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# Involvement of Alternative Oxidase in the Regulation of Growth, Development, and Resistance to Oxidative Stress of *Sclerotinia sclerotiorum*

**Ting Xu[1,2], Fei Yao[1,2], Wu-Sheng Liang[1,2]*, Yong-Hong Li[3], Dian-Rong Li[3], Hao Wang[3], and Zheng-Yi Wang[1,2]**

[1]*Institute of Biotechnology, College of Agriculture and Biotechnology, Zijingang Campus, Zhejiang University, Hangzhou 310058, P. R. China*
[2]*State Key Laboratory of Rice Biology, Hangzhou 310058, P. R. China*
[3]*Hybrid Rapeseed Research Center of Shaanxi Province, Dali 715105, P. R. China*

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***Sclerotinia sclerotiorum*** **is a cosmopolitan, filamentous, fungal pathogen that can cause serious disease in many kinds of crops. Alternative oxidase is the terminal oxidase of the alternative mitochondrial respiratory pathway in fungi and higher plants. We report the presence of this alternative pathway respiration and demonstrate its expression in two isolates of *S. sclerotiorum* under unstressed, normal culture conditions. Application of salicylhydroxamic acid, a specific inhibitor of alternative oxidase, severely inhibited the mycelial growth of *S. sclerotiorum* both on potato dextrose agar plates and in liquid culture media. Inhibition of alternative oxidase could influence the growth pattern of *S. sclerotiorum*, as salicylhydroxamic acid treatment induced obvious aerial mycelia growing on potato dextrose agar plates. Under the treatment with salicylhydroxamic acid, *S. sclerotiorum* formed sclerotia much more slowly than the control. Treatment with hydrogen peroxide in millimolar concentrations greatly decreased the growth rate of mycelia and delayed the formation of sclerotia in both tested *S. sclerotiorum* isolates. As well, this treatment obviously increased their alternative pathway respiration and the levels of both mRNA and protein of the alternative oxidase. These results indicate that alternative oxidase is involved in the regulation of growth, development, and resistance to oxidative stress of *S. sclerotiorum*.**

***Keywords***: *Sclerotinia sclerotiorum*, alternative oxidase, mitochondrial respiratory chain, hydrogen peroxide, oxidative stress

## Introduction

The ascomycete *Sclerotinia sclerotiorum* is a cosmopolitan, filamentous, plant pathogenic fungus. It can invade at least 405 species among 278 genera distributed in 75 families of plants, including both monocotyledons and dicotyledons. Many of its hosts are important crops, such as rapeseed, soybean, peanut, beans, lettuce, sunflower, and celery, in which it causes substantial crop production losses throughout the world every year (Boland and Hall, 1994; Bardin and Huang, 2001). In China, this pathogen is the most potentially dangerous biotic threat to rapeseed. Sclerotinia disease, or stem rot, occurs in all rapeseed-growing areas in China, especially in the region of the Yangtze River. The incidence of sclerotinia stem rot of rapeseed in this region was estimated at 10–20% on average and reached up to 80% during some serious instances (Li *et al*., 2006). In the UK, sclerotinia disease of field lettuce caused by *S. sclerotiorum* is a common problem, with frequently reported losses of 10%. Diseased lettuce heads are unmarketable as they rot very quickly once infected by *S. sclerotiorum* (Young *et al*., 2004). In the USA, annual losses from *S. sclerotiorum* have exceeded $200 million, stimulating Congress to develop a National Sclerotinia Initiative to foster research on the biology and control of this pathogen (Bolton *et al*., 2006).

Mitochondria of fungi and higher plants contain two respiratory electron transport chains. One is the cytochrome pathway (CP) with cyanide-sensitive cytochrome oxidase as the terminal oxidase. Another one is the alternative pathway (AP), which branches from CP in the inner mitochondrial membrane at the site of the ubiquinone pool and uses the alternative oxidase (AOX) as its terminal oxidase. AOX is cyanide-resistant, but sensitive to substituted hydroxamic acids, such as salicylhydroxamic acid (SHAM) (Vanlerberghe, 1997). Research with plants has confirmed that AOX fulfils positive physiological functions in developmental events and in responses to environmental changes. During flowering of the Araceae, such as *Sauromatum guttatum*, the AOX expression level and AP respiration rate were confirmed to dramatically increase in their thermogenic blooms. The energy generated through AP by AOX is the source of thermogenesis. The produced heat leads to a better volatilization of scent compounds to attract insect pollinators (McIntosh, 1994). Besides the special role of AOX in thermogenic plants, AOX may play important roles during resistance or adaption of higher plants against abiotic stresses, as the AOX expression level and AP respiration have been found to be greatly increased in plants exposed to many kinds of abiotic stress, such as chilling, drought, and heat (Ribas-Carbo *et al*., 2000, 2005; Rizhsky *et al*., 2002; Rachmilevitch *et al*., 2007). A proposed role of AOX under abiotic stresses is to prevent deleterious oxidative stress by shunting electrons

*For correspondence. E-mail: liangws@zju.edu.cn; Tel.: +86-571-88982261

from reduced ubiquinone to avoid the over-reduction of CP components, which can afterwards bring about production of endogenous reactive oxygen species (ROS) (Day *et al.*, 1995; McDonald, 2008). Besides the function in abiotic stresses, AOX has also been found to induce resistance of plants against virus infection (Chivasa *et al.*, 1997; Chivasa and Carr, 1998; Fu *et al.*, 2010).

Recently AOX distribution has been widened to all kingdoms of life except the Archaebacteria (McDonald, 2008). However, compared to the wide range of research on AOX in higher plants, there is much less on that in other taxa, including fungi. For fungal AOX, most of the previous research was carried out on a few fungi, such as *Hansenula anomala*, *Neurospora crassa*, *Magnaporthe grisea*, *Ustilago maydis*, *Cryptococcus neoformans*, and *Paracoccidioides brasiliensis* (Minagawa and Yoshimoto, 1987; Lambowitz *et al.*, 1989; Yukioka *et al.*, 1998; Akhter *et al.*, 2003; Juarez *et al.*, 2006; Martins *et al.*, 2011). The information available for AOX in higher plants is often used to explain the roles of AOX in fungi (Day *et al.*, 1995; Joseph-Horne *et al.*, 2001; McDonald, 2008). The role of AOX to prevent oxidative stress in fungi has been described in some reports (Minagawa *et al.*, 1992; Yukioka *et al.*, 1998; Akhter *et al.*, 2003; Juarez et al., 2006; Martins *et al.*, 2011). However, more experimental evidence is still needed to elucidate the roles of AOX in fungi.

Since *S. sclerotiorum* is a plant pathogen that may cause serious damage to diverse crops, high levels of resistance are likely absent in the major crops damaged by this pathogen, and the diseases caused by this pathogen have traditionally been difficult to control (Bolton *et al.*, 2006). There is as yet no research on the biological roles of AOX in *S. sclerotiorum*. This report will provide evidence indicating the involvement of AOX in regulation of growth, development, and resistance to oxidative stress of *S. sclerotiorum*. This knowledge can be helpful for understanding the functions of AOX in the life history of *S. sclerotiorum*, and therefore may supply potential clues for controlling this plant pathogen.

## Materials and Methods

### *S. sclerotiorum* isolates

Two *S. sclerotiorum* isolates (7-3, 44-2) were collected from a rapeseed field. No obvious color and morphological differences were observed between the mycelia and sclerotia of the two isolates on potato dextrose agar (PDA) plates. Inoculation of rapeseed plants with either of the *S. sclerotiorum* isolates caused serious rot symptoms (data not shown). Both *S. sclerotiorum* isolates have been deposited with the Zhejiang University Fungal Collection (Hangzhou, China), accession numbers Ssc-2011-001 and Ssc-2011-002.

### Culture of *S. sclerotiorum* and treatment with effectors

Both *S. sclerotiorum* isolates were cultured by growing in the dark on PDA plates (i.e. 200 g potato infusion, 20 g dextrose, 20 g agar, with distilled water to 1,000 ml) in Petri dishes (9-cm diameter) at 22°C. For continuous culture, a 4-mm diameter mycelial plug was taken from the margins of colonies grown for 3 days and inoculated onto the center of fresh PDA plates.

For culture in liquid media, 3 mycelial plugs (4-mm diameter) from PDA plates were inoculated into a 0.5-L Erlenmeyer flask containing 200 ml potato dextrose (PD) liquid medium (i.e. 200 g potato infusion, 20 g dextrose, with distilled water to 1,000 ml). The flasks were set to continuous oscillation (150 rpm) at 22°C in darkness on rotary shakers.

For treatment experiments on PDA plates or in liquid media, effectors (i.e. SHAM or $H_2O_2$) at the indicated concentrations were added to the PDA or the liquid media before inoculation with the mycelial plugs.

### Measurement of growth rate of *S. sclerotiorum* mycelia

For measurement of growth rate of mycelia of *S. sclerotiorum* cultured on PDA plates, colony diameters were measured at specific times. The diameter of a colony is the mean of two measurements of the diameter of the colony taken at 90° to each other.

The growth rate of *S. sclerotiorum* mycelia cultured in liquid media was determined based on their dry weight. Mycelia were collected by filtration with filter paper in a Buchner funnel under vacuum and then rinsing with 200 ml distilled water. The collected mycelia were set to dry for about 12 h at 80°C in a drying oven. The dry weight of the mycelial sample was taken as the weight that could not be further reduced by the drying procedures.

### Observation of sclerotia formation of *S. sclerotiorum*

Cultivation was as described above and the times to formation of obvious sclerotia were noted.

### Measurement of respiration rate of *S. sclerotiorum* mycelia

Mycelia were collected by Buchner-filtration from *S. sclerotiorum* cultures in liquid media as described above. The collected mycelia were suspended in PD liquid medium and set to a moderate fragmentation with a soymilk grinder (Joyoung Company, China). Oxygen consumption rates of these mycelial samples were measured using a Clark-type oxygen electrode (Hanna Instruments, HI964400M, Woonsocket, RI, USA) at 25°C in a sealed vessel surrounded with a circulating water bath. After measurement the mycelial samples were collected from the vessel and their dry weights were determined as described above. Oxygen consumption rates were calculated from the recordings of the oxygen electrode signals. The AP capacity was the oxygen consumption rate in the presence of 1.0 mM KCN minus the residual oxygen consumption rate in the presence of 1.0 mM KCN and 2.5 mM SHAM. The CP capacity was the oxygen consumption rate in the presence of 2.5 mM SHAM minus the residual oxygen consumption rate.

### RNA extraction and estimation of *aox* gene transcript level in *S. sclerotiorum* by real-time quantitative PCR

Mycelia were collected from *S. sclerotiorum* cultures in PD liquid medium as described above. Total RNA was extracted using the Trizol reagent (Shenggong Company, China) ac-

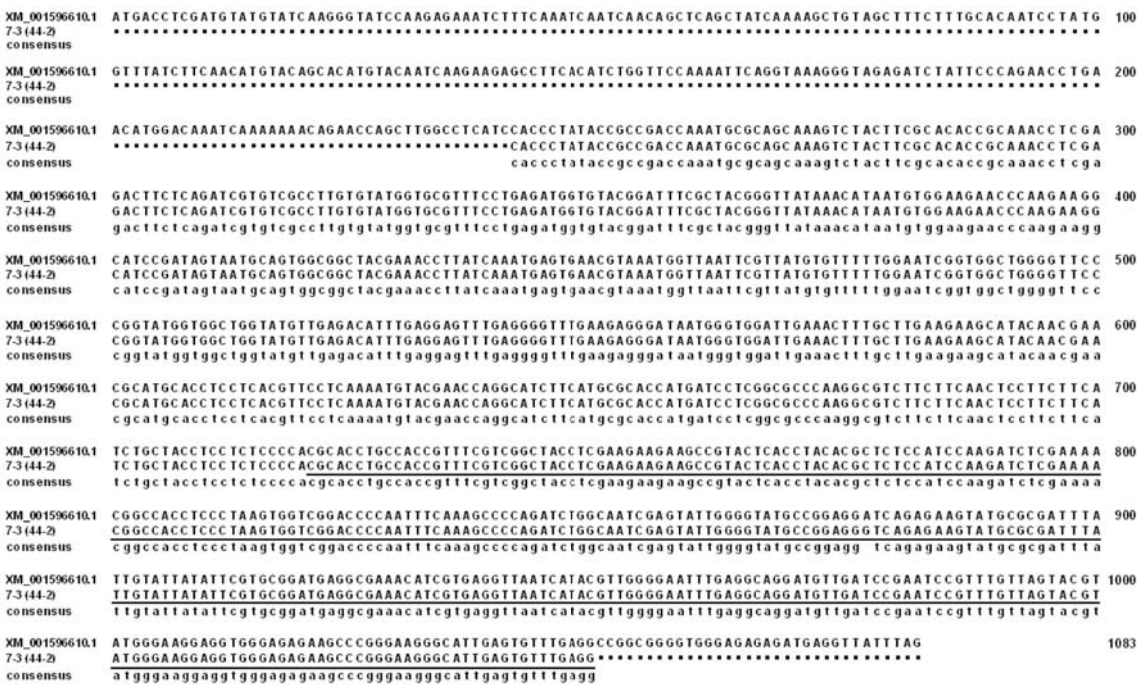


**Fig. 1. Comparison of the partial sequences of the *aox* mRNA of two *S. sclerotiorum* isolates (7-3, 44-2) with the *aox* mRNA of *S. sclerotiorum* 1980 UF-70 registered in GenBank (accession no. XM_001596610.1).** The 809-base fragments of the *aox* mRNA of *S. sclerotiorum* 7-3 and 44-2 had the same sequence, but differed by 1 base from the sequence of XM_001596610.1 in GenBank. Correspondingly, the amino acid $Asp^{293}$ in alternative oxidase (AOX) of *S. sclerotiorum* 1980 UF-70 was changed into Gly in AOX of *S. sclerotiorum* isolate 7-3 and 44-2. Location 149-321 of the protein encoded by the sequence of XM_001596610.1 (nucleotide no. 447-963) is a ferritin-like diiron-binding domain of AOX. The underlined sequence (330 bases) of *aox* mRNA of *S. sclerotiorum* isolate 7-3 was expressed in *E. coli* in our study.

cording to the manufacturer's specifications. Genomic DNA was removed by treatment with DNase I (TaKaRa, China). Complementary DNA was synthesized from the total RNA with random hexanucleotides (TaKaRa) and Primescript reverse transcriptase (TaKaRa).

The real-time quantitative PCR was performed with an iCycler iQ™ Multicolor Real-Time PCR Detection System (Bio-Rad, USA). A set of specific primers for *aox* mRNA of isolates 7-3 and 44-2 of *S. sclerotiorum* was designed for analyzing the transcript level: forward primer: 5′-TTGAA GAGGGATAATGGGTG-3′; reverse primer: 5′-GAAGGA GTTGAAGAAGACGC-3′. Each reaction had a total volume of 20 µl, consisting of 10 µl of SYBR green real-time PCR master mix (Toyobo, Japan), 4 µl of diluted cDNA, and 0.4 µM each of forward and reverse primers. PCR cycling conditions were set as follows: 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec. The histone H3 gene of *S. sclerotiorum* (GenBank accession no. XM_001589836) served as an internal control using a set of specific primers: forward primer: 5′-TACTG GAGGTGTCAAGAAGC-3′; reverse primer: 5′-ACTTGA AGTCTTGGGCGATT-3′. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

### Prokaryotic expression of *aox* mRNA of *S. sclerotiorum* and antibody preparation

Mycelial collection from *S. sclerotiorum* isolate 7-3, total RNA extraction, and complementary DNA synthesis were conducted as described above. Afterwards, a 330-base fragment of the *aox* mRNA of *S. sclerotiorum* isolate 7-3 (the underlined sequence in Fig. 1) encoding the carboxyl terminal region of AOX was amplified by PCR with specific primers. Two copies of this fragment were linked by 5′-GAATTC-3′ and cloned with the vector pMD19-T (TaKaRa). The linked double fragments were excised by restriction enzymes and inserted into the pET-32 Ek/LIC expression vector (Novagen, USA) according to the manufacturer's specifications. All the concerned DNA sequences were confirmed to be correct by DNA sequencing. The recombinant prokaryotic expression vector was expressed in the *Escherichia coli* strain BL21(DE3). The expression product was extracted and purified by $Ni^{2+}$-chelating with an immobilized-metal-affinity chromatography (IMAC) column filled with Chelating Sepharose™ Fast Flow (GE Healthcare, USA). Antibodies against the expressed protein were raised in a male New Zealand white rabbit.

### Estimation of alternative oxidase (AOX) protein level *in S. sclerotiorum* by Western blot

Mycelia were collected from *S. sclerotiorum* cultures in PD liquid media as described above. Mitochondria were extracted and purified according to the methods of Joseph-Horne *et al.* (2000). Estimation of AOX protein level in purified mitochondria by Western blot was carried out as described by Liang and Liang (2002). The purified mitochondria were

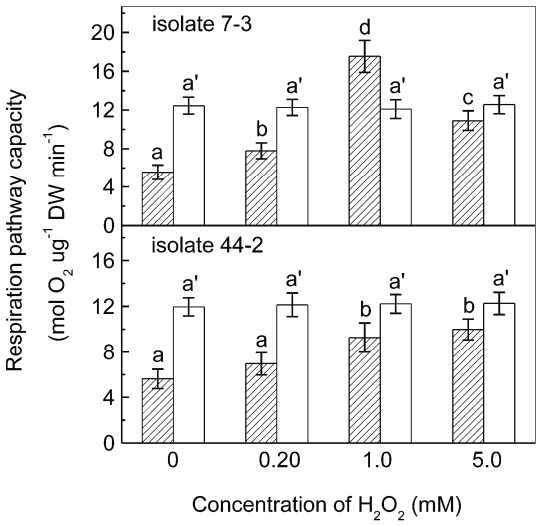


**Fig. 2. Alternative pathway (AP) and cytochrome pathway (CP) capacities in mycelia of two *S. sclerotiorum* isolates with or without $H_2O_2$ treatment.** Mycelia of *S. sclerotiorum* were collected to measure the respiration capacities after culture in potato dextrose liquid media for 3 days. For the effect of $H_2O_2$ on respiration capacities, $H_2O_2$ at indicated concentrations was used to treat both fungal isolates cultured in liquid media for 60 min. Means and standard errors are shown (n>3). Means with different letters above the columns are significantly different (P<0.05). Columns filled with diagonals: AP capacity; Columns without filling: CP capacity.

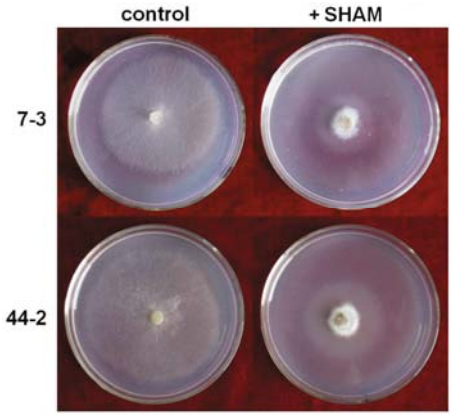


**Fig. 3. Photos showing the effect of inhibition of alternative oxidase (AOX) by salicylhydroxamic acid (SHAM) on growth rate of colonies of *S. sclerotiorum* on potato dextrose agar (PDA) plates in Petri dishes (9-cm diameter).** Experiments were carried out three times and representative results are shown. SHAM: treated with 2.5 mM SHAM.

solubilized with a suitable volume of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA-$Na_2$, 1 mM reduced glutathione, and 0.3% (v/v) Triton X-100. After measurement of their protein concentrations by the method of Bradford (1976) using Bovine serum albumen (BSA) as standard, a suitable volume of solubilized mitochondria was mixed with an equivalent volume of 2× sample loading buffer. After boiling for 3 min the mitochondrial protein samples were subjected to SDS-PAGE with 12% separating gel. The proteins in the gels were electrophoretically transferred onto nitrocellulose membranes. The prepared rabbit polyclonal serum against the prokaryotic expression product was used as the primary antibody to analyze AOX protein on the nitrocellulose membranes. Primary antibody bound on the nitrocellulose membranes was detected with a secondary anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, USA) and visualized using a chemiluminescent reagent system (Lianke Company, China). At the same time, cytochrome c in the mitochondrial protein samples was detected with polyclonal antibody against cytochrome c (Shenggong Company, China) by similar procedures as for AOX protein. Densitometry values for immunoreactive bands were quantified using a GS-700 imaging densitometer (Bio-Rad). After normalization with density of the cytochrome c band from the same mitochondrial protein sample, densities of AOX bands were used to calculate the increase of AOX protein level by $H_2O_2$ treatment.

### Data analysis

All experiments were repeated independently at least three times. Quantitative data were subjected to statistical analysis using the SPSS (version 13) program (Zhang, 2006). Means of the results were compared using ANOVA and P values

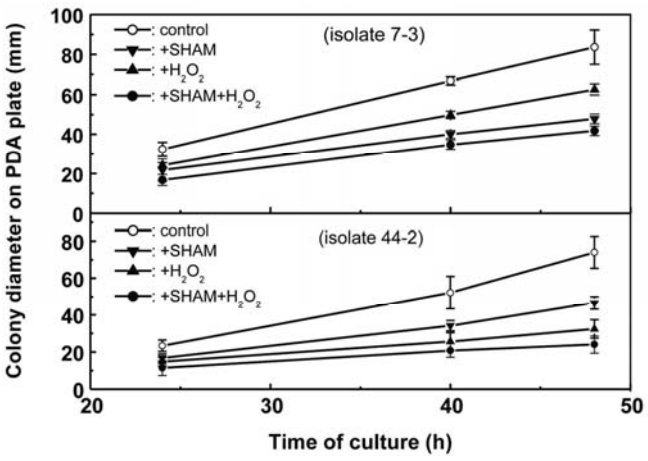


**Fig. 4. Growth rates of colonies of *S. sclerotiorum* on potato dextrose agar (PDA) plates with or without treatment of effectors.** Salicylhydroxamic acid (SHAM): treated with 2.5 mM SHAM. $H_2O_2$: treated with 1.0 mM $H_2O_2$. $H_2O_2$+SHAM: treated with 1.0 mM $H_2O_2$ plus 2.5 mM SHAM. Means and standard errors are shown (n>3).

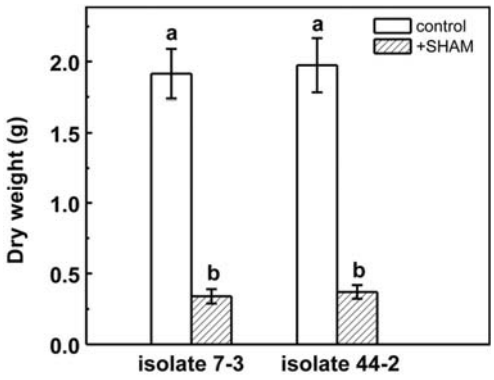


**Fig. 5. Effect of inhibition of alternative oxidase (AOX) by salicylhydroxamic acid (SHAM) on the growth of mycelial biomass of *S. sclerotiorum* cultured in liquid media.** Dry weight of *S. sclerotiorum* was determined after culture in potato dextrose liquid media for 3 days. Means and standard errors are shown (n>3). Means with different letters above the columns are significantly different (P<0.05). SHAM: treated with 2.5 mM SHAM.

<0.05 were considered significant. Duncan's multiple range test was used once the ANOVA was discovered to be significant.

## Results

### Alternative pathway (AP) respiration in *S. sclerotiorum* mycelia under normal conditions

Mycelia of both tested *S. sclerotiorum* isolates, after being cultured in PD liquid medium for 3 days, were collected to measure the AP respiration capacity. Both *S. sclerotiorum* isolates had notable AP respiration capacity under normal culture conditions (Fig. 2).

### Effects of inhibition of alternative oxidase (AOX) on growth rate of *S. sclerotiorum* mycelia

Inhibition of AOX with SHAM greatly decreased the growth rate of colonies of both *S. sclerotiorum* isolates on PDA plates (Figs. 3 and 4). This treatment also dramatically decreased the dry weight of mycelia of both *S. sclerotiorum* isolates during culture in PD liquid media (Fig. 5), which is consistent with its inhibitory effect on the fungal colony growth on PDA plates.

### Effects of inhibition of alternative oxidase (AOX) on growth pattern of *S. sclerotiorum*

During normal mycelial growth of *S. sclerotiorum* on PDA plates, it grew most as substrate mycelia adherent to the top surface of the media. At the late growth stage, near the time to form sclerotia, some aerial mycelia could be seen growing upwards from the top surface of the media on PDA plates. When SHAM was added to inhibit AOX, many more aerial mycelia of *S. sclerotiorum* appeared growing upwards from the top surface of PDA plates than without SHAM treatment (Fig. 6).

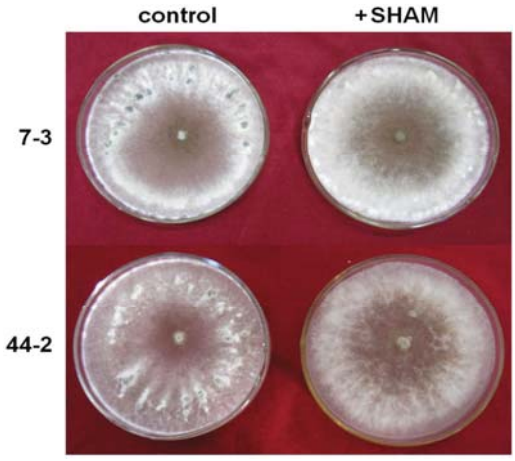


**Fig. 6. Effect of inhibition of alternative oxidase (AOX) by salicylhydroxamic acid (SHAM) on the growth pattern of both *S. sclerotiorum* isolates on PDA plates in Petri dishes (9-cm diameter).** Photos were taken after culture for 7 days. Experiments were carried out three times and representative results are shown. SHAM: treated with 2.5 mM SHAM.

### Effects of inhibition of alternative oxidase (AOX) on sclerotia formation of *S. sclerotiorum*

Sclerotia, which look like feces of mice, are the overwintering bodies and long-term survival structures of *S. sclerotiorum* in the field (Bardin and Huang, 2001; Bolton *et al.*, 2006). On PDA plates, the mycelia of *S. sclerotiorum* may produce many sclerotia after about 5 days. Under the treatment with SHAM to inhibit AOX, *S. sclerotiorum* could still form sclerotia; however, inhibition of AOX greatly delayed their formation in both *S. sclerotiorum* isolates (Fig. 7).

### Effects of treatment with $H_2O_2$ on alternative pathway (AP) respiration in *S. sclerotiorum*

Treatment with $H_2O_2$ at 0.20, 1.0, or 5.0 mM obviously in-

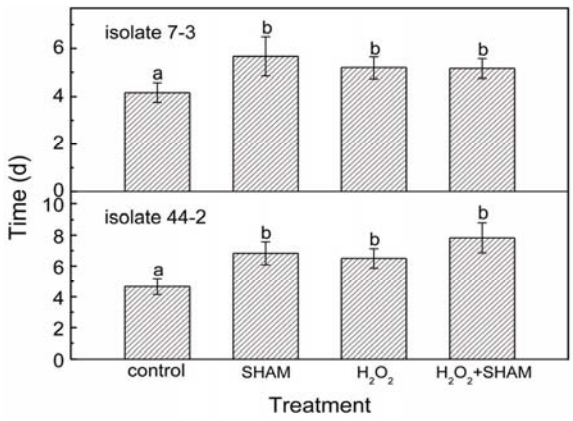


**Fig. 7. Time for *S. sclerotiorum* to form visible sclerotia on potato dextrose agar (PDA) plates with or without treatment of effectors.** Means and standard errors are shown (n>3). Means with different letters above the columns are significantly different (P<0.05). Salicylhydroxamic acid (SHAM): treated with 2.5 mM SHAM. $H_2O_2$: treated with 1.0 mM $H_2O_2$. $H_2O_2$+SHAM: treated with 1.0 mM $H_2O_2$ plus 2.5 mM SHAM.

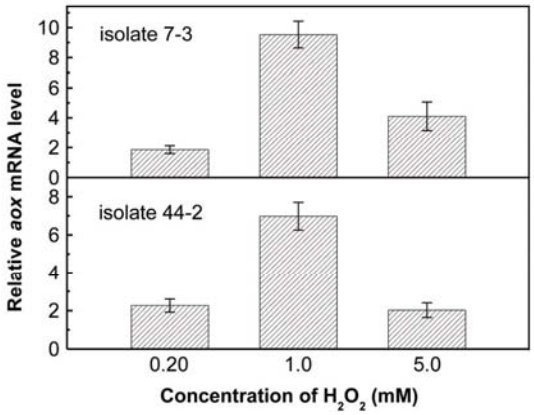


**Fig. 8. Effect of treatment with $H_2O_2$ on *aox* mRNA level in mycelia of *S. sclerotiorum*.** $H_2O_2$ of indicated concentrations was used to treat the two *S. sclerotiorum* isolates cultured in potato dextrose liquid medium for 60 min. Means and standard errors of the results from three independent experiments are shown.

creased the AP respiration rate in *S. sclerotiorum* (Fig. 2). $H_2O_2$ at 1.0 mM induced a greater increase than 0.20 mM, but 5.0 mM $H_2O_2$ had less effect than 1.0 mM. These results indicate that $H_2O_2$ could induce AP respiration in *S. sclerotiorum* in a dose-dependant manner within concentrations to 1.0 mM. In contrast to the inducing effect of $H_2O_2$ on the AP respiration rate, it did not bring about change in the CP capacity of both *S. sclerotiorum* isolates (Fig. 2).

### Effects of treatment with $H_2O_2$ on growth rate of *S. sclerotiorum*

Treatment with 1.0 mM $H_2O_2$ greatly decreased the growth rate of mycelia of both *S. sclerotiorum* isolates on PDA plates (Fig. 4). Treatment with 1.0 mM $H_2O_2$ together with 2.5 mM SHAM caused a stronger decrease in the growth rate of the mycelia than 1.0 mM $H_2O_2$ or 2.5 mM SHAM alone (Fig. 4).

### Effects of treatment with $H_2O_2$ on sclerotia formation of *S. sclerotiorum*

Under treatment with 1.0 mM $H_2O_2$, *S. sclerotiorum* could still form sclerotia. However, $H_2O_2$ treatment delayed the formation of sclerotia of both tested *S. sclerotiorum* isolates on PDA plates (Fig. 7).

### Effects of treatment with $H_2O_2$ on *aox* gene expression level in *S. sclerotiorum* mycelia

Fragments of 809 bases of the *aox* mRNA of *S. sclerotiorum* were cloned and the two isolates showed the same sequences, but differed by 1 base from the registered *aox* mRNA of *S. sclerotiorum* 1980 UF-70 in GenBank (accession no. XM_001596610.1) (Fig. 1). Correspondingly, the amino acid

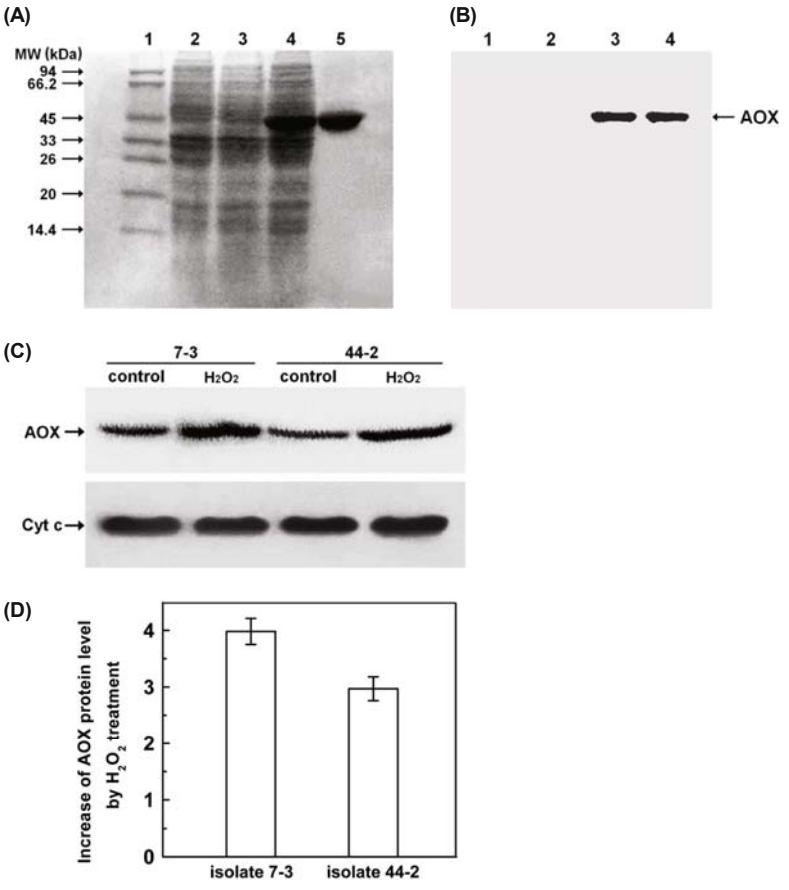


**Fig. 9. (A) Prokaryotic expression of part of *aox* mRNA of *S. sclerotiorum* isolate 7-3 (the underlined sequence in Fig. 1) encoding the carboxyl terminal region of alternative oxidase (AOX).** Lanes: 1, protein molecular weight marker; 2, extract from the *E. coli* strain BL21(DE3); 3, extract from the *E. coli* strain BL21(DE3) harboring the recombinant vector, but without IPTG induction; 4, extract from the *E. coli* strain BL21(DE3) harboring the recombinant vector and induced with IPTG; 5, purified expressed protein (2 μg). (B) Western blot to verify the prepared antibody. Lanes: 1, extract from the *E. coli* strain BL21 (DE3); 2, extract from the *E. coli* strain BL21(DE3) harboring the recombinant vector, but without IPTG induction; 3, extract from the *E. coli* strain BL21 (DE3) harboring the recombinant vector and induced with IPTG; 4, purified expressed protein (2 μg). (C) Western blot to detect the AOX and cytochrome c (Cyt c) proteins in mitochondrial samples purified from mycelia of *S. sclerotiorum*. Both *S. sclerotiorum* isolates were treated with 1.0 mM $H_2O_2$ and cultured in potato dextrose liquid media for 60 min. Each lane was loaded with 25 μg of mitochondrial protein. AOX was detected with the prepared antibody and Cyt c was detected with polyclonal antibody against Cyt c. Experiments were carried out three times and representative results are shown. (D) Increase of AOX protein level by $H_2O_2$ treatment in mycelia of *S. sclerotiorum*. Densitometry values of the immunoreactive bands were quantified using a GS-700 imaging densitometer (Bio-Rad). After normalization with density of the Cyt c band from the same mitochondrial protein sample, densities of AOX bands were used to calculate the increase of AOX protein level by $H_2O_2$ treatment. Means and standard errors are shown (n=3).

Asp[293] in the AOX of *S. sclerotiorum* 1980 UF-70 was changed into Gly in the AOX of *S. sclerotiorum* isolates 7-3 and 44-2.

Treatment with $H_2O_2$ at 0.20, 1.0, or 5.0 mM obviously increased the *aox* mRNA level in mycelia of *S. sclerotiorum* (Fig. 8). $H_2O_2$ at 1.0 mM showed a greater increase than that at 0.20 mM, but $H_2O_2$ at 5.0 mM showed a lower effect than 1.0 mM $H_2O_2$.

Part of the *aox* mRNA of *S. sclerotiorum* isolate 7-3 encoding the carboxyl terminal region of AOX was expressed in *E. coli* and yielded a protein of about 45 kDa (Fig. 9A). Antibody was prepared against the expressed protein (Fig. 9B). With the prepared antibody and mycelia cultured in PD liquid medium, Western blot results indicated that both *S. sclerotiorum* isolates had obvious AOX expression under normal culture conditions (Fig. 9C), which is consistent with their notable AP respiration capacity under these conditions (Fig. 2). What's more, it was found that treatment with 1.0 mM $H_2O_2$ obviously increased the AOX protein level in both *S. sclerotiorum* isolates cultured in liquid medium (Figs. 9C and 9D).

## Discussion

To date, most of the information on AOX in fungi has been from research on a few fungi, such as *H. anomala*, *N. crassa*, *M. grisea*, *U. maydis*, *C. neoformans*, and *P. brasiliensis* (Minagawa and Yoshimoto, 1987; Lambowitz *et al.*, 1989; Yukioka *et al.*, 1998; Akhter *et al.*, 2003; Juarez *et al.*, 2006; Martins *et al.*, 2011). Under conditions without treatment by antimycin A, an inhibitor of CP, the AP respiration was negligible in cells of *H. anomala*. Correspondingly, addition of 2 mM SHAM to growth medium had no effect on its cell growth (Minagawa and Yoshimoto, 1987). Similarly in *N. crassa*, under standard growth conditions without inducers, little or no AOX activity was detected in wild-type strains, and the AOX polypeptides were barely visible by Western blot (Lambowitz *et al.*, 1989). Similarly in *M. grisea*, without treatment with hydrogen peroxide or the fungicide SSF-126 [(E)-2-methoxyimino-N-methyl-2-(2-phenoxyphenyl)-acetamide], which interacts with the cytochrome bc1 complex in CP of mitochondria, little AOX was expressed and the AP respiration was negligible in cells of this fungus (Yukioka *et al.*, 1998). In contrast, *U. maydis* had obvious AP respiration capacity and AOX expression under normal culture conditions (i.e. normal growth temperature and medium richness) (Juarez *et al.*, 2006). Here in this report it was found that both *S. sclerotiorum* isolates also possessed notable AP respiration capacity under normal culture conditions (i.e. without $H_2O_2$ treatment) (Fig. 2), and AOX was obviously expressed in their mycelia (Fig. 9C). Inhibition of AOX with SHAM greatly decreased the mycelial growth rate of *S. sclerotiorum* during culture both in liquid media and on PDA plates (Figs. 3, 4, and 5), and delayed formation of sclerotia on PDA plates (Fig. 7). Therefore, based on the existence and importance of AOX during mycelial growth under normal conditions, fungi may be divided into two groups: in group 1, AOX is negligibly expressed and is not important during normal mycelial growth (e.g. *H. anomala*, *N. crassa*, and *M. grisea*); and in group 2, AOX is obviously expressed and is very important during normal mycelial growth (e.g. *U. maydis* and *S. sclerotiorum*).

As the AP is non-phosphorylating, operation of this pathway in mycelia of *S. sclerotiorum* under normal conditions may cause energy waste. Then, why is AOX still obviously expressed during normal mycelial growth? In higher plants, a hypothesis was proposed that under certain necessary conditions (e.g. when cytosolic energy status is high), operation of AP by AOX *in vivo* can allow glycolysis and the TCA cycle to continue, providing carbon skeletons for biosynthetic reactions in the cell (Day *et al.*, 1995). Therefore, the obvious expression of AOX and the operation of the AP in mycelia of *S. sclerotiorum* under normal conditions may reflect a strong need for carbon skeletons to support biosynthetic reactions in mycelia of the fungus.

Superoxide has been shown to induce the expression of AOX in *H. anomala* (Minagawa *et al.*, 1992). Hydrogen peroxide was observed to obviously induce AOX expression in *P. brasiliensis* (Martins *et al.*, 2011), and induce AOX expression and AP capacity in *M. grisea* (Yukioka *et al.*, 1998), and *S. sclerotiorum* (Figs. 2, 8, 9C, and 9D). The *aox1* mutant strain of *C. neoformans* was found to be more sensitive to the oxidative stressor tert-butyl hydroperoxide (Akhter *et al.*, 2003). These results indicate that ROS can work to activate fungal AOX gene expression, no matter whether its original expression is obvious or not. In higher plants, application of exogenous ROS, such as hydrogen peroxide, has also been confirmed to induce AOX expression (Wagner, 1995; Liang and Liang, 2002; Liang *et al.*, 2002). Inhibition of AOX in mitochondria purified from the pepper (*Capsicum annuum* L.) enhanced superoxide production (Purvis, 1997). Using transgenic cultured cells of the tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) with altered levels of AOX, AOX was confirmed to play a role in lowering mitochondrial ROS formation in higher plant cells (Maxwell *et al.*, 1999). Therefore, ROS may be common messengers for induction of AOX expression and AOX is involved in resistance to oxidative stress in both fungi and higher plants.

The AOX of higher plants has been confirmed to exist as a monomer or homodimer. The monomer is the active form and can be activated by a-keto acids such as pyruvate (Rhoads *et al.*, 1998). But when two subunits are covalently linked by a disulfide bond (i.e. oxidized), the enzyme is then in the form of homodimer, which is essentially inactive and cannot be activated by a-keto acids (Umbach and Siedow, 1993; Rhoads *et al.*, 1998; Liang *et al.*, 2003). In contrast, the AOX of fungi has been confirmed to exist only as a monomer and cannot be activated by a-keto acids (Joseph-Horne *et al.*, 2000; Umbach and Siedow, 2000). The evidence indicating the involvement of AOX in oxidative stress resistance in *H. anomala* (Minagawa *et al.*, 1992), *M. grisea* (Yukioka *et al.*, 1998), *C. neoformans* (Akhter *et al.*, 2003), *P. brasiliensis* (Martins *et al.*, 2011), and *S. sclerotiorum* suggests that absence of activation by a-keto acids does not affect its role in oxidative resistance.

It was found that the expression of *aox* of *P. brasiliensis* was developmentally regulated through the differentiation from the mycelial to the yeast form, and this differentiation was delayed by inhibition of AOX with SHAM (Martins *et*

*al*., 2011). Our results showed that treatment with SHAM to inhibit AOX induced many more aerial mycelia of *S. sclerotiorum* on PDA plates (Fig. 6), which indicates that AOX is involved in the growth pattern regulation of this fungus. Inhibition of AOX might increase oxidative level in cells. But as $H_2O_2$ treatment did not induce aerial mycelia of *S. sclerotiorum* on PDA plates (data not shown), induction of aerial mycelia by inhibition of AOX seemed not to result from oxidative stress. There is a proposal that AOX in higher plants not only acts as a terminal oxidase, but also may be an initiator of cellular reprogramming to adapt to changes in the environment (Clifton *et al*., 2006). More studies are needed to determine the reason and the physiological significance of the regulatory effect of AOX on fungal development.

The *aox1* mutant strain of *C. neoformans* was significantly less virulent than both the wild type and the reconstituted strain in the murine inhalational model, and also had significantly impaired growth within a macrophage-like cell line, which suggests that AOX contributes to the virulence composite of this organism, possibly by improving survival within phagocytic cells (Akhter *et al*., 2003). However, AP-deficient mutants of *M. grisea* with disrupted *aox* gene retained their pathogenicity without significant impairment of virulence while infecting barley leaves (Avila-Adame and Koller, 2002). It is still unclear whether AOX functions in the pathogenicity of *S. sclerotiorum* while infecting its host plants.

There may be important agricultural applications to elucidate the expression properties of AOX in phytopathogenic fungi. For example, quinol oxidation [Qo]-inhibiting fungicides refer to a broad-spectrum class of fungicides that include many current commercial products, such as azoxystrobin, kresoxim-methyl, and metominostrobin. These fungicides act by inhibiting fungal respiration through binding to the Qo center of cytochrome b. Some fungi resistant to Qo-inhibiting fungicides have been reported (Avila-Adame and Koller, 2003; Ishii *et al*., 2009). A part of the resistances has been ascribed to the G143A or G143S mutation of cytochrome b. In addition, as electron transport can be fulfilled by AP when CP is blocked, the expression of AOX has also been found to reduce the sensitivity of fungi to this type of fungicide, although the impact of this resistance by AOX still needs more elucidation (Avila-Adame and Koller, 2003). As AOX is distinctly expressed and an obvious AP respiration exists in mycelia of *S. sclerotiorum* grown under normal conditions, we may need to be more cautious in using Qo-inhibiting fungicides on *S. sclerotiorum* compared to its use on those fungi that barely express AOX and show little AP respiration during normal growth conditions. In fact, AOX has been confirmed to decrease the sensitivity of *S. sclerotiorum* to the Qo-inhibiting fungicide azoxystrobin (unpublished data).

Treatment with 1.0 mM $H_2O_2$ was observed to delay the formation of sclerotia of both tested *S. sclerotiorum* isolates on PDA plates (Fig. 7). The effect of ROS on sclerotia development of *S. sclerotiorum* was also studied recently by Papapostolou and Georgiou (2010a, 2010b). They found that the intra / extracellular $H_2O_2$ level and catalase activity in the sclerotiogenic *S. sclerotiorum* were significantly higher and lower, respectively, than those of the non-differentiating counterpart. Tiron was used as superoxide dismutase mimetic to reduce the superoxide radical level and a decrease in the sclerotial differentiation was observed. The effect of ROS on sclerotia development of *S. sclerotiorum* observed by Papapostolou and Georgiou (2010a, 2010b) seems to be different from that in this report. The reason for the difference remains to be determined.

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