

# Role of the Y134F mutation in *cyp51* and overexpression of *cyp51* in the sensitivity response of *Puccinia triticina* to epoxiconazole

G. Stammler\*, J. Cordero, A. Koch, M. Semar, S. Schlehüser

BASF SE, Agricultural Center, Fungicide Resistance Research, Carl-Bosch-Strasse 64, D-67117 Limburgerhof, Germany

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## ABSTRACT

A collection of 110 isolates of *Puccinia triticina* in 2007 from different European wheat-growing regions were examined for their sensitivity to the demethylation inhibiting (DMI) fungicide epoxiconazole and the expression and mutations of the target gene, *cyp51*. The *cyp51* gene was highly conserved across Europe. A mutation at codon 134 (Y134F) was identified in only five isolates. This mutation had a limited impact, if any, on the sensitivity of *P. triticina* towards epoxiconazole. Over the entire collection of isolates, *cyp51* expression and *in vitro* sensitivity towards epoxiconazole was moderately correlated. A constitutive up-regulation of the *cyp51* gene was detected in some of the isolates with higher ED<sub>50</sub> values. Such up-regulation was not inducible by exposure to different concentrations of epoxiconazole. Isolates of *P. triticina* with the highest ED<sub>50</sub> values and with the Y134F mutation in the *cyp51* gene or with *cyp51* overexpression were equally well controlled *in vivo* by registered field rates of epoxiconazole as compared to an isolate with the lowest ED<sub>50</sub> value.

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## 1. Introduction

*Puccinia triticina*, the causal agent of wheat leaf rust is a biotrophic basidiomycete fungus causing serious disease in wheat worldwide. Commercial cultivars contain several resistance genes known as leaf rust genes (*Lr*) that confer a degree of resistance against wheat leaf rust to the crop. However, new races of *P. triticina* have been shown to have overcome this genetic resistance (Hysing et al., 2006).

Demethylation inhibitor (DMI) fungicides were introduced in agriculture in 1969. They bind to the heme iron part of the cytochrome P450 sterol 14 $\alpha$ -demethylase enzyme (CYP51). This inhibits lanosterol demethylation, which disrupts the ergosterol biosynthesis pathway (Uesugi, 1998).

Lupetti et al. (2002) mentioned the existence of three major molecular mechanisms in fungi pathogenic to humans that are associated with resistance to azole compounds: (I) mutations in the target gene *cyp51* which could confer reduced affinity of DMIs for their binding site, (II) up-regulation of the *cyp51* gene and (III) reduced intracellular accumulation of azoles, caused by enhanced efflux. Such mechanisms have also been identified in various plant pathogenic fungi. For example, the influence of target site

mutations has been described for field isolates of *Blumeria graminis* f. sp. *tritici* (Wyand and Brown, 2005), and f. sp. *hordei* (Délye et al., 1998), *Oculimacula acuformis* and *Oculimacula yallundae* (Albertini et al., 2003) and *Mycosphaerella graminicola* (Leroux et al., 2007; Stammler et al., 2008). Overexpression of *cyp51* has been found in field isolates of e.g. *Blumeriella jaapii* (Ma et al., 2006) and *Monilinia fructicola* (Luo and Schnabel, 2008) while reports on overexpression of the membrane transporter comprising the ATP-binding cassette (ABC) and triazole sensitivity are mainly related to laboratory mutants, especially of *M. graminicola* (Zwiers et al., 2002).

In samples of *P. triticina* collected in 2007, we observed a spectrum of sensitivity (ED<sub>50</sub> values) of *P. triticina* to the DMI epoxiconazole ranging from 0.05 to 2.42 mg l<sup>-1</sup>. As the reasons for these variations in the sensitivity were not well understood, the present study was established to investigate whether overexpression of the target gene *cyp51* or mutations in this gene are responsible for such differences in the ED<sub>50</sub> values, and also to determine the practical relevance of such changes on the field efficacy of epoxiconazole treatments at registered rates.

## 2. Material and methods

### 2.1. Origin of isolates

A total of 110 field strains of *P. triticina* taken from different wheat-growing areas in Europe in 2007 were collected with

\* Corresponding author. Tel.: +49 621 60 27299; fax: +49 621 60 27176.  
E-mail address: [gerd.stammler@basf.com](mailto:gerd.stammler@basf.com) (G. Stammler).

a mobile spore trap and isolated by EpiLogic (Freising, Germany). Ten isolates were collected per region. The isolates are identified by the region code and the running number (e.g. GB2/2, where GB2 identifies the region Cambridge-Dover in Great Britain and/2 the second isolate collected). Detailed data on this are given in Table 1.

## 2.2. Sensitivity of *P. triticina* to epoxiconazole

The sensitivity (ED<sub>50</sub> values) of *P. triticina* field isolates from 2007 used in this study was determined by EpiLogic according to their standard protocol, which is based on a detached leaf assay using formulated active ingredient and is published on the FRAC website (FRAC, 2008).

## 2.3. Maintenance of isolates

Uredospores of *P. triticina* from 15 d-old-cultures were harvested and transferred onto leaf segments (5 cm long) of the susceptible wheat cultivar Monopol. The leaf segments were placed in Petri dishes that contained water agar (0.4% agarose) supplemented with 40 µg ml<sup>-1</sup> benzimidazole and 30 µg ml<sup>-1</sup> streptomycin. The segments were inoculated with spores from pustules of diseased leaves with the help of a paint brush. In order to avoid contamination of isolates, the paint brush was washed twice with ethanol (70%) and three times with water before using it for the next isolate. Plates with freshly inoculated leaves were incubated at 20 °C in a humid chamber for 24 h in darkness followed by incubation at 20 °C and 12 h light/darkness.

## 2.4. PCR amplification and sequencing of the *cyp51* gene

Complete *cyp51* gene sequences were determined for 25 *P. triticina* isolates representing isolates with the highest and lowest sensitivity towards the fungicide epoxiconazole from each region (Table 1).

Total RNA, from pure spores of the reference strain, was isolated using the NucleoSpin<sup>®</sup> DNA Plant Mini Kit (Macherey–Nagel, Düren, Germany) following the instructions of the manufacturer. The primers KES-807-F (5' GGWCTCRACAARTCATTTGC) and KES-808-R (5' GCAATCATSATATGWGCRATCTC) were used to amplify a 272 bp fragment of the *cyp51* gene. Based on this sequence, a second primer pair, (KES-817-F, 5' GATGGCGGTTTTACCCCTTTG and KES-818-R, 5' CGGCCATCCTGTACTGTTG) was used in

combination with the CapFishing Full Length cDNA Isolation Kit (Biocat, Heidelberg, Germany) to obtain the complete *cyp51* gene sequence.

Total DNA was isolated using the NucleoSpin<sup>®</sup> DNA Plant Mini Kit (Macherey–Nagel) following the instructions of the manufacturer. The primers KES-845-F (5'-GTCCACGATGCTTCTGTGATC) and KES-837-R (5'-TCAGAGATGGTTTCTTCGAGTAA), which bind 7 bp upstream of the ATG initiation codon and on the TGA stop codon, respectively, were used to amplify a 2049 bp fragment containing the 2042 bp long *cyp51* gene sequence.

PCR mixtures were composed of 12.5 µl of 2× Phusion<sup>®</sup> Flash High-Fidelity PCR Master Mix (FINNZYMES, Espoo, Finland), 1.25 µl of each primer (10 pmol µl<sup>-1</sup>), 7.5 µl of distilled water and 2.5 µl of template. PCR was performed for 30 s at 98 °C, 40 cycles of 10 s at 98 °C, 30 s at 65 °C, 45 s at 72 °C, followed by a final step of 5 min at 72 °C. PCR products were separated by agarose gel electrophoresis (1.5%) and fragments were purified by using the NucleoSpin<sup>®</sup> Extract II kit (Macherey–Nagel) according to the manufacturers' instructions. The purified fragments were cloned into pJET1.2 vector using the Clone-JET<sup>™</sup> PCR Cloning Kit (Fermentas, St. Leon Rot, Germany) and employed to transform competent *Escherichia coli* cells (DH5α). Two colonies per sample were taken to isolate the plasmid from the *E. coli* cells by using the NucleoSpin<sup>®</sup> Plasmid Kit (Macherey–Nagel). Three µl of plasmid were digested with 0.5 µl of Bgl II and 2 µl enzyme buffer in a total volume of 20 µl per reaction. Plasmids containing the *cyp51* gene were sequenced with the Sanger method using the primers pJet1.2 forward (5'CGACTACTATAGGGAGAGCGGC), pJet1.2 reverse (5'AAGAACATCGATTTTCCATGGCAG) and KES-817-F (5'GATGCGGTTTTACCCCTTTG) with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, USA).

## 2.5. Specific detection of the Y134F mutation in the *cyp51* gene by pyrosequencing

Because of the detection of the mutation Y134F in one isolate (GB2/2), the whole collection of *P. triticina* isolates was analyzed for this codon of the *cyp51* gene by pyrosequencing. Pyrosequencing allows a high-throughput sequencing of small DNA fragments and is therefore a powerful method to detect single nucleotide polymorphisms. Primers KES-849-F (5'-ACCCCTGTATTTCGGAAGTGA) and KES-850-R (5'-Biotin-CCCAGACTTGACGAATTTCTTTT) flank a gene stretch of 81 bp including the codon 134. An amount of 12.5 µl of Thermo-Start<sup>®</sup> PCR Master Mix (2×) (ABgene, Epsom, UK), 1.25 µl of each primer (10 pmol µl<sup>-1</sup>), 7.5 µl distilled water and

**Table 1**  
Origin and sensitivity of different isolates from 2007 of *Puccinia triticina* towards epoxiconazole. Sensitivity was analyzed by EpiLogic (Freising, Germany), ED<sub>50</sub> values are the mean of 3 replications. Test concentrations were 0.00, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 mg a.i. l<sup>-1</sup>. Wheat cultivar was "Kanzler".

Country	Italy	Great Britain	Great Britain	Great Britain	France	France	France	Germany	Germany	Germany	Germany
Region (Code) <sup>a</sup>	Verona-Brescia (I1)	Cambridge-Dover (GB1)	East Anglia (North) (GB2)	Harrogate Newark (GB4)	Calais Lille (F7)	Paris Reims (F11)	Bourges Nevers (F13)	Nürnberg-Freising (D2)	Magdeburg-Halle (D12)	Neustadt Hamburg (D27)	Hannover Kassel (D31)
1	0.06	<b>1.20</b>	<b>0.11</b>	0.43	0.33	<b>0.07</b>	<b>0.25</b>	0.43	<b>0.40</b>	0.58	<b>1.59</b>
2	0.07	<u>0.64</u>	<b>1.64</b>	0.54	0.31	0.29	1.59	0.06	<u>0.07</u>	0.60	<u>0.59</u>
3	0.06	0.56	0.61	0.73	<b>0.22</b>	<b>0.73</b>	1.96	<b>0.05</b>	0.09	0.58	<b>0.37</b>
4	0.05	<b>0.41</b>	0.83	1.18	0.40	0.14	<b>2.20</b>	0.39	0.20	0.62	0.73
5	<b>0.05</b>	0.60	0.78	<b>0.29</b>	<b>0.51</b>	0.42	2.11	0.10	0.23	0.73	1.46
6	0.06	0.59	0.40	0.60	0.28	0.35	<b>2.42</b>	0.06	0.08	0.73	0.59
7	0.07	0.83	0.91	1.14	<b>0.59</b>	0.45	0.25	<b>0.43</b>	<b>0.06</b>	<b>0.91</b>	0.48
8	<b>0.09</b>	0.58	0.78	0.59	<u>0.51</u>	0.21	0.28	<u>0.13</u>	0.06	0.83	0.37
9	<u>0.07</u>	0.42	1.00	1.01	0.56	0.19	0.23	0.05	0.08	0.78	0.76
10	0.05	0.62	1.56	<b>1.46</b>	0.58	0.28	<b>0.22</b>	0.05	0.07	<b>0.39</b>	0.76

Bold + underlined isolates were used to sequence the complete *cyp51* gene ( $n = 25$ ), bold are the most sensitive isolates per region, whereas the bold + underlined are the least sensitive ones.

<sup>a</sup> Region of wheat-growing areas from where isolates of *Puccinia triticina* were collected with a mobile spore trap. The code of the specific region of the country is given in parentheses. Ten isolates were collected from each region. Isolates are described by the region code and the running number (e.g. I1/1...I1/10).

2.5 µl of template in a total volume of 25 µl were incubated for 15 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C, 20 s at 72 °C, with a final extension step of 5 min at 72 °C. The primer KES-851-F (5'-CGGAAGTATGTTGTCT) was designed to sequence an 8 bp fragment containing the nucleotide exchange of either A or T which codes for the point mutation Y134F. Single-strand DNA preparation was carried out using the Vacuum Prep Workstation (Biotage, Uppsala, Sweden) with Streptavidin Sepharose High Performance Beads (Amersham Biosciences, München, Germany) as described by the manufacturers. Samples were transferred to 40 µl annealing buffer (Biotage) containing 1.25 µl of the sequencing primers (10 pmol µl<sup>-1</sup>) and incubated at 80 °C for 2 min. After equilibration to room temperature, the sequencing reaction was performed with the PyroGold Reagents on a PSQ96MA machine (both from Biotage) as described by the manufacturer.

## 2.6. Sequencing of actin and glyceraldehyde 3-phosphate dehydrogenase

For quantification of *cyp51* mRNA, the normalization to stable expressed reference genes is necessary. Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were chosen for this approach. Primers based on the published sequences X77857.1 (*actin*) and CV191603 (*GAPDH*) were designed: KES-911-F (5' CACACTGTCCGATCTACGAAG) and KES-890-R (5' CTTGAC CATCAGGCAATTCGTA) for *actin* and KES-913-F (5'GCTGCCAAGG CTGTCGGCAAAGT) and KES-914-R (5'GAGTAACCCCACTCGTTGT CGTAC) for *GAPDH*. Sequences of the resulting PCR products differed slightly from the published ones. Primers for quantification of *actin* and *GAPDH* with real-time PCR were designed based on our sequences and are described in the next section.

## 2.7. Quantification of *cyp51* mRNA

Pieces of diseased wheat leaf segments (8 d-old) were transferred to a 2 ml reaction tube and immediately frozen in liquid nitrogen. The material was homogenized with a mixer mill (Retsch, Haan, Germany). Total RNA was isolated using the NucleoSpin<sup>®</sup> RNA Plant Mini Kit (Macherey–Nagel) following the instructions of the manufacturer. RNA quality was assessed by gel electrophoresis. About 700–1000 ng of RNA representing a mixture of wheat and *P. triticina* RNA was used to synthesize cDNA by using the Verso<sup>™</sup> cDNA Kit (ABgene) according to the manufacturer's instructions.

Quantitative real-time PCR was used to quantify cDNA levels of the *cyp51* using the primers KES-852-F (5'-TAACTGCCCTGA GTTCCCTAAA) and KES-837-R to obtain a fragment of 98 bp. The expression of the target gene was normalized to the expression of the housekeeping gene *actin*, which was obtained by using the primers KES-928-F (5'-TGGAGCGAGGTTACAGCTTCA) and KES-900-R (5'-TCAGGCAATTCGTAGCTTCTCG), which resulted in a 151 bp fragment. To confirm the data, a second reference gene, *GAPDH*, was amplified with the primers KES-920-F (5'CCCAACTGCCGATGTCTCA) and KES-921-R (5'CTCTGCTAGTGTAGCCCAAGA), which resulted in a 134 bp fragment. Reactions were performed in a total volume of 25 µl containing 12.5 µl of Absolute<sup>™</sup> QPCR SYBR<sup>®</sup> green mix (2×) (ABgene), 0.5 µl of each pair of primer (10 pmol µl<sup>-1</sup>), 2.5 µl of cDNA (diluted in 1:1 ratio) and 9 µl of distilled water. Reference and target genes were amplified in triplicate reactions with the following thermocycling program: 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The reactions were completed with a melting curve. All reactions were performed in an ABI PRISM<sup>®</sup> 7000 system (Applied Biosystems).

The comparative method of 2<sup>-(ΔΔCt)</sup> was used to estimate the relative expression of *cyp51* mRNA by the mathematical equation (Livak and Schmittgen, 2001):

$$2^{-(\Delta\Delta C_t)} = 2^{-[(C_t \text{ cyp51} - C_t \beta\text{-actin})_x - (C_t \text{ cyp51} - C_t \beta\text{-actin})_y]}$$

“x” is the isolate of interest and “y” is the isolate I1/8, which was chosen as a calibrator because it was shown to have a low expression of *cyp51* and was also highly sensitive to epoxiconazole. This comparative method is a useful mathematical model to calculate relative gene expression when the amplification efficiencies of the target and reference genes are similar.

The efficiency and slopes were determined according to Pfaffl et al. (2002) performing two independent biological repetitions of real-time PCR with a five-fold dilution of cDNA from the calibrator isolate (1, 1/5, 1/20, 1/100, 1/500, 1/2500, 1/12 500 and NTC). Efficiency and slopes of GAPDH, *actin* and *cyp51* were nearly equal with values of 2.0 and -3.33, 2.0 and -3.31 and 1.99 and -3.34, respectively.

## 2.8. Correlation between *cyp51* expression level and the sensitivity of *P. triticina* isolates towards epoxiconazole

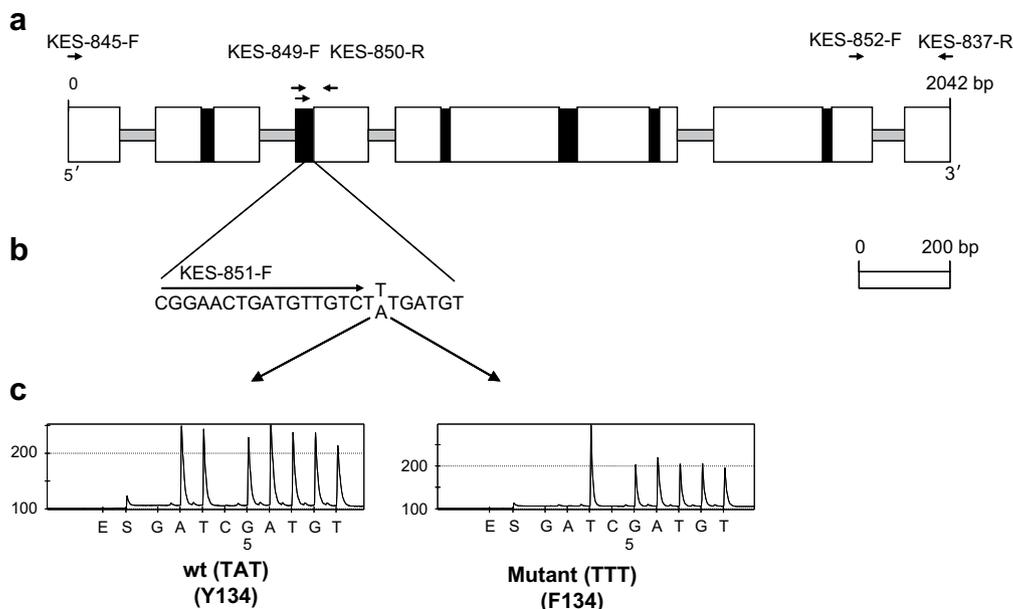
A total of 79 isolates out of the 110 were used to determine the level of correlation between relative expression of *cyp51* and sensitivity of the isolates towards epoxiconazole (ED<sub>50</sub>). Isolates were inoculated on untreated wheat leaf segments and samples were collected after 8 d to isolate total RNA as described in the previous section. The correlation was analyzed by Pearson correlation coefficient analysis. Data from two repetitions of the quantitative RT-PCR determination and the mean ED<sub>50</sub> values were used.

## 2.9. Determination of the *cyp51* expression after epoxiconazole treatments

Two isolates with low and high levels of *cyp51* mRNA expression (GB2/2 and F13/4, respectively), were selected for quantification of the *cyp51* mRNA expression after exposure to epoxiconazole. Fifteen-day-old wheat plants were treated with 0 (control), 0.05, 0.25, 1.25 and 6.25 mg l<sup>-1</sup> of epoxiconazole (Opus<sup>®</sup>, BASF) in a spray chamber with application continued until just before run off. One day after the fungicide treatment, leaf segments (5 cm long) of the treated plants were placed onto 0.4% agarose Petri dishes and infected with the strains mentioned above as described in Section 2.3. Infested leaves were harvested 8 d after infection, when sufficient fungal material had grown for a reliable quantitative RT-PCR as described above.

## 2.10. Greenhouse experiments

Seven-day-old wheat plants of the cultivar “Monopol” were treated in a spray chamber with 400 l ha<sup>-1</sup> using a spray bar with flat fan nozzles to simulate practical conditions. Epoxiconazole (Opus<sup>®</sup>) was applied at a rate of 125 g a.i. ha<sup>-1</sup> of epoxiconazole, according to the registered field rate in Europe. The application timing was 3 d curative (after inoculation). Plants were inoculated separately with three isolates of *P. triticina*: I1/5 (highly sensitive), GB2/2 (Y134F mutant) and F13/4 (with *cyp51* overexpression). Isolate I1/5 was the most sensitive in our collection, the other two isolates were those with the Y134F mutation or *cyp51* overexpression and increased ED<sub>50</sub> values. Inoculation was performed by spraying the plants with 50 ml water, and 500–700 mg of spores per isolate suspended in 35 ml of HFE-7100 solution (3 M, Neuss, Germany) was then sprayed onto the plants using an air brush. Three pots of 8 cm diameter, each containing 10 wheat plants, were used per treatment (untreated



**Fig. 1.** a) Schematic representation of the *cyp51* gene from *Puccinia triticina*: white squares are the coding regions, black ones are the consensus regions (CR1 to CR6 from left to right), grey thin rectangles are the introns and horizontal arrows represent the different primers used. b) Nucleotide sequence of *cyp51* gene where the KES-851-F binds to determine the mutation (Y134F). c) Pyrograms start with the second base of the codon for amino acid 134. The second base in codon 134 of the wild type is an adenine followed by a thymine (third base), the second and third base of the mutant is a thymine, therefore the thymine peak is twice the size of a single thymine.

control, epoxiconazole) and per isolate. The percentage of diseased leaf area was calculated according to the following formula: Control (%) = [(% disease in untreated) – (% disease in treated)/(% disease in untreated)] × 100%. Experiments were repeated and the mean control values were calculated.

### 3. Results

#### 3.1. Analysis of the *cyp51* gene sequence

PCR with the primers KES-845-F and KES-837-R yielded a product of 2049 bp from which 2042 bp was the coding sequence interrupted by five introns (Fig. 1) from 125 to 201, 448 to 532, 698 to 765, 1388 to 1487 and 1879 to 1979 bp coding for a protein of 536 amino acids (gene accession number: FJ976683). A point mutation in one of the isolates with a lower sensitivity (GB2/2) was found. A single A to T exchange at nucleotide 401 resulted in the exchange of tyrosine (Y) to phenylalanine (F) at codon position 134 (Y134F) of CYP51. Sequencing of all other isolates was then directed to this codon as described in the next section. Besides this single amino acid exchange, no other haplotypes were identified in any of the isolates sequenced.

#### 3.2. Influence of the amino acid exchange Y134F in the target protein CYP51 on its sensitivity to epoxiconazole

The pyrogram of a wild type *cyp51* gene showed one peak for adenine and one for thymine in the corresponding sequence coding for amino acid position 134 whereas in isolates carrying the mutation Y134F, the pyrogram showed no peak for adenine but instead one peak twice the size for thymine, which indicated the presence of two thymines at the second and third nucleotide of codon 134. This resulting codon encodes a phenylalanine instead of a tyrosine (Fig. 1).

The mutation was found in only five isolates (GB1/1, GB2/2, GB2/3, D2/1 and D12/1) of 110 *P. triticina* isolates analyzed. Isolates GB1/1 and GB2/2, GB2/3 were from two regions in Great Britain

(Cambridge-Dover and East Anglia) whereas D2/1 and D12/1 were from Germany (Nürnberg-Freising and Magdeburg-Halle, respectively, Table 1 and Fig. 2).

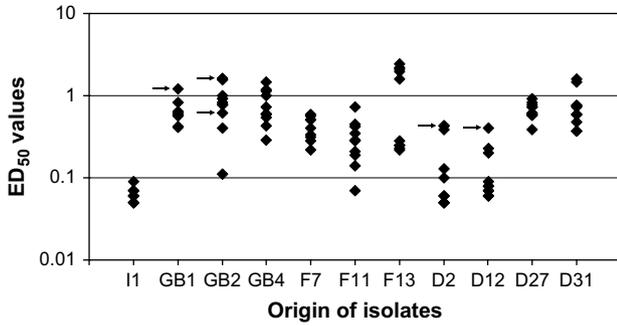
The mutation (Y134F) was present in isolates with increased ED<sub>50</sub> values (GB2/2 and GB1/1) with 1.64 and 1.20 mg l<sup>-1</sup>, respectively, and was also found in three isolates (GB2/3, D2/1 and D12/1) with lower ED<sub>50</sub> values of 0.61, 0.43 and 0.40 mg l<sup>-1</sup>, respectively (Fig. 2), which were in the range of the median (0.43 mg l<sup>-1</sup>) or the mean (0.55 mg l<sup>-1</sup>). There were several isolates with increased ED<sub>50</sub> values that did not contain the mutation. This suggests that another molecular mechanism such as overexpression of *cyp51* gene could be responsible for the reduction in sensitivity.

#### 3.3. Analysis of *cyp51* mRNA expression in various isolates

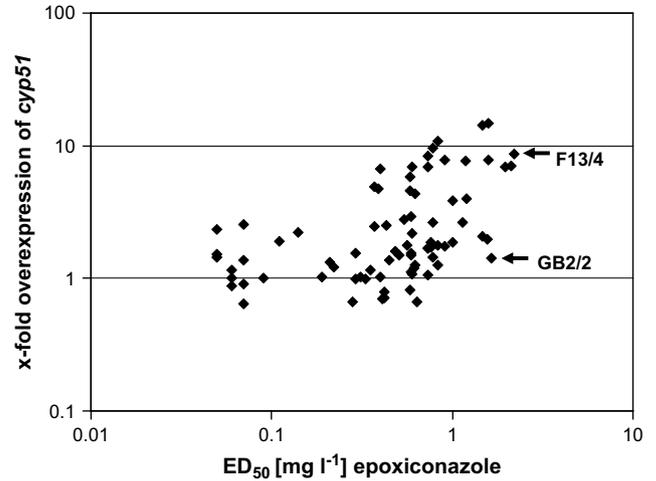
Table 2 shows the *cyp51* mRNA expression of 79 isolates, normalized to actin and GAPDH with the isolate I1/8 as calibrator. Values for both reference genes showed a high correlation ( $r^2 = 0.87$ ). Fig. 3 describes the correlation between expression of the *cyp51* mRNA (here normalized to actin) and sensitivity towards epoxiconazole. There was a correlation between the relative expression of *cyp51* and the sensitivity to epoxiconazole ( $p = 0.001$ ;  $r = 0.56$ ). This indicates a tendency for isolates with increased ED<sub>50</sub> values to be associated with an up-regulation of *cyp51* mRNA, whereas for isolates with lower ED<sub>50</sub> values there is an association with a lower expression of the *cyp51* gene. However, there are also isolates with increased ED<sub>50</sub> values that did not overexpress the *cyp51* gene and many of these also had no Y134F mutation.

#### 3.4. *Cyp51* mRNA expression under exposure to epoxiconazole

Sufficient fungal material could be harvested from leaves treated with concentrations of up to 6.25 mg l<sup>-1</sup> epoxiconazole. The curves in Fig. 4 indicate that the different epoxiconazole treatments did not induce an up-regulation of *cyp51*, either in the strain with low mRNA expression, or in the strain with increased constitutive mRNA expression.



**Fig. 2.** Sensitivity (ED<sub>50</sub> values) of single isolates of *Puccinia triticina* towards epoxi conazole. The isolates are separated by region of origin (e.g. "I1" means Italy, Verona-Brescia, see Table 1). Arrows indicate the ED<sub>50</sub> values of isolates carrying the Y134F mutation.



**Fig. 3.** Correlation of the quantitative expression of *cyp51* (expressed as x-fold amount of the sensitive reference isolate I1/8 with no Y134F mutation, reference gene was *actin*) in different isolates of *Puccinia triticina* and their sensitivity (ED<sub>50</sub> values) to epoxiconazole. Correlation was significant with  $p = 0.001$  and a correlation coefficient of  $r = 0.56$ . The locations of the values of the isolates F13/4 and GB2/2 are indicated by arrows, since these isolates were chosen for experiments on the induction of *cyp51* by epoxiconazole (see Fig. 4).

3.5. Greenhouse tests

All isolates were highly controlled at the registered field rate even under curative conditions, independent of their ED<sub>50</sub> values. Pictures of the leaves show a typical example of disease control under such curative and strong infection conditions. They indicate that the fungus started the infection process, but further development was inhibited after the curative fungicide application (Fig. 5).

4. Discussion

Cytochrome P450 sterol 14 $\alpha$ -demethylase (CYP51) belongs to a group of monooxygenases that form a large single gene superfamily (Yoshida et al., 2000). Mutations in the *cyp51* gene have been ascribed to affect the efficacy of DMI fungicides. In plant pathogens such as *Erysiphe necator* and *B. graminis* f. sp. *hordei*, a substitution of phenylalanine by tyrosine at amino acid position 136 (Y136F) has been identified (Délye et al., 1997, 1998). This amino acid substitution was claimed to cause reduced binding of the inhibitor to the enzyme, as a result of increased hydrophobicity in the enzyme's binding pocket (Délye et al., 1997). In *O. yallundae*, *M. graminicola* and *B. graminis* f. sp. *tritici* the mutation from tyrosine to phenylalanine at the homologous amino acid position was not associated with resistant phenotypes (Albertini et al., 2003; Stammler et al., 2008; Wyand and Brown, 2005). However, to date, the homologous mutation Y134F was the only one found in our study in *P. triticina*, albeit at low frequency (in five out of 110 isolates), and there was also no correlation between the occurrence of this mutation and the sensitivity to epoxiconazole. The existence of the mutation in

isolates with ED<sub>50</sub> values in the range of the median suggests that the impact of Y134F on the sensitivity to epoxiconazole is rather low. This is an important finding because it indicates that the Y134F mutation alone might not confer significant changes in the sensitivity to this triazole.

It seems that the amino acid exchange of tyrosine to phenylalanine could be considered as a natural variation in the CYP51 protein, since the CYP51 protein from different plants such as wheat or rice contains a phenylalanine instead of tyrosine at the homologous codon, while in most fungi and mammals the amino acid at this position is tyrosine (Podust et al., 2001). Apparently this tyrosine (Y134 in *P. triticina*) does not play a relevant role in the enzymatic function of CYP51 and therefore the mutation of tyrosine to phenylalanine does not considerably affect the function of the enzyme.

Since mutations in the target protein are not responsible for the variation in *in vitro* sensitivity, the quantitative expression of the target site was investigated. Up-regulation of the *cyp51* gene has been described to reduce sensitivity to DMIs in *Candida glabrata* (Marichal et al., 1997) and in the plant pathogens *B. jaapii*, *Penicillium digitatum*, *Venturia inaequalis* and *M. fructicola* (Ma et al., 2006; Hamamoto et al., 2000; Schnabel and Jones, 2001; Luo and

**Table 2**  
Relative gene expression of *cyp51* of different isolates of *Puccinia triticina* from 2007 with *actin/GAPDH* as reference genes.

Country	Italy	Great Britain	Great Britain	Great Britain	France	France	France	Germany	Germany	Germany	Germany
Region (Code)	Verona-Brescia (I1)	Cambridge-Dover (GB1)	East Anglia (North) (GB2)	Harrogate Newark (GB4)	Calais Lille (F7)	Paris Reims (F11)	Bourges Nevers (F13)	Nürnberg-Freising (D2)	Magdeburg-Halle (D12)	Neustadt Hamburg (D27)	Hannover Kassel (D31)
1	0.9/1.5	4.0/6.0	1.9/1.4	2.5/3.5	1.0/1.1	2.6/5.7	n.a./n.a.	n.a./n.a.	n.a./n.a.	5.9/5.3	14.9/12.6
2	0.9/1.2	0.7/2.0	1.4/2.4	2.8/3.8	1.0/1.5	1.0/0.9	7.8/16.4	n.a./n.a.	n.a./n.a.	6.9/7.3	1.5/1.7
3	1.0/1.2	1.8/1.6	1.2/1.9	1.7/2.1	1.2/1.1	1.1/1.6	7.0/8.7	n.a./n.a.	n.a./n.a.	4.6/8.5	2.5/4.2
4	2.4/1.6	0.7/1.3	1.8/3.9	7.6/7.7	1.0/1.3	2.2/2.5	8.6/9.0	n.a./n.a.	n.a./n.a.	4.3/6.9	1.7/2.0
5	1.5/2.2	1.1/1.9	2.6/2.9	1.6/1.9	1.5/1.5	0.8/1.8	7.1/9.7	n.a./n.a.	n.a./n.a.	6.9/7.6	14.3/16.4
6	1.2/1.6	1.1/1.7	6.7/8.1	2.2/1.9	n.a./n.a.	1.1/1.3	n.a./n.a.	n.a./n.a.	n.a./n.a.	8.3/11.6	1.5/1.9
7	1.4/2.3	1.3/1.5	1.8/2.7	2.6/4.1	n.a./n.a.	1.4/2.7	n.a./n.a.	n.a./n.a.	n.a./n.a.	7.8/11.3	1.6/2.6
8	<b>1.0/1.0</b>	0.8/2.2	1.5/1.9	2.9/2.6	n.a./n.a.	1.3/1.6	n.a./n.a.	n.a./n.a.	n.a./n.a.	10.8/12.7	4.9/4.9
9	0.6/1.5	0.7/1.3	1.9/2.2	3.9/4.5	n.a./n.a.	1.0/1.7	n.a./n.a.	n.a./n.a.	n.a./n.a.	9.7/10.1	1.9/1.7
10	1.4/2.0	1.3/1.4	2.0/3.8	2.1/3.7	n.a./n.a.	0.7/1.2	n.a./n.a.	n.a./n.a.	n.a./n.a.	4.8/9.2	1.7/2.1

Bold + underlined isolate was taken as calibrator.  
n.a., not analyzed.

Schnabel, 2008). In *P. tritricina*, a significant overexpression in two isolates with increased ED<sub>50</sub> values from eight- to 11-fold higher than the sensitive calibrator isolate was found. However, it cannot be concluded from the present data that this up-regulation of *cyp51* in *P. tritricina* is highly correlated with a lower sensitivity to epoxiconazole, as the correlation coefficient shows. There were some highly sensitive isolates that overexpressed *cyp51* by approximately six-fold, as well as less sensitive isolates that showed no significant overexpression of *cyp51*.

The molecular mechanism for this increase of the *cyp51* mRNA level has not yet been investigated. Possible causes could be related to variations in the *cyp51* promoter or in chromosomal duplication leading to an increase of the copy numbers of the *cyp51* gene (Marichal et al., 1997; Hamamoto et al., 2000; Schnabel and Jones, 2001; Luo and Schnabel, 2008).

Epoxiconazole treatment did not up-regulate *cyp51* in these experiments, which indicates that the expression of *cyp51* is stable and cannot be triggered by epoxiconazole exposure. This fact could have practical relevance since, if it is also true under field conditions, it would mean that a further and transient adaptation in the field as a response to epoxiconazole treatments would not be possible by this mechanism.

Our studies indicate that other, additional mechanisms that have not been investigated for *P. tritricina* so far might play a role in the sensitivity response, such as overexpression of ABC transporters, as found in *C. albicans* (Sanglard et al., 1997; Lyons and White, 2000) and in *P. digitatum* (Nakaune et al., 1998) or in laboratory isolates of *M. graminicola* (Zwiwers et al., 2002).

An additional objective of this work was to find out if epoxiconazole (Opus®) is still able to control field isolates of *P. tritricina* with the highest ED<sub>50</sub> values and those that contained either the mutation (Y134F) or overexpressed the *cyp51* gene. Epoxiconazole provided full control of all isolates at the registered rate, even if applied curatively and under optimal conditions for infection and high infection pressure. This indicates that even isolates of *P. tritricina* with the highest ED<sub>50</sub> values and containing the Y134F mutation or overexpressing the *cyp51* gene were not able to overcome the field efficacy of epoxiconazole. These data are confirmed by numerous BASF field trials in 2007 and 2008 (data not shown) and also by observations under commercial conditions where epoxiconazole treatments provided good control of wheat leaf rust even under severe infection conditions, especially in 2007.

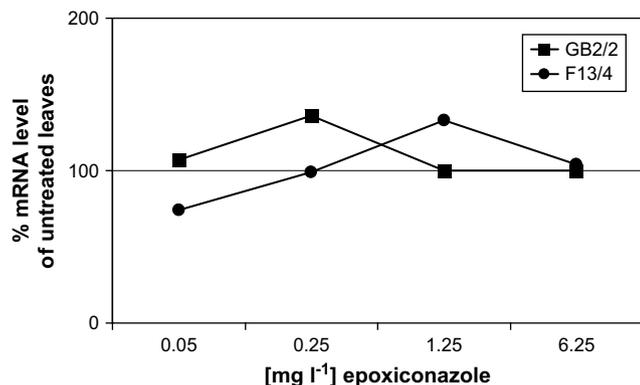


Fig. 4. Expression of *cyp51* in the isolates GB2/2 (low constitutive expression see Fig. 3) and F13/4 (high constitutive expression, see Fig. 3) after exposure on wheat leaves treated with different concentrations of epoxiconazole. Expression data at the different concentrations are given as percentage of the values in the untreated leaves. Values of treated leaves are comparable to the values of the untreated leaves. This demonstrates that *cyp51* is not induced by epoxiconazole exposure.

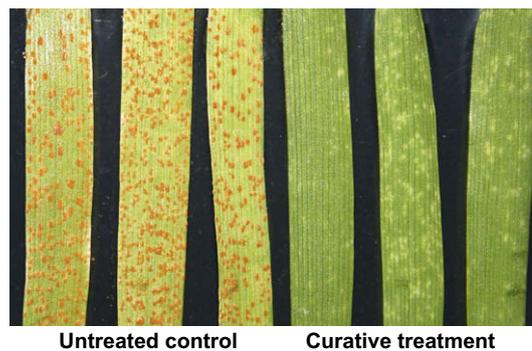
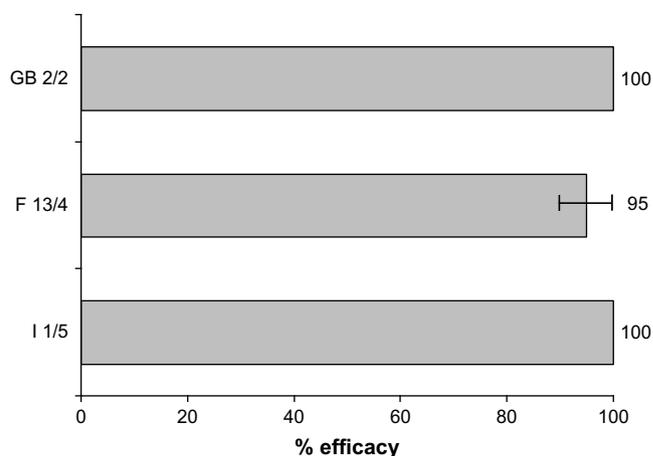


Fig. 5. Efficacy of 125 g ha<sup>-1</sup> epoxiconazole (Opus®) against different isolates of *Puccinia tritricina*. GB2/2 and F13/4 represent isolates with the highest ED<sub>50</sub> values containing the Y134F mutation or showing *cyp51* overexpression, respectively. I1/5 is the most sensitive isolate from this collection. The photograph illustrates the infection of the isolate GB2/2 in the untreated control and the control of rust after curative treatment. The white spots indicate that the fungus started the infection process but further development was stopped after fungicide application.

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