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RESEARCH ARTICLE

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The Cell Wall Integrity MAP Kinase Signaling Pathway Is Required for Development, Pathogenicity, and Stress Adaption of the Pepper Anthracnose Fungus *Colletotrichum scovillei*

Teng Fu, Sung Wook Kang, Yong-Won Song and Kyoung Su Kim

Division of Bio-Resource Sciences and Interdisciplinary Program in Smart Agriculture, Kangwon National University, Chuncheon, Korea

ABSTRACT

The cell wall integrity (CWI) signaling pathway plays important roles in the dissemination and infection of several plant pathogenic fungi. However, its roles in the pepper fruit anthracnose fungus *Colletotrichum scovillei* remain uninvestigated. In this study, the major components of the CWI signaling pathway—CsMCK1 (MAPKKK), CsMKK1 (MAPKK), and CsMPS1 (MAPK)—were functionally characterized in *C. scovillei via* homology-dependent gene replacement. The Δ*Csmck1*, Δ*Csmkk1*, and Δ*Csmps1* mutants showed impairments in fungal growth, conidiation, and tolerance to CWI and salt stresses. Moreover, Δ*Csmck1*, Δ*Csmkk1*, and Δ*Csmps1* failed to develop anthracnose disease on pepper fruits due to defects in appressorium formation and invasive hyphae growth. These results suggest that CsMCK1, CsMKK1, and CsMPS1 play important roles in mycelial growth, conidiation, appressorium formation, plant infection, and stress adaption of *C. scovillei*. These findings will contribute to a better understanding of the roles of the CWI signaling pathway in the development of pepper fruit anthracnose disease.

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Colletotrichum scovillei; pepper fruit anthracnose; cell wall integrity; MAP kinase

1. Introduction

The plant pathogenic fungi are exposed to diverse environmental stresses, including osmotic stress, oxidative stress, nutrient limitations, chemical and mechanical stresses, and stresses from host immune system [1]. The fungal cell wall is a polymeric structure—mainly containing chitin, glucan, galactomannan, and glycosylated proteins—that serves as the primary barrier against environmental stresses to maintain cellular integrity [2,3]. Despite some fundamental diversity in cell wall structure among different species and even different developmental stages of the same fungal species, the maintenance and biosynthesis of the cell wall is mainly dependent on the conserved cell wall integrity (CWI) signaling pathway [1,4]. The CWI signaling pathway plays important roles in cell growth, differentiation, and stress adaption [4,5]. In *Saccharomyces cerevisiae*, the CWI signaling pathway includes several cell surface receptors coupled with a Rho1 GTPase, protein kinase C, and a sequentially activated mitogen-activated protein kinase (MAPK) cascade (Bck1/Mck1-Mkk1/2-Slt2/Mps1) [5].

In plant pathogenic fungi, components of the CWI signaling pathway have been shown to regulate growth, dissemination, infection-related morphogenesis, and suppression of host defense in several fungal pathogens that causes severe foliar and root diseases [6–8]. For example, the *Magnaporthe oryzae* genes *Mck1*, *Mkk2*, and *Mps1* are required for conidiation and appressorium-mediated penetration [7,9,10], while *Botrytis cinerea* Bmp3, orthologous to Mps1, is involved in conidiation, surface sensing, and pathogenicity [6]. However, the roles of the CWI-related MAPKs have not been characterized in the pepper fruit anthranose pathogen *Colletotrichum scovillei*, a species belonging to the *Colletotrichum acutatum* species complex.

Anthracnose disease on pepper fruit caused by *C. scovillei* occurs during pre- and post-harvest stages, leading to huge economic losses in subtropical and temperate countries [11,12]. The *C. scovillei* produces a huge number of conidia under favor conditions, which is a determinant of anthracnose disease dissemination. In the early infection stage, *C. scovillei* develops a non-melanized appressorium, which serves as the major infection structure [13]. The

CONTACT Kyoung Su Kim kims@kangwon.ac.kr

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appressorium then forms a tiny penetration peg, which subsequently differentiates into highly branched invasive hyphae in the cuticle layer of pepper fruits, called the dendroid structure [14,15]. Due to the agricultural impact and unique infection processes, anthracnose disease by *C. scovillei* on pepper fruit caused is becoming an interesting model to study molecular mechanisms governing the developments of fruit fungal disease. So far, several regulatory genes have been characterized to play essential roles in dissemination, appressorium formation, penetration, and invasive hyphae of *C. scovillei* [11,16]. However, the CWI-related MAPKs, known to associate with fungal differentiation, virulence, and stress adaptions in foliar fungal pathogens, have not been investigated in the pepper fruit anthracnose fungus, *C. scovillei*.

This study aims at determining the roles of CWI-related MAPKs in the pepper fruit anthracnose fungus, *C. scovillei*, using deletion mutants (Δ*Csmck1*, Δ*Csmkk2*, and Δ*Csmps1*). The results showed that Δ*Csmck1*, Δ*Csmkk2*, and Δ*Csmps1* were defective in mycelial growth, conidiation, appressorium formation, pathogenicity, and tolerance to CWI and salt stresses, compared to the wild-type. However, the defects of deletion mutants were recovered in each corresponding complemented strain. Together, our results suggest that CWI signaling pathway plays pivotal roles in fungal developments, virulence, and stress adaptions of the pepper fruit anthracnose fungus, *C. scovillei*.

2. Materials and methods

2.1. Fungal strains and culture conditions

The *C. scovillei* strain KC05 was used as the wild-type strain in this study. The wild-type and its transformants were routinely cultured on the minimal medium agar (MMA), complete medium agar (CMA), V8 juice agar (V8A), and potato dextrose agar (PDA) were performed as previously described [11].

2.2. Bioinformatic tools

The amino acid sequences of CWI-related MAPKs and their orthologs used in this study were downloaded from the Comparative Fungal Genomics Platform (CFGP; http://cfgp.riceblast.snu.ac.kr/) and UniProt (https://www.uniprot.org/). Domains of all sequences were predicted using InterProScan (https://www.ebi.ac.uk/interpro/). All amino acid sequences were aligned using the ClustalW. The phylogenetic tree was constructed using the MEGA X software.

2.3. Targeted gene deletion and complementation

The deletion mutants and complemented strains were generated as previously described [11]. Briefly, the upstream and downstream sequences (about 1.5 kb) of each targeted gene were amplified with primers 5 F/5R and 3 F/3R, respectively (Table S1) [17]. The two amplified segments were fused to an *hph* cassette to generate a deletion construct, which was transformed into protoplasts of *C. scovillei* KC05, as previously described [18]. For the generation of complemented strains, genomic copies of the targeted gene were transformed with the geneticin-resistant cassette *G418* to the protoplasts of deletion mutant. The deletion mutants and complemented strains were selected through rounds of screening polymerase chain reaction (PCR) [13,17], then further confirmed through Southern blotting and reverse-transcription (RT)-PCR, respectively.

2.4. Southern blotting and RT-PCR

The Southern blotting and RT-PCR were performed according to previously reported methods. Briefly, the probe DNA (about 500 bp in length) of Southern blotting was labeled with Biotin-High Prime (Roche, Indianapolis, IN, United States) to detect the restriction enzymes-digested genomic DNA in a nylon hybridization membrane [11]. The SuperScript® III First-strand Synthesis System (Invitrogen, Grand Island, NY, United States) was applied to synthesize the first-strand complementary DNA (cDNA) from total RNA (5 μg), extracted using the Easy-Spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, South Korea).

2.5. Assays of fungal developments and pathogenicity

Mycelial growth was tested by measuring colony diameters. mycelial agar plugs (5 mm in diameter) were inoculated onto PDA and incubated for five-days in the dark followed by two days in the light. Conidiation was measured by counting conidia collected with 5 ml of distilled water from seven-day-old V8 agar. The concentrations of conidial suspensions were evaluated using a hemocytometer under a light microscope [11]. To test the conidial germination and appressorium formation, the conidial suspensions (5×10^4 mL^{-1}) were dopped onto the hydrophobic surface of coverslips and incubated in a humid plastic box at 25 °C for 16 h. For pathogenicity assays, conidial suspensions (5×10^5 mL^{-1}) were inoculated onto wounded and intact pepper fruits and incubated in a humid box at 25 °C for six

and nine days, respectively [13]. All data were collected from at least three independent experiments with three replicates per experiment. A Duncan's test ($p < .05$) was used to estimate the significant difference.

3. Results and discussion

3.1. Phylogenetic analysis, domain structure, and targeted gene deletion

To isolate CWI-related MAPKs in *C. scovillei*, amino acid sequences of MCK1 (MGG_00883), MKK2 (MGG_06482), and MPS1 (MGG_04943) were used to blast query the whole genome of *C. scovillei* using the CFGP. Three loci—CAP_011231, CAP_005029, and CAP_001359—were identified and named CsMCK1, CsMKK1, and CsMPS1, respectively. A phylogenetic analysis of CsMCK1, CsMKK1, and CsMPS1 and their orthologs was performed by a neighbor-joining method with 1000 bootstrap replicates [13,19]. The results showed that CsMCK1, CsMKK1, and CsMPS1 from *C. scovillei* are closely related to their homologs in other fungi (Figure 1). Domain prediction showed that all selected CWI-related MAPKs contain a protein kinase domain (IPR000719) (Figure 1). These results suggest that CsMCK1, CsMKK1, and CsMPS1 are conserved among fungi.

To functionally characterize the roles of *CsMCK1*, *CsMKK1*, and *CsMPS1* in *C. scovillei*, each of the targeted genes was deleted *via* homology-dependent replacement (Figure 2(A)). The deletion mutants and complemented strains were confirmed through Southern blotting and RT-PCR, respectively (Figure 2(B,C)).

3.2. Roles of CWI-related MAPKs in mycelial growth and stress adaption

Because the CWI-related MAPK signaling cascade is involved in mycelial growth and stress adaption [8,10,20,21], the colony diameters of mycelial growth in Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* were evaluated on potato dextrose agar (PDA). After five days, colony diameters of Δ*Csmck1* (43.6 ± 1.8 μm), Δ*Csmck1* (43.1 ± 1.6 μm), and Δ*Csmck1* (43.2 ± 1.1 μm) were significantly reduced compared to the wild-type (54.1 ± 1.3 μm) (Figure 3(A,B)). The defect in mycelial growth rate was restored in each corresponding complemented strain (Figure 3(B)). This result suggests that *CsCMK1*, *CsMKK1*, and *CsMPS1* are involved in mycelial growth. To test whether *CsCMK1*, *CsMKK1*, and

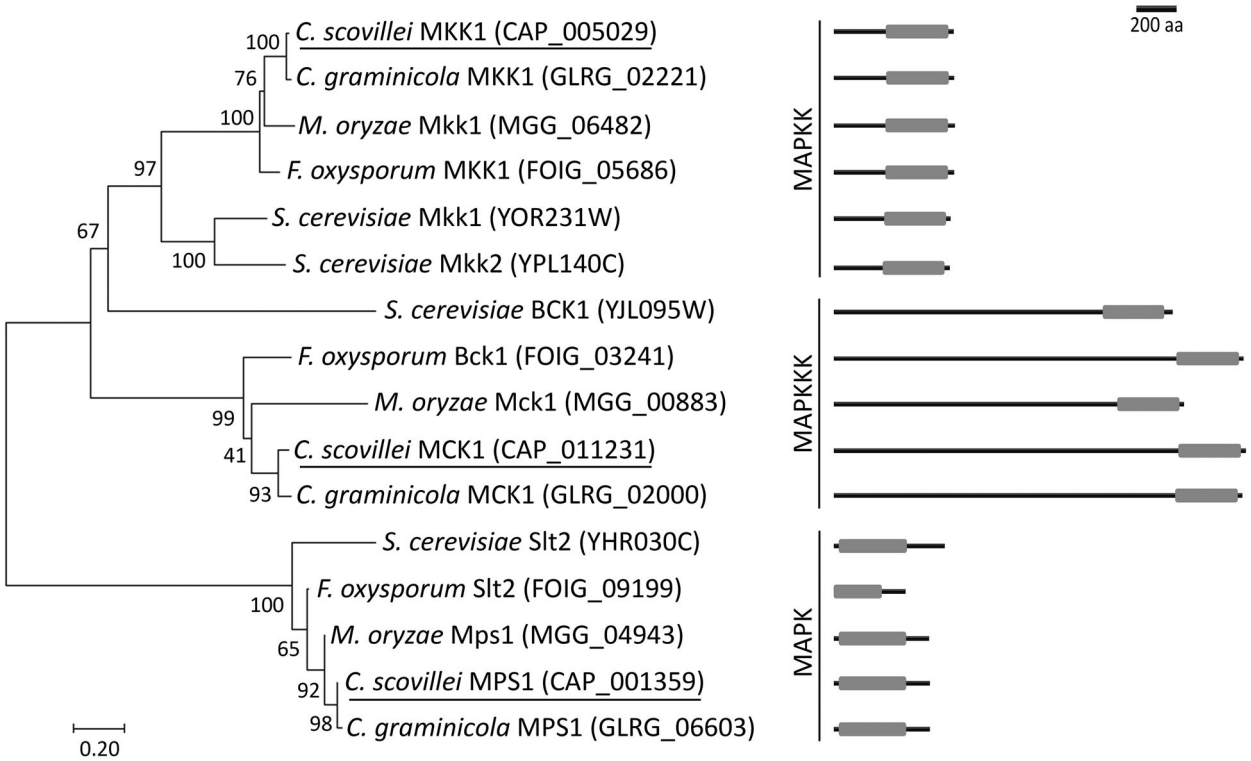

Figure 1. Phylogenetic analysis and domain prediction. Phylogenetic tree of CsCMK1, CsMKK2, CsMPS1, and their orthologs constructed using a neighbor-joining method with 1000 bootstraps in MEGA X. InterProScan was used to predict domains in all protein sequences. All sequences were downloaded from UniProt (https://www.uniprot.org/), NCBI (https://www.ncbi.nlm.nih.gov/), or the Comparative Fungal Genomics Platform (http://cfgp.riceblast.snu.ac.kr/). The protein kinase domain (IPR000719) and amino acid sequence are indicated by a grey box and black line, respectively.

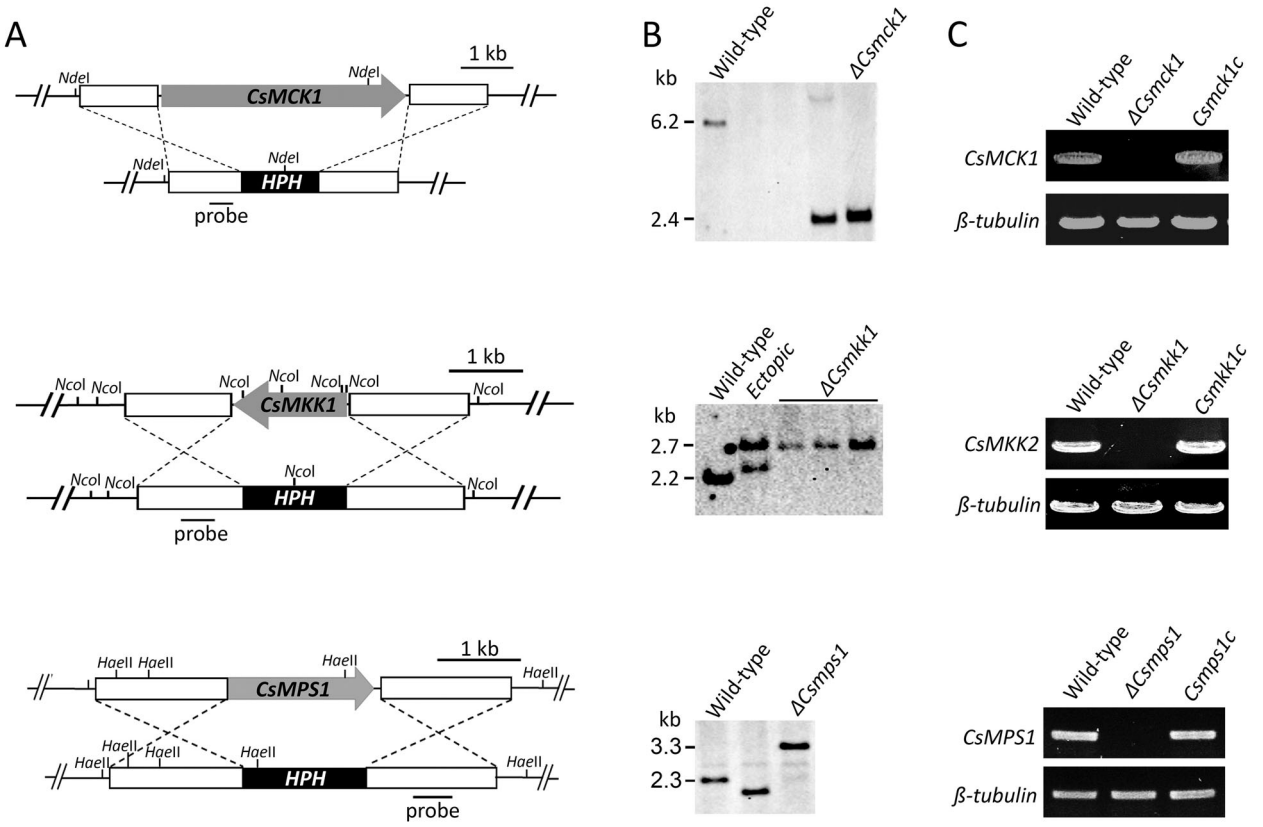

Figure 2. Generation of transformants. (A) Target gene deletion strategy. The open reading frame of the targeted gene was replaced using an *hph* cassette *via* fusion PCR; (B) Verification of deletion mutants. The deletion mutants were selected by rounds of PCR screening with primers SF/SR. The genomic DNA of the wild-type and selected deletion mutants was individually digested with the indicated restriction enzymes and transferred to a nylon membrane. The specific probe (~500 bp), amplified with PF/PR (Table S1), was labeled with Biotin High Prime and hybridized to the nylon membrane; (C) Confirmation of complemented strains. Genomic copies of each target gene were reintroduced to protoplasts of the corresponding deletion mutants. The complemented strains were confirmed *via* reverse transcription PCR with primers RTF/RTR (Table S1).

CsMPS1 are involved in stress adaption of *C. scovillei*, inhibition of mycelial growth was tested on PDA containing stress chemicals, including Congo red, sorbitol, and NaCl. Compared to the wild-type and each complemented strain, the mycelial growth of Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* was significantly inhibited by Congo red and sorbitol but not NaCl (Figure 3(A,B)), suggesting that *CsCMK1*, *CsMKK1*, and *CsMPS1* are involved in tolerance to CWI and osmotic stresses. These results are in accordance with several research previous reported [8,9,20], which highlights the conserved roles of CWI-related MAPKs in fungal growth and stress adaptions.

3.3. Role of CWI-related MAPKs in asexual production

Conidiation play important roles in the dissemination and propagation of plant fungal pathogens [22]. A microscopic visualization indicated that Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* produced fewer conidia compared to the wild-type (Figure 4(A)). A further quantitative measurement showed that the concentrations of conidial suspensions of Δ*Cscmk1* [$(7.0 \pm 1.5) \times 10^4$ mL^{-1}], Δ*Csmkk1* [$(9.0 \pm 1.3) \times 10^4$ mL^{-1}], and Δ*Csmps1* [$(9.0 \pm 1.4) \times 10^4$ mL^{-1}] were significantly lower than the wild-type [$(84.0 \pm 7.9) \times 10^4$ mL^{-1}] (Figure 4(B)). The conidiation defect was recovered in each complemented strain (Figure 4(B)). These results suggest that *CsCMK1*, *CsMKK2*, and *CsMPS1* are involved in conidiation of *C. scovillei*. Considering that generation of conidia from phialide of conidiophores requires cell wall remodeling [23,24], the significantly reduced conidiation in the Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* would result from their defects in cell wall integrity.

3.4. Role of CWI-related MAPKs in appressorium formation

The cell wall mediates signal transduction from the external environment to intracellular events and plays important roles in pre-infection developments

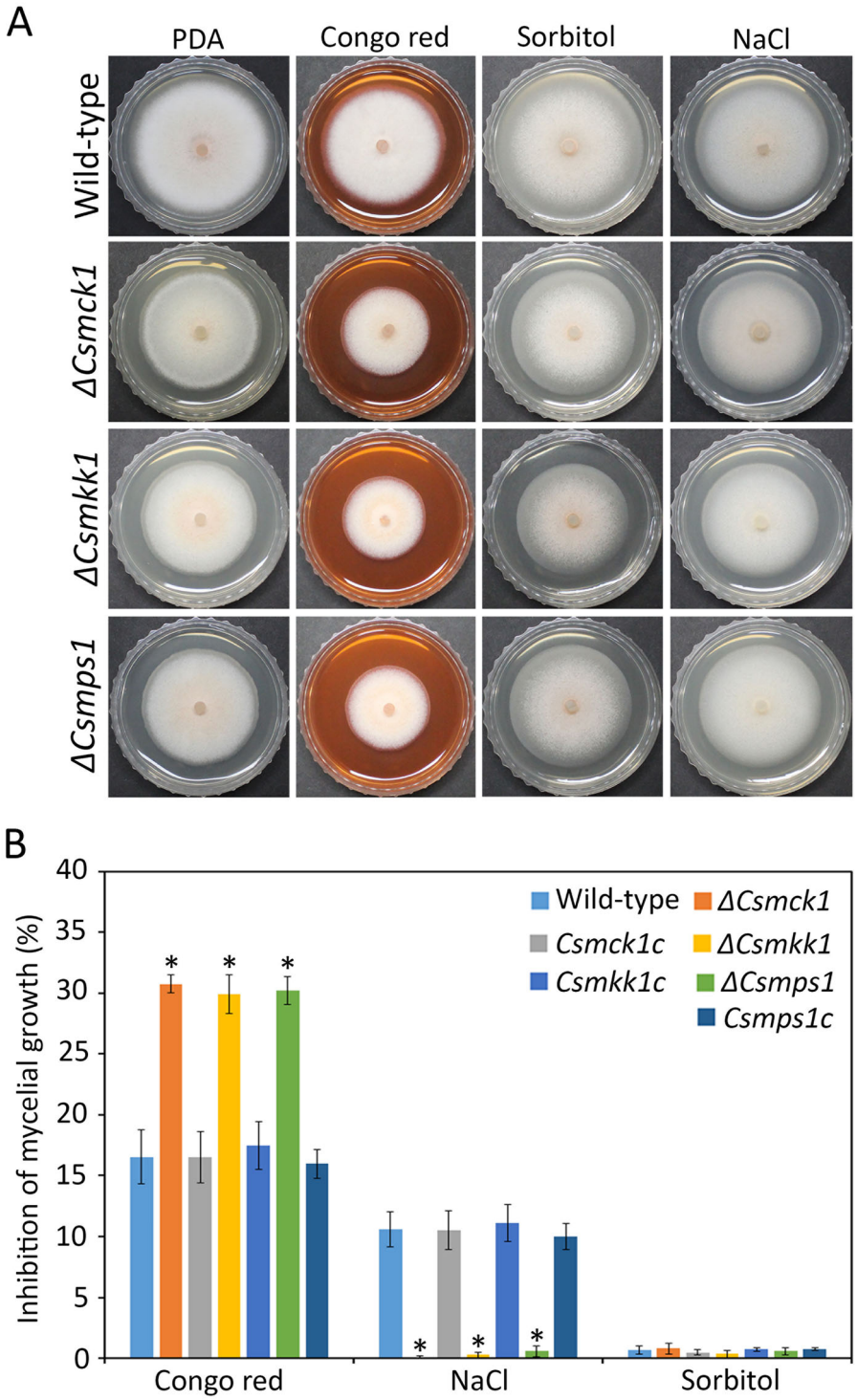

Figure 3. Stress adaptions of mycelia. Mycelial agar plugs were inoculated onto potato dextrose agar (PDA) with or without addition of chemical stressors [Congo red (300 ppm), NaCl (0.4 M), or sorbitol (1 M)] and incubated at 25 °C in the dark. (A) Visualization of colony growth. Photographs were taken after six days; (B) Quantitative measurement of colony growth. Colony diameters were measured after six days. An asterisk (*) indicates a significant difference (Duncan's test, $p < .05$).

[10,25,26]. Thus, the conidial germination and appressorium formation of Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* were examined. The results showed that conidial germination rates of Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* were normal compared to the wild-type. However, Δ*Cscmk1*, Δ*Csmkk1*, and Δ*Csmps1* were completely defective in appressorium formation (Figure 5), whereas the wild-type and complemented strains developed appressorium with > 90% formation rates. This result suggests that *CsCMK1*, *CsMKK1*, and *CsMPS1* are essential for appressorium formation of *C. scovillei*. Intriguingly, loss functions of each CWI-related MAPKs in *M. oryzae* did not abolish appressorium formation [9,10]. However, deletion of *CgMCK1*, *CgMKK1*, or *CgMPS1* in *Colletotrichum gloeosporioides* resulted

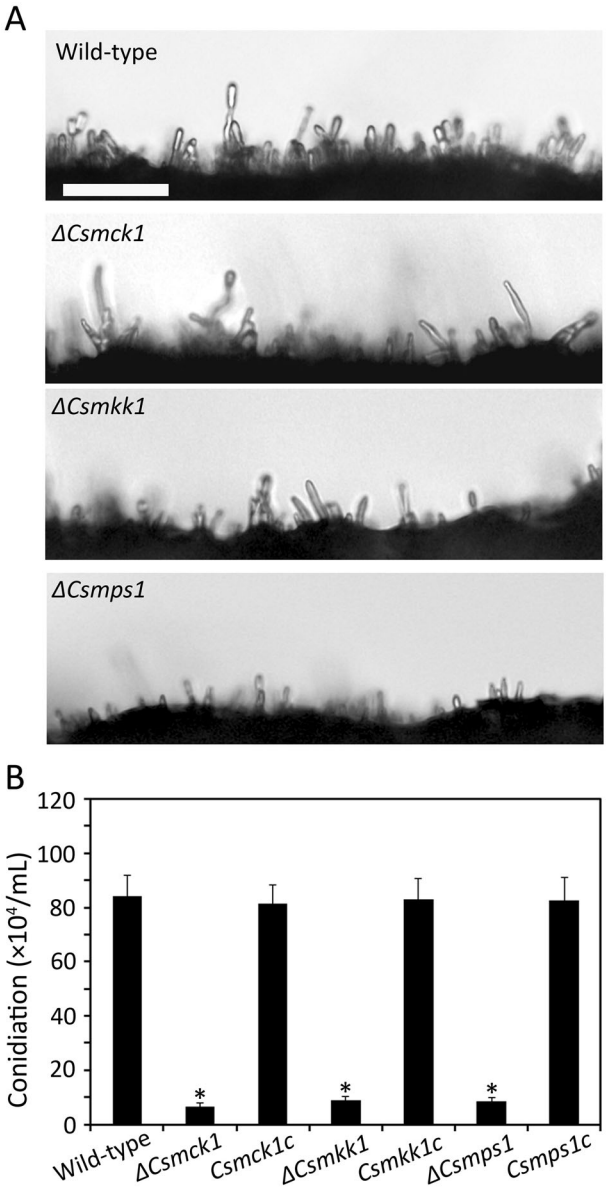

Figure 4. Evaluation of conidiation. (A) Visualization of conidiation. Mycelial agar plugs (5 mm diameter) were inoculated onto oatmeal agar (OMA) and incubated at 25 °C in the dark for three days. The obtained OMA mycelial agar plugs were placed on the hydrophilic surface of a glass slide and incubated in a humid plastic box at 25 °C in the light for 5 h. Scale bar = 50 μm; (B) Quantitative evaluation of conidiation. Three-day-old OMA mycelial agar plugs (5 mm diameter) were inoculated to potato dextrose agar (PDA) and incubated at 25 °C for five days in darkness followed by two days under light. Conidia were harvested with 5 mL of sterilized distilled water.

in a complete defect in appressorium development [20]. These data indicated that CWI-related MAPKs share different mechanisms in the regulation of appressorium formation among different fungal pathogens.

3.5. Role of CWI-related MAPKs in pathogenicity

To determine whether *CsCMK1*, *CsMKK1*, and *CsMPS1* are involved in pathogenicity, conidial

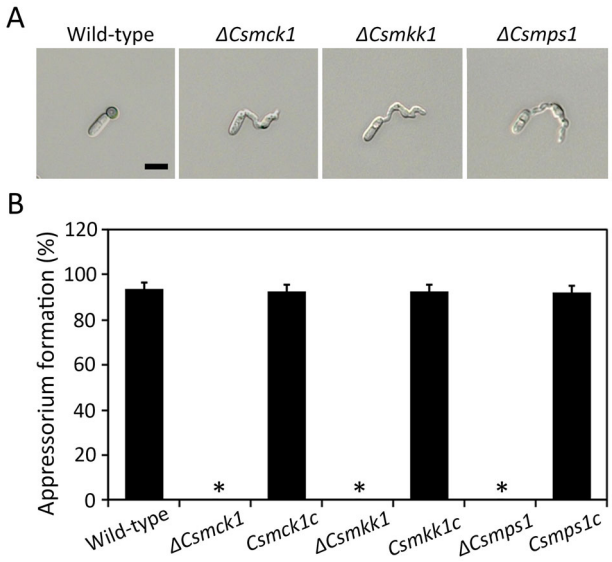

Figure 5. Appressorium formation. Conidia were harvested from seven-day old OMA with sterilized distilled water. The conidial suspensions (5×10^4 conidia mL^{-1}) were dropped onto the hydrophobic surface of coverslips and incubated in a humid plastic box at 25 °C. (A) Visualization of appressorium formation. Photographs were taken after 16 h. Scale bar = 10 μm; (B) Quantitative measurement of appressorium formation. An asterisk (*) indicates a significant difference (Duncan's test, $p < .05$).

suspensions were inoculated onto intact pepper fruits. After nine days, the wild-type and complemented strains caused typical anthracnose disease. However, Δ*Cscmk1*, Δ*Csmkk1* and Δ*Csmps1* failed to cause disease (Figure 6), suggesting that *CsCMK1*, *CsMKK1*, and *CsMPS1* are essential for pathogenicity of *C. scovillei*. To investigate whether *CsCMK1*, *CsMKK1*, and *CsMPS1* are associated with invasive hyphae growth, the conidial suspensions were further inoculated into wounded pepper fruits. After six days, Δ*Cscmk1* and Δ*Csmkk1* failed to cause anthracnose disease, while Δ*Csmps1* led to disease with reduced severity compared to the wild-type and complemented strains (Figure 6). This result suggests *CsMCK1*, *CsMKK1*, and *CsMPS1* are involved in invasive hyphae growth of *C. scovillei*.

Important roles of the CWI-related MAPKs on pathogenicity have been determined in several plant pathogenic fungi [8–10,20]. For example, in the *M. oryzae*, *MCK1*, *MKK2*, and *MPS1* are required for appressorium-mediated penetration [9,10]. In *C. gloeosporioides*, *CgMCK1*, *CgMKK1*, and *CgMPS1* are essential for appressorium formation and invasive hyphae growth [20]. Our data showed that the Δ*Cscmk1* and Δ*Csmkk1* were completely defective on both appressorium formation and invasive hyphae growth (Figures 5 and 6). Although the Δ*Csmps1* failed to form appressorium, it stilled form invasive hyphae, because the Δ*Csmps1* caused anthracnose disease with reduced severity on

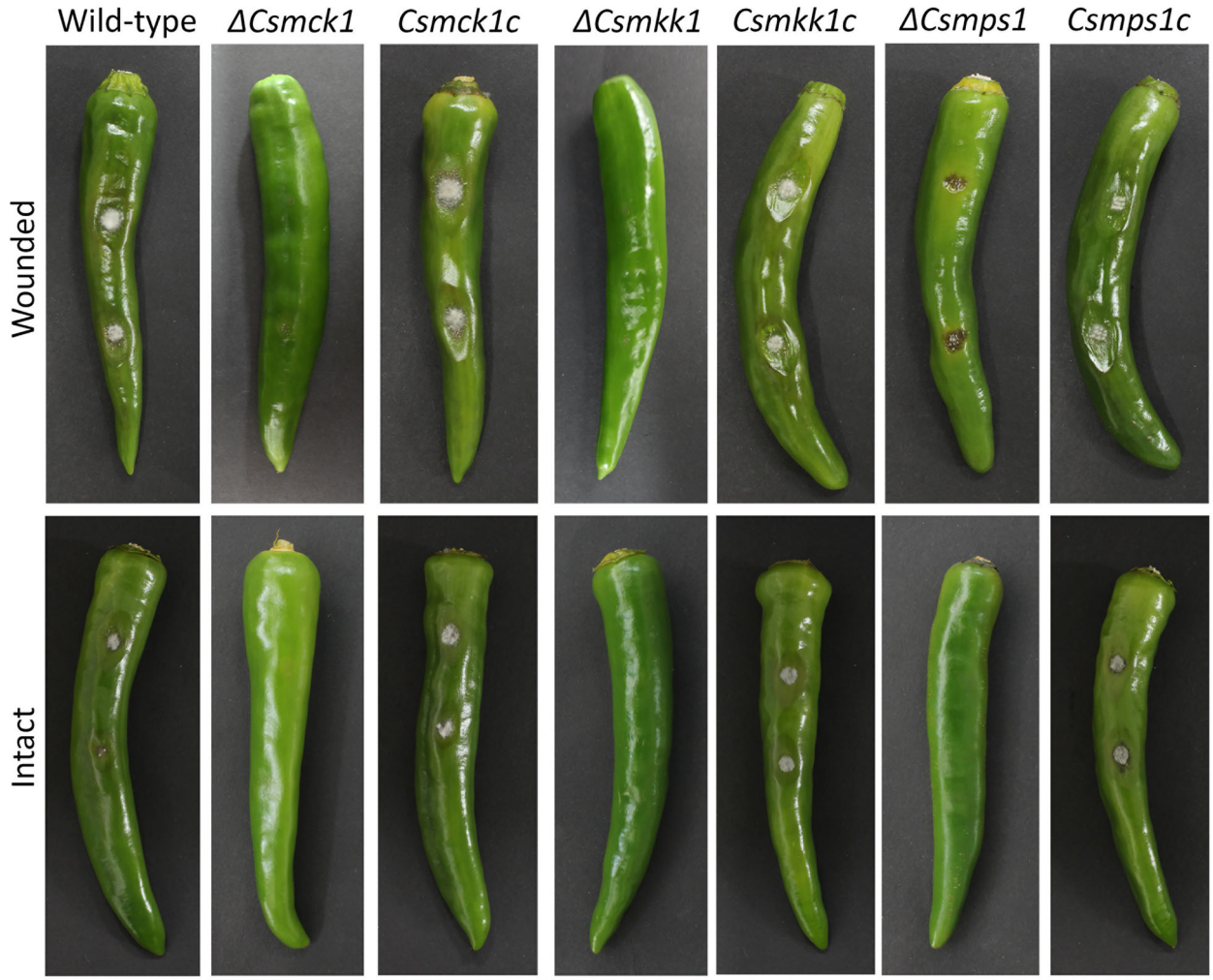

Figure 6. Pathogenicity assay. Drops (20 μL) of conidia suspensions (50×10^4 conidia mL^{-1}) were inoculated onto wounded and intact pepper fruits and incubated in a humid plastic box at 25 °C. Photographs of wounded and intact pepper fruits were taken after six and nine days, respectively.

wounded pepper fruit (Figure 6). Collectively, our results demonstrated that the CWI-related MAPKs, CsMCK1, CsMKK1, and CsMPS1, are important for growth, development, virulence, and stress adaption of the pepper fruit anthracnose fungus *C. scovillei*.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Kyoung Su Kim http://orcid.org/0000-0002-0827-0844

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