Deletion of Cu/Zn Superoxide Dismutase Gene *sodC* Reduces *Aspergillus niger* Virulence on Chinese White Pear

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ABSTRACT. *Aspergillus niger* is a common pathogenic fungus causing postharvest rot of fruit and vegetable, whereas the knowledge on virulence factors is very limited. Superoxide dismutase [SOD (EC 1.15.1.10)] is an important metal enzyme in fungal defense against oxidative damage. Thus, we try to study whether Cu/Zn-SOD is a virulence factor in *A. niger*. Cu/Zn-SOD encoding gene *sodC* was deleted in *A. niger* [MA70.15 (wild type)] by homologous recombination. The deletion of *sodC* led to decreased SOD activity in *A. niger*, suggesting that *sodC* did contribute to full enzyme activity. Δ *sodC* strain showed normal mycelia growth and sporulation compared with wild type. However, *sodC* deletion markedly increased the cell's sensitivity to intracellular superoxide anion generator menadione. Besides, spore germination under menadione and H₂O₂ stresses were significantly retarded in Δ *sodC* mutant compared with wild type. Further results showed that *sodC* deletion induced higher superoxide anion production and higher content of H₂O₂ and malondialdehyde (MDA) compared with wild type, supporting the role of SOD in metabolism of reactive oxygen species (ROS). Furthermore, Δ *sodC* mutant had a reduced virulence on chinese white pear (*Pyrus bretschneideri*) as lesion development by Δ *sodC* was significantly less than wild type. The determination of superoxide anion, H₂O₂, and MDA in *A. niger*-infected pear showed that chinese white pear infected with Δ *sodC* accumulated less superoxide anion, H₂O₂, and MDA compared with that of wild type *A. niger*, implying that Δ *sodC* induced an attenuated response in chinese white pear during fruit–pathogen interaction. Our results indicate that *sodC* gene contributes to the full virulence of *A. niger* during infection on fruit. *Aspergillus niger* is one of the most common species found in fungal communities. It is an important fermentation industrial strain and is also known to cause the most severe symptoms in fruit during long-term storage (Pel et al., 2007). Meanwhile, plants activate their signaling pathways to trigger defense responses to limit pathogen expansion. One of the earliest host responses after pathogen attack is oxidative burst, during which large quantities of ROS are generated by different host enzyme systems, such as glucose oxidase (Govrin and Levine, 2000). ROS such as singlet oxygen, superoxide anion, hydroxyl (OH[•]), and H₂O₂ are released to hinder the advance of pathogens (Gara et al., 2003). ROS can react with and damage cellular molecules, such as DNA, protein, and lipids, which will limit fungal propagation in the host plant (Apel and Hirt, 2004).

Many antioxidant enzymes in fungal pathogens participate in reactive oxygen degradation on plant-released ROS. Among them, SODs are metalloenzymes that form the primary cellular

antioxidant defense system by catalyzing the superoxide disproportionation (a type of redox reaction) to produce molecular oxygen and H₂O₂, which is further catalyzed by peroxidase and catalase (Vallino et al., 2009). SOD activity is usually induced along with overproduction of superoxide anions (Benes et al., 1995; Gupta et al., 1993). Cu/Zn-SOD is a kind of SOD, which is commonly present in the cytoplasm of eukaryotic cells. It is composed of two subunits which contain one Cu²⁺ and one Zn²⁺, respectively. Some progress has been made in the research of Cu/Zn-SOD (SOD1, also known as

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CCS1) in *Saccharomyces cerevisiae* and *Candida albicans* (Gleason et al., 2014). In *S. cerevisiae*, a mutant with deletion of Cu/Zn-SOD is oxygen-sensitive, hypermutable, defective in sporulation (Liu et al., 1992). In the major human fungal pathogen *C. albicans*, Cu/Zn-SOD mutant shows delayed hyphal growth on Spider medium (a medium to stimulate hyphal growth and colony wrinkling in *C. albicans*) and higher sensitivity to menadione, a redox-cycling agent (Hwang et al., 2002). The deletion of *bcsod1* in the necrotrophic fungal pathogen *Botrytis cinerea* causes smaller lesion on pea (*Pisum sativum*) leaves, which proves that this gene is essential for full virulence of *B. cinerea* on plants (Rolke et al., 2004). However, Moore et al. (2002) found that the Cu/Zn SOD *cpsod1* gene in *Claviceps purpurea* is not essential for pathogenicity in rye (*Secale cereal*), and deletion of *cpsod1* caused no increased sensitivity to paraquat, a generator of superoxide, suggesting that the function of Cu/Zn SOD in fungi is not always conserved or the functions of relative genes are redundant. In *A. niger*, we identified *sodC* as a homology of *SOD1* in *S. cerevisiae*. However, how *A. niger* induces responses in fruit and whether *sodC* is a virulence factor for *A. niger* infection on fruit are still unclear.

Some molecular biological manipulation techniques for filamentous fungi have been applied in *A. niger*, such as gene knockout, transformation, and dominant selectable markers (Delmas et al., 2014). In this work, we constructed the deletion mutant Δ *sodC* by homologous recombination. Then, the role of *sodC* gene in fungal resistance to oxidative stress was examined, and the infection ability of Δ *sodC* mutant on chinese white pear fruit was analyzed.

Materials and Methods

STRAINS, PLASMID, AND GROWTH CONDITIONS. *Aspergillus niger* MA 70.15 (Δ *pyrG*, *pyrG* encodes orotidine-5-phosphate decarboxylase, cell lacking this enzyme cannot grow without exogenous uridine, but can resist toxicity of 5-fluoroorotic acid) was used as wild type strain in this work. Generally, *A. niger* was grown on potato dextrose agar medium supplemented with uridine {PDA-Uri [200 g·L⁻¹ potato (*Solanum tuberosum*), 20 g·L⁻¹ agar, 20 g·L⁻¹ glucose, 10 mmol·L⁻¹ uridine]} at 28 °C or liquid PDA-Uri medium.

CONSTRUCTION OF Δ *sodC* MUTANT. The gene *sodC* deletion mutant was constructed according to the procedure by Delmas et al. (2014). To delete the *sodC* gene in *A. niger*, upstream and downstream fragments AB (902 bp) and CD (502 bp) were amplified by polymerase chain reaction (PCR) from *A. niger* MA70.15 genomic DNA. The upstream and downstream fragments contained a common restriction site *Hind*III which was used to ligate the two fragments together and restriction sites *Eco*RI and *Xho*I, respectively. *Eco*RI and *Xho*I restriction sites on the ligated fragment ABCD were used for cloning into the plasmid pC3 (Delmas et al., 2014) to create pC3-*An_ΔsodC* integrative plasmid. Primers are shown as following (bp with underline indicates restriction site): *sodC*-A (5'-CCGGAATT-CATCCAGTGGGTGACCAAGTA-3'); *sodC*-B (5'-CTCAAAGCTTCACCTCCAGAATGCTACTTT-3'); *sodC*-C (5'-GGTGAAGCTTTGAGTAGTCTGCCATTATCCC-3'); and *sodC*-D (5'-CCGCTCGAGGGCTCTTTCCCGATCATAT-3').

Then, the pC3-*An_ΔsodC* integrative plasmid was transformed into the *A. niger* MA70.15 (Δ *pyrG*) strain by polyethylene glycol-mediated transformation of protoplasts according to Ballance and Turner (1985). The gene *pyrG*

encoding for the orotidine-5-phosphate decarboxylase was chosen as a counter-selection marker (Boeke et al., 1984). Transformed clones were confirmed by PCR of genomic DNA to identify the *sodC* deletion mutant (Delmas et al., 2014).

DNA EXTRACTION AND THE CONFIRMATION OF Δ *sodC* STRAIN. Genomic DNA of *A. niger* was extracted using Master Pure Yeast DNA Purification Kit (Epicentre, Madison, WI). Primers *sodC*-A and *sodC*-D flanking the *sodC* gene were used as external primers, and primers *sodC*-F (5'-TCGTTGGATCT-CGTGGTT-3') and *sodC*-R (5'-TCTTGGACTCCTCGTTGC-3') designed on the open reading frame (ORF) of *sodC* gene were used to determine whether the *sodC* gene was deleted or not.

RNA EXTRACTION AND QUANTITATIVE REVERSE TRANSCRIPTASE-PCR (q-PCR). Conidia at 0.1 g of the wild type *A. niger* MA 70.15 and Δ *sodC* grown on PDA-Uri solid medium for 5 d were harvested. Then, they were ground to powder under liquid nitrogen. Total RNA and cDNA were obtained according to the instructions of RNAiso plus (TaKaRa, Tokyo, Japan) (van Leeuwen et al., 2013) and Prime Script RT Master Mix (TaKaRa), respectively. The procedure of q-PCR was as follows: denaturation at 95 °C for 3 min, followed by cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s, cycled for 40 times. The primers used for q-PCR were shown below: *sodC*-S (5'-GAGT-AGTCTGCCATTATCCC-3') and *sodC*-A (5'-CTCGAGGCT-CTTCCCGATCATAT-3'). The expression of actin-encoding gene *An03e03160* was used as a control (Raulo et al., 2016). The primers used for actin-encoding gene were actin-F (5'-ACG-CTTGGACTGTGCCTC-3') and actin-R (5'-CAATGGTT-CGGGTATGTGC-3').

DETERMINATION OF SOD ACTIVITY. Mycelia of the wild type *A. niger* MA 70.15 and Δ *sodC* grown in PDA-Uri liquid medium for 3 d were harvested and washed three times with sterile distilled water. Frozen *A. niger* mycelia at 1 g was ground to powder under liquid nitrogen using a mortar and pestle and 50 mM ice-cold phosphate buffer (pH 7.8, 1 mM EDTA, 1 mM PMSF) was added to 5 mL. The mixture was centrifuged at 12,000 g_n at 4 °C for 10 min to obtain the supernatant which was used for the determination of SOD activity by procedures described by Beauchamp and Fridovich (1971). One SOD unit was defined as the amount of enzyme that inhibits the rate of nitroblue tetrazolium reduction by 50%. Activity was expressed as units per milligram protein. Protein content was measured according to Bradford (1976) using bovine serum albumin as standard.

GROWTH ANALYSIS OF *A. NIGER*. The conidia of the wild type *A. niger* MA 70.15 and Δ *sodC* grown on PDA-Uri solid medium for 5 d were harvested. After removal of mycelia, spore concentration was measured using a haemocytometer and adjusted to the final concentration of 10⁶ spores/mL (Lazar et al., 2008). Then, 5-μL spore suspension of the wild type and Δ *sodC* were inoculated on PDA-Uri solid medium and medium supplemented with 0.02 mM menadione and were cultured at 28 °C in darkness for 4 d. Colony diameter of *A. niger* was determined every 24 h.

The spore suspension (10⁶ spores/mL) of wild type *A. niger* and Δ *sodC* at 200 μL were inoculated to 20-mL PDA-Uri liquid medium, respectively, and were cultured at 28 °C for 150 rpm in the thermostatic shaker. Dry weight of the mycelia of the *A. niger* was measured every 12 h according to the method in (Angelova et al., 2005).

DETERMINATION OF SPORE GERMINATION. Ten microliters of spore suspension (10⁶ spores/mL) for the wild type *A. niger* and

$\Delta sodC$ were placed on a 7-mm-diameter plug of PDA-Uri supplemented with freshly-prepared 0.02 mM menadione and 2 mM H_2O_2 which were then placed on a glass slide. Menadione and H_2O_2 were added to PDA-Uri agar medium when the autoclaved medium was cooled down to 60 °C. Each plug-slide was held in a petri dish with moistened filter paper and then was incubated at 28 °C. Conidia germination (germ tube longer than two times the diameter of the corresponding spore) of the wild type *A. niger* and $\Delta sodC$ which were in PDA-Uri culture medium (for 16 h), medium supplemented with 2 mM H_2O_2 (for 24 h), or medium supplemented with 0.02 mM menadione (for 30 h) were assessed under a compound microscope at 400 $\times$ total magnification with 40 $\times$ of the objective lens and 10 $\times$ of eyepiece lens (Fu et al., 2014).

DETERMINATION OF SUPEROXIDE ANION PRODUCTION AND CONTENT OF H_2O_2 AND MDA IN *A. NIGER* AND CHINESE WHITE PEAR SUBJECTED TO *A. NIGER* INFECTION. A volume of 200- μ L spore suspension (10^6 spores/mL) of wild type and $\Delta sodC$ was inoculated to 20-mL PDA-Uri liquid medium and cultured at 28 °C for 3 d. Then, the medium was supplemented with 0.02 mM menadione and sampled at 12 and 24 h. Freshly cultured *A. niger* mycelia at 1 g was ground to the powder under liquid nitrogen using a mortar and pestle. After *A. niger* inoculation on chinese white pear fruit for 3 d, pear flesh 2 cm far from lesion was sampled for assay and uninoculated pear was used as control. Content of superoxide anion, H_2O_2 , and MDA were determined according to the methods described by Hu et al. (2012).

INFECTION ABILITY OF *A. NIGER* ON CHINESE WHITE PEAR. Chinese white pear fruit were supplied by the Anhui Academy of Agricultural Sciences, Hefei, Anhui, China. Unwounded, healthy fruit of uniform size, color, and weight were selected for experiments. Chinese white pear fruit were washed with tap water and surface-sterilized with 75% ethanol. Then, the surface of the fruit was wounded at 5 sites on each fruit. After air-drying, each of

Fig. 1. The identification of the deletion mutant $\Delta sodC$ and the assays of *sodC* gene expression and superoxide dismutases (SOD) activity in $\Delta sodC$ mutant. (A) Confirmation of *sodC* deletion mutant by polymerase chain reaction (PCR) analysis of genomic DNA from wild type *Aspergillus niger* MA 70.15 and $\Delta sodC$. Lane 1 and 2, genomic PCR of *A. niger* MA 70.15 and $\Delta sodC$ with external primers respectively; lane 3 and 4, genomic PCR of *A. niger* MA 70.15, and $\Delta sodC$ with internal primers, respectively. (B) The expression of *sodC* gene in wild type *A. niger* MA 70.15 (WT, wild type) and $\Delta sodC$. (C) The activity assay of SOD in wild type *A. niger* MA 70.15 (WT) and $\Delta sodC$ mutant. Values are mean $\pm$ SD ($n = 3$). The symbols * and ** in this figure and following ones stand for a significant difference between two data at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 2. Growth analysis of $\Delta sodC$ mutant on potato dextrose agar medium supplemented with uridine (PDA-Uri). (A) The mycelium growth of the wild type *Aspergillus niger* MA 70.15 (WT) and $\Delta sodC$ in the PDA-Uri solid medium for 96 h. (B) The colony diameter of wild type *A. niger* MA 70.15 (WT) and the $\Delta sodC$ in A. Values are mean $\pm$ SD ($n = 9$). (C) The biomass of the wild type *A. niger* MA 70.15 (WT) and the $\Delta sodC$ grown in PDA-Uri liquid culture medium for 96 h. Values are mean $\pm$ SD ($n = 3$).

the wounds (2 mm diameter and 4 mm deep) was injected with 10 μ L of spore suspension (10^6 spores/mL) of wild type *A. niger* and Δ sodC. Each strain was inoculated on three different fruit. Chinese white pear fruit were stored at 25 °C in sealed containers with 200 mL of distilled water on the bottom to maintain the relative humidity which ranged from 85% to 90% (Fu et al., 2014). The lesion diameter caused by *A. niger* was measured every 24 h.

STATISTICAL ANALYSIS. The data in the article are based on three replicates. Statistical significance was tested by one-way analysis of variance (ANOVA) using SPSS Statistics (version 20.0; IBM Corp., Armonk, NY), and the results were expressed as the mean $\pm$ SD. The least significant difference test was performed on all data after ANOVA tests to test for significant ($P < 0.05$ or $P < 0.01$) differences between treatments.

Results

CONSTRUCTION OF THE Δ SODC DELETION MUTANT. The plasmid pC3-*An_ΔsodC* was transformed into *A. niger* MA 70.15 protoplasts. Then, the genomic DNA of transformants was extracted to identify *sodC* deletion mutants. As shown in Fig. 1A, the deletion mutant Δ sodC were confirmed by PCR with the primers sodC-A and sodC-D which flanking *sodC* gene and with primers sodC-F and sodC-R on the ORF of *sodC* gene. Thus, for the genome of wild type, the PCR product on lane 1 is about 2450 bp, and about 1400 bp for lane 2. Because *sodC* gene is deleted in Δ sodC mutant, PCR product using external primers sodC-A and sodC-D on lane 3 is about 650 bp and there is no band on lane 4, confirming that *sodC* was deleted in the mutants. Then, the gene expression of *sodC* in wild type and *sodC* deletion mutant was analyzed. As shown in Fig. 1B, *sodC* gene expression was not detected compared with the high expression in wild type, further confirming that *sodC* gene was deleted in the mutant. To study whether *sodC* gene actually contributes to SOD activity in the *A. niger*, total SOD activity in Δ sodC was determined and compared with that in wild type. As expected, SOD activity in Δ sodC mutant

was significantly lower than that of wild type *A. niger* (Fig. 1C).

GROWTH ANALYSIS OF Δ SODC MUTANT. To evaluate whether the lack of *sodC* would affect fungal growth, we compared growth

Fig. 3. The effect of menadione on the growth of the wild type *Aspergillus niger* MA 70.15 (WT) and Δ sodC mutant. (A) The mycelium growth of the wild type *A. niger* MA 70.15 (WT) and Δ sodC in potato dextrose agar medium supplemented with uridine (PDA-Uri) and PDA-Uri medium supplemented with 0.02 mM menadione for 4 d. (B) The colony diameter of wild type *A. niger* MA 70.15 (WT) and Δ sodC in A. Values are mean $\pm$ SD ($n = 9$).

Fig. 4. The effect of menadione and H₂O₂ on the spore germination of Δ sodC. (A) The spore germination of the wild type *Aspergillus niger* MA 70.15 (WT) and Δ sodC mutant in potato dextrose agar medium supplemented with uridine (PDA-Uri) solid medium (16 h), PDA-Uri solid medium supplemented with 2 mM H₂O₂ (24 h), and supplemented with 0.02 mM menadione (30 h); bar = 100 μ m. (B) Spore germination percentage of the wild type *A. niger* MA 70.15 (WT) and Δ sodC in A. Values are mean $\pm$ SD ($n = 3$).

of $\Delta sodC$ and *A. niger* MA 70.15 on PDA-Uridine medium. As shown in Fig. 2A, within 96 h, there was no difference in the mycelial growth found between the wild type and $\Delta sodC$ on PDA-Uridine solid medium. Colony diameter in the wild type and the $\Delta sodC$ also show that there is no difference in growth (Fig. 2B). Figure 2C shows that $\Delta sodC$ mutant grew normally in PDA-Uridine liquid medium for 96 h in comparison with wild type.

SODC IN *A. NIGER* CONFERS RESISTANCE TO REACTIVE OXYGEN SPECIES. Menadione, a superoxide generating agent, was supplemented to PDA-Uridine medium to evaluate whether the deletion of *sodC* affects the adaption of cells to oxidative stress. As shown in Fig. 3A, the mycelium growth of $\Delta sodC$ was significantly retarded compared with wild type under 0.02 mM menadione. Figure 3B shows that the colony diameter of $\Delta sodC$ was significantly smaller than that of wild type *A. niger*. However, $\Delta sodC$ mutant showed no higher sensitivity to 2 mM H_2O_2 stress compared with wild type (data not shown).

To further analyze the reduced growth of $\Delta sodC$ under menadione stress, spore germination of wild type *A. niger* and $\Delta sodC$ was analyzed under 0.02 mM menadione and 2 mM H_2O_2 stress. As shown in Fig. 4A and B, after 16 h of incubation in control conditions, the spores of the wild type and $\Delta sodC$ all germinated. Under 0.02 mM menadione and 2 mM H_2O_2 stress for 30 and 24 h, respectively, the hyphae were obviously shorter than that in normal conditions, suggesting that 0.02 mM menadione and 2 mM H_2O_2 effectively influenced hyphal growth of *A. niger*. After 30 h of 0.02 mM menadione stress, spore germination percentage of wild type reached 93%, whereas that of $\Delta sodC$ was about 27%. Besides, spore germination percentage of $\Delta sodC$ with 2 mM H_2O_2 stress for 24 h was 91%, which was significantly lower than 99% of the wild type *A. niger*.

DELETION OF SODC ATTENUATES ROS METABOLISM IN *A. NIGER*. To verify the enzymatic activity of *sodC*, superoxide anion production and contents of H_2O_2 and MDA were determined in menadione-treated *A. niger*. As shown in Fig. 5A, superoxide anion production in wild type *A. niger* increased with treatment time of menadione. Meanwhile,

menadione induced significantly higher production of superoxide anion in $\Delta sodC$ cells during the entire treatment. The significant higher level of superoxide anion was also observed in untreated $\Delta sodC$ cells, suggesting that $\Delta sodC$ cells may have

Fig. 5. The effect of menadione on the superoxide anion production (A) and content H_2O_2 (B) and malondialdehyde (MDA) (C) in *Aspergillus niger* MA 70.15 (WT) and $\Delta sodC$ were subjected to 0.02 mM menadione stress and sampled at 12 and 24 h in potato dextrose agar medium supplemented with uridine (PDA-Uridine) liquid medium. Values are mean $\pm$ SD ($n = 3$).

Fig. 6. Pathogenicity of $\Delta sodC$ mutant on *Pyrus bretschneideri*. (A) Photographs of *P. bretschneideri* fruit inoculated with the spores of wild type *Aspergillus niger* MA 70.15 (WT) and the $\Delta sodC$ for 5 d. (B) Lesion diameter caused by wild type *A. niger* MA 70.15 (WT) and $\Delta sodC$ on *P. bretschneideri* fruit as shown in A. Values are mean $\pm$ SD ($n = 15$).

defect in decomposing background level of superoxide anion (Fig. 5A). Besides, menadione also induced higher level of H_2O_2 in $\Delta sodC$ cells in comparison with wild type cells (Fig. 5B). MDA, which is an index of lipid peroxidation, was determined in menadione-treated cell. As shown in Fig. 5C, the content of MDA in wild type increased slightly along with treatment time; however, MDA accumulated more in $\Delta sodC$ cells.

SODC GENE IS INDISPENSIBLE FOR THE FULL VIRULENCE OF *A. NIGER*. To evaluate whether *sodC* gene is involved in the virulence of *A. niger* on fruit, spores were inoculated on chinese white pear fruit. As shown in (Fig. 6A and B), after being infected by *A. niger*, the lesion diameter caused by *A. niger* increased gradually. However, lesion diameter caused by $\Delta sodC$ mutant was significantly smaller than that of wild type, suggesting that *sodC* gene is indispensable for the full virulence of *A. niger*.

LACK OF SODC ALTERS ROS ACCUMULATION AND MDA IN FRUIT UPON *A. NIGER* INFECTION. To study the response of chinese white pear to wild type *A. niger* and $\Delta sodC$ cells, ROS metabolism was analyzed by the determination of superoxide anion production and contents of H_2O_2 and MDA. Superoxide anion production in chinese white pear fruit increased upon *A. niger* infection in both wild type and $\Delta sodC$, whereas wild type *A. niger* induced a stronger response of superoxide anion in comparison with $\Delta sodC$ (Fig. 7A). As shown in Fig. 7B, an accumulation of H_2O_2 occurred in chinese white pear infected with wild type *A. niger*, while the accumulation was attenuated in $\Delta sodC$ -inoculated chinese white pear fruit. Besides, *A. niger* inoculation increased the content of MDA in chinese white pear fruit subjected to both wild type and $\Delta sodC$. However, MDA content in $\Delta sodC$ -inoculated chinese white pear fruit was significantly lower than that of wild type (Fig. 7C).

Discussion

Aspergillus rot (black mold rot) is one of the most important post-harvest diseases of fruit and vegetable, and *A. niger* is known to cause the most severe symptoms (Hasan, 2000). Although a lot of work has been done to improve the ability of being a cell factory for a range of products, such as enzymes and organic acids (Archer, 2000), the knowledge on the factors determining its pathogenesis on fruit is very limited.

In this study, we indentified *sodC* gene as a Cu/Zn-SOD coding gene from *A. niger*. Cu/Zn-SODs are usually located in cytosol and can protect cells from oxidative stress (Holdom et al., 1996). *sodC* gene was deleted in *A. niger* by gene deletion via homologous recombination. The decreased SOD activity in $\Delta sodC$ mutant compared with wild type confirmed that *sodC* did

contribute to the total enzyme activity. As SOD is responsible for the superoxide disproportionation reaction during oxidative stress (Chary et al., 1994), we test the sensitivity of $\Delta sodC$ mutant to oxidative stress. Our results indicate that menadione, a generator of intracellular superoxide anion, could significantly inhibit the mycelium growth of $\Delta sodC$. Further results showed that spore germination of the $\Delta sodC$ was significantly inhibited by menadione and H_2O_2 compared with that of wild type (Fig. 4). All of these indicate that the ability of detoxifying superoxide radicals and even H_2O_2 was attenuated in $\Delta sodC$ mutant and *sodC* gene did contribute to cell's resistance to oxidative stress. Consistently, *B. cinerea* mutants with *SOD1* deletion showed increased sensitivity to ROS-generating agent paraquat (Rolke et al., 2004). Besides, *Sclerotinia sclerotiorum* mutation in *SsSOD1* showed growth rate and sclerotial formation similar to that of the wild type but showed increased sensitivity to heavy metal toxicity and oxidative stress (Xu and Chen, 2013). To further study the attenuated tolerance of $\Delta sodC$ toward menadione, superoxide anion production and contents of H_2O_2 , and MDA are determined in menadione-treated *A. niger*. It is found that ROS and MDA increased in *A. niger* with menadione treatment, while they accumulated more in $\Delta sodC$ cells (Fig. 5). Thus, we provide evidence that $\Delta sodC$ cells have defect in response to menadione stress because of failure in ROS decomposition and increased lipid peroxidation.

ROS burst occurs as one of the earliest cellular response after successful pathogen recognition (Torres et al., 2006). Accordingly, fungi pathogens need to activate their antioxidant

Fig. 7. Superoxide anion production (A) and content of H_2O_2 (B) and malondialdehyde (MDA) (C) in *Pyrus bretschneideri* inoculated with wild type *Aspergillus niger* MA 70.15 (WT) and $\Delta sodC$. *P. bretschneideri* was inoculated with *A. niger* MA 70.15 (WT) and $\Delta sodC$ and were sampled immediately (Control) or on day 3 postinoculation. Values are mean $\pm$ SD ($n = 3$).

defenses to cope with the oxidative stress encountered during infection. SODs constitute the primary cellular antioxidant defense system by detoxifying oxygen radicals and we propose that Cu/Zn-SOD might be a virulence factor for *A. niger* during infection on fruit. In this study, the spores of $\Delta sodC$ mutant were inoculated on chinese white pear fruit. The decreased lesion diameter caused by $\Delta sodC$ suggests that Cu/Zn-SOD contributes to the virulence of *A. niger* (Fig. 6). In normal conditions, antioxidant defense mechanisms are adequate to maintain ROS at basal level and *sodC* deletion did not make much difference on the growth of *A. niger*. During infection on fruit, *A. niger* may need full function of antioxidant system to cope with oxidative stress due to plant response. Cu/Zn-SOD in *A. niger* is required to detoxify dangerous level of superoxide from host's response to fungal infection and thus we observed the decreased virulence of $\Delta sodC$ mutant. Besides *sodC*, manganese SOD *sodB* in *A. niger* may also contribute to the decomposition of superoxide. There are also other examples of SOD that contribute to virulence in phytopathogenic fungi (Rolke et al., 2004). The deletion of *bcsod1* caused smaller lesion and proved that this gene is essential for full virulence of *B. cinerea* on *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) (López-Cruz et al., 2017; Rolke et al., 2004). Besides, it is also found that SOD1 contributes to the virulence of *Fusarium graminearum* in wheat (*Triticum aestivum*) head infection (Yao et al., 2016).

Fruit responses to *A. niger* infection is still unclear. In this work, ROS and MDA content are determined to evaluate whether similar oxidative burst occurs in chinese white pear in response to *A. niger* as observed in other fungi (López-Cruz et al., 2017; Torres et al., 2006). The increased levels of ROS and MDA confirm that *A. niger* infection induces ROS burst in fruits (Fig. 7). Meanwhile, we found that fruit response toward $\Delta sodC$ mutant is attenuated compared with wild type, suggesting the weakened virulence of $\Delta sodC$ cells. However, it is also reported that plants infected with $\Delta bcsod1$ accumulated less H_2O_2 and more superoxide anion than those infected with wild type (López-Cruz et al., 2017). *S. sclerotiorum* deletion in *Sssod1* showed reduced virulence on tomato and tobacco (*Nicotiana tabacum*), but the gene deletion induced a host oxidative burst in adjacent uninfected cells in contrast to reduced ROS production during wild-type infection (Veluchamy et al., 2012). These different findings might be due to different plant materials and different fungi used in researches.

In summary, we show that *sodC* gene in *A. niger* is necessary for the cells' tolerance to exogenous ROS. *sodC* deletion mutant caused reduced virulence on chinese white pear, which further support the hypothesis that SOD is important for the full virulence of *A. niger*.

Literature Cited

- Angelova, M.B., S.B. Pashova, B.K. Spasova, S.V. Vassilev, and L.S. Slokoska. 2005. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. *Mycol. Res.* 109:150–158.
- Apel, K. and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373–399.
- Archer, D.B. 2000. Filamentous fungi as microbial cell factories for food use. *Curr. Opin. Biotechnol.* 11:478–483.
- Ballance, D.J. and G. Turner. 1985. Development of a high-frequency transforming vector for *Aspergillus nidulans*. *Gene* 36:321–331.
- Beauchamp, C. and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44:276–287.
- Benes, S.E., T.M. Murphy, P.D. Anderson, and J.L.J. Houpsis. 1995. Relationship of antioxidant enzymes to ozone tolerance in branches of mature ponderosa pine (*Pinus ponderosa*) trees exposed to long-term, low concentration, ozone fumigation and acid precipitation. *Physiol. Plant.* 94:124–134.
- Boeke, J.D., F. LaCroute, and G.R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* 197:345–346.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Chary, P., D. Dillon, A.L. Schroeder, and D.O. Natvig. 1994. Superoxide dismutase (*sod-1*) null mutants of *Neurospora crassa*: Oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. *Genetics* 137:723–730.
- Delmas, S., A. Llanos, J.L. Parrou, M. Kokolski, S.T. Pullan, L. Shunburne, and D.B. Archer. 2014. Development of an unmarked gene deletion system for the filamentous fungi *Aspergillus niger* and *Talaromyces versatilis*. *Appl. Environ. Microbiol.* 80:3484–3487.
- Fu, L.H., K.D. Hu, L.Y. Hu, Y.H. Li, L.B. Hu, H. Yan, Y.S. Liu, and H. Zhang. 2014. An antifungal role of hydrogen sulfide on the postharvest pathogens *Aspergillus niger* and *Penicillium italicum*. *PLoS One* 9:e104206.
- Gara, L.D., M.C. de Pinto, and F. Tommasi. 2003. The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiol. Biochem.* 41:863–870.
- Gleason, J.E., C.X. Li, H.M. Odeh, and V.C. Culotta. 2014. Species-specific activation of Cu/Zn SOD by its CCS copper chaperone in the pathogenic yeast *Candida albicans*. *J. Biol. Inorg. Chem.* 19:595–603.
- Govrin, E.M. and A. Levine. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10:751–757.
- Gupta, A.S., R.P. Webb, A.S. Holaday, and R.D. Allen. 1993. Overexpression of superoxide dismutase protects plants from oxidative stress (induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants). *Plant Physiol.* 103:1067–1073.
- Hasan, H.A.H. 2000. Patulin and aflatoxin in brown rot lesion of apple fruit and their regulation. *World J. Microbiol. Biotechnol.* 16:607–612.
- Holdom, M.D., R.J. Hay, and A.J. Hamilton. 1996. The Cu,Zn superoxide dismutases of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus terreus*: Purification and biochemical comparison with the *Aspergillus fumigatus* Cu,Zn superoxide dismutase. *Infect. Immun.* 64:3326–3332.
- Hu, L.Y., S.L. Hu, J. Wu, Y.H. Li, J.L. Zheng, Z.J. Wei, J. Liu, H.L. Wang, Y.S. Liu, and H. Zhang. 2012. Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruit. *J. Agr. Food Chem.* 60:8684–8693.
- Hwang, C.S., G.E. Rhie, J.H. Oh, W.K. Huh, H.S. Yim, and S.O. Kang. 2002. Copper- and zinc-containing superoxide dismutase (Cu/Zn-SOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* 148:3705–3713.
- Lazar, E.E., R.B. Wills, B.T. Ho, A.M. Harris, and L.J. Spohr. 2008. Antifungal effect of gaseous nitric oxide on mycelium growth, sporulation and spore germination of the postharvest horticulture pathogens, *Aspergillus niger*, *Monilinia fructicola*, and *Penicillium italicum*. *Lett. Appl. Microbiol.* 46:688–692.
- Liu, X.F., I. Elashvili, E.B. Gralla, J.S. Valentine, P. Lapinskas, and V.C. Culotta. 1992. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. *J. Biol. Chem.* 267:18298–18302.
- López-Cruz, J., C.S. Óscar, F.C. Emma, G.A. Pilar, and G.B. Carmen. 2017. Absence of Cu-Zn superoxide dismutase BCSOD1 reduces *Botrytis cinerea* virulence in *Arabidopsis* and in tomato plants,

- revealing interplay among reactive oxygen species, callose and signalling pathways. *Mol. Plant Pathol.* 18:16–31.
- Moore, S., O.M. de Vries, and P. Tudzynski. 2002. The major Cu,Zn SOD of the phytopathogen *Claviceps purpurea* is not essential for pathogenicity. *Mol. Plant Pathol.* 3:9–22.
- Pel, H.J., J.H. de Winde, D.B. Archer, P.S. Dyer, G. Hofmann, P.J. Schaap, G. Turner, R.P. de Vries, R. Albang, K. Albermann, M.R. Andersen, J.D. Bendtsen, J.A. Benen, M. van den Berg, S. Breestraat, M.X. Caddick, R. Contreras, M. Cornell, P.M. Coutinho, E.G. Danchin, A.J. Debets, P. Dekker, P.W. van Dijk, A. van Dijk, L. Dijkhuizen, A.J. Driessen, C. d'Enfert, S. Geysens, C. Goosen, G.S. Groot, P.W. de Groot, T. Guillemette, B. Henrissat, M. Herweijer, J.P. van den Hombergh, C.A. van den Hondel, R.T. van der Heijden, R.M. van der Kaaij, F.M. Klis, H.J. Kools, C.P. Kubicek, P.A. van Kuyk, J. Lauber, X. Lu, M.J. van der Maarel, R. Meulenberg, H. Menke, M.A. Mortimer, J. Nielsen, S.G. Oliver, M. Olsthoorn, K. Pal, N.N. van Peij, A.F. Ram, U. Rinas, J.A. Roubos, C.M. Sagt, M. Schmoll, J. Sun, D. Ussery, J. Varga, W. Vervecken, P.J. van de Vondervoort, H. Wedler, H.A. Wösten, A.P. Zeng, A.J. van Ooyen, J. Visser, and H. Stam. 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* 25:221–231.
- Raulo, R., M. Kokolski, and D.B. Archer. 2016. The roles of the zinc finger transcription factors *XlnR*, *ClrA* and *ClrB* in the breakdown of lignocellulose by *Aspergillus niger*. *AMB Express* 6:5.
- Rolke, Y., S. Liu, T. Quidde, B. Williamson, A. Schouten, K.M. Weltring, V. Siewers, K.B. Tenberge, B. Tudzynski, and P. Tudzynski. 2004. Functional analysis of H₂O₂-generating systems in *Botrytis cinerea*: The major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on french bean, whereas a glucose oxidase (BCGOD1) is dispensable. *Mol. Plant Pathol.* 5:17–27.
- Torres, M.A., J.D.G. Jones, and J.L. Dangl. 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* 141:373–378.
- Vallino, M., E. Martino, F. Boella, C. Murat, M. Chiapello, and S. Perotto. 2009. Cu,Zn superoxide dismutase and zinc stress in the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn. *FEMS Microbiol. Lett.* 293:48–57.
- van Leeuwen, M.R., P. Krijgsheld, R. Bleichrodt, H. Menke, H. Stam, J. Stark, H.A. Wösten, and J. Dijksterhuis. 2013. Germination of conidia of *Aspergillus niger*, is accompanied by major changes in RNA profiles. *Stud. Mycol.* 74:59–70.
- Veluchamy, S., B. Williams, K. Kim, and M.B. Dickman. 2012. The CuZn superoxide dismutase from *Sclerotinia sclerotiorum* is involved with oxidative stress tolerance, virulence, and oxalate production. *Physiol. Mol. Plant Pathol.* 78:14–23.
- Xu, L. and W. Chen. 2013. Random T-DNA mutagenesis identifies a Cu/Zn superoxide dismutase gene as a virulence factor of *Sclerotinia sclerotiorum*. *Mol. Plant Microbe Interact.* 26: 431–441.
- Yao, S.H., Y. Guo, Y.Z. Wang, D. Zhang, L. Xu, and W.H. Tang. 2016. A cytoplasmic Cu-Zn superoxide dismutase SOD1 contributes to hyphal growth and virulence of *Fusarium graminearum*. *Fungal Genet. Biol.* 91:32–42.