H_2O_2 IS INVOLVED IN cAMP-INDUCED INHIBITION OF SCLEROTIA INITIATION AND MATURATION IN THE SUNFLOWER PATHOGEN *SCLEROTINIA SCLEROTIORUM*

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SUMMARY

Sclerotinia sclerotiorum is a destructive pathogen and has a wide range of hosts, including sunflower. The fungus produces sclerotia, which is a dormant structure and plays a vital role in the infection cycle of *S. sclerotiorum*. The initiation and maturation of sclerotia can be influenced by many factors. In this study, *S. sclerotiorum* isolate X-8 was collected from a diseased sunflower plant, and used to determine the role of H_2O_2 and cyclic adenosine 3', 5'-monophosphate (cAMP) in the sclerotia initiation and maturation. Results showed that the transition of *S. sclerotiorum* from vegetative growth to the initial stage of sclerotia is accompanied by a decrease of H_2O_2 and exogenously supplying H_2O_2 or diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase, in the culture medium promoted or inhibited the initiation of sclerotia, respectively. In addition, the cAMP-induced inhibition of sclerotia initiation and maturation were also accompanied by significantly decrease of H_2O_2. This was achieved by down-regulating the transcripts of *Nox1* and *Nox2* genes, the isoform of NADPH oxidase. Additionally, a decreased enzymatic activity of both superoxide dismutase (SOD) and catalase (CAT) were also detected in *S. sclerotiorum* after cAMP treatment. We concluded that H_2O_2 not only regulates the formation of sclerotia, but also cross-talks with cAMP in regulating the inhibition of sclerotial initiation and maturation.

Keywords: cAMP, sclerotia, H_2O_2, *Nox* genes.

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INTRODUCTION

Sclerotinia sclerotiorum causes destructive diseases in over 400 species of host such as rapeseed, soybean and sunflower (Boland and Hall, 1994; Li *et al*., 2005; Saharan and Mehta, 2008). The symptoms of sunflower White Mold included flower disc rot, leaf rot and stem rot. Severe disease resulted in significant yield losses and reduced seed quality of sunflower in France, United States and China (Gulya *et al*., 1989; Masirevic and Gulya, 1992; Thomas *et al*., 2004 ; Li, 2004; Harveson, 2011).

The pathogen *S. sclerotiorum* produces sclerotia at the end of its infection cycle. The sclerotium is a compact body of aggregated vegetative mycelia, which serves as a dormant structure that can survive in soil for more than eight years, and is the main source of primary inoculum (Adams and Ayers, 1979). The formation of sclerotia is divided into four developmental stages: the vegetative mycelium (VM), sclerotial initiation (SI), sclerotial development (SD) and sclerotial maturation (SM) (Papapostolou and Georgiou, 2010). Numerous factors affect the formation of sclerotia. Rai and his colleagues found that the optimal pH for sclerotia formation is between 4 and 4.5. Oxalate, secreted by *S. sclerotiorum,* could decrease the pH level in mycelium cells, thus promoting sclerotial initiation (Rai and Agnihotri, 1971). Additionally, H_2O_2 was firstly reported to regulate the sclerotia formation in 1975 (Chet and Henis, 1975). The accumulation of lipid peroxides in the SI stage of *Sclerotium rolfsii* could favor the formation of sclerotia (Georgiou, 1997). Applying a hydroxyl radical scavenger in the culture medium inhibited the sclerotia formation of *S. sclerotiorum* (Georgiou *et al*., 2000), and the accumulation of thiols such as glutathione and cysteine, which are involved in redox reactions, are also associated with sclerotia formation (Patsoukis and Georgiou, 2008). Exogenously applied salicylhydroxamic acid, an inhibitor of alternative oxidase, into the culture medium significantly decreased the rate of sclerotia formation (Xu *et al*., 2013). Reactive oxygen species (ROS) are also reported to play an essential role in the virulence in *S. sclerotiorum* (Kim *et al*., 2011).

NADPH oxidases are widely distributed eukaryotic proteins that transfer electrons across biological membranes to catalyze the reduction of molecular oxygen to superoxide (Sumimoto, 2008). BcNoxA and BcNoxB, the

homologs of NADPH oxidases in *Botrytis cinerea*, are both required for sclerotia formation and fungal pathogenicity, and double mutants show additive effects on these processes (Takemoto *et al*., 2011). In contrast, *Nox-1* and *Nox-2* genes in *Neurospora crassa* do not seem to play redundant functions in its development. *Nox-1* elimination results in female sterility, inhibition of asexual development and reduction of hyphal growth. However, a *Nox-2* deficiency did not affect any of these processes, but led to the failure of sexual spore germination, even in the presence of exogenous oxidants (Cano-Domínguez *et al*., 2008). Silencing of Ss*Nox*1 and Ss*Nox*2 not only affects the virulence of *S. sclerotiorum,* but also limits sclerotial development, suggesting that NADPH oxidases regulate the pathogenicity of *S. sclerotiorum* via ROS molecules (Kim, 2014).

Cyclic Adenosine 3', 5'-Monophosphate (cAMP) is an important second messenger molecule. It regulates a variety of physiological mechanisms (Rollins and Dickman, 1998). Increasing endogenous cAMP levels by inhibiting phosphodiesterase activity (with caffeine and 3-isobutyl-1-methyl xanthine) or by activating adenylate cyclase (with NaF) reduced or eliminated sclerotia development in *S. sclerotiorum;* and exogenously supplying cAMP in the culture medium inhibited the formation of sclerotia, indicating that cAMP plays a negative role in the early transition between mycelial growth and sclerotia development (Rollins and Dickman, 1998). On the other hand, the mycelial growth and sclerotia formation were abnormal in a mutant of *S. sclerotiorum* lacking adenylate cyclase, the enzyme converting ATP into cAMP (Jurick and Rollins, 2007). The activity of protein kinase A (PKA), one of the downstream components of the cAMP signaling pathway, was found to be significantly increased in the SI stage of sclerotia formation. Mitogen-activated protein kinases (MAPKs), acting downstream of PKA, were also found to be involved in sclerotia formation in the same fungus compared to other stages (Jurick and Rollins, 2007).

In spite of the above-mentioned evidences on the involvement of cAMP and H_2O_2 molecules in the formation of sclerotia, it is unknown whether these signaling molecules cross-talk in the process of cAMP-induced inhibition sclerotia initiation and maturation. In this study, we addressed two questions, 1) whether H_2O_2 is one of key determinants in the transition from VM to SI stage and 2) whether there is cross-talk between cAMP and H_2O_2 in cAMP-induced inhibition of sclerotia initiation and maturation.

METERIALS AND METHODS

Chemicals. Adenosine 3',5'-cyclic monophosphate (cAMP) and diphenyleneiodonium chloride (DPI) were purchased from Sigma (Cat No. A9501-1G and D2926-10MG); 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Invitrogen (Cat No. D399). Titanium sulfate and 30% H_2O_2 were purchased from Guoyao Chemical Reagent Limited Corporation (Beijing, China); other chemical reagents were purchased from Tianjin Yongda Chemical Reagent Limited Corporation (Tianjin, China).

Fungal isolate, culture conditions, and classification of developmental stages of sclerotia. *S. sclerotiorum* isolate X-8 was isolated from a diseased stem of sunflower, which was collected at Wuyuan, Bayannur city, Inner Mongolia, China. The pure culture of X-8 was identified as *S. sclerotiorum* through PCR with primers ITS1 (CTTGGTCATTTAGACGAAGTAA) and ITS4 (GCATATCAATAAGCGGAGGA) (White *et al*., 1990) and comparing the ITS sequence through blast with sequences in the NCBI database.

Cultures of X-8 were transferred on potato dextrose agar (PDA) and incubated for two to three days. PDA plugs (0.5 cm diameter) were cut from the edge of actively growing colonies and transferred to a PDA plate containing either 0.25 mM H_2O_2, 10 mM cAMP or 1 mM DPI. The plates were incubated at 24°C. The mycelium growth and sclerotia formation rate were monitored over time.

The developmental stages of *S. sclerotiorum* were classified based on the criteria described by Patsoukis and Georgiou (2008). The vegetative mycelium stage (VM) is characterized by rapidly proliferating hyphae; the sclerotial differentiation stage consists of the initiation substage (SI), characterized by the appearance of small distinct sclerotial initials formed from rapidly proliferating interwoven hyphae; the sclerotial development substage (SD), characterized by the enlargment of the sclerotia; the maturation substage (SM), characterized by sclerotial surface delimitation, internal consolidation, and melanin pigmentation, often associated with droplet secretion on the surface of sclerotia.

H_2O_2 detection assay. The H_2O_2 level was determined spectrophotometrically in fungal extracts by comparing the absorbance values obtained as in Li (2000) to a standard curve:

$$H_2O_2\ (\mu mol/g) = C \times Vt/(FW \times V1)$$

Where C is the H_2O_2 value from standard curve (μmol); Vt is the volume of total sample extract (ml); V1is the volume of assayed sample (ml) and FW is the fresh weight of sample (g).

To determine the concentration of H_2O_2, isolate X-8 was sub-cultured on PDA plates covered with cellophane (Haiwang, 22 g). Mycelial samples (0.1 g) were scraped off the cellophane after 60 or 84 h and transferred into 1.5 ml centrifuge tubes. One ml pre-cooled acetone was added to the samples, which were then ground using quartz sand. After centrifugation at 5000 *g* for 10 min, the supernatant was used as the crude extract to measure the concentration of H_2O_2. 5% of titanium sulfate and concentrated ammonia were added to 1 ml of crude extract to form a

precipitate. After centrifugation, 5 ml of sulfuric acid (2 mol/l) was added to the pellet. The dissolved samples were diluted to 10 ml with distilled water, and assayed by the colorimetric method described above. The absorbance values were converted to H_2O_2 concentration values (μmol/g) using the standard curve. Three technical replicates were set for each sample and the experiment was performed three times.

To plot a standard H_2O_2 curve, 30% standard H_2O_2 (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μM) was reacted with 5% titanium sulfate (w/v) in reaction buffer that contained ammonia and acetone, then solved with 2 mol/l sulphuric acid. A regression equation was obtained ($Y = 0.8144X + 0.025$, $R^2 = 0.9953$) by using values collected at 415 nm and used as standard curve.

Catalase (CAT) and superoxide dismutase (SOD) activity assays. Isolate X-8 was sub-cultured onto PDA covered with cellophane. 0.1 g of mycelial sample was scraped off plates after 60 or 84 h of culture, then ground with a mortar and a pestle in sodium phosphate buffer (0.05 M, pH = 7.8) with quartz sand. After centrifugation at 4°C , 5000 *g* for 10 min, the supernatant was collected as crude extract for later enzymatic bioassays. SOD activity was measured spectrophotometrically in the supernatant based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Li, 2000). An aliquot of 0.1 ml crude extract was added into the reaction solution and kept at 25°C in an incubator with light intensity of 4500 Lux. The reaction solution contained 0.2 M phosphate buffer (pH = 7.8), 0.026 M methionine, 7.5×10^{-4} M NBT, 100 μM EDTA-Na_2 and 20 μM riboflavin. Fifteen minutes later, the reaction was stopped by quickly transferring the reaction tube into the dark. The absorbance of the reaction solution was measured spectrophotometrically at 560 nm. One unit of enzymatic activity corresponded to 50% inhibition of NBT reducibility by light.

CAT was assayed according to the protocol described by Li (2000). 0.1 g of mycelial sample was scraped off from PDA plates after 60 or 84 h of culture. The crude extract (0.1 ml, obtained as mentioned above) was added into the reaction mixture [0.2 M phosphate buffer (pH = 7.8), 45.9 μM EDTA-Na_2 and 5.4 mM H_2O_2] and incubated at 25°C. CAT activity was measured by detecting the decomposition of H_2O_2 at 240 nm. One unit of enzymatic activity was arbitrarily set to correspond to a drop of 0.01 absorbance units per minute. Three technique repeats were set for each sample and the experiment was performed three times.

Transcript quantification of *Nox 1* and *Nox2* genes by RT-qPCR. Mycelium (0.1 g) was ground in liquid nitrogen in 1.5 ml centrifuge tubes and mixed with 1 ml of RNAiso Reagent (Bioflux Cat No.BSC51M1). To separate the aqueous phase, 200 μl of chloroform were added and the mixture was centrifuged at 4°C, 10000 *g*, for 15 min. An equal volume of isopropanol was added to the supernatant for RNA precipitation. The precipitate was washed twice with 75% ethanol. After air drying, the pellet was dissolved with DEPC-treated water. The quality of RNA was examined on a 1.2% agarose gel. The cDNA for quantitative reverse-transcriptase PCR (RT-qPCR) analysis was generated by using a commercial kit (BIORED, Cat No.BSB09M1) according to the manufacturer's instructions.

RT-qPCR was performed using a CFX Connect™ Real time System (Bio-Rad) in combination with SYBR Select Master Mix (Life technologies, Carlsbad, USA). The primer pairs Nox1 (forward: GGTTCCAAGTACTCCTCTCA; reverse: CGACATCGGCTCCTACAC) and Nox2 (forward: CACCTTACTGTGTTAGTGTTG; reverse: CCTCGGGACAACAAAAGAAA) were used to amplify the transcript of *Nox1* (165 bp) and *Nox2* (225 bp). Primers EF-1α (forward: CAAGCAAGATCCACCTAAGG; reverse: GCAAGCAATGTGAGCAGTGT) were used to amplify *EF-1α* gene as an internal control (Kim *et al*., 2011).

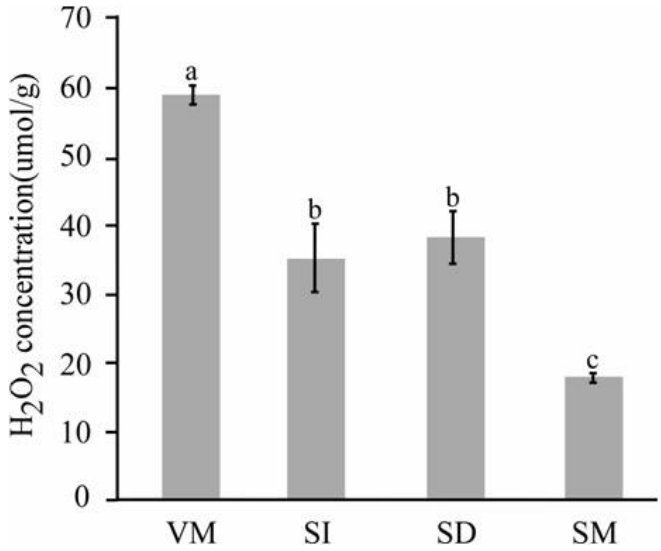

Fig. 1. Endogenous level of H_2O_2 at different developmental stages of sclerotia. VM: vegetative mycelium, SI: sclerotia initial stage, SD: sclerotia development stage, SM: sclerotia maturation stage. The experiments were repeated three times with similar results. Significant differences are indicated with different letters (one-sided Student's T- test, $P \leq 0.05$). Error Bars are from three replicates.

RESULTS

Variation of H_2O_2 levels at different stages of sclerotial formation. To determine whether H_2O_2 was one of the signal molecules involved in the transition from vegetative growth to the initiation of sclerotia, H_2O_2 concentration was measured at different stages of sclerotia development. The concentration of H_2O_2 was 58.55 μmol/g at the VM stage, but dropped to 34.92 μmol/g at the SI stage (Fig. 1). The H_2O_2 value in the SD stage remained at the same level

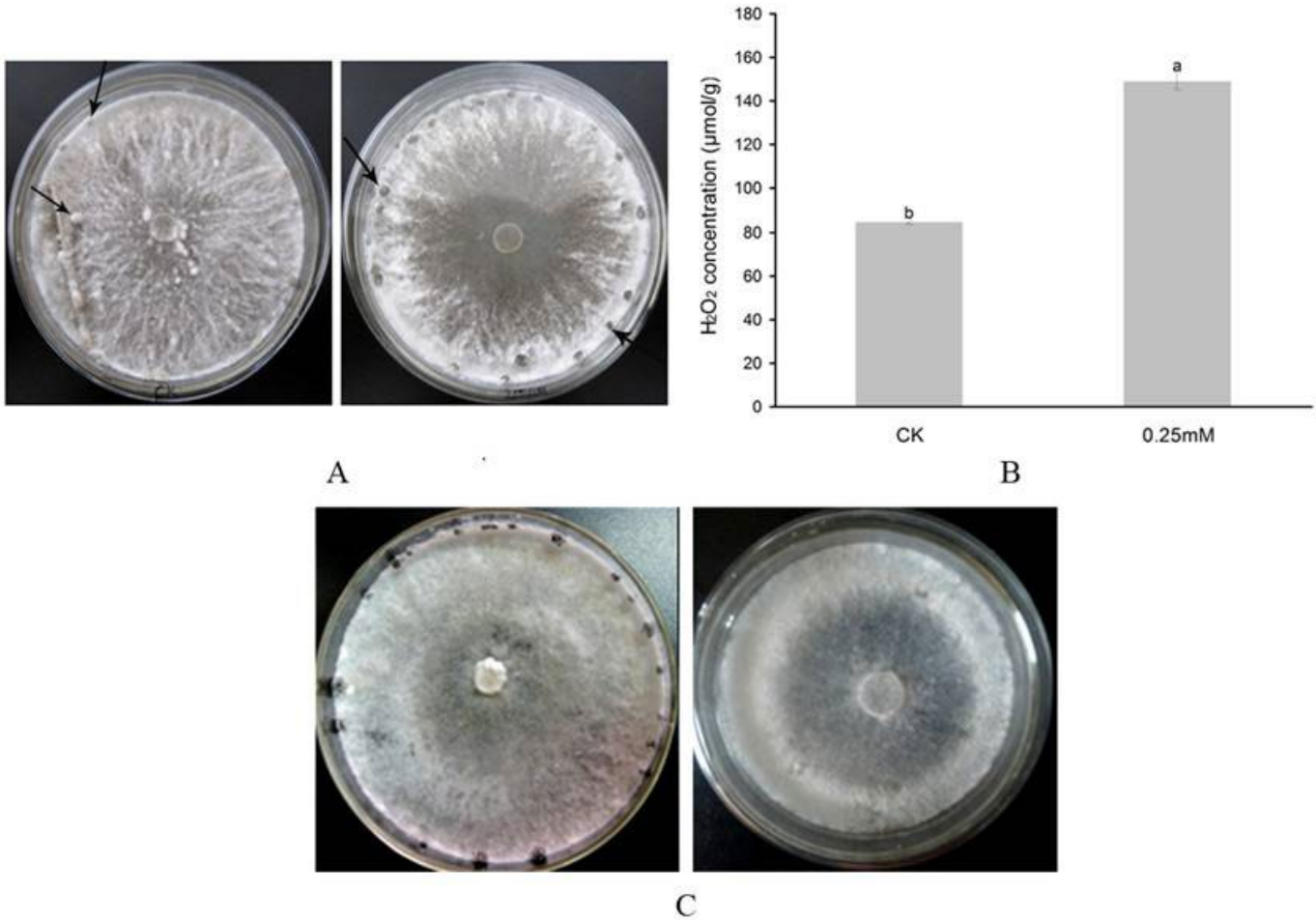

Fig. 2. H_2O_2 promotes sclerotia formation in *S. sclerotiorum*. A. Sclerotia formation after 4 days culture on PDA without (left) and with 0.25 mM H_2O_2 (right) (Arrows point to sclerotia). B. Comparison of endogenous H_2O_2 concentration in mycelium of *S. sclerotiorum* after 4 days culture on PDA medium with and without exogenous 0.25 mM H_2O_2. Significant differences are indicated with different letters (one-sided Student's T- test, $P \leq 0.05$). Error bars are from three replicates. C. Sclerotia formation in *S. sclerotiorum* after 10 days of growth on PDA medium with (right) and without 1 mM DPI (left).

as in the SI stage, but decreased to 17.82 μmol/g at the SM stage. So, both the VM to SI and the SD to SM stage transitions were accompanied by a decrease in the H_2O_2 levels.

To further explore the role of H_2O_2 in the formation of sclerotia, 0.25 mM H_2O_2 was added into the culture medium to test whether it could promote the sclerotia initiation. Indeed, sclerotia were iniatiated more readily and consequently facilitated the production of sclerotia in plates containing 0.25 mM of H_2O_2 (Fig. 2A). So, both the VM to SI and the SD to SM stage transitions were accompanied by a decrease in the H_2O_2 levels. Compared with the control, the concentration of H_2O_2 was increased by 64.85 μmol/g in H_2O_2 supplied sample, confirming the efficacy of treatment (Fig. 2B).

To further test the promoting effect of H_2O_2 on the formation of sclerotia, DPI (1 mM), an inhibitor of NADPH oxidase was added to the medium. Ten days later, no sclerotia initiation or other sclerotia structure formed on the treated plate, whereas sclerotia were observed on the plates without DPI (Fig. 2C). Therefore, the high level of H_2O_2 inside fungal cells at VM stage is at least partly due to increased synthesis by NADPH oxidase and could promote the initiation of sclerotia.

The activity of the ROS-scavenging enzymes CAT and SOD decreases along with sclerotia development. To further determine the involvement of H_2O_2 in the initiation of sclerotia, ROS- scavenge enzymatic activities of CAT and SOD at different stages of sclerotia formation was tested. The activity of CAT was 3.50 U in the VM stage and decreased to 2.57 U at the SI stage and dropped to 1.59 U at the SM stage (Fig. 3A); similarly, the activity of SOD was 64.92 U at the VM stage and decreased to 41.23 at the SI stage and 11.42 U at the SM stage (Fig. 3B). The decrease of both SOD and CAT activities may be linked to the drop of endogenous H_2O_2 concentration during the switch from the VM stage to the SI stage and in the later developmental stages.

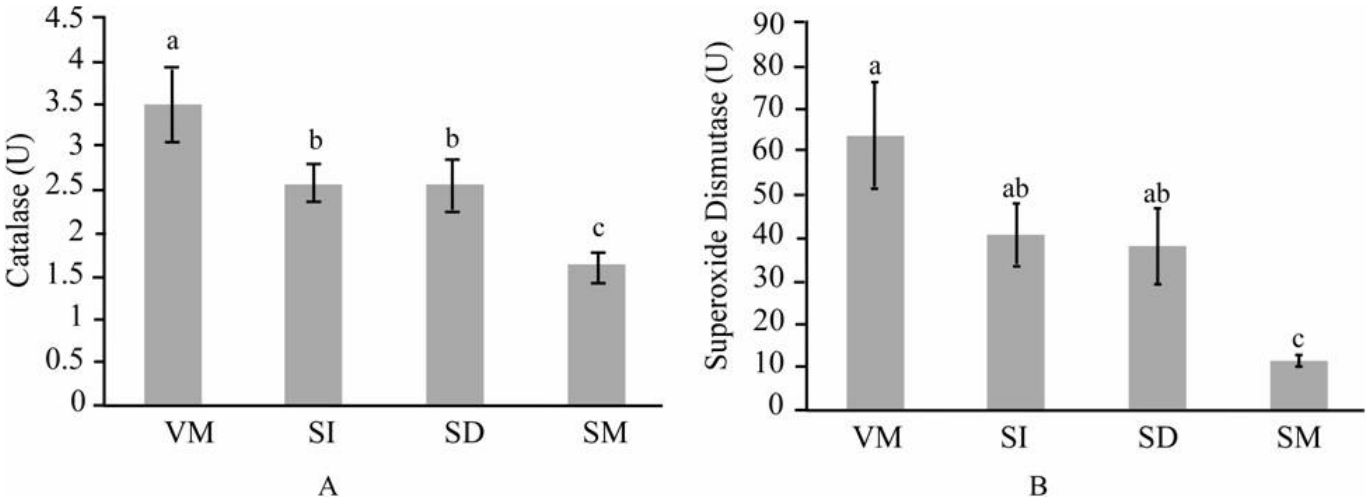

Fig. 3. Enzymatic activities of CAT and SOD at different stages of sclerotia of *S. sclerotiorum*. VM: vegetative mycelium, SI: sclerotia initial stage, SD: sclerotia development stage, SM: sclerotia maturation stage. (A) and (B) are CAT and SOD activities at different stage of sclerotia formation. The experiments were repeated three times with similar results. Significant differences are indicated with different letters (one-sided Student's T- test, $P \leq 0.05$). Error bars is from three replicates.

Exogenous cAMP inhibits the accumulation of H_2O_2. To verify whether cAMP inhibits the initiation of sclerotia via regulation of the accumulation of H_2O_2, isolate X-8, culture on agar medium supplemented with 10 mM cAMP, was used to observe the initiation of sclerotia. Ten days later, unlike the control, no sclerotia initiation or other sclerotia structure were observed on the cAMP-amended plate (Fig. 4A). When sclerotial primordia obtained on regular growth media were transferred onto cAMP-amended media, they could no longer develop into mature sclerotia, contrarily to the ones transferred onto medium not containing cAMP (data not shown). These results suggest that the inhibitory effect of cAMP on sclerotia development persists even after the SI stage. To further test whether H_2O_2 is involved in this process, H_2O_2 concentration was measured in mycelium collected from cAMP-amended plates. As shown in Fig. 4B, after 2 or 3 days of growth on cAMP-amended plates, the H_2O_2 values were 7.78 and 2.93 µmol/g respectively. Both the values are significantly lower than the values of the control plates (62.28 and 56.35 µmol/g, respectively). To further verify the above result, the mycelium collected from cAMP-amended plates was stained with H2DCFDA, a fluorescent indicator of H_2O_2. Compared with untreated samples, which had strong green fluorescence, rather weak fluorescence was observed in cAMP-treated mycelium under microscopy, suggesting less accumulation of H_2O_2 in the cAMP-treated sample (Fig. 4C).

To verify whether cAMP consistently inhibits the maturation of sclerotia via regulation of H_2O_2, we picked out the sclerotia primordia and transferred it onto PDA medium with or with out 10 mM cAMP. Ten days later, the mature sclerotia on the control plate were observed, but no sclerotia were formed on the cAMP-amended plate (Fig. 5). To further test whether H_2O_2 is also involved in this process, H_2O_2 concentration was measured in mycelium collected from cAMP-amended and control plates. After 3 days culture, the value of H_2O_2 on cAMP-amended plates was 3.13 µmol/g, which was significantly lower than the value in the control plate (55.68 µmol/g).

cAMP decreases H_2O_2 levels via regulation of *Nox* genes transcription. *Nox* gene, a homolog of NADPH oxidase, regulates the production of H_2O_2 within cells (Kim *et al.*, 2011). To determine whether such decrease of H_2O_2 levels due to cAMP treatment is achieved via down-regulation of transcripts of *Nox* genes, the relative transcript level of *Nox* genes was quantified through real time PCR. As shown in Fig. 6, after 2 or 3 days growth on cAMP-amended plates, the relative expression value of both *Nox1* and *Nox 2* genes to EF-1α was significantly lower than that of the control (Fig. 6A and 6B), suggesting that the down-regulation of H_2O_2 levels in cAMP treated samples is likely and at least partly due to a transcriptional down-regulation of both *Nox* genes.

cAMP treatment induces the reduction of SOD and CAT activities in the initiation of sclerotia. To verify whether exogenous cAMP could also affect the activity of two important ROS scavenging enzymes, CAT and SOD, when cultured on cAMP-amended medium, the activities were measured in the extracts of *S. sclerotiorum* X-8 (Fig. 7A and 7B). Our results show that both value of CAT and SOD activity in samples collected from the cAMP amended plates were significantly lower than those of control. The decreased value of both SOD and CAT upon treatment with cAMP correlated with the decreased levels of H_2O_2.

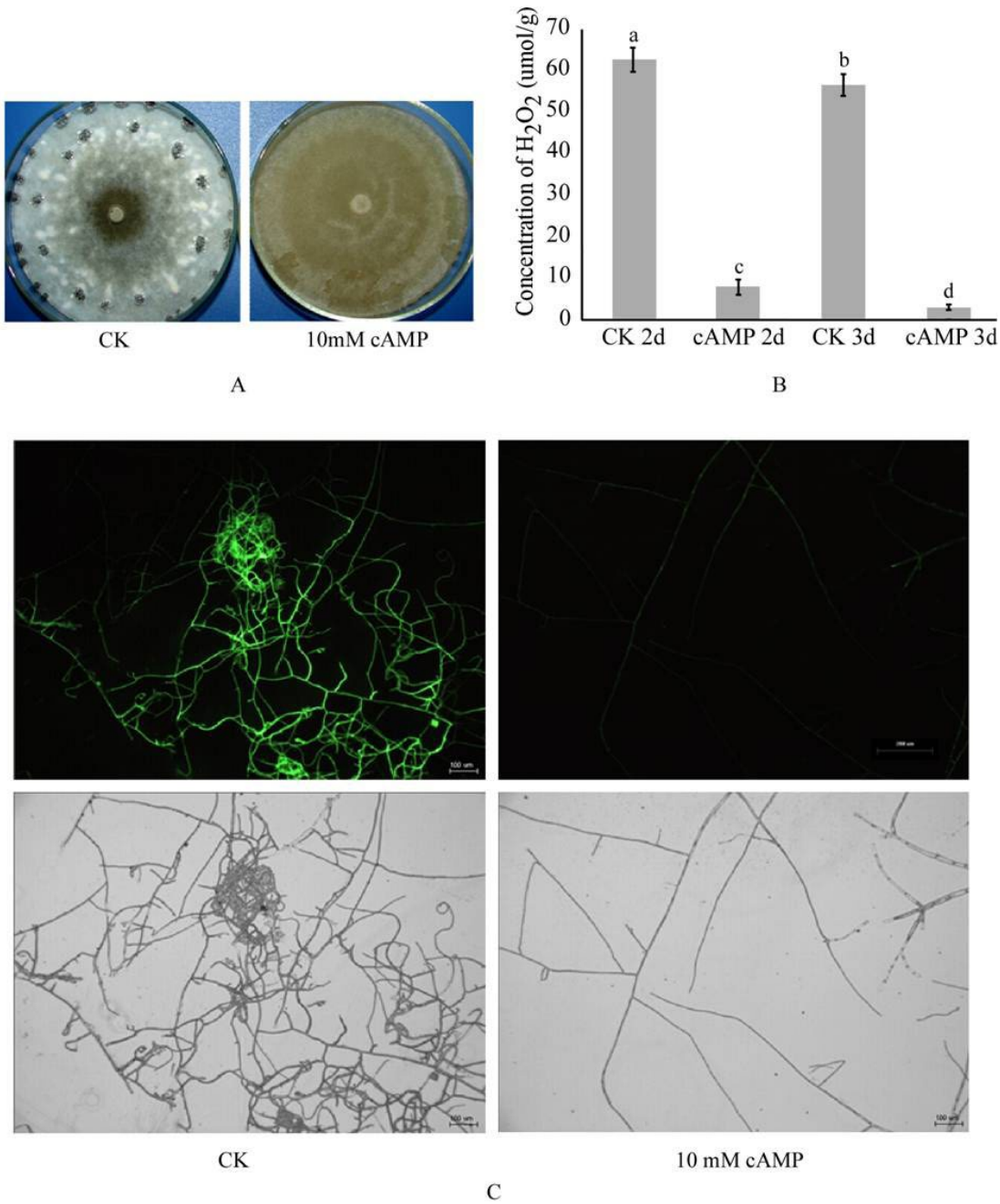

Fig. 4. H_2O_2 is involved in the cAMP-induced inhibition of sclerotia initiation in *S. sclerotiorum*. A. Sclerotia after growth on PDA for 10 days with (right) and without 10 mM cAMP (left). B. Concentration of endogenous H_2O_2 of *S. sclerotiorum* after cultured for 2 or 3 days on PDA with (cAMP) or without (CK) 10 mM cAMP. C. H2DCFDA, a fluorescent indicator of H_2O_2, allows comparing endogenous H_2O_2 level in mycelia cultured for 2 days on medium with (right) or without (left) 10 mM cAMP.

DISCUSSION

It was proposed that the mycelium growth in liquid media is usually exposed to low concentration of oxygen and high concentration of carbon dioxide, which inhibits sclerotial formation by a then unknown mechanisms (Chet and Henis, 1975). Later work showed a positive correlation between lipid oxidation level and sclerotial formation in *Sclerotinia minor* (Georgiou, 1997), indicating that the intracellular redox status of mycelia may regulate sclerotial formation in *S. sclerotiorum*.

In this study, we confirmed that high H_2O_2 levels in vegetative mycelium are required for the initial transition to sclerotia differentiation. After this stage, a decrease is detected during the transition from the VM stage to the SI stage, suggesting higher level of H_2O_2 in the mycelium could promote the initiation of sclerotia. Such transformation would facilitate the decrease of H_2O_2 in mycelium cells, and prevent the cell from programmed cell death (PCD). In fact, exogenous application 0.25 mM H_2O_2 or 1 mM DPI could promote or inhibit sclerotial formation, implying that H_2O_2 plays a role during the very early steps

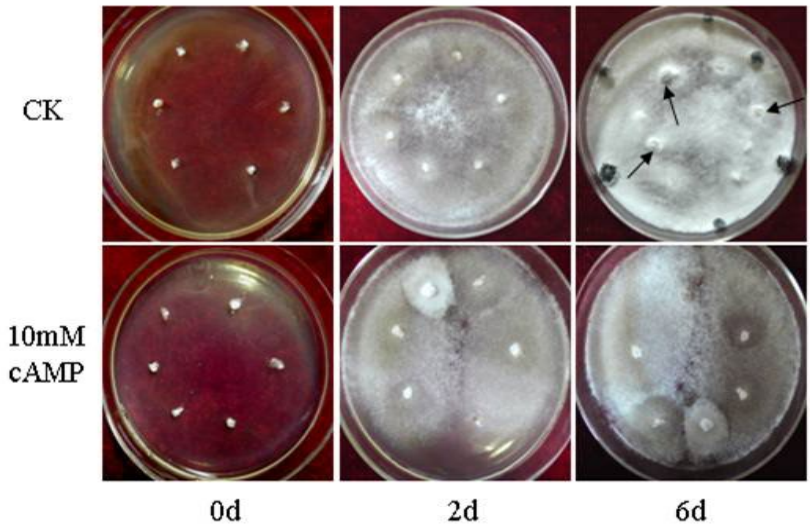

Fig. 5. The inhibition effects of cAMP is persistent after SI stage. Sclerotia maturation after the sclerotia initial transfer on PDA with (low) and without (upper) 10 mM cAMP for 10 days. The arrows point to matured sclerotia.

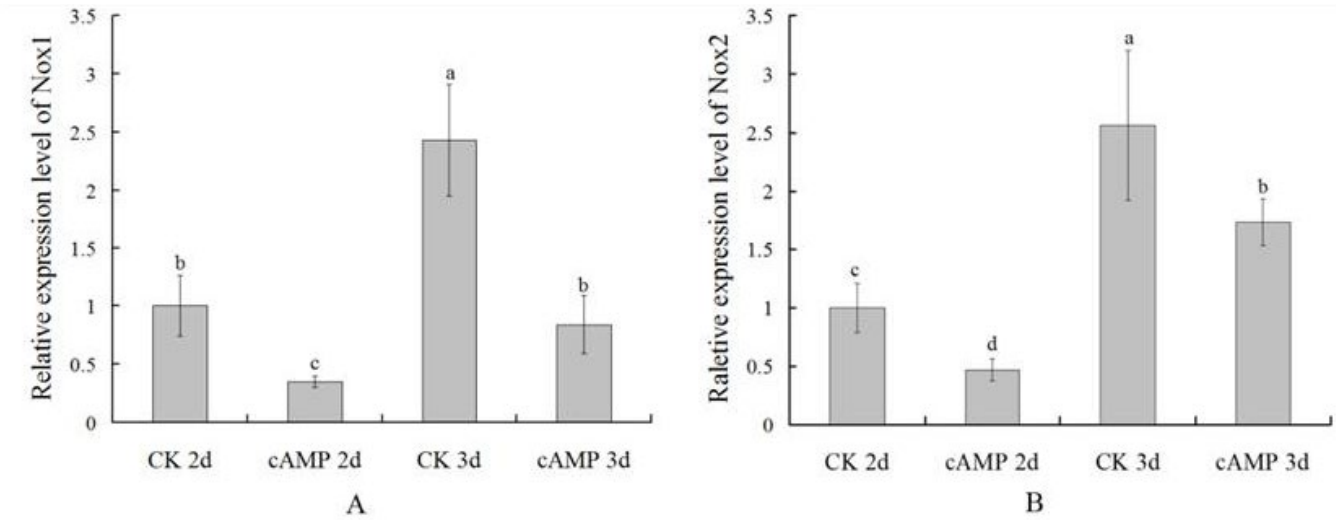

Fig. 6. Transcripts of *Nox1* (A) and *Nox2* (B) after cAMP treatment. *S. sclerotiorum* cultured on medium with or without 10 mM cAMP at the initial stage of sclerotia. The transcripts of *Nox1* and *Nox2* genes were quantified by using quantitative reverse-transcriptase PCR. Transcript quantity after normalization on the transcript of the *EF-1α* gene, an internal control. The bars represent the average level of the three biological and technical repeats. Significant differences are indicated with different letters (one-sided Student's T- test, $P \leq 0.05$). Error bars are from three replicates.

of sclerotial initiation. However, the transfer of sclerotial primordial onto cAMP amended plate also inhibits the formation of sclerotia, indicating the inhibition effect of cAMP persists after the SI stage. Rather lower H_2O_2 levels were detected in the samples which developed from the primordial on cAMP amended plate suggesting the inhibition effects of cAMP on the maturation of sclerotia also goes through the modulation of H_2O_2 levels. This finding is in line with the finding that some hydroxyl radical scavengers and endogenous antioxidants were found to play a negative role in sclerotial development (Georgiou and Petropoulou, 2001a, 2001b). Additionally, the decreased expression pattern of *Nox*1 and *Nox2* gene, together with the decline tendency of the enzymatic activities of SOD and CAT will be the cause and effects of decreased H_2O_2 level at the initial stage of sclerotia formation.

In animal cells, ROS is a secondary messenger and can reduce the basal level of intracellular cAMP by regulating the activity of adenylate cyclase (AC) (Chartoff *et al.*, 2003). This indicates that a cross-talk between cAMP and ROS signaling pathway may exist. Rollins found that exogenous cAMP could suppress sclerotial initiation in *S. sclerotiorum* (Rollins and Dickman, 1998). Adenylate cyclase, which converts ATP to cAMP, also regulates the formation of sclerotia (Kim *et al.*, 2011). Mitogen-activated protein kinases (MAPKs), acting downstream of PKA, were also found to be involved in cAMP-inhibited formation of sclerotia (Jurick and Rollins, 2007). Protein kinase

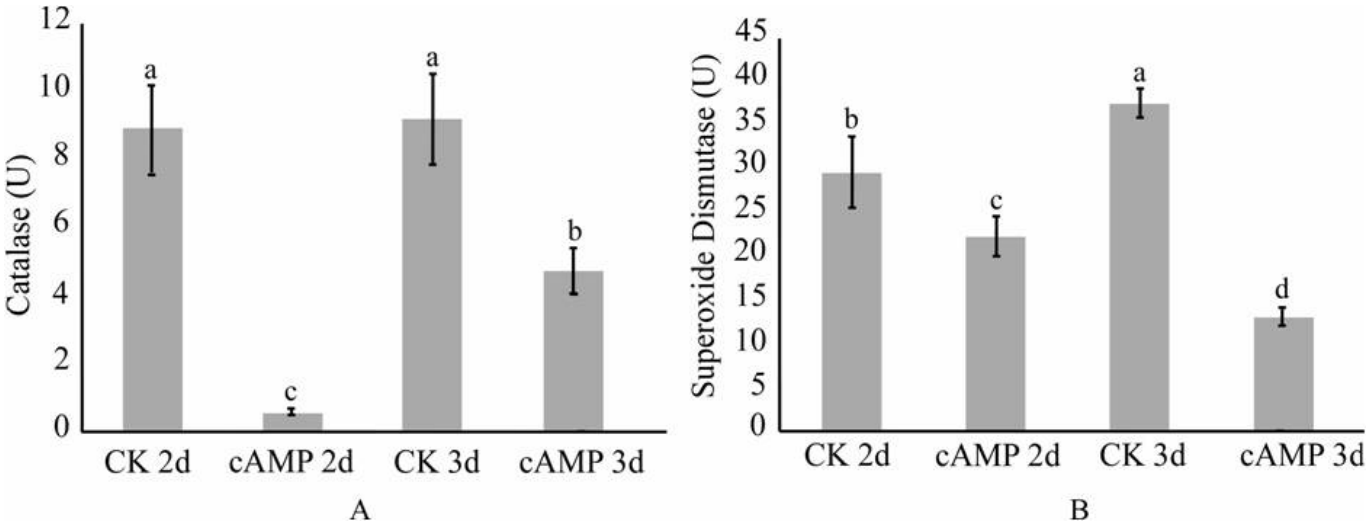

Fig. 7. Enzyme activities of CAT (A) and SOD (B) at the initial stage of sclerotia in the presence or absence of exogenous cAMP. The experiments were repeated for three times with similar results. Significant differences are indicated with different letters (one-side Student's T- test, $P \leq 0.05$). Error bars are from three replicates.

A (PKA) and mitogen activated protein kinases (MAPKs), two downstream effectors of cAMP signaling pathway, were found to be involved in cAMP induced inhibition of sclerotia formation (Kim, 2014). *Botrytis cinerea* has a rather high genetic similarity with *S. sclerotiorum*. In the mutant Δbcg3 of *B. cinerea*, which is defective in a Gα3 subunit, significantly reduced cAMP levels and are associated to promoted sclerotia formation; exogenous cAMP completely suppresses sclerotial formation (Doehlemann *et al.*, 2006), suggesting that cAMP negatively regulates sclerotia formation in *B. cinerea*. This stands in line with our finding that exogenous cAMP in the culture medium does inhibits the initiation of sclerotia and also further developmental stages. However, Harel demonstrated that sclerotia-deficient isolates are always characterized by low PKA activity. Exogenous supplied caffeine, a PKA inducer, significantly enhances PKA activity and promotes the formation of sclerotia in sclerotia-deficient isolates, suggesting that cAMP positively regulates the formation of sclerotia through PKA in *S. sclerotiorum* (Harel *et al.*, 2005). It also has been noted that small G proteins Rap and Ras are involved in the cAMP-induced inhibition of the sclerotial formation pathway (Chen and Dickman, 2005). All these results suggest that cAMP regulates sclerotial formation through different downstream effectors such as Rap, Ras, PKA and MAPKs, and most likely, balanced levels of such crucial messengers are needed throughout the process to obtain full sclerotial development.

For all of above, both ROS and cAMP signal molecules are involved in the initiation and formation of sclerotia, but when our work started, it was not known yet whether there was a cross-talk between them during sclerotia formation, especially in the initiation of sclerotia. Here, we detected the significantly reduction of H_2O_2 levels in *S. sclerotiorum* grown on cAMP-amended medium by both biochemical and cell-imaging techniques. Activity of the ROS-scavenging enzymes SOD and CAT also decreased accordingly, likely as a consequence of the lower ROS levels. Additionally, the transcripts of *Nox1* and *Nox2* were significantly decreased in mycelium grown in the presence of exogenous cAMP, indicating that cAMP likely decreases H_2O_2 *via* down-regulating the transcripts of both *Nox1* and *Nox2* gene. This is in line with the observation that *Nox1* gene expression has a role in the development of multicellular structures of mycelium, just as in the stage of sclerotial initiation.

In this study, we have demonstrated that the elevated level of H_2O_2 at VM stage may be one of the key factors to promote the initiation of sclerotia in *S. sclerotiorum*. Both *Nox1* and *Nox2* genes are the key regulators, promoting the accumulation of H_2O_2 in mycelium cells. The variation of H_2O_2 levels during the transition from the VM to the SI stage could explain the variation of ROS scavenging enzymes such as CAT and SOD. Also, the persistent effects of cAMP on the maturation of sclerotia was also detected and this seems to go through the modulation of H_2O_2 level as well, indicating the cross link between cAMP and H_2O_2 on the regulation of sclerotia initiation and also maturation. Additionally, our results place cAMP signal molecule upstream of H_2O_2 in the signal transduction pathway leading to the VM to SI stage transition, because its excess hampers H_2O_2 accumulation not only at the VM stage, thus inhibiting sclerotial initiation, but also in the later stages, suppressing the maturation of sclerotia. However, what the concentration threshold of H_2O_2 for promoting the transition from mycelium to the initiation of sclerotia; how H_2O_2 modulates the maturation of sclerotia; the relative positions of cAMP, H_2O_2 and other downstream factors, such as PKA and MAPKs, all

along the cAMP-induced sclerotia inhibition pathway need further study.

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