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Involvement of Alternative Oxidase in the Regulation of Sensitivity of *Sclerotinia sclerotiorum* to the Fungicides Azoxystrobin and Procymidone

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Sclerotinia sclerotiorum **is a filamentous fungal pathogen that can infect many economically important crops and vegetables. Alternative oxidase is the terminal oxidase of the alternative respiratory pathway in fungal mitochondria. The function of alternative oxidase was investigated in the regulation of sensitivity of *S. sclerotiorum* to two commercial fungicides, azoxystrobin and procymidone which have different fungitoxic mechanisms. Two isolates of *S. sclerotiorum* were sensitive to both fungicides. Application of salicylhydroxamic acid, a specific inhibitor of alternative oxidase, significantly increased the values of effective concentration causing 50% mycelial growth inhibition (EC_{50}) of azoxystrobin to both *S. sclerotiorum* isolates, whereas notably decreased the EC_{50} values of procymidone. In mycelial respiration assay azoxystrobin displayed immediate inhibitory effect on cytochrome pathway capacity, but had no immediate effect on alternative pathway capacity. In contrast, procymidone showed no immediate impact on capacities of both cytochrome and alternative pathways in the mycelia. However, alternative oxidase encoding gene (*aox*) transcript and protein levels, alternative respiration pathway capacity of the mycelia were obviously increased by pre-treatment for 24 h with both azoxystrobin and procymidone. These results indicate that alternative oxidase was involved in the regulation of sensitivity of *S. sclerotiorum* to the fungicides azoxystrobin and procymidone, and that both fungicides could affect *aox* gene expression and the alternative respiration pathway capacity development in mycelia of this fungal pathogen.**

Keywords: *Sclerotinia sclerotiorum*, alternative oxidase, mitochondrial respiration, azoxystrobin, procymidone, fungicide sensitivity

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Introduction

Sclerotinia sclerotiorum is a cosmopolitan filamentous fungus which is pathogenic to plants. It can invade over 400 plant species which include many economically important crops and vegetables (Boland and Hall, 1994; Bardin and Huang, 2001; Bolton *et al*., 2006). It is the causative agent of sclerotinia stem rot of rapeseed (*Brassica napus* L.) which has been recognized as a major rapeseed disease in many areas of Canada and the USA (Bradley *et al*., 2006; Kutcher and Wolf, 2006). In China the average incidence of this rapeseed disease has been reported as 10–20% but it can rise to 80% during serious outbreaks (Li *et al*., 2006). Sclerotinia stem rot of soybean (*Glycine max* (L.) Merr.) caused by *S. sclerotiorum* was ranked in 1994 as the most severe soybean disease in Argentina and the second most important soybean disease in the USA (Mueller *et al*., 2002). In South Korea, *S. sclerotiorum* has been identified as a cause of stem rot of balloon flower (*Platycodon grandiflorum* A. DC.) which is cultivated as an edible rootcrop and a traditional medicinal herb (Lee *et al*., 2012). Sclerotinia disease of field lettuce (*Lactuca sativa*) caused by *S. sclerotiorum* has been frequently reported to result in losses of 10% in the UK (Young *et al*., 2004). In the USA, annual losses of more than $200 million from *S. sclerotiorum* in agriculture stimulated 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 can possess two respiratory electron transport chains. One is a cytochrome pathway (CP) with cytochrome oxidase as the terminal oxidase. The other is an alternative pathway (AP) with alternative oxidase (AOX) as the 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 fulfills positive physiological functions in developmental events and in responses to environmental changes (Vanlerberghe, 1997; Chivasa and Carr, 1998; Liang and Liang, 2002; Fu *et al*., 2010; Liao *et al*., 2012). Whilst there is a wide range of research on AOX in higher plants, much less is known about AOX in fungi (Xu *et al*., 2012). However, a limited amount of research has revealed that AOX can also play some important roles in the life of fungi (Joseph-Horne *et al*., 2001; Juarez *et al*., 2006; Xu *et al*., 2012). For example, AOX can function in resistance of fungi to oxidative stress. Superoxide has been shown to induce the expression of AOX in *Hansenula anomala* (Minagawa *et al*., 1992). Hydrogen peroxide was observed to obviously induce AOX expression in *Paracoccidioides brasiliensis* (Martins *et al*., 2011), and induce

AOX expression and AP capacity development in *Magnaporthe grisea* (Yukioka *et al*., 1998). An *aox1* mutant strain of *Cryptococcus neoformans* was found to be more sensitive to the oxidative stressor tert-butyl hydroperoxide (Akhter *et al*., 2003).

Besides the role in oxidative stress resistance, AOX may also function in fungal development control. It was found that the expression of *aox* in *P. brasiliensis* was developmentally regulated through the differentiation from the mycelial form to the yeast form, and this differentiation was delayed by inhibition of AOX with SHAM (Martins *et al*., 2011).

One more function of fungal AOX is that it has been reported to be involved in the regulation of fungal sensitivity to some fungicides. Quinol oxidation-inhibiting (QoI) fungicides refer to a broad-spectrum class of fungicides that include many currently commercial products, such as azoxystrobin, kresoxim-methyl, and metominostrobin. This type of fungicides acts by inhibiting fungal respiration through CP by binding to the Qo center of cytochrome *b*, a component of complex III (Bartlett *et al*., 2002). AOX respiration has been found to reduce the sensitivity of some fungi to this type of fungicides (Avila-Adame and Koller, 2003; Wood and Hollomon, 2003; Miguez *et al*., 2004; Banno *et al*., 2009).

Till now high levels of resistance to *S. sclerotiorum* are yet absent in the major crops that are sensitive to this pathogen. Although biological control of *S. sclerotiorum* has been reported (Li *et al*., 2006; Lee *et al*., 2012), use of fungicides is the primary method of controlling the diseases caused by this pathogen (Bradley *et al*., 2006). Various types of fungicides, including QoI fungicides, have been used to control this pathogen (Mueller *et al*., 2002; Bradley *et al*., 2006; Kutcher and Wolf, 2006; Ma *et al*., 2009; Lee *et al*., 2012). However, it is not clear whether AOX is involved in the regulation of *S. sclerotiorum* sensitivity to any of the applied fungicides.

In a previous report we showed that AOX was involved in the regulation of growth, development, and resistance to oxidative stress of *S. sclerotiorum* (Xu *et al*., 2012). In this report we provide evidence that AOX is involved in the regulation of sensitivity of *S. sclerotiorum* to two fungicides azoxystrobin and procymidone. The results are beneficial for gaining a deeper understanding of the expression properties and physiological roles of AOX of *S. sclerotiorum*, and for improving the control of this plant pathogen.

Materials and Methods

S. sclerotiorum isolates

Two *S. sclerotiorum* isolates (7-3, 44-2) were collected from a rapeseed field, and have been deposited with the Zhejiang University Fungal Collection (Hangzhou, China), accession numbers Ssc-2011-001 and Ssc-2011-002 (Xu *et al*., 2012).

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

As previously described (Xu *et al*., 2012), both *S. sclerotiorum* isolates were cultured by growing in the dark on potato dextrose agar (PDA) plates (200 g potato infusion, 20 g dextrose, 20 g agar, with distilled water to a volume of 1 L) 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 (200 g potato infusion, 20 g dextrose, with distilled water to a volume of 1 L). 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. fungicide or SHAM) of tested concentrations were added to the PDA or the liquid media before inoculation with the mycelial plugs.

Determination of sensitivity of *S. sclerotiorum* mycelia to azoxystrobin and procymidone

Azoxystrobin (Methyl (2E)-2-(2-{[6-(2-cyanophenoxy)pyrimidin-4-yl]oxy}phenyl)-3-methoxyacrylate) (Syngenta Crop Protection Inc., USA) was dissolved in distilled water, and procymidone (3-(3,5-dichlorophenyl)-1,5-dimethyl-3-azabicyclo[3.1.0]hexane-2,4-dione) (Zhuyou, Japan) was dissolved in acetone, according to the manufacturer's specifications at a concentration of 100 mg/ml. Suitable volumes of the fungicide solutions were added to PDA medium to obtain final concentrations ranging from 0.1 to 5.0 μg/ml. Six concentrations were tested for each fungicide, with three replicates for each concentration. Mycelial plugs were inoculated onto the PDA plates supplemented with fungicide and were set to culture as described above. As procymidone was dissolved in acetone, the same volume of acetone was added to control PDA media when the effect of procymidone was tested. The colony diameters were measured periodically and used to calculate the EC_{50} values (effective concentration causing 50% mycelial growth inhibition). The diameter of a colony was the mean of two measurements of the diameter of the colony taken at 90° to each other.

When the effectiveness of AOX on the *S. sclerotiorum* sensitivity to fungicides was evaluated, synergy factor (SF) was introduced with an equation $SF = EC_{50(-SHAM)} / EC_{50(+SHAM)}$, in which $EC_{50(-SHAM)}$ refers to EC_{50} values determined with PDA media without addition of SHAM, while $EC_{50(+SHAM)}$ refers to ones determined with PDA media supplemented with 100 μg/ml SHAM to inhibit AOX.

Measurement of respiration rate of *S. sclerotiorum* mycelia

Mycelia were collected from *S. sclerotiorum* cultures in liquid media and their oxygen consumption rates were measured using a Clark-type oxygen electrode (Hanna Instruments, HI964400M, USA) at 25°C as previously described (Xu *et al*., 2012). 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 alternative oxidase (AOX) encoding gene (*aox*) transcript levels in mycelia of *S. sclerotiorum* by real-time quantitative PCR

Mycelia collection from *S. sclerotiorum* cultures in PD liquid medium, total RNA extraction, complementary DNA synthesis and real-time quantitative PCR were performed as previously described (Xu *et al.*, 2012). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

Estimation of alternative oxidase (AOX) protein levels in mycelia of *S. sclerotiorum* by western blot

Mycelia collection from *S. sclerotiorum* cultures in PD liquid medium, mitochondria extraction and estimation of AOX protein levels in purified mitochondria by western blot were carried out as previously described (Xu *et al.*, 2012). The previously prepared rabbit polyclonal serum against AOX of *S. sclerotiorum* was used as the primary antibody to analyze AOX protein on the nitrocellulose membranes (Xu *et al.*, 2012). 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). Cytochrome *c* in the mitochondrial protein samples was simultaneously detected with a polyclonal antibody against cytochrome *c* (Shenggong Company, China) using similar methods. Densitometry values for the immunoreactive bands were quantified using a GS-700 imaging densitometer (Bio-Rad, USA). After normalization with the density of the cytochrome *c* band from the same mitochondrial protein sample, densities of AOX bands were used to calculate the increase in AOX protein levels caused by the fungicide treatment.

Data analysis

All experiments were repeated independently at least three times. Quantitative data were subjected to statistical analysis using SPSS (version 13) program as previously described (Xu *et al.*, 2012). Means of the results were compared using ANOVA and P values <0.05 were considered significant. Duncan's multiple range test was used once the ANOVA was discovered to be significant.

Results

Sensitivity of *S. sclerotiorum* to fungicides

Mycelia of both *S. sclerotiorum* isolates (7-3, 44-2) grew very well on PDA plate at 22°C in darkness. The mycelia extended to cover the PDA plates in about 3 days. However, both of the tested fungicides, azoxystrobin and procymidone, could effectively inhibit the mycelial growth of *S. sclerotiorum*. Azoxystrobin at 0.80 μg/ml and procymidone at 0.40 μg/ml clearly inhibited mycelial growth of both *S. sclerotiorum* isolates on PDA plates (Fig. 1).

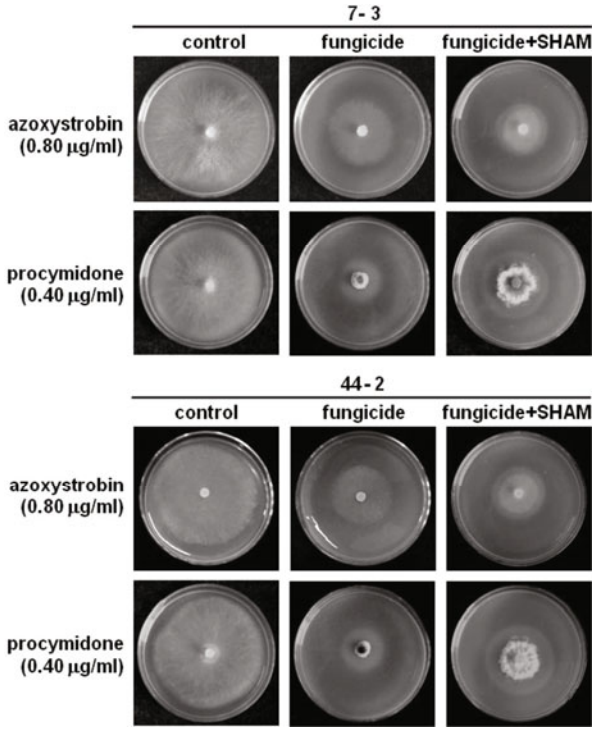

Fig. 1. Effect of fungicides alone or together with 100 μg/ml salicylhydroxamic acid (SHAM) on the mycelial growth of both *S. sclerotiorum* isolates (7-3, 44-2) on PDA plates in Petri dishes (9-cm diameter). Photos were taken after culture for 3 days. Experiments were carried out three times and representative results are shown.

In order to evaluate the sensitivity of *S. sclerotiorum* to the fungicides, the EC_{50} values of both fungicides were measured. Results in Table 1 showed that the EC_{50} values of azoxystrobin in the absence of SHAM for both *S. sclerotiorum* isolates were about 1.1–1.2 μg/ml (equal to 2.7–3.0 μM), while those of procymidone were about 0.2–0.3 μg/ml (equal to 0.7–1.1 μM). These results suggest that both *S. sclerotiorum* isolates were more sensitive to procymidone than to azoxystrobin.

Table 1. The sensitivity of both *S. sclerotiorum* isolates to the fungicides azoxystrobin and procymidone during mycelial growth in the absence or presence of 100 μg/ml salicyhydroxamic acid (SHAM).

Fungicides	*S. sclerotiorum* isolate 7-3			*S. sclerotiorum* isolate 44-2		
	EC_{50}[a] (μg/ml)		SF[b]	EC_{50}[a] (μg/ml)		SF[b]
	-SHAM	+SHAM		-SHAM	+SHAM	
Azoxystrobin	1.102 ± 0.104	0.414 ± 0.035	2.66	1.224 ± 0.113	0.241 ± 0.024	5.08
Procymidone	0.321 ± 0.025	0.542 ± 0.053	0.59	0.243 ± 0.022	0.693 ± 0.061	0.35

[a] EC_{50}: Effective concentration causing 50% mycelial growth inhibition. Data are means and standard errors from three determinations. In every determination six concentrations were tested for each fungicide, with three replicates for each concentration.
[b] Values of synergy factor (SF) were calculated according to the formula: $SF = EC_{50(-SHAM)} / EC_{50(+SHAM)}$

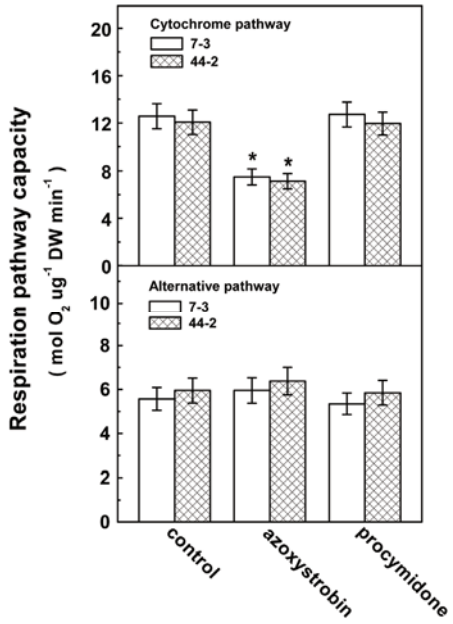

Fig. 2. Immediate effect of azoxystrobin and procymidone on alternative pathway (AP) and cytochrome pathway (CP) capacities in mycelia of both *S. sclerotiorum* isolates (7-3, 44-2). The final concentration of azoxystrobin was 5.51 μg/ml for *S. sclerotiorum* isolate 7-3, and 6.12 μg/ml for isolate 44-2. The final concentration of procymidone was 1.61 μg/ml for *S. sclerotiorum* isolate 7-3, and 1.22 μg/ml for isolate 44-2. These final concentrations of azoxystrobin and procymidone were equal to 5 folds of their EC_{50} values (see Table 1). Higher concentrations of both fungicides were tested and showed the same effects (data not shown). Azoxystrobin and procymidone were dissolved in distilled water and acetone, respectively. H_2O was used for the control. In the experiments to test the effect of procymidone, the final acetone level was less than 0.1%, which had no effect on mycelial repiration rate (data not shown). Means and standard errors of the results from six determinations are shown. Means with a star above the columns are significantly different from control ($P<0.05$).

Involvement of alternative oxidase (AOX) in the regulation of sensitivity of *S. sclerotiorum* to fungicides

When the specific inhibitor of AOX, SHAM, was used at 100 μg/ml to treat both *S. sclerotiorum* isolates together with the two fungicides, it was found to aggravate the toxicity of azoxystrobin to both *S. sclerotiorum* isolates, but it alleviated the toxicity of procymidone to them (Fig. 1).

To evaluate the influence of inhibition of AOX with SHAM on the sensitivity of *S. sclerotiorum* to both fungicides, the SF factor (= $EC_{50(-SHAM)}$ / $EC_{50(+SHAM)}$) of each fungicide was measured. An SF factor greater than 1 suggests that inhibition of AOX with SHAM increased the sensitivity of *S. sclerotiorum* to the fungicide, while a value less than 1 suggests that inhibition of AOX with SHAM decreased the fungal sensitivity to the fungicide (Steinfeld *et al*., 2001). The results in Table 1 indicate that SF factors for azoxystrobin were greater than 1, suggesting that inhibition of AOX with SHAM increased the sensitivity of both *S. sclerotiorum* isolates to azoxystrobin. On the contrary, the SF factors for procymidone were less than 1, indicating that inhibition of AOX with SHAM decreased the sensitivity of both *S. sclerotiorum* isolates to this fungicide. These results suggest that AOX was involved in the regulation of sensitivity of *S. sclerotiorum* to both fungicides. AOX respiration decreased its sensitivity to azoxystrobin, but increased its sensitivity to procymidone.

The immediate effect of fungicides on the capacities of mitochondrial respiration pathways in *S. sclerotiorum* mycelia

As AOX was found to be involved in the regulation of sensitivity of *S. sclerotiorum* to azoxystrobin and procymidone, it was necessary to test whether they had any immediate effect on the mycelial respiration. Results in Fig. 2 indicate that only azoxystrobin reduced the CP capacity in mycelia of both *S. sclerotiorum* isolates, while procymidone showed no immediate impact. Neither fungicide displayed an immediate influence on AP capacity.

The effect of pre-treatment for 24 h with fungicides on the capacities of mitochondrial respiration pathways in *S. sclerotiorum* mycelia

In order to know more about the influence of azoxystrobin and procymidone on the respiration of *S. sclerotiorum* mycelia, the impact of pre-treatment for 24 h with both fungicides on the capacities of both mitochondrial respiration pathways of *S. sclerotiorum* mycelia was investigated. As shown in Fig. 3, azoxystrobin pre-treatment could reduce the CP

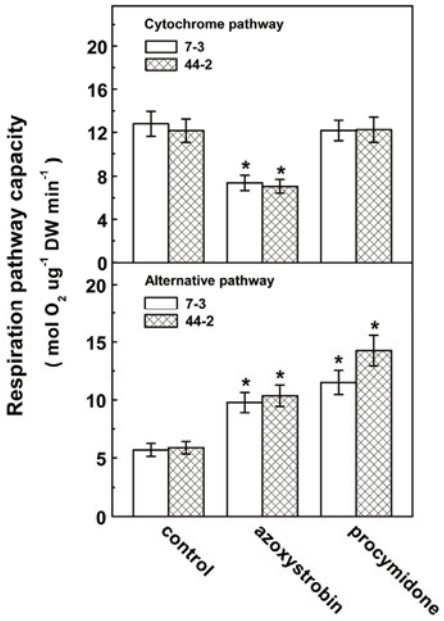

Fig. 3. Effect of pre-treatment for 24 h with azoxystrobin and procymidone on alternative pathway (AP) and cytochrome pathway (CP) capacities in mycelia of both *S. sclerotiorum* isolates (7-3, 44-2). The final concentration of azoxystrobin was 1.102 μg/ml for *S. sclerotiorum* isolate 7-3, and 1.224 μg/ml for isolate 44-2. The final concentration of procymidone was 0.321 μg/ml for *S. sclerotiorum* isolate 7-3, and 0.243 μg/ml for isolate 44-2. These final concentrations of azoxystrobin and procymidone were equal to their EC_{50} values (see Table 1). Both *S. sclerotiorum* isolates were cultured in potato dextrose liquid media. Azoxystrobin and procymidone were dissolved in distilled water and acetone, respectively. H_2O was used as the control for azoxystrobin treatment. The same volume of acetone was added to the media as the control for procymidone treatment. Means and standard errors of the results from six determinations are shown. Means with a star above the columns are significantly different from control ($P<0.05$).

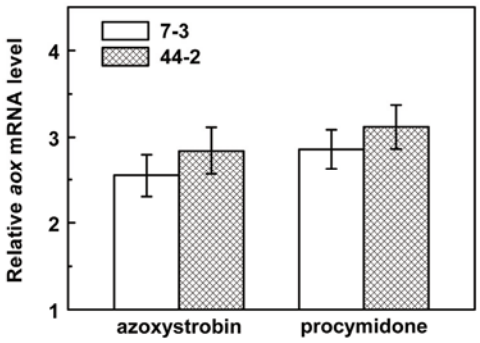

Fig. 4. Effect of pre-treatment for 24 h with azoxystrobin and procymidone on the transcript levels of alternative oxidase encoding gene, *aox*, in mycelia of both *S. sclerotiorum* isolates (7-3, 44-2). The final concentration of azoxystrobin was 1.102 μg/ml for *S. sclerotiorum* isolate 7-3, and 1.224 μg/ml for isolate 44-2. The final concentration of procymidone was 0.321 μg/ml for *S. sclerotiorum* isolate 7-3, and 0.243 μg/ml for isolate 44-2. These final concentrations of azoxystrobin and procymidone were equal to their EC_{50} values (see Table 1). Both *S. sclerotiorum* isolates were cultured in potato dextrose liquid media. Azoxystrobin and procymidone were dissolved in distilled water and acetone, respectively. H_2O was used as the control for azoxystrobin treatment. The same volume of acetone was added to the media for the control of procymidone treatment. The transcript levels of *aox* gene were measured by quantitative real-time PCR and the values were given after normalization with histone H3 gene (GenBank accession no. XM_001589836). Means and standard errors of the results from three independent experiments are shown.

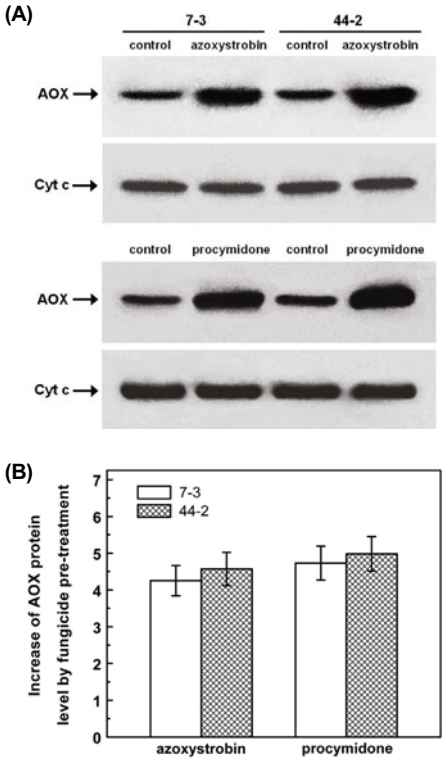

Fig. 5. Effect of pre-treatment for 24 h with azoxystrobin and procymidone on alternative oxidase (AOX) protein levels in mycelia of both *S. sclerotiorum* isolates (7-3, 44-2). The final concentration of azoxystrobin was 1.102 μg/ml for *S. sclerotiorum* isolate 7-3, and 1.224 μg/ml for isolate 44-2. The final concentration of procymidone was 0.321 μg/ml for *S. sclerotiorum* isolate 7-3, and 0.243 μg/ml for isolate 44-2. These final concentrations of azoxystrobin and procymidone were equal to their EC_{50} values (see Table 1). Both *S. sclerotiorum* isolates were cultured in potato dextrose liquid media. Azoxystrobin and procymidone were dissolved in distilled water and acetone, respectively. H_2O was used as the control for azoxystrobin treatment. The same volume of acetone was added to the media for the control of procymidone treatment. (A) Western blot to detect the AOX and cytochrome *c* (Cyt *c*) proteins in mitochondrial samples purified from mycelia of *S. sclerotiorum*. Each lane was loaded with 25 μg of mitochondrial protein. AOX and Cyt *c* were detected with polyclonal antibodies against AOX and Cyt *c*, respectively. Experiments were carried out three times and representative results are shown. (B) Increase of AOX protein levels by fungicide 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 fungicide treatment. Means and standard errors of the results from three independent experiments are shown.

capacity of both *S. sclerotiorum* isolates, while procymidone pre-treatment displayed no influence. However, pre-treatment with azoxystrobin and procymidone both caused an obvious increase in their AP capacity.

The effect of pre-treatment for 24 h with fungicides on *aox* transcript and alternative oxidase (AOX) protein levels in *S. sclerotiorum* mycelia

AP capacity development is generally related to expression of the AOX encoding gene, *aox* (Vanlerberghe, 1997). In order to understand the mechanism of the increased AP capacity by azoxystrobin and procymidone pre-treatment in *S. sclerotiorum* mycelia, the effects of pre-treatment with both fungicides on *aox* transcript and AOX protein levels in mycelia of both *S. sclerotiorum* isolates were checked. Results of real-time quantitative PCR indicated that pre-treatment for 24 h with azoxystrobin and procymidone both significantly increased the *aox* transcript levels in both *S. sclerotiorum* isolates cultured in liquid medium (Fig. 4). In addition, western blot analysis showed that AOX protein levels were also notably enhanced by pre-treatment for 24 h with both fungicides (Figs. 5A and 5B). These results indicate that pre-treatment with azoxystrobin and procymidone can induce *aox* gene expression in *S. sclerotiorum* mycelia.

Discussion

Azoxystrobin and procymidone are fungicides that function by interfering with fungal metabolism. Azoxystrobin is a QoI fungicide and can inhibit mitochondrial electron transport through CP. It possesses the broadest spectrum of activity among all known anti-fungals. Procymidone can inhibit fungal triglyceride biosynthesis and is a fungicide commonly used to control fungal pathogens in fields and greenhouses where rapeseed, lettuce, tomato, cucumber, and garlic are grown (Basallote-Ureba *et al.*, 1998; Griffiths *et al.*, 2003; Paradjikovic *et al.*, 2004; Ma *et al.*, 2009). The results in this work showed that both azoxystrobin and procymidone were highly effective in controlling the growth of both *S. sclerotiorum* isolates (7-3, 44-2) on PDA plates. With the application of SHAM, we further confirmed that AOX was involved in the regulation of sensitivity of *S. sclerotiorum* to both fungicides - AOX respiration decreased their sensi-

tivity to azoxystrobin, but increased their sensitivity to procymidone (Fig. 1 and Table 1).

QoI fungicides such as azoxystrobin act by inhibiting fungal respiration through binding to the Qo center of cytochrome *b*, which can cause a decrease in fungal CP capacity, as shown in Fig. 2. The reduction in the CP capacity of *S. sclerotiorum* mycelia by azoxystrobin is consistent with previous results where another QoI fungicide, SSF-126, reduced the CP capacity in *M. grisea* mycelia (Yukioka *et al*., 1998). Similarly, the induction of azoxystrobin on both *aox* gene expression and AP capacity development in *S. sclerotiorum* mycelia was also consistent with the inducing effect of SSF-126 on *aox* gene expression and AP capacity development in *M. grisea* mycelia (Yukioka *et al*., 1998).

The decrease in *S. sclerotiorum* mycelia sensitivity to azoxystrobin due to AOX respiration in this report (Fig. 1 and Table 1) was also consistent with the observation that AOX respiration reduced the sensitivity of some other fungi, such as *M. grisea* and *Botrytis cinerea*, to QoI fungicides including azoxystrobin (Avila-Adame and Koller, 2003; Banno *et al*., 2009). Therefore, it may be that AOX respiration commonly reduces fungal sensitivity to QoI fungicides. This is believed to result from its ability to switch electron flux from CP to AP when CP is inhibited by QoI fungicides, as observed in *B. cinerea* treated with the QoI fungicide SSF129 (Tamura *et al*., 1999). Although AOX transfers electrons from reduced ubiquinone to oxygen without ATP formation (Vanlerberghe, 1997), AOX respiration can maintain glycolysis and the TCA cycle to some extent and thus provide carbon skeletons for cellular growth and repair when CP is inhibited by QoI fungicides.

To our knowledge, AOX respiration has been reported to be involved in the regulation of fungal sensitivity to only one non-QoI fungicide, fluconazole. AOX was found to reduce the sensitivity of *Candida albicans* to fluconazole which can inhibit the fungal cytochrome P450 enzyme 14α-demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane, causing subsequent accumulation of 14α-methyl sterols. It was inferred that AOX caused the reduction in sensitivity of *C. albicans* to fluconazole potentially through a mechanism that involved decreased intracellular reactive oxygen species production (Yan *et al*., 2009). In this report, we have provided evidence that AOX respiration is involved in the regulation of sensitivity of *S. sclerotiorum* to procymidone. Procymidone belongs to the dicarboximide fungicides which are characterized by the presence of a 3, 5-dichlorophenyl. The mode of action of procymidone has been reported to be inhibition of triglyceride biosynthesis, with a fungitoxic mechanism different from that of QoI fungicides and fluconazole (Griffiths *et al*., 2003). Therefore, as far as we know, procymidone is the second non-QoI fungicide to which AOX respiration has been identified to be involved in the regulation of fungal sensitivity. The function of AOX respiration in the regulation of fungal sensitivity to fluconazole and procymidone also suggests that it may be involved in the fungal sensitivity regulation to a certain amount of non-QoI fungicides.

As a triglyceride biosynthesis inhibitor, procymidone showed no immediate influence on either the AP or the CP capacity in *S. sclerotiorum* mycelia (Fig. 2). However, it induced *aox* gene expression and AP capacity development in *S. sclerotiorum* mycelia after pre-treatment for 24 h (Figs. 3, 4, and 5). This is the first report showing that inhibition of triglyceride biosynthesis can induce *aox* gene expression and AP capacity development in fungi. The mechanism for this inducing effect remains to be determined.

There may be important agricultural applications for the roles of AOX in regulating the sensitivity of pathogenic fungi to fungicides and the influence of fungicides on AOX expression. Although azoxystrobin and procymidone both induced *aox* gene expression and AP capacity development in *S. sclerotiorum* mycelia, AOX respiration showed opposite influences on *S. sclerotiorum* sensitivity to azoxystrobin and procymidone. Therefore, when azoxystrobin is used to control *S. sclerotiorum* it will have a greater effect if its *aox* gene expression and AP capacity development are hindered, or activity inhibited. This may be achieved through the application of certain *aox* expression inhibitors. On the contrary, when procymidone is used to control this fungal pathogen, its *aox* gene expression and AP capacity development should be encouraged by certain *aox* expression activators.

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