Alternative Oxidase Is Involved in the Pathogenicity, Development, and Oxygen Stress Response of *Botrytis cinerea*

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ABSTRACT

Alternative oxidase (AOX) is a ubiquinol terminal oxidase that is involved in fungal mitochondrial oxidative phosphorylation. In this study, we analyzed the roles of AOX in *Botrytis cinerea* by generating BcAOX deletion mutants. The mutants exhibited defects in mycelial growth, sporulation, spore germination, and virulence. Furthermore, the sensitivity of the mutants to quinone outside inhibitor fungicides and oxidative

stress were increased. All phenotypic variations could be restored in the complemented strain. In summary, these results showed that BcAOX is involved in the regulation for vegetative development, adaptation to environmental stress, and virulence of *B. cinerea*.

Keywords: pathogenicity, stress response

Mitochondria of higher plants and fungi have two electron transport chains for respiration. The first is the cytochrome oxidase pathway, the terminal oxidase of which is known to be sensitive to inhibition by cyanide. The other is the alternative oxidation pathway, which branches from the cytochrome oxidase pathway starting at the ubiquinone pool site in the mitochondrial inner membrane and depends on alternative oxidase (AOX) as the terminal oxidase (Day et al. 1995; Joseph-Horne and Hollomon 2000; McIntosh et al. 1998; Vanlerberghe and McIntosh 1997). The alternative respiratory chain confers some resistance to the inhibitors of cytochrome C oxidase, including cyanide, azides, and monoxide in vitro. AOX localizes to the mitochondrial inner membrane and exhibits sensitivity to hydroxamic acids and alkyl gallates (Day et al. 1995; Siedow and Umbach 2000; Vanlerberghe and McIntosh 1997). Research has indicated that AOX has positive physiological roles in certain developmental processes and in adaptation to environmental stresses in plants (Clifton et al. 2005; Giraud et al. 2008; Vanlerberghe et al. 2016; Vanlerberghe and McIntosh 1997). In *Sauromatum guttatum* (Voodoo-lily), the AOX expression level and respiration rate of the alternative oxidation pathway are markedly enhanced in their thermogenic blooms during flowering events. The heat produced contributes to the volatilization of scent compounds to attract pollinating insects (McIntosh et al. 1998). In addition to their significant function in thermogenic plants, AOX may have an important role in the adaption or resistance of higher plants to various abiotic stresses because plants exhibit higher AOX expression levels and faster alternative oxidation pathway respiration rates under abiotic stress conditions (e.g., chilling,

drought, and heat) (Rachmilevitch et al. 2007; Ribas-Carbo et al. 2005; Rizhsky et al. 2002). A proposed function of AOX under abiotic stress is protecting organs from oxidative stress by shunting electrons from reduced ubiquinone to maintain the oxidation–reduction balance of cytochrome pathway components, thereby regulating the accumulation of endogenous reactive oxygen species (ROS) (Day et al. 1995; McDonald 2008). In addition to its function in abiotic stress, plant AOX is involved in inducing plant resistance to certain pathogens (Chivasa and Carr 1998; Fu et al. 2010).

The AOX has been identified in various organisms, including plants, fungi, and some protists (McDonald 2008). However, compared with the comparatively well-studied AOX of higher plants, there are far fewer studies of AOX function in other organisms, including fungi. Previous studies reported that AOX influences the response of fungal cells to various stresses. This is mainly supported by the observation that AOX was transcriptionally up-regulated in *Aspergillus niger*, *Yarrowia lipolytica* (Honda et al. 2012), *Aspergillus fumigatus* (Magnani et al. 2007), *A. niger* (Honda et al. 2012), *Magnaporthe grisea* (Yukioka et al. 1998), *Candida albicans* (Yan et al. 2009), *Hansenula anomala* (Minagawa et al. 1992), and *Paracoccidioides brasiliensis* (Martins et al. 2011) in response to oxidative or osmotic stress. Until recently, AOX in plant-pathogenic fungi *M. grisea* was not reported to be required for pathogenicity (Avila-Adame and Koller 2002; CaÂrdenas-Monroy et al. 2017; Xu et al. 2012). Furthermore, it has been indicated that AOX is required for virulence and might be involved in the infection process of several pathogenic fungi in humans, such as *Cryptococcus neoformans* (Akhter et al. 2003) and *P. brasiliensis* (Hernández Ruiz et al. 2011). *B. cinerea* is a necrotrophic plant pathogenic fungus with a broad host range (Elad et al. 2004; Williamson et al. 2007). Depending on the host, various plant organs, including flowers, tuber, leaves, fruits, and shoots, can exhibit sensitivity to *B. cinerea* infection (Veloukas and Karaoglanidis 2012). In addition, *B. cinerea* can survive as mycelia, conidia, and robust overwintering structures called sclerotia when infection conditions are unfavorable or hosts are not present. An understanding of the biology and virulence mechanisms of *B. cinerea* is critical to laying the groundwork for an efficient

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management strategy against this pervasive pathogen. In this study, we identified the orthologs of AOX in *B. cinerea* based on the protein sequence of AOX in various fungi. A targeted gene

deletion strategy was used to investigate the role of *BcAOX* in the development, oxygen stress response, and virulence of *B. cinerea*.

Fig. 1. Function of alternative oxidase (AOX) on *B. cinerea* growth, sclerotial formation, and conidiation. **A**, B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ were grown on potato dextrose agar (PDA), minimal medium (MM), or complete medium (CM) at 25°C for 3 days. **B**, Comparison of sclerotia formation among $\Delta AOX1$ and $\Delta AOX2$ and after cultivation on PDA, CM, and MM for 4 weeks at 25°C in darkness. **C**, The number of conidia produced by each strain on PDA plates. Bars denote standard errors from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$. **D**, The number of sclerotia was counted on PDA, CM, and MM. Bars denote standard errors from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$. **E**, Characteristics of mycelia and conidia.

MATERIALS AND METHODS

Isolates and media. The *B. cinerea* wild-type strain B05.10, isolated from grape in California via single spore separation, was used as a recipient strain for transformation experiments. The

isolates for this study were grown on potato dextrose agar (PDA; 20% g of potato, 2% g of glucose, 2% g of agar, and 1 liter of water), minimal medium [MM; 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 4 mM $(NH_4)_2SO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 9 μM $FeSO_4$, 10 mM of glucose, and 1 liter of water; pH 6.9], or

Fig. 2. Sensitivity of *B. cinerea* to succinate dehydrogenase inhibitors. **A**, B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ were grown on potato dextrose agar (PDA) with 2 $\mu g/ml$ of boscalid or fluopyram after incubation for 3 days (upper panel). Inhibition of mycelial growth compared with 25°C was measured after each strain was incubated for 3 days on PDA with 2 $\mu g/ml$ of boscalid or fluopyram. Bars denote standard deviations from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$ (lower panel). **B**, Half maximal effective concentration (EC_{50} ; $\mu g/ml$) values and effective concentrations causing 50% mycelial growth inhibition of boscalid and fluopyram against mycelial growth of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$.

complete medium (CM; 10 g of glucose, 2 g of peptone, 1 g of yeast extract, 1 g of casamino acids, 2% nitrate salts, 0.1% trace elements, 0.1 vitamins, and 1 liter of water; pH 6.5) for the mycelia growth assays.

AOX gene deletion and complementation. To study the biochemical function of AOX in *B. cinerea*, we generated a *BcAOX* deletion mutant using a gene replacement vector constructed using a previously described method. First, 670-bp up-stream and 660-bp down-stream flanking fragments of *BcAOX* were amplified from B05.10 genomic DNA (P3/P4 and P5/P6 primer pairs, respectively; Supplementary Fig. S1, Supplementary Table S1). Next, the 1,764-bp *HPH* fragment (with resistance to hygromycin B) was amplified from the pKHT plasmid using the P13/P14 primer pair as described in previously established methods (Duan et al. 2013). Then, the two flanking fragments of *BcAOX* and the *HPH* fragment were mixed in a molar proportion of 1:1:3 for the joint PCR (Yu et al. 2004). The 4376-bp DNA fragment was amplified from the joint PCR product using the P3/P6 primer pair.

Finally, the PCR product was purified and used for transformation of wild-type *B. cinerea* protoplasts.

Based on a previous study, we performed protoplast generation and transformation of B05.10 with some adjustments (Wang et al. 2015; Yang et al. 2013). Mycelial plugs taken from the margin of the colony on PDA were placed in 50-ml flasks containing 30 ml of YEPD (10% peptone, 3% yeast extract, 20% glucose). The mycelia were incubated in a shaker for 18 h at 25°C. Next, fresh mycelia were filtered through a sterile filter and washed twice using distilled water. Then, 0.2 g of fresh mycelia was put into 15 ml of 1.5% lysing enzyme buffer (0.6 M KCl, 50 mM CaCl₂; Sigma-Aldrich, St. Louis, MO). After incubation for 2 h at 30°C and 85 rpm. The protoplasts were collected and the filtrate was washed twice using STC buffer (0.8 M sorbitol, 0.05 M Tris [pH 8.0], and 50 mM CaCl₂) before being resuspended in pretreated SPTC buffer [STC: SPTC=4:1; SPTC was prepared with STC 40% (wt/vol) polyethylene glycol (PEG6000)]. Protoplast transformation was performed according to previously described methods (Yang

Fig. 3. The mycelial growth of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ after incubation for 3 days on PDA supplemented or not supplemented with quinone outside inhibitors (QoIs) azoxystrobin, pyraclostrobin, and kresoxim-methyl alone or together with 100 $\mu\text{g/ml}$ of salicylhydroxamic acid (SHAM).

TABLE 1. Sensitivity of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ to the QoIs azoxystrobin, pyraclostrobin, and kresoxim-methyl during mycelial growth in the absence or presence of 100 $\mu\text{g/ml}$ SHAM^a

Fungicides	Azoxystrobin			Pyraclostrobin			Kresoxim-methyl		
	EC ₅₀ ^b		SF ^c	EC ₅₀ ^b		SF ^c	EC ₅₀ ^b		SF ^c
	-SHAM	+SHAM		-SHAM	+SHAM		-SHAM	+SHAM	
B05.10	38.8107	1.3665	28.40	3.0042	0.0876	34.28	32.4989	0.0834	389.53
$\Delta AOX1$	0.0233	0.0251	0.93	0.0099	0.0103	0.96	0.0035	0.0032	1.10
$\Delta AOX2$	0.0576	0.0514	1.12	0.0071	0.0073	0.97	0.0051	0.0061	0.84
$\Delta AOX1C$	33.0542	1.0253	32.24	2.5814	0.0823	31.37	29.0026	0.0771	376.17

^a EC₅₀ = half maximal effective concentration, QoI = quinone outside inhibitor, SF = synergy factor, SHAM = salicylhydroxamic acid, + = with, and - = without.
^b The effective concentration causing 50% (EC₅₀) mycelial growth inhibition was tested for each fungicide, with three replicates for each concentration.
^c SF values were calculated according to the following formula: SF = EC₅₀ (-SHAM) / EC₅₀ (+SHAM).

et al. 2013). For transformation, 10 µg of the construct in 20 µl of spermidine was added to 1 ml of SPTC buffer containing 1×10⁸ protoplasts/ml and incubated on ice for 30 to 45 min. Next, 1 ml of SPTC was added to the suspension and incubated for 30 min at 25°C. Protoplasts were transferred to 200 ml of RM (casein hydrolysate at 0.5 g/liter, yeast extract at 0.5 g/liter, 0.7 M sucrose, and agar at 13 g/liter) medium (0.5% yeast extract, 0.5% casein hydrolysate, 0.7 M sucrose, and 16 g/liter of agar powder) at 42°C, which was gently mixed and poured in Petri plates (15 ml per plate). Plates were incubated for 16 h at 25°C. Then, the plates were overlaid with 5 to 8 ml of 100 µg/ml hygromycin B SRM medium (0.5% yeast extract, 0.5% casein hydrolysate, 1 M sucrose, and 12 g/liter of agarose). After incubation for 3 to 5 d at 25°C, the transformants were transferred to 100 µg/ml hygromycin B PDA for PCR identification assay. The construct-mediated homologous replacement was confirmed for its *BcAOX* generation effect by PCR amplification of genomic DNA using P1/P2, P7/P8, and P11/P12 primer pairs before being confirmed again by a reverse-transcription

(RT)-PCR analysis of the full cDNA sequences using the P1/P2 primer pair.

To confirm the phenotype of the *BcAOX* deletion mutant caused by *BcAOX* deletion, the $\Delta BcAOX$ was complemented with the full-length wild-type *BcAOX* gene (Duan et al. 2013). The full-length *BcAOX* sequence was amplified from B05.10 genomic DNA using primer pair P3/P6 and then cloned into the pNEO vector to generate the construct pNEO-*BcAOX*-C. Next, the plasmid vector was transformed into the deletion mutants using the previously described technique. Finally, *BcAOX* in the plasmid was sequenced and transformed into $\Delta BcAOX$ protoplasts for complementation. The experiment was performed as described previously, except neomycin (100 µg/ml) was used as a selection marker (Shao et al. 2017).

Intracellular H₂O₂ accumulation assay. To analyze the intracellular H₂O₂ content, 50 µl of 1×10⁵ spore/ml suspensions of each strain were placed in 96-well plates containing Gamborg B5 (GB5) medium supplemented with 2% glucose. After cultivation

Fig. 4. Effects of azoxystrobin alone or together with 100 µg/ml of salicylhydroxamic acid (SHAM) on spore germination of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$. Bars denote standard errors from three experiments.

overnight, the spore suspension of each strain was treated with 0.05 $\mu\text{g/ml}$ of azoxystrobin and 0.05 $\mu\text{g/ml}$ of azoxystrobin containing 100 $\mu\text{g/ml}$ of salicylhydroxamic acid (SHAM). Then, the supernatant was mixed with 0.25 mM of detection solution. The H_2O_2 concentration was measured using a SpectraMax M5 microplate reader at 550 nm (Marschall and Tudzynski 2014). The experiment was conducted twice.

Oxygen consumption rate assay. Ten mycelial plugs from the margin of the fresh colony of each strain were inoculated in 100 ml of alkyl ester broth (AEB) medium and shaken for 36 h in the dark at 175 rpm and 25°C. Before harvesting the mycelia, the culture was supplemented with 20 $\mu\text{g/ml}$ of azoxystrobin and 20 $\mu\text{g/ml}$ of azoxystrobin containing 100 $\mu\text{g/ml}$ of SHAM for 2 h. Then, fresh mycelia from each treatment were used to analyze the oxygen

Fig. 5. Virulence analysis of plant leaves treated or not treated 0.02 $\mu\text{g/ml}$ of azoxystrobin after inoculation with the mycelial plug of the parental strain B05.10, ΔAOX1 , ΔAOX2 , and ΔAOX1C . Agar plugs were used as negative controls. **A**, Disease symptoms on wounded plant leaves after 90 h of inoculation (h.p.i.). **B**, Diameter of the disease lesion on various leaves. Bars denote the standard errors of four repeated experiments. **C**, The control efficacy of azoxystrobin for infection. Values on the bars followed by the same letter are not significantly different at $P = 0.05$. Bars denote standard errors from four experiments.

consumption rate with a dissolved oxygen meter (SX716; Sanxin, Shenzhen, China) (Shirane et al. 1995; Xu et al. 2014). The experiment was conducted twice.

Fungicide sensitivity assay. To analyze the sensitivity of each isolate to different fungicides, mycelial plugs obtained from the margin of a fresh colony were placed on PDA medium amended with boscalid (1.5625 to 50 $\mu\text{g/ml}$), fluopyram (1.5625 to 50 $\mu\text{g/ml}$), azoxystrobin (0.00625 to 50.0 $\mu\text{g/ml}$), pyraclostrobin (0.03125 to 10.0 $\mu\text{g/ml}$), and kresoxim-methyl (0.003125 to 20.0 $\mu\text{g/ml}$). Three replicate plates of each fungicide concentration for each strain were used. After incubation for 3 d at 25°C, the colony diameter of each treatment was measured. The fungicide concentration that resulted in 50% mycelial growth inhibition (half maximal effective concentration [EC_{50}]) for each fungicide against each strain was calculated (Zhang et al. 2007). The experiment was conducted twice.

Virulence assay. Mycelial plugs from the margin of the fresh colony of each strain were inoculated onto strawberry, grape, and cucumber leaves that had been treated with or without 0.02 $\mu\text{g/ml}$ of azoxystrobin. Inoculated leaves were incubated with 17 h of daylight at 25°C. Then, the disease lesions were measured after different times (see figure legends). The experiment was repeated four times.

Statistical analysis. All analyses were performed using statistical software (SAS GLM; SAS Institute, Inc., Cary, NC). The EC_{50} of the isolates was measured by linear regression of the inhibition rate versus $\log_{10}$ of the fungicide concentration. Significance tests adopted least significant difference (LSD) methods.

RESULTS

Identification of the *BcAOX* gene. We identified *BcAOX* (BC1G_05703) in the *B. cinerea* genome via a homology search using BLASTP with the *AOX* gene from *S. cerevisiae* as a query. To analyze the size and existence of introns, RNA was isolated from B05.10 mycelia using an RNA isolation kit (Sangon Biotech. Co., Shanghai, China) and used for cDNA synthesis using a cDNA synthesis kit (TaKaRa Biotech. Co., Dalian, China). The P1/P2 primer pair amplified 1,086-bp and 1,847-bp fragments from the cDNA and genomic DNA, respectively. The sequencing results showed that the *BcAOX* nucleotide sequence was 1,874 bp in length and encoded 361 amino acids. The sequence contained two introns of 64 and 76 bp located at cDNA sequence positions 206 and 342, respectively.

***AOX* deletion and complementation.** To analyze the biology function of *BcAOX* in *B. cinerea*, we generated *BcAOX* deletion mutants ($\Delta BcAOX1$ and $\Delta BcAOX2$) using a homologous recombination method. *BcAOX* deletion mutants were identified from 201 hygromycin-resistant transformants by PCR amplification using different primer pairs. A 628-bp fragment was amplified from the B05.10 and the complemented mutant using the P9/P10 primers, but not the *BcAOX* deletion mutant. An 883-bp fragment was amplified from the *BcAOX* deletion mutant and complemented mutant using the P7/P8 primers, but not B05.10. A 466-bp fragment was amplified from the complemented mutant using the P11/P12 primer, but not the deletion mutant and wild-type strain B05.10. Putative deletion isolates were also examined using RT-PCR analyses. The results indicated that the deletion mutant had completely lost the target transcript and showed that $\Delta BcAOX$ resulted from the anticipated homologous recombination events at the *BcAOX* locus. To confirm phenotypes of $\Delta BcAOX$ caused by *BcAOX* deletion, $\Delta BcAOX$ was complemented by the wild-type *BcAOX* gene that complemented the isolate *BcAOX1C*.

Involvement of *BcAOX* in conidiation and sclerotium production. To analyze the effects of *BcAOX* deletion on vegetative growth, the mycelia growth of each strain was

measured on PDA, CM, and MM. There was no difference in the mycelia growth rate among isolates on PDA, MM, and CM (Fig. 1A). During the sporulation assay, compared with B05.10 and $\Delta BcAOXC$, the sporulation of the *BcAOX* deletion mutant ($\Delta BcAOX1$ and $\Delta BcAOX2$) clearly decreased (Fig. 1C). We explored the function of *BcAOX* in sclerotia production; the *BcAOX* deletion mutant produced more sclerotia compared with the wild-type and the complemented strain after incubation for 4 weeks in the dark on PDA, MM, and CM (Fig. 1B and D). Additionally, the morphology of the mycelia and spores was not observably different between the mutant and wild-type strain (Fig. 1E). These results indicated that *BcAOX* is involved in mycelia growth, sporulation, and sclerotia production of *B. cinerea*.

Effect of *BcAOX* deletion on fungicides sensitivity. We analyzed the sensitivity of the parental strain (B05.10), two *BcAOX* deletion mutants ($\Delta BcAOX1$ and $\Delta BcAOX2$), and the complemented strain ($\Delta BcAOX1C$) to succinate dehydrogenase inhibitors (boscalid and fluopyram) and quinone outside inhibitors (QoIs; azoxystrobin, pyraclostrobin, and kresoxim-methyl). In response to these chemicals, mycelial growth inhibition of $\Delta BcAOX$ with 2 $\mu\text{g/ml}$ of boscalid or fluopyram was similar to that of B05.10 and $\Delta BcAOX1C$ (Fig. 2A). Furthermore, the EC_{50} values of each strain in response to boscalid and fluopyram did not differ significantly (Fig. 2B). However, compared with B05.10 and $\Delta BcAOX1C$, the sensitivity of $\Delta BcAOX$ to all QoIs was significantly increased. Additionally, the sensitivity of each strain to all QoIs did not differ with the addition of SHAM (Fig. 3, Table 1). Moreover, the spore germination of $\Delta BcAOX$ was lower than that of B05.10 and $\Delta BcAOX1C$ during treatment with or

Fig. 6. Oxygen consumption and fluorometric quantification assay. **A**, Oxygen consumption rate of mycelia of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ at 1 h after azoxystrobin alone or with 100 $\mu\text{g/ml}$ salicylhydroxamic acid (SHAM) treat. Bars denote the standard errors of four repeated experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$. **B**, Fluorometric quantification of H_2O_2 of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$. Conidial suspensions ($10^5/\text{ml}$) were incubated for 16 h in a 96-well plate in GB5 medium supplemented with 2% glucose. Just before measurements of relative fluorescence units (RFU), 0.05 $\mu\text{g/ml}$ of azoxystrobin alone or with 100 $\mu\text{g/ml}$ SHAM and 0.01 mM of detection mix were added to the wells.

without 0.05 µg/ml azoxystrobin, but the spore germination rate of each strain did not differ on azoxystrobin-amended media with or without SHAM (Fig. 4). These results indicated that AOX is involved in the sensitivity to QoIs, but not succinate dehydrogenase inhibitors, in *B. cinerea*.

Effect of AOX deletion on the virulence of *B. cinerea*. The effect of *BcAOX* deletion on virulence was analyzed on different

host plants. Mycelial plugs of each strain were inoculated onto host plant leaves. On wounded leaves of cucumber, grape, and strawberry, the disease lesions of *BcAOX* deletion mutant were smaller than those of B05.10 and $\Delta BcAOX1C$ after inoculation for 72 h under 16 h of daylight (Fig. 5A and B). To further analyze the role of respiration in the virulence of $\Delta BcAOX$, we tested the lesions caused by each strain on cucumber and grape leaves

Fig. 7. Sensitivity of B05.10, $\Delta AOX1$, $\Delta AOX2$, and $\Delta AOX1C$ to multiple stresses. **A**, Comparisons were performed on potato dextrose agar (PDA) medium modified with NaCl, KCl, hydrogen peroxide, fludioxonil, procymidone, and paraquat at the concentration indicated. **B**, Inhibition of mycelial growth was analyzed after each strain was incubated for 3 days on PDA supplemented with different compounds. Bars denote standard errors from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

under azoxystrobin or azoxystrobin plus SHAM treatment conditions. As shown in Figure 5A and C, the wild-type strain B05.10 and the complemented strain exhibited larger lesions than $\Delta BcAOX$.

Involvement of AOX in the respiratory rate and H₂O₂ accumulation. During the respiratory assay, the respiratory rate of $\Delta BcAOX$ was significantly decreased with azoxystrobin treatment compared with the wild-type strain and complemented strain. However, when treated with azoxystrobin plus SHAM, the respiration rate of each strain markedly decreased, but it did not differ among isolates (Fig. 6A). During the H₂O₂ accumulation assay, azoxystrobin treatment promoted H₂O₂ accumulation in $BcAOX$ in comparison with the wild-type strain and complemented strain. However, when treated with azoxystrobin plus SHAM, the H₂O₂ accumulation of each strain clearly increased, but it did not differ among the isolates (Fig. 6B).

Sensitivity of the AOX deletion mutant to various environmental stresses. To analyze the role of $BcAOX$ in the tolerance of *B. cinerea* to environmental stresses, we measured the sensitivity of each strain to oxidative and osmotic stress. As shown in Figure 7, there was no difference in the sensitivity of each strain to osmotic stress generated by 1.0 M of NaCl or 1.2 M of KCl, or to oxidative stress induced by 24 mM of H₂O₂ or 5 mM of paraquat and fludioxonil. However, the sensitivity of $\Delta BcAOX$ to procymidone was significantly increased compared to that of B05.10 and $\Delta BcAOXC$. The deletion of $BcAOX$ may affect the Hog pathway and lead to increased sensitivity of $\Delta BcAOX$ to procymidone.

DISCUSSION

The results of this study add to our understanding of alternative respiration in fungi (Joseph-Horne and Hollomon 2000). Because of the biochemical mechanism of commercial fungicides (inhibiting respiration by binding to cytochrome b of the QoI complex) (Jordan et al. 1999), there has been significant interest in the role of the alternative pathway in the electron transfer chain, particularly that of plant-pathogenic fungi. To date, several fungal AOX genes have been identified and characterized in pathogenic fungi (Joseph-Horne and Hollomon 2000; Steinfeld et al. 2001). When AOX orthologs were identified in fungal models, this enzyme was found to be involved in cellular growth and morphological transitions (Martins et al. 2011; Minagawa et al. 1992). Our results further confirm the role of fungal AOX in these pathways. For example, a previous study of *N. rileyi* AOX deletion mutants demonstrated that these mutants also exhibited defects in hyphal morphology, microsclerotia production, and growth yield. The AOX inhibitor, SHAM, inhibits mycelial growth and diminishes the biomass yield of fungi such as *Sclerotinia sclerotiorum* (Zhou et al. 2015). In this study, we found that the mycelial growth rate and sporulation of $\Delta BcAOX$ were clearly decreased. However, the sclerotial production of $\Delta BcAOX$ was significantly increased when compared with that of B05.10 and $\Delta BcAOXC$. These results showed that AOX has important roles in the vegetative development of *B. cinerea*.

A previous study reported that several plant pathogens can resist the full inhibitory action of QoI fungicide during the early stages of application via the activation of alternative respiration (Zhou et al. 2016). Under active conditions of alternative respiration, mitochondrial electron transfer can circumvent the action of cytochrome b in QoIs (Vanlerberghe and McIntosh 1997). Additionally, AOX is essential for eliminating the destruction of the mitochondria respiratory chain, thus promoting the production of a proton motive force via complex I and generating energy for growth (Sierra-Campos et al. 2009). In this study, we analyzed the function of $BcAOX$ for regulating the sensitivity of *B. cinerea* to various fungicides acting on the mitochondrial respiratory chain. The results showed that all

isolates exhibited similar sensitivity to boscalid and fluopyram, which indicated that $BcAOX$ was not involved in the function of the succinate dehydrogenase complex of the cytochrome pathway. However, compared with B05.10 and the complemented isolates, the $BcAOX$ deletion mutant showed increased sensitivity to procymidone. A previous study indicated that procymidone interferes with the Hog1 fungal pathway. Therefore, we inferred that deletion of $BcAOX$ may affect the function of the Hog1 pathway, and that $\Delta BcAOX$ showed increased sensitivity to procymidone. These deductions need to be explored further. In the QoI fungicide assay lacking SHAM treatment, $\Delta BcAOX$ exhibited significantly increased sensitivity to azoxystrobin, pyraclostrobin, and kresoxim-methyl compared to B05.10 and $\Delta BcAOXC$. With SHAM treatment, however, all isolates exhibited similar sensitivity to the three QoIs. Our results are consistent with those of previous studies that indicated that AOX is required for the sensitivity of *M. grisea* and *Ustilago maydis* to QoIs (Avila-Adame and Koller 2002; Caardenas-Monroy et al. 2017). Furthermore, the AOX deletion mutant was also found to exhibit defective virulence in several human-pathogenic fungi, including *P. brasiliensis*, *C. neoformans*, and *C. albicans* (Akhter et al. 2003; Martins et al. 2011). In *M. grisea*, however, the deletion of AOX did not affect the virulence on host leaves (Avila-Adame and Koller 2002, 2003). Until now, AOX has not been reported as directly involved in pathogenicity (Avila-Adame and Koller 2002; Caardenas-Monroy et al. 2017; Xu et al. 2012). Interestingly, the $BcAOX$ deletion mutant of *B. cinerea* exhibited decreased virulence on various host plant tissues with the presence or absence of azoxystrobin. Additionally, the reduced virulence of the $BcAOX$ deletion mutant could be attributable to defects in multiple regulatory factors. First, the growth rate of the $BcAOX$ deletion mutant was slower than that of the wild-type isolate and complemented isolate. Second, we found that the $BcAOX$ deletion mutant had a decreased respiratory rate, which may be involved in the virulence of *B. cinerea*.

Under standard culture conditions, *U. maydis* demonstrated the alternative oxidation pathway respiration capacity (Jurez et al. 2006). A previous study showed that *S. sclerotiorum* isolates also presented significant alternative oxidation pathway respiration capacity under standard culture conditions (Xu et al. 2012). Hydrogen peroxide affected the expression level of AOX genes in *P. brasiliensis* (Martins et al. 2011) and influenced the expression of AOX and alternative oxidation pathway activity in *M. grisea* (Yukioka et al. 1998) and *S. sclerotiorum* (Xu et al. 2012). Furthermore, the deletion of AOX enhanced the sensitivity of *C. neoformans* to tert-butyl hydroperoxide, an oxidative stressor (Akhter et al. 2003). In our study, we found that $BcAOX$ deletion mutants exhibited greatly reduced respiratory rates under azoxystrobin treatment. Additionally, in the presence of azoxystrobin, $\Delta BcAOX$ accumulated more ROS than did B05.10 and $\Delta BcAOXC$. Furthermore, the sensitivity of $\Delta BcAOX$ to oxidative stress generated by paraquat was significantly increased. These results support the expected outcome that AOX is involved in the regulation of the respiration and oxidative balance in *B. cinerea*.

A previous study indicated that mitochondria electron transport has various functions in pathogen fungi and that AOX is involved in the regulation of the electron transport chain (Joseph-Horne and Hollomon 2000). Therefore, we inferred that the effect of the deletion of $BcAOX$ on *B. cinerea* disturbed the mitochondria electron transport, and that the $BcAOX$ deletion mutant resulted in defects in vegetative development, virulence, and various stress responses. The results of this study demonstrated that $BcAOX$ is involved in the development, respiration, and virulence of *B. cinerea*. They also promoted the development of cytochrome pathway research and enhanced our understanding of the biology of *B. cinerea*, which could contribute to controlling gray mold and reducing agricultural damage.

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