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RESEARCH ARTICLE

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The Autophagy Protein CsATG8 is Involved in Asexual Development and Virulence in the Pepper Anthracnose Fungus *Colletotrichum scovillei*

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ABSTRACT

Autophagy serves as a survival mechanism and plays important role in nutrient recycling under conditions of starvation, nutrient storage, ad differentiation of plant pathogenic fungi. However, autophagy-related genes have not been investigated in *Colletotrichum scovillei*, a causal agent of pepper fruit anthracnose disease. *ATG8* is involved in autophagosome formation and is considered a marker of autophagy. Therefore, we generated an *ATG8* deletion mutant, Δ*Csatg8*, via homologous recombination to determine the functional roles of *CsATG8* in the development and virulence of *C. scovillei*. Compared with the wild-type, the deletion mutant Δ*Csatg8* exhibited a severe reduction in conidiation. Conidia produced by Δ*Csatg8* were defective in survival, conidial germination, and appressorium formation. Moreover, conidia of Δ*Csatg8* showed reduced lipid amount and PTS1 selectivity. A virulence assay showed that anthracnose development on pepper fruits was reduced in Δ*Csatg8*. Taken together, our results suggest that *CsATG8* plays various roles in conidium production and associated development, and virulence in *C. scovillei*.

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1. Introduction

Pepper (*Capsicum annuum* L.) is one of the most economically important crops, with the production of dry and green peppers reaching approximately 42.2 million tons worldwide in 2019 [1,2]. Anthracnose disease, caused by fungal species from the *Colletotrichum* genus, is one of the most devastating fungal diseases in pepper [3,4]. Anthracnose disease on pepper fruit and leaves is characterized by sunken lesions and spots [5]. *Colletotrichum scovillei*, belonging to the *Colletotrichum acutatum* species complex, is the main fungal pathogen responsible for pepper yield losses in countries in subtropical and temperate zones [6].

The term autophagy encompasses a number of cellular phenomena [7], including macroautophagy (i.e., nonspecific engulfment of cytosolic components by double-membraned vesicles, which subsequently fuse with the vacuole/lysosome, where the contents are degraded) and pexophagy (i.e., degradation of targeted peroxisomes in the vacuole/lysosome) [8]. Autophagy is a nonselective degradation process that is conserved from yeast to humans [8,9]. Fungal autophagy is involved in the degradation and recycling of unnecessary organelles and long-lived proteins in eukaryotic cells [10–12].

The functional roles of autophagy genes have been well characterized in *Saccharomyces cerevisiae* [8,13–15]. Among the 41 autophagy genes in the genome of *S. cerevisiae*, 19 are essential for autophagosome formation [16]. Autophagy proteins have been classified into five major groups according to their functions: the Atg1 kinase complex, the Atg8 conjugation system, the Atg12 conjugation system, the phosphatidylinositol 3-phosphate kinase complex, and the Atg2–Atg18 complex and Atg9 [17]. Atg4 plays a role as a cysteine protease, and Atg5 is a putative lipase involved in the lysis of autophagic bodies [8,16]. Atg8, used as a marker of autophagy, is associated with the function of the autophagosome membrane [9]. The functional roles of Atg8 are associated with the growth, development, and virulence of several filamentous fungi [8,13,15,18]. For example, in *Aspergillus oryzae*, the deletion of *atg8* caused a rapid increase in hyphal vacuolation under nutrient-starved conditions [8]. In *Magnaporthe oryzae*, Δ*Moatg8* was completely defective in conidiation and virulence [18]. In *Colletotrichum orbiculare*, Δ*Coatg8* exhibited severe defects in conidial germination and appressorium development [13]. However, the functional roles of

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ATG8 have not been evaluated in the pepper fruit anthracnose pathogen, *C. scovillei*.

This study was performed to investigate the functional roles of *CsATG8* in the development and virulence of *C. scovillei* using a deletion mutant (Δ*Csatg8*). The results showed that Δ*Csatg8* was defective in conidiation, conidium survival, conidial germination, appressorium formation, virulence, and tolerance to oxidative stress. Our results suggest that *CsATG8* plays important roles in the growth, development, and virulence of *C. scovillei* in anthracnose disease.

2. Materials and methods

2.1. Fungal strains and culture conditions

The transformants were generated from *C. scovillei* wild-type strain KC05. The fungal strains were cultured on minimal medium agar (MMA; glucose 10 g/l, $NaNO_3$ 2 g/l, KH_2PO_4 1 g/l, $MgSO_47H_2O$ 0.5 g/l, agar 20 g/l, and Trace element solution 0.1 ml/l), oatmeal agar media (OMA; 50 g oatmeal and 25 g agar/l), and V8 media (V8 juice 80 ml/l, 10 N $NaOH_3$ 10 μl/l, agar 15 g/l) at 25 °C and for 5-day dark and next 2-day light. Mycelia for DNA and RNA extraction were grown in completed media broth (CM broth; 0.6% yeast extract, 0.6% casamino acids, and 1% sucrose per liter), and shaken at 150 rpm and 25 °C for 3 days.

2.2. Bioinformatics tools

The sequences of CsATG8 and its homologs were downloaded from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and Comparative Fungal Genomics Platform (CFGP, http://cfgp.riceblast.snu.ac.kr) [18]. Phylogenetic relationships among CsATG8 and other ubiquitin-like domains were analyzed using MEGA 7.0 software. The identities between CsATG8 and its homologs were analyzed using NCBI BLASTP (https://blast.ncbi.nlm.nih.gov/). Domain structures were predicted using InterPro Scan (http://www.ebi.ac.uk/interpro/) and visualized using Illustrator for Biological Sequences, version 1.0.3. Primers were designed using Primer Quest Design Tool (http://sg.idtdan.com/site).

2.3. Targeted gene deletion and complementation

Target gene deletion was performed using a double-joint PCR method with slight modification [19]. The upstream and downstream regions (~1.5 kb) were amplified with primers 5 F/5R and 3 F/3R (Table 1), respectively. The selection marker, the *HPH* cassette, was amplified using primers HPHF/HPHR (Table 1) from pBCATPH [20]. To generate the deletion constructs, those PCR products were fused and amplified using 5 F/3R and NF/NR, respectively (Table 1). The amplified deletion constructs were transformed into wild-type protoplasts using a previously reported method [21,22,23]. To generate complemented strain *Csatg8c*, a full-length sequence of *CsATG8* was co-transformed with geneticin resistant cassette into Δ*Csatg8* protoplasts.

2.4. Vector construction

The carboxyl-terminal amino acid sequence serine-lysine-leucine (SKL) was used as a PTS1 [24]. The *dsRED* gene with a PTS1 was amplified from the pDsRed-gen-36 using the primers DsRED_F and DsRED_SKL_R (Table 1). The pIGPAPA vector was linearized by PCR with the primers DsRED_VF/DsRED_VR. The amplified *dsRED*

Table 1. Primers are used in this study.

Primers	Sequence (5′→3′)
CsATG8 (CAP 002212)	
CsATG8 5 F	CATCGCCATCGCTCACAGG
CsATG8 5 R	TACATAGCAGACCGAAACGCGAAGGAGTAATCGGTCGTCAAG
CsATG8 3 F	GGCGAGGGTCAAGATATAGATGAGGTGGATGGGCGTTCTA
CsATG8 3 R	CCTAAGTCAAGAGCCACGAAA
CsATG8 NF	AGCAGATGCTGGGTCACTA
CsATG8 NR	ATAACCGACCTCAGGTTCCT
CsATG8 SF/cF	TTGAAGAACGGTCAGGTCAAG
CsATG8 SR/cR	TCTCATCCTCATTGCATCAGC
CsATG8 PF	GGCTGTTTGTTGTCTTCA
CsATG8 PR	TAAGTCAAGAGCCACGAA
CsATG8 RTF	GACCGTATTCCCGTTATCTGC
CsATG8 RTR	AGTCGCCAAAGGTGTTCT C
CsATG8 qRTF	GACCGTATTCCCGTTATC
CsATG8 qRTR	CGACGAAGATGAAGATGG
DsRED SKL F	ATGGCCTCCTCCGAGAACG
DsRED SKL R	TTATAACTTGGACAGGAACAGGTGGTGGCGG
DsRED VF	CTGTCCAAGTTATAAAGCGGCCGCCCGGCTGCAG
DsRED VR	CTCGGAGGAGGCCATGGTGGGCAGGTGTGGTATG
HPH F	GGCTTGGCTGGAGCTAGTGGAGG
HPH R	CTCCGGAGCTGACATCGACACCAAC

gene with a PTS1 was cloned into the linearized pIGPAPA using the overlap DNA Cloning Kit (Elpis Biotech, Daejeon, Korea).

2.5. Nucleic acid manipulation

Genomic DNA used in screening PCR was extracted through a quick and safe method [25]. To perform southern blotting, fungal genomic DNA was extracted using a standard method [26]. The genomic DNA isolated from wild-type and candidate mutants were digested with restriction enzyme *Nco*I, and then hybridized with a biotin-labeled DNA probe (about 500 bp) (Roche, Indianapolis, IN). Total RNA was extracted from frozen fungal tissues using the Easy-Spin Total RNA Extraction Kit (Intron Biotechnology, Seoul, South Korea), according to the manufacturer's protocol. First-strand cDNA was synthesized from 5 μg total RNA using the oligo (dT) primer with the SuperScript^{TM} III First-Strand Synthesis System Kit (Invitrogen^{TM} Life Technologies, CA). The *β–tubulin* (CAP_007327) gene was used as the control. The primer sets used for RT-PCR are listed in (Table 1). Real-Time quantitative PCR was performed on the StepOne Real-Time PCR System (Applied Biosystems, CA) using HIPI Real-Time PCR 2 × Master Mix (SYBR Green) (Elpis Biotech).

2.6. Phenotypic analysis

Conidiation was measured by counting the number of conidia harvested with 5 ml of distilled water using a hemocytometer. The conidial length was measured using an Axio Image A2 microscope (Carl Zeiss Microscope Division, Oberkochen, Germany) with ZEN imaging software. Conidial germination and appressorium formation were performed by dropping conidial suspensions (5×10^4 conidia/ml), prepared from filtering through 3 layers of miracloth (Calbiochem, San Diego, CA) and wash for 3 times, onto the hydrophobic surface of coverslips and incubating in a humid box at 25 °C. To evaluate mycelial growth under chemical stresses and starvation, mycelial agar plugs from MMA were inoculated onto CMA containing 20 and 30 mM H_2O_2, and incubated at 25 °C without light for 5 days. To evaluate conidium survival and lipid reservoir, the conidial suspension was stained by Phloxine B and Nile red (Sigma, St Louis, MO). Infection assays were performed by inoculating conidial suspension (20×10^4 conidia/ml) onto healthy intact, wounded pepper fruits and incubating them in a humid box at 25 °C for 5–10 days. For the appressorium penetration and invasive growth assays, conidial drops (5×10^4 conidia/ml) were placed on the surface of pepper fruits and incubated in a moistened box at 25 °C. All experiments were repeated 3 times with 3 replicates.

3. Results

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

Phylogenetic analysis showed that CsATG8 is closely related to its orthologues in fungi. NCBI BLASTP search showed that ATG8 is highly conserved in eukaryotic organisms, with high levels of identity to orthologues from *Colletotrichum gloeosporioides* (98.35%), *Neurospora crassa* (99.17%), *Penicillium rubens* (98.31%), *Botrytis cinerea* (96.58%), *Sclerotinia sclerotiorum* (97.44%), *Fusarium oxysporum* (100%), *Colletotrichum higginsianum* (98.35%), *Pyricularia oryzae* (94.07%), *Yarrowia lipolytica* (90.76%), *Candida albicans* (80.34%), *S. cerevisiae* (79.31%), and *Saccharomyces pombe* (86.21%) and lower levels of identity to the more distant *Arabidopsis thaliana* (80.17%) and *Homo sapiens* (59.48%) orthologues [27,28] (Figure S1(A,B)). Domain prediction showed that all ATG8 proteins contain a small ubiquitin-like domain (IPR029071) (Figure S1(B)). These results indicate that *CsATG8* homologs are highly conserved in fungi.

To investigate the roles of *CsATG8*, we generated a targeted gene deletion mutant by homologous recombination (Figure S2(A)). Δ*Csatg8* was confirmed by Southern blotting and RT-PCR (Figure S2(B,C)). To verify the phenotypes of Δ*Csatg8* caused by the deletion of *CsATG8*, we generated a complemented strain (*Csatg8c*), which was verified by RT-PCR (Figure S2(C)).

3.2. Roles of CsATG8 in conidiation, conidial morphology, reduced lipid amount, and PTS1 selectivity

Δ*Csatg8* showed normal mycelial growth rate but significantly reduced conidiation compared with the wild-type and *Csatg8c* (Figure 1(A,B) and Figure S3), suggesting that *CsATG8* is involved in the conidiation of *C. scovillei*. A vacuole-like structure was observed in 29 ± 3.6% of conidia in Δ*Csatg8*, compared with 5.3 ± 2.5% and 6.3 ± 1.5% of those in the wild-type and *Csatg8c*, respectively (Figure 2(A,B)). It has been reported that peroxisomal matrix proteins are imported into the peroxisome via the PTS1 pathway [24]. Therefore, we expressed the dsRED-SKL fusion protein in wild-type, Δ*Csatg8*, and *Csatg8c*. The cytoplasmic localized RFP signal was detected in 24 ± 3.5% of conidia in Δ*Csatg8* compared with 2.6 ± 1.1% and 6.3 ± 1.5% of those in the wild-type and *Csatg8c*, respectively (Figure 2(C)). Because Lipids droplets are a source of energy in conidia and involved in conidium fitness and germination [29,30], we stained lipid droplets with Nile red and found an accumulation of lipid droplets in

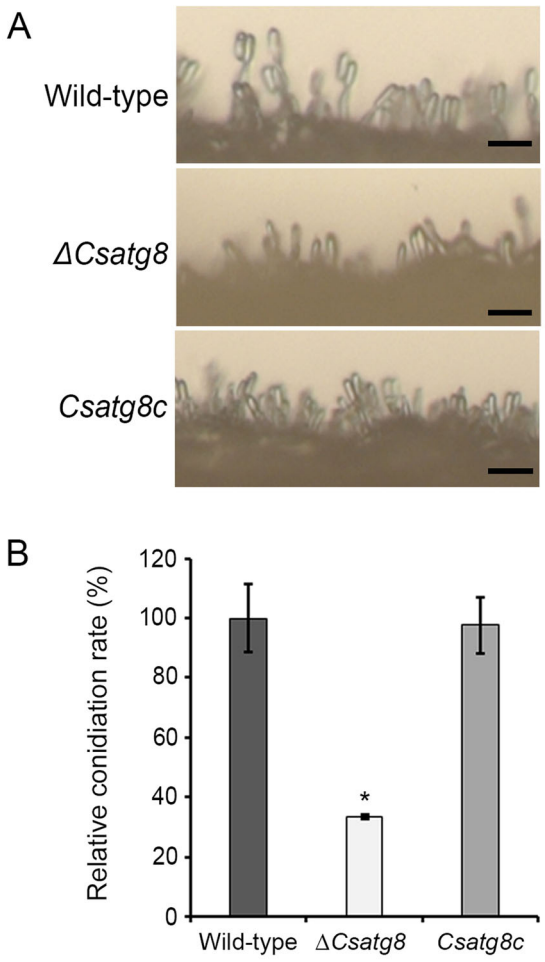

Figure 1. Measurement of conidiation. (A) Observation of conidia developed from conidiophores. Photographs were taken after incubation of 3-day-old mycelial agar plugs in a humid box for 7 h with lightl; (B) Quantitative evaluation of conidiation. The indicated strains were grown on V8 medium for 5 days in the dark and 2 days in the light. $^{*}p < 0.05$ (Tukey's test). Scale bar = 20 μm.

10.5 ± 2.6% of conidia in Δ*Csatg8*, compared with 97.7 ± 1.5% of those in the wild-type (Figure 2(D,E)). This result suggests that *CsATG8* is involved in a proper lipid reservoir. We further examined conidium survival in Δ*Csatg8* by staining with phloxine B [31–34], and the results showed that the proportion of dead conidia was significantly greater in Δ*Csatg8* (20.3 ± 4.5%) than in the wild-type (3 ± 0.5%) and *Csatg8c* (3.6 ± 1.5%) (Figure 3(A,B)). This result suggests that *CsATG8* is involved in conidial survival in *C. scovillei*. Taken together, these results suggest that *CsATG8* is involved in the conidiation, conidium viability, and autophagy-related processes in *C. scovillei*.

3.3. Role of CsATG8 in appressorium formation

Appressorium development is a prerequisite for anthracnose disease [2]. To investigate the role of *CsATG8* in pre-infection, we evaluated the time course of conidial germination and appressorium formation on the hydrophobic surface of coverslips. After 6 h, the germination rate of the wild-type was approximately 72 ± 2.5%, whereas the rate of germ tube formation was only approximately 35 ± 2.0% in Δ*Csatg8*. The defect in conidial germination was recovered in *Csatg8c*. In comparison with the wild-type and *Csatg8c* strains, most conidia of Δ*Csatg8* failed to differentiate into appressoria at 16 h (Figure 4(A,B)). These results suggest that *CsATG8* is involved in conidial germination and appressorium formation in *C. scovillei*.

3.4. Roles of CsATG8 in anthracnose disease formation

To determine the role of *CsATG8* in anthracnose development, we inoculated conidial suspensions onto intact pepper fruits. Δ*Csatg8* was greatly reduced in causing anthracnose disease, whereas both the wild-type and *Csatg8c* caused severe anthracnose disease after 10 days (Figure 5(A)). These observations suggest that *CsATG8* plays an important role in the virulence of *C. scovillei*. We inoculated conidial suspensions onto wounded pepper fruits. After 5 days, Δ*Csatg8* caused a similar level of anthracnose disease as did those of wild-type and *Csatg8c* (Figure 5(B)). This observation reveals that the reduced virulence of Δ*Csatg8* did not result from a defect of invasive hyphae growth. These results suggest that *CsATG8* is important for virulence through the regulation of appressorium-mediated penetration in *C. scovillei*.

3.5. Roles of CsATG8 in tolerance to stress conditions

To determine whether *CsATG8* is involved in tolerance to stress conditions, we evaluated mycelial growth on CMA amended with oxidative stresses (Figure 6(A,B)). Among those stresses, mycelial growth was significantly inhibited by H_2O_2 in Δ*Csatg8* (by 56.6 ± 3.0%) compared with the wild-type (by 44.0 ± 2.0%) and *Csatg8c* (by 42.0 ± 2.0%) (Figure 6(A,B)). This result suggests that *CsATG8* is involved in the tolerance to oxidative stress of *C. scovillei*.

4. Discussion

This study was performed to functionally characterize *ATG8*, one of 23 autophagy-related genes in *C. scovillei*, because ATG8 is a major autophagy protein involved in the development and virulence of plant pathogenic fungi [8,13,15,18].

The function of ATG8 is related to conidiation in several phytopathogenic fungi [13,14,18,35]. In

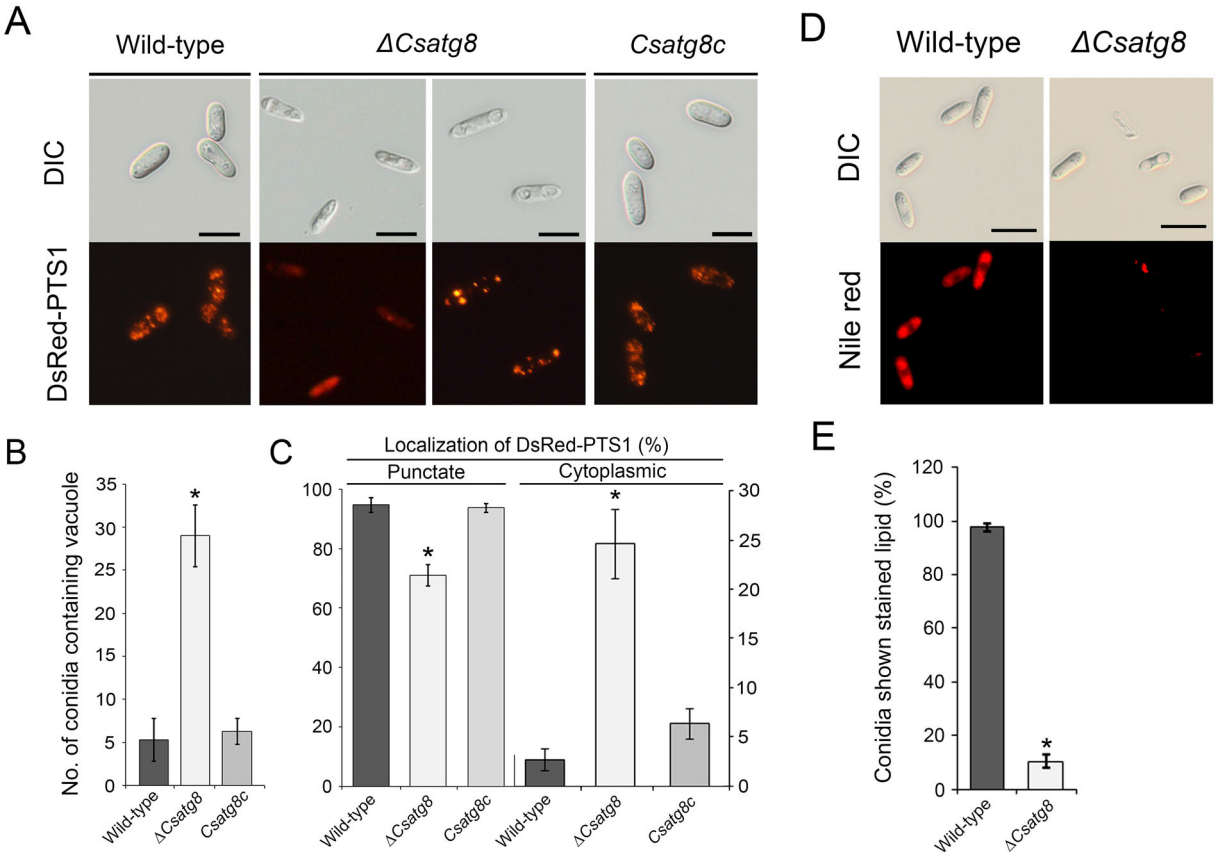

Figure 2. Detection of intracellular vacuoles and lipids. (A) Observation of vacuole-like structures and dsRED:PTS1 localization. Conidia were harvested in MMA medium for 5 days in the dark and 2 days in the light; (B, C) Quantitative examination of vacuole-like structures and the dsRED:PTS1 fusion protein. At least 100 conidia were examined per replicate. $^*p < 0.05$ (Tukey's test); (D, E) Measurement of lipid droplets; (D) Observation of lipid localization. The conidia were stained with Nile red and incubated for 1 h in the dark; (E) Quantitative measurement of lipid droplets. At least 100 conidia were examined per replicate. $^*p < 0.05$ (Tukey's test). Scale bar = 10 μm.

M. oryzae, the deletion of *ATG8* significantly reduced conidiation and conidiophore development [18]. Our results showed that conidiation was significantly reduced in Δ*Csatg8* (Figure 1(A,B)), suggesting that *CsATG8* is involved in conidiation. Interestingly, Δ*Csatg8* produced morphologically abnormal conidia, which were smaller than those of the wild-type (Figure 2(A)). Moreover, the phloxine B assay showed that Δ*Csatg8* produced dead conidia (Figure 3(A)). Defects in the viability of Δ*Csatg8* conidia may reveal a novel function of *ATG8* in plant pathogenic fungi.

ATG8 plays an important role in infection-related morphogenesis [18]. *ATG8* deletion mutants were reported to be defective in conidial germination and appressorium formation in *M. oryzae* and *C. orbiculare* [13,18]. In the present study, conidial germination was significantly lower in Δ*Csatg8* (35 ± 2.0%) than in the wild-type (72 ± 2.5%) after 6 h of incubation. In the conidial viability assay, Δ*Csatg8* showed a conidial survival rate of 79.7 ± 4.5% (Figure 3(B)), indicating that reduced conidial germination was not only due to low conidial viability. Furthermore, Δ*Csatg8* showed reduced appressorium formation (Figure 4(A,B)). These results suggested that *CsATG8* is involved in conidial germination and appressorium formation in *C. scovillei*.

Pexophagy, a type of selective autophagy, is involved in degrading lipids in peroxisomes [12,36]. Nile red staining assay showed that Δ*Csatg8* accumulated fewer lipid droplets than did the wild-type (Figure 2(D,E)), suggesting that *CsATG8* may be involved in the lipid reservoir. Peroxisomal membrane proteins are targeted to the peroxisome membrane by two distinct peroxisomal targeting signals, PTS1 and PTS2 [37,38]. Accompanied by the formation of autophagosomes, peroxisomes are degraded inside vacuoles [13]. RFP:PTS1 signals in Δ*Csatg8* exhibited cytoplasmic localization, whereas the wild-type control showed a punctate localization pattern [36]. CsATG8 may be involved in the formation of intracellular organelles and peroxisomes.

The Δ*Csatg8* showed severely reduced anthracnose disease formation on pepper fruits (Figure 5(A,B)). Previous studies demonstrated that *ATG8* deletion mutants were defective in appressorium-mediated penetration [13,18]. Consistently, Δ*Csatg8* was significantly reduced in penetration ability to

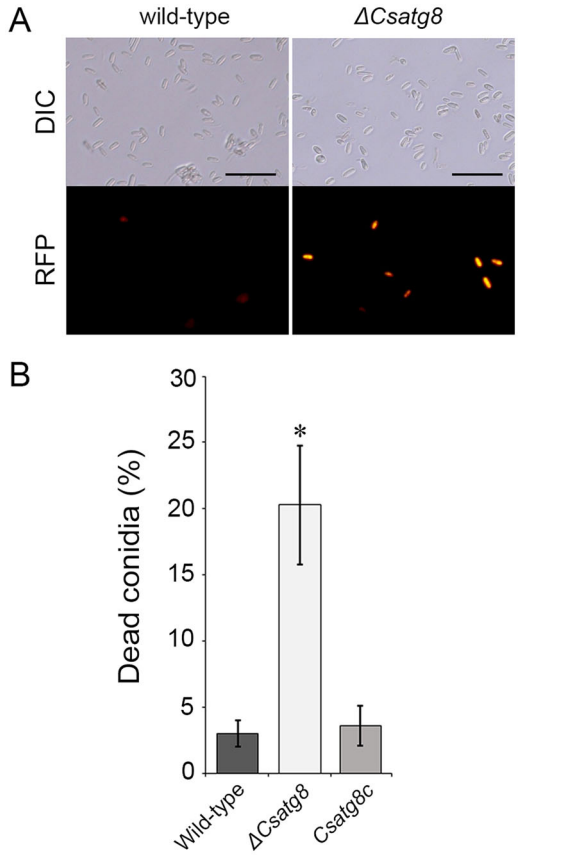

Figure 3. Conidial viability assay. (A) Observation of conidial survival. Conidia harvested from 10-day-old MMA plugs were stained with phloxine B. Dead conidia were stained red. Scale bar = 20 μm; (B) Quantitative evaluation of conidial survival. At least 100 conidia were examined per replicate. $^*p < 0.05$ (Tukey's test).

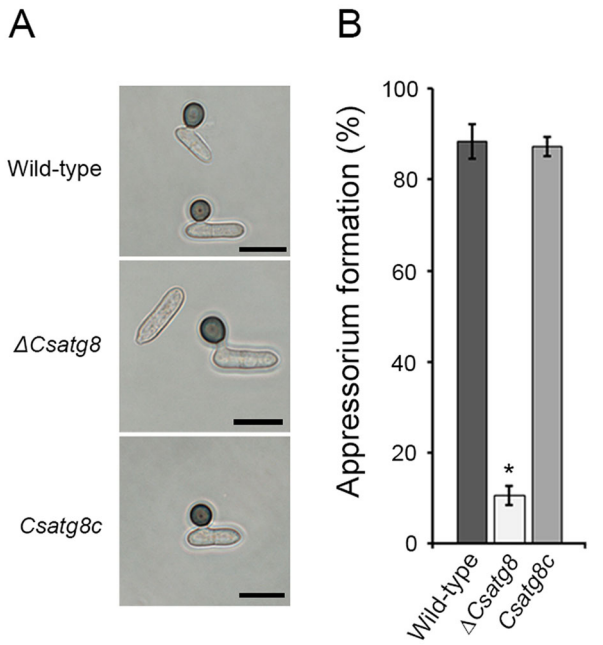

Figure 4. Appressorium formation assay. (A) Photographs of appressorium formation. Conidial suspensions (5×10^4/ml) were placed on the hydrophobic surface of coverslips and incubated in a humid box at 25 °C for 16 h. Scale bar = 10 μm; (B) Quantitative measurement of appressorium formation. At least 100 conidia were examined per replicate. $^*p < 0.05$ (Tukey's test).

Figure 5. Virulence assays. Conidial suspensions (20×10^4/ml) were dropped onto intact and wounded pepper fruits and incubated in a humid plastic box at 25 °C for 14 days.

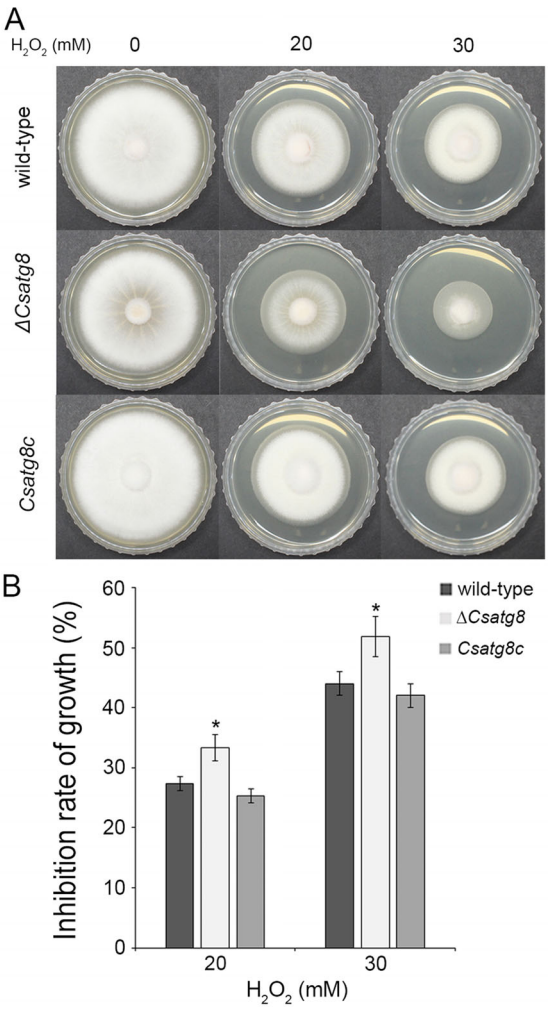

Figure 6. Mycelial growth under conditions of oxidative stress. Mycelial agar plugs were inoculated onto CMA or CMA containing 20 or 30 mM H_2O_2 and then incubated at 25 °C in the dark for 5 days. $^*p < 0.05$ (Tukey's test).

that of the wild-type and *Csatg8c* in the present study (Figure 5(A,B)), suggesting that *CsATG8* is involved in appressorium-mediated penetration. Δ*Csatg8* caused anthracnose disease with similar lesion size on wounded pepper fruits compared to the wild-type. The anthracnose lesions caused by Δ*Csatg8* were black in color, which may be caused by increased pigmentation in Δ*Csatg8*, as mycelia of Δ*Csatg8* were more pigmentated compared to that of wild-type (Figure S3). Therefore, the marked reduction in anthracnose lesions in Δ*Csatg8* should be due to a defect in appressorium-mediated penetration.

Taken together, our results showed that Δ*Csatg8* is defective in conidiation, appressorium formation, virulence, lipid reservoir, PTS1 selection, and tolerance to oxidative stress in *C. scovillei*. Our results suggest that *CsATG8* plays important roles in the development and virulence of *C. scovillei* in anthracnose disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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