Journal homepage www.jzs.univsul.edu.iq
Journal of Zankoy Sulaimani
Part-A- (Pure and Applied Sciences)

Alterations in *CYP51* gene associated with triazole fungicide performance towards the causative agent of leaf blotch of wheat *Mycosphaerella graminicola**

Qasim Abdulla Marzani[1]& Stephen Rossall[2]

[1]*Department of Plant Protection, College of Agriculture, Salahaddin University - Erbil, Southern Region of Kurdistan - Iraq.*
[2]*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK.*
qasim.marzani@gmail.com

Article info

Original: 27/12/2017
Revised: 31/01/2018
Accepted: 07/02/2018
Published online:

Key Words:

CYP51 gene, wheat blotch, Mycosphaerella graminicola, fungicide performance.

Abstract

To explore mutations in the azole performance related *CYP51* gene of *Mycosphaerella graminicola* causes Septoria tritici blotch (STB) in wheat , 18 isolates of *M. graminicola* were collected from wheat fields received from Germany, Scotland and England. The gene was amplified by PCR using four primer sets. Each primer was designed to amplify a part of the gene ranging from 555 to 622 bp. The whole *CYP51* gene amplicon sequences of the isolates were aligned against the *CYP51* of the wild type isolate IPO323 and analyzed using BioEdit software. Fifteen nucleotide sequence alterations were reported in recent UK and German isolates. Some of these mutations were found to be rare, like Y137F, whilst the I381V mutation was found to be increasing with time.

*This work is part of the PhD thesis "*Fungicide resistance and efficacy for control of Pyrenophora teres and Mycosphaerella graminicola on barley and wheat*"

Introduction

Wheat is a grass which belongs to the plant family Poaceae and is native to arid countries of western Asia [1]. The first primitive wheat was harvested and cultivated in the so-called Fertile Crescent of south-western Iran, north-eastern Iraq, and south-eastern Turkey (Kurdistan). Wheat is being grown in some 100 countries around the world now. Wheat is the most popular crop which is widely grown, traded and consumed worldwide [2]. Although, since the green revolution, global cereal yields have increased dramatically. The yield still not at the required level to satisfy the global requirement [3]. Furthermore, the demand for wheat is growing faster than any other crop, due to the ever-increasing of global population.

Cereals are vulnerable to many biotic attacks including those by fungi, bacteria, viruses, nematodes and insects. Considerable reductions in grain yield and quality results from the damage they generate. The major threats to human food and crop production, since agriculture became the main source of human food supply, are from yield losses caused by plant pathogens.

Coalescence analysis of pathogen DNA sequence data indicates that *Mycosphaerella graminicola* (Fuckel) J. Schorot. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.), is an important pathogen of wheat worldwide, emerged about 10500 years ago during the domestication of wheat in the Fertile Crescent of the Middle East, from an ancestral population which still exists and has a wide host range [4].

Septoria tritici blotch (STB) caused by *M. graminicola* is the most worldwide economically important foliar disease of wheat [5], [6], and [7]. Plant diseases are managed through practicing cultural methods, host resistance and the use of fungicide applications. Due to the lack of highly resistant cultivars, the use of chemicals remains an essential tool to increase yield in crops in spite of the raise of environmental concerns [8].

Site-specific fungicides, such as methyl-benzimidazole carbamate (MBC), sterol demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors have been very effective. Due to target-site mutations, resistance has developed to both MBCs and QoI fungicides. Practical fungicide resistance began to occur shortly after the introduction of single-site fungicides. Incidences of resistance to important diseases have been well-documented [9]. Fungicide resistance has been found in many pathogens and in different groups of fungicides. Reduced sensitivity to DMIs was reported by 1994 for at least 13 plant pathogens. In most cases the resistance was polygenic, although in some cases was monogenic [10]. A clear erosion in triazole efficacy against *M. graminicola* has shown that higher doses are now required to achieve effective disease control [11]. In plant pathogens, three major mechanisms of resistance to DMIs have been reported. These include: 1) point mutations in the CYP51 gene encoding the sterol 14α-demethylase that result in reduced affinity of DMIs for their target ; 2) over-expression of the CYP51 gene, resulting in elevated levels of CYP51p; and 3) reduced accumulation of DMIs in fungal cells through up-regulation of active efflux proteins. The latter mechanism involves ABC (ATP-binding cassette) transporters or major facilitators and can mediate multidrug resistance to various classes of fungicides. In European countries, DMI resistance resulted mostly from changes in the CYP51 gene, at least until 2007 [12]. To date, 22 different amino acid alterations (substitutions and deletions) have been detected in the CYP51 gene in *M. graminicola* populations in Western Europe [13]; [14]; [15]; and [16]. Previous studies indicated the existence of 8 categories of *M. graminicola* strains (TriR1-TriR8) displaying reduced sensitivity to DMIs [14]; [17]; and [18]. These different resistance types (R-types) are associated with either single or combinations of single nucleotide polymorphism (SNPs) or amino acid deletions in the CYP51 gene. Changes from glycine to aspartate (G460D/S) at position 460, a tyrosine to phenylalanine (Y137F) at position 137, and valine to alanine (V136A) at position 136 have been described as R2, R3, and R5 phenotypes, respectively. The R4 genotype is characterised by a mutation Y461S/H or ΔY459/G460, while genotypes R6, R7- and R7+ are characterised by a SNP that leads to 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-) [14]. The mutations V136A and I381V occur only in combinations with mutations or a deletion of the amino acids tyrosine or glycine in the YGYG region (positions 459–461), while mutations or the YG-deletion at 459–462 could also occur as a single event [15]. There are also other single mutations such as D107V, D134G, S524T or combinations of them (V136A + I381V or I381V without a mutation at 459–462) described by [15] for the first time in isolates of *M. graminicola* and have never been detected before in the CYP51 gene. However, these classifications, with the new emerging mutations, have been modified recently by the Leroux group to include more R-groups ranging from R1-R12 [12].

This research aims to isolate a collection of *M. graminicola* strains from infected wheat leaves, derived mainly from the UK and Germany, to detect alterations in the CYP51 gene, encoding the sterol 14α - demethylase target for triazole fungicides and for comparison with some older stock isolates.

Materials and methods

Mycosphaerella graminicola isolates

Wheat leaves, infected with leaf blotch, were received from wheat fields in England, Scotland, and Germany. The leaves were surface sterilized with an 8% Domestos solution (Domestos®, Johnson Diversy Ltd., Northampton, UK)**,** to give a sodium hypochlorite concentration of 0.5 %, for 5 minutes, washed three times with sterile distilled water and then dried with sterile filter papers. Leaf segments were attached (pycnidia facing up) to glass slides with the aid of Vaseline, then placed in a sterile damp chamber for 24 h. Conidia

oozing from pycnidia were picked up using a fine point glass needle and then transferred to fresh PDA, amended with antibiotics (penicillin 30 mg L^{-1} and streptomycin 133 mg L^{-1}), by streaking the surface of medium with the inocula. The inoculated plates were incubated at 20° C for 3-5 d. Colonies were purified on the same medium and three isolates, each from a separate leaf, were chosen from each region. Isolates were consecutively numbered and further sub-cultured for the purpose of making spore suspensions and preserving in glycerol stock cultures for long-term cold storage at -80° C. Older isolates, from previous years, were also included in the study as reference strains. All *M. graminicola* isolates used in this study are described in Table1.

Culture preservation

To detect the *CYP51* mutations in *M. graminicola* isolates, PCR-based methods were used. Fungal isolates were grown in 30 ml of potato dextrose broth (PDB), placed in 100 ml conical flasks. The inoculated liquid cultures were incubated in a controlled environment incubator shaker (New Brunswick Scientific, Edison, USA) at 20°C for 2-3 weeks depending on the isolate. The resultant mycelia were placed in Falcon tubes, centrifuged at 2065 g for 5 min, washed twice with water and then placed in a freezer at -80° C. Fungal samples were placed in liquid nitrogen and then freeze-dried for 48 h (Christ-Alpha 2-4 LD, Germany).

Detection of CYP51 mutations

DNA extraction

DNA extraction was performed following this procedure: 20 mg of freeze-¬dried mycelium was taken from each isolate, placed in microtubes (2 ml screw cap tubes) with 0.5 g of 2 mm glass beads and then placed in liquid nitrogen for 30 seconds. To disrupt the fungal tissue, the tubes were placed in a tissue-lyser (FastPrepTM FP 120, Thermo Electron) and run at the highest speed (6.5 Hz) for 40 seconds. The fungal DNA was then extracted following the manufacturer's protocol for the extraction kit (DNeasy® Plant Mini Kit (50), QIAGEN, GmbH) and quantified using a NanoDrop® Nd-1000 spectrophotometer (Thermo Scientific).

DNA amplification (PCR)

Four distinct PCR reactions were performed to amplify the CYP51 gene by using four primer sets (synthesized by Eurofins, UK) designed and used by [14], each primer was designed to amplify a part of the gene ranging from 555 to 622 bp, to make PCR products overlapping each other (Table 2). At the beginning of the CYP51 gene, an additional upward 200 bp sequence was amplified with CYP1 and CYP2 primers. Amplifications were performed in a total volume of 25 µL which consisted of 0.4 µM of each primer, 0.2 mM dNTPs, 1x GoTaq PCR reaction buffer (Promega, Madison, USA), 1.5 mM $MgCl_2$, 0.5 U DNA polymerase (GoTaq® Flexi DNA Polymerase, Promega). PCR was performed in Flexigene cycler using the following conditions: initial preheat for 2 min at 95°C, followed by 37 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min followed by a final step 72°C for 15 min. Amplified DNA fragments were resolved and visualized on a 1.5% agarose gel. The gel was prepared with 1x TAE buffer and ethidium bromide was added for a final concentration of 0.5 µg mL^{-1}. Four microliters of each PCR product was loaded into the gel well alongside 4 µL of a DNA size marker (100 bp ladder). Electrophoresis was performed at 90 V for 60 to 80min, after which it was visualised under ultra violet (UV) illumination and photographs taken.

Table -1: *Mycosphaerella graminicola* isolates used in this study

No	Isolate	Origin	Fungicide history	Sensitivity to QoIs
1	Tibb-2	Tibbermore, Scotland	Untreated	Unknown
2	Nuf-Un-2	Nufarm-England	Untreated	Unknown
3	Nuf-Pz-2	Nufarm-England	Prochloraz	Unknown
4	Roy-Un-2	Royston-England	Untreated	Unknown
5	King-Un-2	Devon-England	Untreated	Unknown
6	King-Pz-2	Devon-England	Prochloraz	Unknown
7	Skedd-2	Fife-Scotland	Untreated	Unknown
8	Head-2	Headly Hall, Yorkshire	Untreated	Unknown
9	Ger-3-2	Germany	Unknown	Unknown
10	Ger-4-2	Barlt-Germany	Unknown	Unknown
11	Pittend	Kinross-Scotland	Untreated	Unknown
12	Ire-3	Ireland	Untreated	Wild type
13	HA-3	Harper Adams	Unknown	G143A
14	G303	Rothamsted (Herts)	Treated	G143A
15	Roy-Pz-1	Royston-England	Prochloraz	Unknown
16	S331	Loughborough	Unknown	Wild type
17	Ctrl-1	Rothamsted (Herts)	Untreated	Wild type
18	Lars-37	Somerset	Untreated	G143A

Gene sequencing

To detect the differences, the PCR products were sequenced (Eurofins, UK), the four sequence parts of the gene were then gathered and then the whole sequence of the CYP51 gene for each isolate were aligned beside the sequence of the CYP51 gene of wild type isolate IPO323 and analysed using BioEdit software (Biological sequence alignment editor, version 7.0.9).

Table -2: Primers used to amplify the four parts of CYP51 gene in *M. graminicola*

Primer name	Primer direction	Sequence(5'-3')	Product length	Tm(C)
CYP1(F)	Forward	GAAACAGCGTGTGTGAGAGC	564	59.4
CYP2(R)	Reverse	GCGTTGACGTCCTTCAGTTT		57.3
CYP3(F)	Forward	CTGCTGGGAAAGAAGACGAC	555	59.4
CYP4(R)	Reverse	TCTTCTTCTGCGCATAATCG		55.3
CYP5(F)	Forward	GGGATTCACACCGATCAACT	614	57.3
CYP6(R)	Reverse	AGTTTCGAGAGGTTGGCGTA		57.3
A(F)	Forward	CACTCTTCATCTGCGACCGAGTC	622	64.2
B(R)	Reverse	CTGCTGTAATCCGTACCCACCAC		64.2

Results and discussions

Detection of CYP51 mutations

Sequencing the CYP51 gene, encoding the sterol 14α-demethylase target for triazole fungicides, identified several point mutations within 18 *M. graminicola* isolates. These mutations included SNPs and amino acid deletions. Amino acid changes were at positions 24 (valine to aspartate) in isolate S331, at position 50 (leucine to serine) in 15 isolates, change serine to tyrosine at position 51 also in isolate S331, and 9 isolates had changes from serine (S) to asparagine (N) at position 188. At the position 379, the change from alanine (A) to glycine (G) was observed in 5 isolates, the change from isoleucine (I) to valine (V) at position 381 dominated the changes, combined with other changes and deletions at positions 459, 460, 461, and 513 (Figure 1).

The overall SNP changes and deletions occurring in the CYP51 gene for each isolate are shown in Table 3. Based on the changes and deletions of this study and by referring to the previous classifications of Leroux group [12] which were based on genotyping and *in vitro* phenotyping, isolates can be categorised into 9 variants, which express phenotypic variation in sensitivity to triazoles. Sensitive isolates (S) included those with no important mutations mentioned previously by other researchers (included isolate S331 only). The Y137F mutation was found in one isolate only (isolate Ctrl-1) and therefore supposed to be classified as R3 genotype. However, the S524T mutation although not detected in this study because the primers used to amplify the CYP51 gene did not extend to cover the 524 position of the gene, previous sequence results carried out by [11] confirm the existence of this mutation in this isolate. Therefore with the existence of the S524T mutation, a new name (R3+) had to be given to this variant. The R4a variants included isolates with Y461H (isolate Ire-3) but when combined with V136C was given a different name as R4a+(isolate Roy-Pz-1). The combination of Y461H with V136A considered R5a variant included 2 isolates (Skedd-2 and Lars-37) while the latter mutation when combine with the Y459/G460 deletion is characterised as R5b variant (isolate Nuf-Pz-2).

The R6a variants are characterised by the combination of the I381V with the mutations at positions 459 or 461. This includes isolate Nuf-Un-2, Roy-Un-2 and King-Pz-2. The R7 group represents isolates with the I381V mutation combined with the double deletions at 459 and 460 positions of the CYP51 gene. The latter variant when combine with the A379G mutation is characterised as R8 variant (Table 3).

```
                    10        20        30        40        50        60        70        80        90       100
                    |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
IPO323 CYP51        MGLLQEVLAQFDAQFGQTSLWKLVGLGFLAFSTLAILLNVLSQLLFRGKLSDPPLVFHWVPFIGSTITYGIDPYKFFFSCREKYGDVFTFILLGKKTTVC
1-Tibb-2            ..................................................S.................................................
2-Nuf-Un-2          ..................................................S.................................................
3-Nuf-Pz-2          ..................................................S.................................................
4-Roy-Un-2          ..................................................S.................................................
5-King-Un-2         ..................................................S.................................................
6-King-Pz-2         ..................................................S.................................................
7-Skedd-2           ..................................................S.................................................
8-Head-2            ..................................................S.................................................
9-Ger-3-2           ..................................................S.................................................
10-Ger-4-2          ..................................................S.................................................
11-Pittend          ..................................................S.................................................
12-Ire-3            ..................................................S.................................................
13-HA3              ..................................................S.................................................
14-G303             ..................................................S.................................................
15-Roy-Pz-1         ..................................................S.................................................
16-S331             ......................D...........................T.................................................
17-Ctrl-1           ..................................................S.................................................
18-Lars-37          ..................................................S.................................................

                    120       130       140       150       160       170       180       190       200
                    .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
IPO323 CYP51        LKDVNAEEIYSPLTTPVFGKDVVYDCPNSKLMEQKKFVKYGLTTSALQSYVTLIAAETRQFFDRNNPHKKFASTSGTIDLPPALAELTIYTASRS
1-Tibb-2            ..................................................................N.....................
2-Nuf-Un-2          ..................................................................N.....................
3-Nuf-Pz-2          ......................A...........................................N.....................
4-Roy-Un-2          ..................................................................N.....................
5-King-Un-2         ..................................................................N.....................
6-King-Pz-2         ..................................................................N.....................
7-Skedd-2           ......................A...........................................N.....................
8-Head-2            ..................................................................N.....................
9-Ger-3-2           ..................................................................N.....................
10-Ger-4-2          ..................................................................N.....................
11-Pittend          ..................................................................N.....................
12-Ire-3            ..................................................................N.....................
13-HA3              ..................................................................N.....................
14-G303             ..................................................................N.....................
15-Roy-Pz-1         ......................C...........................................N.....................
16-S331             ..................................................................N.....................
17-Ctrl-1           ......................F...........................................N.....................
18-Lars-37          ......................A...........................................N.....................

                    220       230       240       250       260       270       280
                    .|....|....|....|....|....|....|....|....|....|....|....|....|..
IPO323 CYP51        VREGFDSSFADLYHYLDMGFTPINFMLPWAPLPQNRRRDYAQKKMSETYMSIIQKRRESKTGEHEEDSK
1-Tibb-2            ..........................................................
2-Nuf-Un-2          ..........................................................
3-Nuf-Pz-2          ..........................................................
4-Roy-Un-2          ..........................................................
5-King-Un-2         ..........................................................
6-King-Pz-2         ..........................................................
7-Skedd-2           ..........................................................
8-Head-2            ..........................................................
9-Ger-3-2           ..........................................................
10-Ger-4-2          ..........................................................
11-Pittend          ..........................................................
12-Ire-3            ..........................................................
13-HA3              ..........................................................
14-G303             ..........................................................
15-Roy-Pz-1         ..........................................................
16-S331             ..........................................................
17-Ctrl-1           ..........................................................
18-Lars-37          ..........................................................
```

Figure -1: Amino acid sequences of the CYP51 gene of 18 *M. graminicola* isolates aligned with the wild type isolate IPO323 (continued in next page).

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             280       290       300       310       320       330       340       350       360
              |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
IPO323 CYP51  DSKGANTRTAMPFPTRRLLILRCSWPASTLHLRPSPGSLSASHPAPTSKTNSSKNKRICSVTPTAVSRSSHTPTSRNSPSSIKSSKKPFV
1-Tibb-2      ....................................................................................
2-Nuf-Un-2    ....................................................................................
3-Nuf-Pz-2    ....................................................................................
4-Roy-Un-2    ....................................................................................
5-King-Un-2   ....................................................................................
6-King-Pz-2   ....................................................................................
7-Skedd-2     ....................................................................................
8-Head-2      ....................................................................................
9-Ger-3-2     ....................................................................................
10-Ger-4-2    ....................................................................................
11-Pittend    ....................................................................................
12-Ire-3      ....................................................................................
13-HA3        ....................................................................................
14-G303       ....................................................................................
15-Roy-Pz-1   ....................................................................................
16-S331       ....................................................................................
17-Ctrl-1     ....................................................................................
18-Lars37     ....................................................................................

                  370       380       390       400       410       420       430       440       450       460
                  .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
IPO323 CYP51  SKKPFVVVKETLRIHAPIHSILRKVKSPMPIEGTAYVIPTTHTLLAAPGTTSRMDEHFPDCLHWEPHRWDESPSEKYKHLSPTTALGSIAEEKEDYGYGL
1-Tibb-2      ................G.V.....................................................................--...
2-Nuf-Un-2    ..................V.....................................................................H..
3-Nuf-Pz-2    ....................................................................................--...
4-Roy-Un-2    ..................V.....................................................................H..
5-King-Un-2   ................G.V.....................................................................--...
6-King-Pz-2   ..................V.....................................................................S....
7-Skedd-2     ....................................................................................S..
8-Head-2      ................G.V.....................................................................--...
9-Ger-3-2     ..................V.....................................................................--...
10-Ger-4-2    ................G.V.....................................................................--...
11-Pittend    ................G.V.....................................................................--...
12-Ire-3      ....................................................................................S..
13-HA3        ..................V.....................................................................--...
14-G303       ..................V.....................................................................--...
15-Roy-Pz-1   ....................................................................................H..
16-S331       ....................................................................................
17-Ctrl-1     ....................................................................................
18-Lars-37    ....................................................................................H..

                  470       480       490       500       510
                  .|....|....|....|....|....|....|....|....|....|....|
IPO323 CYP51  VSKGAASPYLPFGAGRHRCIGEQFAYVQLQTITATMVRDFKFYNVDGSDNVV
1-Tibb-2      ..................................................K..
2-Nuf-Un-2    ...................................................
3-Nuf-Pz-2    ..................................................K..
4-Roy-Un-2    ...................................................
5-King-Un-2   ..................................................K..
6-King-Pz-2   ...................................................
7-Skedd-2     ...................................................
8-Head-2      ..................................................K..
9-Ger-3-2     ..................................................K..
10-Ger-4-2    ..................................................K..
11-Pittend    ..................................................K..
12-Ire-3      ...................................................
13-HA3        ..................................................K..
14-G303       ..................................................K..
15-Roy-Pz-1   ...................................................
16-S331       ...................................................
17-Ctrl-1     ...................................................
18-Lars-37    ...................................................
```

Figure 1 (continued from previous page) Amino acid sequences of the *CYP51* gene of 18 *M. graminicola* isolates aligned with the wild type isolate IPO323.

The results of screening 18 *M. graminicola* isolates revealed that *CYP51* mutations are widespread across the UK as well as in German populations of *M. graminicola*. This was previously suggested by many authors in recent European populations of *M. graminicola* [19]; [20]; and [21] . In this study, screening of point mutations in the *CYP51* gene, revealed the existence of 9 genotypes (variants) of strains (S, R3+, R4a, R4a+, R5a, R5b, R6a, R7 and R8 displaying different sensitivities to DMIs. Previous studies have confirmed the presence of up to 1-12 different sub-populations that respond differently to different triazoles [12];[14]; [15]; [17]; and [18]). Other research groups, including the Rothamsted group led by Fraaije, however, do not agree with this R-group classification as it is based on multiple, unrelated parameters.

The results of current research have shown the possibility of 15 different alterations (substitutions or deletions) in the *CYP51* gene in positions from 24 to 513 (Table). Earlier work by [14] showed 16 different

mutations and deletions in the same range of sequence and at the same positions. However, it would appear that mutations in the *CYP51* gene represent a continuous process which has continued over last 20 years.

Table - 3: SNPs and deletions in the *CYP51* gene of 18 *M. graminicola* isolates.

Isolate	Amino acid position												Genotype
	24	50	51	136	137	188	379	381	459	460	461	513[a]	
Tibb-2	V	**S**[b]	S	V	Y	**N**	**G**	**V**	-[c]	-	Y	**K**	R8
Nuf-Un-2	V	**S**	S	V	Y	S	A	**V**	Y	G	**H**	N	R6a
Nuf-Pz-2	V	**S**	S	**A**	Y	**N**	A	**I**	-	-	Y	**K**	R5b
Roy-Un-2	V	**S**	S	V	Y	S	A	**V**	Y	G	**H**	N	R6a
King-Un-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
King-Pz-2	V	**S**	S	V	Y	S	A	**V**	**S**	G	Y	N	R6a
Skedd-2	V	**S**	S	**A**	Y	S	A	**I**	Y	G	**S**	N	R5a
Head-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Ger-3-2	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
Ger-4-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Pittend	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Ire-3	V	**S**	S	V	Y	S	A	**I**	Y	G	**S**	N	R4a
HA-3	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
G303	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
Roy-Pz-1	V	L	S	**C**	Y	S	A	**I**	Y	G	**H**	N	R4a+
S331	**D**	L	**T**	V	Y	S	A	**I**	Y	G	Y	N	S
Ctrl-1	V	L	S	V	**F**	S	A	**I**	Y	G	Y	N	R3+
Lars-37	V	**S**	S	**A**	Y	S	A	**I**	Y	G	**H**	N	R5a

[a] *primers used did not extend to cover further areas of the CYP51 gene and therefore, the S524T not detected*
[b] *bold letters represent changes*
[c] *deletion of amino acid*

Since the process began new changes have emerged from year to year. To date, more than 20 different combinations of mutations have been detected and the trend continues to increase [22]. In the current study two new alterations, V24D and S51T (both in isolate S331) have been detected and their effects on the sensitivity of *M. graminicola* to DMIs is not known. Interestingly, within the population of isolates tested, it was also found that substitution Y137F was present in only one isolate (Ctrl-1), an older isolate, which was isolated in 2001 and donated much later to Dr Rossall, as a triazole-sensitive strain, by the Rothamsted research group. This finding was also supported by [14] where they stated that Y137F is rare or even absent in modern *M. graminicola* populations. It has been suggested that isolates carrying Y137F are less sensitive to triadimenol, an azole fungicide introduced in the late 1970s and now no longer used for *M. graminicola* control. The substitution from isoleucine to valine at position 381 was also detected frequently. This was previously found to be unique to *M. graminicola* [23] and is still the predominant substitution in Western Europe [15]. Furthermore, sequence results showed the high level of I381V genotypes (9 out of 12 of 2008 isolates, 75%) in samples screened. This is in agreement with that of [23] who observed a high frequency of I381V genotypes (70%) in samples screened *in planta* using allele-specific q-PCR. Similarly, [23] found the prevalence of the I381V mutation in the *CYP51* gene in populations of *M. graminicola* and they added that this frequency increased from 40% in 2004 to 67% in 2006. Similar to these findings, [25] also confirmed the occurrence of a significant change in *M. graminicola* genotype composition over the last 2 decades; where wild type isolates disappeared while genotypes R3 to R6 predominated. However, the recently-emerged CYP51 genotypes, carrying combinations of mutations D134G, V136A, Y461S, and S524T, revealed a substantial impact on sensitivity to the most widely-used triazoles, which include epoxiconazole

and prothioconazole [26]. However, in the current study the primers used to amplify the *CYP51* gene did not extend to cover the 524 position of the gene and it is therefore not known whether this change exists in isolates that were screened for mutations in this work. With hindsight, use of more extensive primers to detect other mutations would have been beneficial to this work. The primers used were those which had been utilised previously by Leroux group [14].

Previous studies found four residues altered in *M. graminicola* isolates in regions predicted to impact on substrate/inhibitor recognition [21] with other alterations at non-conserved residues implicated in reduced azole sensitivity. In agreement with this, biological data obtained by [27] has demonstrated a clear relationship between substitutions in putative substrate recognition sites (SRSs), SRS-1 (V136A/C and Y137F) and SRS-5 (A379G and I381V) associated with isolate azole sensitivity. Therefore alterations at non-conserved residues are likely to be compensatory, required to maintain enzyme activity when residues important for function are changed. In response to this, particular amino acid changes only occur consecutively, as A379G is only found in isolates carrying the I381V substitution. Some alterations are never found in combination such as V136A and I381V. This is in agreement with the results presented here and supports the same concept that was observed in the results obtained in screening all *M. graminicola* isolates. However, an exception to this rule was found by [15], who found a UK isolate which had the V136A mutation, combined with I381V, Y461H and the new D134G mutation. Recently, [12] have also found the V136A mutation combined with I381V in isolates of *M. graminicola* collected in 2009 in the UK and France.

Conclusions

In *M. graminicola* isolates, multi-allele alterations (substitutions and deletions) were detected. A total of 15 alterations were detected in 12 positions in *CYP51* gene. The substitution characterised V136A was found to be selected by prochloraz while genotypes characterised as I381V were differentially selected by tebuconazole. The study confirmed previous findings that these alterations contribute as major factors to cause resistance in the azole group of fungicides.

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