Field Resistance to Strobilurin (Q_oI) Fungicides in *Pyricularia grisea* Caused by Mutations in the Mitochondrial Cytochrome b Gene

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ABSTRACT

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Gray leaf spot caused by *Pyricularia grisea* is a highly destructive disease of perennial ryegrass turf. Control of gray leaf spot is dependent on the use of preventative fungicide treatments. Strobilurin-based (Q_oI) fungicides, which inhibit the cytochrome bc₁ respiratory complex, have proven to be very effective against gray leaf spot. However, in August 2000, disease was diagnosed in Q_oI-treated perennial ryegrass turf on golf courses in Lexington, KY, Champaign, IL, and Bloomington, IL. To determine if resistance was due to a mutation in the fungicide target, the cytochrome b gene (*CYTB*) was amplified from baseline and resistant isolates. Nucleotide sequence analysis revealed an intronless coding region of 1,179 bp. Isolates that were resistant to Q_oI fungicides possessed one of two different mutant alleles, each of which carried a single point

mutation. The first mutant allele had a guanine-to-cytosine transition at nucleotide position +428, resulting in a replacement of glycine 143 by alanine (G143A). Mutant allele two exhibited a cytosine-to-adenine transversion at position +387, causing a phenylalanine-to-leucine change (F129L). Cleavable amplified polymorphic sequence analysis revealed that neither mutation was present in a collection of baseline isolates collected before Q_oI fungicide use and indicated that suspected Q_oI-resistant isolates found in 2001 in Indiana and Maryland possessed the F129L mutation. The *Pyricularia grisea* isolates possessing the G143A substitution were significantly more resistant to azoxystrobin and trifloxystrobin, *in vitro*, than those having F129L. DNA fingerprinting of resistant isolates revealed that the mutations occurred in just five genetic backgrounds, suggesting that field resistance to the Q_oI fungicides in *Pyricularia grisea* is due to a small number of ancestral mutations.

Additional keywords: cleavable amplified polymorphic sequence analysis, *Magnaporthe grisea*.

Despite its recent emergence, gray leaf spot of perennial ryegrass caused by *Pyricularia grisea* (teleomorph = *Magnaporthe grisea*) has rapidly become one of the most destructive of all turf grass diseases (22). A significant factor in the epidemiology of gray leaf spot is the rapidity of host death after infection. It is common for large expanses of turf to be completely devastated within a week or two after symptoms first appear. For this reason, curative treatments are ineffective in controlling gray leaf spot, once it has been diagnosed. Instead, preventative fungicide sprays are recommended for management of the disease.

The strobilurin-based (Q_oI) fungicides have proven very effective in controlling gray leaf spot (25). The specific target for the Q_oI fungicides is the quinol-oxidizing (Qo) site of the mitochondrial enzyme cytochrome b. Binding of these chemicals at the Qo site blocks electron transport, thereby inhibiting respiration (1). Field resistance to Q_oI fungicides has been observed in Europe and Asia among pathogens of a number of crops, including wheat (*Blumeria graminis* f. sp. *tritici*) (18), banana (*Mycosphaerella fijiensis*) (17), and grape (*Plasmopara viticola*) (6), as well as barley (*Blumeria graminis* f. sp. *hordei*) (7) and apple (*Venturia inaequalis*) (19). Two mechanisms of resistance to Q_oI fungicides have been reported. The first involves mutation of the cytochrome b gene, resulting in peptide sequence changes that prevent fungicide binding. Several point mutations associated with resistance have been identified in *Saccharomyces cerevisiae*, many of which result in impaired respiration and, hence, reduced fitness (3). Prior to the present study, field resistance in phytopathogenic fungi was exclusively associated with cytochrome b gene (*CYTB*) mutations

that result in a replacement of glycine by alanine at amino acid position 143 (G143A) (6,7,17-19). More recently, resistance in *Pythium aphanidermatum* and *Alternaria solani* has been associated with a mutation resulting in a F129L change (G. Olaya, *personal communication*).

A second resistance mechanism, which functions *in vitro*, has been identified in a number of phytopathogenic fungi, including *Magnaporthe grisea* (27,28), *Septoria tritici* (30), *Botrytis cinerea* (20), and *Venturia inaequalis* (14). This involves the activity of an alternative oxidase (AO) enzyme, which oxidizes ubiquinone while reducing oxygen to water. This bypasses the Q_oI-induced block in the electron transport chain allowing growth (16). In *Magnaporthe grisea*, the AO gene is expressed *in vitro* in response to oxidative stress; however, AO transcription is repressed *in planta*, enabling Q_oI fungicides to be used for controlling disease caused by *Magnaporthe grisea* (27) and other phytopathogenic fungi.

Recently, there have been two reports of field resistance to Q_oI fungicides having arisen in the United States. In 2000, resistance was detected in populations of *Pyricularia grisea* causing gray leaf spot disease in perennial ryegrass (26), and in the cucurbit pathogen *Didymella bryoniae* (12). Resistance to Q_oI fungicides in *Pyricularia grisea* was first detected during a fungicide trial in a perennial ryegrass fairway on a golf course in Lexington, KY. Gray leaf spot disease progress curves in the treated plots were the same as in the water controls (26). Concurrently, there was significant gray leaf spot damage to fairways that had been treated with a Q_oI fungicide. These observations were consistent with the hypothesis that resistance to Q_oI fungicides had arisen in the gray leaf spot population (26). Apparent cases of resistance also were found at golf courses in Champaign and Bloomington, IL. *In vitro* sensitivity assays performed on *Pyricularia grisea* isolates collected from symptomatic, Q_oI-treated perennial ryegrass turf in Kentucky and Illinois revealed that many suspect isolates were

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over 1,000-fold less sensitive to Q_oI fungicides than baseline isolates collected before these chemicals were labeled for use on turf grass (26). In growth chamber inoculation experiments, the amount of disease caused by resistant isolates on fungicide-treated plants was equivalent to that on water-treated plants (26).

Based on the observation that the Q_oI-resistant *Pyricularia grisea* isolates were able to germinate in very high concentrations of fungicide, we hypothesized that resistance also was conferred by mutation of the target enzyme. To test this hypothesis, we isolated and characterized the *CYTb* gene from several resistant isolates and compared these sequences with *CYTb* genes from baseline isolates. We also wanted to determine if resistance has been selected multiple times, as a result of widespread fungicide use. Therefore, we performed restriction fragment length polymorphism and DNA fingerprint analyses on the resistant isolates to determine if mutations were present in a number of different genetic backgrounds.

MATERIALS AND METHODS

Fungal isolates. Fungal isolates used in this study and their hosts and places of origin are listed in Table 1. Cultures were isolated from diseased leaves as follows. Detached leaves were placed on the surface of water agar in a petri dish. The lid was replaced to maintain high humidity. The following day, a sealed Pasteur pipette tip was brushed across the sporulating lesions to pick up conidia, which then were spread across the surface of 4% water agar. Approximately 20 h later, a single germinated conidium was transferred to oatmeal agar. Subsequent to single-sporing, all isolates were routinely cultured on oatmeal agar in the absence of fungicide selection. Permanent stocks were made by drying cultures in cellulose paper disks and storing at -20°C (23).

Amplification of an internal fragment of the cytochrome b gene. Oligonucleotide primers (PgcytbF1 and PgcytbR1) (Table 2)

were designed based on the *Pyricularia grisea* cytochrome b gene sequence deposited in the EMBL database (accession no. X87999). Polymerase chain reaction (PCR) was performed in a total reaction volume of 50 µl containing 1× buffer (Perkin-Elmer), 1.5 mM MgCl₂, 12.5 pmol of each dNTP, 20 pmol of each primer, 20 ng of template DNA, H₂O to 49.5 µl, and 0.5 µl of AmpliTaq Gold (Perkin-Elmer). Cycling parameters were 95°C for 10 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, and a final extension of 5 min at 72°C.

Cloning and sequence analysis of a full-length copy of the cytochrome b gene. The cytochrome b gene fragment amplified by PCR was ³²P-labeled using an oligolabeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) and used to probe a cosmid library of *Pyricularia grisea* strain ML33. This resulted in the identification of cosmid M11A2. Using this clone as a template, the sequences of the 5' and 3' ends of the *CYTb* gene were determined by primer walking out from the previously determined sequence. The full-length *CYTb* gene sequence then was used to design oligonucleotide primers (IDT Technologies, Coralville, IA) for amplification of the entire gene from the Q_oI-resistant (Q_oI^R) *Pyricularia grisea* isolates. Sequences of the primers used in this study are provided in Table 2.

DNA sequence analysis. PCR products were purified using the CONCERT PCR prep kit (Gibco-BRL) and ≈200 ng of purified product was sequenced with BigDye (Applied Biosystems, Foster City, CA) or DTCS (Beckman-Coulter, Fullerton, CA) chemistries. DNA sequence was acquired using ABI310 (ABI) or CEQ2000XL (Beckman-Coulter) capillary sequencers. Additional oligonucleotide primers were synthesized to enable acquisition of the complete *CYTb* gene sequence information on both DNA strands. Primer sequences are listed in Table 2.

Screening isolates for mutations in the cytochrome b gene. The G143A mutation resulted in the creation of an *Fnu4HI* restriction site (...GGTGC... to ...GCTGC...), while F129L resulted in

TABLE 1. *Pyricularia grisea* isolates used in this study

Isolates ^a	Year	Host ^b	Place of isolation ^c	Collector
Baseline				
Guy11	1988	Rice	French Guyana	J.-L. Nottoghem (11)
82T11	1982	Rice	Texas	A. Marchetti
LpKY96-1	1996	Prg	Kentucky	P. Vincelli (5)
HO	1996	Prg	Pennsylvania	P. Dernoeden (5)
CHRF, CHW, FH	1996	Prg	Maryland	P. Dernoeden (5)
RGNJ	1996	Prg	New Jersey	B. Clarke (5)
WF9722, WF9723, WF9724	1997	Prg	Illinois	H. Wilkinson (5)
LpKY97-1	1997	Prg	Kentucky	P. Vincelli (5)
GG9, GG11, GG12, GG13	1997	Prg	Kentucky	M. Farman (5)
KS320, KS330, KS331	1997	Prg	Kansas	N. Tisserat (5)
LpOH1, LpOH3	1997	Prg	Ohio	J. Rimelspach (5)
LpKY98-1, LpKY98-2	1998	Prg	Kentucky	M. Farman (5)
Resistant				
LpRI00-1, LpRI00-2	2000	Prg	Rhode Island	N. Jackson (5)
<u>Her^R1A1, Her^R1A2, Her^R2-2, Her^R2000-1, Her^R2000-2,</u> <u>Her^R2000-3, Her^R2000-4, Her^R2000-5, GG2a, GG2b</u>	2000	Prg	f/w 2, GGCC ^d , Lexington, KY	M. Farman/P. Vincelli
<u>GG5-2b-1, GG5-2b-2, GG5-4a,</u> <u>GG5-4b, GG5-4c</u>	2000	Prg	f/w 5, GGCC ^d , Lexington, KY	M. Farman/P. Vincelli
<u>ILL00-1</u>	2000	Prg	Champaign, IL	A. Hamblin
<u>ILL-BL-00</u>	2000	Prg	Bloomington, IL	A. Hamblin
IN0024	2000	Prg	Indiana	P. Harmon/R. Latin
IN0025	2000	Prg	Indiana	P. Harmon/R. Latin
<u>GG3H 1-1, GG3H 1-6, GG3H 5-1, GG3H 4-2, GG2 6ft-1</u>	2001	Prg	f/w 2, GGCC, Lexington, KY	Y.-S. Kim/M. Farman
<u>GG14 3-1, GG14 3-5, GG14 2-1, GG14 2-4, GG14 11-1</u>	2001	Prg	f/w 14, GGCC, Lexington, KY	Y.-S. Kim/M. Farman
<u>IN0106</u>	2001	Prg	Indiana	P. Harmon/R. Latin
<u>IN0107</u>	2001	Prg	Indiana	P. Harmon/R. Latin
<u>LpMD01-1</u>	2001	Prg	Maryland	P. Dernoeden
<u>LpOH01-1</u>	2001	Prg	Ohio	J. Rimelspach

^a Baseline collected before labeling of Q_oI fungicides for use on turf grasses; underlined text indicates isolates that were collected from areas of turf with a confirmed record of Q_oI fungicide treatment.

^b Prg = perennial ryegrass.

^c f/w = fairway.

^d GGCC = Griffin Gate Country Club.

the elimination of a *StyI* restriction site (...CCTAGG... to ...CAT-AGG...). Screening for each mutation was carried out using *Fnu4HI* and *StyI* for cleavable amplified polymorphic sequence (CAPS) analysis of PCR products. PCR was performed using genomic DNA extracted from conidiospores as follows. A toothpick was scraped over the surface of a conidiating culture grown on oatmeal agar. The spores were suspended in 10 µl of sterile H₂O. This step was repeated and the suspension then was frozen at -80°C. After thawing, the PCR reagents were added directly to the suspension. Cycling conditions were as described previously. PCR product (5 µl) was digested with each enzyme and the restriction fragments were resolved by electrophoresis in 4% agarose gels (3:1; Nusieve: LE agarose, BioWhittaker Products, Rockland, ME) using a Kilobase Plus size marker (Invitrogen, Carlsbad, CA).

In vitro tests for Q₀I fungicide sensitivity. Fungicide sensitivity was represented as the concentration at which germination was inhibited by 50% (EC₅₀ value). All fungicide sensitivity tests were performed in the presence of salicylhydroxamic acid (SHAM) at 100 µg/ml, which was required to suppress resistance due to alternative oxidase (13,26). Conidial germination was the best indicator of sensitivity to Q₀I fungicides because, in a preliminary test, in vitro mycelial growth of five baseline isolates in the presence of azoxystrobin at 5 µg/ml was reduced only 22 to 56% in the absence of SHAM and 61 to 78% in its presence (P. Vincelli and E. W. Dixon, unpublished data), indicating that mycelial growth of *Pyricularia grisea* was rather insensitive to azoxystrobin, even in the presence of SHAM. Technical-grade azoxystrobin (Syngenta, Wilmington, DE) and trifloxystrobin (Novartis Crop Protection, Inc., Research Triangle Park, NC) were dissolved in acetone and diluted 100-fold in sterile deionized water to create stock solutions. A stock solution of SHAM was prepared by dissolving the chemical in methanol and diluting 100-fold in sterile, deionized water to give a final concentration of 50 mg/ml. To determine effects of fungicides on conidial germination, chemical solutions, fresh conidial suspension, and sterile deionized water were mixed in individual wells of a 24-well plate. Fungicide dilutions and reagent or spore additions were performed in a manner that maintained uniform concentrations of spores and solvents (0.1% methanol and 0.1% acetone) in each test well. After mixing chemicals and conidia in test wells (one well for each

fungicide concentration tested), three 30-µl droplets of each chemical concentration were placed into an individual 90-mm polystyrene petri dish and each droplet was covered with a coverslip (14). The dishes were sealed with Parafilm and incubated in the dark for 18 to 24 h. After incubation, conidia were scored at ×100 magnification as either ungerminated or germinated (germ tube at least the length of the conidium, or with an appressorium). For sampling conidia, the coverslip in each replicate droplet was divided into quadrants and conidia were counted from microscope fields arbitrarily located within each quadrant (avoiding the center and edge of the coverslip) until 100 conidia were counted.

Arbitrarily selected baseline isolates and suspect isolates were first tested in a series of 10-fold dilutions of azoxystrobin or trifloxystrobin with and without SHAM; the ranges used for both fungicides were 0.001 to 10 µg/ml for baseline isolates and 0.1 to 100 µg/ml for suspect isolates. From these preliminary evaluations using the 10-fold dilution series, ranges of concentrations appropriate for testing each group of isolates were identified. Germination then was determined for each isolate over a threefold dilution series within those ranges. For isolates that were either fully germinated or ungerminated over a given threefold dilution series, tests were repeated with threefold dilutions over a different range of concentrations. In all tests, germination in fungicide-free controls was 85 to 95%. Relative germination (RG) then was calculated for each replicate as 100 × percent germination in the presence of fungicide divided by mean percent germination of that isolate in the absence of fungicide. RG for selected baseline isolates was determined several times; RG for all suspect isolates was determined at least twice in completely separate tests. These data were analyzed by analysis of variance (ANOVA) following a completely randomized design (15). To determine the EC₅₀ value for each fungicide, RG was regressed against log₁₀[fungicide]. EC₅₀ then was determined by rearranging the regression equation to solve for log₁₀[fungicide] at RG = 50.

DNA fingerprinting. Each genomic DNA sample (200 ng) was digested with *PstI* and electrophoresed in a 0.7% agarose gel in 0.5× Tris borate-EDTA buffer. Following electrophoresis, gels were depurinated, denatured, and neutralized according to standard protocols (21), followed by transfer to a Hybond N+ membrane (Amersham Pharmacia Biotech). The immobilized DNAs then were probed separately with two different probes: (i) Cosmid 16-1-B and (ii) a 0.9-kb internal fragment from the Pot2 transposon (9). The hybridization and subsequent washing conditions were as described by Thornbury and Farman (21). Hybridization signals were detected by overnight exposure of phosphorimage screens (Amersham Pharmacia Biotech).

RESULTS

Isolation and nucleotide sequence analysis of the cytochrome b gene from Q₀I^R and sensitive *Pyricularia grisea* isolates. The *Pyricularia grisea* isolates collected from fungicide-

TABLE 2. Oligonucleotide primers used in this study

Primer name	Sequence
PgCytb_F1	5'-AGTCCTAGTGTAATGGAAGC-3'
PgCytb_F2	5'-GATACTgCTggTTCAAgC-3'
PgCytb_F3	5'-CAGATTTAGGTAGATCTAGAGG-3'
PgCytb_F4	5'-CATAGACGTGATATTTGGG-3'
PgCytb_R1	5'-ATCTTCAACGTGTTTATGACAC-3'
PgCytb_R2	5'-ATCTTgCCCTATTCAAgg-3'
PgCytb_R3	5'-CTATGTAGATAACGAACAAATCACC-3'
PgCytb_R4	5'-TACAAAGCAAGGTGGAGG-3'

TABLE 3. Nucleotide substitutions in the *Pyricularia grisea* cytochrome b gene

Isolates	Nucleotide substitution ^a	Resistant (R) or sensitive (S)
Perennial ryegrass pathogens		
Baseline isolates		
CHW, HO, FH, LpKY97-1, GG9, IN0107	...	S
Resistant isolates		
GG5-2b-1, GG5-4B, ILL00-1, LpMD01-1, IN0106	C387A (225)	R
1A1, Her ^R 2000-2, Her ^R 2000-3, Her ^R 2000-4, Her ^R 2000-5, GG2a, GG2b, GG5-2b-2, GG5-4a, ILL-BL00	G428C (266)	R
Rice pathogens		
Guy11, ML33, 926-20, CH104-3, IT11	A677T (515)	S
Reference sequence		
EMBL: X87999 (host unknown)	A677T (515), A706T (544), C728T (566)	Unreported

^a Relative to the nucleotide sequence of the cytochrome b gene in the baseline isolates from perennial ryegrass. Numbers in parentheses correspond to the nucleotide position within the polymerase chain reaction amplicon.

treated turf were essentially immune to levels of azoxystrobin comparable to labeled rates (26). Such high levels of resistance are indicative of a target site mutation. Therefore, we sought to characterize the cytochrome b gene to determine if there were mutations that were specific to the resistant isolates. Amplification of an internal portion of the *CYTB* gene using primers CytbF1 and CytbR1 (Table 2) produced a fragment of ≈ 850 bp for all the isolates tested. Amplified fragments obtained from 7 baseline isolates and 17 resistant isolates were purified and sequenced using the primers listed in Table 2.

In relation to the *CYTB* genes from the baseline isolates, the reference sequence in the EMBL database had three nucleotide substitutions at positions 515, 544, and 566 within the PCR product (Table 3). Each of these changes is predicted to alter the amino acid sequence of the CYTB peptide (Fig. 1). To determine if the deviations from the reference sequence were specific to *Pyricularia grisea* isolates from perennial ryegrass, *CYTB* genes from five rice-infecting isolates were amplified and sequenced. This revealed that the perennial ryegrass and rice pathogens' *CYTB* genes were identical at positions 544 and 566 but showed that the adenine base at position 515 was unique to the isolates from perennial ryegrass (Table 3).

Sequencing of *CYTB* gene fragments from the isolates found on QoI-treated turf revealed additional mutations that were not found in any of the baseline isolates. Thirteen of the resistant isolates had a single guanine-to-cytosine substitution that was at base position 266 within the PCR product. Alignment of a conceptual translation of this sequence with cytochrome b protein sequences predicts that this mutation results in a substitution of alanine for glycine at amino acid position 143 (G143A). The four remaining azoxystrobin-resistant isolates lacked the G143A mutation. These isolates possessed an adenine instead of cytosine at position 225, predicting a change of phenylalanine 129 to leucine (F129L). None of the isolates had both mutations.

To determine if the G143A and F129L substitutions were the only changes to the cytochrome b protein, we sought to characterize the complete gene. A full-length *CYTB* gene copy was isolated from a cosmid library of the rice-pathogenic isolate

ML33. Sequence analysis revealed that the coding region of the *CYTB* gene in this isolate is 1,179 bp in length and contains no introns. The sequences of the 5' and 3' untranslated regions of the gene in ML33 were used to design primers for amplification of the 5' and 3' ends of the *CYTB* gene from 13 susceptible and 16 resistant isolates. Sequence analysis of these PCR products revealed no additional differences in the opening reading frame, or in the untranslated regions of the promoter and terminator. Alignment of the complete predicted amino acid sequence with other CYTB proteins enabled identification of the likely translational start site. This confirmed that the mutations found in the resistant isolates did indeed affect amino acids 129 and 143 of the *Pyricularia grisea* protein (data not shown). Full-length sequences of each of the *CYTB* alleles characterized in this study have been deposited as GenBank Accession nos. AY245424 through AY245427.

Cytochrome b is encoded by the mitochondrial genome. It is possible for resistant isolates to harbor both resistant and susceptible mitochondria, a condition known as heteroplasmy. However, our sequence analyses revealed very clear chromatogram peaks at the critical nucleotide positions and, therefore, gave no indication of a heteroplasmic condition in any of the resistant mutants (data not shown).

Relationship between cytochrome b mutations and the levels of resistance to QoI fungicides. Vincelli and Dixon previously reported that *Pyricularia grisea* isolates collected in 2000 from azoxystrobin-treated perennial ryegrass had decreased sensitivities to azoxystrobin and trifloxystrobin, compared with baseline isolates that were collected before use of QoI fungicides (26). Based on their data, there appeared to be two classes of mutant with different fungicide sensitivities (classes I and II, Table 4). The first class was resistant to high levels of azoxystrobin and trifloxystrobin, resulting in EC₅₀ values that were approximately 1,300- and 1,000-fold higher, respectively, than with the baseline isolates (Tables 4 and 5). These highly resistant isolates all had the G143A mutation. The second class of mutants produced a 140-fold increase over baseline in the EC₅₀ of azoxystrobin and a 30-fold increase in the EC₅₀ for trifloxystrobin (Table 5). These

Isolate	A.A.	peptide sequence
Baseline	123	L <u>M</u> MAIG <u>F</u> LGIVLPYGQMSLW <u>G</u> ATVITNLISAIPWIGQDIVEFIWGGFSVNNATLNRFFALH
F129L		L
G143A		A
rice		
X87999		
Baseline	184	FVLPFVLAALVLMHLIALHDTAGSSNPLGVSGNYDRITFAPY <u>F</u> LFKDLITIF <u>I</u> FIFVLS <u>A</u> F
F129L		
G143A		
rice		F
X87999		F L V

Fig. 1. Comparison of amino acid sequences in *Pyricularia grisea* cytochrome b peptides. Shown are internal residues of cytochrome b proteins predicted by conceptual translation of the respective nucleotide sequences. The top sequence represents a portion of the peptide in the baseline isolates. Below this are cytochrome b (CYTB) sequences in the F129L and G143A mutants, rice-pathogenic isolates, and the sequence in the EMBL database. Amino acid substitutions are shown below the baseline sequence in their corresponding positions. The affected residues are underlined for clarification.

isolates all had the F129L mutation. The in vitro sensitivity levels also were correlated with the type of mutation in the isolates collected in 2001 (Table 4, underlined isolates). G143A mutants from both years had levels of sensitivity similar to the two fungicides. However, the F129L mutants collected in 2001 appeared to be more sensitive to trifloxystrobin than equivalent mutants identified the previous year (Tables 4 and 5). Therefore, three of the F129L mutants collected in 2000 were retested (GG5-2b-1, GG5-4b, and ILL00-1). In several independent experiments, these isolates all exhibited almost baseline sensitivity to this Qo inhibitor (Table 4, values not underlined). This strongly suggests that the previously reported EC₅₀ values are inaccurate and that the F129L mutants have little, or no, resistance to trifloxystrobin in vitro.

Several additional experiments were performed to determine the cause of the discrepancy, including comparison of fresh fungicide or SHAM stocks with the ones used in the previous experiments. However, in each case, the EC₅₀ values were in the same range as the values obtained for the 2001 isolates (P. Vincelli and E. W. Dixon, *unpublished data*). It is highly unlikely that the initial stock solutions were incorrectly prepared; however, this possibility cannot be completely excluded.

CAPS analysis of cytochrome b mutations in other perennial ryegrass-infecting isolates of *Pyricularia grisea*. The G143A mutation results in creation of a third *Fnu*4HI site in the PCR product and F129L eliminates the single *Sty*I site (Fig. 2A). This enabled us to survey for each mutation rapidly using CAPS

analysis of *CYTB* amplification products. A possible reason that resistance emerged so rapidly in the gray leaf spot pathogen population was that the mutation was already present before QoI fungicides were used on turf grass. Unfortunately, there were very few isolates collected in the United States before strobilurin use; therefore, it is not possible to test this in a rigorous manner. Nevertheless, we used CAPS analysis to survey a collection of 22 baseline *Pyricularia grisea* isolates that were obtained from perennial ryegrass prior to fungicide use. All of the PCR products

TABLE 5. Average in vitro EC₅₀ values for two QoI fungicide chemistries against wild-type and mutant *Pyricularia grisea* isolates collected in 2000 and 2001^a

Mutation	Azoxystrobin		Trifloxystrobin	
	EC ₅₀	Resistance	EC ₅₀	Resistance
2000				
Wild-type	0.025 ± 0.007	...	0.028 ± 0.004	...
G143A	33.53 ± 9.94	1,341	27.5 ± 13.85	982
F129L	3.48 ± 2.56	139	0.87 ± 0.43	31
2001				
Wild-type	0.023 ± 0.006	...	0.022 ± 0.006	...
G143A	25.73 ± 6.62	1,119	15.04 ± 7.23	683
F129L	3.44 ± 0.54	149	0.074 ± 0.023	3

^a EC₅₀ = concentration at which spore germination is inhibited by 50% (measured in µg/ml). Resistance = increase in resistance levels in vitro = EC₅₀ mutant/EC₅₀ wild-type.

TABLE 4. Correlation of mutation type with in vitro sensitivity to two QoI fungicide chemistries

Isolate ^a	Mutation ^b	Sensitivity (EC ₅₀) ^c	
		Azoxystrobin	Trifloxystrobin
Baseline isolates			
LpKY96-1	—	<u>0.025, 0.026, 0.038</u>	<u>0.028</u>
LpOH97-1	—	<u>0.020, 0.023, 0.021</u> , 0.031	<u>0.029, 0.032, 0.023</u> , 0.024
LpKY98-1	—	0.015, 0.030	0.011, 0.020
IN0025	—	<0.1, 0.021	<0.1, 0.020
IN0107	—	0.025, 0.020	0.027, 0.023
LpOH01-1	—	0.016, 0.024	0.023, 0.031
Resistant isolates			
Class I ^d			
Her ^R 1A2	G143A	24.89, 17.77	15.98, 11.82
Her ^R 2000-1	G143A	24.74, 24.85	15.03
Her ^R 2000-2	G143A	<u>44.76, 39.32</u>	<u>111.27, 43.15, 25.14</u>
Her ^R 2000-3	G143A	13.56	7.03
Her ^R 2000-4	G143A	24.74, 30.58	15.66, 23.74
Her ^R 2000-5	G143A	<u>37.35, 34.64</u>	<u>22.83, 32.28</u>
GG2b	G143A	<u>27.00, 44.55</u>	<u>21.41, 61.16</u>
GG5-2b-2	G143A	<u>21.95, 18.7</u> , 31.02	<u>22.09, 17.89</u> , 21.04
GG5-4a	G143A	13.76, 18.3	10.12
GG5-4c	G143A ^e	23.26, 21.42	12.53
ILL-BL-00	G143A	<u>23.09, 20.65</u>	<u>14.53, 14.77</u> , 34.35
GG3H 1-1	G143A	30.25, 29.26	8.54, 12.03
GG3H 1-6	G143A	35.22, 25.03	7.12, 14.78
GG3H 5-1	G143A	38.53, 33.0	9.04, 12.79
GG3H 4-2	G143A	31.09, 26.19	7.57, 13.19
Class II			
GG5-2b-1	F129L	<u>2.01, 1.24</u> , 3.28, 2.35	<u>1.46, 0.89</u> , 0.11, 0.073, 0.049
GG5-4b	F129L	<u>2.57, 3.55, 2.39</u> , 3.05, 3.94	<u>1.01, 1.1</u> , 0.12, 0.092
ILL-00-1	F129L	<u>3.61, 8.96</u> , 2.75	<u>0.44, 0.31</u> , 0.071
LpMD01-1	F129L	2.26, 3.57	0.11
GG14 3-1	F129L	3.74, 3.38	<1.0, 0.081
GG14 3-5	F129L	4.08, 3.86	<1.0, 0.046
GG14 2-1	F129L	4.08, 4.08	0.075, 0.076
GG14 2-4	F129L	2.96, 3.28	0.077
GG14 11-1	F129L	3.36, 4.11	0.089, 0.066
IN0106	F129L	2.93, 3.24	0.040, 0.046

^a Underlined isolates were obtained in 2001.

^b Underlined data were reported in a previous paper by Vincelli and Dixon (26), the remaining data are new. Indicates that the mutation was confirmed by sequencing.

^c EC₅₀ = concentration at which spore germination is inhibited by 50% (measured in µg/ml). All values are the means of three replicates. Commas separate values obtained from independent experimental trials.

^d Resistant isolates were classified based on their fungicide sensitivity levels.

^e GG5-4c is clonally related to GG5-4a.

Fig. 2. Cleavable amplified polymorphic sequence analysis of *CytB* alleles. **A**, The G143A mutation resulted in creation of a third *Fnu*4HI site in the cytochrome b gene (*CYTb*) amplification product, whereas F129L caused loss of the single *Sty*I site. Site changes are circled. Molecular sizes are indicated in brackets beneath each fragment. **B**, Mutations were detected by digesting *CYTb* amplification products with the restriction enzymes *Fnu*4HI or *Sty*I, followed by electrophoresis through 1% agarose gels. Shown is a digital image of the ethidium bromide-stained gel on a UV transilluminator. Isolate names are shown above the respective lanes. The cytochrome b alleles revealed by the analysis are denoted W, G, and F for wild type, G143A, and F129L, respectively. "M" indicates the marker lanes and molecular sizes are shown (in base pairs).

from the baseline isolates gave digestion patterns consistent with the wild-type allele (data not shown); therefore, we were unable to obtain definitive evidence that the G143A and F129L mutations preexisted use of Q_oI fungicides on turf grasses.

In 2001, we collected several new *Pyricularia grisea* isolates from Q_oI-treated turf on Griffin Gate Golf Course in Lexington, KY. In addition, several isolates were acquired from other researchers who had obtained them from turf having a history of Q_oI fungicide use. The *CYTb* gene was amplified from these isolates by PCR, using conidiospore DNA as a template. Representative results from a CAPS analysis of some of these isolates are shown in Figure 2B. The second lane from the left contains the amplification product from IN0107, a wild-type control isolate. The isolates from Griffin Gate golf course (GG2 6ft-1 and GG3H 1-1) had the G143A mutation, whereas GG14 3-1 had F129L. The latter mutation also was found in IN0106 and LpMD01-1 (Fig. 2), isolates that came from Indiana and Maryland, respectively. All of the mutations predicted by CAPS analysis were confirmed by sequence analysis of the complete *CYTb* gene. The remaining isolates obtained from cooperating researchers lacked mutations (Fig. 2B). The *CYTb* genotypes of most of the resistant isolates analyzed in this study are summarized in Table 6.

Genetic relationships between Q_oI^R isolates. The discovery of two independent mutations in the Q_oI^R isolates analyzed in this study reveals that resistance to Q_oI fungicides has arisen at least twice in the gray leaf spot pathogen population. However, the genetic relationships between isolates possessing the same type of mutation were not clear. It is possible that isolates with the same mutation were derived from a single Q_oI^R progenitor. Alternatively, spontaneous mutation to Q_oI resistance may arise quite frequently in the gray leaf spot pathogen population. To address this issue, we used restriction fragment length polymorphism (RFLP) analysis and DNA fingerprinting to investigate the genetic background of the resistant isolates. If the resistant individuals were descendants of a single progenitor, they should have similar fingerprints and share RFLP alleles. Conversely, if multiple mutations have arisen, the mutants would likely have different fingerprint profiles, which should reflect the various patterns present in the baseline population.

A previous DNA fingerprint analysis of 19 members of the baseline population, using the repetitive Pot2 element as probe, revealed 10 Pot2 fingerprint groups (FGs) with *Bam*HI-digested genomic DNA (M. Farman, *unpublished data*). One of the largest groups, FG2, was represented by five isolates (allele frequency = 0.26; Table 6). Other groups differed from FG2 based on differences at between one and five restriction fragments. The six baseline isolates shown in the rightmost lanes of Figure 3 represent 5 of the 10 groups. These groups also were differentiable based on their *Pst*I fingerprints (data not shown). Among the 20 Q_oI^R isolates that we analyzed, 15 had identical Pot2 fingerprints matching the profile of FG2 (Fig. 3); 2 belonged to FG4 (Fig. 3, lanes 2 and 9), another previously characterized group; and 2 isolates defined two new FGs, FG11 (Fig. 3, lane 1) and FG12 (data not shown).

To provide additional insight into the genetic background of the resistant isolates, we used the single-copy RFLP probe 16-1-B that identified two alleles in the baseline population. Alleles 1 and 2 were represented by *Pst*I restriction fragments of 8.2 and 10.5 kb, respectively (data not shown). Their frequencies in the baseline population were 0.79 for allele 1 and 0.21 for allele 2 (Table 6). Among the 15 resistant isolates analyzed, 12 possessed the type 2 allele, which was rarer in the baseline population. Interestingly, the 12 DNA samples that revealed the type 2 allele at the 16-1-B locus were not the same 12 that had the FG2 Pot2 profile. Based on the combined Pot2 and 16-1-B marker data, the resistant isolates fell into eight distinct multilocus haplotypes (Table 6).

DISCUSSION

In 2000, the occurrence of gray leaf spot disease on Q_oI-treated turf was significant because it represented the first possible occurrence in the United States of resistance to this class of fungicides. To determine if these outbreaks were due to changes in the sensitivity of *Pyricularia grisea* to Q_oI fungicides, in vitro testing was performed. This revealed that the isolates collected from fungicide-treated turf were up to 1,300 times less sensitive to azoxystrobin than baseline isolates collected before the use of Q_oI fungicides (26). In the present study, sequence analysis of the cytochrome b gene from many *Pyricularia grisea* isolates with de-

TABLE 6. Molecular marker haplotypes of baseline and Q_oI-resistant isolates

Isolates	CYTb mutation ^a	Fingerprint group ^b	16-1-B haplotype ^c	Multilocus haplotype ^d
Baseline				
WF9724	—	1	1	A
HO	—	1	2	B
LpKY96-1, GG12, WF9722, WF9723	—	2	1	C
GG13	—	2	2	D
FH, KS331, LpKY97-1, LpOH1, RGNJ	—	3	1	E
LpOH3	—	4	2	F
GG11	—	5	1	G
KS330	—	6	1	H
KS320	—	7	2	I
CHW	—	8	1	J
GG9	—	9	1	K
CHRF	—	10	1	L
Resistant				
Her ^R 1A1	G143A	11	2	A ^R
GG3H 1-1	G143A	12	2	B ^R
Her ^R 2000-1B	G143A	4	2	C ^R
GG5-2b-1	F129L	4	2	D ^R
Her ^R 2000-2, Her ^R 2000-3	G143A	2	1	E ^R
ILL00-1	F129L	2	1	F ^R
Her ^R 2000-4, Her ^R 2000-5, GG2a, GG2b, GG5-2b-2, GG5-4a, GG5-4c, ILL-BL-00, GG2 6ft-1	G143A	2	2	G ^R
GG5-4b, GG14H 3-1, IN0106, LpMD01-1	F129L	2	2	H ^R

^a CYTb = cytochrome b gene.

^b Pot2 probe, *Bam*HI digest.

^c 1 = 8.2-kb allele, 2 = 10.5-kb allele.

^d Superscript "R" = Q_oI-resistant.

creased sensitivity revealed that they all have mutations in the gene for cytochrome b, the target enzyme for the Q_oI fungicides. Two different nucleotide substitutions were detected in the *CYTb* gene from these resistant isolates, both of which are predicted to result in changes in the amino acid sequence of the cytochrome b protein.

There is substantial evidence that the mutations causing the F129L and G143A substitutions are causal to the decreased sensitivity. First, both types of mutation have been implicated in Q_oI resistance in other organisms. The G143A change is associated with Q_oI resistance in a number of phytopathogenic fungi, including *Venturia inaequalis* (19,29), *Podosphaeria fusca* (8), *Pseudoperonospora cubensis* (8), *Mycosphaerella fijiensis* (17), *Blumeria graminis* f. sp. *tritici* (18), and *Blumeria graminis* f. sp.

hordei (7). In addition, the cytochrome b gene of *Mycena galopoda*, a strobilurin-producing basidiomycete that is naturally resistant to these compounds, has an alanine residue at amino acid position 143 (10). The F129L change has been documented in Q_oI-resistant mutants of *Saccharomyces cerevisiae* (4) and the alga *Chlamydomonas reinhardtii* (2). The F129L substitution identified in *Pyricularia grisea* was the first example of this mutation conferring field resistance to the Q_oI fungicides in a phytopathogenic fungus. Since our discovery, however, F129L changes also have been identified in *Pythium aphanidermatum* from turf and *Alternaria solani* from potato (G. Olaya, personal communication).

The type of mutation (F129L or G143A) present in a given mutant was highly predictive of its in vitro sensitivity to different Q_oI fungicides (Table 4). The G143A mutants exhibited a high

Fig. 3. Pot2 fingerprinting of Q_oI-resistant mutants. Genomic DNA was digested with *Pst*I and electrophoresed in a 0.7% agarose gel (≈200 ng DNA/lane). The gel was washed and blotted to a nylon membrane, which then was probed with a ³²P-labeled 0.9-kb internal fragment of the Pot2 transposon. Shown is the PhosphorImage obtained following exposure of the hybridized blot. DNA samples from Q_oI-resistant isolates were loaded on the left and samples from baseline (Q_oI-sensitive) isolates are loaded on the right. Isolate names are noted above the respective lanes. Gray shading is used to highlight the baseline isolates. Fingerprint group affiliations are indicated below each isolate name. Relevant diagnostic restriction fragments differences are arrowed.

level of cross-resistance to azoxystrobin and trifloxystrobin. The F129L substitution conferred a lower level of resistance to azoxystrobin and little, or no, resistance to trifloxystrobin (Table 4). Similar differences in *in vitro* sensitivities also were observed in tests performed in an independent laboratory (G. Olaya, *personal communication*). Thus, it appears that G143A confers cross-resistance between different strobilurin chemistries, whereas F129L does not. *In vivo* tests indicate that the differential fungicide-mutant interactions also may be manifested in planta (26). For example, in two separate growth chamber experiments, azoxystrobin was ineffective in controlling disease caused by five G143A isolates but still provided partial control of three F129L mutants. Trifloxystrobin also was ineffective against the former isolates but gave almost complete protection against two of the F129L mutants and partial protection against the third (26). *In vivo* tests performed in an independent lab found that azoxystrobin and trifloxystrobin failed to control both G143A and F129L mutants (G. Olaya, *personal communication*). Thus, the relative efficacies of the two fungicides may be sensitive to environmental factors. Nevertheless, the results published by Vincelli and Dixon (26) are reproducible, having been confirmed in a subsequent repetition of the *in vitro* experiments (P. Vincelli and E. W. Dixon, *unpublished data*). These results notwithstanding, it is important to consider that the differences in efficacy against F129L mutants measured in growth chamber assays may not accurately reflect the relative performance of the two fungicides under field conditions. In this regard, it would be premature to base any recommendations for fungicide use on these findings.

The discovery of two mutation types in the Q_oI^R isolates indicates that a minimum of two mutational events underlie the outbreak of resistance in the gray leaf spot pathogen population. Incorporation of the *CYTB* gene data with the information from the genetic markers in the nuclear genome revealed that 9 of the 20 Q_oI^R isolates analyzed had identical haplotypes at the Pot2, 16-1-B, and *CYTB* loci. In contrast, only 1 of 18 baseline isolates had the same combination of haplotypes (GG13, Table 6). Indeed, the "baseline" frequency of the 16-1-B² allele alone was much lower than in the resistant population (0.21 versus 0.85). This indicates that, if *CYTB* mutations have arisen multiple times in the gray leaf spot pathogen population, they did not occur randomly but were somehow favored in the FG2 genetic background. An alternative interpretation, however, is that most, if not all, of the Q_oI-resistant isolates characterized in this study were derived from a small number of ancestral mutants. Initially, the fact that resistance is found in seven different genetic backgrounds would appear to indicate that resistance has arisen multiple times. However, given the predominance of the FG2 and FG4 haplotypes within the resistant population, and comparing their abundance with their respective baseline frequencies, it is plausible that the G143A and F129L mutations arose one time, with each occurring in a distinct background. The extant haplotypes then may have arisen through genetic recombination, with concomitant mitochondrial transfer. Although it appears that the mutants displaying the FG11 and FG12 Pot2 profiles might have independent origins, it is quite likely that these fingerprints are mutational derivatives of the FG2 profile, especially considering that the Pot2 probe is part of a transposon (9). If recombination has occurred, the most likely route would be through hyphal anastomosis because mating is precluded by the absence of the MAT1-1 mating-type allele in the gray leaf spot pathogen population (24; M. Farman, *unpublished data*). Of course, our ability to make more definitive conclusions about the *CYTB* mutation frequency will require identification of a larger number of informative genetic markers, preferably in the mitochondrial genome itself.

The loss of Q_oI fungicide efficacy at certain sites in the Midwest only 2 years after these chemicals were first labeled for use on turf grasses, and a similar breakdown on the East Coast only one year later, suggests that resistance may already have

been present in the pathogen population before this class of fungicides was employed for controlling gray leaf spot. The isolate from Maryland (LpMD01-1) is a member of FG2 (data not shown) and is, therefore, clonally related to the resistant isolates that are predominant in the Midwest. However, due to the wide separation of these sites and the lack of Q_oI resistance reports between these locations, it seems unlikely that resistance spread to the East Coast from Illinois and Kentucky. Assuming that the LpMD01-1 mutant does not have an independent origin, these observations suggest that a resistant subpopulation of FG2 already was established throughout the central and eastern United States prior to Q_oI fungicide use and that the localized resistance outbreaks were due to particularly high disease pressure in these locations. If true, it is likely that in the near future, heavy disease pressure will lead to a widespread breakdown in Q_oI-based control of gray leaf spot throughout the central United States. This possibility highlights the importance of adhering to Q_oI use guidelines, by employing fungicide mixes and rotating fungicide classes, to minimize the likelihood of catastrophic failures in disease control.

The Q_oI fungicides also are used to control blast in the United States rice crop. Fortunately, resistance to these chemicals has not yet been detected for blast. *Pyricularia grisea* isolates causing gray leaf spot on perennial ryegrass do not infect rice and are quite distinct genetically (5,24). As a result, the Q_oI-resistant isolates occurring on perennial ryegrass should not pose an immediate threat to the United States rice crop. However, the extremely high sequence similarity between cytochrome b genes in the two host-specific forms of *Pyricularia grisea*, and the lack of discernable fitness effects associated with the mutations identified in this and another study (Y.-S. Kim and M. L. Farman, *unpublished data*), suggest that *de novo* resistance-conferring mutations also could arise in the rice-infecting *Pyricularia grisea* population. Finally, it is possible that mutant mitochondria can be introduced into the rice-infecting population through mating or hyphal anastomosis. For this reason, it is important that the current Q_oI resistance outbreak in perennial ryegrass is contained through sound cultural practices and by adhering to the manufacturers' guidelines on fungicide use and application rates. Such precautions should help to minimize the spread of resistant *CYTB* alleles.

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