Calcium Signaling Is Suppressed in *Magnaporthe oryzae* Conidia by *Bacillus cereus* HS24

Wenxiang Huang,^{1,2} Xingyu Liu,^{1,2} Xiaosi Zhou,^{1,2} Xiaoli Wang,³ Xinyu Liu,^{1,2} and Hongxia Liu^{1,2,†}

¹ Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

² Key Laboratory of Integrated Management of Crop Diseases and Pests (Nanjing Agricultural University), Ministry of Education, Nanjing 210095, China

³ Jiangsu Provincial Key Construction Laboratory of Probiotics Preparation, Huaiyin Institute of Technology, Huaian 223003, China

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ABSTRACT

Rice yield is greatly reduced owing to rice blast, a polycyclic fungal disease caused by the ascomycete *Magnaporthe oryzae*. Previously, *Bacillus cereus* HS24, isolated from a rice farm, showed a strong antimicrobial effect toward *M. oryzae*. To better exploit it as a biocontrol agent, HS24 was studied for the mechanism that it uses to suppress rice blast. Conidium germination in *M. oryzae* was significantly inhibited by HS24, whereby inhibition reached 97.8% at the concentration of 10⁷ CFU/ml. The transcription levels of *Ca²⁺/calmodulin-dependent protein kinase II*, *PMC1*, and *CCH1*, key genes involved in the *M. oryzae* Ca²⁺ signaling pathway, were significantly decreased in HS24-treated conidia at high concentration. The treatment of *M. oryzae* with the corresponding Ca²⁺ signaling pathway inhibitors KN-93, verapamil, and cyclopiazonic acid significantly reduced conidium

germination. This inhibitory effect was found to be concentration dependent, similar to the HS24 treatment. We also found that HS24 was able to decrease the intracellular free Ca²⁺ concentration in *M. oryzae* conidia significantly. The addition of exogenous Ca²⁺ did not diminish the inhibitory effect of HS24 on the reduction of intracellular free Ca²⁺ concentration and the level of conidium germination. In conclusion, *B. cereus* HS24 at high concentration prevents extracellular Ca²⁺ from entering the conidia in *M. oryzae*, causes a significant reduction of intracellular free Ca²⁺ concentration, and results in the inhibition of conidium germination.

Keywords: *Bacillus cereus* HS24, biological control, conidium germination, Ca²⁺ signaling pathway, *Magnaporthe oryzae*

Rice (*Oryza sativa* L.) is the staple food of more than half of the world's population. However, rice yield is greatly reduced owing to rice blast, a polycyclic fungal disease caused by *Magnaporthe oryzae*. Infection by this pathogen consists of a series of distinct processes (Hebert 1971). First, conidia of the pathogen adhere to the rice epidermis, and second, they germinate to form germ tubes under suitable environmental conditions. When the tips of germ tubes receive hydrophobic signals from the host, appressoria develop and invade the rice cells underneath. Infectious hyphae ramify intra- and intercellularly throughout host tissues. Visible symptoms become apparent 4 to 5 days after the initial adhesion. Secondary conidia are produced from the original lesion and spread to other parts of the host and/or other hosts (Galhano and Talbot 2011).

At present, rice blast management relies primarily on the utilization of resistant rice varieties and frequent fungicide application. However, owing to the limited choices of resistant rice varieties and the desire to reduce pesticide applications (Miah et al. 2013), these two control methods are not always available. Additionally, fungicide-resistant strains emerge periodically because of selective pressure and the genetic complexity and diversity of *M. oryzae* (Chuma et al. 2011). Hence, biological control (biocontrol) using antagonistic microorganisms has emerged as a promising and alternative control strategy, and it has become the focus of extensive research in rice blast management. For many

biocontrol agents, multiple modes of action have been identified, and therefore, biocontrol may exert less selective pressure on pathogens than conventional control methods (Hyakumachi et al. 2014). In addition, microbial pesticides may be more environmentally sustainable than chemical pesticide alternatives (Schaffrath et al. 2000).

So far, four types of microbes have been studied as microbial pesticides against *M. oryzae*, namely the genus *Bacillus*, the genus *Pseudomonas*, fungi, and the genus *Actinomyces*; among them, the genus *Bacillus* is the most intensively reported (Wen et al. 2013). Yang et al. (2008) reported that the fresh culture of two *Bacillus etiohyticus* strains IPe2 and IPe14 had biocontrol efficacies of 64.4 and 56.4%, respectively, in reducing rice blast incidence (Yang et al. 2008). The water-soluble formulation of a *Bacillus megaterium* strain isolated in Thailand by Kanjanamaneeathian et al. (2009) was effective in controlling rice blast and increasing yield (Kanjanamaneeathian et al. 2009). Suryadi et al. (2013) found that *Bacillus firmus* E65 had a significant inhibitory effect on mycelium growth in *M. oryzae*, with an inhibition level of 73 to 85% on solid culture media (Suryadi et al. 2013). Leelasuphakul et al. (2006) demonstrated the biocontrol effect of *Bacillus subtilis* NSRS89-24 culture filtrate on rice blast. The active antimicrobial substance purified from the culture filtrate was identified as dextranase, which can cause the cell wall to dissolve and thereby, reduce the pathogenicity of *M. oryzae* (Leelasuphakul et al. 2006). Tendulkar et al. (2007) purified a surfactant from *Bacillus licheniformis* BC98 fermentation broth, which caused abnormal *M. oryzae* germ tube expansion and further led to failure in appressorium formation. This purified surfactant could also change the morphology of *M. oryzae* and inhibit mycelial growth (Tendulkar et al. 2007). Zhang et al. (2014) found that *B. subtilis* KB-1122 could cause a series of deformities in *M. oryzae*, including germ tube abnormality, cell wall rupture, and mycelium collapse. Serine, threonine, and tyrosine kinases were found to be up-regulated in KB-1122 when interacting with *M. oryzae*, which

[†]Corresponding author: H. Liu; hxliu@njau.edu.cn

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suggested that they may be involved in the collapse of mycelium in *M. oryzae* (Zhang et al. 2014). All of these studies focused on the inhibitory effect of biocontrol agents on the development of *M. oryzae*, such as inhibition of conidium germination, germ tube deformity, mycelium growth inhibition, and mycelium collapse. However, little work has been done to elucidate the molecular mechanisms underlying the various phenotypic changes in *M. oryzae*.

Conidium germination is the earliest essential step for the growth, development, and infection of *M. oryzae*. Previous work revealed that Ca^{2+} enhances the attachment of pycnidiospores in the plant-parasitic fungus *Phyllosticta ampellicida*, and it also regulates pycnidiospore germination and appressorium formation (Shaw and Hoch 1999). In other eukaryotes, such as plants, Ca^{2+} is a ubiquitous secondary messenger in signal transduction pathways (Pitt and Ugalde 1984; Rudd and Franklin-Tong 1999). In fungi, Ca^{2+} has been implicated in a variety of processes (Shaw and Hoch 2001), including spore attachment in *Colletotrichum lindemuthianum*, *Candida albicans*, and *P. ampellicida* (Jones and O'Shea 1994; Shaw and Hoch 1999; Young and Kauss 1984); spore germination in *Metarhizium anisopliae* and *P. ampellicida* (St Leger et al. 1990); tip growth in *Saprolegnia ferax* and other fungi (Garrill et al. 1993; Jackson and Heath 1993); appressorium differentiation in *M. anisopliae*, *Zoophthora radicans*, and *Colletotrichum trifolii* (Magalhães et al. 1991; St Leger et al. 1990); and zoospore encystment and germination in *Phytophthora cinnamomi* and the genus *Pythium* (Donaldson and Deacon 1992; Gubler et al. 1989).

The Ca^{2+} signaling pathway is also involved in conidium germination in filamentous fungi (Liu et al. 2015; Nguyen et al. 2008; Wang et al. 2003). The fluctuation of the intracellular free Ca^{2+} concentration has an important regulatory effect on the metabolic activity in fungi. When the cells are subjected to environmental stimuli, the influx of extracellular Ca^{2+} and the release of intracellular Ca^{2+} from reservoir increase the free Ca^{2+} concentration in the cytoplasm, and the elevated Ca^{2+} concentration initiates the calcium signaling pathway (Locke et al. 2000). Key signaling factors (Ca^{2+} channel protein Cch1, plasma membrane Ca^{2+} ATPase [Ca^{2+} -ATPase Pmc1], and Ca^{2+} /calmodulin-dependent protein kinase II [CaMKII]) play an important role in the regulation of cellular Ca^{2+} concentration. The Ca^{2+} channel protein Cch1 is an important component of the high-affinity Ca^{2+} influx system, which localizes on the plasma membrane. When the high-affinity Ca^{2+} influx system is activated, it allows Ca^{2+} to flow from the extracellular space into the cell (Locke et al. 2000). Ca^{2+} -ATPase Pmc1 is a key protein localized on the vacuole membrane responsible for directing the cytosolic Ca^{2+} to vacuole (Kmetzsch et al. 2013). CaMKII is a serine/threonine-specific protein kinase, which is activated when the level of cytoplasmic Ca^{2+} is increased to regulate Ca^{2+} metabolism (Payne et al. 1988).

Bacillus cereus HS24 was isolated from rice rhizosphere in a field that was moderately affected by *M. oryzae*. HS24 demonstrated a biocontrol efficiency of 77.5% in a 45-day greenhouse inoculation

assay with *M. oryzae* Guy11 (Tokpah et al. 2016). To better exploit it as a potential biocontrol agent, we studied the mechanisms underlying the suppression by *B. cereus* HS24 of *M. oryzae* Guy11 and demonstrate that HS24 inhibits conidium germination in *M. oryzae* by blocking its Ca^{2+} signaling pathway. This study not only provides valuable information on how the rhizobacterium *B. cereus* suppresses *M. oryzae*, but also, it sheds light on the potential interactions between *B. cereus* and fungi in general.

MATERIALS AND METHODS

Strains and culture conditions. *B. cereus* HS24 and *Bacillus* sp. HL16 were inoculated onto a Luria–Bertani (LB) agar plate (Bertani 2004), and they were incubated at 28°C for 24 h in a constant temperature incubator. A single colony of each strain was picked and inoculated into 200 ml of LB broth medium. The strains were cultured for 24 h at 28°C under shaking conditions (200 min⁻¹), and they were diluted to 1×10^5 , 1×10^6 , and 1×10^7 CFU/ml using sterile water. *M. oryzae* Guy11 (donated by Zhengguang Zhang, Nanjing Agricultural University) was inoculated onto a potato dextrose agar plate and cultured in a constant temperature incubator at 28°C for 4 days in darkness. The fungal colony was then inoculated onto a straw decoction and corn agar plate (Zhang et al. 2011), and it was cultured at 28°C in darkness for 3 to 4 days. This was followed by incubation under continuous fluorescent light for 7 days to induce the production of conidia. Ten milliliters of sterile water was added to each plate, and a brush was used to collect conidia by gently brushing the surface of the plate. The conidial suspension was filtered through three layers of lens paper. The conidia were counted with a hemacytometer under a microscope (Zhang et al. 2009), and then, conidium concentration was adjusted to 1×10^5 conidia per milliliter for use.

Effects of HS24 on conidium germination, appressorium formation, germ tube length, and turgor pressure. One milliliter of *M. oryzae* conidial suspension was centrifuged in four 1.5-ml centrifuge tubes at 12,000 min⁻¹ for 10 min to obtain conidial precipitates. One milliliter of sterile water, LB broth, and HL16 as the control groups and 1 ml of HS24 at different concentrations (1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 CFU/ml) were added to each of the four centrifuge tubes to resuspend the conidial precipitates. Each treatment was repeated three times. A droplet (20 μ l) of conidial suspension from each treatment group was placed on a hydrophobic microscope cover glass with wet filter paper and incubated at 28°C in darkness. Conidium germination, germ tube length, and appressorium formation were monitored at different time points postincubation under a microscope. The percentage of conidia that germinated and formed appressoria was determined for each replicate. Germ tube length was measured at 6 h post-incubation (hpi), and turgor pressure inside mature appressoria was assessed by cytorrhysis assay at 24 hpi (Odenbach et al. 2007). More than 100 conidia were assessed for each treatment, and each treatment was repeated at least three times.

Effects of HS24 on conidium attachment. Conidia of *M. oryzae* were treated with sterile water or HS24 at the concentration of 1×10^7 CFU/ml. Then, droplets (20 μ l) of conidial suspension from each treatment were placed on hydrophobic coverslips with wet filter paper and incubated at 28°C in darkness. At 2 hpi, conidia on each coverslip were counted under a microscope before and after being washed with the same water flow. Attachment was calculated as the number of conidia after being washed relative to the number before being washed.

Effect of Ca^{2+} signaling inhibitors on conidium germination and germ tube length. Ca^{2+} signaling inhibitors at different concentrations (ethylene glycol tetraacetic acid, abbreviated to EGTA; Adamas: 0, 50, 100, 150, and 200 mM; verapamil; MEC: 0, 100, 200, 300, and 400 μ M; KN-93; MEC: 0, 20, 40, 60, 80, and 100 μ M; and cyclopiazonic acid [CPA]; Cayman: 0, 20, 40, 60, 80, and 100 μ M) were added to the conidial suspension. Twenty

TABLE 1. Primers used for PCR amplification of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), Pmc1, and CCH1 genes in *Magnaporthe oryzae*

Gene and direction	Sequence
<i>EF1α</i>	
Forward	CAAGAACGTGTCCGTCAAG
Reverse	CCATAGGAGGGTCGTTCTT
<i>CaMKII</i>	
Forward	ACACCATCATAAGGTACGGC
Reverse	CGAAGCTCTGACCGTAGTAG
<i>PMC1</i>	
Forward	TCTATGTGCGGAGGTTTCTG
Reverse	ACAAAATAGCAATCCATCGCC
<i>CCH1</i>	
Forward	GCAGAGGAGACGGATACTTT
Reverse	ACGAGAAACCATAGTGCAGA

microliters of mixture from each treatment was dripped onto a microscope cover glass with wet filter paper and incubated at 28°C in darkness. Conidium germination, appressorium formation, and germ tube length were examined at 6 hpi.

RNA extraction and gene transcription analysis. *M. oryzae* conidia cocultured with sterile water or HS24 were

collected at 2 hpi for RNA extraction. Reverse transcription was done using the PrimeScript RT reagent kit (TaKaRa). Transcription of Ca^{2+} signaling-related genes was examined by reverse transcription quantitative PCR (RT-qPCR) on an ABI 7500 Fast Real-Time system (Applied Biosystems) following previously established procedures (Guo et al. 2010). The primers used in this study were designed using the NCBI (<https://www.ncbi.nlm.nih.gov>) as shown in Table 1.

Determination of Ca^{2+} concentration in conidia. The free Ca^{2+} in the conidia of *M. oryzae* was stained with Fluo 3-AM (Sigma) at the concentration of 150 μM as described previously (Zhang et al. 1998). The change of Ca^{2+} concentration was indicated by relative fluorescence intensity calculated as the fluorescence intensity of each treatment divided by the fluorescence intensity of the water treatment.

Statistics and data analysis. All treatments were repeated at least three times, and >100 conidia were observed in each replicate. Data were analyzed with SPSS 17.0 (IBM Corporation) by one-way analysis of variance followed by means separation using least significance difference (LSD) test ($P < 0.05$).

RESULTS

Effects of HS24 on conidium germination, appressorium formation, germ tube length, and turgor pressure. The inhibitory effect of HS24 on conidium germination, germ tube

Fig. 1. Effect of *Bacillus cereus* HS24 at various concentrations on conidium germination, germ tube growth, and appressorium formation at 8 h post-incubation. *Magnaporthe oryzae* conidia were treated with sterile water, Luria-Bertani (LB) broth, *Bacillus* sp. HL16 at a concentration of 1×10^7 CFU/ml (a negative control of bacterium), and HS24 at the concentrations indicated. **A**, Conidium development was observed with HS24 at **A, a**, 1×10^7 , **A, b**, 1×10^6 , **A, c to f**, 1×10^5 , **A, g**, 1×10^4 , **A, h**, 1×10^3 , and **A, i**, 1×10^2 and with **A, j**, sterile water. Conidia were examined by differential interference contrast microscopy. Scale bar = 30 μm . **B**, Conidium germination and **C**, appressorium formation with sterile water, LB broth, HL16, and HS24 at various concentrations. Values are means and standard deviations of three replicates. Means separation letters indicate statistical significance ($P < 0.05$) based on one-way analysis of variance test. Scale bar = 30 μm .

Fig. 2. Effect of *Bacillus cereus* HS24 on conidium germination in *Magnaporthe oryzae* at different time points. Conidia were treated with Luria-Bertani (LB) broth, sterile water (control), or HS24 at the concentrations of 1×10^5 , 1×10^6 , or 1×10^7 CFU/ml. **A**, Conidium germination evaluated with LB broth, sterile water, or HS24. **B**, Conidia examined microscopically at 24 h post-inoculation (hpi). Values are means and standard deviations of three replicates. Means separation letters indicate statistical significance ($P < 0.05$) based on one-way analysis of variance followed by the least significant difference test. Scale bar = 20 μm .

growth, and appressorium formation was dependent on its concentration (Fig. 1). At 8 hpi, HS24 at 10^7 CFU/ml completely suppressed conidium germination. HS24 at 10^6 CFU/ml allowed 14.4% of the conidia to germinate, but no appressoria were seen. The conidia that did germinate under this condition developed germ tubes nearly as long as the controls. HS24 at 10^5 CFU/ml allowed 74.4% of the conidia to germinate, but only 63.3% of the germings developed appressoria, with delayed accumulation of melanin in 31.0% of the appressoria (Fig. 1A). Most of the germings produced short germ tubes under this condition, and germ tubes with normal length could only be seen occasionally (Fig. 1A). HS24 at 10^4 CFU/ml and lower concentrations (concentrations of 10^3 and 10^2 CFU/ml) supported similar levels of germination, appressorium formation, germ tube growth, and melanin accumulation in appressoria to the controls (Fig. 1B).

Conidium germination after treatment by HS24 at 10^5 CFU/ml was not significantly different from the controls at 24 hpi. Germination increased from 17.9% at 8 hpi to 56.0% at 24 hpi when treated with HS24 at 10^6 CFU/ml. Only a few conidia germinated (2.1%) when treated with HS24 at 10^7 CFU/ml at 24 hpi (Fig. 2).

Taken together, we conclude that HS24 at the concentration of 1×10^7 CFU/ml can significantly inhibit conidium germination in *M. oryzae*. The inhibitory effect on conidium germination and appressorium formation became weaker as the concentration of HS24 decreased (Fig. 1). We also found that, although appressorium formed in germinated conidia treated with HS24 at 10^5 CFU/ml, the germ tubes were significantly shorter than the controls (Fig. 3A and B). Circular melanin-containing mature appressoria were abundantly observed when treated with HS24 at 10^5 CFU/ml. No significant changes of turgor pressure were observed between water and HS24-treated *M. oryzae* samples when the appressoria were treated with glycerol at various concentrations (Fig. 3C). In addition, no significant difference in conidium germination was found between the treatments of sterile water, LB broth, and HL16;

therefore, we used the sterile water as the control for the rest of the experiment. HS24 at the concentration of 1×10^7 CFU/ml was used in all of the following experiments.

Effect of HS24 on conidium attachment. Conidia need to attach to the host surface before invading the host cells (Shaw and Hoch 2000). To clarify whether HS24 inhibited conidium germination through reducing conidium attachment, we used consistent water flow to wash the hydrophobic coverslips, which contained conidia that had been treated with water or HS24 (1×10^7 CFU/ml) for 2 h. The results showed that there was no significant difference in attachment between the water and HS24-treated

Fig. 4. Attachment of conidia of *Magnaporthe oryzae* incubated with water or *Bacillus cereus* HS24 for 2 h. **A**, Attachment of conidia on coverslips detected after being washed by a consistent water flow. **B**, Conidia on coverslips before or after being washed. Values are means and standard deviations of three replicates. Means separation letters indicate statistical significance ($P < 0.05$) based on one-way analysis of variance followed by the least significant difference test. hpi, hours postincubation. Scale bar = 20 μ m.

Fig. 3. Effect of *Bacillus cereus* HS24 on germ tube length and turgor pressure inside appressoria of *Magnaporthe oryzae* at the concentration of 1×10^5 CFU/ml. **A**, Germ tubes examined microscopically. Scale bar = 20 μ m. **B**, Germ tube length measured at 6 h postincubation (hpi). **C**, Appressorium cytorrhysis measured at 24 hpi. Values are means and standard deviations of three replicates. Means separation letters indicate statistical significance ($P < 0.05$) based on one-way analysis of variance followed by the least significant difference test.

samples (Fig. 4), which indicates that HS24 does not affect conidium attachment.

Effects of Ca^{2+} signaling inhibitors on conidium germination, germ tube elongation, and appressorium formation. Previous work revealed that Ca^{2+} plays a key role in spore germination in fungi (Shaw and Hoch 1999; St Leger et al. 1990; Wang et al. 2003). We treated *M. oryzae* conidia with four Ca^{2+} signaling inhibitors: Ca^{2+} -chelating agent EGTA, CaMKII

inhibitor KN-93, calcium channel protein CCH1 inhibitor verapamil, and Ca^{2+} -ATPase PMCA1 inhibitor CPA. The results showed that all inhibitors tested reduced conidium germination, and the effect became stronger when the concentration of inhibitors increased (Fig. 5A, C, E, and G). The concentration-dependent manner of the inhibitory effect by Ca^{2+} signaling inhibitors was in line with that by HS24. In addition, germ tube elongation was also affected by the inhibitors (Fig. 5B, D, F, and H). We also observed

Fig. 5. Effect of exogenous addition of inhibitors on conidium germination and germ tube length of *Magnaporthe oryzae* at 6 h postincubation. Effect of various concentrations of EGTA, KN-93, verapamil, and cyclopiazonic acid on A, C, E, and G conidium germination and B, D, F, and H, germ tube length. Values are means and standard deviations of three replicates.

that the germinating conidia produced less mature appressoria by 40.0% at 6 hpi compared with the water controls (Fig. 6).

Down-regulation of genes involved in *M. oryzae* Ca²⁺ signaling pathways by HS24. To further test whether the above Ca²⁺ signaling-associated genes in *M. oryzae* conidia were affected by HS24, the transcription levels of *CaMKII*, *PMC1*, and *CCH1* were determined by RT-qPCR. Our results showed that these three genes were significantly down-regulated in *M. oryzae* by HS24 at 1 × 10⁷ CFU/ml compared with the water-treated group (Fig. 7).

HS24 leads to the appearance of granular vesicles in *M. oryzae* conidia. It was reported that the treatment with Ca²⁺ signaling inhibitor verapamil resulted in the appearance of granular vesicles in the uniform protoplasm in *M. oryzae* conidia (Wang et al. 2003). In this study, we performed a similar test to determine

whether HS24 is able to induce granular vesicles in *M. oryzae* conidia. Our results showed that granular vesicles were found in the HS24 (1 × 10⁷ CFU/ml)-treated conidia but not in the water-treated conidia (Fig. 8A). This result further illustrated that HS24 may suppress *M. oryzae* conidium germination through interfering with its Ca²⁺ signaling pathway at a high concentration.

HS24 reduces the intracellular Ca²⁺ concentration. Because HS24 inhibits the expression of three key genes involved in Ca²⁺ signaling, we further tested whether HS24 could change the intracellular free Ca²⁺ concentration in conidia. It was observed that HS24-, verapamil-, and EGTA-treated conidia demonstrated weaker fluorescence intensity compared with the sterile water-treated conidia. The exogenously added Ca²⁺ did not restore the Ca²⁺ concentration to a normal level in HS24 and verapamil treatment groups. However, it restored the Ca²⁺ concentration in the EGTA treatment group (Fig. 8). These results indicate that HS24 interferes with calcium channels, such as *Cch1*, to reduce Ca²⁺ concentration in conidia. Furthermore, exogenous addition of various concentrations of Ca²⁺ under HS24 treatment failed to compensate for the inhibitory effect of HS24 on conidium germination (Fig. 9).

DISCUSSION

In this research, we mainly studied the inhibitory effect of *B. cereus* HS24 on *M. oryzae* with the aim to better understand the

Fig. 6. Effect of low concentrations of calcium signaling inhibitors on appressorium formation of *Magnaporthe oryzae* at 6 h postincubation. **A**, Effect of EGTA, KN-93, verapamil, and cyclopiazonic acid at low concentrations on appressorium formation. **B**, Morphology of appressoria examined microscopically. Values are means and standard deviations of three replicates. Scale bar = 20 μm.

Fig. 7. Transcription of Ca²⁺ signaling involved genes in *Bacillus cereus* HS24-treated conidia of *Magnaporthe oryzae*. Conidia were treated with sterile water or HS24 (1 × 10⁷ CFU/ml) for 2 h, and then, total RNA was extracted. Reverse transcription quantitative PCR was used to measure transcription. Values are means and standard deviations of three replicates. *Significant difference ($P < 0.05$) based on one-way analysis of variance followed by the least significant difference test.

Fig. 8. Free Ca²⁺ concentration determination and appearance of granular vesicles in *Bacillus cereus* HS24-treated conidia of *Magnaporthe oryzae*. **A**, Ca²⁺ fluorescence staining in conidia treated with sterile water or HS24 (1 × 10⁷ CFU/ml). Scale bar = 20 μm. **B**, Relative fluorescence intensity of free Ca²⁺ in conidia. The concentration of verapamil was 500 μM, the concentration of EGTA was 200 mM, and the Ca²⁺ concentration was 100 mM. Values are means and standard deviations of three replicates. Means separation letters indicate statistical significance ($P < 0.05$) based on one-way analysis of variance followed by the least significant difference test.

Fig. 9. Influence of exogenous Ca²⁺ on conidium germination of *Bacillus cereus* HS24-treated conidia of *Magnaporthe oryzae*. HS24 concentration was 1 × 10⁷ CFU/ml. Values are means and standard deviations of three replicates. **Significant difference ($P < 0.01$) based on one-way analysis of variance followed by the least significant difference test.

mechanism of HS24 as an agent for biocontrol of rice blast. The results showed that HS24 (1×10^7 CFU/ml) inhibits conidium germination through interfering with the Ca^{2+} signaling pathway in *M. oryzae*.

We observed that the inhibitory effect on conidium germination by HS24 is concentration dependent. The concentration of 10^7 CFU/ml of HS24 completely suppressed conidium germination at 24 hpi. In contrast, a lower concentration (10^6 CFU/ml) allowed some conidia to germinate, and the concentration of 10^5 CFU/ml showed no inhibitory effect at 24 hpi. This led us to question whether the germinated conidia treated with HS24 at 10^5 CFU/ml would grow normally. We found that the germ tubes of conidia treated with HS24 at high concentration were significantly shorter than those treated at low concentration or with water. To test whether the shortened germ tubes would form normal-functioning appressoria, we monitored appressorium formation and the turgor pressure inside the appressoria. The results showed that circular melanin-containing mature appressoria were abundantly observed at 24 hpi. In addition, no significant changes of turgor pressure were detected between water- and HS24-treated samples. These findings indicate that *B. cereus* HS24 at lower concentration has no effect on the eventual appressorium formation and turgor pressure inside the appressoria. Based on these results, we chose 10^7 CFU/ml as the working concentration of HS24 for the Ca^{2+} signal-associated trails.

Intracellular Ca^{2+} is an important secondary messenger in all organisms. When the Ca^{2+} concentration in the environment varies from 1 to 100 mM, there is a slight change in the concentration of Ca^{2+} inside the cytoplasm; this is usually in the range of 50 to 200 nM (Cui and Kaandorp 2006; Cui et al. 2009a, b). The calcium homeostasis system, which is composed of various calcium channels and ion pumps as well as many related proteins and enzymes, plays an important role in maintaining the optimum Ca^{2+} concentrations in the cytoplasm and organelles, such as vacuoles, endoplasmic reticulum, and Golgi apparatus (Bonilla and Cunningham 2002; Cui and Kaandorp 2006; Kmetzsch et al. 2013). Normally, the plasma membrane Ca^{2+} influx system is activated in response to various external environmental changes, such as alkaline stress, cold/heat stress, oxidative stress, and ethanol stress (Courchesne et al. 2011; Kraus et al. 2005; Popa et al. 2010; Teng et al. 2013; Wang et al. 2011). The transient increase in intracellular Ca^{2+} concentration can also be induced by Ca^{2+} secretion from the internal calcium pool (Locke et al. 2000). Elevated Ca^{2+} concentration in filamentous fungi cells directly affects a wide range of cellular processes, such as sporulation, conidium germination (St Leger et al. 1990), mycelium tip growth, mycelium branching (Jackson and Heath 1993), gene expression, and circadian rhythms. Increased Ca^{2+} concentration also regulates the signaling cascade, and it activates the calcineurin pathway to reduce the Ca^{2+} concentration to basal level (Kozubowski et al. 2009; Kraus et al. 2005). Key signaling factors, such as Ca^{2+} channel protein Cch1, Ca^{2+} -ATPase Pmc1, and CaMKII, play an important role in the regulation of cellular Ca^{2+} concentration. In this study, we found that the transcription of the above Ca^{2+} signaling-associated genes was reduced by HS24. We also found that the corresponding Ca^{2+} signaling inhibitors decreased *M. oryzae* conidium germination, which is consistent with previous findings (Kim et al. 1998). Taken together, these results suggest that the inhibition by HS24 may be through interfering with the Ca^{2+} signaling pathway in *M. oryzae*.

Free Ca^{2+} labeling with fluorescent dye in conidia revealed that HS24-, verapamil-, and EGTA-treated conidia had less free Ca^{2+} than the water-treated conidia. Exogenous addition of Ca^{2+} restored the cytoplasmic Ca^{2+} concentration in EGTA-treated conidia. The chelation of exogenously added EGTA with free Ca^{2+} resulted in a dramatic decrease of the calcium concentration in the environment and caused the reduction of Ca^{2+} concentration in conidia; when enough exogenous Ca^{2+} was added into the environment, the intracellular Ca^{2+} concentration increased to a normal level. In contrast, exogenous addition of Ca^{2+} did not restore the Ca^{2+}

concentration in the HS24 and verapamil treatment groups. Consistently, the reduced germination level of HS24-treated conidia was not reversed by adding exogenous Ca^{2+} . These results indicate that the Ca^{2+} channel could be disturbed by HS24 and that exogenous Ca^{2+} is prevented from entering the conidia. Thus, the reduction of cytoplasmic Ca^{2+} concentration by HS24 could be ascribed to the reduced transcription of Cch1, an essential component of the high-affinity Ca^{2+} influx system for the entry of exogenous Ca^{2+} into conidia.

The Ca^{2+} -ATPase Pmc1 plays a role in directing redundant intracellular Ca^{2+} to the vacuole, and CaMKII regulates Ca^{2+} metabolism when cytoplasmic Ca^{2+} concentration is increased. Thus, rather than the lower expressions of Ca^{2+} -ATPase Pmc1 and CaMKII, the reduced expression of Cch1 could be responsible for the reduction of the cytoplasmic Ca^{2+} concentration to suppress conidium germination. Nevertheless, the reduced transcription levels of these three genes showed that HS24 impacted multiple Ca^{2+} signaling components, which are all crucial for conidium germination. Taking the above results together, we draw the conclusion that HS24 blocks the extracellular Ca^{2+} from entering the conidia by inactivating the high-affinity Ca^{2+} influx system protein Cch1, which may result in the lower concentration of Ca^{2+} in the conidia and the suppression of conidium germination in *M. oryzae*.

It has been reported that hyphal elongation requires the presence of high concentrations of Ca^{2+} at the tip of hyphae (Jackson and Heath 1993; Silverman-Gavrila and Lew 2000). Interestingly, the hyphal tip in *M. oryzae* also produces an appressorium-like structure when attached to a hydrophobic surface (Han et al. 2015). It has been reported that increased calcium concentration not only enhances conidium germination but also, promotes germ tube elongation (Shaw and Hoch 2000). In this study, our results are in line with these findings. We found that the conidium germination was delayed by HS24 at the concentration of 10^5 CFU/ml. The conidium germination level increased from 39.2% at 4 hpi to the maximum at 24 hpi. HS24 at 10^6 CFU/ml supported longer germ tube growth compared with HS24 at 10^7 CFU/ml; HS24 at 10^4 CFU/ml and lower concentrations allowed longer germ tube growth than HS24 at 10^5 CFU/ml. These observations suggest that calcium is required for germ tube growth. Shorter germ tubes and reduced appressorium formation induced by HS24 at 10^5 CFU/ml compared with HS24 at 10^6 CFU/ml (Fig. 1), which is consistent with those in pCa3 media in comparison with in pCa5 media (figure 2 in Shaw and Hoch 2000). This may be pathogen's adaption to decreased calcium for infection.

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