Calcium/Calmodulin-Dependent Signaling for Prepenetration Development in *Colletotrichum gloeosporioides*

Kwang-Heum Uhm, Il-Pyung Ahn, Soonok Kim, and Yong-Hwan Lee

School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea.

Current address of I.-P. Ahn: Plant Biotechnology Division, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon 441-100, Korea.

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ABSTRACT

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Colletotrichum gloeosporioides forms a specialized infection structure, an appressorium, for host infection. Contacting hard surface induces appressorium formation in *C. gloeosporioides*, whereas hydrophobicity of the contact surface does not affect this infection-related differentiation. To determine if the calcium/calmodulin-dependent signaling system is involved in prepenetration morphogenesis in *C. gloeosporioides* pathogenic on red pepper, effects of calcium chelator (EGTA), phospholipase C inhibitor (neomycin), intracellular calcium modulators (TMB-8 and methoxy verapamil), and calmodulin antagonists (chlorpromazine, phenoxy benzamine, and W-7) were tested on conidial germination and appressorium formation. Exogenous addition of Ca^{2+} , regardless of concentration, augmented conidial germination, while appressorial differentiation decreased at higher concentrations. Inhibition of appressorium formation by EGTA was partly restored by the addition of calcium ionophore A23187 or CaCl_2 . Calcium channel blockers and calmodulin antagonists specifically reduced appressorium formation at micromolar levels. These results suggest that biochemical processes controlled by the calcium/calmodulin signaling system are involved in the induction of prepenetration morphogenesis in *C. gloeosporioides* pathogenic on red pepper.

Additional keywords: anthracnose, *Glomerella cingulata*.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., a homothallic Ascomycete, causes anthracnose diseases on more than 197 plant species including crops, vegetables, weeds, and trees (10). This fungus is also the causal agent of pepper anthracnose, one of the most devastating fruit diseases of red pepper (*Capsicum annuum* L.) in Korea. For the infection to take place, a series of infection-related morphogenesis, initiated by spore adhesion to the host surface, spore germination, germ tube elongation, appressorium formation, and penetration by an infection peg, must precede. Previous studies with *C. gloeosporioides* and *C. trifolii* have shown that prepenetration morphogenesis, particularly appressorium formation, is induced by environmental cues involving thigmo-signals of the contact surface such as hydrophobicity and rigidity (34), and chemicals including host surface wax and ethylene (16).

These environmental signals, usually recognized by membrane-spanning receptors, are transferred into cells to express the appropriate cellular responses. The relaying of the signal is referred to as signal transduction and is mediated by second messengers including cyclic nucleotides (18,19,31), calcium (8,18), and inositol phosphate (3,9).

Cytosolic calcium ion is regarded as an indispensable cation for cell growth and development and appressorial development in many organisms (13). The Ca^{2+} signaling pathway in cells is initiated via conformational changes of the GTP-binding protein (G-protein) linked to a membrane receptor (17). Phospholipase C (PLC) is activated by the G-protein and hydrolyzes phosphatidyl

inositol-1,4-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (30). IP_3 in turn causes the release of endogenous Ca^{2+} from intracellular calcium stores (1). Increments of intracellular Ca^{2+} result in the activation of calmodulin, one of the most ubiquitous and conserved Ca^{2+} receptor protein. Ca^{2+} binding induces conformational changes in calmodulin, resulting in the activation of many enzymes, including the multifunctional, dedicated calmodulin-dependent protein kinase and protein phosphatases (15,32). Phenotypically, calmodulin mediates numerous fungal cell responses involving development (22), cytokinesis (20), and regulation of enzymatic activities (23).

In parallel, several reports suggest the involvement of the calcium/calmodulin signaling system of *Colletotrichum* spp. in the culmination of appressorium formation and disease occurrence over the past several years. Extensive studies using a wide range of pharmacological agents, which disrupt Ca^{2+} influxes or interfere with the calcium-binding protein, have strongly suggested the involvement of Ca^{2+} signaling systems in the appressorium formation of *C. trifolii* (34) and *C. gloeosporioides* pathogenic on avocado (15). More recently, calmodulin genes were cloned and characterized in these fungi. Calmodulin gene was highly expressed by hard-surface contact, ethylene, germination, and appressorium formation (15). Furthermore, reducing the functional transcript accumulation of this gene by expressing antisense copy resulted in the reduction of appressorium formation and perturbation of disease progress (35). These results suggest that calmodulin modulates the prepenetration events from spore attachment to appressorium formation in these fungi. However, no report has yet been made on the infection-related morphogenesis and related signaling system in *C. gloeosporioides* pathogenic on red pepper. Dissecting signaling pathways that transmit host surface-mimic signals into the cells might provide the new target sites for the development of effective control measures (5,26).

In this study, we used several inhibitors thought to act on various steps in the calcium/calmodulin-dependent signaling

Corresponding author: Y.-H. Lee; E-mail address: yonglee@plaza.snu.ac.kr

The first and second authors contributed equally.

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pathway to elucidate the role of Ca^{2+} /calmodulin in the prepenetration morphogenesis of *C. gloeosporioides*. Our pharmacological data strongly indicate that the calcium/calmodulin-dependent signaling system is involved in the prepenetration development of *C. gloeosporioides* pathogenic on red pepper.

MATERIALS AND METHODS

Fungal isolate and cultural condition. *C. gloeosporioides* isolate virulent on pepper fruits was obtained from the National Institute of Agricultural Science and Technology, Rural Development Administration, Korea. Conidia were harvested from 7-day-old cultures grown on potato dextrose agar (PDA; Difco Laboratories, Detroit) at 25°C in the dark. Fungal stock was maintained as conidial suspension amended with 20% glycerol at -70°C until use.

Conidial germination and appressorium formation. To induce conidial germination or appressorium formation, conidia were collected from PDA by mild scraping with sterile distilled water and washed twice by centrifugation. Fifty microliters of spore suspension (1×10^5 conidia per ml) was placed onto hydrophobic and hydrophilic surfaces of GelBond (FMC Corp., Rockland, ME), microscopic coverslips (Marienfeld, Germany), and polystyrene petri dishes (GCEM Corp., Korea), which were then placed in a humid plastic box and incubated at room temperature for 12 h. Conidia were considered germinated when germ tube length was equal to or greater than spore length. Appressorium was considered mature when melanization was evident. Percentages of germinated and germinating conidia to form appressoria were estimated from at least 100 conidia with three replicates per treatment. Experiments were repeated at least three times independently.

Chemical treatments. To test the effects of calcium-related chemicals, 45 µl of conidial suspension (1×10^5 conidia per ml) was placed on the hydrophobic side of GelBond and an additional 5 µl of chemical solutions to be tested was added. Conidial germination and appressorium formation were measured on the substrata as described previously.

Preparation of chemicals. All chemicals used in this study were purchased from Calbiochem (La Jolla, CA) unless otherwise indicated. Tested chemicals were dissolved completely in proper solvents. EGTA (Sigma Chemical, St. Louis), a calcium chelator, was dissolved in distilled water and adjusted to pH 7.5 with 10 N NaOH. Neomycin, a PLC inhibitor, was dissolved in distilled

water. A23187, a calcium ionophore, and an intracellular calcium release inhibitor, TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethyl-amino)octyl ester], were dissolved in dimethyl sulfoxide (DMSO). Methoxy verapamil, a blocker of calcium channel anchored in the plasmamembrane, was dissolved in distilled water. Three calmodulin antagonists were prepared by dissolving W-7 [N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide], phenoxy benzamine, and chlorpromazine in DMSO, ethanol, and distilled water, respectively.

RESULTS

Kinetics of conidial germination and appressorium formation. Conidia of *C. gloeosporioides* germinate only if surface contact is made. Following germination, when the germ tube encounters a hard surface, appressorium formation is initiated. In our observation, 2 to 3 h after contacting the hydrophobic surface of GelBond, conidia began to develop germ tubes (Fig. 1). More than 60 and 90% of the germinated conidia produced appressoria at 6 and 12 h after incubation, respectively. All hard surfaces tested induced appressorium development at high frequencies, including hydrophilic surface of GelBond (Table 1), whereas soft surfaces such as water agar (2%) failed to induce appressorium formation (data not shown).

Effect of exogenous calcium, calcium chelator, and ionophore. To investigate the effect of exogenous Ca^{2+} concentration on appressorium formation and conidial germination, CaCl_2 and EGTA were tested (Table 2; Fig. 2). Although addition of CaCl_2 enhanced germination at the tested concentrations, 10 and 100 mM CaCl_2 treatments inhibited appressorium formation by approximately 40% and to near abolishment, respectively. Addition of EGTA reduced conidial germination and appressorium formation in a dosage-dependent manner. The inhibition of appressorium formation by EGTA was partly restored by the addition of A23187 or Ca^{2+} .

TABLE 1. Conidial germination and appressorium formation of *Colletotrichum gloeosporioides* on different substrata^a

Substrata	% Conidial germination (±SD)	% Appressorium formation (±SD)
GelBond (hydrophobic face)	75.7 ± 7.0	98.3 ± 2.1
GelBond (hydrophilic face)	66.7 ± 6.0	81.7 ± 6.7
Coverslips	60.3 ± 0.6	94.7 ± 2.5
Polystyrene	69.0 ± 4.0	98.0 ± 1.0

^a Conidia of *C. gloeosporioides* were collected from potato dextrose agar, washed twice with distilled water, and placed on the hydrophobic surface of GelBond at a final concentration of 1×10^5 conidia per ml.

TABLE 2. Effects of calcium chelator, ionophore, and CaCl_2 on conidial germination and appressorium formation of *Colletotrichum gloeosporioides*^a

Chemical	% Conidial germination (±SD)	% Appressorium formation (±SD)
EGTA (10 mM)	15.7 ± 2.1	0.3 ± 0.6
(1 mM)	46.3 ± 2.5	71.7 ± 4.0
(0.1 mM)	67.0 ± 1.7	61.3 ± 7.6
CaCl_2 (100 mM)	81.3 ± 2.5	1.7 ± 0.6
(10 mM)	89.0 ± 2.6	54.7 ± 2.3
(1 mM)	86.0 ± 1.0	88.3 ± 1.5
(0.1 mM)	88.7 ± 0.6	88.0 ± 1.0
EGTA (10 mM) + A23187 (10 µM)	15.3 ± 2.1	40.0 ± 10.0
EGTA (10 mM) + CaCl_2 (1 mM)	59.6 ± 2.6	29.3 ± 3.2
Control	66.3 ± 3.2	91.0 ± 1.7

^a Conidia of *C. gloeosporioides* were collected from potato dextrose agar, washed twice with distilled water, and placed on the hydrophobic surface of GelBond at a final concentration of 1×10^5 conidia per ml. Germination and appressorium development were determined microscopically at 12 h after incubation.

Fig. 1. Time course of conidial germination and appressorium formation of *Colletotrichum gloeosporioides* on the hydrophobic surface of GelBond.

Effect of PLC inhibitor. The phosphoinositide signaling system is related to calcium signaling through IP₃, an intracellular calcium activator. IP₃ is formed from PIP₂ by activated PLC and released into the cytosol (9). To determine if phosphoinositide signaling participates in the appressorium formation, neomycin was tested (Fig. 3). Exogenous addition of neomycin, a PLC inhibitor, specifically inhibited appressorium formation by approximately 55% at 10 μ M. However, germination was suppressed more severely at higher concentrations.

Effect of calcium channel modulators. Intracellular Ca²⁺ concentration may be regulated by the release of Ca²⁺ from intracellular stores or by Ca²⁺ channels located in the cell membrane

(6). To determine if the modulation of calcium channels affects the conidial germination and appressorium formation of *C. gloeosporioides*, two calcium channel modulators were tested (Fig. 4). TMB-8, an inhibitor of calcium release from intracellular calcium store, specifically inhibited appressorium formation by 75 and 92% at 10 and 100 μ M, respectively, but did not affect conidial germination. Methoxy verapamil, a calcium channel blocker located in the plasmamembrane, also specifically inhibited appressorium formation by 50% over a wide range of concentrations without affecting conidial germination.

Effect of calmodulin antagonists. The action of Ca²⁺ in cells is mainly mediated by calmodulin, the most potent Ca²⁺-binding protein. Calmodulin antagonists compete with Ca²⁺ for binding to calmodulin, and consequently inhibit Ca²⁺/calmodulin signaling. To investigate whether calmodulin participates in the appressorium formation of *C. gloeosporioides*, the effects of several different antagonists were evaluated. All three calmodulin antagonists, chlorpromazine, phenoxy benzamine, and W-7, have specific inhibitory effects on the appressorium formation (Figs. 2 and 5). Phenoxy benzamine and W-7 specifically inhibited appressorium formation, whereas conidial germination remained unaffected over a wide range of concentrations. Chlorpromazine also selectively impaired appressorium formation at 1 and 10 μ M concentrations; however, conidial germination and appressorium formation were both inhibited at 100 μ M.

DISCUSSION

Extensive research has been conducted to identify environmental cues and cellular signaling systems in the infection-related morphogenesis of plant pathogenic fungi during the last decade. Accumulated data suggested that physicochemical signals, including rigidity and hydrophobicity of the contact surface and host surface chemicals, are the determinants inducing infection structure formation in several plant pathogenic fungi (12,19,34). In this study, however, *C. gloeosporioides* pathogenic on red pepper required rigidity, not hydrophobicity of the contact surface, for the induction of appressorium formation. Similar results have been described in the appressorium formation of *C. graminicola* (4). On the other hand, hydrophobicity of the contact surface is one of the determining signals for appressorium formation in *C. trifolii* (34) and a well-studied rice blast fungus, *Magnaporthe grisea* (19).

Fig. 2. Inhibition of appressorium formation of *Colletotrichum gloeosporioides* in the presence of **A**, H₂O, **B**, 100 mM CaCl₂, and **C**, 10 μ M W-7. Conidial suspension with chemicals was placed on the hydrophobic surface of GelBond and incubated in a moistened box at 25°C for 12 h. a, appressorium; c, conidium; and g, germ tube.

Fig. 3. Effects of neomycin on conidial germination and appressorium formation in *Colletotrichum gloeosporioides*. Conidial suspensions with different concentrations of neomycin were placed on the hydrophobic surface of GelBond and incubated in a moistened box at 25°C for 12 h.

These variances suggest the possibility of different mechanisms in pathogenesis including host-sensing among different species of *Colletotrichum* as well as other plant pathogenic fungi.

Addition of calcium selectively inhibited appressorium formation at higher concentrations. In addition, treatment of calcium chelator (EGTA) inhibited appressorium formation and conidial germination. These results indicate that maintaining the level of intracellular Ca^{2+} concentration is important for appressorium formation in *C. gloeosporioides*. This is further supported by the fact that EGTA inhibition of appressorium formation was partly restored by the addition of exogenous calcium or calcium ionophore (A23187). Taken together, these results strongly suggest that sustained calcium homeostasis, rather than transient change of Ca^{2+} , in the cytosol is important for appressorium formation in this fungus. Similar phenomena have been suggested for appressorium formation in *C. trifolii* (34) and other fungi such as *Zoophthora radicans* (24), *Metarhizium anisopliae* (29), and *Phyllosticta ampellicida* (27). Treatment with neomycin, a PLC inhibitor, resulted in the reduction of appressorium formation in *C.*

Fig. 4. Effects of calcium channel modulators on conidial germination and appressorium formation in *Colletotrichum gloeosporioides*. Conidial suspensions with different concentrations of chemicals were placed on the hydrophobic surface of GelBond and incubated in a moistened box at 25°C for 12 h.

Fig. 5. Effects of calmodulin antagonists on conidial germination and appressorium formation in *Colletotrichum gloeosporioides*. Conidial suspensions with different concentrations of chemicals were placed on the hydrophobic surface of GelBond and incubated in a moistened box at 25°C for 12 h.

gloeosporioides. Release of calcium from intracellular calcium stores by IP₃ is indispensable for the induction of appressorium formation. To understand the possible involvement of this mechanism more directly, the effects of calcium channel modulator on conidial germination and appressorium formation of *C. gloeosporioides* were tested. Both calcium channel modulators, TMB-8 and methoxy verapamil, specifically reduced appressorium formation over a wide range of concentrations, further supporting the results that maintenance of cytosolic Ca²⁺ within a certain range is indispensable for appressorium formation in this fungus. In addition, greater reduction of appressorium formation was induced by TMB-8 than by methoxy verapamil. This indicates that Ca²⁺ release from internal calcium stores is more important than Ca²⁺ influx mediated through the plasmamembrane gate on the appressorium formation. Similar results were observed in our previous study on appressorium formation of *Magnaporthe grisea* (18). However, both conidial germination and appressorium formation were inhibited without selectivity in *Phyllosticta ampellicida* (27) and *C. trifolii* (34). It should be noted that TMB-8 affects not only Ca²⁺ channels but other signal transduction pathways such as protein kinase C (28). Therefore, the results should be interpreted cautiously.

Although inhibition of prepenetration morphogenesis by the calmodulin antagonists has been described previously in the filamentous fungi, notable differences exist in their inhibitory patterns. All three calmodulin antagonists specifically inhibited appressorium formation at micromolar levels in this study, whereas conidial germination remained unaffected. Specific inhibition of appressorium formation by calmodulin antagonists was reported in *C. trifolii* (34) and *Magnaporthe grisea* (18). On the other hand, nonspecific inhibitions of conidial germination and appressorium formation were reported in *Phyllosticta ampellicida* (27). These variable inhibitory effects on appressorium formation among the fungal species might be due to the alternatives of biochemical signaling pathways via calmodulin rather than to the structural differences of calmodulin in these fungi. This is supported by the fact that calmodulin genes are highly conserved and nearly identical in their nucleotide sequences in several fungi (15,21,35).

Comparative analyses on Ca²⁺ signaling for appressorium formation between *C. gloeosporioides* isolates pathogenic on red pepper in the current study and on avocado unveiled significantly different responses against calmodulin antagonists. Specific inhibition on appressorium formation was observed in a red pepper isolate, whereas nonspecific inhibition on conidial germination and appressorium formation was observed in an avocado isolate (15). Furthermore, in *C. gloeosporioides* pathogenic on avocado, treatment of a PLC inhibitor, U73122, resulted in the synchronous inhibition of conidial germination and appressorium formation at 64 nM. However, selective inhibition of appressorium formation by another PLC inhibitor, neomycin, was observed at 10 µM in this study. Although different inhibitory effects were observed through treatments of PLC inhibitors, the validity of these effects remains unclear because different chemicals (U73122 and neomycin) were used in each experiment. In addition, appressorium formation of *C. gloeosporioides* pathogenic on avocado was selectively triggered by the surface wax of its host avocado, but not by other plant surface waxes. However, appressorium formation of *C. gloeosporioides* pathogenic on red pepper in the current study did not show such host-specific responses. Treatments of plant waxes from red pepper fruits and leaves, leaves of cabbage (*Brassica oleracea*), and Japanese ivy (*Parthenocissus tricuspidata*) enhanced appressorium formation (data not shown). Taken together, these results suggest that differences in signaling for appressorium formation exist between *C. gloeosporioides* pathogenic on avocado and on red pepper. This speculation may be further supported by the complexity on the taxonomy of *C. gloeosporioides*. von Arx (33) subsumed 594 species into a large

one, *C. gloeosporioides*, based on the morphological characteristics. As a result, this species is composed of not only mycologically but genetically and pathogenically diverse strains (2,11). This complexity has also been demonstrated in the vegetative compatibility grouping and DNA fingerprinting analyses of this fungus (7,14,25). High level of species heterogeneity might induce strain-specific responses in the signaling cascades regulating the prepenetration morphogenesis in *C. gloeosporioides*.

In conclusion, maintaining calcium homeostasis in cells is important for appressorium formation in *C. gloeosporioides* pathogenic on red pepper. Calcium homeostasis in the cytosol appears to be controlled by complex biochemical mechanisms including calcium channels in the cell membrane, cytosolic stores, and binding with calmodulin. Although the precise mechanisms of signaling pathways are still unclear, we demonstrate that the calcium/calmodulin-dependent signaling system is involved in the appressorium formation of *C. gloeosporioides* pathogenic on red pepper.

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