Characterization of *Colletotrichum graminicola* Isolates Resistant to Strobilurin-Related QoI Fungicides

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ABSTRACT

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Isolates of *Colletotrichum graminicola* were collected from annual bluegrass or bent grass turf in Japan and the United States, and their sensitivities to QoI fungicides (QoIs) as well as their cytochrome *b* sequences were characterized. Five isolates sampled from turf treated repeatedly with azoxystrobin were highly QoI resistant under both in vivo and in vitro test conditions. The nucleotide sequences of a large cytochrome *b* gene segment involving the binding site of QoIs were fully homologous for all resistant isolates and contained the G143A target site mutation known to confer QoI resistance in other pathogens. QoI-sensitive isolates collected prior to treatments with QoIs were more diverse with regard to their cytochrome *b* gene sequences and their phenotype responses to QoIs. All wild-type isolates retained a glycine in position 143 of cytochrome *b*. Three of the four QoI-sensitive isolates were, in addition, distinguished by leucines in positions 95, 130, and 141, which were exchanged to threonine in all resistant but also in one of the sensitive isolates. In addition to a more pronounced divergence of cytochrome *b* sequences, the sensitive wild-type isolates also were diverse with regard to the induction of alternative respiration in response to QoI action, as indicated by comparisons of QoI sensitivities displayed in the absence or presence of the alternative oxidase inhibitor salicylhydroxamic acid. These different phenotype responses expressed under in vitro test conditions had no or only a slight impact on anthracnose control in protective applications of azoxystrobin. Isolate responses in vitro were very similar for trifloxystrobin, indicating cross-resistance among the class of QoIs. Our results imply that *C. graminicola* falls into the class of pathogens with a potential for rapid selection of highly QoI-resistant phenotypes. Frequent monitoring of population sensitivities will be required to determine the status of population responses toward practical QoI resistance.

Colletotrichum graminicola (Ces.) G.W. Wils. infects a broad range of grasses, including annual bluegrass (*Poa annua*) and bent grasses (*Agrostis* spp.) of turf (43). As documented in recent studies, *C. graminicola* isolates display a high degree of host specificity (7,9,20). The pathogen causes foliar and basal stem rot anthracnose, a disease responsible for severe thinning of turf and routinely managed with fungicides (43).

Strobilurin-related QoI fungicides such as azoxystrobin and trifloxystrobin have been introduced for the control of several turf diseases, including anthracnose (1,16). Respective fungicides inhibit respiration by binding to the Qo center of cytochrome *b*, functioning as a crucial component of

the mitochondrial complex III (8,37,44). Two mechanisms of resistance to Qo inhibitors (QoIs) have been characterized in detail. Numerous fungal organisms can respond to QoI action by the expression of alternative respiration, a pathway circumventing electron transport through the cytochrome *b* target site of QoIs (3,23,26). The induction of this alternative pathway of respiration can serve as a highly effective rescue mechanism when pathogen sensitivities are tested in the absence of a host, but disease control is not impaired when host surfaces are protected by a QoI fungicide prior to infection (5,32–34,46). Thus far, alternative respiration has not been directly affiliated with the occasionally rapid selection of QoI-resistant subpopulations of pathogens. Instead, mutational changes of the cytochrome *b* target site are responsible for several cases of practical resistance to QoI fungicides (8,18,19).

Target site mutations of cytochrome *b* conferring resistance to QoIs have been described for *Saccharomyces cerevisiae* and some other organisms prior to the commercial introduction of QoI fungicides (11,18,26,45). For laboratory mutants of *Venturia inaequalis* and, more recently,

Magnaporthe grisea, a G143A amino acid exchange in cytochrome *b* was found to render a high level of QoI resistance (4,6,14,45). Although this particular mutation had not been reported for resistant *S. cerevisiae* mutants, it has been affiliated with the emergence of practical QoI resistance (8,18,19). Full documentation of G143A target site-resistant field isolates has been provided for powdery mildew of wheat (10,15,36,40) and cucumber (21), for downy mildew of cucumber (21) and grapevine (42), and for black sigatoka of banana (39). The same mechanism has been reported for *M. grisea* (14), *V. inaequalis* (8,18), and *Dydimella bryoniae* (8).

A different F129L cytochrome *b* mutation conferring QoI resistance was identified in field isolates of *M. grisea* (14), *Pythium aphanidermatum* (31), and *Alternaria solani* (35). This F129L mutation had been reported for *S. cerevisiae*, where it rendered a high level of resistance to the natural QoI myxothiazole but a lower degree of resistance to the strobilurin-related QoIs tested (22,26).

In the present study, responses to QoIs are characterized for baseline isolates of *C. graminicola* collected prior to the exposure of turf to QoI fungicides and for isolates collected from turf repeatedly treated with azoxystrobin, and respective isolate responses are compared with the sequences of cytochrome *b* as the target site of QoIs. A preliminary account of this study has been presented recently (30).

MATERIALS AND METHODS

Materials and fungal cultures. Azoxystrobin (technical) and trifloxystrobin (technical) were provided by their respective manufacturers. Salicylhydroxamic acid (SHAM) was purchased from Aldrich Co. (Milwaukee, WI). Potato dextrose broth (PDB) and potato dextrose agar (PDA) was from Difco Laboratories (Pittsburgh). Disposable petri dishes were from Fisher Scientific (Pittsburgh). All other chemicals and biochemicals were purchased from Sigma-Aldrich (St. Louis), if not stated otherwise.

With the exception of isolate 98-155, *C. graminicola* isolates were collected from either annual bluegrass or creeping bent grass (*Agrostis palustris* L.) in Japan and the United States (Table 1). Isolate 98-155 was collected in 1998 from the ornamental nonhost *Soleirolia soleiroliae* (Req.) Dandy

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(Irish moss, Baby's tears) grown in a private garden in California. Isolates K-3625 and K-3626 were collected from creeping bent grass in 1995 and 1996, respectively, at golf courses located in different regions of Japan. Isolate 99-78 originated from annual bluegrass in Pennsylvania in 1999. All four isolates were representatives of *C. graminicola* populations never treated with a QoI fungicide (Table 1).

Isolates K3627 and K3628 originated from diseased bent grass that had been treated in 1999 with azoxystrobin at two different golf courses in Japan serving as experimental sites for the commercial development of the fungicide. Isolates 97-72, 97-73, and 97-74 were collected in 1997 from annual bluegrass showing symptoms of anthracnose at a private golf course in Delaware where azoxystrobin had been tested for anthracnose control for three seasons. The sites exposed to azoxystrobin prior to the collection of *C. graminicola* isolates had received a total of 10 to 15 azoxystrobin treatments.

All isolates were derived from a single conidium. They were identified as *C. graminicola* according to conidia morphology and the appearance of anthracnose symptoms on infected turf grasses (7,9). The isolates were preserved in a nonmetabolizing state on cellulose filter paper and were stored in vials at 4°C.

Sensitivity tests in vivo. Annual bluegrass (Valley Seed Services, Fresno, CA) and creeping bent grass (cv. Penncross) were grown in plastic pots (10 by 10 cm) containing loamy-sandy soil. Absorbent clay was used to anchor seed and to provide moisture for seed germination. Pots were seeded and then watered 2 to 3 times per day using a fog nozzle. During the pregermination phase, pots were covered with paper to reduce evaporation. Grasses were grown for 2 to 3 weeks in a greenhouse (27 to 32°C, 14-h photoperiod) and were cut twice to a height of 1.5 to 2 cm with a handheld clipper.

For tests assessing the performance of azoxystrobin, grasses were sprayed with

azoxystrobin (Heritage 50WG) to evenly cover leaves, using a spray-paint gun. The spray coverage and fungicide concentration used (1 g of Heritage 50WG per liter of water) reflected the dose recommended for the control of anthracnose on turf. Grasses were inoculated 24 h after the fungicide was applied by misting leaves with a conidial suspension of *C. graminicola* (2×10^5 conidia ml⁻¹). Conidia were produced by growing mycelial cultures on PDA (10 g of PDA + 10 g of agar/liter) at 20°C and with 12-h fluorescent light photoperiods. After 7 to 10 days of incubation, conidia were harvested by gently rubbing the flooded culture surfaces (10 ml of water) with a glass rod and filtering conidial suspensions through two layers of cheesecloth.

The inoculated turf grasses were transferred to a dark dew chamber for 24 h (28°C). The plants were moved into a greenhouse (27°C, day and 24°C, night with 12- to 14-h photoperiods) and the potting medium was kept moist by placing pots on wet capillary matting. Foliar anthracnose symptoms developed 10 to 14 days after inoculation. Disease severity was assessed visually and recorded as percentage of foliar tissue showing anthracnose symptoms for an entire pot. Mean disease severity values were determined in three independent experiments with three replicates each, and data from the three experiments were combined and analyzed.

Sensitivity tests in vitro. Sensitivities of *C. graminicola* isolates to azoxystrobin and trifloxystrobin were determined for the stage of colony growth. The fungicides were dissolved in acetone, and PDA was amended to final concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg ml⁻¹. For sensitivity tests in the presence of SHAM as an inhibitor of alternative respiration, SHAM was dissolved in methanol:acetone (1:1 [vol/vol]) and PDA was amended to a final concentration of 100 µg ml⁻¹, including control plates not amended with a QoI. This dose of SHAM provided full synergism of QoI action for QoI-

sensitive isolates. Inhibition of mycelial growth with SHAM alone never exceeded 30%. The final solvent concentrations of 0.1% had no effect on mycelial growth.

Agar plugs (2 by 2 mm) containing actively growing mycelium of 4-day-old cultures of *C. graminicola* were placed on respective PDA surfaces. The diameters of colonies were measured after six days of incubation at 20°C. Inhibitor doses required for 50% inhibition of mycelial growth (ED₅₀ values) were determined by regressing mean relative growth (colony diameters on QoI-amended medium/diameters on nonamended media × 100) against the log of QoI concentrations. Sensitivity tests were repeated three times, with three replicate colonies measured each time. All data were combined for analysis.

Characterization of cytochrome *b* sequences. In order to determine the partial sequence of the cytochrome *b* gene, suitable polymerase chain reaction (PCR) primers were designed by sequencing parts of the gene of isolate 99-78. Basic procedures used were as described previously (3,17). Mycelium was grown in PDB for 5 days under shaking (220 rpm) at 20°C, harvested by filtration through miracloth, and washed with water. Mycelial pads were ground with mortar and pestle in liquid nitrogen, and DNA was extracted using the cetyltrimethylammonium bromide-based method (12). Degenerate primers were designed according to highly conserved regions of cytochrome *b* genes. The primer pair 5'-AA(TC)GC(TAC)AC-(AT)TTAAA(CT)AGA-3' and 5'-GG(AT)-AT(TA)(CG)(TA)(CT)CTTA(GA)(TA)AT-(TA)GC-3' amplified a 344-bp fragment in the vicinity of the C-terminus of cytochrome *b*, and the respective sequence allowed to design the homologous primer 5'-CATTCTGGAACTATAGCAGC-3' (P1).

In order to design a suitable PCR primer close to the N-terminus of cytochrome *b*, RNA was extracted from mycelium utilizing the Tri Reagent-RNA/DNA/Protein isolation system (Molecular Research Cen-

Table 1. Pathogenicity and in vivo sensitivities of *Colletotrichum graminicola* isolates to azoxystrobin

Isolate	Origin			Host specificity ^a		Disease severity ^b			
	Country	Host	Exposure ^c	Bent grass	Bluegrass	Control	Treated	P ^d	Control (%) ^e
K-3627	Japan	Bent grass	+	+	–	69 (15)	62 (6)	0.34	10.2
K-3628	Japan	Bent grass	+	+	–	79 (6)	77 (4)	0.58	2.5
97-72	United States	Bluegrass	+	–	+	78 (7)	80 (7)	0.65	0
97-73	United States	Bluegrass	+	–	+	89 (1)	89 (4)	0.78	0
97-74	United States	Bluegrass	+	–	+	85 (4)	85 (3)	0.80	0
K-3625	Japan	Bent grass	–	+	–	44 (3)	0.3	<0.001	99.3
K-3626	Japan	Bent grass	–	+	–	40 (8)	0.1	0.002	99.7
98-155	United States	Irish moss	–	–	+	45 (9)	7 (4)	0.001	84.4
99-78	United States	Bluegrass	–	–	+	65 (11)	1 (1)	0.001	98.5

^a Symptoms of foliar anthracnose were produced (+) or not produced (–) on the respective hosts.

^b Disease severity is expressed as percentage of foliar tissue exhibiting anthracnose symptoms. Mean values of disease severity with standard deviations given in parentheses.

^c Exposure of turf grasses was a total of 10 to 15 applications of azoxystrobin (+) or no applications (–).

^d Probability levels for comparisons of means between grasses showing anthracnose symptoms and treated or not treated with azoxystrobin (*t* test).

^e Percentages of control are given as 100 - relative disease severity (= mean severity of treated grasses/mean severity of nontreated grasses × 100).

ter, Inc., Moonachie, NJ). Primer P1 was used in combination with the degenerate primer 5'-GA(GA)CA(TC)AT(TAC)ATG-(AC)G(AT)GA(TC)GT-3' to amplify a 626-bp reverse transcriptase (RT)-PCR product. The second homologous primer, 5'-CATAGTAACAGCTTCTGC-3' (P2), was derived from the sequence of this fragment. The pair of homologous primers P1 and P2 allowed us to amplify RT-PCR products from all nine isolates of *C. graminicola*. The 192 amino acids of cytochrome *b* (GenBank accession no. AY285743) corresponded to positions 82-273 of the *S. cerevisiae* protein.

RT-PCR products were prepared for all isolates and sequenced in both directions with an ABI automated DNA sequencer with fluorescent-dye-labeled dideoxy terminators at the Cornell DNA Service facility. Sequence comparisons were done using the Lasergene software (DNASTAR, Inc., Madison, WI) and the BLAST algorithm (2) provided by the National Center for Biotechnology Information.

RESULTS

Pathogenicity and in vivo sensitivities to azoxystrobin. With the exception of isolate 98-155, all isolates of *C. graminicola* were collected from either creeping bent grass or annual bluegrass in Japan and the United States (Table 1). All isolates were only pathogenic on the grass they had been isolated from (Table 1) and, thus, exhibited the host specificity described previously (7,9,20). Isolate 98-155 collected from Irish moss caused anthracnose on annual bluegrass but not on creeping bent grass (Table 1).

The effectiveness of azoxystrobin in preventing anthracnose development was strictly dependent on the history of azoxystrobin use prior to sampling. For all five isolates retrieved from turf treated repeatedly with azoxystrobin, anthracnose control achieved with azoxystrobin at a dose reflecting the recommended application rate was insignificant (Table 1). In contrast, all four isolates collected prior to QoI treatments were effectively controlled at the same dose (Table 1). Anthracnose caused by these four isolates was not con-

trolled when azoxystrobin was applied at the same dose 24 h after inoculation (*data not shown*), indicating poor after-infection activity of the fungicide under the test conditions employed. There was no indication of decreased virulence of isolates resisting a high dose of azoxystrobin under in vivo test conditions (Table 1).

Sensitivities tests in vitro. The apparent azoxystrobin resistance of the isolates collected subsequent to repeated applications of the fungicide (Table 1) was fully confirmed in in vitro tests. Mycelia of all isolates resisting azoxystrobin in vivo displayed ED₅₀ values of >10 µg ml⁻¹ in the absence and presence of SHAM employed as an inhibitor of alternative respiration (Table 2). At the highest dose of 10 µg ml⁻¹, only one of the resistant isolates (K-3627) was significantly more sensitive to azoxystrobin when SHAM was present. However, a high level of azoxystrobin resistance was retained (Table 2). The same high level of azoxystrobin resistance was expressed during the stage of conidia germination in the presence of flavone known to inhibit the induction of alternative respiration (34; *data not shown*). The results indicated that the mechanism responsible for a high level of azoxystrobin resistance was unrelated to alternative respiration.

Responses to SHAM were more diverse among the four isolates of *C. graminicola* displaying a sensitive phenotype in the presence of a host than those displaying a resistant phenotype (Tables 1 and 2). For isolates K-3625 and K3626, SHAM had small yet insignificant synergistic effects on the activity of azoxystrobin (Table 2), indicating that alternative respiration was largely ineffective in the rescue of cells from QoI action. However, effects of SHAM were more pronounced for isolate 99-78, in particular at the highest dose of azoxystrobin tested (Table 2). These differences under in vitro test conditions with mycelia were not expressed in preinoculation tests in vivo, where all four isolates were equally sensitive to azoxystrobin (Table 1).

In contrast, SHAM failed to synergize azoxystrobin action for isolate 98-155 (Table 2). Therefore, this different re-

sponse to SHAM was evaluated in greater detail. In the absence of SHAM, the dose responses of isolates 99-78 and 98-155 were very similar (Fig. 1). In the presence of SHAM, synergistic effects of SHAM on the sensitivity of isolate 99-78 became progressively more pronounced as the dose of azoxystrobin increased, whereas SHAM had no effect on the dose response of isolate 98-155 (Fig. 1). This difference between isolates 99-78 and 98-155 was reflected in a slight yet significant ($P = 0.05$) decrease in efficacy of disease control (Table 1). Respective dose responses were different for the azoxystrobin-resistant isolate 97-72. Here, slight inhibition of mycelium by azoxystrobin was observed only at the highest dose of 10 µg ml⁻¹, in both the absence and presence of SHAM (Fig. 1).

The various patterns of in vitro sensitivities to azoxystrobin were largely the same for trifloxystrobin. Isolate 99-78 responded as sensitive, with synergistic action of SHAM expressed at increasingly higher

Fig. 1. Dose responses of representative *Colletotrichum graminicola* isolates to azoxystrobin. Sensitivities were tested in the absence (closed symbols) or presence (open symbols) of salicylhydroxamic acid at 100 µg ml⁻¹. Isolates tested were 97-72 (triangles), 98-155 (circles), and 99-78 (squares). Relative growth is expressed as percentage of inhibition of mycelial growth – 100. Azoxystrobin concentrations are given as µg ml⁻¹.

Table 2. In vitro sensitivities of *Colletotrichum graminicola* isolates to azoxystrobin^a

Isolate	ED ₅₀ (µg ml ⁻¹)			Inhibition (%) at 10 µg ml ⁻¹		
	-SHAM	+SHAM	P ^b	-SHAM	+SHAM	P ^b
K-3627	>10	>10	...	4 (5)	28 (8)	0.03
K-3628	>10	>10	...	24 (6)	32 (10)	0.42
97-72	>10	>10	...	17 (5)	24 (7)	0.24
97-73	>10	>10	...	17 (10)	21 (9)	0.50
97-74	>10	>10	...	10 (10)	11 (8)	0.95
K-3625	0.05 (0.03)	0.02 (0.01)	0.16	95 (4)	100 (0)	0.19
K-3626	0.08 (0.04)	0.04 (0.04)	0.49	82 (15)	100 (0)	0.17
98-155	0.12 (0.02)	0.12 (0.04)	0.96	69 (2)	70 (4)	0.79
99-78	0.06 (0.05)	0.01 (0.002)	0.07	66 (11)	100 (0)	0.03

^a Mean inhibitor doses required for 50% inhibition of mycelial growth (ED₅₀ values) and percentages of inhibition of mycelia tested in the absence (–) and presence (+) of salicylhydroxamic acid (SHAM). Standard deviations are in parenthesis.

^b Probability levels (*t* test) for comparisons of mean ED₅₀ values and percentages of inhibition determined in the presence and absence of SHAM.

concentrations of trifloxystrobin (Fig. 2). Isolate 98-155 was inhibited by trifloxystrobin but, very similarly to azoxystrobin, QoI action was not influenced by SHAM (Fig. 2). Isolate 97-72, as a representative azoxystrobin-resistant isolate, was inhibited at trifloxystrobin concentrations $>1 \mu\text{g ml}^{-1}$, with an ED_{50} value of $4.9 \mu\text{g ml}^{-1}$ determined in the presence of SHAM (Fig. 2). In comparison to the fully sensitive isolate 99-78 displaying an ED_{50} value of $0.02 \mu\text{g ml}^{-1}$ in the presence of SHAM (Fig. 2), the resistance factor for isolate 97-72 was 245 for trifloxystrobin and $>1,000$ for azoxystrobin. Although the resistance factor was smaller for trifloxystrobin, the results demonstrated full cross-resistance for the two QoIs tested.

Amino acid and nucleotide sequences of cytochrome *b*. PCR products reflecting amino acids 82 to 273 and spanning the functional core of cytochrome *b* were sequenced for all isolates of *C. graminicola*. The deduced amino acids were highly conserved, with only 11 amino acid exchanges (5.7%) detected among all nine isolates (Fig. 3). The high degree of amino acid homology was reflected by the nucleotide sequences (92% identity) and confirmed the classification of all isolates as *C. graminicola*.

Regardless of their origin, from either Japan or the United States, and their different host specificities (Table 1), all five QoI-resistant isolates exhibited identical amino acid (Fig. 3) and nucleotide sequences (*data not shown*). Sequences of the sensitive isolates K3625 and K3626 collected in Japan also were identical, but they differed from QoI-resistant isolates in six amino acid positions (Fig. 3). Foremost

Fig. 2. Dose responses of representative *Colletotrichum graminicola* isolates to trifloxystrobin. Sensitivities were tested in the absence (closed symbols) or presence (open symbols) of $100 \mu\text{g ml}^{-1}$ SHAM. Isolates tested were 97-72 (triangles), 98-155 (circles), and 99-78 (squares). Relative growth is expressed as percentage of inhibition of mycelial growth – 100. Trifloxystrobin concentrations are given as $\mu\text{g ml}^{-1}$.

was the G143A exchange affiliated with several other pathogens developing practical resistance to QoIs (8,18,19). In addition, the two sensitive isolates from Japan were distinguished from resistant isolates by three leucine-to-threonine exchanges in positions 95, 130, and 141, a cysteine-to-threonine exchange in position 118, and an isoleucine-to-methionine exchange in position 232 (Fig. 3).

As expected, the sensitive isolates 98-155 and 99-78 had retained a glycine in position 143 (Fig. 3). However, only isolate 98-155 shared the three leucines in positions 95, 130, and 141 with the two sensitive isolates collected in Japan, whereas isolate 99-78 displayed all three leucine-to-threonine exchanges found in the homologous group of azoxystrobin-resistant isolates (Fig. 3). A similar pattern applied to the isoleucine-to-methionine exchange in position 232; however, here, the exchange pattern was reversed for the two sensitive U.S. isolates (Fig. 3). Other amino acid permutations were detected in positions 132, 199, 227, and 243, but they were restricted to

the QoI-sensitive isolates collected in the United States (Fig. 3).

Eight amino acid exchanges detected in cytochrome *b* of the *C. graminicola* isolates were based on single nucleotide transversions, whereas three exchanges, including the leucine-to-threonine exchange in position 130, were based on two nucleotide transversions (Table 3). The nucleotide most prominently involved in transversions was thymine, with T-to-A and T-to-C transversions observed most frequently (Table 4). For transversions involving guanine, the G-to-C transversions responsible for G143A exchanges were most prominent (Table 4). Predominance of thymine transversions reflected the high thymine content of the sequenced cytochrome *b* portion (Table 4). Although transversions involving thymine and guanine were slightly higher or lower than reflected by the overall nucleotide usage (Table 4), this difference was not significant ($P = 0.18$). The results indicated that all four nucleotides had been subjected to transversions at a frequency reflecting their overall usage.

			90	100	110
G143A	(res)	HSNTASAF	FFLVYTHVGR	GMYYGSYRAP	RTLWVVMGTM
K3625	(sen)	HSNTASAF	FFLVYLHVGR	GMYYGSYRAP	RTLWVVMGCM
K3626	(sen)	HSNTASAF	FFLVYLHVGR	GMYYGSYRAP	RTLWVVMGCM
98-155	(sen)	HSNTASAF	FFLVYLHVGR	GMYYGSYRAP	RTLWVVMGAM
99-78	(sen)	HSNTASAF	FFLVYTHVGR	GMYYGSYRAP	RTLWVVMGTM
		120	130	140	150
G143A	(res)	MTVAMMGMGF	TGYVLPYGQM	STWAAATVITN	TMSAIPWIGQ
K3625	(sen)	MTVAMMGMGF	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ
K3626	(sen)	MTVAMMGMGF	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ
98-155	(sen)	MTVAMMGMGF	LGYVLPYGQM	SLWGATVITN	TMSAIPWIGQ
99-78	(sen)	MTVAMMGMGF	TGHVLPYGQM	STWGATVITN	TMSAIPWIGQ
		160	170	180	190
G143A	(res)	DIVEFVWGGF	SVNNATLNRF	FALHFVLPFV	LAALVLMHLM
K3625	(sen)	DIVEFVWGGF	SVNNATLNRF	FALHFVLPFV	LAALVLMHLM
K3626	(sen)	DIVEFVWGGF	SVNNATLNRF	FALHFVLPFV	LAALVLMHLM
98-155	(sen)	DIVEFVWGGF	SVNNATLNRF	FALHFVLPFV	LAALVLMHLM
99-78	(sen)	DIVEFVWGGF	SVNNATLNRF	FALHFVLPFV	LAALVLMHLI
		200	210	220	230
G143A	(res)	ATHDVTGSSN	PLGVSGNYDR	MPFAPYYLFK	DLMTMFMFVF
K3625	(sen)	ATHDVTGSSN	PLGVSGNYDR	MPFAPYYLFK	DLITMFMFVF
K3626	(sen)	ATHDVTGSSN	PLGVSGNYDR	MPFAPYYLFK	DLITMFMFVF
98-155	(sen)	ATHDVTGSSN	PLGVSGNYDR	MPFAPYYTFK	DLMTMFMFVF
99-78	(sen)	ATHDVTGSSN	PLGVSGNYDR	MPFAPYYLFK	DLITMFMFVF
		240	250	260	270
G143A	(res)	GLSLFVFFMP	NVLGDSDNYM	MANPMQTPAA	MVPE
K3625	(sen)	GLSLFVFFMP	NVLGDSDNYM	MANPMQTPAA	MVPE
K3626	(sen)	GLSLFVFFMP	NVLGDSDNYM	MANPMQTPAA	MVPE
98-155	(sen)	GLSTFVFFMP	NVLGDSDNYM	MANPMQTPAA	MVPE
99-78	(sen)	GLSLFVFFMP	NVLGDSDNYM	MANPMQTPAA	MVPE

Fig. 3. Amino acid sequences of cytochrome *b* segments expressed by isolates of *Colletotrichum graminicola* responding resistant (res) or sensitive (sens) to azoxystrobin. The sequences of all resistant isolates (K3627, K3628, 97-72, 97-73, and 97-74) were fully homologous and are combined as G143A. The amino acids exchanged are presented in bold.

DISCUSSION

For several plant pathogens, the selection of QoI-resistant subpopulations has progressed more rapidly than anticipated, and a G143A mutation of the cytochrome *b* target site was identified as the cause of resistance in the majority of reported cases (8,15,18,19,21,39,40,42). The results described here indicate that *C. graminicola* belongs to the group of pathogens prone to rapid resistance development. Highly resistant G143A target site mutants were selected in Japan and the United States in response to the treatment of turf with a total of 10 to 15 azoxystrobin applications at each location. The unimpaired virulence of respective mutants had, most likely, contributed to their rapid selection.

The cytochrome *b* sequences of all five QoI-resistant isolates of *C. graminicola* described here were fully homologous, regardless of their origin and their different host specificities. In addition to the G143A amino acid exchange, the QoI-resistant isolates were distinguished by amino acid exchanges in other positions. The most consistent difference identified for three of the four sensitive isolates was a leucine-to-threonine exchange in positions 95, 130, and 141. An arbitrary nature of these three additional amino acid permutations is unlikely, in particular because the L130T exchange was based on two nucleotide transversions.

The results indicate that amino acid exchanges in addition to the resistance-conferring G143A mutation might have been involved in the occasionally rapid selection of fully competitive QoI-resistant subpopulations. Although speculative at present, such additional amino acid exchanges might compensate for functional impairments caused by cytochrome *b* mutations toward decreased QoI binding af-

finities. A functional impairment of mutated cytochrome *b* has been reported for several point mutations conferring QoI resistance to *S. cerevisiae* (11,26). Additional amino acid exchanges existing prior to G143A mutations might enable mutated mitochondria to retain full respiratory functionality, facilitating their selection to a homoplasmic stage. If this hypothesis is correct, one of the three QoI-sensitive isolates of *C. graminicola* described here would already carry the three leucine-to-threonine exchanges found in all QoI-resistant G143A mutants, and a single mutation in the 143 position of cytochrome *b* would be sufficient to render QoI resistance without compromising competitiveness of resistant mutants.

The three leucines in positions 95, 130, and 141 under discussion are highly conserved in the cytochrome *b* of other fungal pathogens (39,45). Although analyses of sequence data surrounding the G143A mutation site of cytochrome *b* remain scarce and restricted to a small number of isolates, diversions from the highly conserved leucines in respective positions have been described for other G143A target site mutants. For example, the leucines in positions 130 and 141 were conserved in a QoI-sensitive isolate of *Podosphaera fusca* (syn: *Sphaerotheca fuliginea*), but both were exchanged in the two QoI-resistant G143A mutants described (21). Amino acids in the same positions departed from the normally conserved leucine in the cytochrome *b* of two sensitive but also three QoI-resistant isolates of *Pseudoperonospora cubensis* (21). For *Erysiphe graminis* f. sp. *tritici*, leucine in position 141 was exchanged to a histidine in both a sensitive and a G143A mutant (40). Interestingly, the selection of fully competitive QoI-resistant isolates was rapid for all three pathogens (10,21,40). Our finding that one of four QoI-sensitive isolates of *C. graminicola* already contained all three leucine-to-threonine exchanges consis-

tently found in the G143A-resistant isolates might have contributed to the rapid selection of fully competitive QoI-resistant mutants.

A second, not yet fully understood question concerns the origin of G143A mutations conferring QoI target site resistance. In contrast to previous targets of fungicide action, the gene encoding the cytochrome *b* target of QoIs is of mitochondrial origin and, thus, is represented by multiple alleles. Mutational changes of mitochondrial genes are facilitated by a relatively high mutation rate of mitochondrial DNA (25,38). One of the major causes of such accelerated mutations is the oxidative damage of nucleotides by reactive oxygen species generated during mitochondrial respiration (25,38), with guanine and thymine known to be most prone to oxidation (13). If not repaired, oxidative damage of nucleotides can lead to mismatches during DNA replication and, thus, to potentially permanent nucleotide transversions in mitochondrial genes (25,38). In all cases reported thus far and including the resistant *C. graminicola* mutants described here, the G143A exchanges were caused by a single guanine-to-cytosine transversion.

Oxidative damage of guanine responsible for mutational nucleotide mismatches is known to generate 8-hydroxyguanine, which mispairs with adenine during DNA replication, leading to the replacement of guanine by thymine (13,41). Such guanine transversions were not apparent in the cytochrome *b* sequences described in this study. However, products originating from a second oxidation of 8-hydroxyguanine have been reported to mispair with guanine (28) and, thus, would account for the guanine-to-cytosine transversions identified for all G143A target site mutants resisting high levels of QoIs. In addition to guanine, thymine also is prone to oxidative damage. Oxidation of the methyl group in thymine generates 5-formyluracil, a nucleotide reported to potentially mispair with guanine,

Table 3. Amino acid and nucleotide polymorphism in cytochrome *b* genes of nine *Colletotrichum graminicola* isolates

Position ^a	Amino acid ^b	Codon ^c
95	L/T	TTA/CTA
118	C/T	TGT/ACT
118	C/A	TGT/GCT
130	L/T	TTA/CTG
132	Y/H	TAT/CAT
141	L/T	TTA/CTA
143	G/A	GGT/GCT
199	M/I	ATA/ATC
227	L/T	TTA/CTA
232	I/M	ATA/ATC
243	L/T	TTA/CTA

^a Amino acid positions of cytochrome *b* deduced from nucleotide sequences.

^b Amino acid exchanges relative to the amino acids expressed by the QoI-sensitive reference isolate K3625. Amino acids of the reference isolate are given first, followed by the amino acids identified in eight other isolates at respective positions.

^c Nucleotides encoding the amino acids in reference isolate K3625 and respective amino acids exchanged in the other isolates.

Table 4. Nucleotide usage and nucleotide transversions in the cytochrome *b* alleles of nine *Colletotrichum graminicola* isolates

Nucleotides	Nucleotide usage ^a		Transversions ^b	
	<i>n</i>	%	<i>n</i>	%
Thymine	228	39.6	24	50.0
T:A	...	...	13	...
T:C	...	...	10	...
T:G	...	...	1	...
Adenine	157	27.3	10	20.8
A:G	...	...	5	...
A:C	...	...	3	...
A:T	...	...	2	...
Cytosine	90	15.6	10	20.8
C:T	...	...	9	...
C:G	...	...	1	...
Guanine	101	17.5	4	8.4
G:C	...	...	3	...
G:A	...	...	1	...

^a Nucleotides used in the sequenced part of the cytochrome *b* allele expressed by the QoI-sensitive reference isolate K3625.

^b Nucleotide transversions identified in the partial cytochrome *b* genes of eight other isolates.

thymine, or cytosine (24,29). The various point mutations generated by such mismatches would account for all nucleotide transversions of thymine described here for cytochrome *b* of *C. graminicola* and for most of the several leucine-to-threonine exchanges in cytochrome *b*.

Inhibition of electron transport at the level of cytochrome *b* is known to increase the concentration of reactive oxygen species in mitochondria (23,26) and, thus, can be expected to transiently increase the oxidative damage of nucleotides responsible for mutagenic mismatches during DNA replication. Whether accelerated mutagenesis of mitochondrial DNA under the action of a QoI is involved in the generation of target-site-resistant cytochrome *b* alleles remains unknown at present, but it cannot be excluded. For example, the spontaneous emergence of two distinct target site mutants both based on the transversion of guanine has been reported recently for *M. grisea* subjected to azoxystrobin-mediated oxidative stress (4,6). An accelerated adaptation of fungicide-resistant populations to new fungicides in general has been described for *V. inaequalis* (27).

The results described here for *C. graminicola* have impact on the management of azoxystrobin resistance in the control of anthracnose. Our comparison of azoxystrobin with trifloxystrobin revealed that cross-resistance exists among the class of QoI fungicides. Similar to other pathogens (36), the highly resistant isolates of *C. graminicola* described here had reached an apparent homoplasmic stage and were not impaired in their competitive fitness. Once such resistant isolates reach a high frequency in response to treatment with QoIs, the fungicides will become ineffective in the control of anthracnose. Our observation that the G143A mutation was accompanied by three additional leucine-to-threonine exchanges in cytochrome *b* found in three of the four sensitive isolates indicates that target site mutants lacking these additional amino acid exchanges might retain a heteroplasmic cytochrome *b* allele population for a longer period of time and might return to a more sensitive stage once they are relieved from the selection pressure. For example, some QoI-resistant isolates of *E. graminis* f. sp. *tritici* containing a mixed allele population for cytochrome *b* were found to slowly return to a QoI-sensitive stage once they were relieved from the selection pressure (15). In such cases, restricting the number of QoI applications over a season of anthracnose management would compose a valuable tool in resistance management.

In summary, our data document that *C. graminicola* belongs to the group of pathogens with the potential for rapid emergence of fully competitive QoI-resistant mutants. Once such target site mutants are selected to a high frequency, QoIs will lose their effectiveness and should no longer be used

in the management of anthracnose. The question of how fast this stage of ineffectiveness will be reached at particular sites will depend on several parameters, such as the QoI use history, the particular population structure of *C. graminicola*, and environmental conditions favoring rapid population growth. Guidance in these questions can be provided only by continuous monitoring programs, either with bioassays according to the sensitivity data provided in this study, or with PCR-based tests evaluating the frequency of G143A mutations within a given population of *C. graminicola*.

As reported for several pathogens (5,32–34,46), the variable interference of alternative respiration with QoI action had no impact on the protective performance of azoxystrobin in the control of anthracnose. This predictive limitation of bioassays based on mycelial growth must be considered in the design of respective monitoring procedures. PCR-based tests aimed at the G143A exchange conferring QoI resistance have been described recently (15,42). For *C. graminicola*, such tests should include the additional amino acid exchanges potentially involved in predisposing sensitive isolates toward fully functional G143A mutations.

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