Curr Genet (2003) 42: 332–338
DOI 10.1007/s00294-002-0356-1

RESEARCH ARTICLE

Cruz Avila-Adame · Wolfram Köller

Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin

Received: 5 February 2002 / Revised: 13 July 2002 / Accepted: 11 October 2002 / Published online: 30 January 2003

Abstract The class of Qo-inhibiting fungicides (QoIs) act as respiration inhibitors by binding to the Qo center of cytochrome b. The longevity of these fungicides has been challenged by the selection of fungal sub-populations resisting high doses of QoI fungicides, with a G143A amino acid exchange in the cytochrome b target site identified as the most common cause of resistance. In contrast, the mechanism of alternative respiration, as another mechanism of fungal QoI resistance, has thus far not been affiliated with practical resistance. In the present study, azoxystrobin-resistant mutants of *Magnaporthe grisea* were generated and characterized. Emergence of these spontaneous mutants was facilitated when resting melanized mycelia were allowed to escape full inhibition by azoxystrobin. This escape was related to the intactness of alternative respiration, indicating that residual expression of this rescue mechanism was involved in the spontaneous emergence of target-site mutants. The two mutants characterized resisted high doses of the QoI, azoxystrobin, with resistance factors exceeding 1,000. Two different mutations of the cytochrome b gene were identified as exchanges of guanine, leading to a G143A or a G143S amino acid exchange. Resistance of both target-site mutants remained stable during four consecutive disease cycles in the absence of azoxystrobin. Several parameters tested to measure fitness penalties inherent to the mutational changes revealed that the G143A mutant was not compromised. In contrast, the conidia production of the G143S mutant was significantly lower under both saprophytic and pathogenic conditions of reproduction.

Keywords Azoxystrobin · Cytochrome b · Qo inhibitors · Fungicide resistance

Communicated by U. Kück

C. Avila-Adame · W. Köller (✉)
Department of Plant Pathology, Cornell University,
New York State Agricultural Experiment Station,
Geneva, NY 14456, USA
E-mail: wk11@cornell.edu

Present address: C. Avila-Adame
Departamento de Fitopatologia,
Colegio de Postgraduados Campus Tabasco,
H. Cárdenas, Tabasco, CP 86500, México

Introduction

Although several classes of modern agricultural fungicides have been developed over the past three decades (Köller 1999), their longevity has been severely restricted by the development of resistance (Dekker 1995; Köller 1991; 2001). The prevailing model explaining the selection of fungicide-resistant plant pathogen populations considers random and rare mutations as the cause for pre-existing but infrequent resistant phenotypes prior to the introduction of a new fungicide. Such rare mutants gain in competitiveness under the selection force of a new fungicide and are selected to frequencies at which disease control of the entire population becomes unsatisfactory (Milgroom et al. 1989; Skylakakis 1987; Wolfe 1982).

The class of strobilurin-related QoIs, which act as inhibitors of respiration by binding to the Qo center of cytochrome b, comprises the most recent addition to the arsenal of modern fungicides (Bartlett et al. 2002; Sauter et al. 1999; Ypema and Gold 1999). The risk of QoI resistance development was anticipated prior to their commercial introduction, with two potentially relevant mechanisms receiving initial attention in risk assessment studies. As summarized recently (Köller et al. 2001), several mutational changes of the cytochrome b target site conferring resistance to Qo inhibitors had been described for *Saccharomyces cerevisiae*. During a mutagenesis study with the apple scab pathogen, *Venturia inaequalis*, a G143A amino acid exchange of the cytochrome b target site was found to confer resistance to the QoI, kresoxim-methyl (Köller et al. 2001; Zheng et al. 2000). Although this particular G143A mutation had

not been reported for *S. cerevisiae* before (Köller et al. 2001), it has since been identified in several subpopulations of plant pathogenic fungi-resisting QoIs under practical conditions of disease control (Bartlett et al. 2002; Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b). Resistance of respective target-site mutants was a stable trait, suggesting that the mutated mitochondrial alleles encoding cytochrome b had been selected to predominant frequencies.

The induction of alternative respiration in response to QoI action was investigated as a second potential mechanism of fungal resistance to this class of fungicides (Joseph-Horne and Hollomon 2000; Joseph-Horne et al. 2001; Köller et al. 2001; Yukioka et al. 1998). Although alternative respiration was found to be expressed by wild-type isolates of several pathogens under saprophytic conditions of development, the mechanism appeared to be of minor importance in the control of respective diseases with QoIs (Olaya and Köller 1999; Olaya et al. 1998; Zheng et al. 2000; Ziogas et al. 1997). Thus far, the alternative respiration mechanism has not been affiliated with QoI resistance of pathogens under practical conditions of disease control (Heaney et al. 2000). However, a recent study employing an alternative oxidase-deficient mutant of *Magnaporthe grisea* revealed that alternative respiration provided rescue from full QoI action when infected barley leaves were treated with azoxystrobin. Only the wild-type isolate of *M. grisea*, but not an alternative oxidase-deficient mutant, continued to develop disease symptoms at relatively high azoxystrobin doses, leading to incomplete control of the disease in a post-infection mode of inhibitor application (Avila-Adame and Köller 2002).

The results presented here indicate that such residual growth of *M. grisea* mycelia facilitated by alternative respiration might have contributed to the relatively frequent generation of spontaneous and stable cytochrome b target site mutants resisting high doses of azoxystrobin.

Materials and methods

Fungal isolates and mutants

Vegetative propagation of *M. grisea* mycelia was on oatmeal medium (Difco Laboratories, Detroit, Mich.) or complete medium (1% sucrose, 0.6% yeast extract, 0.6% casein enzymatic hydrolysate, solidified with 1.5% agar; Crawford et al. 1986). All strains and mutants were preserved in a non-metabolizing state on cellulose filters, following standard procedures (Crawford et al. 1986).

Strain 4091-5-8 of *M. grisea*, pathogenic on goosegrass, weeping lovegrass and barley (DeZwaan et al. 1999), was used as a wild-type strain. The AOXMg-minus mutant M-145 was derived from strain 4091-5-8 by gene disruption (Avila-Adame and Köller 2002). Stable mutants of *M. grisea* resistant to azoxystrobin were generated by transferring plugs of fully melanized mycelia from the center of 6-day-old cultures to complete medium amended with 10 μg azoxystrobin ml^{-1} plus 150 μg ml^{-1} of the alternative oxidase inhibitor, salicylhydroxamic acid (SHAM). Small mycelial colonies developed after 7 days of incubation at 25 °C. Fifty small plugs derived from the melanized areas of such slowly developing colonies were transferred to complete medium amended with the same inhibitors. Several colonies distinguished by rapid growth after the second transfer were allowed to sporulate on oatmeal medium; and monoconidial isolates were prepared on fungicide-free medium. The experiment was repeated twice; and two monoconidial mutants were obtained resisting high concentrations of azoxystrobin in the presence of SHAM.

Sensitivity tests

Sensitivities to azoxystrobin were carried out with mycelia following the procedures described by Avila-Adame and Köller (2002). In brief, treatments included azoxystrobin in the presence or absence of 150 μg SHAM ml^{-1}. Radial growth was measured for colonies grown at 25 °C for 6 days. ED_{50} values were calculated by regressing the mean relative growth values (radial growth on strobilurin-amended medium divided by radial growth on unamended medium ×100) against log azoxystrobin concentration. All tests were repeated at least twice, with three mycelial colonies each. Relative growth values were calculated by combining respective data obtained for all replications.

Sensitivities of germinating conidia were determined with conidia derived from mycelia grown on oatmeal agar for 8–10 days. Suspensions of conidia (6 μl containing 3×10^4 conidia ml^{-1}) were placed on the surface of 1.5% water/agar or water/agar amended with 10 μg azoxystrobin ml^{-1} plus 100 μg SHAM ml^{-1}. Conidia were allowed to germinate for 24 h at 25 °C; and germination was assessed for 100 conidia. Germination frequencies were determined as means derived from three replications, with three replicates each.

In order to compare conidia production of the wild-type strain with two spontaneous mutants growing under saprophytic conditions, conidia were suspended from mycelial colonies grown on oatmeal agar for 8–10 days. Conidia produced by single colonies were suspended in water and counted. Production of conidia was determined as the mean of three replications, with three replicate colonies each.

Infection of barley leaves

Infection assays were performed with excised leaves of barley cv Barsoy, following procedures described elsewhere in full detail (Avila-Adame and Köller 2002; Balhadère et al. 1999). In brief, leaf sections were excised from the third and fourth leaves of 4-week-old plants. Conidial suspensions in 0.25% gelatin were applied to the upper side of leaf sections as 10-μl droplets (5×10^4 conidia ml^{-1}; DeZwaan et al. 1999). Inoculated leaf segments were placed on surfaces of 1.5% water-agar in closed petri dishes and incubated at 25 °C under a 13-h period of fluorescent light.

Disease symptoms were scored 5 days after inoculation. Fully mature lesions distinguished by a necrotic center were allowed to complete sporulation, as described by Avila-Adame and Köller (2002). Infection and sporulation tests were done at least twice, with a minimum of six leaf segments infected in each experiment. For the analysis of data, results from all replications and replicates were combined.

Molecular characterization of spontaneous azoxystrobin-resistant mutants

The cytochrome b gene of *M. grisea* (lodged in GenBank under accession number X87999) was used as a template for sequence analysis. A primer pair was designed amplifying a 628-bp fragment of cytochrome b. This fragment included the G143A position reported for several pathogens (Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b; Zheng et al. 2000) and the locations of all point mutations identified in *S. cerevisiae* (Köller et al. 2001; Zheng et al. 2000). Primer sequences were 5′-GATCATATAGAGCTCCTCG-3′

(P1) and 5′-CTAC CTAAATCTGTAACAGG-3′ (P2) for the 5' and 3' region of the cytochrome b gene, respectively. PCR amplifications were performed using both total DNA and cDNA.

DNA and RNA were extracted from mycelia grown in liquid complete medium for 4 days at 28 °C, with shaking at 220 rpm. Mycelia were harvested by filtration through miracloth (Calbiochem-Novabiochem Corp., La Jolla, Calif.) and washed with water. Mycelia were ground with mortar and pestle in liquid nitrogen. Total DNA was extracted by a CTAB-based method (Csaikl et al. 1998) and RNA was extracted using the Tri-Reagent RNA/DNA/protein isolation system (Molecular Research Center, LPS, Moonachie, N.J.).

PCR amplifications with genomic DNA as a template were performed in a Mastercycler (Eppendorf Scientific, Westbury, N.Y.) in 50-µl reactions containing assay buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 1 µg of total DNA, 1.5 mM $MgCl_2$, 0.6 µM of each primer, 0.2 mM of dNTP and 2 units of Taq polymerase (Eppendorf Scientific, Westbury, N.Y.). The reaction started with denaturation at 94 °C for 3 min and continued for 35 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min for denaturation, annealing and extension, respectively. The reaction was completed with a final extension at 72 °C for 5 min.

Reverse transcription was carried out in a 25-µl reaction volume containing 30 units of the AMV reverse transcriptase, 6 µg of total RNA, 1 mM of dNTP, 0.25 µg of the 3' primer μg^{-1} RNA, 40 units of Rnasin ribonuclease inhibitor, 10 mM of dithiothreitol and 5 µl of AMV RT 5× reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM spermidine). All components were purchased from Promega Corporation, Madison, Wis. The mixture of RNA, 3' primer, Rnasin ribonuclease inhibitor and water were mixed, incubated at 70 °C for 5 min and chilled on ice for 5 min. All other components were added to the mixture and incubated at 42 °C for 60 min. The reaction was terminated at 95 °C for 5 min. PCR amplification was performed as for genomic DNA, by adjusting the components to 50 µl, using 6 µl of the reverse transcription reaction as a template and by modifying the annealing temperature to 53 °C.

PCR-RFLP analysis was performed for the wild-type isolate and the strobilurin-resistant mutants. The primer pair, P1 and P3 (5'-CCACCTCAAATGAATTCAAC-3'), was used to amplify a 193-bp section of the 628-bp PCR product used as template. The 193-bp fragments containing the nucleotides encoding the amino acid at position 143 were separated on a 0.7% agarose gel in TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM disodium EDTA) and purified with the QIAEX II gel extraction kit (Qiagen, Valencia, Calif.). Purified PCR products were digested with the restriction enzyme, Fnu4H I (New England BioLabs, Beverly, Mass.), according to the manufacturer's protocol and visualized on ethidium bromide-stained agarose gels (1.2%). Sizes of DNA fragments were determined by comparison with the 1-kb Plus DNA ladder (Life Technologies, Grand Island, N.Y.).

Sequencing of amplified cytochrome b fragments was carried out with an ABI automated sequencer with fluorescent-dye-labeled didoxy terminators, at DNA Services, NYSAES, Cornell University, N.Y. Sequence comparisons were performed using Lasergene software (DNAStar, Madison, Wis.) and the Basic local alignment search tool (BLAST) algorithm (Altschul et al. 1997) provided by the National Center for Biotechnology Information.

Statistical data analysis

Comparison of the variables evaluated was carried out by analysis of variance (ANOVA), using the SAS general linear model (SAS Institute, Cary, N.C.). Multiple-means comparisons were supported by Tukey's test and two-means comparisons by the SAS *t*-test.

Results

Generation of azoxystrobin-resistant mutants

In previous studies, the sensitivity of *M. grisea* mycelia to azoxystrobin was determined by transferring mycelial fragments from actively expanding perimeters of colonies to inhibitor-containing media (Avila-Adame and Köller 2002). At an azoxystrobin concentration of 10 µg ml^{-1} plus 150 µg SHAM ml^{-1}, development of colonies from such non-melanized mycelia was fully prevented for both the wild-type strain (Fig. 1A) and the AOXMg-minus mutant (Fig. 1C). In contrast, small colonies developed for the wild-type strain when fragments of fully melanized mycelia were subjected to the same inhibitory conditions (Fig. 1A). For the AOXMg-minus mutant, the inhibition of older melanized mycelia was as complete as for non-melanized mycelia (Fig. 1C). In a second round of mycelial transfers from the small colonies developing from melanized mycelia of the wild-type

Fig. 1A–C Development of *Magnaporthe grisea* mycelium in the presence of azoxystrobin (10 µg ml^{-1}) plus salicylhydroxamic acid (SHAM; 150 µg ml^{-1}). Growth was initiated either with non-melanized mycelia derived from the perimeters of actively expanding colonies (*lower specimens*) or with melanized mycelia derived from older colony sections (*upper specimens*). Non-melanized and melanized mycelial segments from small colonies developing from melanized wild-type mycelia (**A**) were transferred to growth media containing the same mixture of inhibitors (**B**). Development of both non-melanized and melanized mycelium of the AOXMg-minus mutant was fully prevented under identical conditions (**C**)

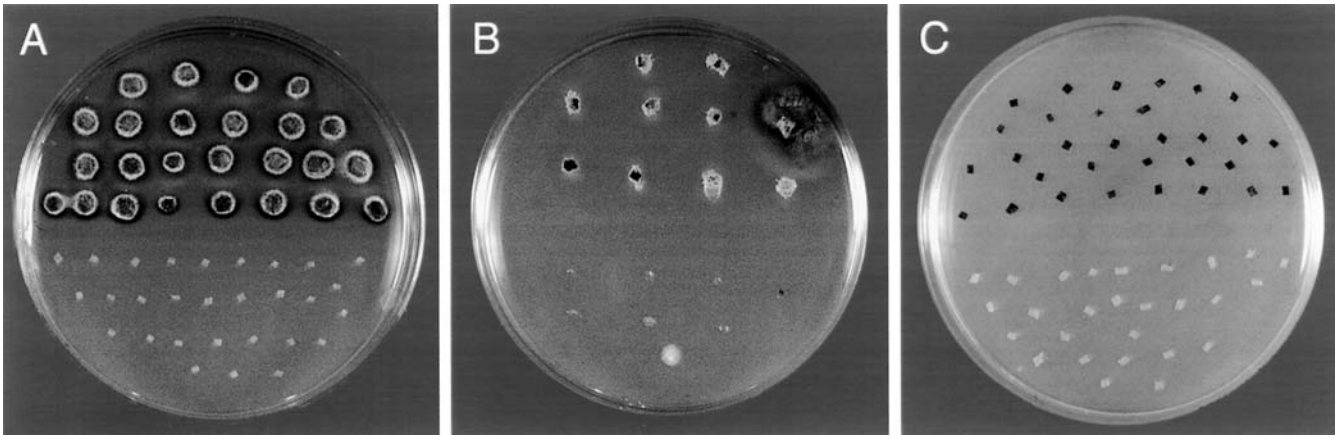

Table 1 Mycelial sensitivities of a wild-type strain and mutants of *Magnaporthe grisea* to azoxystrobin. The AOX-minus mutant (*M-145*; Avila-Adame and K ler 2002) and two cytochrome b target-site mutants (*G143A*, *G143S*) were derived from wild-type strain 4091-5-8. Values given are mean concentrations providing 50% inhibition (ED_{50}) and relative growth (*RG*) at a dose of 10 μg azoxystrobin ml^{-1}. Figures in parentheses are standard deviations. Means within a column not followed by a common letter are significantly different (Tukey's test, $\alpha = 0.05$). Sensitivity tests were conducted in the absence (−SHAM) and presence (+*SHAM*) of 150 μg salicylhydroxamic acid ml^{-1}

Strain	ED_{50} (μg azoxystrobin ml^{-1})		RG at 10 μg azoxystrobin ml^{-1}	
	−SHAM	+SHAM	−SHAM	+SHAM
Wild type	1.8 (1.5) a	0.010 (0.001) a	36 (4.7) a	0 a
M-145	0.01 (0.001) b	0.009 (0.001) a	0 b	0
G143A	>10	>10	77 (7.1) c	82 (4.4) b
G143S	>10	>10	83 (2.1) c	87 (3.9) b

strain (Fig. 1A), some colonies originating from melanized mycelia grew rapidly (Fig. 1B). Residual colony growth was also apparent in transfers of non-melanized mycelia, although rapid growth indicating spontaneous mutations toward a high level of azoxystrobin resistance was never observed (Fig. 1B).

From a total of 100 transfers made from melanized mycelia, conidia of two spontaneous mutant colonies yielded isolates expressing stable resistance to azoxystrobin. As reported by Avila-Adame and Köller (2002), disruption of a single alternative oxidase gene rendered the AOX-minus mutant, M-145, >150-fold more sensitive to azoxystrobin (Table 1). This phenotypic change could be mimicked by inclusion of the alternative oxidase inhibitor, SHAM, in respective sensitivity tests (Table 1). Two monoconidial cultures prepared from mutant colonies were highly resistant to azoxystrobin not only in the absence but also in the presence of SHAM, with <25% inhibition achieved at the highest azoxystrobin dose tested (Table 1). This high level of resistance exceeded a factor of 1,000, even in the presence of SHAM (Table 1), indicating that alternative respiration was not involved in the phenotypic change observed.

Characterization of mutants

Sequence analysis of the cytochrome b target site involved a 628-bp PCR product of cytochrome b amplified from either genomic DNA or cDNA. In both cases, two different point mutations at position 143 were identified for the two mutants (Fig. 2A). One mutation was the G143A amino acid exchange reported for a laboratory mutant of *V. inaequalis* (Zheng et al. 2000) and for several resistant fungal isolates selected in response to applications of Qo-inhibiting fungicides (Bartlett et al. 2002; Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b). This mutation was based on a GGT/GCT transversion. The second point mutation based on

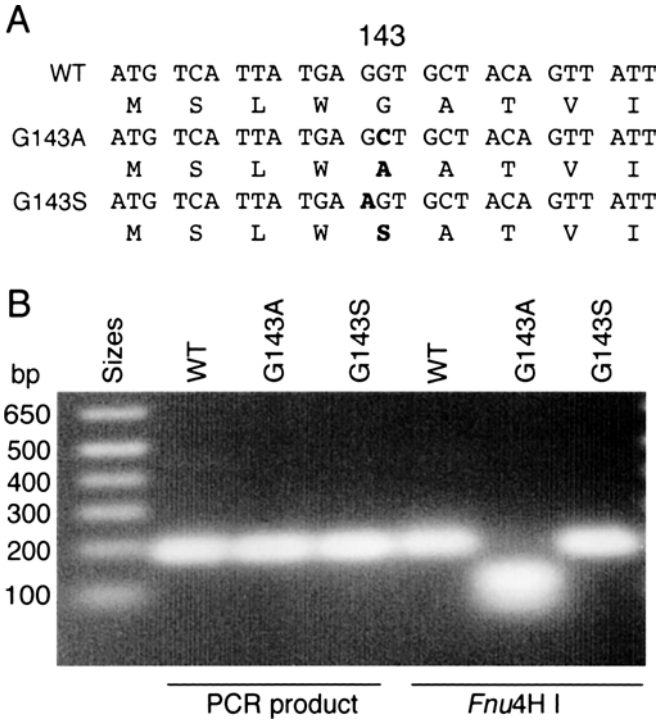

Fig. 2A, B Characterization of the cytochrome b sequence expressed in *M. grisea* mutants resuming mycelial growth in the presence of azoxystrobin plus SHAM. **A** Base-pair exchanges and resulting amino replacements are illustrated for the wild-type strain (*WT*) and the two target-site mutants, *G143A* and *G143S*. **B** The base-pair exchange for the G143A mutant was confirmed by digesting a 193-bp cytochrome b PCR product with *Fnu*4H I

a GGT/AGT transversion translated into the novel G143S amino acid exchange in the cytochrome b target site (Fig. 2A). No other mutational changes were detected within the sequenced DNA, which reflected approximately 50% of the cytochrome b coding region and contained all mutational changes conferring the QoI resistance of *S. cerevisiae* to Qo inhibitors (Di Rago et al. 1995; Köller et al. 2001; Zheng et al. 2000).

Further support for the two different cytochrome b mutations was provided by PCR-RFLP employing Fnu4H I as a restriction enzyme. The endonuclease recognized the sequence GCNGC, which was characteristic of the G143A, but not the G143S or the wild-type cytochrome b sequence. Digestion of the 193-bp PCR products containing amino acid position 143 produced the expected smaller DNA fragments for the G143A mutant, while fragments derived from the wild-type strain or the stable G143S mutant remained unaltered (Fig. 2B).

Stability and pathogenic fitness of target-site mutants

In order to evaluate the stability of the two cytochrome b target site mutants, non-treated barley leaves were inoculated with conidia derived from mycelial cultures. Conidia isolated from mature lesions were employed in three additional rounds of leaf infections. Sensitivity

Table 2 Stability of target site-resistant mutations of *M. grisea* during consecutive cycles of barley leaf infections. QoI-resistant cytochrome b target-site mutants (*G143A*, *G143S*) were derived from wild-type strain 4091-5-8. *Relative germination*is given for conidia derived from mature lesions on 10 μg azoxystrobin ml^{-1} plus 100 μg salicylhydroxamic acid ml^{-1}. Figures are the means of three replications, with standard deviations never exceeding 4% of the means

Strain	Relative germination			
	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Wild type	0.0	0.0	0.0	2.7
G143A	94.6	97.0	95.2	98.9
G143S	97.4	95.3	92.3	98.6

tests conducted after each cycle revealed that neither mutant had reverted back to azoxystrobin sensitivity during the three consecutive rounds of pathogenic reproduction in the absence of the QoI, azoxystrobin (Table 2). The results suggested that both mutated mitochondrial cytochrome b alleles had been selected to a stable state of azoxystrobin resistance.

Fitness parameters tested under both saprophytic and infectious conditions of development revealed that the G143A mutant was not different from the wild-type strain. The respective mutant was not impaired during saprophytic development, as measured by colony size and conidia formation, nor in its pathogenicity and virulence on barley leaves, as measured by the frequency of mature lesion development, the size of mature lesions and the number of conidia recovered from such lesions. In contrast, some fitness parameters of the G143S mutant departed significantly from the wild-type strain. These included both the growth rate and sporulation of mycelium under saprophytic conditions and the number of conidia formed in mature lesions.

Discussion

Two potential mechanisms of fungal resistance to QoIs were described prior to their introduction as commercial fungicides: (1) circumvention of the inhibited cytochrome b target site by activating alternative respiration and (b) mutational amino acid exchanges of the cytochrome b target site, lowering the affinity of inhibitor-binding (Köller et al. 2001). Although the alternative respiration pathway of electron transfer was identified in numerous wild-type isolates of plant pathogenic fungi (Joseph-Horne and Hollomon 2000; Joseph-Horne et al. 2001; Köller et al. 2001), the mechanism has yet not been affiliated with practical resistance to Qo-inhibiting fungicides. In contrast, a G143A mutation of cytochrome b was found to be affiliated with practical Qo inhibitor resistance in the control of several plant diseases (Bartlett et al. 2002; Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b). The speed with which mutants containing the G143A target site alteration were selected to detectable frequencies was unexpectedly high in some cases and required as few as one to four QoI applications, in the case of cucumber powdery mildew in Japan (Ishii et al. 2001).

The occasionally rapid emergence of QoI-resistant target-site mutants in pathogen populations might relate to the results reported in this study for *M. grisea*. When resting and melanized mycelia of the wild-type isolate were exposed to conditions fully inhibitory to young and actively growing mycelium, small mycelial colonies continued to develop. This residual growth was not observed when melanized mycelia of our AOXMg-minus mutant were subjected to the same conditions, indicating the involvement of alternative respiration in this process. Although a lowered uptake of SHAM into melanized mycelia of the wild-type strain might have caused incomplete inhibition of alternative respiration, the precise mechanism responsible for the sustained colony growth remains unknown. However, the residual growth of wild-type mycelia in the presence of azoxystrobin was a prerequisite which was sufficient for the spontaneous generation of the stable cytochrome b target site mutants, G143A and G143S, described here.

Both spontaneous target-site mutations conferred a high level of QoI resistance, which was independent from alternative respiration. Resistance of the two mutants was stable during four cycles of pathogen reproduction in the absence of a QoI, suggesting that mutated mitochondrial alleles of the cytochrome b gene had been selected to a homoplasmic stage in both cases. Predominance was confirmed by PCR-RFLP for the G143A mutant. For the G143S mutant, a considerable presence of wild-type cytochrome b alleles cannot be excluded.

Interestingly, the novel G143S mutation described here has not yet been identified in any QoI-resistant subpopulation of plant pathogens (Bartlett et al. 2002; Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b). The lack of this phenotype in QoI-resistant subpopulations of pathogens might be explained by the impaired fitness we found for the G143S mutant, which was most apparent in a lowered production of conidia. Impaired cytochrome bc_1 activities, which have been reported for several QoI-resistant *S. cerevisiae* mutants (Köller et al. 2001), might account for this fitness penalty. The spontaneous G143A mutant of *M. grisea* reflecting the QoI-resistant genotypes identified in several resistant pathogen subpopulations (Heaney et al. 2000; Ishii et al. 2001; Sierotzki et al. 2000a, 2000b) was not impaired in any of the fitness parameters tested and, therefore, should be expected to fully compete within a population context.

The spontaneous generation of stable QoI-resistant target site mutants of *M. grisea* in response to azoxystrobin departs from the model implying that mutations responsible for preexisting fungicide-resistant phenotypes are infrequent and constant over time (Milgroom et al. 1989; Skylakakis 1987; Wolfe 1982). The mitochondrial nature of the gene encoding the cytochrome b target site of QoIs might explain the spontaneous

emergence of stable target-site mutants at a relatively high mutation frequency. It has been established that the lack of histones in mitochondrial DNA and its close vicinity to the electron transport chain leads to an elevated rate of mitochondrial DNA damage. Prominent in the damage and, thus, mutagenesis of mitochondrial DNA is the oxidation of guanine to 8-hydroxyguanine by reactive oxygen species, leading to mispairing during DNA replication if not repaired (Bohr and Anson 1999; Sawyer and Van Houten 1999; Singh et al. 2001).

The concentration of reactive oxygen species responsible for guanine oxidation is known to increase under the action of a QoI fungicide and to serve as a signal for the expression of alternative respiration in *M. grisea* (Yukioka et al. 1998) and other fungi (Joseph-Horne and Hollomon 2000; Joseph-Horne et al. 2001; Köller et al. 2001). Elevated levels of reactive oxygen can also be expected to increase the rate of mutagenic oxidation of guanine, leading to guanine replacements. It is interesting to note that all G143A mutations giving rise to highly QoI-resistant target mutants of plant pathogens and including both *M. grisea* mutants described here are based on the replacement of guanine in the GGT codon of glycine in position 143 (Heaney et al. 2000; Ishii et al 2001; Sierotzki et al. 2000a, 2000b).

Potentially increased mutation rates toward QoI target-site resistance, due to a direct response of the organism to inhibitor action, might be considered as an example for adaptive mutations (Rosenberg 2001), rather than supporting the model of constant and infrequent mutations leading to rare preexisting phenotypes expressing resistance (Milgroom et al. 1989; Skylakakis 1987; Wolfe 1982). The prerequisite for the selection of favorably mutated mitochondrial cytochrome b alleles would require the continuation of residual ATP production. As described here and in a previous study (Avila-Adame and Köller 2002), such conditions can be provided by the residual expression of alternative respiration as a mechanism of rescue from complete inhibition.

Acknowledgements This work was supported, in part, by financial support to C.A.-A. provided by the Fulbright–Garcia Robles foundation, the Institute of International Education, CONACYT–Mexico and the Colegio de Postgraduados–Mexico.

References

Altschul SF, Madden TL, Schäffer AA, Zhang, J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Avila-Adame C, Köller W (2002) Disruption of the alternative oxidase gene in *Magnaporthe grisea* and its impact on host infection. Mol Plant-Microbe Interact 15:493–500

Balhadère PV, Foster AJ, Talbot NJ (1999) Identification of pathogenicity mutants of the rice blast fungus *Magnaporthe grisea* by insertional mutagenesis. Mol Plant-Microbe Interact 12:129–142

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B (2002) The strobilurin fungicides. Pest Manag Sci 58:649–662

Bohr VA, Anson RM (1999) Mitochondrial DNA repair pathways. J Bioenerg Biomembr 31:391–398

Crawford MS, Chumley FG, Weaver CG, Valent B (1986) Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisae*. Genetics 114:1111–1129

Csaikl UM, Bastian H, Brettschneider R, Gauch S, Meir A, Schauerte M, Scholz F, Sperisen C, Vornam B, Ziegenhagen B (1998) Comparative analysis of different DNA extraction protocols: a fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. Plant Mol Biol Rep 16:69–86

Dekker J (1995) Development of resistance to modern fungicides and strategies for its avoidance. In: Lyr H (ed) Modern selective fungicides. Fisher, Jena, pp 23–38

DeZwaan TM, Carroll AM, Valent B, Sweigard JA (1999) *Magnapothe grisea* Pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues. Plant Cell 11:2013–2030

Di Rago JP, Hermann-Le Denmant S, Paques F, Risler J, Netter P, Slonimski PP (1995) Genetic analysis of the folded structure of yeast mitochondrial cytochrome b by selection of intragenic second-site revertants. J Mol Biol 248:804–811

Heaney SP, Hall AA, Davies SA, Olaya G (2000) Resistance to fungicides in the QoI-STAR cross-resistance group: current perspectives. Proc Br Crop Prot Conf Pests Dis 2000:755–764

Ishii H, Fraaije BA, Sugiyama T, Noguchi K, Nishimura K, Takeda T, Amano T, Hollomon DW (2001) Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology 91:1166–1171

Joseph-Horne T, Hollomon DW (2000) Functional diversity within the mitochondrial electron transport chain of plant pathogenic fungi. Pest Manag Sci 56:24–30

Joseph-Horne T, Hollomon DW, Wood PM (2001) Fungal respiration: a fusion of standard and alternative components. Biochim Biophys Acta 1504:179–195

Köller W (1991) Fungicide resistance in crop protection. In: Pimentel D (ed) CRC handbook of pest management in agriculture. CRC Press, Boca Raton, Fla., pp 679–720

Köller W (1999) Chemical approaches to managing plant pathogens. In: Ruberson JR (ed) Handbook of pest management. Dekker, New York, pp 337–376

Köller W (2001) Fungicide resistance. In: Maloy OC, Murray TD (eds) Encyclopedia of plant pathology. Wiley, New York, pp 483–488

Köller W, Avila-Adame C, Olaya G, Zheng D (2001) Resistance to strobilurin fungicides. In: Clark JM, Yamaguchi I (eds) Agrochemical resistance extent, mechanism, and detection. American Chemical Society, Washington, D.C., pp 215–229

Milgroom MG, Levin SA, Fry WE (1989) Population genetics theory and fungicide resistance. In: Leonard KJ, Fry WE (eds) Plant disease epidemiology: genetics, resistance and management, vol 2. McGraw-Hill, New York, pp 340–367

Olaya G, Köller W (1999) Baseline sensitivities of *Venturia inaequalis* populations to the strobilurin fungicide kresoxim-methyl. Plant Dis 83:274–278

Olaya G, Zheng D, Köller W (1998) Differential responses of germinating *Venturia inaequalis* conidia to kresoxim-methyl. Pestic Sci 54:230–236

Rosenberg SM (2001) Evolving responsively: adaptive mutation. Nat Rev Genet 2:504–515

Sauter H, Steglich W, Anke T (1999) Strobilurins: evolution of a new class of active substances. Angew Chem Int Ed 38:1328–1349

Sawer DE, Van Houten B (1999) Repair of DNA damage in mitochondria. Mutat Res 434:161–176

Sierotzki H, Parisi S, Steinfeld U, Tenzer I, Poirey S, Gisi U (2000a) Mode of resistance to respiration inhibitors at the cytochrome bc_1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Manag Sci 56: 833–841

Sierotzki H, Wullschleger J, Gisi U (2000b) Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* field isolates. Pestic Biochem Physiol 68:107–112

Singh KK, Sigala B, Sikder HA, Schwimmer C. (2001) Inactivation of *Saccharomyces cerevisiae OGG1* DNA repair gene leads to an increased frequency of mitochondrial mutants. Nucleic Acids Res 29:1381–1388

Skylakakis G (1987) Changes in the composition of pathogen populations caused by resistance to fungicides. In: Wolfe MS, Caten CE (eds) Populations of plant pathogens: their dynamics and genetics. Blackwell, Oxford, pp 227–237

Wolfe MS (1982) Dynamics of the pathogen population in relation to fungicide resistance. In: Dekker J, Georgopoulos SG (eds) Fungicide resistance in crop protection. Centre for agricultural publishing and documentation, Wageningen, pp 139–148

Ypema HL, Gold RE (1999) Kresoxim-methyl. modification of a naturally occurring compound to produce a new fungicide. Plant Dis 83:4–19

Yukioka H, Inagaki S, Tanaka R, Katoh K, Miki N, Mizutani A, Masuko M (1998) Transcriptional activation of the alternative oxidase gene of the fungus *Magnaporthe grisea* by a respiratory-inhibiting fungicide and hydrogen peroxide. Biochim Biophys Acta 1442:161–169

Zheng D, Olaya G, Köller W (2000) Characterization of a laboratory mutant of *Venturia inaequalis* resistant to the strobilurin-related fungicide kresoxim-methyl. Curr Genet 38:148–155

Ziogas BN, Baldwin BC, Young JE (1997) Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. Pestic Sci 50: 28–34