

## Following the dynamics of strobilurin resistance in *Blumeria graminis* f.sp. *tritici* using quantitative allele-specific

real-time PCR measurements with the fluorescent dye

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Strobilurin-resistant isolates of *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici*, the cause of wheat powdery mildew, were more than 10-fold less sensitive to azoxystrobin than sensitive isolates. In all resistant isolates, a mutation resulting in the replacement of a glycine by an alanine residue at codon 143 (G143A) in the mitochondrial cytochrome *b* gene was found. Allele-specific primers were designed to detect this point mutation in infected wheat leaves. Using quantitative fluorescent allele-specific real-time polymerase chain reaction (PCR) measurements, strobilurin-resistant A143 alleles could be detected amongst strobilurin-sensitive G143 alleles at a frequency of at least 1 in 10 000, depending on the amount of target and nontarget DNA. Most isolates tested were dominant homoplasmic for either the A143 or G143 allele, although mixed populations of alleles could be detected in some isolates. In some of these isolates, strobilurin resistance was not always stable when they were maintained for many generations in the absence of selection. The allele-specific real-time PCR assay was also used to follow the dynamics of A143 alleles in field populations of *B. graminis* f.sp. *tritici* before and after application of fungicides. As expected, the A143 allele frequency only increased under selection pressure from a strobilurin fungicide. After three sprays of azoxystrobin, a pronounced selection for the strobilurin-resistant allele, with an increase in average frequency from 2.2 to 58%, was measured. The use of quantitative real-time PCR diagnostics for early detection of fungicide resistance genes at low frequency, coupled with risk evaluation, will be invaluable for further resistance risk assessment and validation of antiresistance strategies.

**Keywords:** allele-specific real-time PCR, cytochrome *b*, *Erysiphe graminis*, fungicide resistance, mitochondria, strobilurin fungicides

### Introduction

Powdery mildew, caused by the obligate biotrophic fungus *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici*, is a serious threat in European wheat-growing areas. In the UK alone, annual losses of £24 million were estimated for the period 1985–89 (Cook *et al.*, 1991). Good farming practices, including crop rotation with resistant cultivars, can reduce infection, but fungicides are the main control measure for wheat powdery mildew epidemics. Together, these practices have resulted in a decline in severity of the

disease in England and Wales between 1989 and 1998 (Hardwick *et al.*, 2001).

Although many different fungicides are available to control powdery mildews, rapid development of fungicide resistance in these pathogens is a particular problem. For *B. graminis*, practical resistance problems have been reported for benzimidazoles (Vargas, 1973), hydroxypyrimidines (Hollomon, 1975), sterol 14 $\alpha$ -demethylation inhibitors (DMIs) (Buchenauer & Hellwald, 1985) and, since 1998, strobilurins (Heaney *et al.*, 2000). Overall, resistance risk is generally high for single-site inhibitors where the resistance mechanism is monogenic, whilst polygenic-based resistance evolves more slowly and rarely causes product failures.

Resistance to benzimidazoles based on point mutations in the  $\beta$ -tubulin target gene have been reported for many

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plant pathogenic fungi, including *Rhynchosporium secalis* (Wheeler *et al.*, 1995) and *Tapesia* spp. (Albertini *et al.*, 1999). Although mutations at 10 sites within the  $\beta$ -tubulin gene can confer benzimidazole resistance (Davidse & Ishii, 1995), only a few have been found in field populations of fungi, probably due to reduced fitness and/or pathogenicity linked with certain mutations. For DMI resistance, an increased efflux mechanism operated by ATP-binding cassette transporters has been reported for *Aspergillus nidulans* (Del Sorbo *et al.*, 1997) and *Penicillium digitatum* (Nakaune *et al.*, 1998). For *P. digitatum*, DMI resistance can also be triggered by a transcriptional enhancer of the sterol 14 $\alpha$ -demethylase target gene (CYP51) (Hamamoto *et al.*, 2000). For *Uncinula necator* (D lye *et al.*, 1997) and *B. graminis* f.sp. *hordei* (D lye *et al.*, 1998), an identical point mutation in CYP51 was associated with DMI resistance. Increased multidrug efflux in combination with different mutations in CYP51 contributes to the stepwise increase in resistance to DMIs in *Candida albicans* (Sanglard *et al.*, 1998).

Several amino acid substitutions in two regions of the mitochondrial cytochrome *b* gene, which confer resistance to strobilurins and other respiration inhibitors at the Qo centre of the cytochrome *bc*<sub>1</sub> enzyme complex, have been identified in yeast and fungi naturally resistant to strobilurins (Di Rago *et al.*, 1989; Kraiczy *et al.*, 1996). For *Venturia inaequalis* (Zheng *et al.*, 2000), *Mycosphaerella fijiensis* (Sierotzki *et al.*, 2000a), *B. graminis* f.sp. *tritici* (Sierotzki *et al.*, 2000b; Fraaije *et al.*, 2000) and the oomycete *Plasmopara viticola* (Heaney *et al.*, 2000), an exchange of glycine to alanine at position 143 (G143A) of the cytochrome *b* gene has been reported to confer high resistance levels to strobilurins. Lower resistance levels to strobilurins, due to altered alternative respiration in the absence of the host plant, were found in *Mycosphaerella graminicola* (anamorph *Septoria tritici*) (Ziogas *et al.*, 1997) and *V. inaequalis* isolates (Zheng *et al.*, 2000). However, these isolates were controlled *in vivo* by strobilurins.

DNA-based tests to screen for fungicide resistance can be sensitive and cost-effective, especially for obligate biotrophic pathogens unable to grow on artificial media. Rapid tests which measure the frequency of resistant alleles, both without and under selection pressure of fungicides, can offer opportunities to improve resistance risk assessment, optimize resistance management and support new products (Brent & Hollomon, 1998). Polymerase chain reaction (PCR) linked with allele-specific probes (Koenraadt & Jones, 1992), PCR-restriction fragment length polymorphism (Luck & Gillings, 1995) and allele-specific PCR (D lye *et al.*, 1997) have been used to detect fungicide resistance.

The objective of this study was to develop a DNA diagnostic based on real-time allele-specific PCR using the fluorescent dye SYBR Green I (Schneeberger *et al.*, 1995). The potential application of this assay to monitor *in planta* the dynamics of strobilurin-resistant A143 alleles in isolates as well as in populations of *B. graminis* f.sp. *tritici* under selection pressure of fungicides is demonstrated and discussed.

## Materials and methods

### Fungal isolates

All *B. graminis* f.sp. *tritici* isolates in this study were purified as single spore or colony isolates. These isolates were maintained at 17°C on untreated leaf pieces (wheat cv. Riband) on 0.5% (w/v) water agar containing benzimidazole (50 p.p.m.) in plastic boxes, and subcultured at 10-day intervals. Thirty-three isolates from either commercial crops (Table 1) or the field trial undertaken in this study (Table 2) were analysed by both bioassay and PCR-based assays.

### Fungicide sensitivity assays

Leaf pieces (2–3 cm) of wheat (cv. Riband) were floated on solutions of fungicides for 16 h at 17°C. To avoid vaporization effects, each concentration of azoxystrobin (10-fold dilutions ranging from 0.000 05 to 5.0  $\mu$ g mL<sup>-1</sup>) was tested in a close-fitting polystyrene box (Stewarts Plastics, Croydon, UK). Leaves were inoculated by blowing viable conidia into a settling tower (Hollomon, 1975), and kept initially in darkness for 6 h at 20°C to synchronize spore germination. After 72 h of incubation under white light at 17°C, leaves were cleared and stained with 0.05% trypan blue in glycerol, lactic acid and water (1:1:1). The number of colonies on each leaf piece (*n* = 3) was counted under a microscope, and final mean counts per leaf were compared with those of fungicide-untreated leaves to determine the dose needed to reduce growth by 50% (ED<sub>50</sub>).

### Field experiment

A field of wheat (cv. Claire) grown in Herefordshire, UK, was divided into three plots (36 × 30 m). One plot remained untreated throughout the season and other plots were treated three times (at growth stages (GS) 31, 32 and 43), at approximately 14-day intervals, with either 0.66 L ha<sup>-1</sup> Amistar (250 g L<sup>-1</sup> azoxystrobin; Syngenta, Bracknell, Berkshire, UK) or 0.66 L ha<sup>-1</sup> Folicur (250 g L<sup>-1</sup> tebuconazole; Bayer plc, Bury St Edmunds, Suffolk, UK). To monitor the frequency of strobilurin-resistant alleles in populations of *B. graminis* f.sp. *tritici*, nine samples on an equidistant 3 × 3 grid pattern were taken from all plots each time. The distance between sampling points within a plot was approximately 7.5 m, while spacing between sampling points of different plots was 15 m. Plots were sampled just before each spray and approximately 14 days after the final treatment, at GS 30, 32, 43 and 65, respectively. On each sample date, 10 leaves visually infected with powdery mildew were collected within a radius of 1 m from each sampling point. The leaves were collected from the most recently emerged leaf layers that had mildew pustules, to maximize the probability that infection and pustule development occurred after the previous fungicide application. Isolates of *Blumeria graminis* f.sp. *tritici* were obtained from these leaves and DNA was extracted from the bulk sample. Mildew was assessed visually on 25 tillers from all plots at GS 65.

**Table 1** *Blumeria graminis* f.sp. *tritici* isolates characterized from commercial wheat crops

Isolate designation	Location	Year	Source <sup>a</sup>	Strobilurin sensitivity <sup>b</sup>	R-allele frequency (%) <sup>c</sup>
ResU1	SW England	1985	1	S	ND
Lars5	SW England	1985	2	S	ND
W26	SW England	1987	2	S	0
W45	N Germany	1987	3	S	0
W43	Hungary	1988	2	S	0
JAS501	N Germany	1998	3	R + S <sup>d</sup>	37
JAS506	N Germany	1998	3	R + S <sup>d</sup>	24
DE60-4	NE England	1999	4	S	0
DE74-1	S England	1999	4	S	0
DE75-2	S England	1999	4	S	0
MW16	UK	1999	4	S	0
Gblet1	UK	1999	4	R	100
S1	Germany	2000	5	R	ND
S11/3	Germany	2000	5	R	100
L-CL1-ST1	SW England	2000	2	R	100
AR49-2-S2	E England	2000	2	R	100

<sup>a</sup>Source names, 1, IACR-Rothamsted, Harpenden, UK; 2, IACR-Long Ashton Research Station, Long Ashton, UK; 3, Syngenta, Jealott's Hill Research Station, Bracknell, UK; 4, Dow Agrosciences, Letcombe Laboratory, Wantage, UK; 5, BASF, Limburgerhof, Germany.

<sup>b</sup>Strobilurin sensitivity: S, sensitive, ED<sub>50</sub> ranging from < 0.005 to 0.05 µg mL<sup>-1</sup> azoxystrobin; R, resistant, ED<sub>50</sub> ranging from 0.05 to > 5.0 µg mL<sup>-1</sup>.

<sup>c</sup>Frequency of strobilurin-resistant A143 alleles of cytochrome *b*; ND, not determined.

<sup>d</sup>Isolates JAS501 and JAS506 isolated as single spores from infected leaves in the summer of 1998 were initially found resistant to strobilurins. However, in November 1999, after being grown for more than 30 generations without selection pressure of fungicides, both isolates were sensitive to strobilurins.

## DNA extraction

DNA extraction from conidia, infected and healthy wheat leaves, and measurements of plasmid DNA concentrations using the fluorescent dsDNA-specific dye PicoGreen (Molecular Probes, Leiden, the Netherlands) were carried out as described by Fraaije *et al.* (1999).

## Cloning and sequencing part of the cytochrome *b* gene

For *Saccharomyces cerevisiae* and some other fungi, two regions of the cytochrome *b* gene covering amino acid positions 127–153 and 255–276 have point mutations which confer resistance to strobilurins (Zheng & Köller, 1997). After aligning nucleotide sequences from *S. cerevisiae* (Norbrega & Tzagoloff, 1980), *Neurospora crassa* (Citterich *et al.*, 1983), *Podospora anserina* (Cummings *et al.*, 1989) and *A. nidulans* (Waring *et al.*, 1981), consensus primers CBF1 and CBR3 (Table 3) were designed to amplify the coding sequence for amino acid codons 127–276 of the cytochrome *b* gene from *B. graminis* f.sp. *tritici*. After PCR, excess primers were removed with the High Pure PCR Product Purification Kit (Boehringer-Mannheim, Germany) and the PCR products ligated directly into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* JM109 cells (Promega) according to a standard protocol (Sambrook *et al.*, 1989), and plasmid DNA extracted using the RPM kit (Bio101 Inc, Carlsbad, CA, USA). Finally, the nucleotide sequence was determined

in a dideoxy chain termination method reaction (Sanger *et al.*, 1977).

## PCR standard protocol

Standard PCR was carried out in a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with 1.25 units of Red Hot DNA polymerase (ABgene, Epsom, UK) using 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 75 mM Tris-HCl (pH 9.0), 0.01% Tween-20, 1.5 mM MgCl<sub>2</sub>, 125 µM of each dNTP, 0.5 µM of each primer and 100 ng template DNA in a final volume of 100 µL. Conditions to amplify part of the cytochrome *b* gene from *B. graminis* f.sp. *tritici* were: 94°C for 3.5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1.5 min PCR was terminated with a DNA extension at 72°C for 8.5 min. PCR products were separated on ethidium bromide-stained 1.3% (w/v) agarose gels run in 1x Tris-borate-EDTA buffer and exposed to UV light to visualize DNA fragments.

## Quantitative fluorimetric allele-specific real-time PCR assay

Primer sets 143GF/PMR4 and 143CF/PMR5 (Table 3) were used to amplify preferentially one allele by matching the desired allele and mismatching the other allele at the 3' end of the primer. Primer set 142F/PMR5, which amplifies a nonallele-specific fragment of the cytochrome *b* gene from all isolates of *B. graminis* f.sp. *tritici*, was used to check for inhibition of the PCR. Reverse primers were

Isolate designation <sup>a</sup>	Treatment and timing <sup>b</sup>	Strobilurin sensitivity <sup>c</sup>	R-allele frequency (%) <sup>d</sup>
A3(1)-1	Untreated	R	100
A5(3)-2	Untreated	S	0
A7(3)-1	Untreated	S	0
B5(0)-1	Untreated	R	100
B5(0)-2	Untreated	R	100
B8(1)-1	After first tebuconazole spray	S	0
B3(2)-1	After second tebuconazole spray	S	0
B3(2)-3	After second tebuconazole spray	S	0
B8(2)-3	After second tebuconazole spray	R	100
B6(3)-2	After third tebuconazole spray	R	100
C9(0)-1	Untreated	R	100
C9(0)-2	Untreated	R	100
C5(1)-1	After first azoxystrobin spray	R	100
C6(1)-1	After first azoxystrobin spray	R	100
C4(2)-1	After second azoxystrobin spray	R	100
C1(3)-1	After third azoxystrobin spray	R	100
C3(3)-1	After third azoxystrobin spray	R	97

<sup>a</sup>Isolate designation; letters show from which plots A (untreated), B (tebuconazole-treated) and C (azoxystrobin-treated) isolates were obtained; first number represents sample point; number between brackets indicates timing before (0) or after spraying (1, 2 and 3); and the last number indicates which pustule was further tested.

<sup>b</sup>Samples before first or after first, second or third fungicide spray were collected at GS 30, 32, 43 and 65, respectively.

<sup>c</sup>Strobilurin sensitivity: S, sensitive, ED<sub>50</sub> ranging from < 0.005–0.05 µg mL<sup>-1</sup> azoxystrobin; R, resistant, ED<sub>50</sub> ranging from 0.05 to > 5.0 µg mL<sup>-1</sup>.

<sup>d</sup>Frequency of strobilurin-resistant A143 alleles of cytochrome *b*.

designed to have mismatches at the 3' end in comparison with other fungal cytochrome *b* gene sequences, including partial cytochrome *b* gene sequences of *S. tritici* and *Phaeosphaeria nodorum* (anamorph *Stagonosporum nodorum*) (B.A. Fraaije, unpublished data). This was done to prevent amplification of cytochrome *b* gene sequences of nontarget organisms present in DNA extracted from wheat leaves. For each reaction, 150 µM of each dNTP, 0.3 µM of each primer, 0.35 units of Red Hot DNA polymerase and 1x SYBR Green I (Molecular Probes, Leiden, the Netherlands), 1:15 diluted, were used. DNA template (100 ng) was obtained from *B. graminis* f.sp. *tritici*-infected and uninfected leaves. Assays (25 µL) were performed in capped MicroAmp Optical 96-well reaction plates (PE Applied Biosystems, Fosters City, CA, USA). Amplification and detection were performed in a PRISM 7700 Sequence Detection System (PE Applied Biosystems) under the following conditions: one cycle at 50°C for 5 s, one cycle at 94°C for 2 min 30 s, and 35 cycles at 94°C for 30 s, 57°C (primer set 143GF/PMR4) or 62°C (143CF/PMR5) for 40 s, and 72°C for 40 s. Increase of fluorescent emission signal from SYBR Green I ( $\Delta Rn$ ) was registered at 72°C without normalization of the signal. The Sequence Detector software version 1.6.3 (PE Applied Biosystems) was used to analyse the data. Samples, all tested in duplicate, were regarded as positive at any given cycle when  $\Delta Rn$  of a given sample exceeded at least 10 times the standard deviation of the fluorescent emission of the no-template control reaction. This threshold was set manually and the cycle reaching this point was

**Table 2** *Blumeria graminis* f.sp. *tritici* isolates characterized from field plots

**Table 3** Oligonucleotides tested for amplifying fragments of the cytochrome *b* gene

Primer designation	Sequence (5'–3')
Forward primers	
CBF1	TATTATGAGAGATGTAATAATGG
142F	GGCAGATGAGCCACTGG
143CF	CAGATGAGCCACTGGGC
143GF	CAGATGAGCCACTGGGG
Reverse primers	
CBR3	CCTAATAATTTATTAGGTATAGATCTTA
PMR4	TAATATTGCATAGAAGGGCAG
PMR5	ACTCCGGTACAATAGCAGCC

called the cycle threshold (*Ct*). For each sample, the amount of target DNA was quantified using appropriate calibration curves in which *Ct* was plotted against the amount of target DNA. To generate these curves, wheat-leaf DNA samples (100 ng) were spiked with different amounts of target DNA, ranging from 0.0014 to 1440 pg of plasmid DNA, and these were run simultaneously in each experiment. Plasmid PW26, which contains the G143 allele of the cytochrome *b* gene from isolate W26, and primer set 143GF/PMR4 were used to detect and quantify the amount of G143 alleles in isolates and field populations of *B. graminis* f.sp. *tritici*. To measure the amount of A143 alleles, plasmid P501 and primer set 143CF/PMR5 were used.

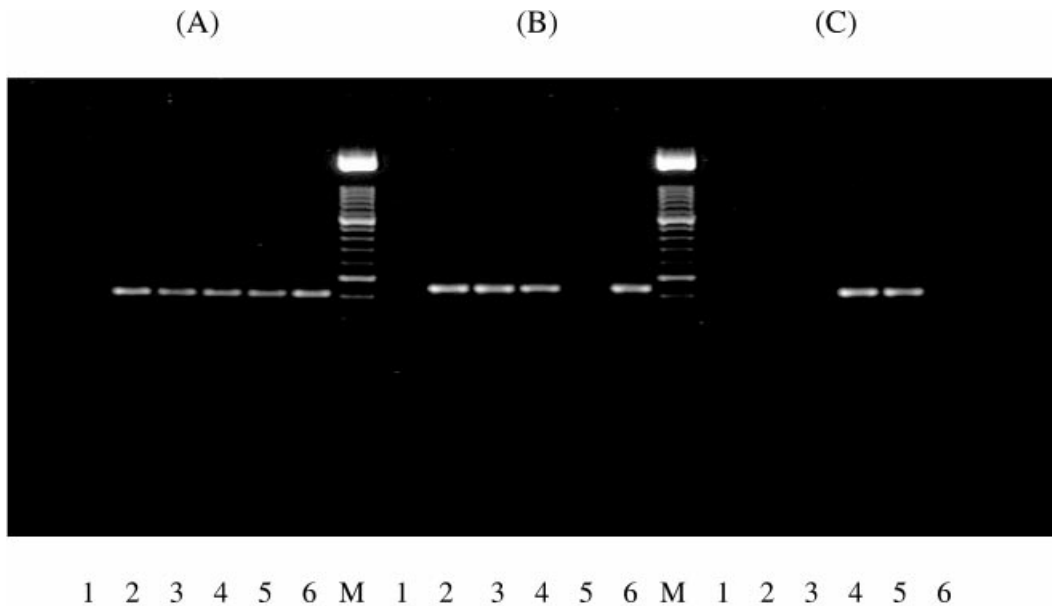


Figure 1 Detection of strobilurin resistance in *Blumeria graminis* f.sp. *tritici* isolates on wheat leaves by allele-specific PCR using primer combinations 142F/PMR5 (A), 143GF/PMR4 (B) and 143CF/PMR5 (C). PCR samples (2  $\mu$ L) were analysed in agarose gel electrophoresis. Lane 1, uninfected wheat leaves of cv. Riband; lane 2, isolate DE60.4 (S); lane 3, isolate DE74.1 (S); lane 4, isolate JAS501 (R + S); lane 5, isolate S11/3 (R); lane 6, isolate DE75.2 (S); lane M, 100-bp DNA size markers. S, strobilurin-sensitive; R, strobilurin-resistant phenotype.

## Results

### Fungicide sensitivity testing

In total, 33 isolates of *B. graminis* f.sp. *tritici* were tested for strobilurin sensitivity (Tables 1 and 2). All 19 strobilurin-resistant isolates with  $ED_{50} \geq 0.05 \mu\text{g mL}^{-1}$  (including isolates JAS501 and JAS506 when tested in 1998) were able to grow at a dose-rate of azoxystrobin of  $0.5 \mu\text{g mL}^{-1}$ . In 1999, isolates JAS501 and JAS506, like the remaining strobilurin-sensitive isolates, were found to be sensitive, with  $ED_{50} < 0.05 \mu\text{g mL}^{-1}$ , and unable to grow on leaves treated with  $0.5 \mu\text{g mL}^{-1}$  azoxystrobin.

### Cytochrome *b* gene partial cloning and sequencing

For all *B. graminis* f.sp. *tritici* isolates tested, primer set CBF1/CBR3 generated a single product of 675 bp at 55°C. The amplified sequence (GenBank accession number AJ293566) contained no introns and encoded 207 amino acids (positions 76–282) of the cytochrome *b* protein. Comparison with other fungal amino acid sequences of cytochrome *b* revealed a high identity between *B. graminis* f.sp. *tritici* and *V. inaequalis* (90.8%), *N. crassa* (87.9%), *P. anserina* (87.4%) and *A. nidulans* (87.0%). The strobilurin-resistant isolates JAS501, JAS506, GBlot1 and S1 showed a guanine to cytosine (G-to-C) transversion at nucleotide 228 of the cloned fragment (AJ293567), which resulted in the G143A amino acid exchange. G143A was not present in the sequences of the strobilurin-sensitive isolates W26, DE60.4 and ResU1. In comparison with the amino acid sequences published by Sierotzki *et al.* (2000b), a glutamine residue was found in

all isolates instead of a histidine residue at position 138. Glutamine at position 138 is well conserved in other fungal cytochrome *b* amino acid sequences (Degli Esposti *et al.*, 1993). Plasmids PW26 and P501, containing the 675-bp fragment of the cytochrome *b* gene from isolates W26 and JAS501, respectively, were used in the fluorimetric real-time allele-specific PCR assays.

### Allele-specific PCR assay for detection of G143A in isolates

Using an annealing temperature of 57°C, primer set 142F/PMR4 amplified a 411-bp fragment of the cytochrome *b* gene from infected leaves of all isolates tested, but not from uninfected leaves (Fig. 1). Primer sets 143GF/PMR4 and 143CF/PMR5 discriminated between strobilurin-sensitive G143 and resistant A143 alleles of cytochrome *b*; they did not amplify DNA from uninfected wheat leaves (Fig. 1) or other wheat-associated fungi tested, including *S. tritici*, *Phaeosphaeria nodorum* and *Tapesia* spp. (data not shown). At 55°C, primer set 143GF/PMR4 amplified a 439-bp product from strobilurin-sensitive isolates. For strobilurin-resistant isolates JAS506 (data not shown) and JAS501, the 439-bp fragment was also amplified in large amounts (Fig. 1). At 62°C, primer set 143CF/PMR5 amplified a 409-bp product from strobilurin-resistant isolates only (Fig. 1).

### Quantitative detection of G143 and A143 alleles using allele-specific real-time PCR

To quantify the G143 and A143 alleles in isolates, real-time allele-specific PCR assays were performed using the

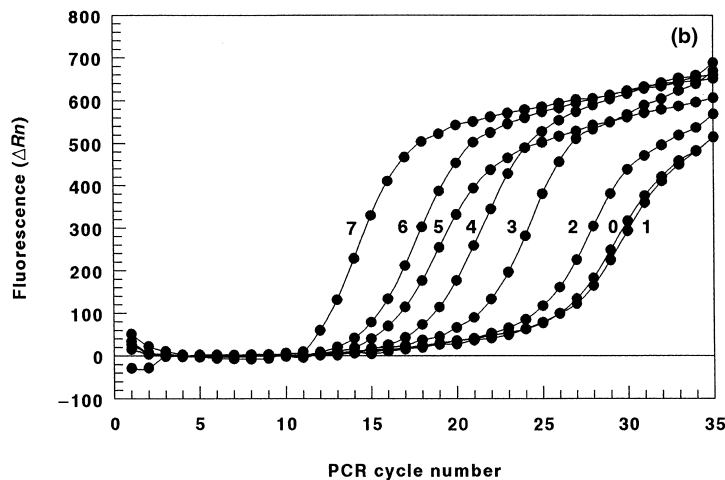
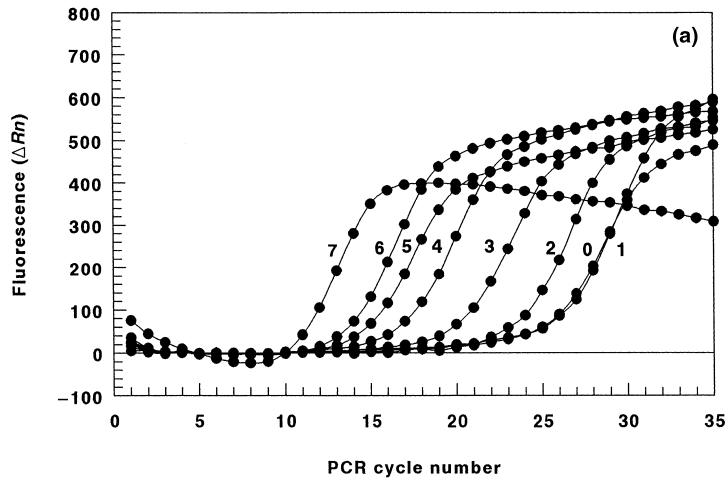


Figure 2 Fluorescent allele-specific real-time PCR signals of wheat DNA samples spiked with DNA of plasmid P501 containing either G143 (a) or A143 alleles (b). Curve 0 = unspiked wheat DNA sample; curves 1–7 represent samples to which 0.00144, 0.0144, 0.144, 1.44, 7.2, 14.4 and 144 pg, respectively, of plasmid DNA were added.

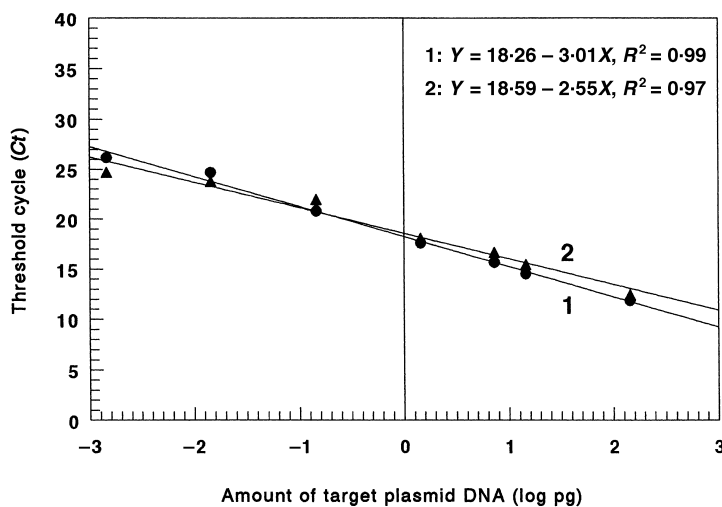


Figure 3 The relationship between threshold cycle in fluorescent allele-specific real-time PCR and amount of DNA of plasmid P501, containing either G143 (1, ●) or A143 (2, ▲) alleles, in spiked wheat DNA samples.

fluorescent dsDNA-specific dye SYBR Green I to measure the amount of amplified DNA. After spiking 100 ng of wheat DNA with serial dilutions of plasmid DNA, containing either the G143 (plasmid PW26) or A143 allele (plasmid P501), fluorescent signals with different *Ct* values were obtained (Fig. 2). For all experiments using

primer sets 143GF/R4 and 143CF/R5, the amount of input target plasmid DNA between 0.001 and 1000 pg, added to 100 ng of wheat DNA, was linearly correlated with *Ct* for detection of G143 and A143 alleles, respectively (Fig. 3). The detection threshold for both assays was between 0.001 and 0.005 pg of plasmid target DNA,

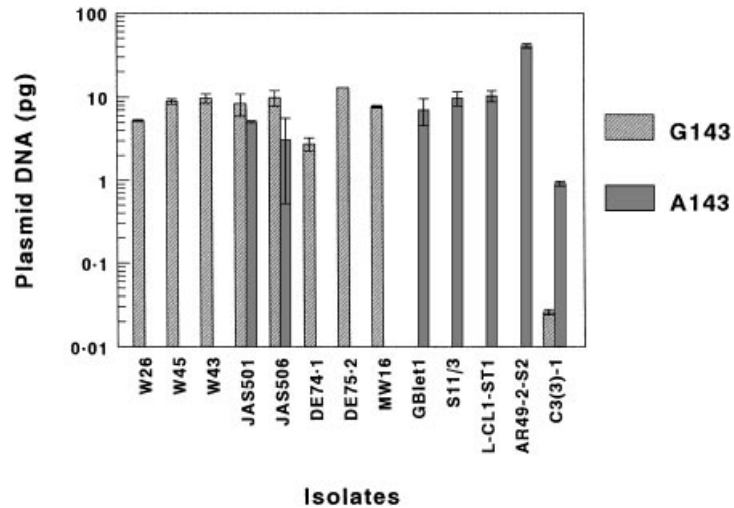


Figure 4 Amounts of A143 and G143 alleles in isolates of *Blumeria graminis* f.sp. *tritici*. Results expressed in pg plasmid DNA, containing either A143 or G143 alleles, using fluorescent allele-specific real-time PCR measurements. The average and standard errors of two measurements are given.

because wheat DNA, alone or spiked with less target plasmid DNA, could produce  $\Delta Rn$  signals with identical Ct and shape (Fig. 2). To exclude false-positive samples, only samples with a fluorescent signal equivalent to  $\geq 0.010$  pg of allele-specific plasmid target DNA were considered positive in this study. Primer-dimers and/or weak non-specific amplification of nontarget DNA visualized in gel electrophoresis with larger sample volumes (data not shown) could cause these signals. For detection of G143 and A143 alleles, false-positive nonallele-specific signals from PW26 and P501 could also be obtained when an excess of plasmid DNA  $> 500$  pg per assay was tested.

#### Detection of G143A in isolates of *Blumeria graminis* f.sp. *tritici* using fluorescent allele-specific real-time PCR assays

For all *B. graminis* f.sp. *tritici* isolates characterized in Tables 1 and 2, G143 and A143 alleles were detected and quantified using fluorimetric allele-specific real-time PCR measurements (Fig. 4). For most strobilurin-resistant isolates, the frequency of A143 alleles was 100%, while A143 alleles were not detected in sensitive isolates (Tables 1 and 2). A low level of G143 alleles was detected in the strobilurin-resistant isolate C3(3)-1, while JAS501 and JAS506, isolated and tested positive for strobilurin resistance in 1998, contained more G143 than A143 alleles (Fig. 4). However, the DNA of these isolates tested in PCR was obtained in July 1999, and bioassays performed in November 1999 showed that these isolates, which have been grown for more than 30 generations without selection pressure of fungicides, have become sensitive to strobilurins ( $ED_{50} < 0.05 \mu\text{g mL}^{-1}$ ).

#### Frequency of A143 alleles in field populations of *Blumeria graminis* f.sp. *tritici* before and after application of fungicides

For each population sampled, the amounts of G143 and A143 alleles were measured and the resulting frequencies

Table 4 Mildew foliar disease levels (% leaf area affected) in different plots at GS65. Four leaf layers were assessed separately

	Untreated	Tebuconazole-treated	Azoxystrobin-treated
Flag leaf	0.7	0.1	0.4
Leaf 2	2.3	0.2	1.4
Leaf 3	5.3	0.1	3.6
Leaf 4	7.7	0.2	4.6

of A143 alleles calculated (Fig. 5). The average frequency of the A143 allele in *B. graminis* f.sp. *tritici* populations from the untreated plot increased only slightly during the growing season, from 1.3 to 3.2% and, with the exception of sample 6(3), stayed well below 10%. The average frequency of the A143 allele in populations sampled from all plots before spraying was 2.2%. The A143 allele frequency increased under selection pressure from azoxystrobin, especially after the third spray, after which the average frequency was 58%. Additionally, for all populations sampled, the A143 allele frequencies were between 33 and 82%. Only in five out of 27 populations sampled throughout the season from the tebuconazole-treated plot was the frequency of the A143 allele higher than 10%. However, there was no clear relationship between the number of tebuconazole sprays and the increase in frequency of A143 alleles. Instead, the higher A143 allele frequencies obtained for some populations sampled from the tebuconazole-treated plot were probably due to sporadic immigration of strobilurin-resistant isolates from the adjacent azoxystrobin-treated plot. In comparison with the untreated plot, there was a greater probability of finding immigrants in the tebuconazole-treated plot, because most previously established mildew populations were eradicated (Table 4). The frequencies reflected mixed populations of strobilurin-resistant and -sensitive isolates, as sensitive and resistant isolates with high levels of G143 and A143 alleles, respectively, were isolated before and after application of fungicides (Table 2).

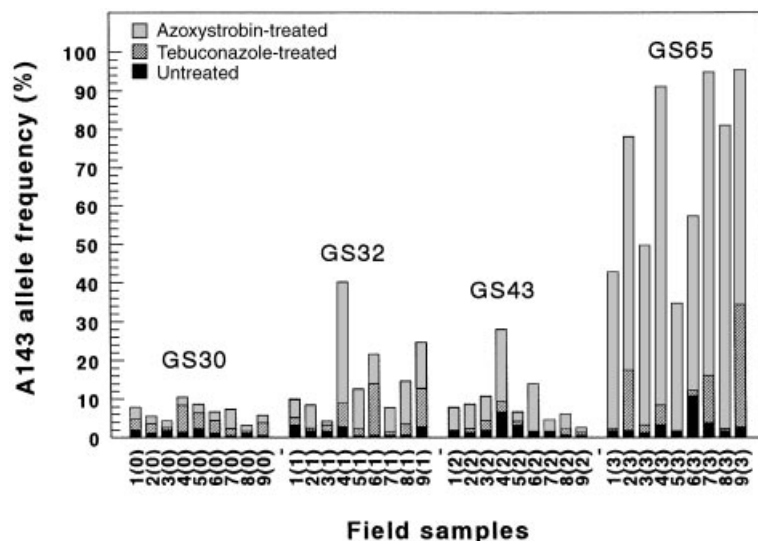


Figure 5 Monitoring strobilurin resistance associated with G143A in field populations of *Blumeria graminis* f.sp. *tritici* using fluorescent allele-specific real-time PCR measurements. On the x-axis, the first number represents the sample point, while the number of fungicide sprays is shown in parentheses.

## Discussion

In all 19 strobilurin-resistant isolates of *Blumeria graminis* f.sp. *tritici* tested (Tables 1 and 2), obtained from different locations, the mutation causing a G143A amino acid exchange in the mitochondrial cytochrome *b* gene was detected by sequencing or allele-specific PCR. So far, G143A has been found in all isolates with high resistance levels to strobilurins (Fraaije *et al.*, 2000; Sierotzki *et al.*, 2000b). Although different amino acid changes in the cytochrome *b* gene can confer QoI resistance, G143A is predominantly found in fungi sensitive to QoI inhibitors and probably causes resistance by affecting the inhibitor binding site (Kraiczky *et al.*, 1996).

Results of this study and earlier work (Fraaije *et al.*, 2000) suggest that a mixed heteroplasmic population of strobilurin-sensitive and resistant mitochondria can be present in a single isolate of *B. graminis* f.sp. *tritici*. This is plausible as cells typically contain hundreds of mitochondria with multiple genomes. In this study, most resistant isolates showed a dominant homoplasmic population of A143 alleles. Zheng *et al.* (2000) reported for *V. inaequalis* that cells with heteroplasmic cytochrome *b* alleles reverted rapidly to a more homoplasmic state, with mainly G143 alleles, without selection pressure of strobilurin fungicides. This indicates that a fitness penalty is associated with G143A in *V. inaequalis*. For *B. graminis* f.sp. *tritici*, the fitness penalty for G143A might be low, as competition studies with mixed populations of strobilurin-sensitive and -resistant isolates showed that the frequency of resistant spores was not affected over six generations (Heaney *et al.*, 2000). However, within a single isolate of *B. graminis* f.sp. *tritici* there might be a fitness penalty associated with G143A. For instance, in this study, single-spore isolates JAS501 and JAS506 reverted to the sensitive phenotype after being grown for more than 30 generations without selection pressure from strobilurins. With the possibility of mixed populations ruled out, this is probably a result of heteroplasmy of G143A or,

less likely, back mutation and subsequent selection. Long exposure time to strobilurins will probably rapidly select for homoplasmic A143 populations within cells through mitotic fusion, segregation and transmission, especially in the areas of greatest metabolic need (Yaffe, 1999). Mitochondria are most likely uniparental maternally inherited and, therefore, homoplasmic maternal cells will produce homoplasmic offspring. Without any fitness penalty associated with G143A, antiresistance strategies should aim to slow down resistance by decreasing the mitochondrial inheritance rate of the A143 allele by using alternating fungicides from different cross-resistance groups.

Quantitative real-time PCR assays using SYBR Green I (Hiratsuka *et al.*, 1999) and fluorescent probes, e.g. TaqMan assays (Lee *et al.*, 1993), molecular beacons (Täpp *et al.*, 2000) and Scorpions (Thelwell *et al.*, 2000), have been used successfully to detect mutations. The advantage of using fluorescent probes is that different alleles can be detected simultaneously. The fluorimetric allele-specific real-time PCR assay described in this paper is rapid, cost-effective and applicable for high-throughput routine testing. When using TaqMan probes to detect and quantify G143A in isolates and populations of *B. graminis* f.sp. *tritici*, similar results were obtained (B.A. Fraaije, unpublished data). Heteroplasmic G143A mutations can be detected at a frequency of at least 1 in 10 000, most likely sensitive enough to detect a point mutation in a mitochondrial genome within a single spore.

As shown in this study, allele-specific gene frequencies determined with PCR can improve the ability to study the evolution of fungicide resistance at the population level by directly monitoring the genotype. With optimal sampling procedures and clear phenotype-to-genotype relationships, detection threshold frequencies of resistant alleles might be identified, above which additional sprays of strobilurins alone, or in mixtures with a fungicide from a different cross-resistance group, would fail to control disease. Similar research could explore other pathogen-fungicide combinations, where practical resistance



problems have not yet emerged, but where early detection at low frequency of resistant alleles, coupled with risk evaluation, would allow implementation of antiresistance strategies in order to prolong the cost-effectiveness and lifetime of fungicides.

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