Kelch Repeat Protein Clakel2p and Calcium Signaling Control Appressorium Development in Colletotrichum lagenarium

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Kelch repeat proteins are important mediators of fundamental cellular functions and are found in diverse organisms. However, the roles of these proteins in filamentous fungi have not been characterized. We isolated a kelch repeat-encoding gene of Colletotrichum lagenarium Clakel2, a Schizosaccharomyces pombe tea1 homologue. Analysis of the clakel2 mutant indicated that Clakel2 is required for the establishment of cellular polarity essential for proper morphogenesis of appressoria and that there is a plant signal-specific bypass pathway for appressorium development which circumvents Clakel2 function. Clakel2p was localized in the polarized region of growing hyphae and germ tubes, and the localization was disturbed by a microtubule assembly blocker. The clakel2 mutants formed abnormal appressoria, and those appressoria were defective in penetration hypha development into cellulose membranes, an artificial model substrate for fungal infection. Surprisingly, the clakel2 mutants formed normal appressoria on the host plant and retained penetration ability. Normal appressorium formation on the artificial substrate by the clakel2 mutants was restored when cells were incubated in the presence of CaCl2, or exudates from cucumber cotyledon. Furthermore, calcium channel modulators inhibited restoration of normal appressorium formation. These results suggest that there could be a bypass pathway that transduces a plant-derived signal for appressorium development independent of Clakel2 and that a calcium signal is involved in this transduction pathway.

The ability to generate cell polarity in eukaryotic organisms is important to regulate processes such as cell division, cell differentiation, and cell migration. The general principles of cell polarization are relatively well understood in yeasts. In budding and fission yeast, the onset of polarized growth is controlled by the determination of a growth origin that is usually marked by the deposition of a landmark protein (9). In the fission yeast Schizosaccharomyces pombe, tea1p, a kelch repeat protein, could be a candidate landmark protein localized at microtubule plus ends (3). The tea1 mutants grow as monopolar cells and form bent or T-shaped cells under certain conditions (28). tea2 encodes a kinesin-like protein (6), and the tea2p protein is loaded onto microtubule plus ends using tea2p’s intrinsic motor activity (7). The polarity of hyphae is a defining feature of filamentous fungi, allowing them to efficiently colonize and exploit new substrates. In fungal hyphae, which grow at the tip, apical extension is based on the intracellular transport of vesicles along the cytoskeleton. KipA, related to Tea2p in Aspergillus nidulans, is also required for polarized growth (19). Myo5, the actin-based myosin motor of Ustilago maydis, is required for morphogenesis, dimorphic switch, and pathogenicity, indicating that the cytoskeleton is important for the polarized growth of fungal hyphae (51).

Colletotrichum lagenarium is a plant-pathogenic fungus that causes anthracnose disease of cucumber. The infection process of this fungus involves a series of key steps and changes in fungal morphology. Melanization of the appressorium, a specialized infection structure, is essential for penetration of host leaves (20, 22, 32, 40, 45). The establishment of polarity occurs at several points during infection by C. lagenarium: germ tube emergence from the conidium, germ tube elongation, penetration peg emergence from the appressorium, and branch emergence from hyphae growing inside the host plant. Although the regulation of cell polarity is likely to play a key role in the morphological transitions that occur during pathogenesis, little is known about the mechanistic details and structural requirements for these transitions in plant pathogens.

A number of environmental signals encountered by a plant-pathogenic fungus on a host surface mediate the signal cascades that lead to appressorium development. In many fungal pathogens, infection-related morphological changes are linked to signal transduction through the cyclic AMP (cAMP) and mitogen-activated kinase (MAPK) pathways (25). For example, in the rice blast fungus Magnaporthe grisea, cAMP is crucial for appressorium differentiation (24, 29), while in C. lagenarium, cAMP-protein kinase A (PKA) signaling regulates germination but is not required for appressorium differentiation (54). The MAPK cascades also play pivotal roles in the infection process of fungi (52). In C. lagenarium, three MAPKs were characterized. MAF1, the Saccharomyces cerevisiae SLT2 orthologue, regulates appressorium development, and the maf1 mutant has reduced pathogenicity on host plants (16). CMK1, the S. cerevisiae Fus3/Kss1 orthologue, is involved in germination, appressorium formation, and infection hypha growth (42). OSC1, the S. cerevisiae HOG1 orthologue, is involved in a response to high osmotic stress (17). Moreover, CST1, the S. cerevisiae STE12 transcription factor orthologue is involved in penetration peg formation by appressoria (46).

Calcium signaling machinery is also involved in transducing
a wide variety of external signals through numerous stimulus-response pathways within the cellular transduction network (4, 37). In Colletotrichum trifolii (50) and Colletotrichum gloeosporioides (14, 48), a calcium signal is important for appressorium formation, and in Fusarium graminearum (35) and Neurospora crassa (34), calcium serves as a hyphal branching signal.

In this study, we investigated the involvement of a cell polarity factor in the infection-related morphogenesis of plant-pathogenic fungi. We isolated from C. lagenarium the ClaKEL2 gene, an S. pombe tea1 homologue, encoding a kelch repeat protein. Analysis of the clakel2 knockout mutant indicated that ClaKEL2 is required for proper morphogenesis of appressoria and that there is a bypass pathway independent of ClaKEL2 that transduces a plant-derived signal for appressorium development through calcium signaling. These findings provide the first information on the function of kelch repeat proteins in filamentous fungi.

MATERIALS AND METHODS

Strains and culture conditions. Strain 104-T (stock culture of the laboratory of Plant Pathology, Kyoto Prefectural University) of C. lagenarium (Passerini) Ellis et Halsted [syn. C. orbiculare (Berkeley et Montagne) Arx] was used as the wild-type strain (21). The wild-type C. lagenarium strain and its mutants were maintained on potato dextrose agar (PDA) medium (3.9%, wt/vol; Difco, Detroit, MI) or synthetic dextrose (SD) medium (0.667% [wt/vol] yeast nitrogen base without amino acids [Difco], 0.5% [wt/vol] glucose, 2% [wt/vat] agar) at 24°C in the dark. Escherichia coli strain DH5α was used as a host for gene manipulation and Agrobacterium tumefaciens strain C58C1 was used as a transfer DNA donor for fungal transformation. These bacterial strains were maintained on Luria-Bertani medium (36) at 37°C, and AB minimal medium (26) at 28°C, respectively.

Fungal transformation. C. lagenarium was transformed using the A. tumefaciens-mediated transformation protocol according to a method described previously (47). Hygromycin-resistant transformants were selected on medium containing 100 µg/ml hygromycin B (Wako Chemicals, Osaka, Japan), 100 µg/ml cefotaxim (Wako Chemicals, Osaka, Japan), and 100 µg/ml spectinomycin (Wako Chemicals, Osaka, Japan). Bialaphos-resistant transformants were selected on SD medium containing 4 µg/ml bialaphos (Meiji Seika Kaisha, Ltd., Tokyo, Japan), 100 µg/ml cefotaxim, and 100 µg/ml spectinomycin. Sulfonurea-resistant transformants were selected on SD medium containing 4 µg/ml chlorimuronethyl (Chem Service, West Chester, PA), 100 µg/ml cefotaxim, and 100 µg/ml sulfonurea.

Cloning and sequencing. All primers used in this study are listed in Table S1 in the supplemental material. The gene containing the kelch motif ClaKEL2 was isolated by PCR using degenerate primers. The genomic DNA of C. lagenarium was used as a template for PCR. For isolation of ClaKEL2, three degenerate primers, dKEL2S2, dKEL2S3, and dKELAS1, were designed based on the amino acid sequence of the putative kelch repeat protein in other filamentous fungi, including M. grisea, F. graminearum, A. niger, and N. crassa. The first PCR was performed with primers dKEL2S2 and dKELAS1. Nested PCR was performed with primers dKEL2S2, dKEL2S3, and dKELAS1, designed based on the amino acid sequence of the putative kelch repeat protein in other filamentous fungi, including M. grisea, F. graminearum, A. niger, and N. crassa. The first PCR was performed with primers dKEL2S2 and dKELAS1. Nested PCR was performed with primers dKEL2S3 and dKELAS1. PCR conditions were as follows: 5 cycles of 94°C for 30 s, 40°C for 2 s, and 74°C for 30 s, followed by 30 cycles of 94°C for 30 s, 58°C for 2 s, and 74°C for 30 s. The second PCR was performed with primers dKEL2S3 and dKELAS1. The amplified products were digested with XhoI site of pGreenII0000 (13) and designated as pGr2H1. The 1.027-kb fragment corresponding to the ClaKEL2 gene was cloned into pBSKEL2H::EGFP construct in which the kanamycin resistance gene replaced the chromosomal resistance gene and the bialaphos resistance gene was inserted into pBSKEL2H. The pBSKEL2HTn containing the transposon 439 bp from the start codon was selected from randomly cloned inserts. Finally, the Xhol fragment containing ClaKEL2 and the transposon from pBSKEL2HTn were cloned into the A. tumefaciens binary vector pBluescript II SK−. The 2.2-kb EcoRI::TN −<KAN−2> construct was digested with EcoRI and introduced into pBI-SCD1pGFP containing the selection marker into the target DNA. The obtained transformant containing the hygromycin resistance gene from pCB1636 was introduced into the Xhol site of pGreenII0000 (13) and designated as pGr2H1. The pEGFP ORF encoding glycine residues in the N-terminal region and the terminator of the glucococase gene of Aspergillus awamori (49) were amplified by PCR with primers pEGFPSG and GA-AS1. The primer pEGFPSG contains a terminal HindIII site, whereas GA-AS1 contains a terminal Sall site. The amplified product was digested with HindIII and Sall, introduced into the HindIII and Sall site of pGr2H1, and designated as pGr2H1GlyGFP. The BamHI-KpnI fragment containing pEGFP and the hygromycin resistance gene from pGr2H1GlyGFP was introduced into the BamHI-KpnI site of the A. tumefaciens binary vector pBI-G3C::KAN-2. The 439-bp pEGFP from the start codon was inserted into pBI-G3C::KAN-2, and named pBI-KEL2::GFP.

For the mRFP1::<TUB1::EGFP fusion gene, the GFP-expressing A. tumefaciens binary vector pBluescript II SK− was used as a construct. The pBI-SCD1pGFP::EGFP vector contains the sulfonylurea resistance gene from pCB1551 (39) and the EGFP ORF linked by the replacement of the stop codon by a short spacer coding for five glycine residues. The EGFP was controlled by the 221-bp 5′ upstream region of the SCD1 gene of C. lagenarium (43). The last codon of EGFP was fused to 15 nucleotides coding a glycine linker followed by an EcoRI BamHI site and the terminator of the glucococase gene of A. awamori. The full region of the α-TUB1 gene was amplified by PCR with primers α-TUB1S and α-TUB1AS from genomic DNA of C. lagenarium. The α-TUB1S primer contains a terminal EcoRI site, whereas the α-TUB1AS primer contains a terminal BamHI site. The amplified product was digested with EcoRI and BamHI, introduced into the EcoRI and BamHI site of pBI-SCD1pGFP, and designated pBI-SCD1pGFP::TUB1S. The full region of the amplified monomeric red fluorescent protein gene (mRFP1) was amplified by PCR with primers mRFPS and mRFPN from the plasmid pCFbD::mRFP1 (44). The 504-bp fragment contains a terminal XbaI site, whereas the mRFPASgL vector contains a terminal EcoRI site. The amplified product was digested with EcoRI and XbaI, introduced into the EcoRI and SpeI sites of pBI-SCD1pGFP::TUB1S, and named pBI-SCD1pmRFPI::α-TUB1S.

Genomic DNA blot analysis. Total DNA of C. lagenarium was isolated from mycelia, and DNA blot analysis was performed according to a method described previously (41). DNA digestion, gel electrophoresis, labeling of probes, and hybridization were performed according to the manufacturer's instructions and standard methods (36). DNA probes were labeled with digoxigenin (DIG)-dUTP using the BioBEST DIG labeling kit (Takara Bio). Hybridized DNA was detected by anti-DIG-alkaline phosphatase Fab fragments (Roche Diagnostics, Tokyo, Japan), and light emission resulting from the enzymatic dephosphorylation of CDP-Star detection reagent (GE Healthcare, Tokyo, Japan) by alkaline phosphatase was recorded on X-ray film.

Pathogenicity tests. The inoculation assay on cucumber (Cucumis sativus L. “Suyo”) was performed as described previously (46).

Microscopy. For appressorium formation and penetration assays in vitro, conidia were harvested from 5- to 7-day-old PDA cultures and suspended in distilled water. The conidial suspension, adjusted to 105 conidial/ml, was dropped onto an eight-well multilayer glass slide (ICN Biomedicals, Aurora, OH) or onto a 25-µl drop of a cellulosic membrane (Wako Chemicals, Osaka, Japan) and incubated at 24°C in the dark (21). Germlings were observed with a Nikon Eclipse E600 microscope with differential interference contrast optics (Nikon, Tokyo, Japan). For observation of GFP and mRFP1 fluorescence, cells were viewed on a fluorescent microscope with a GFP(R)-BP filter (460- to 500-nm excitation...
filter, 505-nm dichroic mirror, and 510- to 560-nm barrier filter) and a G-2A filter (510- to 560-nm excitation filter, 575-nm dichroic mirror, and >590-nm barrier filter), respectively.

Preparation of chemicals. For the application of exogenous calcium ions, CaCl$_2$ was dissolved in distilled water to make a 1 M solution. Nifedipine (Wako Chemicals, Osaka, Japan), a voltage-dependent Ca$^{2+}$ channel blocker, was dissolved in dimethyl sulfoxide (DMSO) to make a 5 mM stock solution. TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride] (Sigma-RBI), an inhibitor of intracellular calcium release, was dissolved in DMSO to give a 5 mM stock solution. Exudates from cucumber cotyledons were collected in distilled water droplets (10 $\mu$L) incubated on the surface of cotyledons for 1 h at 24°C. The collected exudates were filtered through a MILLEX-GV filter unit with a 0.22-$\mu$m pore-size filter (Millipore, Tokyo, Japan).

Chemical treatments. To test the effects of calcium-related chemicals or plant exudates on fungal development, conidia were resuspended to a concentration of 10$^5$ conidia/mL in one of the chemical solutions or cucumber exudate, and 20-$\mu$L exudates on fungal development, conidia were resuspended to a concentration of 10$^5$ conidia/mL in one of the chemical solutions or cucumber exudate, and 20-$\mu$L exudates were then placed on a polystyrene petri dish. Conidial germination and appressorium formation were observed after incubation at 24°C for 24 h.

Nucleotide sequence accession number. The ClaKEL2 sequences were deposited in the DDBJ database under accession number AB297533.

RESULTS

Isolation of the ClaKEL2 gene. The ClaKEL2 gene containing a kelch motif was isolated by PCR using degenerate primers designed from conserved amino acid sequences of the kelch motif of S. cerevisiae Kel1p (33), S. pombe tea1p (28), and hypothetical proteins of the following filamentous fungi: N. crassa (accession number XP_324802), M. grisea (accession number XP_366799), A. nidulans (accession number XP_662168), and F. graminearum (accession number EAA70872). Amplified products were cloned into pBluescript II SK$^+$ and sequenced. The DNA sequence of one fragment showed a high degree of similarity to tea1 of S. pombe and KEL1 of S. cerevisiae. A primer pair designed from this DNA sequence was then used to screen a C. lagenarium cDNA library.

Structural analysis of the ClaKEL2 gene. The sequence of ClaKEL2 was determined (Fig. 1A). The transcriptional initiation site was suggested by RACE analysis. The ATG codon 449 bp downstream of the transcriptional start point is the probable translational initiation site of ClaKEL2. The upstream region of the predicted ORF of these genes contained upstream ORFs (uORFs) of 174 bp in ClaKEL2. The sequence of three introns located from nucleotides 283 to 351, 768 to 836, and 1120 to 1168 were verified by comparison of the nucleotide sequence of the cDNA and genomic sequence. Based on the preferred translation initiation codon and knowledge of the introns, ClaKEL2 was predicted to encode a protein of 1,578 amino acids. Analysis of the sequence revealed repeats of the kelch motif, named after the Drosophila protein in which these repeats were first identified (5, 53). The kelch motif is a segment of 44 to 56 amino acids, and the motif reveals eight key conserved residues, including four hydrophobic residues followed by a double glycine element, separated from two characteristically spaced aromatic residues (1). Six repeats were found in the amino terminus in Clakel2p (Fig. 1B).

The deduced amino acid sequence of ClaKEL2 had a high degree of homology with the amino acid sequence of tea1p of S. pombe (E value of 2e-77) and Kel1p of S. cerevisiae (E value of 2e-58).

ClaKEL2 is involved in appressorium morphogenesis. We isolated clakel2 disruption mutants to define the function of ClaKEL2. The A. tumefaciens binary gene replacement vector pBIG3CEKEL2Tn was introduced into the wild-type strain 104-T by A. tumefaciens-mediated transformation methods, and gene replacements of ClaKEL2 were verified by DNA gel blot analysis (Fig. 2). The clakel2 mutants did not differ from the wild type in their growth rate on PDA medium, but both mutants had reduced conidiation (data not shown). The wild type usually produced orange conidial masses in the central region of colonies on PDA medium. In contrast, the mutants produced black conidial masses on PDA, and some conidia germinated on PDA medium (data not shown). Conidia from clakel2 mutants germinated, but the appressoria were morphologically aberrant. About 90% of the appressoria produced by the wild type had a spherical form (Fig. 3A and B). The clakel2 mutant produced normal appressoria similar to those of the wild type during early stages of appressorium formation on glass slides (data not shown). However, about 90% of the mutant appressoria displayed abnormal lateral germination at a later stage, whereas about 90% of the appressoria produced by the wild type remained spherical in shape (Fig. 3A and B). Furthermore, reintroduction of ClaKEL2 into the clakel2 mutant restored normal appressorium morphogenesis (Fig. 3A and B).

Clakel2p localized to regions of polarized growth. To further elucidate the cellular role of Clakel2p, a translational fusion with the GFP reporter gene was constructed. The ClaKEL2-GFP fusion gene expressed under its native ClaKEL2 promoter was introduced into the clakel2 mutant. Using DNA gel blot analysis, we confirmed that the obtained transformant kel2K2G contained one copy of the fusion gene (data not shown). Temporal localization of Clakel2p during appressorium formation was investigated in detail with fluorescence microscopy. In ungerminated conidia before the start of incu-

FIG. 1. Organization of the ClaKEL2 gene in C. lagenarium strain 104-T. (A) Schematic representation of ClaKEL2. Exons are indicated as gray boxes. The predicted kelch repeat-encoding regions are presented as slashed boxes. Three introns of ClaKEL2 are shown as gray bars among four exons. The predicted uORF is presented as a dotted box. The transcriptional initiation site was determined by 5' RACE and is indicated by a bent arrow. The ORF of ClaKEL2 starting with the predicted start codon ATG, which is designated as +1, and ending with a TAG termination codon at 4926 is indicated. The uORF is predicted to be from −312 to −112. (B) Alignment of the kelch repeats in Clakel2p. Identical or similar residues are shown shaded in black. The numbers on the left refer to the positions of the amino acids in the kelch motif. The kelch motif reveals eight key conserved residues, including four hydrophobic residues (h) followed by a double glycine element (GG) separated from two characteristically spaced aromatic residues (a).
bination, no GFP fluorescence was detected (Fig. 4A). At 1 to 2 h, localized GFP fluorescence was observed at the cell periphery at one end of the conidia (Fig. 4B). In germinating conidia, localized GFP fluorescence was observed at the tips of germ tubes (Fig. 4C). During appressorium formation, GFP fluorescence became faint when germ tubes ceased apical growth and differentiated into swollen appressoria (Fig. 4D). In undifferentiated vegetative hyphae, GFP signals were specifically detected at the hyphal tips (Fig. 4E).

Localization of Clakel2p is dependent on microtubules. The localization of tea1p in S. pombe strictly depends on functional microtubules (3, 11, 33). We investigated whether tubulin components of the cytoskeleton were involved in transporting Clakel2p to cell apices. We constructed a monomeric red fluorescent protein (mRFP1) (8) and an α-tubulin fusion gene. The A. tumefaciens binary vector pBl-SCD1p-mRFP1:α-TUB1 containing the sulfonurea resistance gene (SUr) was introduced into strain kel2K2G, and the transformant was named kel2K2GRT. We depolymerized microtubules using the tubulin-depolymerizing drug benomyl. At 1 h after the addition of benomyl (10 μg/ml), RFP fluorescence labeled segmented microtubules or microtubule stubs, and concomitantly, 73% of the Clakel2p-GFP signals in the tips of vegetative hyphae disappeared (Fig. 5A). In contrast, in the presence of DMSO, the solvent for benomyl, RFP fluorescence labeled long filamentous microtubules, and Clakel2p localized to hyphal tips in 94% of the vegetative hyphae (Fig. 5A).

Next, we investigated the localization of Clakel2p during conidial germination. At 3 h after incubation of conidia in Czapek medium (0.05% [wt/vol] MgSO₄·7H₂O, 0.1% [wt/vol] K₂HPO₄, 0.05% [wt/vol] KCl, 0.2% [wt/vol] NaN₃, 0.001% [wt/vol] FeSO₄·7H₂O, 20 mM glucose, pH 6.0), Clakel2p-GFP was localized at the tip of germ tubes in 97% of conidia, similar to the polar localization of the protein in vegetative hyphae (Fig. 5A). In contrast to vegetative hyphae, localization of Clakel2p-GFP in conidia was not affected by the addition of benomyl, even though microtubules were segmented. The protein was observed at germ tube tips in 95% of conidia after 3 h of incubation (Fig. 5B).

Normal appressorium morphogenesis of the clakel2 mutant is restored on the plant surface. To elucidate whether ClaKEL2 is required for pathogenicity, clakel2 mutants were inoculated onto host cucumber cotyledons. The clakel2 mutants produced lesions similar to those caused by the wild-type 104-T, despite their abnormal appressorium formation on glass slides (Fig. 6A). To examine the infection-related morphogenesis of clakel2 mutants, we placed conidial suspensions onto the abaxial surface of cucumber cotyledons and observed subsequent growth with light microscopy. Surprisingly, on cucumber cotyledons, about 50% of the clakel2 mutants formed normal spherical appressoria, as opposed to the aberrant ones formed on glass slides, and these normal appressoria also penetrated the cucumber epidermis with the same frequency as the wild-type (Fig. 6B and C).

To test the penetration ability of appressoria formed by the clakel2 mutant, conidia were incubated on cellulose membranes. More than 80% of wild-type appressoria formed infection hyphae that penetrated and grew inside the cellulose membrane (Fig. 7A and B). In contrast, about 90% of appressoria formed by the clakel2 mutants failed to penetrate or form infection hyphae inside cellulose membranes (Fig. 7A and B). Furthermore, reintroduction of ClaKEL2 into the clakel2 mutant restored its ability to penetrate cellulose and form infection hyphae. Thus, more than 80% of appressoria formed by the ClaKEL2-complemented transformant kel2K2G successfully penetrated into the membranes (Fig. 7A and B).

Calcium signaling restores appressorium morphogenesis of clakel2 mutants. To elucidate the factors that restore appressorium morphogenesis in clakel2 mutants in planta, we examined whether germ tubes developing from clakel2 conidia treated with exudates from cucumber cotyledons were able to produce normal appressoria on an artificial substratum. Conidia were incubated at 24°C for 24 h in the cucumber exudates in a plastic petri dish. The exudates induced normal appressorium formation in clakel2 mutants. Thus, about 73% of the appressoria of clakel2 mutants incubated in cucumber exudates showed normal appressoria, while 60% of the appressoria incubated in distilled water underwent lateral germination (Fig. 8A).

Next, we examined whether exogenous application of intracellular second messengers induced normal appressorium formation in clakel2 mutants on artificial substrata, based on the hypothesis that plant-derived signals induce normal appresso-
Conidia were incubated in the presence of intracellular second messengers such as cAMP, 2-diocanoyl-rac-glycerol (a diacylglycerol analog), and CaCl$_2$ in a plastic petri dish. More than 90% of the appressoria from the clakel2 mutant showed lateral germination in the presence of cAMP (10 mM) or 2-dioctanoyl-rac-glycerol (20 µg/ml) (data not shown), while about 70% of the appressoria from the clakel2 mutants showed normal appressoria when incubated in the presence of CaCl$_2$ (5 mM) (Fig. 8A).

To verify whether signals derived from the cucumber cotyledon were transduced through calcium signaling, conidia were incubated in the presence of the calcium channel modulators nifedipine (an external Ca$^{2+}$ ion blocker) or TMB-8, an inhibitor of intracellular calcium ion release. In the wild type, these calcium channel modulators neither inhibited appressorium formation nor induced lateral germination (Fig. 8B). In the clakel2 mutant, CaCl$_2$ or cucumber exudates effectively induced normal appressorium formation (Fig. 8B), but nifedipine (50 µM) inhibited the normal appressorium formation induced by CaCl$_2$. Thus, about 60% of the appressoria from the clakel2 mutants showed normal appressoria when incubated in the presence of CaCl$_2$ (5 mM) (Fig. 8A).

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TMB-8 (50 μM). TMB-8 inhibited normal appressorium formation by the clakel2 mutant exposed to cucumber exudates. About 70% of the appressoria that formed in the presence of cucumber exudates had a normal shape, while TMB-8 decreased this frequency to about 40%. On the other hand, TMB-8 did not inhibit normal appressorium formation induced with CaCl2. About 60% of the appressoria formed in CaCl2 had a normal shape, while no less than 60% of the appressoria formed in the presence of both CaCl2 and TMB-8 had a normal shape (Fig. 8B).

**DISCUSSION**

C. lagenarium develops a series of infection-related structures, and this process is closely linked to pathogenesis. Understanding the mechanism underlying infection-related morphological transitions would help to provide a model for the cell biology of other filamentous fungi. In this study, we reported roles of a novel kelch repeat protein in the morphological development of the plant-pathogenic fungus C. lagenarium and found that kelch repeat proteins were involved in appressorium morphogenesis and in pathogenicity. Moreover, we found that appressorium development is triggered through a calcium signaling pathway.

Clakel2p is a member of the kelch family of proteins. We identified and characterized a kelch repeat protein in the filamentous fungus C. lagenarium. At present, more than 20 kelch repeat proteins have been cloned and characterized from viruses, plants, fungi, and mammals (1). A database search of the genome sequences of filamentous fungi such as N. crassa, M. grisea, F. graminearum, and A. nidulans revealed that filamentous fungi have a number of predicted kelch family proteins. However, the roles of these proteins in filamentous fungi are
not well known. In fission yeast, the kelch repeat proteins tea1p and tea3p are important for establishing cell polarity (2, 28). Tea1p associates in the polarisome complex with other polarity factors such as formin (11). In filamentous fungi, it is reported that sepA, encoding formin, is required for the maintenance of cell polarity during hyphal growth and asexual morphogenesis (12). However, the functional characterization of the tea1p homologue in filamentous fungi has not been reported.

The kelch repeat-encoding gene ClaKEL2 was isolated by PCR using degenerate primers designed from conserved amino acid sequences of the kelch motif obtained from fungal genome databases. Analysis of the consensus pattern of the kelch motif in Clakel2p revealed that ClaKEL2 encoded six copies of the kelch motif at the N terminus. On the basis of the position of the kelch motif and the presence of other protein domains in the amino acid sequences, the kelch family of proteins is classified into five structural subgroups, based on the position of the \(\beta\)-propeller and other protein domains within the primary sequences: (a) proteins with an N-terminal dimer and C-terminal \(\beta\)-propeller (N-dimer/C-propeller proteins); (b) \(\beta\)-propeller proteins; (c) N-propeller/C-dimer proteins; (d) N-propeller proteins; and (e) propeller proteins (1). Structural analysis of the deduced amino acid sequence of ClaKEL2 revealed the presence of six repeats of the kelch motif at the N terminus and a coiled-coil region at the C terminus, indicating that Clakel2p belongs to the N-propeller/C-dimer protein group. Thus, Clakel2p has the same structural features as tea1p and Kel1p proteins of yeasts.

Functional role of Clakel2p in C. lagenarium. We found evidence that the Clakel2p plays an essential role in the morphological transitions of C. lagenarium. During germination, Clakel2p was localized to the presumed site of germ tube emergence and remained restricted to the apex of the elongating germ tube, and weak fluorescence was observed in the peripheral area of the appressoria. These results suggest that Clakel2p is involved in polarized growth. In accordance with this finding, most conidia of clakel2 mutants formed abnormal appressoria on glass slides. In contrast, hyphal growth of the clakel2 mutants on PDA medium was similar to that of the wild type. Also, conidiation of the clakel2 mutants was similar to the wild type, though the amount of spore production was reduced. It seems that the polarized growth of filamentous fungi and yeasts is regulated by different molecular machineries. For example, polarized growth of hyphae in filamentous fungi is mediated by the Spitzenkörper, while in S. cerevisiae, cell polarity is controlled by the polarisome, and in Candida albicans, the Spitzenkörper is specific for hyphae and is not implicated in yeast and pseudohyphal growth (10). Thus, different molecular mechanisms may control the polarized growth of hyphae and appressorium formation.

When microtubules were depolymerized with benomyl, Clakel2p-GFP was no longer found in most of the hyphal tips examined. This finding suggested that Clakel2p localization at hyphal tips was dependent on microtubule function. Interestingly, in contrast to vegetative hyphae, most of the Clakel2p-GFP fusion protein remained at the tip of germ tubes emerging from conidia when microtubules were depolymerized with
benomyl, suggesting that the localization of ClaKEL2p was independent of microtubules during conidial germination. In S. pombe, the function of tea1p is dependent upon its transport to the cell ends on the growing plus ends of microtubules (3, 11, 38). Recently, the tea1p-binding partner, tea4p, was identified in fission yeast (27). tea4p was required for the establishment of bipolar growth, and was localized at the cell ends. Like tea1p, tea4p also localized to the plus ends of microtubules, indicating that localization of tea4p may be similarly regulated by a microtubule-dependent mechanism. In budding yeast, the tea4p homologue, Bud14p, was also identified (15).

Bud14p physically interacted with Kel1p, and localization at the bud cortex depended on Kel1p. Accumulation of Bud14p was dependent on the actin cytoskeleton. Thus, tea4p and Bud14p are structurally conserved and function as binding partners for tea1p and Kel1p, respectively, but the cytoskeleton components involved in proper localization of tea4p and Bud14p are quite different. In A. nidulans, conidia treated with benomyl germinate, and subsequent fungal structures grow in a polarized manner (31). Furthermore, conidia of C. lagenarium are able to germinate, and the germ tube forms an appressorium in the presence of benomyl (43). These findings suggest that microtubules are not essential for germination. Conidial germination may have a microtubule-independent mechanism to determine the site of polarized growth, whereas vegetative hyphae may have a microtubule-dependent mechanism to determine polarization.

The regulation of appressorium formation on an artificial substratum differs from that on the host plant. Appressoria of clakel2 mutants showed lateral germination on cellulose membranes, the same as on glass slides. Because these abnormal appressoria never formed infection hyphae, we assumed that the clakel2 mutants could not penetrate into host plant cells. However, in pathogenicity tests on cucumber, the clakel2 mutants caused lesions the same as the wild type because of the increased number of normal appressoria. These results suggest that ClaKEL2 is essential for normal appressorium formation on artificial substrates but is dispensable for appressorium formation on the host plant surfaces. In general, prepenetrative morphological changes in plant-pathogenic fungi are triggered by external signals such as physical or chemical signals from the plant surface (18). Appressorium formation in C. lagenarium is considered to be controlled by different signaling pathways, one of which is independent of ClaKEL2. In fission yeast, tea1p is required for polarity in cells growing vegetatively, but not in shmooing cells. In the presence of a mating pheromone as an external signal, tea1p is delocalized from microtubules and is not localized to the cell ends (30). Moreover, the levels of tea1p are reduced three- to fourfold by 5 h after the addition of pheromone (28). Possibly the machinery for forming appressoria that involves ClaKEL2p is overridden by external plant signals. Normal appressorium formation on artificial substrates was restored in the clakel2 mutants when cells were supplied with cucumber exudates, implying that a chemical component could induce appressorium formation in C. lagenarium independently of ClaKEL2p function. Exogenous calcium ions also induced normal appressorium formation in clakel2 mutants, suggesting that regulation of cytosolic calcium is involved in appressorium formation. In Colletotrichum spp. a wide range of pharmacological experiments have indicated that the calcium signal transduction pathway is involved in appressorium formation (48, 50). In C. gloeosporioides, which is pathogenic on red pepper, inhibition of incremental increases in cytosolic Ca²⁺ concentration affected appressorium formation. The calcium channel blockers, methoxy verapamil and TMB-8, specifically inhibited appressorium formation (48). Also in C. trifolii, similar results were obtained using nifedipine and TMB-8 (50).

In the present study, pharmacological experiments revealed that calcium ion channel modulators inhibited normal appressorium formation by the clakel2 mutant when cells were incubated with cucumber exudates. This result suggests that ap-
pressiorium formation triggered by chemical components on the host surface and the calcium signaling pathway are linked. Moreover, normal appressorium formation triggered by host surface components was more sensitive to TMB-8 than to nifedipine. This suggests that intracellular Ca\(^{2+}\) concentration regulated by Ca\(^{2+}\) release from intracellular stores was more important than external Ca\(^{2+}\) influx for appressorium formation on cucumber leaves. Furthermore, nifedipine and TMB-8 did not inhibit appressorium formation by the wild type. This suggests that appressorium formation by \textit{C. lagenarium} is not regulated by calcium signaling acting as the downstream target of \textit{clakel2} but is instead controlled by two independent signaling pathways, one involving calcium signaling and the other involving \textit{ClakEL2}.

Other signal transduction pathways such as the cAMP and MAPK signaling pathways are also known to be involved in the infection-related morphogenesis of fungal pathogens (25). In \textit{C. lagenarium}, the \textit{s. cerevisiae SLT2-related MAPK gene MAF1} regulates appressorium development (16), and in yeast activation of \textit{Stl2p} is regulated by \textit{PKC1} which encodes a homologue of Ca\(^{2+}\)-dependent subtypes of mammalian PKC (23). Moreover, it has been suggested that \textit{KEL1} functions in parallel with \textit{PKC1} (33). Therefore, we speculate that in \textit{C. lagenarium} the \textit{MAF1}-related MAPK pathway could be involved in calcium signaling in parallel with \textit{ClakEL2}. Our next target is to identify specific components of the calcium signaling pathway that leads to appressorium formation. Screening for forward mutations of the \textit{clakel2} mutant that cause defects in appressorium development on plant surfaces should enable us to identify such genes.

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