Research Article

Received: 12 October 2020 Revised: 19 January 2021 Accepted article published: 17 March 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6372

A world-wide analysis of reduced sensitivity to DMI fungicides in the banana pathogen *Pseudocercospora fijiensis*

Pablo Chong,[a,b] Josué Ngando Essoh,[c,d] Rafael E Arango Isaza,[e,f] Paul Keizer,[g] Ioannis Stergiopoulos,[h] Michael F Seidl,[i] Mauricio Guzman,[j] Jorge Sandoval,[j] Paul E Verweij,[k] Gabriel Scalliet,[l] Helge Sierotzski,[l] Luc de Lapeyre de Bellaire,[d,m] Pedro W Crous,[i,n] Jean Carlier,[o,p] Sandrine Cros,[p] Harold J G Meijer,[b] Esther Lilia Peralta[a] and Gert H J Kema[b,q*]

Abstract

BACKGROUND: *Pseudocercospora fijiensis* is the causal agent of the black leaf streak disease (BLSD) of banana. Bananas are important global export commodities and a major staple food. Their susceptibility to BLSD pushes disease management towards excessive fungicide use, largely relying on multisite inhibitors and sterol demethylation inhibitors (DMIs). These fungicides are ubiquitous in plant disease control, targeting the CYP51 enzyme. We examined sensitivity to DMIs in *P. fijiensis* field isolates collected from various major banana production zones in Colombia, Costa Rica, Dominican Republic, Ecuador, the Philippines, Guadalupe, Martinique and Cameroon and determined the underlying genetic reasons for the observed phenotypes.

RESULTS: We observed a continuous range of sensitivity towards the DMI fungicides difenoconazole, epoxiconazole and propiconazole with clear cross-sensitivity. Sequence analyses of PfCYP51 in 266 isolates showed 28 independent amino acid substitutions, nine of which correlated with reduced sensitivity to DMIs. In addition to the mutations, we observed up to six

* *Correspondence to: Gert H. J. Kema, Wageningen University and Research, Wageningen Plant Research, PO Box 16, 6700 AA, Wageningen, The Netherlands. E-mail: gert.kema@wur.nl*

Funding information
Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (SENESCYT), Grant/Award Number: xxx; Escuela Superior Politécnica del Litoral (ESPOL), Grant/Award Number: xxx; Centro de Investigaciones Biotecnológicas del Ecuador (CIBE), Grant/Award Number: xxx; Syngenta AG, Grant/Award Number: xxx; Dutch Dioraphte Foundation, Grant/Award Number: xxx.

a *Centro de Investigaciones Biotecnológicas del Ecuador, CIBE, Laboratorio de Fitopatología, Escuela Superior Politécnica del Litoral, ESPOL., km 30.5 via perimetral, Guayaquil, 090112, Ecuador*

b *Wageningen Research, Wageningen University and Research, Wageningen, The Netherlands*

c *Unité de Recherches sur les Systèmes de Production Durables (SYSPROD), Laboratoire de Phytopathologie, Centre Africain de Recherches sur Bananiers et Plantain, CARBAP, Douala, Cameroun*

d *UPR GECO, CIRAD, Montpellier, France*

e *Escuela de Biociencias, Universidad Nacional de Colombia, Sede Medellín (UNALMED), Medellín, Colombia*

f *Corporación para Investigaciones Biológicas, Unidad de biotecnología Vegetal (CIB), Medellín, Colombia*

g *Biometris, Wageningen University and Research, Wageningen, The Netherlands*

h *Department of Plant Pathology, University of California, Davis, California, USA*

i *Hugo R. Kruytgebouw, Utrecht University, Utrecht, The Netherlands*

j *Departamento de Fitoprotección, Corporación Bananera Nacional (CORBANA S.A.), Limón, Costa Rica*

k *Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

l *Disease control group, Syngenta Crop Protection AG, Stein, Switzerland*

m *GECO, Montpellier University, Montpellier, France*

n *Lab of Evolutionary Phytopahtology, CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands*

o *UMR BGPI, CIRAD, Montpellier, France*

p *BGPI, Montpellier University, Cirad, Inrae, Montpellier SupAgro, Montpellier, France*

q *Laboratory of Phytopathology, Wageningen University and Research, Wageningen, The Netherlands*

© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

insertions in the *Pfcyp51* promoter. Such promoter insertions contain repeated elements with a palindromic core and correlate with the enhanced expression of *Pfcyp51* and hence with reduced DMI sensitivity. Wild-type isolates from unsprayed bananas fields did not contain any promoter insertions.

CONCLUSION: The presented data significantly contribute to understanding of the evolution and global distribution of DMI resistance mechanisms in *P. fijiensis* field populations and facilitate the prediction of different DMI efficacy. The overall reduced DMI sensitivity calls for the deployment of a wider range of solutions for sustainable control of this major banana disease.
© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Supporting information may be found in the online version of this article.

Keywords: azoles; bananas; black Sigatoka; *cyp51*; fungicide resistant; palindrome; promoter insertions; *Pseudocercospora fijiensis*; mutations; *Mycosphaerella fijiensis*

INTRODUCTION

Banana is the most popular fruit around the world.[1] Commercial banana production is dominated by 'Cavendish' cultivars that dominate the export trade (95%) but are also important for domestic markets in many countries.[2–4] This global patchwork of manifold Cavendish plantations amalgamates to form an unparalleled monoculture that facilitates the expansion of black leaf streak disease (BLSD), caused by the Dothideomycete fungus *Pseudocercospora fijiensis*. The fungus colonizes the foliage resulting in characteristic necrotic spots that eventually coalesce into large blotches and defoliate the plants.[5] This initiates premature ripening, which is a major secondary post-harvest loss.[6] The extreme susceptibility of Cavendish bananas to BLSD requires frequent fungicide application, in most environments at weekly intervals, for appropriate disease control.

Extensive use of fungicides can have detrimental effects on biodiversity and the occupational health of plantation workers[7,8] Fungicides also exert an enormous selection pressure on *P. fijiensis* populations which gradually alleviates sensitivity and reduces overall efficacy.[9–12] Sterol demethylation inhibitors (DMIs) are the most commonly applied systemic fungicides for BLSD management.[13] These fungicides interact with the catalytic site of the sterol 14α-demethylase enzyme, also known as CYP51.[13] This protein is a key player in ergosterol biosynthesis, catalysing the demethylation of lanosterol via its heme-bound iron atom in the substrate recognition site (SRS).[14–16] Continuous use of DMIs has contributed to the selection and dissemination of reduced sensitivity in *P. fijiensis* populations.[12,13,17–23] The link between DMI overuse and the occurrence of reduced efficacy and concurring genetic variation at the target site has been demonstrated in many fungal species.[11,16,24–26]

The commonest observed genetic mechanisms are non-synonymous point mutations in the coding region of the *cyp51* gene resulting in modified versions of the CYP51 protein, and changes in the *cyp51* gene promotor resulting in elevated expression levels.[11,12,15,24,27–39] Point mutations in the *cyp51* coding region mostly result in amino acid changes within the SRS regions (SRS1–6).[13,14] SRS1–6 are peptide chain regions at the protein core that interact with the target substrate; they do not inactivate the enzyme but compromise the fungicide-binding affinity.[14,29] The most common substitutions in the *P. fijiensis cyp51* gene (*Pfcyp51*) are at positions Y136 (Y137) and A313 (A311), inside the putative SRS1 and SRS4, respectively, and substitutions at the Y461 (Y459) and Y463 (Y461) positions.[11–13,40] Interestingly, *P. fijiensis* isolates from Costa Rica with an accumulated number of mutations in the *Pfcyp51* gene also contain repeated element insertions in the promoter that contribute to enhanced gene expression and elevated half-maximal effective concentration (EC_{50}) values.[11,12]

Despite existing information regarding *Pfcyp51* genetic variation,[12,22,41,42] the relationship with a global DMI sensitivity analysis is currently lacking. Here, we first analyse the molecular effects underlying reduced sensitivity towards DMIs by phenotyping the sensitivity of 592 isolates collected from Cameroon, Colombia, Costa Rica, the Dominican Republic, Ecuador, the Philippines, Guadalupe and Martinique. These data are then further supported by sequence analysis of *Pfcyp51* and its promoter region of a subset of 266 isolates. Finally, we validate the positive correlation between the use of DMIs and specific modifications in the promoter and coding region of *Pfcyp51* leading to reduced sensitivity at a global scale.

MATERIALS AND METHODS

Pseudocercospora fijiensis isolates and inoculum

A suite of 592 *P. fijiensis* isolates was collected, mostly from Cavendish, from 2012 to 2014, in different regions of Cameroon, Colombia, Costa Rica, Dominican Republic, Ecuador, the Philippines, Guadalupe and Martinique corresponding to major banana-producing regions as well as non-treated and recently colonized areas (Table 1). To affirm species identity and determine the population structure, a set of 155 isolates was selected based on sensitivity range and origin for genotyping by sequencing (GBS) using DArTseq (www.diversityarrays.com/). DNA samples were processed in digestion/ligation reactions[43] and the technology was optimized for *P. fijiensis* by replacing a single PstI-compatible adaptor with two separate adaptors corresponding to two different restriction enzyme overhangs. The PstI-compatible adapter was designed to include the Illumina flow cell attachment sequence.[44] DArTseq markers were quality filtered (Qpmr >2.7, Reproducibility = 1, CallRate >0.66), resulting in 6586 polymorphic DArTseq markers. Based on the presence or absence profiles of these markers, the Jaccard-distance between isolates was determined using R (http://www.R-project.org).[45] Subsequently, complete hierarchical clustering analysis was performed, as implemented in R.[45]

To affirm the taxonomical identity of the collection, a random set of 28 isolates was also tested with elongation factor-1α, which was amplified with primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′)[46] and analysed using the NCBI genome database and the *P. fijiensis* v2.0 JGI genome portal. Further corroboration was obtained from the *Pfcyp51* sequencing of 266 isolates from the collection.

The sequences of five wild-type isolates (X845, X846, X847, X849 and X851) from different countries were used to compare variation among *Pfcyp51* but these strains were not phenotyped in this study. We regarded these isolates as DMI sensitive, based on their

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

TABLE 1. Origins and characteristics of the *Pseudocercospora fijiensis* isolates used in this study

Country/collection	Year of collection	Isolates DMI sensitivity tested	*Pfcyp51* sequenced	Population characteristics	DMI and total fungicide application per year of collection
Colombia CIB UBALMED	Late 2012	98	34	Treated farms and a subset of 13 isolates from non-treated zones	DMI estimated application: 7 cycles from a total of 32 cycles
Costa Rica CORBANA	Early 2014	107	33	Treated farms	DMI estimated application: 7 cycles from a total of 56 cycles
Dominican Republic CIRAD	Early 2013	25	23	Treated farms	Data undetermined
Ecuador CIBE-ESPOL	Early 2011	101	40	Treated farms and a subset of 25 isolates from non-treated zones	DMI estimated application: 13 cycles from a total of 30 cycles
Philippines PRI-WUR	Early 2013	98	28	Treated farms and 1 isolate from non-treated zones	DMI estimated application: 12 cycles from a total of 54 cycles
Guadalupe CIRAD	Early 2013	30	3	Low exposure	DMI estimated application: 6 cycles from a total of 10 cycles
Martinique CIRAD	Early 2013	42	5	Low exposure	DMI estimated application: 9 cycles from a total of 11 cycles
Cameroon CIRAD	Mid 2014	90	94	Treated farms and a subset of 25 isolates from non-treated zones	DMI estimated application: 7 cycles from a total of 45 cycles
Individual sensitive isolates WUR[a]	2009	1	6	Non-treated	Non-treated zones
Total	8 collections	592	266		

[a] Indonesia, Gabon, Burundi, Taiwan, Philippines and Cameroon.
DMI, demethylation inhibitor.

response to propiconazole and cyproconazole.[12] Inoculum for all experiments was prepared using the protocol of Peláez *et al.*[47] with modifications as described by Chong *et al.*[11]

Fungicide testing

Propiconazole, difenoconazole and epoxiconazole were used for this experiment because they are the most prominent DMIs for BLSD control across the eight countries. Syngenta Crop Protection AG provided technical grade quality samples of propiconazole and difenoconazole and epoxiconazole was obtained from Sigma Aldrich. Preparation of the fungicide doses was conducted following the protocol of Chong *et al.*[11] Initially, 592 isolates were tested in duplicate, against each fungicide with eight concentrations (0, 0.004, 0.016, 0.04, 0.16, 0.64, 2.56 and 10.24 mg L^{-1}). In a second screening, a subset of 253 isolates was re-evaluated with two technical and three biological repetitions, due to technical difficulties with the recovery of some isolates. Finally, a third test was performed with 21 top-ranking *P. fijiensis* isolates with EC_{50} values above 10 mg L^{-1} in the initial test[48] against extended final concentrations using 0, 0.64, 2.56, 10.24, 15.36, 20.48, 30.72 and 40.96 mg L^{-1}. In all experiments, we added dimethyl sulfoxide (1%, v/v) to the final concentration and incubated plates in the dark at 27°C for 10 days. Mycelium growth was determined using an Infinite® 200 PRO (TECAN) microplate reader, which was calibrated at room temperature (wavelength 690 nm, multiple reads per well in a 5 × 5 circle-filled form, bandwidth 9 µm, five flashes at 1 mm exclusion from well walls). EC_{50} was determined by plotting the growth profiles from the optical density (OD) readings, adjusted for the background. Monotone regression spline functions[49] were applied to fit the curve profiles using GenStat 18th edition software (VSN International). The EC_{50} sensitivity threshold ranges for all fungicides were arbitrarily chosen based on the clustering analyses of the $\log_2(EC_{50})$ means standard error of the differences and the genetic information of the *Pfcyp51*. The EC_{50} sensitivity thresholds selected for the isolate groupings were: sensitive, less than 0.1 mg L^{-1}; tolerant, 0.1–0.99 mg L^{-1}; and resistant, 1 mg L^{-1} or above.

Pfcyp51

Sequencing

To our knowledge no *cyp51* paralogs have been described in *P. fijiensis* as corroborated by genome analyses (Blast 1 and 2, Supporting Information). Because *Pfcyp51* is orthologous to *Zymoseptoria tritici* (SEPTTR) *cyp51B*, mutations in the *Pfcyp51* are labelled using SEPTTR mutation references as proposed in the fungicide target-site unified nomenclature.[50] The coding region of *Pfcyp51*, including 227 base pairs (bp) of its promoter, were amplified using the amplification primers CYP51_Pfijien_F1 (5′-AAGGTCATATCGCAGG-3′) and CYP51_Pfijien_R1 (5′-GAATGTTATCGTGTGACA-3′). The polymerase chain reaction (PCR) program consisted of an initial denaturation step at 94°C (5 min), followed by 34 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s) and an extension at 68°C (90 s). A final extension step was performed at 72°C (7 min). The expected amplicons ranged from 2 to 2.2 kb and were directly sequenced by Macrogen using the amplification primers and sequencing primers: CYP51_Pfijien_F2 (5′-ACAGAAACATCACCTCC-3′, CYP51_Pfijien_F3 (5′-ATTGCTTCACTTTCATCC-3′), CYP51_Pfijien_F4 (5′-CTCTACCAC GATCTCGAC-3′) and CYP51_Pfijien_R2 (5′-GATATGGATATAGTTGT-3′). The sequences were assembled using SeqMan (Lasergene v8 software from DNASTAR®).

© 2021 The Authors.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Contigs were aligned and analysed using CLC Genomic software v7.5.2 from Qiagen. The wild-type *P. fijiensis* isolate CIRAD86 genome version 2.0 (http://fungi.ensembl.org/Pseudocercospora_fijiensis_cirad86/Info/Index) was used as reference to determine the number and type of mutations per isolate. We used MEME,[51] GLAM2[52] and ESEfinder 3.0[53] software to analyse the promoter region of *Pfcyp51*.

Sensitivity analyses

A single biological determination of EC_{50} was performed in duplicate for all fungicides on all 592 isolates. Subsequently, three biological replicates of a representative subset of 253 isolates were phenotyped due to the aforementioned technical difficulties with the recovery of the remaining isolates of the initial collection. Among this subset, we could not determine EC_{50} for all fungicides of 45 isolates because they were outside the tested doses range. The data were first analysed with a full factorial analysis of variance (ANOVA) model comparing main effects and interactions for the experimental variables isolates (592-1) and fungicides (3-1). Prior to analysis the data were $\log_2$-transformed to obtain homogeneity of variance and a better approximation by the normal distribution. Then, a Finlay–Wilkinson model (FW)[54,55] was used to describe the interaction between two factors (isolates and fungicides) in a more parsimonious nonlinear form. In FW models one of the factors act as a product with a linear relation to the other. This relation can depend either on the fungicide factor or isolate factor with the model EC_{50}; $y_{ijk} = \text{Fungicide}_i + b_i \times \text{Isolate}_j + \varepsilon_{ijk}$ or $y_{ijk} = \text{Isolate}_i + b_i \times \text{Fungicide}_j + \varepsilon_{ijk}$. This results in 'sensitivities' (b_i) for fungicides or isolates indicated by the steepness of the slope. In this study, the fungicides factor was chosen for the FW model because it uses only three lines to describe the general sensitivity response of the isolates towards each fungicide.

Pfcyp51 sequence sensitivity correlations

From 266 *Pfcyp51* sequences, 23 substitutions, binary variables, and a promoter palindromic factor with six levels were established. The fungicides and country of origin were considered as explanatory factors, with three and eight levels, respectively. The FW estimates of the EC_{50} sensitivities were taken as the response or dependent variable in a regression model, where the mutations, promotor, country and fungicide are used as explanatory factors. To analyse main effects of the substitutions alone, these were first fitted with a step-forward approach to select the most explanatory substitutions without the expected moderating and/or confounded effect of the promotor or the other factors. These selected substitutions were subsequently subjected to an all-subset selection procedure, where we could decide which subset of significant substitutions forms the most stable combination. These most explanatory substitutions variables were used to refit the model, now with the promoter and fungicide factor added as main effects. In the next three steps, possible first-order interaction terms with the mutations were added with forward selection followed by backward elimination. Each of these rounds tries iteratively to include subsequent interaction terms based on a forward inclusion ratio and overall significance and retains only the best fitting combinations. First among the mutations themselves, then mutations with promotor and finally mutations with fungicide and country. The model resulting from this process is refitted to arrive at a final model with backward elimination to see if any previously included interaction term has become superfluous. The 23 mutations were pairwise tested for interaction with Fisher's exact test on independence, which can be used to judge the plausibility to accept or discard certain results from the subsequent model fitting.

RESULTS

Different species of the genus *Pseudocercospora* cause similar symptoms on banana. Moreover, these species also morphologically resemble *P. fijiensis* and can coexist in the same leaf.[19,56] To assess the potential occurrence of other *Pseudocercospora* species in our collection, we used GBS for 155 isolates (Figure S1) and confirmed the identity of 28 isolates from the collection by sequencing the elongation factor-1α gene. Finally, *Pfcyp51* sequences of 265 isolates served as a third confirmation. All these isolates were identified as *P. fijiensis*. Hence, we assume that the isolates of the entire global collection were correctly identified based on classical morphology and ascospore germination patterns (data not shown). The GBS analysis used hierarchical clustering based on 6586 polymorphic DArTseq markers and identified a clear clustering pattern reflecting the geographical origin of the *P. fijiensis* isolates, which was independent of the degree of sensitivity to DMIs (Figure S1).

P. fijiensis DMI sensitivity

The *P. fijiensis* collection was tested for sensitivity against the DMIs difenoconazole, epoxiconazole and propiconazole (Table S1). In general, we observed a cross-resistance between these fungicides as shown in Figure S2(A) where the raw $\log_2(EC_{50})$ fitted *versus* estimates illustrates this as a positive band. The FW model, using the fungicides parameter, expressed the sensitivity of each fungicide toward all isolates with an explanatory power of $P < 0.001$. Figure S2(B) depicts the FW model with three lines: the isolate mean responses to each fungicide. The model shows a clear difference between difenoconazole and the two other fungicides (whose lines are nearly parallel). Hence, the structure of the populations based on their sensitivity response (resistant, tolerant, or sensitive) might differ between products (Figures S2B and S3).

A summary of the overall sensitivity category by fungicide is shown in Table S2. Almost all *P. fijiensis* isolates from Costa Rica belong to the resistant category—with highest recorded EC_{50} values—and a minority was classified as tolerant for difenoconazole (1.87%), epoxiconazole (2.08%) and propiconazole (0.94%), whereas no sensitive isolates were observed (Table S2). Similarly, the Philippines and Colombia also show a high incidence of resistant isolates for difenoconazole (58.16% and 71.43%), epoxiconazole (54.08% and 48.98%) and propiconazole (72.45% and 69.39%). By contrast, most isolates from Ecuador were classified as tolerant for difenoconazole (53.47%), epoxiconazole (52.48%) and propiconazole (53.47%). In Cameroon, many isolates were tolerant for difenoconazole (44.57%) and epoxiconazole (50%), but the sensitivity for propiconazole was almost equally distributed among resistant (39.13%), tolerant (27.17%) and sensitive (33.70%) strains. In the Dominican Republic, many strains displayed resistance to difenoconazole (44%) and propiconazole (52%), but most isolates were only tolerant to epoxiconazole (52%). A complete description of distribution across sensitivity classes is shown in Figures 1, S2 and S3 and Tables 2 and S3. The lowest EC_{50} values were observed in isolates from Guadalupe, Martinique and Cameroon. All isolates from untreated areas in Cameroon, Colombia and Ecuador were sensitive (Figure 1 and Table S2), whereas all other isolates from these countries showed an almost continuous range of EC_{50} values (Figure 1 and Table S2).

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

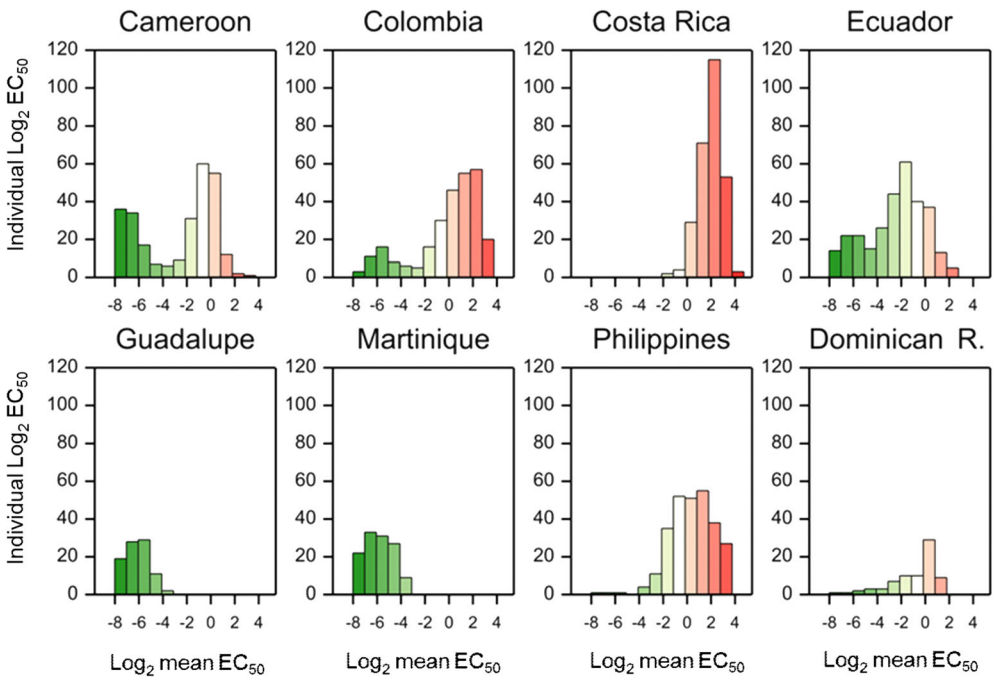

FIGURE 1. Observed sensitivity differences to three demethylation inhibitor (DMI) fungicides (difenoconazole, epoxiconazole and propiconazole) among *Pseudocercospora fijiensis* isolates from seven countries. Data are presented as the frequency of individual half maximal effective concentration (EC_{50}) data that match against the EC_{50} means for the combined response to the tested DMIs (log_2).

TABLE 2. Fisher's protected least significant difference test showing the difference in sensitivity from *Pseudocercospora fijiensis* populations by origin

Country	Mean $log_2(EC_{50})$	Homogeneous group	Isolate count
Guadalupe	−6.015	A	30
Martinique	−5.833	A	42
Ecuador	−2.655	B	101
Cameroon	−2.655	B	90
Dominican Republic.	−0.924	C	25
Colombia	0.220	D	95
Philippines	0.388	E	98
Costa Rica	2.010	F	111

FIGURE 2. Amino acid substitutions identified in the *Pseudocercospora fijiensis* 14α-demethylase enzyme. In total 28 amino acid changes were observed, located at 20 positions in the sequence of *Pfcyp51*. The substitutions with red labels are in the vicinity of the substrate recognizing site (SRS).

© 2021 The Authors.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

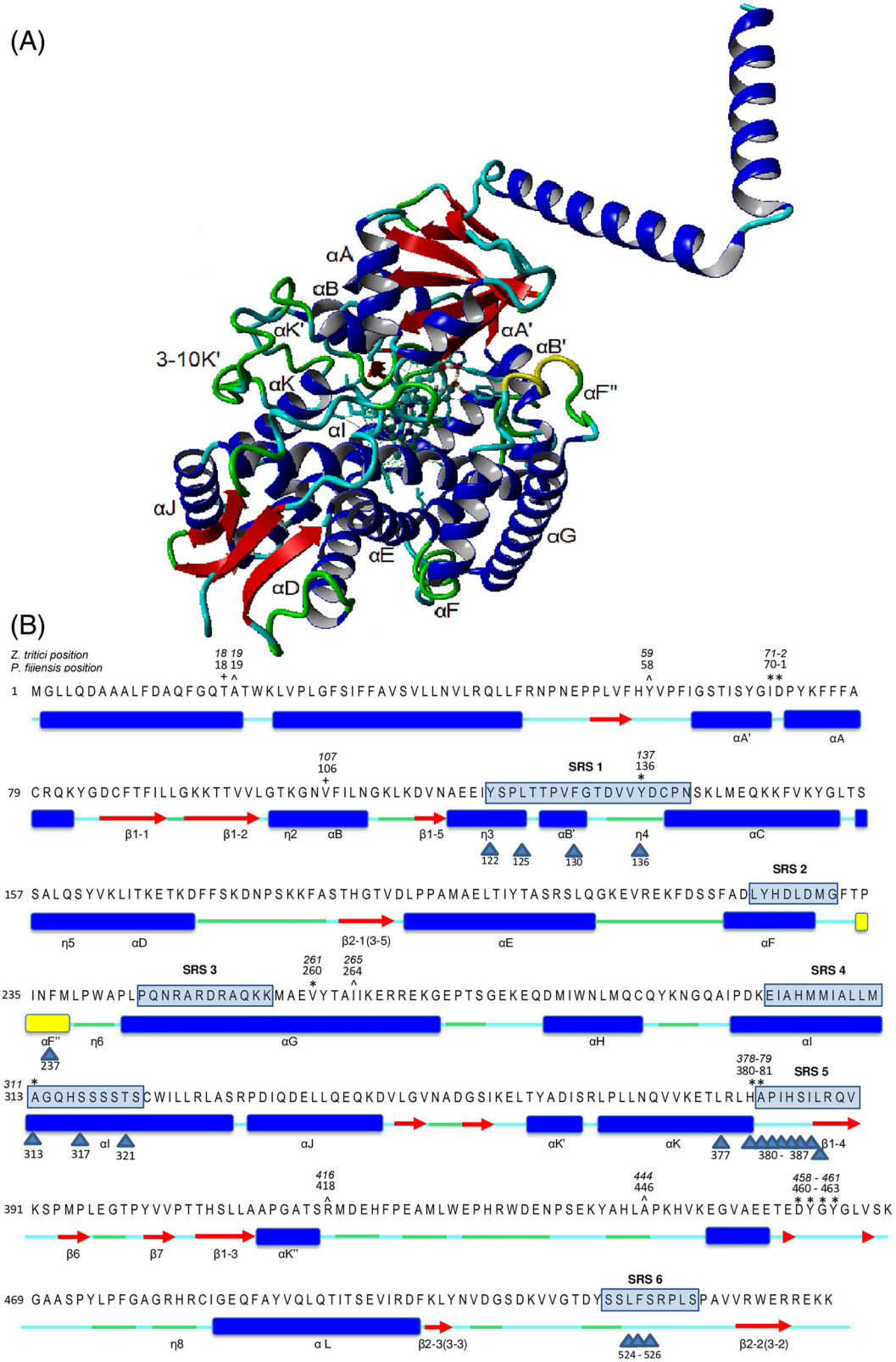

FIGURE 3. Schematic representations of CYP51. (a) Three-dimensional model based on *Pseudocercospora fijiensis* CIRAD86 (genotype G1). (b) PfCYP51 secondary structure model annotated based on Cañas *et al.*[13] and Chen *et al.* (2010)[71] (variation in nomenclature between authors is show in parentheses). Helix structures are shown as blue cylinders, *β* sheets are indicated in red, turns in green and random coils in cyan. Main *α* helixes are depicted in capital letters and the putative substrate recognition sites (SRS) indicated as boxes. The changes in amino acids identified in *Pfcyp51* are depicted as: (ᐟ) only in demethylation inhibitor (DMI)-sensitive isolates, (*) only in resistant strains and (+) present in both. Residues that potentially locate within 7 Å of the propiconazole docking site are labelled with blue triangles.

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

TABLE 3. Changes in the CYP51 protein sequences of *Pseudocercospora fijiensis* isolates per country

Country/amino acid substitution	Colombia	Costa Rica	Cameroon	Dominican Republic	Ecuador	Guadalupe	Philippines	Martinique	Individual Isolates	Total
(*n*)	34	33	94	23	40	3	28	5	6	266
Promoter insertion	24 (70.6%)	26 (78.8%)	62 (66%)	17 (74%)	5 (12.5%)	0	8 (28.6%)	0	0	142 (53.38%)
T18I	34 (100%)	33 (100%)	0	23 (100%)	40 (100%)	3 (100%)	15 (53.6%)	5 (100%)	2 (33.3%)	155 (58.27%)
A19E	1 (2.90%)	0	0	0	1 (2.5%)	0	0	0	0	2 (0.75%)
Y59F	0	0	0	0	0	0	0	0	1 (16.6%)	1 (0.38%)
I71M	0	0	0	0	0	0	2 (7.10%)	0	0	2 (0.75%)
D72E	0	0	0	0	0	0	2 (7.10%)	0	0	2 (0.75%)
V107D	34 (100%)	33 (100%)	94 (100%)	23 (100%)	40 (100%)	3 (100%)	28 (100%)	5 (100%)	5 (83.3%)	265 (99.62%)
V117L	0	0	0	0	0	0	0	0	1 (16.6%)	1 (0.38%)
Y137F	21 (61.8%)	19 (57.6%)	1 (1.06%)	2 (8.7%)	0	0	4 (14.3%)	0	0	47 (17.67%)
K172R	0	0	0	0	0	0	4 (14.3%)	0	1 (16.6%)	5 (1.88%)
V261L	0	2 (6.1%)	0	0	0	0	0	0	0	2 (0.75%)
I265T	0	0	0	0	0	0	0	1 (20%)	0	1 (0.38%)
A311G	9 (26.5%)	19 (57.6%)	64 (68.1%)	19 (82.6%)	33 (82.5%)	0	27 (96.4%)	0	0	171 (64.29%)
H378N	0	3 (9.1%)	0	0	0	0	0	0	0	3 (1.13%)
A379G	1 (2.9%)	7 (21.2%)	0	3 (13%)	0	0	0	0	0	11 (4.14%)
R416G	0	0	0	1 (4.3%)	0	0	0	0	0	1 (0.38%)
A444S	0	0	0	0	0	0	22 (78.6%)	0	1 (16.7%)	23 (8.65%)
D458E	0	0	0	0	0	0	15 (53.6%)	0	0	15 (5.64%)
D458V	0	0	49 (52.1%)	0	0	0	0	0	0	49 (18.42%)
ΔY459	0	0	0	0	0	0	2 (7.1%)	0	0	2 (0.75%)
Y459D	2 (5.9%)	2 (6%)	0	0	2 (5%)	0	2 (7.1%)	0	0	8 (3.01%)
Y459N	2 (5.9%)	0	0	0	0	0	15 (53.6%)	0	0	17 (6.02%)
Y459S	0	0	0	0	0	0	2 (7.1%)	0	0	2 (0.75%)
G460A	0	1 (3%)	0	0	0	0	0	0	0	1 (0.38%)
G460D	0	0	4 (4.3%)	0	0	0	0	0	0	4 (1.50%)
Y461D	21 (61.8%)	22 (66.7%)	6 (6.4%)	14 (60.9%)	1 (2.5%)	0	6 (21.4%)	0	0	70 (26.32%)
Y461H	3 (8.8%)	1 (3%)	0	2 (8.7%)	10 (25%)	0	0	0	0	16 (6.02%)
Y461N	0	2 (6.1%)	5 (5.3%)	3 (13%)	20 (50%)	0	0	0	0	30 (11.28%)
Y461S	0	4 (12.1%)	0	2 (8.7%)	0	0	0	0	0	6 (2.26%)

© 2021 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

The overall response of the global population is shown in Figures 1, S2 and S3 and Tables 1 and S2. The additional sensitivity analyses of the 21 top-ranking resistant isolates (EC_{50} values up to 40.96 mg L^{-1}; Figure S4) revealed that isolates CaM10_6, CaM1_5 and CaM3_1 from Costa Rica have EC_{50} values above 3 mg L^{-1} for epoxiconazole and over 6 mg L^{-1} for difenoconazole and propiconazole (Figure S4). In particular, isolate CaM10_6 displays EC_{50} values above 20 and 18 mg L^{-1} for difenoconazole and propiconazole, respectively.

Pfcyp51 diversity

We identified 60 unique genotypes with a total of 28 mutations in the coding region of *Pfcyp51* (Figures 2 and 3 and Table S3) compared with the sensitive reference isolate CIRAD86.[57] The amino acid changes were dispersed over 20 positions. Strikingly, all isolates shared a non-synonymous mutation resulting in the amino acid change V107D (Table S4). The number of mutations per position per country is summarized in Table 3. Except for Y137F, all amino acid substitutions are derived from single base mutations. In Y137F, the wild-type codon is TAC at position 405 bp and the altered codons are TTC and TTT, which are present in 29 isolates from different populations (Costa Rica, Cameroon, Colombia and Philippines) and in 11 isolates from Costa Rica, respectively. The list of the codons for each substitution is summarized in Table S5.

At the global scale, the most frequently observed amino acid changes are V107D (265 isolates), A311G (171 isolates), T18I (155 isolates), Y461D (70 isolates), D458V (49 isolates), Y137F (47 isolates) and Y461N (30 isolates) (Table 3). The largest number of specific mutations was present among Philippine isolates. Mutations resulting in I71M, D72E, D458E, ΔY459 and Y459S were unique for the Philippine collection, whereas mutations leading to K172R and A444S were shared with a sensitive isolate from Taiwan. Unique mutations were also observed in isolates from other countries. For instance, V261L, H378N and G460A were exclusive for isolates from Costa Rica, whereas amino acid changes D458V and G460D were found only in isolates from Cameroon. Just a few mutations leading to amino acid changes were only found once, such as I265T in an isolate from Martinique and R416G in an isolate from the Dominican Republic (Table 3). By contrast, other mutations are ubiquitous such as T18I, present in all isolates from Latin America and the Caribbean and in 15 isolates from the Philippines. The same mutation existed in two sensitive wild-type isolates from the Philippines and Indonesia (Table 3) but was absent among Cameroon isolates.

The number of amino acid substitutions per individual genotype varied from one to seven (Table S3). Most of the non-sensitive isolates gained four amino acid changes when compared with the reference isolate. The most common combination was T18I + V107D + A311G + Y461D, present in genotypes G29 to G32, identified in 24 isolates from the Dominican Republic (14 isolates), Costa Rica (five isolates), Colombia (two isolates), Philippines (two isolates) and Ecuador (one isolate). Genotype G25, was the only isolate from Cameroon and contained the modification Y137F (Table S3). Thirty-two isolates share only a single substitution (V107D), when compared with the CIRAD86 reference. The two- and three-way combinations T18I + V107D, T18I + A19E + V107D, T18I + Y59F + V107D, T18I + V107D + I265T, T18I + V107D + R416G, T18I + V107D + A444S and V107D + V117L + A444S were all present in *P. fijiensis* isolates sensitive to DMIs. By contrast, substitutions Y137F, A311G, H378N, D458E, D458V, ΔY459, Y459D, Y459N, Y459S, G460A, G460D, Y461D, Y461H, Y461N and Y461S were present only in isolates with reduced sensitivity to DMIs. Interestingly, genotypes G8, G12–14, G18, G19, G36, G41, G49, G52, G53, G57, G58 and G60 show a differential impact on the sensitivity for the three fungicide with higher EC_{50} values for propiconazole (Table S3).

Promoter insertions

In total, we sequenced *Pfcyp51* in 266 isolates of which 142 carry an insertion in the promoter (Tables 3 and S2). Previously, these insertions have been correlated with overexpression of *Pfcyp51* and hence, reduced sensitivity to DMIs.[11, 12] For instance, *P.*

FIGURE 4. Logo made in MEME[50] of the repeated inserts elements found in the promoter of 142 *Pseudocercospora fijiensis* strains. Element 'A' and its palindrome is common in all repeat candidates that were identified by the software.

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

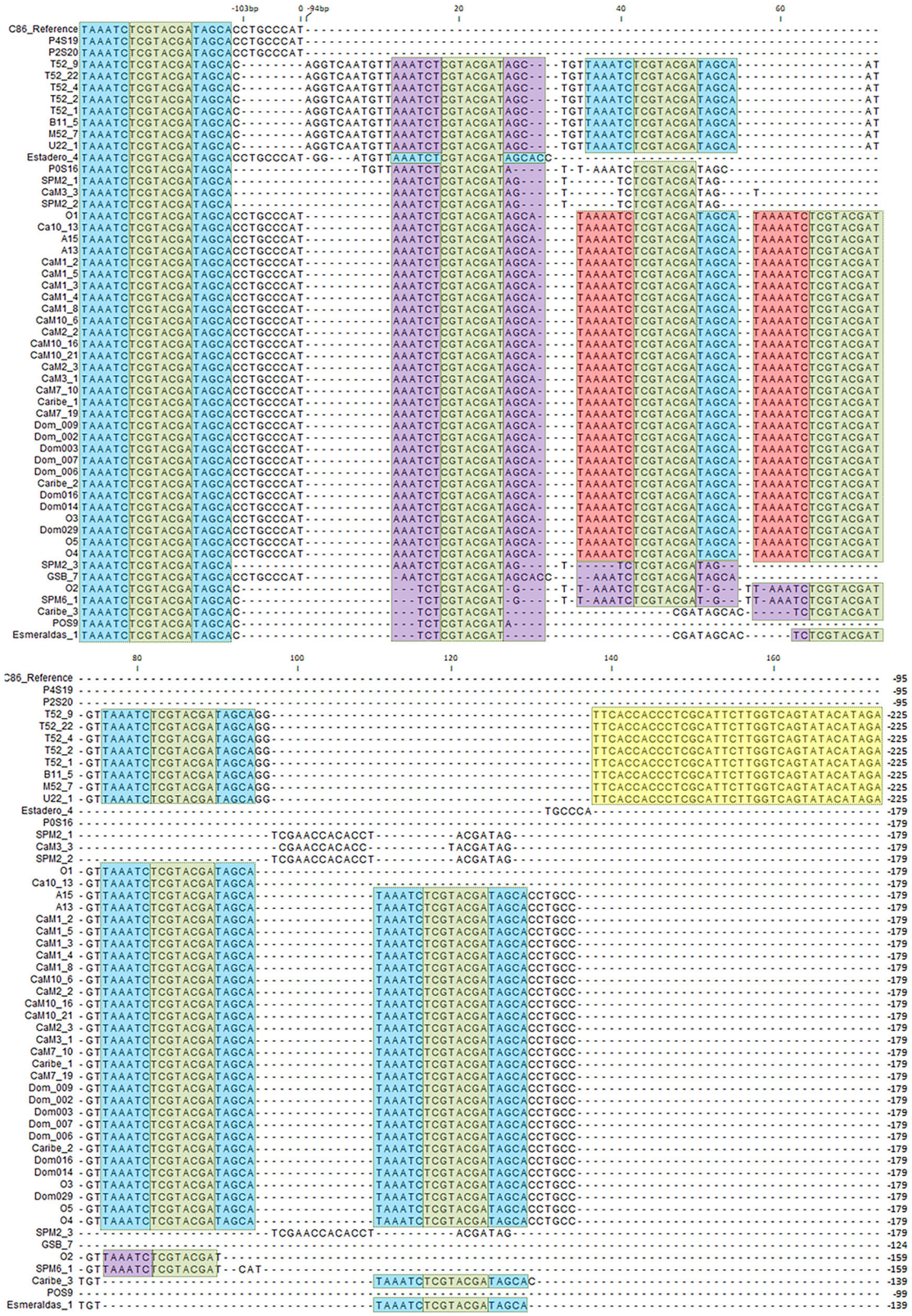

FIGURE 5. Analysis of the insertions in the promoter of the *Pfcyp51* gene in *Pseudocercospora fijiensis* strains from various countries. Insertions are generally located from 94 to 103 bp upstream of the start codon of the gene. Element 'A' is marked with blue together with the palindromic arrangement TCGTACGA marked in green. Alterations of element 'A' are marked with red and partial constructions of the element with purple. Part of the novel insertion just identified in Philippines isolates, element 'B', is marked with light yellow. Negative values on the right represent the position from the beginning of the insertion related to the start codon of the gene.

© 2021 The Authors.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

fijiensis isolates with the combination T18I + V107D + Y137F + Y461D (genotypes G22, G23 and G24) differed in DMI sensitivity, depending on the number of insertions in their promoters (Table S3). Similarly, genotypes G35 and G36 (T18I + V107D + A311G + Y461N) do not differ in PfCYP51 substitutions; nonetheless, the three G36 isolates with promoter insertions display higher EC_{50} values, and highest values are observed in isolates carrying three insertions (Table S3).

In 98 isolates, the insertions substitute a stretch of 8–27 bp starting at position 103 or 102 bp upstream of the start codon, for example, in the Philippine isolate T52_22 an 8 bp region is substituted by an insertion of 123 bp at position −102 bp. Others have accumulated multiple substitutions, such as isolate CaM3_3 from Costa Rica, which has one 16 bp exchange for a 9 bp fragment at position −103 bp and a second substitution of 7 bp with a 76 bp fragment, localized at −94 bp (Table S4). In addition, 38 isolates contain an insertion at position −94 bp. Two isolates from Cameroon, P2S20 and P4S19, have a substitution followed by an insertion at position 157 bp upstream of the start codon. The Philippine isolates (M52_4, M52_9, M52_23 and U22_3) show a deletion of eight nucleotides, 'CATGGACC', in the promoter region beginning 97 bp upstream of the start codon. Generally, most insertions comprise one or more copies (or partial/modify/reverse copies) of the genomic element, 'TAAATCTCGTACGA-TAGCA'.[12] This is present as a single element in reference isolate CIRAD86, originally located a few nucleotides downstream in the promoter, indicated as element 'A'[11, 12] (Figures 4 and 5).

Despite the geographical differences in isolates, we identified very similar insertions in the *Pfcyp51* promoter. Overall, limited numbers of substitutions and insertions were observed although at variable positions (Figure S5). Some isolates contain a partial construction of element 'A' in their insertions, whereas others have a reverse or modified element due to a few additional nucleotides. Interestingly, promoter element 'A' and all its variants contains a palindromic DNA core sequence, 'TCGTACGA', present at least twice in the isolates that contain an insertion (Figures 4, 5 and S5) and up to six copies in the resistant isolates (Tables S3 and S4). For example, Philippine isolate T52_22 possesses three copies of element 'A' and one partial copy, resulting in four copies of the palindrome. In a similar way, the Ecuadorian isolates RCQS_3 and RCQS_16 possess one copy of the 'A' element, but three of the palindromic sequences, two of them in partial stretches of 'A' (Figure S5 and Table S4). The smallest insertion, found in isolate POS9 from Cameroon encodes a single 'A' element, but two copies of the palindrome (Figure 5). The presence of three or more palindromic insertions correlates with strongly reduced DMI sensitivity (Tables S3 and S4). Interestingly, mutation Y137F only occurred in isolates with multiple promoter insertions (three or more 'TCGTACGA'). The detailed gene configurations of representative isolates with reduced sensitivity are presented in Tables S3 and S4 and Figure 6. Although geographically different isolates show very similar insertions in the *Pfcyp51* promoter, we found an additional and unique insertion in Philippine isolates. This 39 bp insertion, 'TTCACCACCCTCGCATTCTTGGTCA-GTATAC-ATAGACCT', which we indicate as the 'B' element, is present in eight Philippine isolates (Figures 6, 7 and S5). The 'B' element also encompasses a palindromic 6 bp DNA fragment 'GTATAC'.

Pfcyp51 variability supports reduced DMI sensitivity

Substitutions A311G, Y137F, H378N, Y461D and D458V are the main explanatory modulations related to increasing EC_{50} values and hence, reduced sensitivity to the tested DMIs (Table 4). Additional candidate mutations for a main effect were A379G, A444S, T18I, Y461N and D458E based on a ratio of 20 for inclusion compared with the mean square error ($P < 0.00001$). However, these were less consistent, so a combination among the substitutions could be more plausible. Retaining the first five mutations and adding the main effect of the *Pfcyp51* promotor and the fungicide treatment resulted in an even higher EC_{50} predictive power. Figure 7 shows that the number of insertions in the *Pfcyp51* promoter corresponds with reduced fungicide sensitivity, indicated by the number after the five binary position representing the mutational main effect

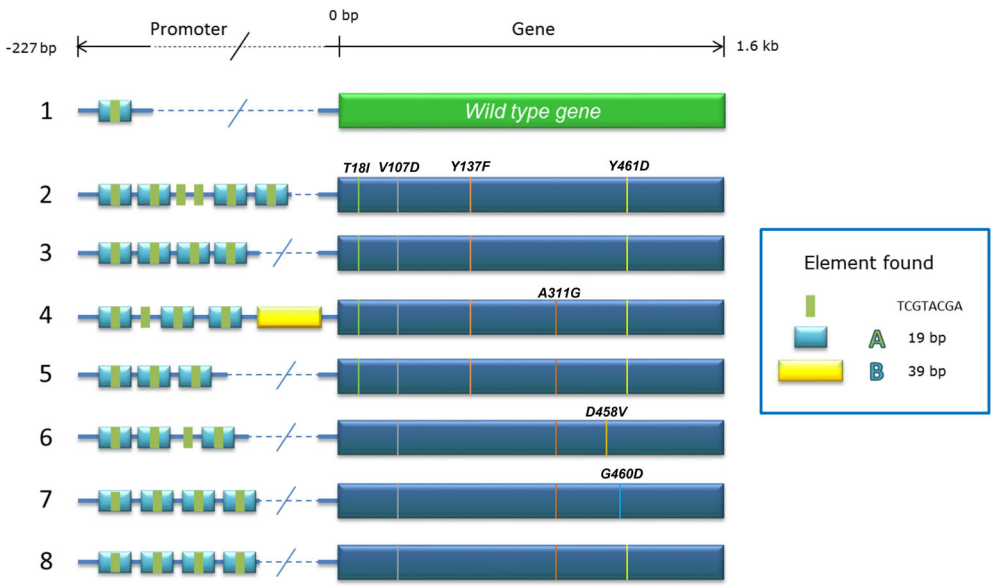

FIGURE 6. Representation of the *Pfcyp51* gene. Genomic configuration of elements of the most representative resistant genotypes are shown with insertions in the promoter of the *Pfcyp51* gene. Vertical lines in the coding domain of the *Pfcyp51* gene represent the different CYP51 codon position substitutions: (1) reference genotype G1; (2) resistant genotype G24; (3) resistant genotype G23; (4) resistant genotype G43 (Philippines); (5) resistant genotype G42; (6) resistant genotype G13; (7) resistant genotype G25; and (8) resistant genotype G18.

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

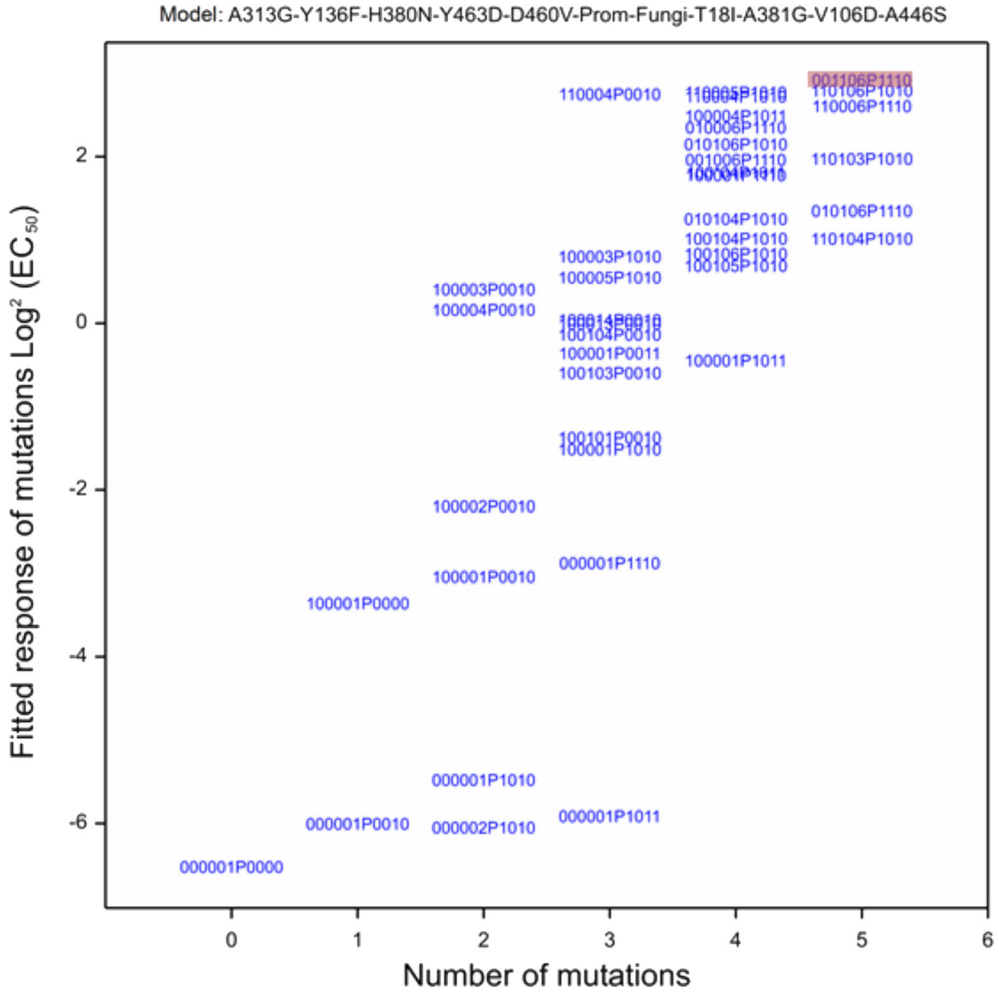

FIGURE 7. Predicted interaction of the accumulation of specific CYP51 substitutions with the sensitivity response on propiconazole fungicide. The genotype number codes are represented by the presence/absence of substitutions (1/0 matrix) with the exception of the *Pfcyp51* palindromic promoter insertions that have six levels. The 11 number codes follow the chosen fungicide correlated model: (1) A313G (A311G); (2) Y136F (Y137F); (3) H380N (H378N); (4) Y463D (Y461D); (5) D460V (D458V); (6) promoter insert numbers; (7) fungicide name; (8) T18I (T18I); (9) A381G (A379G); (10) V106D (V107D); and (11) A446S (A444S). The substitutions are placed from left to right in order of importance where the first is the most interactive and the last is the least interactive. For practical reasons number code 7 has been labelled for the fungicide (P for propiconazole). For example, model resistant genotype code 001106P1110 (marked in light red) has five substitutions: H380N (H378N), Y463D (Y461D), T18I (T18I), A381G (A379G) and V106D (V107D) with six promoter palindromic inserts and it has been predicted as resistant ($\log_2 EC_{50} > 0$) in the interaction with the fungicide propiconazole.

and before the fungicide letter (P). The inclusion of the fungicide factor demonstrates the main effect of the treatment but only propiconazole (P) is shown in Figure 7 as the differences were too small for difenoconazole and epoxiconazole.

Next, all first-order interactions were evaluated and added if significant, followed by backward selection to check out the specific combinations that had most predictive power. Substitutions T18I, A379G and A444S are again indicated but in combination with one or the other and a new mutation V107D was put forward in this context. Also, the interaction between Y137F and A311G, which were both already suspected to confer a main effect in the model, is assessed as important. This combination again reduces the sensitivity to the DMIs as can be seen from the parameter estimate, and seemingly this is attributed to Y137F, similar to its combination with A379G that resulted in a lower sensitivity. Finally, the interactions between promotor insertions with all mutations were checked, but none was found to specifically affect any DMI fungicide sensitivity. This means that either the interaction with the fungicide is already covered by a mutation or that no specific mutation was involved with a specific fungicide interaction. Figure 7 represents the effect of the accumulation of these crucial mutations for DMI sensitivity. In the lower left is the sensitive reference 'without' mutation and the 'simplest' promotor of *Pfcyp51*. The upper right strain(s) have the maximum number of accumulated mutations as an additive effect together with most promotor insertions.

DISCUSSION

Control of fungal plant pathogens strongly relies on a limited set of fungicides.[24] DMI fungicides are cornerstone antifungals for industry and contemporary disease management strategies in humans, livestock and plants.[10–16,24–39] In this paper we address their use for BLSD control and describe the global occurrence and mechanisms involved in reduced sensitivity of *P. fijiensis*. This consequently leads to excessive fungicide applications to maintain control of BLSD in bananas. The dispersal and magnitude of DMI fungicide resistance in *P. fijiensis* urgently requires an

 © 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

TABLE 4. Regression analyses of *Pfcyp51* mutations on DMIs efficacy

Accumulated analysis of variance

Substitution change	Degrees of freedom	Sum squares	Mean squares	Variance ratio
+ A313G (A311G)	1	1876.24	1876.24	2489.04
+ Y136F (Y137F)	1	2268.64	2268.64	3009.60
+ H380N (H378N)	1	508.66	508.66	674.79
+ Y463D (Y461D)	1	116.14	116.14	154.07
+ D460V (D458V)	1	110.48	110.48	146.57
+ Promoter	5	205.53	41.11	54.53
+ Fungicides	2	64.44	32.22	42.74
+ T18I.A381G (T18I.A379G)	1	51.55	51.55	68.39
+ V106D. A446S (V107D.A444S)	1	148.27	148.27	196.70
+ Y136F. A313G (Y137F.A311G)	1	222.94	222.94	295.75
+ Y136F.A381G (Y137F.A379G)	1	44.60	44.60	59.17
Residual	627	472.63	0.75	
Total	643	6090.13	9.47	

This table shows the fitted model[a] with the relevant factors (amino acid substitutions and promoter insertions, *F*-test <0.001) that remain from 23 factors evaluated. Factors are in descending order of importance base on the accumulated analyses of the variance ratio. The threshold of including a variable was heuristically set to a variance ratio of 10, which gave 11 factors as predictor for the loss of sensitivity to demethylation inhibitors. This final model[a] was checked by backward elimination to see if any previously included terms became superfluous.
[a] Fitted model: A313G–Y136F–H380N–Y463D–D460V–Prom–Fungi–T18I–A381G–V106D–A446S.

understanding of the underlying mechanisms to develop new and sustainable control strategies. Here, we analysed an unparalleled set of *P. fijiensis* isolates obtained from populations in countries with varying disease management practices, hence our *P. fijiensis* isolate sets cover and represent a broad array exposed to various BLSD management scenarios. This enables global analysis of the causal relationship between DMI fungicide applications and the occurrence of reduced sensitivity in *P. fijiensis* using prime genetic dynamics. The distribution of EC_{50} values across all isolates revealed a continuous range of DMI sensitivity for all tested fungicides (Figure S3). This complicates setting clear cut-off values to discern statistically significantly different sensitivity groups, hence we used EC_{50} criteria to define these groups. These permitted analyses based on the non-synonymous mutations in *Pfcyp51*, its promoter characteristics, and the origin of the samples.

Resistance mechanisms in isolates with reduced sensitivity were always correlated with mutations in *Pfcyp51* and insertions in its promoter region. All resistant isolates exclusively originated from commercial banana farms, especially from Costa Rica, Colombia and the Philippines, where banana production is economically very important and the number of fungicide applications per season is high (Figure 1 and Table S2). Costa Rica has a long history of BLSD control associated with a continuously increasing number of fungicides applications per year.[9,12,18] In parallel, we isolated the most resistant isolates from Costa Rica and have recently proven the association between their genetic constitution and DMI sensitivity.[12] Evidently, continuous fungicide applications expose populations to extreme selection pressure and a consequent shift towards resistance, for example 99% of the Costa Rican isolates had EC_{50} values higher than 1 mg L^{-1} for propiconazole. Although field doses are sometimes 100 times higher, depending on the carrier solution of the active ingredient, the actual propiconazole doses inside banana leaf range between 1 and 5 mg L^{-1} during the first 4 days of application.[48] Most isolates from remote areas were sensitive, likely due to the absence of selection pressure. Indeed, recent findings for strobilurins suggested that these remote areas are genetically isolated from large commercial banana plantations, as indicated by their population genetic parameters.[57] For DMIs, similar mechanisms seem to be operational. Hence, the rare occurrence of reduced sensitivity in overall sensitive populations seems to be largely due to genetic drift.

Although we lack detailed information on the actual number of DMI applications per sampling location, it seems that the number of resistant isolates increases parallel with the overall number of fungicide applications (Figure 1 and Table S2) underpinning the selective pressure exerted by multiple applications of DMIs in the 30 years since their introduction.[58] The relatively low percentage of resistant isolates in Ecuador (difenoconazole 16.83%, epoxiconazole 8.91% and propiconazole 21.78%) might reflect the climate of the country, with long dry seasons, reducing BLSD development and consequently resulting in a lower frequency of fungicide applications.[18] Changing weather conditions across the globe affect BLSD control, and fungicide applications typically reduce during dry spells.[7,17,23] Therefore, it would be worth continually monitoring DMI sensitivity in *P. fijiensis* populations in these areas, including Ecuador. Incursions of *P. fijiensis* into Martinique and Guadalupe happened in 2010 and 2012, respectively.[20,59] For that reason, exposure to DMIs has been limited, hence the selective pressure is lower, which accords with our results as all *P. fijiensis* isolates were sensitive. The null hypothesis is that these islands were colonized by wild-type *P. fijiensis* isolates. The alternative hypothesis considers that, in the absence of selection pressure, the fitness costs of reduced sensitivity, driven by accumulated mutations, forces the population back to sensitivity. In most fungi, however, such reversion to sensitivity has not been observed except in *Magnaporthe oryzae* and *Cercospora beticola*.[60] For example, ceasing DMI applications for a period of 4 years in the 'San Pablo' farm in Costa Rica hardly affected the frequency of resistant *P. fijiensis* strains in the population.[61] More recently, we observed near fixation of resistance to strobilurin fungicides in *P. fijiensis* populations of three commercial plantations in Costa Rica,[57] but sensitivity in nearly all strains from an

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

untreated trial site at San Carlos, approximately 100 km away.[12,57] Moreover, we have identified exclusively wild-type isolates in non-sprayed areas such as, Bohol in the Philippines, Ebonji and Tombel in Cameroon,[61] Bejuquillo in Colombia and Esmeraldas in Ecuador.[12] Therefore, we consider the abovementioned alternative hypothesis unlikely. Furthermore, GBS analysis shows that genetic variation across all isolates is better explained by their geographical origin rather than the degree of DMI sensitivity. This suggests that the evolution of resistant genotypes occurs independently and hence favours the null hypothesis.[62]

The sequencing data for *Pfcyp51* across all populations highlight a peculiarity of the CIRAD86 reference isolate—originating from Cameroon—that was selected for the first genetic linkage map and genome sequencing.[63] It encodes V106 (SEPPTR D107), whereas the sequences of all 268 genotyped isolates encode D106. With the suggested centre of origin of *P. fijiensis* in Southeast Asia, we propose that the wild-type genotype is D106 rather than V106, which is also supported by the corresponding position D107 of the *Ztcyp51B* orthologue.[50] This may indicate that the proposed additive role of V107D for DMI resistance is an artefact, based on a mutation in the reference CIRAD86 and underscores the need for more genomic information from the centre of origin.

It is apparent that the genetic effects of the DMI application on *P. fijiensis* populations are solely targeted on modifications on the *Pfcyp51*.[11] Most *Pfcyp51* modulations parallel the DMI fungicide resistance response and are comparable with those identified in other organisms. Substitutions V137A and I379V are correlated with reduced sensitivities to triadimenol in *Erysiphe necator* and to tebuconazole in *Z. tritici*, respectively.[24] The accumulation of mutations tends to confer increased resistance to DMI.[24] Here, we were unable to determine such specific substitutions for any of the tested fungicides, which might be due to the high number of factors analysed (individual mutations, mutation combination and seven levels of promoter insertions) and hence, further studies may identify unique mutation/efficacy interactions. Sensitive isolates also show variation in *Pfcyp51* with a maximum of three aa changes. Overall, the maximum number of amino acid substitutions was found in Philippines isolates, which accumulated up to seven amino acid substitutions in the coding region of *Pfcyp51*. Such a high degree of polymorphism in CYP51 was previously reported for *Oculimacula* (*Tapesia*) *acuformis* and *O. yallundae*.[35] The substitutions resulting in A19E, I71M, D72E, V261L, I265T, H378N, R416G, D458E, D458V, Y459N, Y459S, ΔY459 and G460D were hitherto unknown in *P. fijiensis*, although other changes in positions 461 and 462 (SEPTTR 459 and 460) were reported to affect DMI sensitivity.[12,13,40] Substitutions A19E, Y59F, V117L and R416G were solely detected in DMI sensitive isolates, suggesting that these represent natural random variation, uncorrelated with DMI sensitivity. Notably, substitution *I265T* – although also detected in a DMI-sensitive isolate – was correlated with additive reduced efficacy of DMIs. Similarly, substitutions T18I and A444S are present in both sensitive and resistant isolates, but also correlated with additive effects in isolates with reduced sensitivity. These observed additive effects might be explained as compensatory substitutions for DMI sensitivity as illustrated by aa changes at positions 459 to 461 in ZtCYP51, compensating the I381V substitution that was, by itself, enzymatically lethal as corroborated by complementation experiments in *Saccharomyces cerevisiae*.[25] Nevertheless, these modifications urge for additional studies to elucidate their contribution to *P. fijiensis* survival.

Substitutions A311G, Y137F, H378N, Y461D and D458V are directly correlated with resistance[24,50] (Table 4 and Figure 7). Substitution *Y137F* was also linked with DMI resistance in *Penicillium italicum*, *Uncinula necator* and *Blumeria graminis* f. sp. *hordei*.[35,38] A substitution at Y136 (SEPTTR Y137), or its equivalent in other species, is the most frequently observed modification of CYP51 in pathogenic fungi.[24,50] Interestingly, Y137F may originate from two sequential codons. The wild-type codon is TAC, whereas the modified codons are TTC and TTT, but these mutations could also have arisen independently. The latter is unique for the Costa Rican population.

Substitutions at positions 136, 313 and 381 (SEPTTR 137, 311 and 379) are all in the SRS. Changes in positions 460 to 463 (SEPTTR 458 to 461) are usually not described in the vicinity of the SRS[24] but may compromise the three-dimensional structure of the protein resulting in an affinity change (Figure 3). The deletion of ΔY459 provoked a shift in positions 523 to 526 (SEPTTR 521–524) introducing S523 into the active site and pushing S526 from its original position (Figure 3). Additional studies are required to elucidate how these changes affect the structure of the catalytic centre.

The presence of repeated elements and insertions in the promotor region of *Pfcyp51* explains overexpression of the gene.[12] As previously shown, promoter insertions positively correlate with resistance to DMIs[12] (Table 4 and Figure 7). None of the sensitive isolates contained insertions although they were very common in tolerant and resistant isolates. As in many other species, these insertions were also associated with non-synonymous mutations in the coding region.[27,29,31,33,37,64] All these inserts vary in size and nature across species and are not located at equal positions and clearly result from independent events, which raise the question about their origin. They might be remains of transposable element activity, some of which contain powerful promoters.[24,65] In *P. fijiensis* we found three independent promoter insertions, at −94, −103 and − 157 bp from the start codon. The latter was present in only two isolates from Cameroon. However, all isolates with insertions containing tandem copies of the palindromic sequence in the 'A' element were at least tolerant to the tested DMIs (above 0.1 mg L^{-1}) (Tables S3, S4 and Figure S5).

Palindromic motifs constitute an important group of regulatory elements in eukaryotes in which they act as cis elements.[66] Many transcription factors bind palindromic sequences with high affinity.[67,68] For example, the transcription factor ADR1 binds as a monomer to palindromic sequences to regulate expression of the ADH2 gene in *S. cerevisiae*.[69] In *Cercospora nicotianae* the transcription factor CRG1 binds to a palindrome sequence present in genes that confer resistance to cercosporin.[70] The group of bZIP transcription factors target palindromic DNA sequences as dimers, thereby regulating, for example, secondary metabolism.[65] The importance of the palindromic sequences might explain the existence of isolates with different 'A' element configurations but with similar DMI resistance levels (Figures 5 and S5). A second palindromic sequence, inserted in the 'B' element, was present in the *Pfcyp51* promotor of Philippine isolates. Because of the absence of intermediate isolates containing only this 'B' element, the correlation with *Pfcyp51* gene expression could not be established. Other isolates also containing four copies of the 'A' element but without the 'B' element had similar EC_{50} values.

In summary, the 'A' element and particularly its palindromic core is important for the regulation of gene expression, most likely as a transcriptional enhancer.[12, 37, 39] The mechanism and components involved, however, remain to be elucidated. Future work will aim to characterize the mechanism and identify the involved TFs and additional determinants.[39] Promoter insertions of the 'A'

 © 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

element tend to confer higher EC_{50} values regardless of the DMI fungicide and might be the reason why we were unable to determine specific substitutions discriminating for the tested fungicides. This might suggest that the effect of the promoter insertion can mask the specific interaction between a substitution and a particular fungicide and induce some degree of cross-resistance among DMI fungicides. Interestingly, only isolates with PfCYP51 substitutions in positions 136, 313, 380, 381 and 460–463 (SEPTTR 137, 311, 379 and 458 to 460) show insertions in the promoter region. This suggests that the selection for overexpression occurs only after the emergence of *Pfcyp51* point mutations leading to reduced sensitivity. Our previous transformation study indicated that insertions alone do not significantly increase DMI resistance.[12] For this reason, we conclude that the main resistance factors are the mutations in the *Pfcyp51* gene and that the insertions in the promoter region induce additive effects.

Three isolates from Costa Rica, CaM10_6, CaM1_5 and CaM3_1, revealed extraordinarily high EC_{50} values that remain unexplained solely by the *Pfcyp51* promoter configuration, which was similar to other, less-resistant isolates from Costa Rica. This may suggest the presence of additional genetic components that may indirectly modulate resistance as observed in *O. yallundae*.[36] These may include minor fungicide resistance genes, genes linked with detoxification, stress responses or growth rates. Nonetheless, a previous analysis using an unbiased genetic approach by means of crosses between resistant and sensitive isolates (CaM10_6 x Bo_1) confirmed *Pfcyp51* as the single explanatory gene for reduced DMI sensitivity.[11] The current study significantly contributes to the understanding of the origin and dissemination of DMI sensitivity mechanisms in *P. fijiensis* populations and facilitates the prediction of the efficacy of new generations of fungicides.

ACKNOWLEDGMENTS

This work was supported in part by the Ecuadorian government through the Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (SENESCYT), Ecuadorian University, Escuela Superior Politécnica del Litoral (ESPOL), Centro de Investigaciones Biotecnológicas del Ecuador (CIBE) and Syngenta AG. PC is a graduate student in the Wageningen University and Research (WUR) banana program, RA was supported by the Universidad National de Colombia, sede Medellín. GHJK and HJGM are supported by the Dutch Dioraphte Foundation. We gratefully acknowledge María Isabel Jiménez, María Jama and Rufino Meza for their help in collecting and providing the Ecuadorian samples, and to Vicente Rey from AUGURA-Cenibanano for his help in collecting Colombian Isolates. Finally, we thank Caucasella Díaz, Tatiana Chavez, Carla Maté Goldar and Aikaterini Vichou for their contribution to the laboratory work, and Pieter Vereijken for his support in data analyses. Banana research at WUR is financially supported by the Dutch Dioraphte Foundation.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Kema GHJ and Drenth A eds, *Achieving Sustainable Cultivation of Bananas*, Vol. **1**. Burleigh Dodds Science Publishing, Cambridge (2018).
2 Ploetz RC, Kema GHJ and Ma LJ, Impact of diseases on export and smallholder production of banana. *Annu Rev Phytopathol* **53**:269–288 (2015).
3 Shively G and Hao J, *A Review of Agriculture, Food Security and Human Nutrition Issues in Uganda*. Purdue University, Department of Agricultural Economics, West Lafayette, USA: Contract No.: 12-3, (2012).
4 Zeitoun M, Allan T, Al Aulaqi N, Jabarin A and Laamrani H, Water demand management in Yemen and Jordan: addressing power and interests. *Geograph J* **178**:54–66 (2012).
5 Mourichon X, Carlier J, Fouré E. Sigatoka leaf spot diseases. *Musa Disease Fact Sheet No 8*. (1997). https://www.bioversityinternational.org/e-library/publications/detail/sigatoka-leaf-spot-diseases/
6 Stover RH and Simmonds NW, *Bananas*, 3rd edn. Wiley, New York, p. 468 (1987).
7 Risède J-M, Lescot T, Cabrera JC, Guillon M, Tomekpé k, Kema GHJ, et al. Challenging Short and Mid-Term Strategies to Reduce the Use of Pesticides in Banana Production. ENDURE, editor. (2010. Available: https://agritrop.cirad.fr/553854/1/document_553854.pdf. [14 May 2020]
8 van Wendel de Joode B, Barbeau B, Bouchard MF, Mora AM, Skytt A, Cordoba L *et al*., Manganese concentrations in drinking water from villages near banana plantations with aerial mancozeb spraying in Costa Rica: results from the Infants' Environmental Health Study (ISA). *Environ Pollut* **215**:247–257 (2016).
9 Chong P, Carr C, Murillo G, Guzmán M, Sandoval J and Kema GHJ, An historical treatise and critical review of BLSD control in banana production, in *The Origin, Versatility and Distribution of Azole Fungicide Resistance in the Banana BLSD Pathogen Pseudocercospora fijiensis*, ed. by Chong P. Ph.D. Wageningen University, Wageningen, pp. 23–58 (2016).
10 de Lapeyre de Bellaire L, Essoh Ngando J, Abadie C, Chabrier C, Blanco R, Lescot T *et al*., Is chemical control of *Mycosphaerella* foliar diseases of bananas sustainable? *Acta Hortic* **828**:161–170 (2009).
11 Chong P, Vichou AE, Schouten HJ, Meijer HJG, Arango Isaza RE and Kema GHJ, *Pfcyp51* exclusively determines reduced sensitivity to 14alpha-demethylase inhibitor fungicides in the banana BLSD pathogen *Pseudocercospora fijiensis*. *PLoS ONE* **14**:e0223858 (2019).
12 Diaz-Trujillo C, Chong P, Stergiopoulos I, Cordovez V, Guzman M, De Wit P *et al*., A new mechanism for reduced sensitivity to demethylation-inhibitor fungicides in the fungal banana BLSD pathogen *Pseudocercospora fijiensis*. *Mol Plant Pathol* **19**:1491–1503 (2018).
13 Cañas GP, Velasquez MJA, Florez JMR, Rodríguez P, Moreno CX and Arango R, Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. *Pest Manage Sci* **65**:892–899 (2009).
14 Lepesheva GI and Waterman MR, CYP51-the omnipotent P450. *Mol Cell Endocrinol* **215**:165–170 (2004).
15 Akins RA and Sobel JD, Antifungal targets, mechanisms of action, and resistance, in *Candida albicans*. Antimicrobial Drug Resistance, ed. by Mayers D, Sobel J, Ouellette M, Kaye K and Marchaim D. Springer, Cham, pp. 347–407 (2009).
16 Warrilow AG, Parker JE, Kelly DE and Kelly SL, Azole affinity of sterol 14alpha-demethylase (CYP51) enzymes from *Candida albicans* and *Homo sapiens*. *Antimicrob Agents Chemother* **57**:1352–1360 (2013).
17 Ploetz R, Black Sigatoka. *Pestic Outlook* **11**:19–23 (2000).
18 Marín DH, Romero RA, Guzmán M and Sutton TB, Black Sigatoka: an increasing threat to banana cultivation. *Plant Dis* **87**:208–222 (2003).
19 Churchill AC, *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. *Mol Plant Pathol* **12**:307–328 (2011).
20 Guzmán M, Orozco M, Pérez L. Sigatoka leaf spot diseases of bananas: dispersion, impact and evolution of management strategies in Latin America-Caribbean region. XX Reunião Internacional da Associação para a Cooperação em Pesquisa e Desenvolvimento Integral das Musáceas (Bananas e Plátanos) 9 a 13 de setembro de 2013 - Fortaleza, CE, Brasil (2013).
21 Vincellin P. Some principles of fungicide resistance. Agriculture & Natural Resources. *PPFS-MISC-02*. (2014). Available: https://plantpathology.ca.uky.edu/files/ppfs-misc-02.pdf [14 May 2020]
22 Robert S, Ravigne V, Zapater MF, Abadie C and Carlier J, Contrasting introduction scenarios among continents in the worldwide invasion of the banana fungal pathogen *Mycosphaerella fijiensis*. *Mol Ecol* **21**:1098–1114 (2012).

© 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

23 Lapeyre L, Eric F, Abadie C and Carlier J, Black leaf streak disease is challenging the banana industry. *Fruits* **65**:327–342 (2010).
24 Cools HJ, Hawkins NJ and Fraaije BA, Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathol* **62**:36–42 (2013).
25 Becher R and Wirsel SG, Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. *Appl Microbiol Biotechnol* **95**:825–840 (2012).
26 Villani SM, Hulvey J, Hily JM and Cox KD, Overexpression of the CYP51A1 gene and repeated elements are associated with differential sensitivity to DMI fungicides in *Venturia inaequalis*. *Phytopathology* **106**:562–571 (2016).
27 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).
28 Bean TP, Cools HJ, Lucas JA, Hawkins ND, Ward JL, Shaw MW *et al.*, Sterol content analysis suggests altered eburicol 14alpha-demethylase (CYP51) activity in isolates of *Mycosphaerella graminicola* adapted to azole fungicides. *FEMS Microbiol Lett* **296**:266–273 (2009).
29 Cools HJ, Bayon C, Atkins S, Lucas JA and Fraaije BA, Overexpression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Manage Sci* **68**:1034–1040 (2012).
30 Albarrag AM, Anderson MJ, Howard SJ, Robson GD, Warn PA, Sanglard D *et al.*, Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in *cyp51A*, upregulation of *cyp51A*, and integration and activation of transposon Atf1 in the *cyp51A* promoter. *Antimicrob Agents Chemother* **55**:5113–5121 (2011).
31 Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M *et al.*, A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob Agents Chemother* **51**:1897–1904 (2007).
32 Verweij PE, GHJ K, B BZ and Melchers WJ, Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*. *Pest Manage Sci* **69**:165–170 (2013).
33 Ma Z, Proffer TJ, Jacobs JL and Sundin GW, Overexpression of the 14α-demethylase target gene (CYP51) mediates fungicide resistance in *Blumeriella jaapii*. *Appl Environ Microbiol* **72**:2581–2585 (2006).
34 Eddouzi J, Parker JE, Vale-Silva LA, Coste A, Ischer F, Kelly S *et al.*, Molecular mechanisms of drug resistance in clinical *Candida* species isolated from Tunisian hospitals. *Antimicrob Agents Chemother* **57**:3182–3193 (2013).
35 Albertini C, Gredt M and Leroux P, Polymorphism of 14α-demethylase gene (CYP51) in the cereal eyespot fungi *Tapesia acuformis* and *Tapesia yallundae*. *Eur J Plant Pathol* **109**:117–128 (2003).
36 Dyer PS, Hansen J, Delaney A and Lucas JA, Genetic control of resistance to the sterol 14α-demethylase inhibitor fungicide prochloraz in the cereal eyespot pathogen *Tapesia yallundae*. *Appl Environ Microbiol* **66**:4599–4604 (2000).
37 Schnabel G and Jones AL, The 14α-demethylase (CYP51A1) gene is overexpressed in *Venturia inaequalis* strains resistant to Myclobutanil. *Phytopathology* **91**:102–110 (2001).
38 Délye C, Laigret F and Corio-Costet MF, 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).
39 Bolton MD, Ebert MK, Faino L, Rivera-Varas V, de Jonge R, Van de Peer Y *et al.*, RNA-sequencing of *Cercospora beticola* DMI-sensitive and -resistant isolates after treatment with tetraconazole identifies common and contrasting pathway induction. *Fungal Genet Biol* **92**:1–13 (2016).
40 Chong P, Arango R, Stergiopoulus I, Guzmán M, Crous P, Silva G *et al.*, Analysis of azole fungicide resistance in *Mycosphaerella fijiensis*, causal agent of black Sigatoka, in *Proceedings of the 16th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds; April 25–29, 2010*, ed. by Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE and Lyr H. Friedrichroda, Germany: Braunschweig: DPG Selbstverlag., pp. 217–222 (2011).
41 Hayden HL, Carlier J and Aitken EAB, Genetic structure of *Mycosphaerella fijiensis* populations from Australia, Papua New Guinea and the Pacific Islands. *Plant Pathol* **52**:703–712 (2003).
42 Halkett F, Coste D, Platero GG, Zapater MF, Abadie C and Carlier J, Genetic discontinuities and disequilibria in recently established populations of the plant pathogenic fungus *Mycosphaerella fijiensis*. *Mol Ecol* **19**:3909–3923 (2010).
43 Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H *et al.*, Diversity arrays technology: a generic genome profiling technology on open platforms. *Methods Mol Biol* **888**:67–89 (2012).
44 Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES *et al.*, A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* **6**:e19379 (2011).
45 R-Development-Core-Team, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2008). Available: https://www.R-project.org/. [14 May 2020].
46 Carbone I and Kohn LM, A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**:553–556 (1999).
47 Peláez JE, Vásquez LE, Díaz TJ, Castañeda DA, Rodríguez E and Arango R, Use of a micro title plate dilution assay to measure activity of antifungal compounds against *Mycosphaerella fijiensis*, Morelet. *Rev Fac Nac Agron Medellín* **59**:3425–3433 (2006).
48 Ciba-Geigy, *Tilt Technical Dossier*. Ciba-Geigy, Basle, Switzerland (1991).
49 Ramsay JO, Monotone regressions splines in action. *Stat Sci* **3**:425–441 (1988).
50 Mair W, Lopez-Ruiz F, Stammler G, Clark W, Burnett F, Hollomon D *et al.*, Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. *Pest Manage Sci* **72**:1449–1459 (2016).
51 Bailey TL and Elkan C eds, *Fitting a Mixture Model by Expectation Maximization to Discover Motifs in Biopolymers. Proceedings of the 2nd International Conference of Intelligent Systems for Molecular Biology*. AAAI Press, Menlo Park, CA (1994).
52 Frith MC, Saunders NF, Kobe B and Bailey TL, Discovering sequence motifs with arbitrary insertions and deletions. *PLoS Comput Biol* **4**:e1000071 (2008).
53 Cartegni L, Wang J, Zhu Z, Zhang MQ and Krainer AR, ESEfinder: a web resource to identify exonic splicing enhancers. *Nucleic Acids Res* **31**:3568–3571 (2003).
54 Finlay KW and Wilkinson GN, The analysis of adaptation in a plant-breeding programme. *Aust J Agr Res* **14**:742–754 (1963).
55 Eberhart SA and Russell WA, Stability parameters for comparing varieties. *Crop Sci* **6**:36–40 (1966).
56 Arzanlou M, Groenewald JZ, Fullerton RA, Abeln ECA, Carlier J, Zapater M-F *et al.*, Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. *Persoonia* **20**:19–37 (2008).
57 Arango RE, Diaz C, Dhillon B, Aerts A, Carlier J, Crane CF *et al.*, Combating a global threat to a clonal crop: banana BLSD pathogen *Pseudocercospora fijiensis* (synonym *Mycosphaerella fijiensis*) genomes reveal clues for disease control. *PLoS Genetics* **12**:e1005876 (2016).
58 Stover RH, Sigatoka leaf spots: Thirty years of changing control strategies 1959–1989, in *INIBAP Proceedings International Worshop*, ed. by Fullerton RA. INIBAP, San José, Costa Rica, pp. 66–74 (1990).
59 Ioos R, Hubert J, Abadie C, Duféal D, Opdebeeck G and Iotti J, First report of black Sigatoka disease in banana caused by *Mycosphaerella fijiensis* on Martinique Island. *Plant Dis* **95**:359 (2011).
60 Hollomon DW, Fungicide resistance: facing the challenge. *Plant Prot Sci* **51**:170–176 (2015).
61 Guzmán M. Estrategias para el manejo de la resistencia de *Mycosphaerella fijiensis* Morelet, agente causal de la Sigatoka negra en banano, a fungicidas triazoles. Master's thesis, Universidad de Costa Rica; Costa Rica (2007).
62 Rieux A, Halkett F, de Lapeyre de Bellaire L, Zapater MF, Rousset F, Ravigne V *et al.*, Inferences on pathogenic fungus population structures from microsatellite data: new insights from spatial genetics approaches. *Mol Ecol* **20**:1661–1674 (2011).
63 Manzo-Sanchez G, Zapater MF, Luna-Martinez F, Conde-Ferraez L, Carlier J, James-Kay A *et al.*, Construction of a genetic linkage map of the fungal pathogen of banana *Mycosphaerella fijiensis*, causal agent of black leaf streak disease. *Curr Genet* **53**:299–311 (2008).
64 Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, van der Lee HA *et al.*, Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS ONE* **7**:e31801 (2012).
65 Sun X, Xu Q, Ruan R, Zhang T, Zhu C and Li H, PdMLE1, a specific and active transposon acts as a promoter and confers *Penicillium digitatum* with DMI resistance. *Environ Microbiol Rep* **5**:135–142 (2013).
66 Knox BP and Keller NP, Key players in the regulation of fungal secondary metabolism, in *Biosynthesis and Molecular Genetics of Fungal*

 © 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Secondary Metabolites, Vol. **2**, ed. by Martín J-F, García-Estrada C and Zeilinger S. Springer, New York, pp. 13–28 (2015).
67 Qian J, Lin J and Zack DJ, Characterization of binding sites of eukaryotic transcription factors. *Genomics Proteomics Bioinf* **4**:67–79 (2006).
68 Narlikar L and Hartemink AJ, Sequence features of DNA binding sites reveal structural class of associated transcription factor. *Bioinformatics* **22**:157–163 (2006).
69 Thurkral SK, Eisen A and Young ET, Two monomers of yeast transcription factor ADR1 bind a palindromic sequence symmetrically to activate ADH2 expression. *Mol Cell Biol* **11**:1566–1577 (1991).
70 Chung K-R, Daub ME, Kuchler K and Schüller C, The CRG1 gene required for resistance to the singlet oxygen-generating cercosporin toxin in *Cercospora nicotianae* encodes a putative fungal transcription factor. *Biochem Biophys Res Commun* **302**:302–310 (2003).
71 Chen C-K, Leung SSF, Guilbert C, Jacobson MP, McKerrow JH, and Podust LM. Structural characterization of CYP51 from trypanosoma cruzi and trypanosoma brucei bound to the antifungal drugs posaconazole and fluconazole. *PLoS Neglected Tropical Diseases* **4**:e651 (2010). http://dx.doi.org/10.1371/journal.pntd.0000651

 © 2021 The Authors.
Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.