Disruption of the Alternative Oxidase Gene in *Magnaporthe grisea* and Its Impact on Host Infection

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Plants and numerous fungi including *Magnaporthe grisea* protect mitochondria from interference by respiration inhibitors by expressing alternative oxidase, the enzymatic core of alternative respiration. The alternative oxidase gene *AOXMg* of *M. grisea* was disrupted. Several lines of evidence suggested that the disruption of *AOXMg* was sufficient to completely curb the expression of alternative respiration. In the infection of barley leaves, several *AOXMg*-minus and, thus, alternative respiration-deficient mutants of *M. grisea* retained their pathogenicity without significant impairment of virulence. However, differences between the wild-type strain and an *AOXMg*-minus mutant were apparent under oxidative stress conditions generated by the treatment of infected barley leaves with the commercial respiration inhibitor azoxystrobin. Symptom development was effectively suppressed on leaves infected with the alternative respiration-deficient mutant, while lesions on leaves infected with the wild-type strain continued to develop at much higher inhibitor doses. However, respective lesions rarely developed to the stage of full maturity. The results did not conform to a previous model implying that expression of alternative respiration is silenced during pathogenesis by the presence of constitutive plant antioxidants. Rather, alternative respiration provided protection from azoxystrobin during both saprophytic and infectious stages of the pathogen. The nature of similar oxidative stress conditions in the ecology of *M. grisea* remains an open question.

Plants and numerous fungi can complement or replace the normal route of mitochondrial electron transport by the expression of alternative respiration (Day et al. 1995; Joseph-Horne and Hollomon 2000; McIntosh et al. 1998; Vanlerberghe and McIntosh 1997). This alternative pathway is resistant to the cytochrome *c* oxidase inhibitors cyanide, azide, and carbon monoxide and relies on the expression of alternative oxidase (AOX) activity. AOX, which is susceptible to inhibition by hydroxamic acids and alkylgallates, is encoded by a nuclear gene, is transcribed in the cytosol, and is transported into the inner mitochondrial membrane (Day et al. 1995; Siedow and Umbach 2000; Vanlerberghe and McIntosh 1997).

One of the primary proposed functions of alternative respiration in both plants and fungi is to provide cells and mitochondria with a protective mechanism against reactive oxygen species produced during the disturbance of cytochrome-mediated electron transport (Maxwell et al. 1999; Møller 2001). In plants, expression of alternative respiration is achieved by either the induction of AOX transcription or the activation of constitu-

tively expressed AOX already present in mitochondrial membranes (Siedow and Umbach 2000; Vanlerberghe and McIntosh 1997). Although evidence for the constitutive presence of AOX in fungal mitochondria was provided for *Gaeumannomyces graminis* (Joseph-Horne et al. 1998), the phenomenon was not apparent for other fungal organisms (Affourtit et al. 2000; Umbach and Siedow 2000; Yukioka et al. 1998). Alternatively, reactive oxygen species such as H₂O₂ have been identified as inducers of fungal AOX expression (Minagawa et al. 1992; Mizutani et al. 1996; Yukioka et al. 1998). Additional functions of fungal alternative respiration including roles in plant-pathogen interactions have been proposed but remain poorly defined at present (Joseph-Horne and Hollomon 2000; Joseph-Horne et al. 2001).

Increased interest in fungal alternative respiration was spurred by the recent introduction of strobilurin-related fungicides acting as specific respiration inhibitors by binding to the Qo center of cytochrome *b* (Clough et al. 1995; Jordan et al. 1999; Köller 1999; Ypema et al. 1999). Under saprophytic conditions of pathogen growth, these Qo-inhibitors induce the expression of alternative respiration, allowing sustained growth and development in the presence of a fully inhibited cytochrome *b* target site (Joseph-Horne and Hollomon 2000; Köller et al. 2002). Surprisingly, a similar rescue mechanism from inhibitor action was not apparent when plant diseases were controlled with the same inhibitors (Olaya et al. 1998; Olaya and Köller 1999; Ziogas et al. 1997). This discrepancy between pathogen responses during saprophytic and pathogenic stages was explained by proposing that the induction of alternative respiration is suppressed in the presence of a host. According to the model, constitutive plant antioxidants such as flavone enter fungal cells and will decrease the levels of reactive oxygen species required for the induction of AOX (Mizutani et al. 1996; Tamura et al. 1999; Yukioka et al. 1998).

Suppression of AOX induction during the infectious stages of plant pathogens would imply that alternative respiration constitutes a dispensable trait in the infection of plants. Thus far, the model of AOX silencing has only been based on indirect evidence (Mizutani et al. 1996; Olaya et al. 1998; Yukioka et al. 1998). The present study explores the role of alternative respiration in host infection by employing AOX-disrupted mutants of *Magnaporthe grisea* (Hebert) ME Barr and by characterizing both their virulence and their responses to oxidative stress inflicted by the Qo-inhibiting fungicide azoxystrobin.

RESULTS

Disruption of the alternative oxidase gene *AOXMg*.

A DNA fragment containing the coding region of *AOXMg* as well as flanking regions (Fig. 1) was isolated from the genome of *M. grisea*. In a Southern analysis with endonucleases lack-

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ing restriction sites in *AOXMg*, single DNA fragments hybridized with a probe reflecting a central and highly conserved portion of *AOXMg* (Fig. 2). The results indicated the presence of a single *AOXMg* copy and the lack of other closely related alternative oxidase genes. Transformation with the disruption vector pAOXHYG (Fig. 1) yielded six hygromycin-resistant transformants fully inhibited at 0.1 $\mu\text{g ml}^{-1}$ azoxystrobin, the dose employed in the negative selection of alternative respiration-deficient mutants. At the same dose, mycelial growth of the wild-type isolate was only inhibited by 37%.

The results of a Southern analysis were consistent with the disruption of *AOXMg* in all six transformants (Fig. 3). Genomic DNA fragments generated with *Bgl*II, an endonuclease lacking restriction sites in both *AOXMg* and the hygromycin resistance cassette of pAOXHYG, were upshifted in size by the expected 2.5 kb when probed with a 727-bp portion of *AOXMg*. The same fragments isolated from the mutants hybridized with a probe reflecting the hygromycin-resistance cassette of the transformation vector. Additional copies of the vector were not detected in any of the transformants (data not shown), indicating single insertion events in all six mutants.

Responses of mycelia to azoxystrobin.

Mycelia of all *AOXMg*-minus mutants were ≥ 180 -fold more sensitive to the Qo inhibitor azoxystrobin than the wild-type strain when median effective doses (ED_{50} values) were compared (Table 1). Respective sensitivity differences were even more pronounced at high azoxystrobin doses, as indicated by the >400 -fold difference recorded for ED_{90} values (Table 1). Moreover, growth of the wild-type isolate was inhibited by only 64% at the highest tested azoxystrobin concentration of 10 $\mu\text{g ml}^{-1}$, while complete growth inhibition of all *AOXMg*-minus mutants was already achieved at the 100-fold lower dose of 0.1 $\mu\text{g ml}^{-1}$.

In the presence of the AOX inhibitor salicylhydroxamic acid (SHAM), ED_{50} values of the *AOXMg*-minus mutants were not altered, while the sensitivity of the wild-type strain became indistinguishable from the mutants (Table 1). The same applied to respective ED_{90} values, with the exception of mutant M-155 responding slightly more sensitive when tested in the presence of SHAM (Table 1). The results indicated that increased azoxystrobin sensitivities of the *AOXMg*-minus mutants were explained by their complete deficiency of an alternative respiration response counteracting the action of azoxystrobin.

Further support for the disruption of a single alternative oxidase gene involved in the expression of alternative respiration was derived from the genetic analysis of three *AOXMg*-minus

mutants. The result expected from the presence of a single alternative oxidase gene was a 1:1 cosegregation for the two traits of hygromycin-resistance and the alternative respiration-mediated resistance to azoxystrobin. This expectation was fully confirmed in tests of progeny sensitivities (Table 2). Rare and insignificant occasions of unexpected genotypes were most likely explained by rare homologous recombinations between the disrupted and the intact *AOXMg*.

Pathogenicity and virulence of *AOXMg*-minus mutants on barley leaves.

The complete lack of alternative respiration in the *AOXMg*-minus mutants when challenged with the Qo inhibitor azoxystrobin provided the opportunity to evaluate the role of this alternative route of electron transfer during pathogenesis. All six *AOXMg*-minus mutants caused lesions on barley leaves (Fig. 4) and, thus, were pathogenic. Several parameters of virulence were evaluated for the three mutants characterized genetically (Table 2) and were compared with the wild-type strain. Parameters included infection efficacies, appressoria formation, stages of lesion development, and the sizes as well as conidiation of fully mature lesions of type 3 (Table 3). For all five measures of virulence investigated, differences observed between the *AOXMg*-minus mutants and the wild-type strain were not significant. Although mean values of conidia formation were numerically lower for all three mutants tested, the observed effect was statistically insignificant (Table 3). All results indicated that, in the infection of barley leaves by *M. grisea*, expression of alternative respiration was a dispensable trait under the infection conditions employed.

Fig. 1. Diagram of *AOXMg* of *Magnaporthe grisea* (GenBank accession number AF325683) and strategy of *AOXMg* disruption. Closed boxes in *AOXMg* indicate exons and open boxes indicate introns. An internal 792-bp *Hind*III-*Kpn*I section of *AOXMg* was deleted and replaced by a selectable marker gene containing the hygromycin B phosphotransferase gene (*hph*) of *Escherichia coli* and the *oliC* promoter and the *trpC* terminator of *Aspergillus nidulans*.

Fig. 2. Southern blot analysis of alternative oxidase genes in *Magnaporthe grisea*. Genomic DNA was digested with endonucleases as indicated and probed with a 167-bp polymerase chain reaction product reflecting a central region of *AOXMg*.

Expression of alternative respiration in response to oxidative stress.

In order to compare the impact of alternative respiration on the response to oxidative stress of mycelia developing inside the host with mycelia growing under saprophytic conditions (Table 1), barley leaves infected with *M. grisea* were treated with the same Qo inhibitor, azoxystrobin. Increasing inhibitor doses led to increasing levels of symptom suppression on barley leaves infected with the arbitrarily selected *AOXMg*-minus mutant M-145 (Fig. 5). The ED_{50} dose determined was $0.2 \mu\text{g ml}^{-1}$. For the wild-type strain tested under identical conditions, the dose required to achieve 50% of symptom suppression was $7.1 \mu\text{g ml}^{-1}$ (Fig. 5) and, thus, was 36-fold higher.

The results indicated that very similar to saprophytic conditions (Table 1), mycelium of the wild-type strain developing inside the host was capable of coping effectively with oxidative stress inflicted by azoxystrobin. However, the effectiveness of the alternative respiration rescue was reduced under the in planta conditions of disease development. While ED_{50} values determined for the wild-type strain and the mutant M-145 growing under saprophytic conditions were separated by a factor of 180 (Table 1), the respective factor was 5-fold lower for the suppression of symptom development (Fig. 5).

Although alternative respiration provided an effective mechanism for protecting the wild-type strain of *M. grisea* from the inhibitory action of azoxystrobin on symptom development, the stage of lesion maturity was clearly affected under such conditions. Lesions of the wild-type strain continued to develop, but they rarely reached the stage of fully mature type 3 lesions (Table 4). In fact, the ED_{50} value determined for the suppression of mature lesion development was $0.2 (\pm 0.05) \mu\text{g ml}^{-1}$ for the wild-type strain and $0.1 (\pm 0.04) \mu\text{g ml}^{-1}$ for the alternative respiration-deficient mutant M-145. In contrast to the

suppression of symptom development (Fig. 5), this difference was small and of low statistical significance ($P = 0.09$), indicating that lesions were effectively prevented from reaching a fully mature stage for both the wild-type strain and the alternative respiration-deficient mutant. However, the wild-type strain retained the capacity to develop abundant type 2 lesions at relatively high doses of azoxystrobin, while the much fewer lesions continuing to develop on leaves infected with *AOXMg*-minus mutant were restricted to reaching the least mature lesion type 1 (Table 4).

DISCUSSION

Until recently, research on the route of alternative respiration in fungal organisms had been dedicated to saprophytes such as *Neurospora crassa* and several yeasts (Joseph-Horne and Hollomon 2000; Joseph-Horne et al. 2001). The introduction of a new class of commercial fungicides acting as specific respiration inhibitors by binding to the Qo center of cytochrome *b* (Clough et al. 1995; Jordan et al. 1999; Köller 1999; Köller et al. 2002; Ypema et al. 1999) has triggered increasing interest in exploring this alternative route of fungal electron transfer in plant pathogenic fungi. Consequently, an increasing number of AOX genes have been fully or partially cloned and sequenced (Joseph-Horne et al. 2000; Steinfeld et al. 2001).

The expression of alternative respiration in a plant pathogenic fungus was first documented for *M. grisea* responding to the Qo respiration inhibitor metominostrobin (Mizutani et al. 1996). It was soon recognized that expression of alternative respiration provided a rescue from Qo inhibitor action in several other plant pathogens (Joseph-Horne and Hollomon 2000; Köller et al. 2002). However, it was also recognized that this rescue mechanism was not counteractive to complete control of diseases provided by the same inhibitors (Olaya and Köller 1999; Olaya et al. 1998; Ziogas et al. 1997). It was proposed that, during the infection of host plants, endogenous plant antioxidants suppress the expression of alternative oxidase induction by lowering the concentration of reactive oxygen species as the signal for AOX induction (Mizutani et al. 1996; Tamura et al. 1999; Yukioka et al. 1998). This model of gene silencing during pathogenesis suggested that alternative respiration is a dispensable trait during host-pathogen interactions.

The present study describes, for the first time, the results of a direct approach to defining the role of fungal alternative respiration in plant infection. Under saprophytic growth conditions, disruption of the alternative oxidase gene *AOXMg* in *M. grisea* fully abolished the expression of alternative respiration in response to inhibition of the normal cytochrome-mediated

Fig. 3. Southern blot analysis of *AOXMg* of *Magnaporthe grisea* in alternative respiration-deficient mutants. Genomic DNA was digested with *Bgl*III. **A**, Fragments were probed with the 727-bp *Eco*RI-*Hind*III segment of *AOXMg* located upstream of the hygromycin resistance cassette inserted to generate the *AOXMg*-minus mutants. **B**, The same fragments were probed with a 1.5-Kb segment of the hygromycin resistance cassette.

Table 1. Azoxystrobin sensitivities of mycelia of the wild-type strain and *AOXMg*-minus mutants of *Magnaporthe grisea*

Genotype	$ED_{50} (\mu\text{g ml}^{-1})^y$		$ED_{90} (\mu\text{g ml}^{-1})^y$	
	-SHAM	+SHAM	-SHAM	+SHAM
Wild-type	1.8 a	0.010 a	>10 a	0.027 a
M-95 ^z	0.010 b	0.009 a	0.025 b	0.023 ab
M-98 ^z	0.009 b	0.010 a	0.023 b	0.026 ab
M-145 ^z	0.010 b	0.009 a	0.023 b	0.024 ab
M-155 ^z	0.009 b	0.008 a	0.022 b	0.021 b
M-259 ^z	0.009 b	0.007 a	0.022 b	0.022 ab
M-337 ^z	0.009 b	0.009 a	0.023 b	0.023 ab

^y Azoxystrobin doses providing 50% (ED_{50}) and 90% (ED_{90}) growth inhibition, respectively. Mean values were derived from combining data of two separate experiments with three replicates each. Sensitivities were determined in the absence of (-SHAM) and the presence of (+SHAM) salicylhydroxamic acid. Means not followed by a common letter are significantly different (Tukey's test $\alpha = 0.05$).

^z Derived from the disruption of *AOXMg*.

chain of electron transport. Based on several lines of evidence, additional alternative oxidase genes, which is a common phenomenon in plants (Daisuke et al. 1997; Whelan et al. 1996; Yusukey et al. 1997) and also has been suggested for *Candida albicans* (Huh and Kang 1999), could not be identified in *M. grisea*. Southern analysis gave no indication for the presence of additional genes closely related to *AOXMg*, the AOX inhibitor SHAM had no effect on the sensitivity of *AOXMg*-minus mutants to the Qo inhibitor azoxystrobin, full inhibition of AOX with SHAM rendered the wild-type isolate to respond as sensitive to azoxystrobin as the *AOXMg*-minus mutants, and genetic analysis confirmed the presence of a single AOX gene induced in response to the Qo inhibitor azoxystrobin. The results strongly suggest that disruption of *AOXMg* fully abolished the expression of alternative respiration in *M. grisea* responding to the inhibition of normal cytochrome-mediated respiration.

AOXMg-minus mutants and, thus, alternative respiration-deficient mutants retained pathogenicity and full virulence on barley leaves. Consequently, our results gave no indications for a crucial role of alternative respiration in the infection of the barley host under normal infection conditions. However, post-infection exposure of the wild-type strain and an alternative respiration-deficient mutant to azoxystrobin demonstrated that the induction of alternative respiration provided an effective rescue mechanism from oxidative stress for mycelium of *M. grisea* developing inside a host. Our results were not in support of a model implying the silencing of alternative respiration during pathogenesis by the presence of constitutive plant anti-oxidants suppressing the induction of alternative respiration (Mizutani et al. 1996; Yukioka et al. 1998). Although the effectiveness of the rescue mechanism was 5-fold lower under in planta than under saprophytic conditions of pathogen development, alternative respiration was not entirely silenced but rather continued to provide effective protection from azoxystrobin during host infection. In spite of the alternative respiration-mediated rescue from oxidative stress during both saprophytic and infectious conditions, a penalty inherent to the rescue response was apparent in the fact that the maturity stage of lesions developing in the presence of azoxystrobin was severely impaired. These results conform to the finding that *Mycosphaella graminicola* mycelium developing inside wheat leaves was inhibited, but remained viable, after infected leaves were treated with azoxystrobin (Rohel et al. 2001).

Regardless of the effectiveness of alternative respiration in the protection from oxidative stress, the inherent penalty of slower mycelial growth during saprophytic stages or decreased lesion maturity during infectious stages appears to be governed by the reduced ATP production the route of alternative respiration provides (McIntosh et al. 1998). Consequently, the capacity of *M. grisea* to respond to oxidative stress by the expression of alternative respiration appears to constitute a survival mechanism rather than a penalty-free rescue mechanism under both saprophytic and infectious stages of pathogen development. The nature of the environmental conditions in the ecol-

ogy of *M. grisea* imposing oxidative stress similar to the Qo inhibitor azoxystrobin remains an open question.

MATERIALS AND METHODS

Fungal cultures.

The *M. grisea* strains 4091-5-8 (mating type 1-2, pathogen of barley, weeping lovegrass, and goosegrass) and 4136-4-3 (mating type 1-1, pathogen of weeping lovegrass and goosegrass) were provided by S. Kang (Pennsylvania State University) (DeZwaan et al. 1999; Valent et al. 1987, 1991). Mycelia were grown on oatmeal agar (Difco Laboratories, Detroit, MI, U.S.A.) or on the complete medium described by Crawford and associates (1986) (1% sucrose, 0.6% yeast extract and 0.6% casein enzymatic hydrolysate, solidified with 1.5% agar). Sexual crosses involving mutants of strain 4091-5-8 were performed with strain 4136-4-3 according to the procedure described by Valent and associates (1986). Strains and mutants were preserved as described elsewhere (Valent et al. 1991).

Disruption of the alternative oxidase gene *AOXMg*.

AOXMg derived from *M. grisea* DNA (GenBank accession number AF325683) was used to construct the transformation vector pAOXHYG composed of pBluescript-SK⁻ (Promega Corporation, Madison, WI, U.S.A.), a 2.65-kb insert of the hygromycin resistance construct from pOHT containing the *hph* gene of *Escherichia coli* and both the *oliC* promoter and the *trpC* terminator from *Aspergillus nidulans* (Yao and Köller 1995) and two fragments of the *AOXMg* (727 and 354 bp) flanking the hygromycin resistance cassette (Fig. 1). The vector was prepared by cloning the 1873-bp *EcoRI*-*SacII* fragment of *AOXMg* (Fig. 1) in pBluescript-SK⁻ to generate pAOX. The plasmid was double digested with *HindIII* and *KpnI* to delete a 792-bp fragment from the *AOXMg* segment. The hygromycin cassette was ligated into the *HindIII*-*KpnI*-digested pAOX (Fig. 1) to produce the transformation vector.

The pAOXHYG vector was used in circular form to transform protoplasts (6.5 µg per 10⁸ protoplasts) following the one-step gene disruption technique (Rothstein 1983). Protoplast preparation and polyethylene glycol-based transformation was performed following a combination of established protocols (Leung et al. 1990; Shi et al. 1995). Subsequent to transformation, protoplasts were plated on regeneration medium (1.5% agar, 20% sucrose, 0.6% yeast extract, 0.6% casein enzymatic hydrolysate) containing 200 µg ml⁻¹ hygromycin B and 25 µg ml⁻¹ ampicillin. Plates were incubated at 25°C, and individual transformant colonies 1 to 2 mm in size developing after 5 days were transferred to complete media amended with 200 µg ml⁻¹ hygromycin.

Characterization of *AOXMg*-minus mutants.

Selection of *AOXMg*-minus mutants was accomplished by sensitivity tests on complete medium amended with 0.1 µg of azoxystrobin per ml (Zeneca Agrochemicals, Richmond, CA,

Table 2. Segregation analysis of random ascospore progenies^a

Mutants ^c	Sensitivity distributions ^b				χ^2
	Hyg ^s Azo ^s	Hyg ^r Azo ^s	Hyg ^s Azo ^r	Hyg ^r Azo ^r	
M-145	0	89	96	1	0.27
M-259	3	93	95	3	0.21
M-337	5	94	100	1	0.36

^a Derived from crosses between three *AOXMg*-minus mutants and the wild-type strain 4136-4-3.

^b Sensitive (^s) or resistant (^r) responses of progenies to hygromycin B (Hyg) or azoxystrobin (Azo), respectively. Values are the number of genotypes tested. The χ^2 analysis tested a 1:1 segregation ratio ($\alpha = 0.05$) for the genotypes Hyg^rAzo^s and Hyg^sAzo^r.

^c Derived from the disruption of *AOXMg*.

U.S.A.). Transformants showing no or slow growth after 5 days of incubation at 25°C were selected for further analysis. This negative selection process yielded 28 putative *AOXMg*-minus mutants out of a total of 1,007 transformants tested.

Disruption of *AOXMg* in transformants selected according to increased azoxystrobin sensitivities was tested by polymerase chain reaction following routine procedures (Sambrook et al. 1989) using partially purified DNA (Wu et al. 1997). One pair of primers employed amplified a 628-bp fragment of the *AOXMg* at the 5' region. The forward primer was 5'-ACGAA-CACTGAACGTACC-3' (P1) and the reverse primer was 5'-TG-GAGGTGTCTCAGCATG-3' (P2). A second pair of primers amplified a 666-bp fragment of gene at the 3' end. The forward primer was 5'-AACGAGCGCATGCACCT-3' (P3) and the reverse primer was 5'-GTGTCAGTCTTGCGTAGT-3' (P4).

Primers P2 and P3 were located in the *AOXMg* fragment that had been replaced by the hygromycin cassette in the disruption vector. Consequently, lack of amplification indicated disruption of *AOXMg*. Of the 28 putative *AOXMg*-minus mutants, six lacked the expected amplification products.

AOXMg disruption and vector insertion sites were verified for the six mutants by Southern analysis using genomic DNA extracted by the cetyltrimethylammonium bromide-based method described by Csai et al. (1998). Genomic DNA (1.5 to 4 µg) was digested with *Bgl*II, separated on a 0.7% agarose gel in 1× Tris-acetate-EDTA buffer, and transferred onto a nylon membrane (Micron Separation Inc., Westborough, MA, U.S.A.) according to a standard protocol (Sambrook et al. 1989). The 727-bp *Eco*RI-*Hind*III *AOXMg* fragment located upstream of the hygromycin resistance cas-

Fig. 4. Pathogenicity of *AOXMg*-minus mutants of *Magnaporthe grisea* on barley leaves. Disease symptoms shown are lesions of type 3, developing 5 days after inoculation with the wild-type strain 4091-5-8 or with the six *AOXMg*-minus mutants. A control leaf segment was challenged with inoculation droplets void of conidia.

sette of pAOXHYG (Fig. 1) and a 1.5-kb fragment of the hygromycin resistance cassette obtained by digestion of pOHT with *Eco*RI were used as probes labeled with 32 P (Prime-a-Gene System Kit, Promega, Madison, WI, U.S.A.; [α^{32} P] dCTP, Du Pont-NEN, Boston).

Fig. 5. Infection efficacy of the *Magnaporthe grisea* wild-type strain 4091-5-8 (WT) and an *AOXMg*-minus mutant (M-145) on barley leaves treated with increasing doses of azoxystrobin 36 h after inoculation.

Azoxystrobin sensitivity of mycelia under saprophytic growth conditions.

Azoxystrobin sensitivities were evaluated for the stage of mycelial growth on complete media. Azoxystrobin concentrations were 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 $\mu\text{g ml}^{-1}$. Tests were performed in the presence of and absence of the alternative oxidase inhibitor SHAM (Aldrich, Milwaukee, WI, U.S.A.) employed at a concentration of 150 $\mu\text{g ml}^{-1}$. This SHAM dose was found to fully inhibit alternative respiration-mediated resistance responses. Azoxystrobin was dissolved in acetone, and SHAM was dissolved in methanol/acetone (1:1, vol/vol). All media contained 0.2% (vol/vol) of acetone and 0.1% (vol/vol) of methanol in addition to respective inhibitors. These solvent concentrations had no effect on mycelial growth. For sensitivity tests, mycelial plugs (2×2 mm) were excised from the colony edge of 4- to 5-day-old mycelial cultures grown on oatmeal agar and transferred to complete medium amended with either azoxystrobin, SHAM, or the mixture of both. Diameters of mycelial colonies were measured after incubation for 6 days at 25°C.

All sensitivity tests were conducted twice with three replicates each. When mixtures of azoxystrobin and SHAM were tested, nontreated controls reflected colonies grown in the presence of SHAM. At the dose of 150 μg of SHAM per ml employed in these controls, SHAM inhibited growth by <28%. ED_{50} and ED_{90} values were calculated by regressing the mean relative growth values (colony diameter on strobilurin-amended medium divided by the diameter on unamended medium and multiplied by 100) within the range of 10 to 90 against the log of azoxystrobin concentrations.

Table 3. Virulence of the wild-type strain and *AOXMg*-minus mutants of *Magnaporthe grisea* on barley leaves

Genotypes	Infection efficacy (%) ^b	Appressorium formation (%) ^c	Types of lesions (%) ^a			Type 3 lesions	
			1	2	3	Lesion size (mm ²)	Conidia per mm ² (× 10 ³)
Wild-type	92 (13) ^d	80 (10)	15 (6) ^d	44 (27) ^d	41 (21) ^d	6.9 (1.5) ^d	2.7 (2.4) ^d
M-145 ^e	98 (4)	86 (6)	23 (11)	46 (14)	31 (25)	7.4 (1.0)	1.5 (0.8)
M-259 ^e	99 (2)	82 (6)	31 (28)	28 (9)	41 (36)	7.3 (1.5)	1.7 (1.3)
M-337 ^e	97 (3)	84 (4)	31 (22)	29 (13)	40 (30)	6.8 (1.2)	1.2 (0.7)
<i>P</i> ^f	0.70	0.64	0.72	0.46	0.96	0.80	0.28

^a Type 1, brown lesions of up to 1 mm in size; type 2, eyespot lesions of up to 3×2 mm in size; type 3, sporulating eyespot lesions with necrotic center.

^b Inoculation sites distinguished by development of lesions.

^c Appressorium formation was determined on a polystyrene surface. Mean values were derived from three replicates, each with 100 germinating conidia evaluated. Standard deviations are in parentheses.

^d Mean values are combined from three separate experiments with 54 leaf segments containing three individual inoculation sites each. Standard deviations are in parentheses.

^e Derived from the disruption of *AOXMg*.

^f Derived from ANOVA analysis.

Table 4. Effects of azoxystrobin on lesion maturation^a

Azoxystrobin ($\mu\text{g ml}^{-1}$)	Wild-type isolate ^b lesion type ^c				Mutant ^b lesion type ^c			
	No lesion	1	2	3	No lesion	1	2	3
0	1	5	19	29	5	10	17	22
0.03	3	4	21	26	4	20	12	18
0.1	0	4	27	23	17	10	15	12
0.3	3	9	35	7	41	7	6	0
1.0	14	19	21	0	45	8	1	0
3.0	22	18	14	0	48	6	0	0
10.0	28	17	9	0	47	7	0	0

^a In response to treatment of barley leaves infected with the wild-type strain or the *AOXMg*-minus mutant M-145 of *Magnaporthe grisea*.

^b Values represent the number of lesions formed in three separate experiments with a total of 54 sites inoculated with the wild-type strain or the *AOX*-minus mutant M-145. Barley leaf segments were treated with azoxystrobin 36 h after inoculation.

^c Type 1, brown lesions of up to 1 mm; type 2, eyespot lesions of up to 3×2 mm; and type 3, sporulating eyespot lesions with necrotic center.

Sensitivities of sexual progenies derived from crosses between *AOXMg*-minus mutants and strain 4136-4-3 were tested as follows. Mycelial cultures developing from single arbitrarily selected ascospores were grown on inhibitor-free complete medium. Sensitivity tests were conducted with mycelium growing on media amended with single discriminatory doses of azoxystrobin ($1.0 \mu\text{g ml}^{-1}$) or hygromycin *B* ($200 \mu\text{g ml}^{-1}$). At the discriminatory azoxystrobin dose, respective *AOXMg*-minus mutants were completely inhibited, while mycelium of the wild-type strain 4136-4-3 continued to grow after 6 days of incubation at 25°C . Likewise, *AOXMg*-minus mutants developed mycelium at the discriminatory hygromycin *B* dose used, while the wild-type strain 4136-4-3 was fully inhibited.

Pathogenicity and virulence tests.

Infections of detached barley (cv. Bar soy) leaf segments followed the procedure described by Balhadère and associates (1999). Leaf sections (4 cm) were excised from the second and third leaves of barley plants grown for 4 weeks in a cycle of light for 14 h at 20°C and dark for 10 h at 16°C (65% relative humidity). Conidial suspensions in water were prepared from mycelial cultures grown for 8 to 10 days on oatmeal agar. Suspensions were diluted into 0.25% gelatin (type B, bovine skin, bloom 75; Sigma Chemical Company, St. Louis) to yield a density of $5 \times 10^4 \text{ ml}^{-1}$. Leaf sections were inoculated by placing $10 \mu\text{l}$ conidial suspensions onto the upper surface (Seaward et al. 1998). Inoculated leaf segments were placed on the surface of 1.5% water agar in petri dishes and were incubated at 25°C under a 13-h fluorescent light regimen. Inoculation drops were removed after 48 h. Infection experiments were done three times for each of the strains, with 54 leaf segments inoculated at three separate sites each.

Disease symptoms were scored 5 days after inoculation. The lesion types that developed resembled the lesion types described on rice (Valent et al. 1991). Type 1 lesions were brown pinpoint lesions of up to 1 mm in diameter. Type 2 lesions were eyespot lesions of up to 3.0×2.0 mm in size distinguished by a brown center and surrounded by a dark-brown margin. The type 3 lesions were eyespot lesions of similar or slightly larger sizes characterized by a necrotic center and sometimes surrounded by a yellow halo (Fig. 4). Lesion sizes were measured with a pair of calipers. To complete full conidia formation, infected leaves were placed on wet filter paper inside of sealed plastic bags after lesion scoring was completed and were incubated for 24 h at 25°C . Conidia were removed by stirring excised lesions for 60 min in 5 ml of 0.005% Tween 20. The numbers of conidia formed were determined with a hemacytometer. Conidia were not formed in type 1 lesions, were scarce and inconsistent in type 2 lesions, and were most abundant and uniform in type 3 lesions.

For testing the effects of azoxystrobin on lesion development, leaf segments were inoculated and incubated as described above. Inoculation droplets were removed after 36 h, and the infected leaf segments were soaked for 90 min in 100 ml of 0.005% Tween 20 containing azoxystrobin at the concentrations of 0, 0.03, 0.1, 0.3, 1.0, 3.0, and $10.0 \mu\text{g ml}^{-1}$. Segments were dried on paper towels, after which incubation of leaf segments was continued. Lesions were scored 5 days after inoculation.

Formation of appressoria was quantified by germinating conidia on the inductive surface of polystyrene (Kuo and Hoch 1996). Conidial suspensions ($50 \mu\text{l}$) in 0.25% gelatin (10^4 conidia ml^{-1}) were placed on the surface of polystyrene petri dishes and were covered with a glass coverslip. Appressoria formation was determined after 24 h incubation at 25°C . Three replicates, each with 100 germinating conidia examined, were evaluated.

Data analysis.

Statistical data analysis was supported by programs provided by Statistical Analysis System (SAS Institute Inc., Cary, NC, U.S.A.). Differences between the wild-type strain and *AOXMg*-minus mutants for all variables studied were analyzed according to general linear model procedures. Multiple-means comparisons were done according to the Tukey test; the *t* test was employed in two-means comparisons. Distributions of sensitivity frequencies displayed by the progenies of sexual crosses were analyzed by a χ^2 test.

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