Research Article

Received: 23 November 2012 Revised: 28 February 2013 Accepted article published: 15 April 2013 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.3562

Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms†

Helena K Schmitz,[a] Carlos-Antonio Medeiros,[b] Ian R Craig[c] and Gerd Stammler[d]*

Abstract

BACKGROUND: Since the invasion of *Phakopsora pachyrhizi* (Syd. & P. Syd.) in Brazil, there have been detrimental yield losses of soybeans [*Glycine max* (L.) Merr.]. Disease management is mainly based on fungicide treatment. The sensitivity of single *P. pachyhrizi* isolates towards different demethylation-inhibitors (DMIs) and quinone-outside-inhibitors (QoI) was surveyed and the corresponding resistance mechanisms were analysed.

RESULTS: The QoI-response remained stable, while a loss of sensitivity towards DMIs occurred. Molecular analyses of *cytochrome b* showed an intron after codon 143 which is reported to prevent the development of a G143A mutation. Analysis of *cyp51* revealed that point mutations and overexpression are involved in the sensitivity reduction towards DMIs. Of the detected mutations, Y131F and Y131H, respectively, and K142R are likely homologous to mutations found in other pathogens. As suggested by modelling studies, these three mutations as well as additional mutations F120L, I145F and I475T correlate to increased effective doses of 50%, ED_{50}-values, towards all tested DMIs. Furthermore, a constitutive up-regulation of the *cyp51*-gene up to ten-fold was noticed in some of the DMI-adapted isolates, while all sensitive isolates responded as the wild type.

CONCLUSION: The G143A mutation is thought to result in significant as well as stable resistance factors towards QoIs, while other mutations play only a minor role. Since G143A development is prevented in *Phakopsora pachyhrizi*, a stable control of soybean rust with QoIs in future is rather likely. In contrast, a shifting in sensitivity towards DMIs has been observed, which is due to multiple independent mechanisms.

Keywords: demethylation-inhibitors; quinone-outside-inhibitors; overexpression; point mutation; *sterol 14α-demethylase*; *cyp51*; *cytochrome b*; *Phakopsora pachyrhizi*; soybean rust

1 INTRODUCTION

Soybean [*Glycine max* (L.) Merr.,] is one of the most important crops worldwide ranking only behind wheat (*Triticum aestivum* L.), corn (*Zea mays* L.) and rice (*Oryza sativa* L.) in terms of total area under cultivation [FAOSTAT (http://faostat.fao.org)]. Soybeans are not only used for human consumption but, more importantly, for livestock feeding and industrial purposes [ASA (http://www.soystats.com)]. The United States are the most important producers of soybeans (35% of the world production), followed by Brazil (26%) and Argentina (20%) [FAOSTAT (http://faostat.fao.org)]. In Brazil, soybeans account for 36% of cultivated area and are the most dominant crop being cultivated, followed by corn (20% of cultivated area) and sugarcane [(*Saccharum officinarum* L.) 14%; FAOSTAT (http://faostat.fao.org)].

Soybean yield is reduced by a number of biotical and abiotical factors. One of the most important diseases that can threaten soybeans is *Phakopsora pachyrhizi* Syd. & P. Syd. (Asian soybean rust).[1,2] *P. pachyrhizi* originated in Asia leading to vast yield losses.[3] The phytopathogen first appeared in Brazil in 2001, leading to additional costs of US$ 2 billion.[4] Cultivation practices

* *Correspondence to: Gerd Stammler, BASF SE, Speyerer Strasse 2, 67117 Limburgerhof, Germany. E-mail: gerd.stammler@basf.com*

† *Paper was given in part at 58. German Phytopathology Conference, 10.–14. September 2012, in Braunschweig and represents the main results of the PhD thesis "In vivo und molekularbiologische Untersuchungen zur Sensitivität von Phakopsora pachyhrizi gegenüber Demethylierungs-Inhibitoren und Qo-Inhibitoren" by HK Schmitz, 2013 (online available at http://opus.ub.uni-hohenheim.de/volltexte/2013/828/).*

a *Universität Hohenheim, Otto-Sander-Straße 5, 70599 Stuttgart, Germany*

b *BASF SA, Rodovia SP 340 - Km 144 Estacao Experimental Agricola, 13830-000 Santo Antonio de Posse, Brazil*

c *BASF SE, Carl-Bosch-Strasse 38, 67056 Ludwigshafen, Germany*

d *BASF SE, Speyerer Strasse 2, 67117 Limburgerhof, Germany*

such as late sowing, choice of soybean cultivar or elimination of secondary hosts have been applied to reduce the severity of the disease.[5–8] Other attempts included breeding for *P. pachyrhizi* resistance and the introduction of a 'free host period', in which no soybeans are cultivated for three months.[9–11] However, the application of fungicides is the most employed and most efficient method for controlling *P. pachyrhizi*, and in particular demethylation-inhibitors (DMIs) and quinone-outside-inhibitors (QoI) have provided the best and most consistent results.[12] Other phytopathogens developed adaptations towards both, DMIs and QoIs, which resulted in reduced efficiency of these plant protection compound classes. The most important resistance mechanism against QoIs are point mutations (F129L, G137R, G143A) within the *cytochrome b* (*cyt b*) gene, which have been found in many pathogens.[13–16] Resistance mechanisms against DMIs are variable and complex, involving point mutations, over-expression of the target gene *cyp51* and up-regulation of efflux-transporter. A number of plant pathogens show a more or less significant adaptation to DMIs, such as *Blumeria graminis* (DC.) Speer, *Mycosphaerella graminicola* (Fuckel) J. Schröt., *Puccinia triticina* Erikss. or *Uncinula necator* (Schwein.) Burrill.[17–23]

In this study, the sensitivity of *P. pachyrhizi* isolates towards DMIs and QoIs was analysed by the detached leaf test. Subsequently, *cyp51* and *cyt b*, which are the respective target enzymes of DMIs and QoIs, were investigated using molecular genetic techniques.

2 MATERIAL AND METHODS

2.1 Origin of *P. pachyrhizi* isolates

P. pachyrhizi strains were isolated from soybean leaf samples from different regions of Brazil in 2009/2010. Dry infected leaves were hydrated in a humid chamber for ca. 20 to 24 h. Spores from sporulating uredia were then either transferred to a Tween water (0.03%) drop on a six-day-old soybean leaf (BBCH scale 101[24]) via inoculation needle or were suspended by shaking hydrated leaves in Tween water. Following this, suspensions were applied with an airbrush (0.3 mm nozzle) to the abaxial surfaces of unifoliate leaves, which had been placed on water agar (0.4%), which included 1H-benzimidazole (40 mg L^{-1}) and streptomycin sulphate (30 mg L^{-1}). Isolates were separated by transferring spores from only one uredium to a new soybean leaf via the inoculation needle. After inoculation, soybean leaves were incubated in a humid chamber in the dark for 20 to 24 h followed by 12 h light at 20 °C. A total of 90 strains were isolated in this way and were transferred in three week intervals. *P. pachyrhizi* isolates were propagated for detached leaf tests in a glasshouse.

2.2 Detached leaf tests

Sensitivities of Brazilian *P. pachyrhizi* isolates ($n = 38$) and one strain originating from Taiwan were tested against different fungicides in detached leaf tests in comparison to a reference strain isolated in 2004. Dilution series of cyproconazole (Alto®, Syngenta AG, Basel, Switzerland), epoxiconazole (Opus®, BASF SE, Ludwigshafen, Germany), metconazole (Caramba®, BASF SE) tebuconazole (Folicur®, Bayer CropScience AG, Monheim, Germany) azoxystrobin (Amistar®, Syngenta AG) (all 0/0.01/0.1/1/10 mg L^{-1}) and pyraclostrobin (Comet®, BASF SE) (0/0.03/0.3/3/30 mg L^{-1}) were applied on soybean plants (cultivar Dekalb 22–52) at growth stage 101 (BBCH scale) just before runoff. Treated plants were placed in a growing chamber for drying at 22 °C overnight, before leaves were harvested. The next day, leaves were cut and placed on water agar (0.4%), which included 1H-benzimidazole (40 mg L^{-1}) and streptomycin sulphate (30 mg L^{-1}). Spore suspensions of *P. pachyrhizi* isolates in Tween water (0.03%) were prepared and applied with an airbrush (0.3 mm nozzle) to the abaxial surfaces of the unifoliate leaves. From each fungicide and concentration two soybean leaves were inoculated (approximately 0.5 mL spore suspension per leaf). Subsequently, plates were incubated in a humid chamber in the dark for 20 to 24 h followed by 12 h light at 20 °C. Three weeks after inoculation, infected leaf area was evaluated by visual scoring, assessing thinly dispersed pustules in combination with total necrosis of the leaf as a disease severity of 100%. The effective doses of 50% (ED_{50}-values), the concentration at which 50% of the fungus growth was inhibited, were calculated.

2.3 Nucleic acid extraction and reverse transcription

DNA of *P. pachyrhizi* spores was extracted using the NucleoSpin DNA Plant II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the instructions of the manufacturer for SDS based DNA extraction.

Total RNA of *P. pachyrhizi* spores was extracted using the Spectrum Plant Total RNA Kit and on-column DNase digestion was performed using On-Column DNase I Digest Set (both Sigma Aldrich, St Louis, MO, USA) following the instructions of the manufacturer. RNA of each isolate was extracted at least twice at subsequent dates. RNA concentrations were measured on a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and RNA quality was assessed employing the Agilent RNA 6000 Nano Kit on the Agilent 2100 Expert Bioanalyser (both Agilent Technologies, Santa Clara, CA, USA) using the Plant RNA Nano Assay as described by the manufacturer. The RIN (RNA Integrity Number) obtained in this way varied between six and eight. RIN values above five are recommended for expression analysis.[25]

Each RNA (500 ng) was reverse transcribed to cDNA using the oligo-dT primer of the Verso cDNA Kit (Thermo Scientific Inc, Waltham, MA, USA) as described by the manufacturers. Reverse transcription conditions were as follows: 42 °C for 30 min, 45 °C for 15 min, 50 °C for 15 min and 95 °C for 2 min.

2.4 Sequence analysis

Using Phusion Hot Start, High-Fidelity DNA Polymerase Master Mix (Finnzymes OY, Espoo, Finland) and primer pair KES 1098 fw (5′ ATGTCTTCCAGCGTTATAATCG 3′) and KES 1133 rv (5′ TCGAGGGAAAGGAGTTGATC ′3), respectively, the *cyp51* gene (2155 bp, GenBank accession no. KC741475) was amplified (primer end concentration 500 nM). The following polymerase chain reaction (PCR) programme was applied for amplification: an initial heating for 15 min at 98 °C was followed by 35 cycles at 98 °C for 5 s, 63 °C for 5 s and 72 °C for 45 s, and a final amplification step at 72 °C for 1 min. PCR products were gel electrophoretic separated, extracted using NucleoSpin Extract II (Macherey-Nagel GmbH & Co. KG) and cloned in XL-1 Blue Competent Cells (Agilent Technologies) using the CloneJET PCR Cloning Kit (Fermentas GmbH, St Leon-Rot, Germany) following the instructions of the manufacturers. Clones were sequenced using primers pJet1.2 fw (5′ CGACTCACTATAGGGAGAGCGGC 3′), pJet1.2 rv (5′ AAGAACATCGATTTTCCATGGCAG 3′) and KES 961 (5′ TGGGAA-GAGGGTATGATGTATTGG 3′). Sequences were analysed using the Lasergene Software package (DNASTAR, Madison, WI, USA).

Cyt b (2598 bp, GenBank accession no. GQ33420) was cloned in the same manner, using the primer pair KES 1158 fw

Table 1. Oligonucleotide primers used in this study and their purposes

Name	Sequence 5′–3′	Purpose
KES 498 fw	GGACACTAGTATGGGCGATTG	Pyrosequencing of *cyt b*, 124 bp fragment with KES 499 rv
KES 499 rv [a,b]	CATGTGAGGCGGTCTCATT	Pyrosequencing of *cyt b*, 124 bp fragment with KES 498 rv
KES 500 fw	TTGTAATAATAGCGACAGC	Pyrosequencing of *cyt b* (F129L), sequencing primer
KES 501 fw	TATGGACAGATATCACTATG	Pyrosequencing of *cyt b* (G143A), sequencing primer
KES 1132 fw	TAGGTTATGTACTACCGTAT	Pyrosequencing of *cyt b* (G137R), sequencing primer
KES 1496 fw [a,b]	GTTTTAACTTTTTGATGGCCTGAA	Pyrosequencing of *cyp51* (Y131F, Y131H), 101 bp fragment with KES 1493 rv
KES 1493 rv	CTGCTGCATTAGGATCGAGTTG	Pyrosequencing of *cyp51* (Y131F, Y131H), 101 bp fragment with KES 1496 fw
KES 1494 rv	CGAGTTGGGTACATCR	Pyrosequencing of *cyp51* (Y131F, Y131H), sequencing primer
KES 1564 fw [a,b]	ACCCTTTGTTGATGAGAAGTGTC	Pyrosequencing *cyp51* (K142R, I145F), 396 bp fragment with KES 1563 rv
KES 1563 rv	ACGTCCTTAGTGACAGATTTTACATTT	Pyrosequencing of *cyp51* (K142R, I145F), 396 bp fragment with KES 1564 fw
KES 1562 rv	GTAAGGCCAGCCTTG	Pyrosequencing of *cyp51* (K142R, I145F), sequencing primer
KES 1595 fw	AGGAAGCATGGAAATGTGTTTAC	Pyrosequencing of *cyp51* (F120L), 273 bp fragment with KES 1596 rv
KES 1596 rv [a,b]	TCCTTACCAAAGACAGGTGTAGC	Pyrosequencing of *cyp51* (F120L), 273 bp fragment with KES 1595 fw
KES 1594 fw	GAAGAAGCTTACACCCAT	Pyrosequencing of *cyp51* (F120L), sequencing primer
KES 1599 fw [a,b]	GGTCAATGCCGAAGAAGCTTA	Pyrosequencing of *cyp51* cDNA, 233 bp fragment KES 1600 fw
KES 1600 rv	CATGGACGTCCTTAGTGACAGAT	Pyrosequencing of *cyp51* cDNA, 233 bp fragment with KES 1599 fw
KES 1601 rv	TTACCAAAGACAGGTGTAG	Pyrosequencing of *cyp51* (F120L, cDNA), sequencing primer
KES 1461 fw	ACAGTTTCACCACAACCGCC	Expression analysis reference gene, *aktin*
KES 1462 rv	TGACCGTCGGGAAGTTCG	Expression analysis reference gene, *aktin*
KES 1216 fw	TGATAGACTGAGGCGTGAACAGG	Expression analysis, *cyp51*
KES 1178 rv	AATAGAGGTGTTTGGAGTCGATCGTAATC	Expression analysis, *cyp51*
KES 1465 fw	GGTATGGCTTTCCGAGTTCCA	Expression analysis reference gene, *gapdh*
KES 1466 rv	TCAGTTGATACCAAATCATCCTCAG	Expression analysis reference gene, *gapdh*
KES 1463 fw	CTGCGAACAACTATGCTCGTG	Expression analysis reference gene, *α-tubulin*
KES 1464 rv	CACGAAGAAGCCTTGGAGTCC	Expression analysis reference gene, *α-tubulin*

[a] Synthesis by Eurogentec (Seraing, Belgium).
[b] 5′ Biotin labelling.

(5′ CAGTAGCCTAAAGAAGGGTGTTAA ′3) and KES 1159 rv (5′ CCCGTTGAATATCTTGACATCTTAC ′3) for amplification and pJet1.2 fw, pJet1.2 rv, KES 1175 fw (5′ AATTCTACTAGATCCCCT 3′) and KES 1176 fw (5′ AATTCTACTAGATCCCCT 3′) for the sequencing reaction. The PCR programme for amplification was: initial heating at 98 °C for 15 s, 35 cycles at 98 °C for 10 s, 64 °C for 5 s and 72 °C for 45 s, followed by a final amplification step at 72 °C for 1 min.

For point mutation studies of the *cyp51* gene, pyrosequencing assays were developed using the Pyrosequencing Assay Design Software (Qiagen, Hilden, Germany). Standard PCR using Maxima Hot Start PCR Master Mix (Fermentas GmbH) and the primer pairs listed in Table 1 were employed for amplification of all gene fragments under the following conditions: initial heating for 15 min at 95 °C, 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 20 s, followed by a final amplification step at 72 °C for 5 min. Every template was applied in duplicate. Single strand preparation was performed by immobilizing PCR products to Streptavidin Sepharose Beads (GE Healthcare, UK), and subsequently cleaned up with ethanol (70%), denaturated with sodiumhydroxid (0.2 M) and finally washed in tris-acetat (10 mM) implementing the Vacuum Prep Worktable (Qiagen) following the instructions of the manufacturer. After transferring immobilized single strand samples to annealing buffer amended with the according sequencing primer, the samples were heated at 80 °C for 2 min in an incubator. Samples, which were cooled to room temperature, were then pyrosequenced using PyroMark Gold Q96 Reagents on a PSQ 96MA machine (both Qiagen) as described by the manufacturers. Therefore, sequencing primers listed in Table 1 were used. For assay validation, standard mixtures were performed. Every run included standards of 100% wild type and mutation, respectively, as well as 30/70%, 50/50% and 70/30% mixtures of wild type and mutation. While cDNA of 48 isolates was studied for mutations, analysis of DNA was performed for all 90 isolates.

2.5 Expression analysis

Expression studies were performed on a Rotor-Gene Q 2-Plex machine (Qiagen) and analysed with the GenEx software (MultiD Analyses, Göteburg, Sweden) following MIQE guidelines.[26] Six genes (*actin*, *α-tubulin*, *gapdh*, *sdhc*, *sdhd* and *cyt b*) were validated as reference genes with geNorm and NormFinder (GenEx software), finding *actin*, *α-tubulin* and *gapdh* to perform most stable (data not shown). Quantitative polymerase chain reaction (qPCR) efficacy was assessed by standard curves for all genes and was 0.97 for *actin*, 0.98 for *α-tubulin*, 0.94 for *gapdh* and 1.04 for *cyp51* (data not shown). All cDNA samples were used in duplicate. Threshold cycles (ct) were adjusted to the same level in each run. For qPCR performance, MESA FAST qPCR MasterMix Plus dTTP for SYBR Assay No ROX (Eurogentec, Seraing, Belgium) was used, together with the primer (200nM) listed in Table 1. Conditions for qPCR were as follows: initial heating to 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s and a final step at 72 °C for 20 s. Subsequently, specificity was verified by a melting curve. The fluorescence level was acquired during the elongation step. In a first approach *cyp51*-expression was measured from *P. pachyrhizi* spores of four DMI adapted isolates treated with epoxiconazole (3 mg L^{-1}) in a detached leaf test. In addition, expression of both DMI-adapted and sensitive isolates was analysed from the untreated

control. In a second approach, expression analysis of non-treated spores was performed for 48 isolates. To prevent false positive results, the over-expression limit was set at expression three-fold higher than the reference strain.

2.6 Modelling studies

A structural model of the CYP51 protein from the *P. pachyrhizi* reference strain was constructed using the homology modelling tool in MOE (Version 2010.1, Chemical Computing Group Inc., Montreal, Canada). The template for this modelling was the crystallographically determined structure of human CYP51 (PDB ID: 3LD6), which has a sequence identity with the fungal CYP51 of 38%. The homology model was built using the software's default parameter settings. The resistant mutations identified in this work were then manually implemented into the three-dimensional structure of the reference strain protein.

To investigate the effect of the resistant mutations on DMI binding, the following four fungicide compounds were docked into the homology model: cyproconazole, epoxiconazole, metconazole and tebuconazole. The docking was performed using Glide (Version 5.7, Schrödinger, LLC, New York, NY, USA, 2011) following ligand preparation with LigPrep (Version 2.5, Schrödinger, LLC, 2011). Default parameter settings were used for both steps. No constraints were applied during the docking run, but of the resulting poses only those with an azole-heme binding distance less than 2.8 Å were retained.

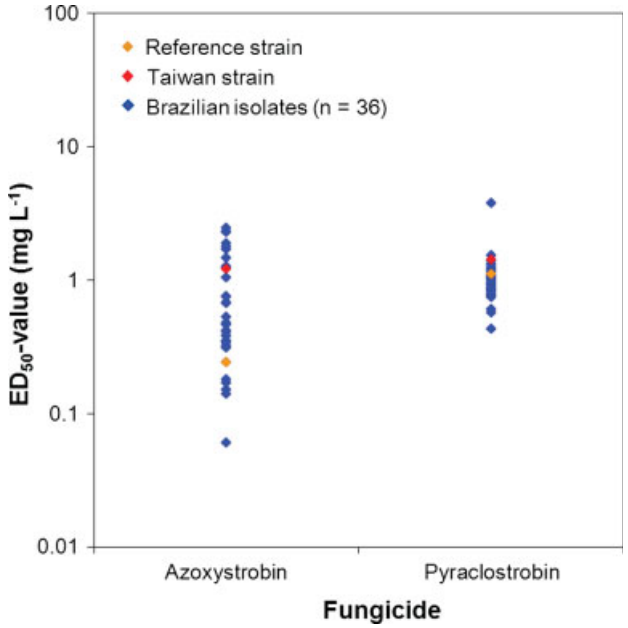

Figure 1. Sensitivity of *Phakopsora pachyrhizi* towards azoxystrobin and pyraclostrobin. ED_{50}-values from all tested isolates ranged from 0.14 mg L^{-1} to 2.47 mg L^{-1} (azoxystrobin) and 0.43 mg L^{-1} to 1.53 mg L^{-1} (pyraclostrobin). The reference strain and the Taiwan strain did not differ in their sensitivity.

3 RESULTS

3.1 Sensitivity of *Phakopsora pachyrhizi* to QoI and DMI fungicides

In detached leaf tests, QoIs adequately controlled all isolates showing narrow ranges of ED_{50}-values, ranging from 0.14 mg L^{-1} to 2.47 mg L^{-1} for azoxystrobin (median: 0.41 mg L^{-1}) and 0.43 mg L^{-1} to 1.53 mg L^{-1} for pyraclostrobin (median: 0.95 mg L^{-1}), respectively (Fig. 1). The sensitivity of the reference strain was within these ranges, showing ED_{50}-values of 0.24 and 1.11 mg L^{-1}, respectively.

The sensitivity of the different *P. pachyrhizi* strains towards DMIs covered a broader range. While the reference strain was controlled by ED_{50}-values of 0.01 mg L^{-1} for all DMIs, most Brazilian isolates needed higher concentrations to be completely controlled, depending on the isolate and fungicide. Most isolates were controlled by fungicide concentrations as high as 4 mg L^{-1} (Fig. 2). The ranges for all DMIs were 0.01 to 10 mg L^{-1} with medians of 2.2 mg L^{-1} for cyproconazole and metconazole, 2.3 mg L^{-1} for epoxiconazole, and 2.5 mg L^{-1} for tebuconazole. A number of isolates tested in this study could not be effectively controlled (growth $>$ 50% at the highest fungicide rate) by cyproconazole, metconazole and tebuconazole. In contrast epoxiconazol effectively controlled all isolates, with the exception of two, at a concentration of 10 mg L^{-1} (Fig. 2). On the basis of inhibition curves different ED_{50}-value classifications could be made, which may slightly differ between different DMIs. Isolates with ED_{50}-values of $<$ 0.1 mg L^{-1} were considered as sensitive, ED_{50}-values of 0.1 to 0.5 mg L^{-1} as sensitive to medium, ED_{50}-values of 0.5 to 4–7 mg L^{-1} as medium to high, and ED_{50}-values of 4–7 to 10 mg L^{-1} were considered as high. Since the reference strain has never been in contact with fungicide treatment in the field, this strain and isolates with similar ED_{50}-values ($\leq$ 0.01 mg L^{-1}) were referred to highly sensitive. In contrast, isolates which needed fungicide treatment of $\geq$ 10 mg L^{-1} to be controlled, were referred to highly DMI adapted or resistant.

3.2 Analysis of the *cyt b* gene sequence

Sequencing DNA and cDNA of the *P. pachyrhizi* reference strain revealed that the *cyt b* gene sized 2524 bp, containing one intron of 1336 bp. The intron was located directly after the amino acid at position 143 (Fig 3). No mutations have been observed in wild type codons, i.e. F129, G137 and G143, which could potentially lead to reduced sensitivity against QoIs. Identity of the pronounced sequence was verified by BLAST search.

3.3 Analysis of the *cyp51* gene sequence

Amplification of *P. pachyrhizi* with KES 1098 fw and KES 1133 rv resulted in a 2155 bp fragment, of which 2107 bp encode the corresponding CYP51 protein. The *cyp51* gene was found to be disrupted by five introns of different size (Fig. 3). Sequencing of the reference strain revealed some variation between different clones of one transformation reaction, since several nucleotide and/or amino acid substitutions occurred. Clearly, these substitutions did not constitute mutations leading to reduced sensitivity towards DMIs, because the reference strain is highly sensitive. Isolates which showed reduced sensitivity were also sequenced and revealed this kind of variation. Amino acid substitutions at positions 120 (F120L), 131 (Y131H/F), 142 (K142R), 145 (I145F) and 475 (I475T) could be linked to increased ED_{50}-values (Fig. 2), since these substitutions were only found in isolates which show decreased DMI sensitivity. Several *cyp51*-clones of sequenced isolates did not only show one identical *cyp51*-haplotype with amino acid substitutions, but also other haplotypes including the wild type within one transformation reaction, suggesting that more than one gene copy is present in a single isolate (data not shown). With the exception of I145F, all mutations occur in combinations (i.e. Y131H + F120L,

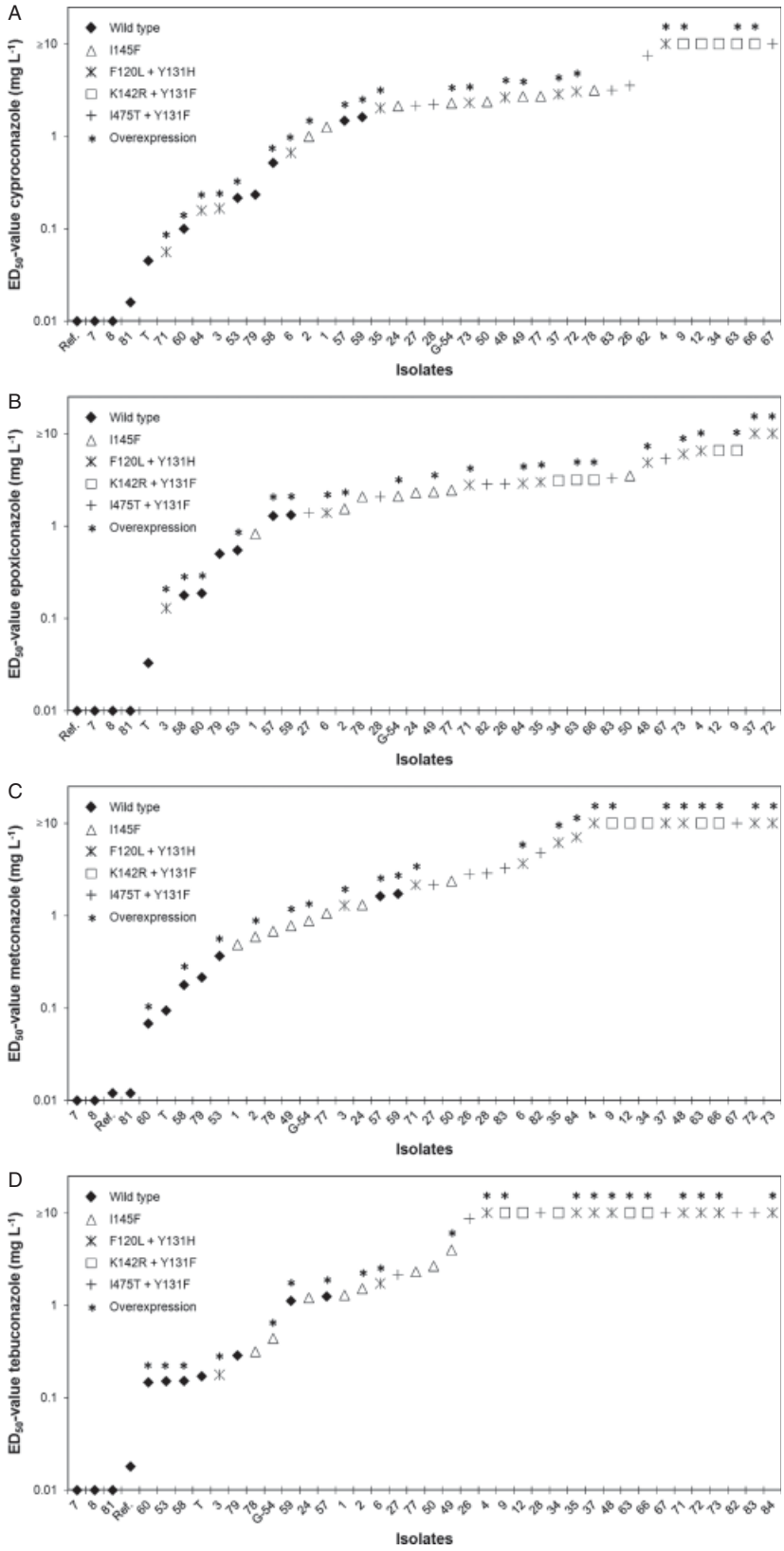

Figure 2. Sensitivity of *Phakopsora pachyrhizi* towards different triazoles and corresponding resistance mechanism. Isolates are sorted by rising ED_{50}-values of cyproconazole (A), epoxiconazole (B), metconazole (C) and tebuconazole (D). Symbols represent different amino acid substitutions in the *cyp51* gene and over-expression of *cyp51* is highlighted by an additional star. Threshold for over-expression is determined as three-fold higher expression compared to *cyp51* expression of the reference strain (Ref.).

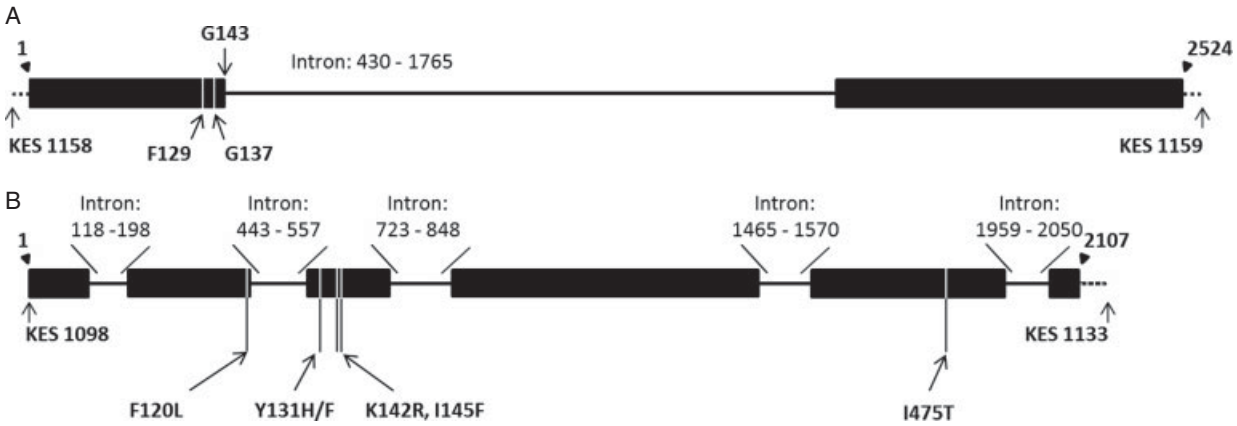

Figure 3. Schematic gene structure of *Phakopsora pachyrhizi cyt b* (A) and *cyp51* (B). *Cytochrome b* DNA is 2524 bp in size and interrupted by a 1336 bp intron which is located directly after codon 143. Location of used primers in this study and codons of most important mutations (i.e. F129L, G137R, G143A) in other phytopathogens are indicated. The 2107 bp sized *cyp51* DNA is interrupted by five introns of different size (81 bp, 115 bp, 126 bp, 105 bp, 92 bp). Mutations found in this study (F120L, Y131H/F, K142R, I145F, I475T) are marked.

Y131F + K142R, Y131F + I475T). Despite this, substitutions are not necessarily coupled within one haplotype since haplotypes with only one substitution can be found. Pyrosequencing never showed 100% mutation in *P. pachyrhizi* DNA but values of approximately 33% or 50%, respectively. The same mutations as in DNA were detected in *P. pachyhrizi* cDNA. However, mutation frequency in cDNA was usually higher than in DNA (Table 2).

The occurrence of I145F is related to ED_{50}-values between 0.3 mg L^{-1} and 4 mg L^{-1} for the fungicides used in this study. A threshold of 1.4 and 3 mg L^{-1} accounts for substitutions of Y131F + I475T and Y131F + K142R, respectively. The widest range of ED_{50}-values is observed in the presence of Y131H + F120L, ranging from 0.06 mg L^{-1} to 10 mg L^{-1}.

The mutations found in the current study were widely distributed within Brazil (Fig. 4). With exception of K142R, which was located in the central region around Senador Canedo, the mutations were not restricted to specific regions. Combinations of Y131H + F120L and Y131F + I475T appeared as the most frequent mutations, though Y131F + I475T was only found in sites where more than ten isolates originated from. Y131H + F120L occurred as the most widely distributed mutation, whereas I145F was the least observed mutation and was mainly located in Santo Antônio de Posse. Isolates with the wild type sequence were present at all sites. Isolates with different haplotypes and *cyp51* expression originated from one sample (data not shown), indicating that ED_{50}-values of the population exists in a field rather than one clone.

3.4 Expression of *cyp51*

In a first approach, *cyp51* expression was analysed from *P. pachyrhizi* spores of DMI-sensitive and -adapted isolates treated with 3 mg L^{-1} of epoxiconazole in a detached leaf test. Expression of *cyp51* using *actin*, α*-tubulin* and *gapdh* as reference genes was four- to eight-fold increased for adapted isolates, in comparison to the reference isolate (Fig. 5). Though DMI-adapted isolates differ in their *cyp51* expression, three out of four isolates over-expressed *cyp51*. All sensitive isolates (ED_{50}-values of < 0.1 mg L^{-1}) expressed *cyp51* at normal level. *Cyp51* expression was increased regardless of whether the isolates were treated with epoxiconazole or not.

In a second approach, *cyp51* expression of all fungicide-tested isolates was studied (Table 2). Expression ranges from three- to ten-fold increase in comparison to the reference strain. Over-expression was found in isolates with wild type *cyp51* gene as well as in isolates showing mutations in the *cyp51* gene. A combination of over-expression and mutation correlates with increased ED_{50}-values towards DMIs (Fig. 2).

3.5 Modelling of *cyp51*

The homology model of *P. pachyrhizi* CYP51 was used to identify the locations of the resistance mutations F120L, Y131F/H, K142R, I145F, and I475T in the three-dimensional structure of the protein. The amino acids at positions 120 and 131 are located within the binding site of the enzyme (Fig. 6). Residue K142 makes a salt bridge with one of the carboxylate groups of the heme, while I145 and I475 are respectively located near enough to the edge and the lower face of the heme to make non-directional van-der-Waals interactions (Fig. 7).

The docking of four DMIs (cyproconazole, epoxiconazole, metconazole and tebuconazole) suggests that F120 and Y131 are in van-der-Waals contact with these inhibitors, with minimum heavy-atom to heavy-atom distances between 3.6 Å and 4.1 Å. Amino-acids K142, I145 and I475 are not in contact with the DMIs considered here.

4 DISCUSSION

As observed by other groups,[27–29] no loss of QoI sensitivity was detected in our study. Results obtained by detached leaf test were confirmed by molecular biological analyses. The *cyt b* gene was sequenced, demonstrating that an intron of 1336 bp occurred just after codon 143 of the 2524 bp nucleotide sequence. As proposed by Grasso *et al.*,[15] an intron after codon 143 results in deficient splicing of the mRNA, which would be lethal in the case of a G143A mutation. A number of pathogens were analysed for G143A, but it was never found adjacent to the subsequent intron.[15] Since an intron is found in rusts directly after codon 143, the G143A mutation has never been found.[15] Nevertheless, there is a possibility that other mutations may evolve such as F129L or G137R as found in other plant pathogens.[14,16] While the G137R mutation is responsible for reduction in fitness, both F129L and G137R mutations can be controlled by field application rates.[14,16,30] Thus, should F129L or G137R develop in *Phakopsora pachyhrizi*,

Table 2. Frequency of mutations in DNA and cDNA, and relative expression of *cyp51*, using *actin*, *α-tubulin* and *gapdh* as reference genes

	Frequency of mutations (%)												
	F120L		Y131H		Y131F		K142R		I145F		I475T		
Isolate	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	Expression *cyp51*(−fold)
Ref.	—	—	—	—	—	—	—	—	—	—	—	—	1.0
T	—	—	—	—	—	—	—	—	—	—	—	—	0.9
G—54	—	—	—	—	—	—	—	—	47	79	—	—	3.0
1	—	—	—	—	—	—	—	—	50	66	—	—	1.9
3	33	88	32	93	—	—	—	—	—	—	—	—	5.9
4	35	89	34	97	—	—	—	—	—	—	—	—	6.9
5	29	83	35	99	—	—	—	—	—	—	—	—	4.0
6	34	94	31	87	—	—	—	—	—	—	—	—	8.2
7	—	—	—	—	—	—	—	—	—	—	—	—	0.5
8	—	—	—	—	—	—	—	—	—	—	—	—	0.5
9	—	—	—	—	34	73	36	79	—	—	—	—	3.8
12	—	—	—	—	34	64	36	68	—	—	—	—	2.9
13	33	89	33	98	—	—	—	—	—	—	—	—	9.0
24	—	—	—	—	—	—	—	—	48	66	—	—	2.8
26	—	—	—	—	55	42	—	—	—	—	50	40	0.7
27	—	—	—	—	55	78	—	—	—	—	54	72	1.0
28	—	—	—	—	51	85	—	—	—		52	82	1.7
34	—	—	—	—	32	88	35	81	—	—	—	—	2.5
35	34	91	33	95	—	—	—	—	—	—	—	—	7.2
37	36	91	32	98	—	—	—	—	—	—	—	—	8.4
48	34	89	33	95	—	—	—	—	—	—	—	—	5.8
49	—	—	—	—	—	—	—	—	50	65	—	—	8.3
50	—	—	—	—	—	—	—	—	50	57	—	—	2.7
53	—	—	—	—	—	—	—	—	2	0	—	—	3.1
54	—	—	—	—	—	—	—	—	47	71	—	—	5.3
56	—	—	—	—	—	—	—	—	—	—	—	—	2.1
57	—	—	—	—	—	—	—	—	—	—	—	—	8.6
58	—	—	—	—	—	—	—	—	—	—	—	—	4.8
59	—	—	—	—	—	—	—	—	—	—	—	—	10.0
60	—	—	—	—	—	—	—	—	—	—	—	—	9.5
62	—	—	—	—	33	66	37	59	—	—	—	—	2.2
63	—	—	—	—	33	72	34	77	—	—	—	—	4.4
66	—	—	—	—	—	—	34	69	—	—	—	—	3.7
67	—	—	—	—	43	81	—	—	—	—	45	80	1.3
71	32	88	34	95	—	—	—	—	—	—	—	—	5.9
72	31	89	30	96	—	—	—	—	—	—	—	—	7.4
73	32	89	32	98	—	—	—	—	—	—	—	—	10.1
77	—	—	—	—	—	—	—	—	47	65	—	—	1.5
78	—	—	—	—	—	—	—	—	48	64	—	—	1.6
79	—	—	—	—	—	—	—	—	—	—	—	—	1.9
80	—	—	—	—	—	—	—	—	—	—	—	—	10.3
81	—	—	—	—	—	—	—	—	—	—	—	—	0.6
82	—	—	—	—	52	70	—	—	—	—	49	61	2.0
83	—	—	—	—	52	74	—	—	—	—	47	76	1.2
84	34	88	32	100	—	—	—	—	—	—	—	—	8.0

the impact of these mutations is suggested to be low. However, neither of these mutations have been detected in monitoring studies in any plant pathogenic rust species so far.

For the DMIs used in this study, a broad range of ED_{50}-values between the reference strain and the Brazilian isolates have been detected, with resistance factors up to 1000. Sensitivity monitoring in Brazil revealed a lower range of ED_{50}-values than we observed, since a slightly different methodology was applied following FRAC recommendations with populations of *P. pachyrhizi* [FRAC (http://www.frac.info)].[11] Due to a mixture of sensitive and resistant strains, fungicides show improved performance rather in fungi populations than in single isolates. Single strains represent both best and worst cases for managing pathogens in the field. Detached leaf tests are appropriate for discriminating the sensitivity of different isolates towards DMIs. However, the growth conditions of the isolate are optimized in detached leaf

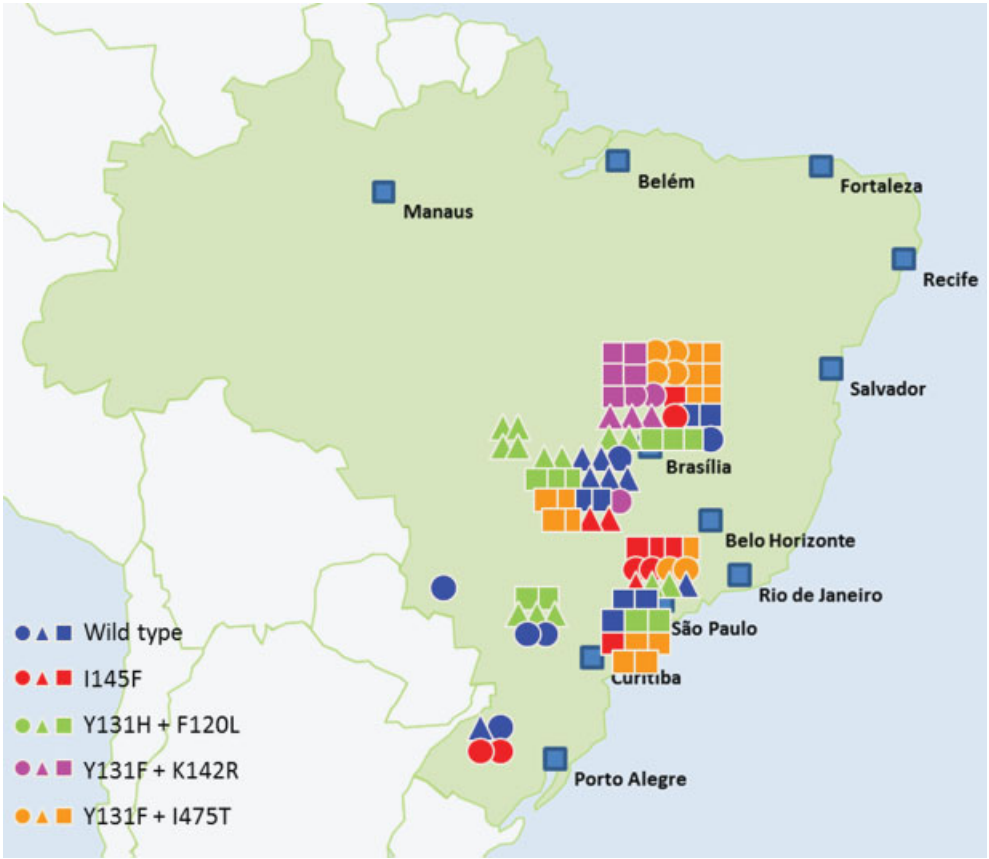

Figure 4. Origin of *Phakopsora pachyrhizi* isolates in Brazil, classified by *cyp51* amino acid substitutions and *cyp51* expression. While colours represent amino acid substitutions, different shapes correspond to wild type expression (circle), over-expression (square) or not analysed (triangle).

tests and are different to conditions in the field. Natural defence mechanisms such as hypersensitive response (HR) and systemic acquired resistance (SAR) of the plant against the pathogen might be missing or significantly reduced in a detached leaf. Furthermore, the mechanical barrier of detached leaves might be reduced due to a poor cuticle, which makes it easier for the pathogen to penetrate and infect. Thus, the pronounced ED_{50}-values and resistance factors in this study may differ to a certain extent in the field. Nevertheless, the recent study emphasizes that a significant sensitivity loss of *P. pachyrhizi* towards DMIs developed. Other studies have analysed disease severity and yield rather than ED_{50}-values to assess the efficiency of fungicide treatments (compounds, quantity, timing) in the field.[27–29] DMI- and QoI-fungicides, or a combination of these, performed best resulting in adequate control of *P. pachyhrizi*.

The *P. pachyrhizi* reference strain revealed several nucleotide substitutions from which some were located within introns. These variations were also found in Brazilian isolates and the Taiwan strain. Since there was a difference in sensitivity of most Brazilian isolates and the reference strain in detached leaf tests, these substitutions do not account for adaptation towards DMIs. Sequence analyses of sensitive and adapted *P. pachyhrizi* isolates revealed mutations at codons 120, 131, 142, 145 and 475. Since mutated and non-mutated wild type *cyp51* were found in one transformation reaction of single isolates it could be concluded that more than one *cyp51* gene copy exists in the genome of *P. pachyrhizi*. Multiple *cyp51* copies are also described for other fungal pathogens, e.g. *Aspergillus* sp. or *Fusarium* sp., although these copies differ in their DNA sequences and protein function.[31,32] The comparison of the frequency of mutations in DNA (i.e. in the genome) and cDNA (i.e. the expressed mRNA) showed that mutated genes were always more expressed than the wild type. When we assume that mutated *cyp51* is less sensitive to DMI inhibition, the selective (over-) expression of the mutated *cyp51* allele leads to a synergistic effect on the adaptation to DMIs.

Alignment of *P. pachyrhizi cyp51* indicated that the Y131F mutation is homologous to Y134F in *Puccinia triticina*, Y136F in *Uncinula necator* and *Blumeria graminis* f. sp. *hordei* or Y137F in *Mycosphaerella graminicola*.[18–20,22] In addition, Y131F in *P. pachyrhizi* is homologous to Y132H in *Candida albicans* (C.P. Robin) Berkhout and K142R is homologous to K143R/E in *C. albicans* and K147Q in *B. graminis*.[33–35] To our knowledge, no homologous mutations in other fungi are reported for *P. pachyrhizi* mutations F120L, I145F and I475T.

In *B. graminis* f. sp. *hordei*, *M. graminicola* and *P. triticina* it is known that Y136F, Y137F and Y134F, respectively, confer sensitivity losses to DMIs to a greater or lesser extent depending on the DMI.[22,34,36] High resistance factors against triadimenol were associated with the corresponding mutation in *M. graminicola* and *U. necator*.[18,20]

The binding site of CYP51 is buried in the core of the protein, such that the substrate must pass along an access channel that is formed by movement of an α-helix.[37] The substitution of Y136F in *B. graminis* and Y137F in *M. graminicola* prevents DMI-binding due to a change in polarity.[36] In other organisms mutations corresponding to *P. pachyhrizi* F120L and Y131H/F are located in

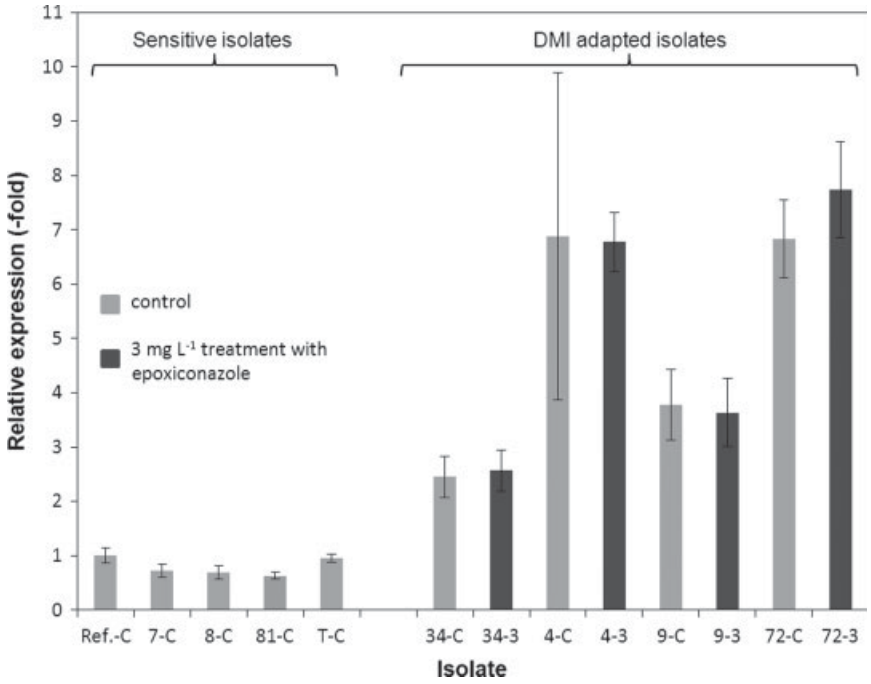

Figure 5. Relative expression of *cyp51*, using *actin*, *tubulin* and *gapdh* as reference genes. *Cyp51* expression of triazole-sensitive isolates (reference strain, isolates 7, 8, 81 and Taiwan strain) and triazole-adapted isolates were analysed from an untreated control (-C) and a 3 mg L^{-1} treatment with epoxiconazole (-3). The over-expression limit was set at expression three-fold higher than the reference strain to prevent false positive results. Expression of *cyp51* is increased to a different extent in some of the adapted isolates (i.e. isolates 4, 9 and 72). Over-expression is not induced by treatment with 3 mg L^{-1} epoxiconazole. Values represent the mean of two replications (on RNA level), with standard deviations shown.

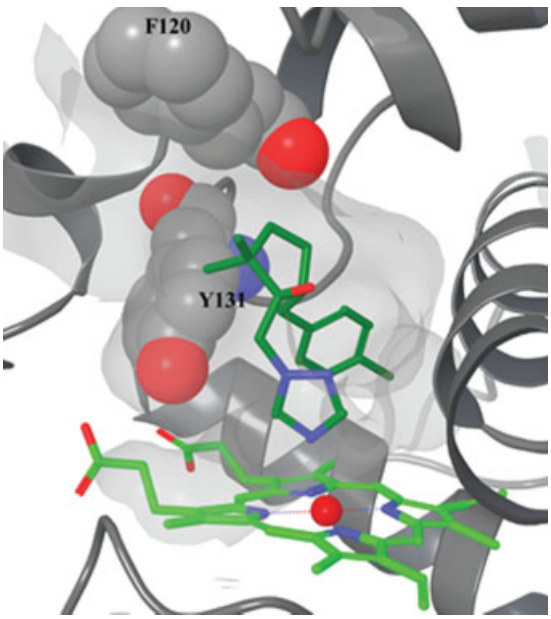

Figure 6. Location of F120 and Y131 in the binding site of the *Phakopsora pachyrhizi* CYP51 homology model. Amino-acids F120 and Y131 are depicted as space-filling spheres with carbon atoms coloured grey. The protein backbone is represented in grey ribbon, and the heme as a green tube with the central iron atom coloured red. A docked pose of metconazole is shown as a dark green tube for reference.

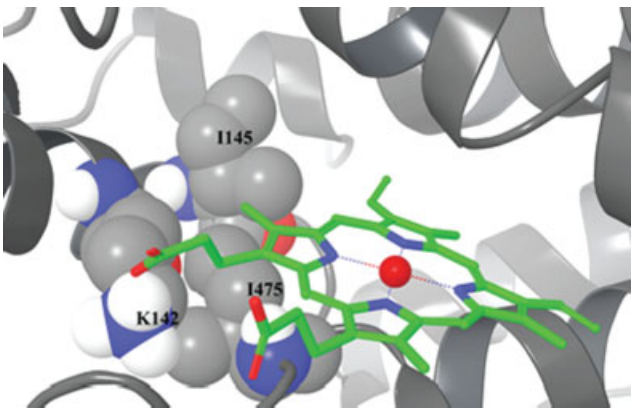

Figure 7. Location of K142, I145, and I475 adjacent to the heme in the *Phakopsora pachyrhizi* CYP51 homology model. Amino-acids K142, I145, and I475 are depicted as space-filling spheres with carbon atoms coloured grey. The protein backbone is represented in grey ribbon, and the heme as a green tube with the central iron atom coloured red.

one of the highly conserved substrate recognition sites, suggesting the same function.[23] The modelling of *P. pachyrhizi* CYP51 in this study suggests that Y131 and F120 can make non-directional van-der-Waals interactions with DMIs. Since these mutations result in smaller amino-acid side-chains, they may affect DMI binding by increasing the size of this part of the binding site, therefore weakening the van-der-Waals contacts. The mutation Y131F/H also represents a change in polarity that could influence inhibitor binding.[36] In contrast, mutations K142R, I145F, and I475T make no direct interaction with the DMIs considered here, but may indirectly affect DMI binding by altering the position of the heme. In particular, the change in volume associated with I475T may enforce a change in protein conformation that is propagated to the heme via the adjacent C474. Additionally, a conformational change of the heme may have an impact on the function of the enzyme.

Besides mutations within the *cyp51* gene, other mechanisms causing sensitivity changes to DMIs have been reported, such as *cyp51*-over-expression.[17,22,38–40] Over-expression of *cyp51* has also been found in isolates of *P. pachyrhizi* in this study. All highly sensitive isolates did not show any over-expression of *cyp51*, while about half of the strains with lower sensitivity to all DMIs an up-regulation up to ten-fold was noticed. Most of the isolates over-expressing *cyp51* responded to DMI treatment with highest ED_{50}-values. In particular two strains (isolates 37 and 72) showed high ED_{50}-values to all four DMIs. Interestingly, over-expression was never found in isolates containing the Y131F + I475T haplotype, but in all cases with Y131H + F120L haplotype. Whether this over-expression is connected with the mutation or if these two resistance mechanisms are independently acquired was not fully elucidated in this study. Over-expression mechanisms may include insertions within the promotor region, up-regulation of transcription factors or gene duplication.[38,39,41,42]

The sensitivity response of some of the strains might not only be related to alterations of the target site *cyp51* based on mutations or over-expression. The sensitivity loss of these isolates might be caused by other additional mechanisms such as enhanced efflux. This has been described for some pathogens as a mechanism for DMI adaptation.[23,43,44]

Taken together our data show that the Brazilian population of *P. pachyrhizi* is sensitive to QoI fungicides. Therefore the risk of QoI resistance development in future seems to be rather low. However, the population has adapted to DMIs and mutations in *cyp51* as well as over-expression of the mutated allele have been identified as important mechanisms for the acquired sensitivity loss, though additional and so far unrecognized mechanisms might also play a role in the sensitivity response.

ACKNOWLEDGEMENT

The authors thank Margit Holzmann for excellent technical assistance.

REFERENCES

1 Carmona MA, Gally ME, Lopez SE, Asian soybean rust: incidence, severity, and morphological characterization of *Phakopsora pachyrhizi* (uredinia and telia) in Argentinia. *Plant Dis* **89**:109 (2005).
2 Hartman GL, West ED, Herman TK, Crops that feed the world 2. Soybean – worldwide production, use, and constraints caused by pathogens and pests. *Food Security* **3**:5–17 (2011).
3 Ono Y, Buritica P, Hennen JF, Delimitation of *Phakopsora*, *Physopella* and *Cerotelium* and their species on *Leguminosae*. *Mycol Res* **96**:825–850 (1992).
4 Yorinori JT, Paiva WM, Frederick RD, Costamilan LM, Bertagnolli PF, Hartman G, *et al*, Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. *Plant Dis* **89**:675–677 (2005).
5 Kawuki RS, Adipala E, Tukamuhabwa P, Yield loss associated with soya bean rust (*Phakopsora pachyrhizi* Syd.) in Uganda. *J Phytopath* **151**:7–12 (2003).
6 Corrêa da Silva JV, Juliatti FC, Vaz da Silva JR, Barros FC, Soybean cultivar performance in the presence of soybean Asian rust, in relation to chemical control programs. *Eur J Plant Pathol* **131**:409–418 (2011).
7 Twizeyimana M, Ojiambo PS, Hartman GL, Bandyopadhyay R, Dynamics of soybean rust epidemics in sequential plantings of soybean cultivars in Nigeria. *Plant Dis* **95**:43–50 (2011).
8 Hu M, Wiatrak P, Effect of planting date on soybean growth, yield, and grain quality: Review. *Agron J* **104**:785–790 (2012).
9 Hartman GL, Miles MR, Frederick RD, Breeding for resistance to soybean rust. *Plant Dis* **89**:664–666 (2005).
10 Kendrick MD, Harris DK, Ha B-K, Hyten DL, Cregan PB, Frederick RD, *et al*, Identification of a second Asian soybean rust resistance gene in Hyuuga soybean. *Phytopathology* **101**:535–543 (2011).
11 Godoy CV, Changes in performance of SBR fungicides over the years and new management strategies adopted in Brazil, in 2009 National Soybean Rust Symposium. New Orleans, December 9–11 (2009). Available: http://www.plantmanagementnetwork.org [5 July 2012].
12 Ivancovich A, Soybean rust in Argentina. *Plant Dis* **89**:667–668 (2005).
13 Heaney SP, Hall AA, Davies SA, Olaya G, Resistance to fungicides in the QoI-STAR cross-resistance group: current perspectives, in *Proceedings, Brighton Crop Protect Conference – Pests Dis*, BCPC, Farnham, UK, pp. 755–762 (2000).
14 Kim Y, Edward WD, Vincelli P, Farman ML, Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial *Cytochrome b* gene. *Phytopathology* **92**:891–900 (2003).
15 Grasso V, Palermo S, Sierotzki H, Garibaldi A, Gisi U, *Cytochrome b* gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag Sci* **62**:465–472 (2006).
16 Sierotzki H, Frey R, Wullschlege J, Palermo S, Karlin S, Godwin J, *et al*, *Cytochrome b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manag Sci* **63**:225–233 (2007).
17 Cools HJ, Bayon C, Atkins S, Lucas JA, Fraaije BA, Overexpression of the *sterol 14α-demethylase* gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Manag Sci* **68**:1034–1040 (2012).
18 Délye C, Laigret F, Corio-Costet M, A mutation in the *14α-demethylase* gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Appl Environ Microbiol* **63**:2966–2970 (1997).
19 Délye C, Bousset L, Corio-Costet M, PCR cloning and detection of point mutations in the *eburicol 14α-demethylase* (*CYP51*) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. *Curr Genet* **34**:399–403 (1998).
20 Leroux P, Albertini C, Gaultier A, Gredt M, Walker A, Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag Sci* **63**:688–698 (2007).
21 Stammler G, Carstensen M, Koch A, Semar M, Strobel D, Schlehuber S, Frequency of different *CYP51*-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. *Crop Prot* **27**:1448–1456 (2008).
22 Stammler G, Cordero J, Koch A, Semar M, Schlehuber S, Role of the Y134F mutation in *cyp51* and overexpression of *cyp51* in the sensitivity response of *Puccinia triticina* to epoxiconazole. *Crop Prot* **28**:891–897 (2009).
23 Leroux P, Walker A, Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag Sci* **67**:44–59 (2010).
24 Munger P, Bleiholder H, Hack H, Hess M, Stauss R, van den Boom T, *et al*, Phenological growth stages of the soybean plant (*Glycine max* L. MERR.): Codification and description according to the BBCH scale. *J Agron Crop Sci* **179**:209–217 (1997).
25 Feige S, Pfaffl MW, RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Asp Med* **27**:126–139 (2006).
26 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, *et al*, The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**:611–622 (2009).
27 Miles MR, Levy C, Morel W, Mueller T, Steinlage T, van Rij N, *et al*, International fungicide efficacy trials for the management of soybean rust. *Plant Dis* **91**:1450–1458 (2007).
28 Mueller TA, Miles MR, Morel W, Effect of fungicide and timing of application on soybean rust severity and yield. *Plant Dis* **93**:243–248 (2009).
29 Scherm H, Christiano RS, Esker PD, Del Ponte EM, Godoy CV, Quantitative review of fungicide efficacy trials for managing soybean rust in Brazil. *Crop Prot* **28**:774–782 (2009).
30 Semar M, Strobel D, Koch A, Klappach K, Stammler G, Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the *cytochrome b* gene. *J Plant Dis Protect* **114**:117–119 (2007).
31 Mellado E, Diaz-Guerra TM, Rodriguez-Tudela JL, Identification of two different *14α-sterol demethylase*-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol* **39**:2431–2438 (2001).
32 Yin Y, Liu X, Li B, Ma Z, Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology* **99**:487–497 (2009).

33 Sanglard D, Ischer F, Koymans L, Bille J, Amino acid subsitutions in the cytochrome P-450 *lanosterol 14α-demethylase* (*CYP51A1*) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother* **42**:241–253 (1998).
34 Wyand RA, Brown JK, Sequence variation in the *cyp51* gene of *Blumeria graminis* associated with resistance to *sterol demethylase* inhibiting fungicides. *Fungal Genet Biol* **42**:726–735 (2005).
35 Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, *et al*, Contribution of mutations in the cytochrome P450 *14α-demethylase* (*Erg11p*, *Cyp51p*) to azole resistance in *Candida albicans*. *Microbiology* **145**:2701–2713 (1999).
36 Mullins JG, Parker JE, Cools HJ, Togawa RC, Lucas JA, Fraaije BA, *et al*, Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS ONE* **6**:e20973 (2011).
37 Boscott PE, Grant GH, Modeling cytochrome P450 *14α demethylase* (*Candida albicans*) from P450cam. *J Mol Graphics* **12**:185–192 (1994).
38 Hamamoto H, Hasegawa K, Nakaune R, Lee YJ, Makizumi Y, Akutsu K, *et al*, Tandem repeat of a transcriptional enhancer upstream of the *sterol 14α-demethylase* gene (*CYP51*) in *Penicillium digitatum*. *Appl Environ Microbiol* **66**:3421–3426 (2000).
39 Schnabel G, Jones AL, The *14α-demethylase* (*CYP51A1*) gene is over expressed in *Venturia inaequalis* strains resistant to myclobutanil. *Phytopathology* **91**:102–110 (2001).
40 Luo C, Schnabel G, The cytochrome P450 *lanosterol 14α-demethylase* gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. *Appl Environ Microbiol* **74**:359–366 (2008).
41 Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhäuser J, Rogers PD, A gain-of-function mutation in the transcription factor *Upc2p* causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryot Cell* **7**:1180–1190 (2008).
42 Marichal P, van den Bossche H, Odds FC, Nobels G, Warnock DW, Timmerman V, *et al*, Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob Agents Chemother* **41**:2229–2237 (1997).
43 Sanglard D, Kuchler K, Ischer F, Pagani J, Monod M, Bille J, Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* **39**:2378–2386 (1995).
44 Stammler G, Semar M, Sensitivity of *Mycosphaerella graminicola* (Anamorph: *Septoria tritici*) to DMI fungicides across Europe and impact on field performance. *OEPP/EPPO Bulletin* **41**:149–155 (2011).

