ALLELE-SPECIFIC REAL-TIME PCR FOR QUANTIFICATION AND DISCRIMINATION OF STEROL 14 α-DEMETHYLATION-INHIBITOR-RESISTANT GENOTYPES OF *MYCOSPHAERELLA GRAMINICOLA*

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SUMMARY

The level of resistance of *Mycosphaerella graminicola* to sterol 14 α-demethylation inhibitors (DMIs) is characterised by point and deletion mutations in the *CYP51* gene that encodes sterol 14 α-demethylase. Rapid and pre-symptomatic detection of these mutations is required for effective control by fungicides. In this study, an allele-specific real-time PCR method was developed. An additional mismatched nucleotide at the third position from the single nucleotide polymorphism at the 3-prime end of each allele-specific primer stops non-specific PCR amplification. Minor groove binding specific probes were designed to quantify general strains of *M. graminicola* and strains that contain isoleucine at position 381 of the CYP51 protein sequence. Specific amplification of the target alleles was reproducible. A high level of discrimination between genotypes using pure fungal DNA was confirmed *in vivo*, based on leaf samples collected from different wheat growing regions in France. A high level of I381V-genotypes (>70%) was found in all samples. The results showed that allele-specific real-time PCR allows pre-symptomatic and accurate quantitative detection of DMI-resistant genotypes of *M. graminicola*, with a shorter turnaround time compared to conventional methods. The simplicity and effectiveness provided by intentional mismatch primers offer a broad range of applications for laboratory and field analysis.

Key words: Allele-specific PCR, SNP, IMP, *Mycosphaerella graminicola*, 14 α–demethylation inhibitors

INTRODUCTION

Mycosphaerella graminicola (Fuckel) Schröter in Cohn (anamorph *Septoria tritici*) is the causal agent of septoria leaf blotch, which is regarded as a major disease of wheat because of its impact on wheat production worldwide. Epidemics can cause 30-40% losses in yield (Palmer and Skinner, 2002). Owing to the lack of highly resistant cultivars, fungicide application is currently the main form of disease management. Several families of fungicides can be used against *M. graminicola*, and amongst these, sterol 14 α-demethylation inhibitors (DMIs) have been key agents for the last 25 years and are still able to check disease development. Several European studies have shown significant shifts in the sensitivity of *M. graminicola* populations to DMIs in the last 20 years (Leroux *et al*., 2006, 2007). The molecular mechanisms responsible for DMI resistance in several important human and plant fungal pathogens have been well studied (de Waard, 1996; Lupetti *et al*., 2002) and four major mechanisms have been reported: overexpression of the *CYP51* gene encoding sterol 14 α-demethylase (Schnabel and Jones, 2000); decreased intracellular accumulation of DMIs (de Waard and Van Nistelrooy, 1980); mutations in *CYP51* resulting in reduced affinity of DMIs for their target (Sanglard *et al*., 1998; Marichal *et al*., 1999; Cools *et al*., 2005; Leroux *et al*., 2006, 2007); and inactivation of the sterol $\Delta^{5(6)}$ desaturase (Kelly *et al*., 1996). The latter is not associated with DMI resistance in field isolates of plant pathogens (Cools *et al*., 2002).

Recently, the sensitivity of *M. graminicola* populations to DMI fungicides was classified into different R-types that are associated with single nucleotide polymorphisms (SNPs) or amino acid deletions in *CYP51*. SNPs which lead to a glycine to aspartate (G460D) change at position 460, a tyrosine to phenylalanine (Y137F) change at position 137, and a valine to alanine (V136A) change at position 136 have been characterised as genotypes R2, R3 and R5 respectively. The R4 genotype is characterised by a mutation (Y461S/H) or ΔY459/G460, while genotypes R6, R7- and R7+ are characterised by an SNP that leads to the substitution of valine for isoleucine at position 381 (I381V), in combination with either a point mutation Y459S/D/N or Y461S/H (R6), or the double amino acid deletion ΔY459/G460 with the mutation A379G (R7+) or without A379G (R7-) (Leroux *et al*., 2006, 2007; Stammler *et al*., 2008a). The greatest differences in fungicide resistance levels have been observed among these *M. graminicola* genotypes. For example, isolates carrying

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I381V (R6, R7- and R7+) are less sensitive to tebuconazole but sensitive to prochloraz, whereas isolates carrying substitution V136A (R5) are most resistant to prochloraz (Leroux *et al*., 2007; Fraaije *et al*., 2007, 2008; Stammler *et al*., 2008b). The pre-symptomatic detection and quantification of SNPs that are related to different resistance levels in *M. graminicola* are very important for the effective control of strains that are highly resistant to DMIs, by allowing better timing, choice, and dose of fungicide applications. However, it is not possible to achieve these objectives with conventional methods, which cannot be applied until visible symptoms appear.

Real-time PCR is frequently used for DNA quantification and SNP discrimination (Qin and Yung, 2007). It involves only one setup step and does not require any post-reaction handling for genotype scoring (Chen and Sullivan, 2003). Allele-specific PCR using minor-groove-binding (MGB) probes has been developed by Fraaje *et al*. (2007) to detect and quantify *M. graminicola* wild type isolates (I381) or general genotypes carrying a mutation V381. However, probes designed to detect Y459/G460 and ΔY459/G460 alterations were not specific enough due to cross-reactivity, making determination of frequencies of these mutations in populations impossible (Fraaje *et al*., 2007). Therefore, current methods for determining the frequency of individual R-types of *M. graminicola* still depend on sequencing *CYP51* (Brunner *et al*., 2008; Stammler *et al*., 2008a). This assay is an enzymatic cascade involving multiple enzymes which increases the cost, time and complexity of the protocol (Chen and Sullivan, 2003).

PCR can be adapted for rapid detection of single-base changes in genomic DNA, by using specifically designed oligonucleotides (Sommer *et al*., 1989; Sarkar and Sommer, 1990). SNPs can be distinguished by using a specific primer in which the nucleotide at the 3' end is complementary to one allele, but forms a mismatch with the second allele (Solemani *et al*., 2003; Wilkins *et al*., 2006). However, the specificity of allele-specific PCR (AS-PCR) depends on two characteristics: (i) the ability of the mismatch to prevent extension of the primer by the polymerase; and (ii) the difference between the annealing temperature (T_m) for the binding of the primer to the perfect and mismatched sequences (Guo *et al*., 1997). For both of these reasons, a single 3' mismatch has limited discrimination. However, the use of proofreading polymerases and/or the deliberate incorporation of a mismatched nucleotide three bases in from the 3' end can increase the sensitivity of these SNP assays (Papp *et al*., 2003; Zhang *et al*., 2003; Wilkins *et al*., 2006). In this study, we evaluated the efficiency of the intentional mismatch primer (IMP) method to detect and quantify the different DMI-resistant genotypes of *M. graminicola* that infect wheat cultivars in France.

MATERIALS AND METHODS

Fungal isolates. Twenty-seven isolates of *M. graminicola* were obtained from our laboratory, ISA (Institut Supérieur d'Agriculture), Lille, France and from INRA (Institut National de la Recherche Agronomique), Versailles, France (Table 2). Sporidia (yeast-like cells) were activated by transfer to fresh potato dextrose agar medium (39 g l^{-1}; Sigma, USA). After 10 days of incubation at 18°C with a 12 h photoperiod, mycelia and spores were scraped off the surface and grown in liquid yeast–sucrose medium (yeast extract 10 g l^{-1}, sucrose 10 g l^{-1}; Sigma, USA) for 7 days at 18°C with a 12-h photoperiod and shaking (150 rpm). Mycelia and spores were collected by centrifugation at 8000*g* for 2 min, and washed twice with sterile distilled water.

Plant materials. Wheat (*Triticum aestivum* L.) leaf samples were provided by Bayer CropSciences (BCS, France). Leaf samples were collected from 14 localities in the major wheat growing regions of France during April, May, and June 2007 (Table 1). The wheat leaves carried a natural infection of *M. graminicola* and this produced symptoms identical to those of septoria leaf blotch. Each sample consisted of 40 leaves collected randomly from one foliar layer (F). Foliar layer number (Fn) was determined by counting the position of the leaf from the top of the plant. At least three repetitions of each cultivar were analysed. Leaves were stored at -80°C until lyophilisation.

DNA extraction. Fungal or leaf samples stored at –80°C were placed in liquid nitrogen before they were lyophilised in a Virtis 12 XL lyophiliser for 48 h. The dried samples were then ground using an MM 300 Mixer Mill (Qiagen, USA). DNA was extracted using the DNeasy 96 Plant kit (Qiagen, USA) according to the manufacturer's protocol. DNA was quantified by measurement of UV absorption at 260 nm (Cary 50 UV–Vis spectrophotometer; Varian, France). DNA samples were adjusted to 10 ng μl^{-1} before being used for real-time PCR.

SNP-specific primer design. All the *CYP51* EST sequences, in particular EF418622 (wild type), EF418624 (R2), EF418625 (R3), EF418623 (R4), EF418626 and EF418627 (R5), EF418628 and EF418629 (R6), and EF418630 (R7- and R7+), available from NCBI (http://www.ncbi.nlm.nih.gov/dbEST/index.html) were aligned by ClustalW (http://align.genome.jp/sit-bin/clustalw) and used for primer design. PCR primers were designed using Primer Express™ version 3 (Applied Biosystems, USA). Forward and reverse primers, MG-for and MG-rev, respectively (Fig. 1), were designed that bound to non-polymorphic sequences to quantify the general level of *M. graminicola* infection.

Allele-specific PCR primers (ASPs) were designed for each putative SNP in such a way that the last nucleotide at the 3' end of the forward primers was complementary to only one allele of the putative SNP. The efficiency of the IMPs was tested by intentionally adding a mismatch at the third nucleotide from the 3' end in all the ASPs. All the reverse primers had the same sequence as the wild-type, except that for R7-, which carried an intentional mismatch at the third nucleotide from its 3' end, to prevent the amplification of R6 strains. To prevent the amplification of R7 strains by the R6 primers, the R6 reverse primer was designed such that the last three nu-

Table 1. Leaf samples collected from wheat growing localities in France during April to June 2007.

Locality code	Locality	Cultivar	Collection date	Foliar layer
02	Courmont	Istabraq	20 April	F5
02	Coulonges	Dinosor	20 April	F5
21	Agencourt	Trémie	10 April	F5
21	Magny	Senkara	11 April	F4
37	Esvres	Autan	03 April	F5
37	Beaumont	Autan	16 April	F4
41	Suevres	Courtot	10 April	F4
45	Le Bardon	Nirvana	10 April	F4
47	St Pierre Sur Droft	Apache	10 April	F4
49	Soucelles	Orvantis	10 April	F5
51	Auberive	Aubusson	17 April	F5
51	Bouy	Cordiale	20 April	F5
51	Bisseuil	Cordiale	23 April	F5
54	Hageville	Charger	05 April	F6
60	Lavaquerie	Trémie	19 April	F5
62	Izel Les Hameaux	Cordiale	16 April	F4
62	Izel	Cordiale	16 April	F4
62	Wavrans/Aa	Cordiale	17 April	F4
76	Hautot	Charger	25 April	F5
76	St Sylvain	Shango	25 April	F5
80	Hangest	Cordiale	16 April	F5
86	Sammarcolles	Cezanne	05 April	F5
02	Courmont	Istabraq	12 June	F2
21	Agencourt	Trémie	21 May	F3
21	Magny/Tille	Senkara	22 May	F3
37	La Chapelle Blanche	Autan	04 June	F2
37	Ligueil	Autan	04 June	F2
37	Beaumont La Ronce	Autan	05 June	F1
47	St Pierre S/ Dropt	Apache	21 May	F2
49	Soucelles	Orvantis	22 May	F1
51	Auberive	Aubusson	04 June	F3
51	Bouy	Cordiale	14 June	F3
51	Bisseuil	Cordiale	19 June	F1
54	Hageville	Charger	23 May	F3
60	Lavaquerie	Trémie	05 June	F3
62	Izel Les Hameaux	Cordiale	04 June	F3
62	Wavrans/Aa	Cordiale	04 June	F3
62	Izel Les Hameaux	Cordiale	12 June	F3
76	Hautot-Le Vatois	Charger	13 June	F3
76	St Sylvain	Shango	13 June	F3
80	Hangest En	Cordiale	30 May	F2
80	Santerre	Cordiale	30 May	F2
86	Sammacolles	Cezanne	21 May	F2

cleotides at the 3' end corresponded to the position of the double amino acid deletion ΔY459/G460, which characterises R7 populations. IMPs were designed for all genotypes of *M. graminicola* classified by Leroux *et al*. (2007) except for that of R4, which shares individual alleles with R5, R6 and R7. In addition a primer that was specific for the point mutation A379G was designed using the same sequence related to R7 (GenBank accession No. EF418628). This ASP was considered to be an R(7+)-specific primer (Fig. 1).

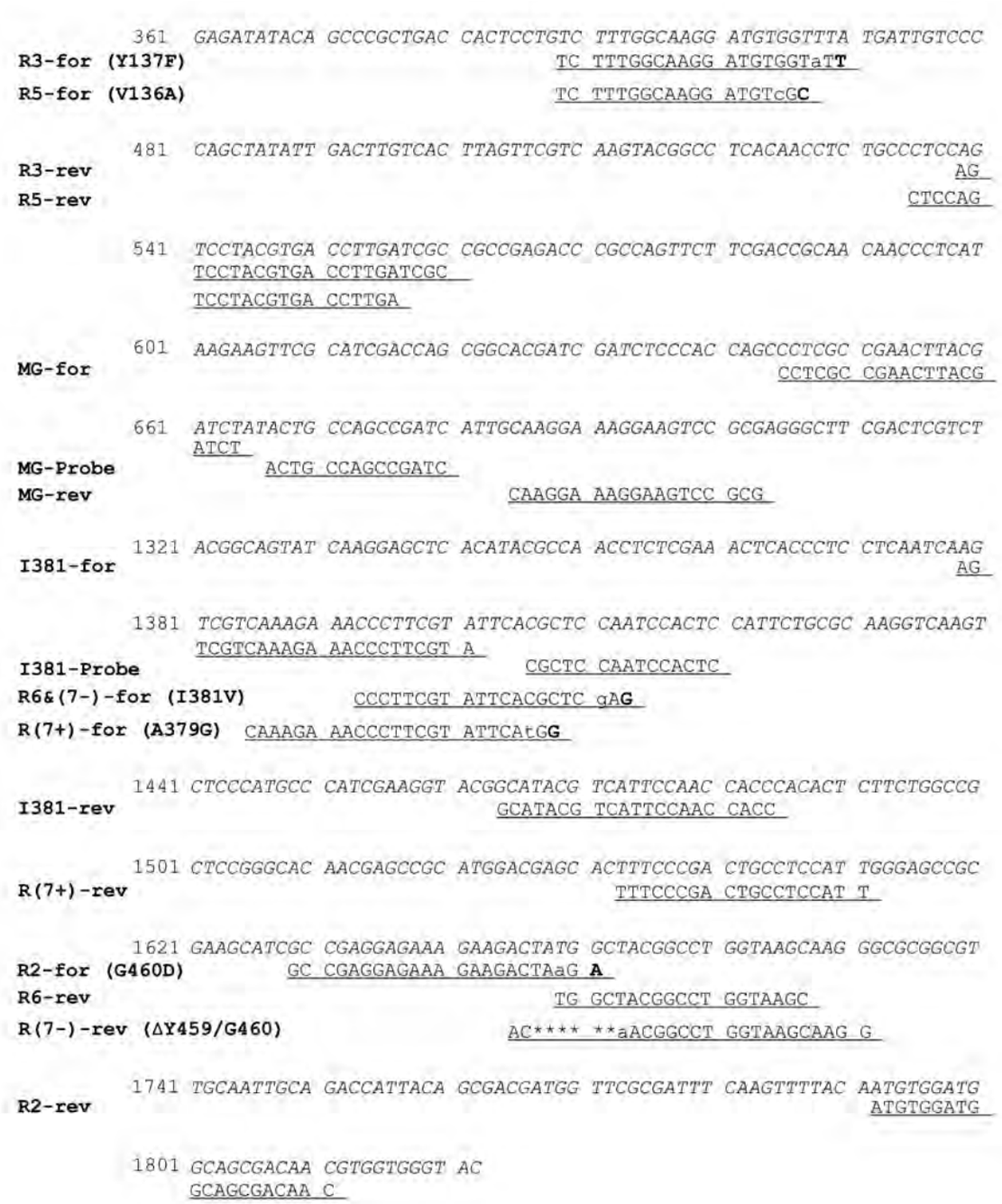

Fig. 1. Schematic alignment of the *CYP51* gene encoding *M. graminicola* sterol 14 α-demethylase. The bp numbering and italic nucleotides are those of the wild-type sequence (GenBank EF418622). The underlined sequences indicate the primers and probes which are shown in 5' to 3' orientation, except the reverse primers (rev) which are shown as the reverse complement. The forward primer (for) has the putative SNP at its 3' end. SNP sites are in bold. Lower case nucleotides are intentional mismatches. Asterisks represent the position of deleted nucleotides. I381 indicates primers or probe used to amplify I381- genotypes. MG indicates primers or probes used to quantify the general level of *M. graminicola* infection R2, R3, R5, R6, R7- and R7+ are DMI-resistant genotypes as described according to Leroux *et al*. (2006, 2007) and Stammler *et al*. (2008a).

Quantification of individual genotypes of *M. graminicola*. SYBR Green was used as the detection system for all of the SNP-specific primers for R2, R3, R5, R6, R7-, and R7+, as well as to quantify the general infection level of *M. graminicola* (Fig. 1). A reaction mixture of 25 µl was used, which contained 12.5 µl SYBR Green PCR master mix (Applied Biosystems, USA), 0.3 µM each primer, 50 ng of DNA, and water to a volume of 25 µl. The conditions for the quantitative PCR were as described by Selim *et al.* (2007).

Quantification of I381-genotypes of *M. graminicola*. A specific fluorescent 3' MGB probe for I381-genotypes (I381-probe) was designed to hybridise to isoleucine at position 381 of CYP51. To quantify the general infection level of *M. graminicola*, an MGB-specific probe (MG-probe) was designed that bound to the non-polymorphic region (Fig. 1). The 3' MGB DNA probes were also designed using Primer Express™ version 3 (Applied Biosystems, USA). The MG-probe and I381-probe were labelled at their 5' ends with the fluorescent dyes FAM (6-carboxyfluorescein) and VIC, respectively (Fig. 1). A TaqMan duplex assay was done in a reaction mixture of 25 µl that contained: 12.5 µl Universal TaqMan PCR Master Mix (Applied Biosystems, USA), 0.3 µM each primer, 0.2 µM each probe, 50 ng of DNA, and water up to a volume of 25 µl. The conditions of quantitative PCR were as follows: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. All quantitative PCRs of the *M. graminicola* genotypes was carried out using an ABI PRISM 7300 sequence detection system (Applied Biosystems, USA).

Primers and probes for the reference and mutant-specific real time PCR assays were designed to be used at the same hybridisation temperature. Thus, it was possible to run all AS-PCR reactions for the same sample on one plate to circumvent plate-to-plate variations.

Standard curves for each target were generated by plotting known amounts of DNA against C_t values. The resulting regression equations were used to calculate the amounts of DNA in test samples.

Statistical analyses. These were performed using JMP SAS-based software (JMP Start Statistics, SAS Institute, 2005). Student's *t* test was used to detect differences between sample collection dates of each allele-specific mutation. $P \leq 0.05$ was considered to indicate statistical significance.

RESULTS

Evaluation of AS-PCR efficiency *in vitro*. The specificity of each of the AS-PCR reactions was first tested using pure DNA samples from twenty-seven strains of *M. graminicola* (Table 2). Seven of them, K1882, 04-DJ1, 04-JL3B, 04-XT3, 04-DJ2, 04-XT1, and 04-XZ3 had been identified previously by Leroux *et al.* (2007) as wild type, R2, R3, R4, R5, R6, and R7 respectively, by sequencing the *CYP51* gene. These seven strains were used as positive controls. Samples of DNA from uninfected wheat leaves were used as negative controls.

A threshold cycle (C_t) was determined for each sample using the exponential growth phase and the baseline signal from fluorescence versus cycle number plots. For each SNP and each strain, C_t values were obtained (Table 2) and ΔC_t was determined by subtracting the average of C_t values of matched and mismatched amplification. In general, higher ΔC_t values reflected higher efficiency of AS-PCRs. The limit of the method was obvious when a PCR assay was run in the presence of only the mismatched nucleotide at the 3' end (ASP) with ΔC_t values between 2 to 14 cycles (data not shown), whereas the ΔC_t values obtained with IMP primers (Table 2) showed a high level of discrimination between specific and non-specific amplification with ΔC_t values of 18.00, 18.00, 18.39, 17.87, 17.08 and 18.30 for R2, R3, R5, R6, R7- and R7+ respectively. The efficiency of any IMP primers was not affected when using mixtures of different genotype DNA with different ratios (data not shown).

The I381-probe, which was designed to detect the isoleucine amino acid at position 381 of the *M. graminicola* CYP51 protein sequence to characterise wild type and all I381-genotypes, showed a very high level of discrimination between I381 and V381-genotypes, since no fluorescence emission was detected with all of the V381-genotypes (Table 2). Amplification was obtained over all strains tested with MG-primers (MG-for and MG-rev) using either SYBR Green as the fluorescent dye or using the MG-probe labelled with the FAM dye at its 5' end (Table 2 and Fig. 1).

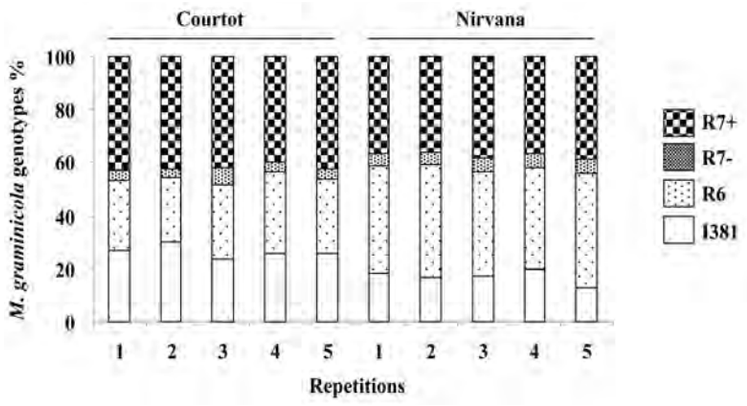

Fig. 2. Validation and reproducibility of SNP-specific IMPs for quantifying DMI-resistant genotypes of *M. graminicola*. Five replicates of wheat leaf samples collected from Courtot and Nirvana cultivars from Suevres and Le Bardon localities, respectively. Samples were collected from leaf layer four (F4) during the wheat growing season in April 2007.

Table 2. Validation of SNP-specific IMPs of each DMI-resistant *M. graminicola* genotype.

Strain number	Phenotypes	TaqMan		SYBR Green						
		MG	I381	MG	R2	R3	R5	R6	R7-	R7+
ST6	wt or R1 or R4	18.50	**20.48**	16.13	30.65	34.91	33.08	30.67	30.47	31.43
TO248	wt or R1 or R4	18.51	**19.43**	15.56	30.17	38.42	34.60	32.46	35.46	34.20
TO249	wt or R1 or R4	18.60	**20.00**	15.77	30.99	36.88	35.96	32.53	33.53	33.59
TO250	R3	18.53	20.62	16.34	30.97	**18.16**	34.44	30.21	31.40	33.18
TO253	R3	18.66	20.57	16.00	30.46	**18.83**	34.06	29.59	30.95	32.67
TO254	R6	18.57	ND	16.15	30.03	34.81	33.07	**18.03**	30.40	34.01
TO255	R3	18.62	20.56	16.43	30.36	**18.88**	34.54	28.98	32.96	32.05
TO256	R7-	18.58	ND	16.99	30.89	34.40	35.50	31.64	**17.21**	32.30
TO257	R7+	18.49	ND	17.11	30.08	34.29	34.76	31.97	31.02	**18.11**
TO304	R6	18.70	ND	16.00	30.42	39.00	33.34	**17.72**	30.83	31.94
TO305	R6	18.56	ND	16.20	30.46	37.97	33.39	**17.93**	30.03	32.05
TO306	R7+	18.54	ND	17.07	30.04	35.93	33.49	31.17	31.18	**18.23**
TO307	R6	18.52	ND	16.04	30.84	33.03	33.14	**17.57**	30.00	31.67
TO308	R7+	18.48	ND	16.93	30.30	33.98	33.06	30.99	30.47	**17.85**
TO312	R7-	18.55	ND	17.02	30.74	33.56	31.81	30.20	**17.02**	31.55
TO410	R7+	18.44	ND	17.12	30.52	36.10	34.05	30.60	30.48	**18.47**
TO411	R6	18.62	ND	16.20	30.40	35.44	33.26	**18.04**	30.44	30.58
TO412	R6	18.60	ND	15.90	30.45	35.85	34.75	**18.08**	30.32	32.40
TO413	R6	18.53	ND	16.34	30.50	35.06	33.76	**18.04**	30.44	33.02
TO417	R7+	18.52	ND	17.29	30.14	37.67	35.35	30.29	30.87	**19.05**
K1882	wt	18.54	**20.32**	16.00	31.00	34.60	34.50	31.02	35.40	33.70
04-DJ1	R2	18.60	20.00	16.30	**18.00**	35.90	33.50	30.57	34.80	33.12
04-JL3B	R3	18.59	20.86	16.33	30.60	**17.70**	33.00	30.15	31.70	32.14
04-XT3	R4	18.49	**20.00**	16.20	30.20	37.02	34.80	32.50	34.77	34.02
04-DJ2	R5	18.52	20.61	16.31	30.16	35.90	**17.00**	32.48	35.00	33.95
04-XT1	R6	18.50	ND	16.15	30.15	36.70	34.00	**17.51**	35.65	34.03
04-XZ3	R7-	18.60	ND	16.00	30.00	39.01	33.90	32.20	**17.01**	33.28
T. *aestivum*		ND	ND	ND	ND	ND	ND	ND	ND	ND
ΔC_t^*			>40		18.00	18.00	18.39	17.87	17.08	18.30

* ΔC_t, difference between average C_t of PCR with matched and mismatched DNA.

Strain numbers in bold represent wild type (wt), R2, R3, R5, R6 and R7 are *M. graminicola* genotypes as published by Leroux *et al*. (2007). TaqMan represents MGB-specific probes used to quantify general strains of *M. graminicola* (MG) or I381-genotypes (I381), respectively. C_ts of specific amplification are in bold.

Validation of AS-PCR efficiency *in vivo*. To validate the efficiency of IMP primers tested with *M. graminicola* pure DNA, it was necessary to confirm that they functioned correctly with strains infecting wheat *in vivo*. Wheat leaf samples collected from fourteen localities in France, with or without visible symptoms, were analysed to determine the infection level of *M. graminicola* genotypes (I381, R6, R7- and R7+). The difference between general estimation of *M. graminicola* genotypes (MG) and (I381) was expressed as V381-genotypes.

Reproducibility of the intentional mismatch primers or I381-probe was evaluated using five repetitions from the same field on two wheat cultivars, Courtot and Nirvana, which were collected during April 2007 from the same foliar layer (F4), from two localities in France, Suevres and Le Bardon, respectively. Figure 2 shows constant results over all repetitions for R6, R7-, R7+ and I381-genotypes with the average of 28/41, 4/5, 42/37 and 26/17 % in the two localities (Suevres/Le Bardon), respectively.

The general infection level of *M. graminicola* was estimated in 50 ng of DNA extracted from leaf samples collected from foliar layer F4 to F6 during April, and from the top three layers (F1 to F3) during May and June, with an average of 3169 and 2002 pg, respectively. Results of all samples collected from the fourteen localities during April to June showed that the frequency of V381-genotypes was often greater than 70% with high frequencies of R6 and R7+ (Fig. 3). No significant differences were observed between the percentages of R6,

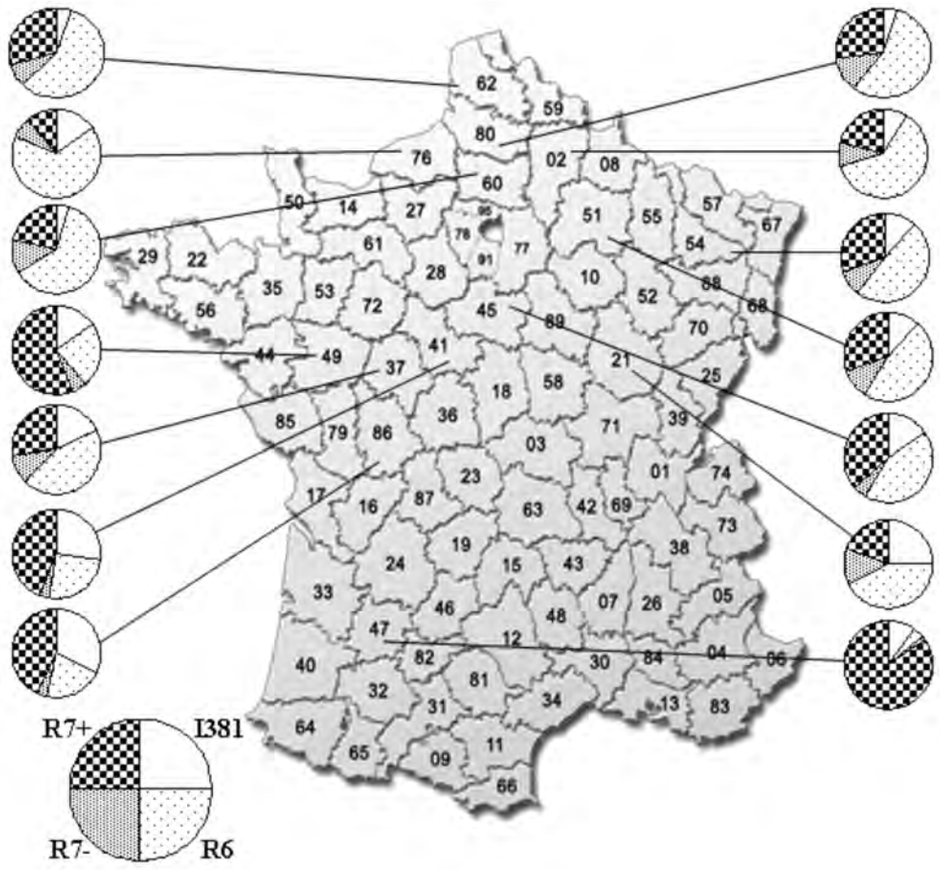

Fig. 3. Average frequency of DMI-resistant genotypes of *M. graminicola* during April, May and June of the 2007 season. Leaf samples were collected from the major French wheat growing regions.

R7-, R7+ and I381-genotypes over the two sampling dates with an average of 46/45, 9/9, 31/30 and 14/15 % in April/May and June respectively (Fig. 4).

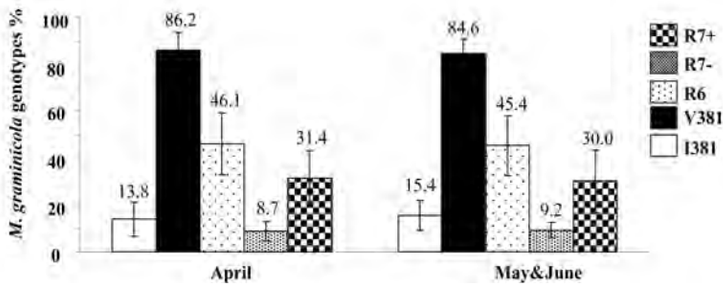

Fig. 4. Average frequency of V381 individual genotypes (R6, R7-, R7+) of *M. graminicola* in leaf samples collected during April and May to June of the 2007 season. Bars indicate standard deviation.

DISCUSSION

Currently, control of *M. graminicola* relies mainly on fungicide application. DMIs have been major agents against *M. graminicola* over the past 25 years, but recently significant resistance to DMIs has been found (Mavrolidi and Shaw, 2005; Leroux *et al*., 2006, 2007). Sequence analysis of *CYP51* (encoding *M. graminicola* sterol 14 α–demethylase) has shown that DMI resistance is associated with amino acid alterations and/or SNPs (Cools *et al*., 2002; Leroux *et al*., 2006, 2007; Stammler *et al*., 2008a, 2008b). Conventionally, methods used to detect DMI-resistant strains have been based mainly on the evaluation of mature pycnidia isolated from necrotic lesions such as germ-tube elongation tests (Leroux *et al*., 2007) and microtitre assays (Stammler *et al*., 2008b). However, these methods cannot be applied until visible symptoms appear. In addition, they usually require a degree of taxonomic expertise to distinguish symptoms caused by *M. graminicola* leaf blotch from those of other foliar diseases and abiotic factors. The accuracy and reliability of these methods therefore depends largely on the experience and skill of the person performing the test (McCartney *et al*., 2003). Accurate detection and identification of these mutations in the early stages of epidemics will be useful for disease control through better timing, choice and dosage of fungicide applications. For example, V381-genotypes are equally or more sensitive to prochloraz than V136A-

genotypes (Fraaije *et al*., 2007; Leroux *et al*., 2007). Although an allele-specific PCR using MGB-probes to determine general frequency of I381 and V381 genotypes of *M. graminicola* has been reported (Fraaje *et al*., 2007), this method cannot be used to determine the frequency of individual R-types.

In the present study, real-time AS-PCR was developed to quantify the individual genotypes of *M. graminicola* that were sensitive or resistant to DMIs. This approach is particularly suitable for the estimation of very low SNP allele frequencies in large populations of genotypes (Schwarz *et al*., 2004). In theory, chain extension during PCR will only occur if the 3' nucleotide is complementary to the template nucleotide at the SNP locus. ASPs that only contain an SNP at the 3' end of the forward primers had little effect on the discrimination between the different *M. graminicola* populations. Normally, the difference in binding affinity between a perfectly matched target DNA and one with a mismatched base is too small to allow complete discrimination (Taton *et al*., 2000). In addition, an efficient yield of primer-extended products from 3' mismatched primers, using DNA polymerase that lacks proofreading function, has been reported (Zhang *et al*., 2003). However, the discrimination of wild-type versus mutant sequences can be improved by using allele-specific primers with artificially mismatched bases in the 3'-terminal regions (Glaab and Skopek, 1999; Bates and Taylor, 2001). Therefore, all ASPs in this study were modified by adding an intentional mismatch at the third nucleotide from their 3' ends.

The IMPs tested with different isolates of *M. graminicola* were found to amplify their targets consistently and clearly. The potential of the IMP method has been discussed (Okimoto and Dodgson, 1996; Papp *et al*., 2003; Wilkins *et al*., 2006). Moreover, allele-specific SNP discrimination was enhanced by using a specific MGB probe that was designed to detect isoleucine at position 381 of CYP51, which characterises wild type and R1 to R5 genotypes of *M. graminicola*. The I381-probe in the current study is different from that designed by Fraaje *et al*. (2007) in which the polymorphic site was placed approximately in the middle third of the probe, and also the probe was designed to contain more cytosines than guanines to avoid quenching of the fluorophore which can increase if more guanines are present (Seidel *et al*., 1996).

It was not possible to design the V381-specific probe on the same strand of DNA that was used to design the I381-probe because the point mutation responsible for the amino acid change A379G is just five nucleotides downstream of the SNP that is related to the mutation I381V. However, high specificity of the MG-probe in detecting general populations of *M. graminicola* was obtained. The duplex TaqMan assays using I381 and MG probes for the same DNA sample allowed quantifying the V381-genotypes from the difference between them.

The robustness of the AS-PCRs was confirmed using wheat leaf samples from different wheat growing regions in France. The results showed that *M. graminicola* genotypes could be scored clearly and with a high level of reproducibility. In the 14 wheat-growing regions in the present study, the frequency of V381-genotypes was >70%, which agrees with the frequency of >50% reported in previous surveys carried out in 2005, 2006, 2007 and 2008 (Leroux *et al*., 2006; Cousin and Moronval, 2008; Brunner *et al*., 2008; Stammler *et al*., 2008a). The current status seems to be the result of a changing population over the last two decades. The high frequency of amino acid substitution I381V in combination with other mutations such as A379G and ΔY459/G460 supports the hypothesis that some combinations of *CYP51* mutations have evolved through intragenic recombination and have subsequently reached high frequencies due to reduced azole sensitivity compared with the parental haplotypes (Brunner *et al*., 2008).

In conclusion, the studies of Leroux and colleagues in 2006 and 2007 have allowed the development of allele-specific primers that are related to different DMI-resistant genotypes of *M. graminicola*. This method is simple, rapid (approximately 3 h), inexpensive and reproducible. Moreover, early and pre-symptomatic detection and quantification of DMI-resistant genotypes of *M. graminicola*, provided by IMP primers, offers a broad range of applications for laboratory and field analysis, e.g., early detection of mutants in populations under natural selection pressure and estimation of the mutation frequency during fungicide application.

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