, R. N., Schoenike, B., and Andrews, J. H. 1997. Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification of the fungus on leaf surfaces. *Biotechniques* 23:686-690.
- Wang, Z. Y., Jenkinson, J. M., Holcombe, L. J., Soanes, D. M., Veneault-Fourrey, C., Bhambra, G. K., and Talbot, N. J. 2005. The molecular biology of appressorium turgor generation by the rice blast fungus *Magnaporthe grisea*. *Biochem. Soc. Trans.* 33:384-388.
- Xu, J. R., Staiger, C. J., and Hamer, J. E. 1998. Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 95:12713-12718.
- Yamauchi, J., Takayanagi, N., Komeda, K., Takano, Y., and Okuno, T. 2004. cAMP-pKA signaling regulates multiple steps of fungal infection cooperatively with Cmk1 MAP kinase in *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* 17:1355-1365.