A new mechanism for reduced sensitivity to demethylation-inhibitor fungicides in the fungal banana black Sigatoka pathogen Pseudocercospora fijiensis

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SUMMARY

The Dothideomycete Pseudocercospora fijiensis, previously Mycosphaerella fijiensis, is the causal agent of black Sigatoka, one of the most destructive diseases of bananas and plantains. Disease management depends on fungicide applications, with a major contribution from sterol demethylation-inhibitors (DMIs). The continued use of DMIs places considerable selection pressure on natural P. fijiensis populations, enabling the selection of novel genotypes with reduced sensitivity. The hitherto explanatory mechanism for this reduced sensitivity was the presence of non-synonymous point mutations in the target gene Pfcyp51, encoding the sterol 14α-demethylase enzyme. Here, we demonstrate a second mechanism involved in DMI sensitivity of P. fijiensis. We identified a 19-bp element in the wild-type (wt) Pfcyp51 promoter that concatenates in strains with reduced DMI sensitivity. A polymerase chain reaction (PCR) assay identified up to six Pfcyp51 promoter repeats in four field populations of P. fijiensis in Costa Rica. We used transformation experiments to swap the wt promoter of a sensitive field isolate with a promoter from a strain with reduced DMI sensitivity that comprised multiple insertions. Comparative in vivo phenotyping showed a functional and proportional up-regulation of Pfcyp51, which consequently decreased DMI sensitivity. Our data demonstrate that point mutations in the Pfcyp51 coding domain, as well as promoter inserts, contribute to the reduced DMI sensitivity of P. fijiensis. These results provide new insights into the importance of the appropriate use of DMIs and the need for the discovery of new molecules for black Sigatoka management.

Keywords: DMI, fungicide, Pfcyp51 promoter.

INTRODUCTION

Black Sigatoka, caused by the ascomycete Pseudocercospora fijiensis (Morelet, 1969) Deighton (1976) [previously Mycosphaerella fijiensis Morelet (1969)], is one of the most devastating and economically significant diseases of export bananas and plantains. Disease management is mainly based on the extensive application of primarily single-site fungicides. However, the continuous sexual reproduction of P. fijiensis generates genetically highly diverse and hence versatile populations that quickly adapt to changing environments, including extensive fungicide treatments (Arango Isaza et al., 2016; Conde-Ferraz et al., 2007; Hayden and Carlier, 2003; Rivas et al., 2004; Romero and Sutton, 1997). As a result, reduced fungicide efficacy develops frequently and spreads rapidly (Arango Isaza et al., 2016). This situation has contributed to a grave increase in the number of fungicide applications, which can reach over 50 applications per year (maximum of 10 applications with sterol 14α-demethylation-inhibitors, DMIs) in most banana export countries (Chong-Aguirre, 2016; De Lapeyre De Bellaire et al., 2010; FRAC, 2010; Martínez-Bolanos et al., 2012), thereby frequently comprising a 30% share of the production costs (Marín et al., 2003). This practice poses a threat to the occupational health of plantation workers and the environment if guidelines are not followed. It is thus imperative to understand the mechanisms by which reduced fungicide efficacy develops to enable
adequate long-term disease management strategies with optimized chemical input.

Azole fungicide applications against black Sigatoka started in 1987 and have been widely used since 1991 when propiconazole, one of the major contemporary DMIs, was introduced to the market (Chong-Aguirre, 2016; Romero and Sutton, 1997). Currently, several DMIs, such as difenoconazole, bitertanol and epoxiconazole, are used in disease management programmes, either alone or in mixtures with other fungicides with different modes of action. DMIs inhibit the activity of the CYP51 enzyme which is involved in the 14α-demethylation of the ergosterol precursor eburicol (24-methylene-24,25-dihydrolanosterol). Ergosterol regulates cellular membrane fluidity and permeability and is essential for cell viability (Lepesheva and Waterman, 2011). However, reduced efficacy of single-site fungicides surfaced rapidly in P. fijiensis after the introduction of quinone outside inhibitors (Qois or strobilurins), methyl benzimidazole carbamates (MBCs) and DMIs for disease control in banana production (Amil et al., 2007; Arango Isaza et al., 2006, 2009; Romero and Sutton, 1997). Previous studies on P. fijiensis have revealed the correlation between the reduced efficacy of propiconazole and point mutations in the coding domain of the Pfcyp51 gene, which cause non-synonymous amino acid substitutions surrounding the substrate recognition sites (SRSs) at positions Y136, A313, Y461 and Y463 (Cañas-Gutiérrez et al., 2009; Chong-Aguirre, 2016). Until now, this was the only explanatory mechanism for reduced sensitivity towards azoles in P. fijiensis. Here, we introduce an additional mechanism that drives reduced sensitivity to DMIs in P. fijiensis. We identified the presence of one or more repetitive elements in the promoter region of Pfcyp51 amongst P. fijiensis field isolates with reduced DMI sensitivity, and catalogued such variants in 225 field isolates originating from various (treated and untreated) banana plantations in Costa Rica. Comparison with 14 control isolates from Ecuador, Asia and Africa showed a positive correlation between the presence and copy number of the Pfcyp51 promoter elements, Pfcyp51 overexpression and reduced DMI sensitivity. We subsequently established the functional relationship between the number of promoter inserts, increased target expression and reduced DMI sensitivity through Pfcyp51 promoter swapping experiments between wild-type (wt) isolates and P. fijiensis strains with reduced DMI sensitivity. We thereby formally demonstrated a novel mechanism involved in reduced fungicide efficacy of DMIs to P. fijiensis, in addition to the described target site mutations in the coding sequence of Pfcyp51.

RESULTS

In vitro sensitivity to propiconazole

The P. fijiensis isolates that were tested for sensitivity to propiconazole were classified into three groups: (1) sensitive isolates with 50% inhibitory concentration (EC50) ≤ 0.10 mg/L; (2) moderately resistant isolates with EC50 = 0.10–1.0 mg/L; and (3) resistant isolates with EC50 > 1.0 mg/L (Table 1). Among the 25 isolates tested for sensitivity to propiconazole, seven were sensitive, 14 were moderately resistant and four were resistant. Clear cross-resistance between propiconazole and cyproconazole was observed, as the majority of isolates showed similar EC50 values (Table 1; Fig. S1, see Supporting Information).

Pseudocercospora fijiensis isolates with reduced sensitivity always contain repetitive elements in the Pfcyp51 promoter

Detailed comparison between the Pfcyp51 promoter sequences from resistant isolates and the reference P. fijiensis isolate CIRAD86 revealed that resistant isolates possessed an insertion in the promoter at 103 bp upstream from the start codon. Meanwhile, some isolates with reduced sensitivity showed a shorter insertion than resistant strains at the same position. Likewise, sensitive isolates did not show any insertion. Insertions comprised repeats of 19-bp elements ‘TAAATCTCGTACGATAGCA’ present once in the Pfcyp51 promoter at 122 bp upstream from the start codon, at scaffold 7:2121794–2121813 of the CIRAD86 reference (Pseudocercospora fijiensis v2.0, Joint Genome Institute) (Figs 1 and 2).

Some isolates contained part of the element in their insertions, whereas others had a modified element with a few additional nucleotides. In addition to the 19-bp element, slightly modified 16-bp (TAAAATCTCGTACGAT) and 20-bp (TAAAATCTCGTACGATAGCA) elements were also present in the Pfcyp51 promoter. For example, in resistant isolates Ca1_5, Ca5_16, Ca6_11 and Ca10_13 (Table 1; Text S1, see Supporting Information), the basic 19-bp element was repeated up to six times (four fully conserved and one partial, mostly in tandem insertion) and thrice in the moderately resistant P. fijiensis isolates Z8_12 and Z8_18. DNA sequence analysis of the resistant isolates from Costa Rica (Ca5_16, Ca6_11 and Ca10_13) revealed that these contained identical mutations in the coding region of the Pfcyp51 gene, and that the overall length of the Pfcyp51 promoter inserts accumulated to 100 bp (Table 1).

Repetitive elements in the promoter of Pfcyp51 up-regulate its expression

To test whether Pfcyp51 gene expression is affected by the presence of repetitive elements, we quantified the expression in mycelium by real-time reverse transcription-polymerase chain reaction (RT-PCR), normalized to the expression of the actin gene (Pfact), relative to wt controls. Pseudocercospora fijiensis isolates Ca5_16, Ca6_11 and Ca10_13, all containing six repeat elements in the Pfcyp51 promoter, showed a 3.3–5.6-fold increase in Pfcyp51 gene expression relative to control isolate E22, and a
Table 1  Origin and characteristics of the Pcp51 gene and its promoter in 25 Pseudocercospora fijiensis isolates used in this study, including their sensitivity to propiconazole and cyproconazole (half-maximal effective concentration, EC_{50}).

| Origin  | Isolate | Promoter insertion | Repetitive units | CYP51 modulations | Propiconazole EC_{50} (mg/L) | SD | Cyproconazole EC_{50} (mg/L) | SD |
|---------|---------|--------------------|------------------|-------------------|-----------------------------|----|-----------------------------|----|
| Burundi | X849    | wt                 | 1                | V106D             | 0.004                       | 0.002 | 0.006                       | 0.001 |
| Cameroon | C_86    | wt                 | 1                | T18I V106D        | <0.001*                     | <0.001* | <0.001*                     | <0.001* |
| Ecuador | RS_13   | wt                 | 1                | T18I V106D A313G Y463N | 0.112                       | 0.052 | 0.121                       | 0.060 |
| Ecuador | E_22    | wt                 | 1                | T18I V106D A313G Y463N | 0.011                       | 0.009 | 0.352                       | 0.208 |
| Ecuador | GS_10   | wt                 | 1                | T18I V106D A313G Y463N | 0.481                       | 0.047 | 0.666                       | 0.253 |
| Ecuador | GS_4    | wt                 | 1                | T18I V106D A313G Y463N | 0.284                       | 0.011 | 0.514                       | 0.059 |
| Ecuador | RN_3    | wt                 | 1                | T18I V106D A313G Y463N | 0.420                       | 0.285 | 0.843                       | 0.242 |
| Ecuador | RN_5    | wt                 | 1                | T18I V106D A313G Y463N | 0.197                       | 0.085 | 0.231                       | 0.067 |
| Ecuador | SaR_2   | wt                 | 1                | T18I V106D A313G Y461D | 0.214                       | 0.121 | 0.611                       | 0.284 |
| Ecuador | SaR_5   | wt                 | 1                | T18I V106D A313G Y463N | 0.214                       | 0.121 | 0.611                       | 0.284 |
| Gabon   | X851    | wt                 | 1                | V106D             | <0.001*                     | <0.001* | 0.101                       | 0.002 |
| Indonesia | X845    | wt                 | 1                | T18I Y58F V106D | 0.009                       | 0.011 | 0.003                       | 0.001 |
| Philippines | X846   | wt                 | 1                | T18I V106D V161L | 0.007                       | 0.006 | 0.002                       | 0.001 |
| Taiwan  | X847    | wt                 | 1                | V106D             | <0.001*                     | <0.001* | <0.001*                     | <0.001* |
| Costa Rica | Z4_14  | wt                 | 1                | T18I V106D A313G Y463D | 0.214                       | 0.005 | 0.674                       | 0.294 |
| Costa Rica | Z8_17  | wt                 | 1                | T18I V106D A313G Y463S | 0.158                       | 0.092 | 0.633                       | 0.264 |
| Costa Rica | Z4_16  | wt                 | 1                | T18I V106D A313G Y463S | 0.166                       | 0.047 | 0.521                       | 0.080 |
| Costa Rica | Z4_7   | wt                 | 1                | T18I V106D A313G Y463S | 0.266                       | 0.115 | 0.489                       | 0.153 |
| Costa Rica | Z4_11  | wt                 | 1                | T18I V106D A313G Y463H | 0.112                       | 0.033 | 0.561                       | 0.225 |
| Costa Rica | Cal_5  | wt                 | 1                | V106D             | 0.144                       | 0.209 | 1.883                       | 1.055 |
| Costa Rica | Z8_12  | wt                 | 1                | V106D             | 0.188                       | 0.040 | 0.403                       | 0.146 |
| Costa Rica | Z8_18  | wt                 | 1                | V106D             | 0.153                       | 0.056 | 0.784                       | 0.514 |
| Costa Rica | Cal_10 | wt                 | 1                | V106D             | 3.346                       | 0.725 | >10*                        | -- |
| Costa Rica | Ca5_16 | wt                 | 1                | V106D             | 2.292                       | 0.420 | >10*                        | -- |
| Costa Rica | Ca6_11 | wt                 | 1                | V106D             | 2.75                        | 0.13  | 7.979                       | 3.293 |

*Out of dose range for calculations.
SD, standard deviation; wt, wild-type.
Fig. 1 The Pfcyp51 structure. (A) Alignment of the promoter regions of the Pfcyp51 gene of Pseudocercospora fijiensis isolates collected from the Zent (Z), Cartagena (Ca), San Pablo (SP) and the wild-type (wt) San Carlos (ZTSC) banana plantations in Costa Rica; isolate CIRAD86 (C86) is the reference wt isolate; the repeat element present in all isolates at position 122 bp is shown by the green arrows and additional repeated elements identified in various P. fijiensis isolates are shown as red arrows (see Table 1 for origin of isolates). (B) Configuration of the Pfcyp51 promoter and coding domains of the wt P. fijiensis isolates used to generate transformants. The promoter region is shown on the left as a blue line with different coloured boxes; green, blue and orange boxes represent the 19-bp, 20-bp and 16-bp promoter repeat elements, respectively; rectangular boxes on the right represent the coding regions of the Pfcyp51 gene in these isolates: green represents the sensitive wt and blue denotes the resistant donor (resistant wt) coding region. Vertical lines in the coding regions represent amino acid substitutions.
smaller difference from the other control strain CIRAD86 that only contained the basic 19-bp element (Fig. 3). In contrast, no significant difference was found between the control isolate CIRAD86 and P. fijiensis isolate Z8_12, which has three repeat elements. The up-regulation of Pfcyp51 was constitutive and independent of the addition of propiconazole to the culture medium (data not shown).

**Pfcyp51** promoter insertions accumulate in *P. fijiensis* strains with reduced fungicide sensitivity originating from frequently sprayed commercial banana plantations in Costa Rica

To identify the number of repeat element copies in the Pfcyp51 promoter, we performed PCR analyses on 225 isolates originating from four banana plantations in Costa Rica that have been studied previously (Arango Isaza et al., 2016): three plantations (Cartagena, Zent and San Pablo) with intensive fungicide applications and one unsprayed plantation (ZTSC or San Carlos). Comparison of the amplicon sizes by gel electrophoresis and sequence data revealed banding patterns that corresponded to two, three and six promoter repeats (Fig. 4).

Isolates containing six repeat elements dominated (50 of 82) the Cartagena population, followed by isolates with two copies (29 of 82), whereas isolates with only the original 19-bp element were scarce (three of 82). In contrast, the Zent population was dominated by isolates with only the 19-bp element in the Pfcyp51 promoter (59 of 84), but isolates containing two and six promoter repeats were also found (11 and 14 of 84, respectively). The San Pablo population was dominated by a genotype with three promoter repeats (23 of 43), which was not observed in the other populations, in addition to strains with one (eight of 23) and two (two of 23) promoter repeats. None of the genotypes with accumulated promoter repeats were observed in the San Carlos populations that exclusively comprised *P. fijiensis* strains with the original 19-bp element in the Pfcyp51 promoter (Fig. 4).

Sequence analyses revealed that the accumulated promoter repeat elements varied from 42 bp (two elements), 59 bp (three elements) up to 100 bp (six elements). All repeat elements were inserted exactly 103 bp upstream of the start codon of Pfcyp51 and were 20 bp (TAAAATCTCGTACGATAGCA), 19 bp (TAAAATCTCGTACGATAGCA) or 16 bp (TAAAATCTCGTACGAT) in length and concatenated in tandem, or were separated by a few nucleotides. Elements of 20 and 19 bp only differ by one extra adenine, whereas the 16-bp element represents a shorter version of the 19-bp insert (Fig. 1). The 19-bp element was found in isolates with one, two and three copies, whereas, in isolates with six Pfcyp51 promoter inserts, the 19-bp element was always accompanied by single inserts of the 16- and 20-bp units. Hence, the 19-bp element was the most common insertion across all isolates analysed (Fig. 1).

**Analysis of the Pfcyp51 coding sequence**

As expected, sequence analyses of different isolates revealed the presence of non-synonymous mutations in the coding region of Pfcyp51. These resulted in amino acid changes Y136F, A313G and Y463D/H/N which have been reported previously and associated with reduced sensitivity to propiconazole (Cañas-Gutiérrez et al., 2009). Here, we identified nine new amino acid substitutions (T18I, Y58F, V106D, V116L, K171R, A381G, A446S, G462A and Y463S) (Table 1). All isolates contained the T18I and V106D substitutions. Apart from these, the most frequent amino acid substitutions, A313G and Y463N/D/S/H, were observed in 11 and 16 of 25 isolates, respectively. These mutations were often found in combination with Y136F and A381G. Thus, the most frequently observed haplotypes amongst the 25 isolates were T18I, V106D, Y136F, A313G and Y463D/N/S, which were found in combination
with two, three or six copies of the \textit{Pfcyp51} repeat element. Strains with the T18I, V106D, Y136F and Y463D \textit{Pfcyp51} modifications showed the least sensitivity to the tested fungicides. In addition, several other combinations of amino acid substitutions were observed in the analysed cohort of \textit{P. fijiensis} isolates, including A313G and Y463S/H/D/N; A381G and G462A; Y136F and Y463D; Y136F, A381G and Y463D; and K171R and A446S.

**Functional analysis of the \textit{Pfcyp51} promoter insertions**

We discovered a range of promoter insertions in \textit{P. fijiensis} isolates from banana plantations that had been treated with fungicides. These promoter insertions, in particular the six repeat inserts, conferred enhanced expression of \textit{Pfcyp51}. The isolates carrying these insertions also displayed reduced sensitivity to DMI fungicides, but also carried a \textit{Pfcyp51} mutation in the coding sequence, which was hitherto the only explanatory mechanism for reduced DMI sensitivity. To disentangle the relationship between mutations in the coding sequence and the promoter insertions, we introduced the \textit{Pfcyp51} promoter from the resistant \textit{P. fijiensis} isolate Ca5_16 with six repeat elements into the sensitive wt E22 isolate from Ecuador (Table 1; Fig. 5).

Transformation of wt \textit{P. fijiensis} isolate E22 resulted in 250 green fluorescent protein (GFP)- and hygromycin (\textit{hph})-positive transformants. The transformants were characterized by PCR to differentiate isolates with six repeats in the \textit{Pfcyp51} promoter at the correct integration site from ectopic transformants (Fig. 5). Two independent transformants, Swap 26 and Swap 121, showing the Ca5_16 promoter amplicon and positive for the correct integration site, were selected for further analyses (Fig. 5). Subsequently, we performed qRT-PCR analyses on Swap 26 and Swap 121, together with the \textit{P. fijiensis} control isolates comprising the recipient wt isolate E22 and the wt resistant isolates Ca5_16 and Ca10_13 and an ectopic transformant. Consistent with previous results, the resistant isolates Ca5_16 and Ca10_13 expressed...
Pfcyp51 at a higher level than did the wt E22 recipient isolate. Moreover, the expression of Pfcyp51 was significantly increased in both Swap 26 and Swap 121 compared with wt strain E22 and the ectopic isolate, and not significantly different from the resistant donor isolate Ca5_16 (Fig. 6). Hence, these results demonstrate that replacement of the Pfcyp51 promoter from a sensitive P. fijiensis isolate by the promoter from a resistant strain results in the overexpression of Pfcyp51.

To determine whether the observed effect was independent ofazole fungicides, we challenged the transformants with difenoconazole, epoxiconazole and propiconazole in 96-well plates and calculated the EC50 values. A consistent growth pattern was
observed for all controls (0 mg/L). The wt strain Ca10_13 grew up to 2.56 mg/L of difenoconazole or epoxiconazole, and 10.24 mg/L of propiconazole (wt isolate Ca5_16 was removed because of contamination). The sensitive wt isolate E22 and the ectopic transformant only grew up to 0.016 mg/L of difenoconazole and 0.04 mg/L of epoxiconazole or propiconazole. The Swap 26 and Swap 121 transformants grew on DMI concentrations that were at least fourfold higher than those of the sensitive wt control isolate E22. For difenoconazole, transformants Swap 26 and Swap 121 displayed a twofold and over fourfold (4.25) increase in EC50 compared with the sensitive wt isolate E22, respectively (Fig. 6).

For epoxiconazole, Swap 26 displayed a 4.48-fold reduction in sensitivity, whereas Swap 121 displayed a slightly higher 8.36-fold reduction. Finally, EC50 for propiconazole of the wt strain E22 was 4.65-fold and 5.23-fold lower than that of Swap 26 and Swap 121, respectively. The ectopic transformant displayed a similar sensitivity to wt E22 regardless of the fungicide used (Fig. 6). These data confirm that Pfcyp51 promoter modifications contribute to reduced DMI efficacy in P. fijiensis.

**DISCUSSION**

Disease management in agricultural crops is commonly based on an integrated approach comprising host resistance, agronomic measures and crop protection agents whenever necessary (Matthews et al., 2014). As a result of the ubiquity of ‘Cavendish’ clones, which represent over 90% of the global banana trade, and their vulnerability to P. fijiensis, disease control in banana almost entirely relies on crop protection agents and prophylactic measures. Despite the use of decision support systems accompanied by leaf surgery and the removal of infected foliage to reduce the inoculum potential, the cornerstone for P. fijiensis control remains chemical crop protection, with the emphasis on azole fungicides (Price et al., 2015). Consequently, the selection pressure on the pathogen has been enormous and has resulted in the appearance of P. fijiensis populations with reduced fungicide sensitivity, which calls for a better understanding of its origin and dissemination.

The presence of mutations in the Pfcyp51 gene has been related previously to propiconazole resistance in P. fijiensis (Cañas-Gutiérrez et al., 2009). Here, we have focused on the promoter region as an important determinant for Pfcyp51 gene expression, and describe the identification of a 19-bp element, whose concatenation up-regulates Pfcyp51 expression and confers reduced DMI sensitivity. Our data represent the first report of the targeted genetic modification of P. fijiensis to demonstrate a new mechanism for DMI sensitivity modulation in this organism.

PICYP51 substitutions Y136F, A313G, A381G, Y461D, Y463D, Y463H and Y463N were found in the present study, in accordance with previous observations for P. fijiensis exposed to...
propiconazole (Cañas-Gutiérrez et al., 2009), as well as in exposure to other azoles in Zymoseptoria tritici, Candida albicans, Pyrenophora teres f. sp. teres and Aspergillus fumigatus (Akins and Sobel, 2009; Cools and Fraaije, 2013; Mair et al., 2016; Mellado et al., 2007). Unexpectedly, we identified a 100-bp insertion in the PfCyp51 promoter region in addition to the coding region mutations in most P. fijiensis isolates from the Cartagena population. These insertions comprise six copies of a repetitive element, whereas a single copy of this element is present in all sensitive isolates. Isolates with reduced sensitivity usually have two, three or more copies of this element (Chong-Aguirre, 2016).

Unlike in P. teres f. sp. teres (Mair et al., 2016) and Erysiphe necator (Rallos and Baudoin, 2016), which showed overexpression of Cyp51, but no promoter modification, changes in the promoter region of the cyp51 gene have been described in other fungi. Such changes comprise repeated promoter elements, truncated derivatives of a LINE-like retrotransposon in Blumeriella jaapii (Ma et al., 2006), a MITE-like transposon named PdMLE1 in Penicillium digitatum (Sun et al., 2013), a larger transposon of 1.8 kb in A. fumigatus (Albarraq et al., 2011; Verweij et al., 2013) or transcription factor binding sites in Venturia inaequalis (Villani et al., 2016). More detailed studies are required in P. fijiensis to determine whether the repeat elements observed here correspond to the movement of a transposon sequence, or whether PfCyp51 expression is possibly co-regulated by transposons. However, unlike previous reports of promoter insertions with 199 bp to 5.6 kbp sequence transposons in V. inaequalis (Schnabel and Jones, 2001; Villani et al., 2016), the PfCyp51 promoter insertion merely comprises 19-bp elements, or minor 16-bp and 20-bp variants, which accumulate up to 100 bp in length, shorter than the insertions in V. inaequalis and Z. tritici (Cools et al., 2012), where no transposons were reported. The insertions in the PfCyp51 promoter are shorter than any promoter insertions reported in A. fumigatus (Snelders et al., 2012; Verweij et al., 2007) and Pyrenepeziza brassicaceae (Carter et al., 2014). In other organisms, e.g. Escherichia coli, overexpression of a desired gene was achieved by tandem repeats of core promoter sequences called ‘MCnPtaCs’ (Li et al., 2012). In this way, a larger number of mutations in the coding region could be controlled, which would compromise the activity of the enzyme and hence reduce sensitivity (Cools et al., 2012; Leroux and Walker, 2011). Possibly, this also applies to P. fijiensis, as we did not find strains with reduced sensitivity and insertions in the promoter, but no mutations in the coding region. Isolates from wt populations lacked promoter insertions, but, occasionally, possessed mutations in the coding region.

We studied the regulatory nature of the inserted sequences in P. fijiensis in silico and showed that the 19-bp (TAAATCTCGTAC GATAGCA) repeat element is the most common feature. Within populations, we identified a clear genetic diversity in the number of promoter repeats. The frequency of isolates with more repeats was higher in banana plantations with up to eight DMI cycles, such as Cartagena, Zent and San Pablo. Although expected, it is also striking that all isolates from the untreated San Carlos plantation contained the single 19-bp element. For the first time, using a targeted reverse genetics approach in P. fijiensis, we have validated that the presence of six copies of this element in the promoter increases the expression of PfCyp51 by at least three-fold compared with wt isolates and other isolates with reduced sensitivity and up to three repeat elements. Previously, Cañas-Gutiérrez et al. (2009) were unable to show such expression in experiments with P. fijiensis in response to propiconazole, and considered it to be either non-existent or an unimportant mechanism in this fungus. However, this was probably because of the use of fewer isolates that showed a limited reduction in sensitivity. Hence, we now propose that promoter repeats constitute a genetic adaptation mechanism to the high selective pressure imposed on P. fijiensis by the continuous use of different DMI fungicides.

Although P. fijiensis is a difficult fungus to transform (Diaz-Trujillo et al., 2011), and although site-specific recombination levels seem to be very low, promoter swapping was successfully applied in our study. The introduction of the promoter from a P. fijiensis isolate with strongly reduced sensitivity into a sensitive isolate by site-specific recombination resulted in a transformant with increased expression of PfCyp51, and consequently reduced sensitivity to threeazole fungicides, as a result of promoter replacement. The Swap 26 and Swap 121 transformants were at least four times less sensitive than the recipient wt isolate E22, but not as resistant as the wt resistant isolate Ca10_13 or the donor wt isolate Ca5_16, which had similar (Y136F and Y463D) coding domain mutations. Hence, we expect that the reverse experiment, replacing the wt promoter (with inserts) from an isolate with reduced sensitivity with a promoter from a sensitive wt, should result in an increase in sensitivity. Finally, swapping the wt PfCyp51 coding domain of a sensitive strain with this domain of an isolate with reduced sensitivity, thereby generating a strain with a wt coding domain, but multiple promoter inserts, which we have never encountered in nature, should result in increased sensitivity. However, the discovery of additional mechanisms for DMI sensitivity requires genetic studies, genome-wide associations or mapping analyses (Chong-Aguirre, 2016). We expect, however, that the combination of overexpression conferred by promoter insertions and PfCyp51 target site mutations will explain most DMI sensitivity modulations.

DMIs are and will probably remain a cornerstone of global black Sigatoka disease management. However, the risks of bad practices or excessive applications will exert a significant selection pressure on P. fijiensis populations, making these increasingly insensitive. Hence, DMI applications may lose their competitive advantage compared with other less environmentally friendly
compounds. The practical spin-off of this study is that we can now use a simple PCR assay to monitor, evaluate and predict reduced DMI sensitivity in *P. fijiensis* field populations. Although we have focused here on *P. fijiensis*, DMIs are evidently under pressure because of overall reduced sensitivity issues (Chen et al., 2016; Hayashi et al., 2003; Leroux and Walker, 2011; Liu et al., 2015; Mullins et al., 2011; Sun et al., 2013, 2014; Villani et al., 2016), and are therefore increasingly being studied in various other fungal pathogens (Alvarez-Rueda et al., 2011; Becher and Wirsel, 2012; Carter et al., 2014; Cools et al., 2012; Frenkel et al., 2014; Li et al., 2012; Luo and Schnabel, 2008; Mair et al., 2016; Nikou et al., 2009; Rallois and Baudoin, 2016; Verweij et al., 2013). This fosters research and development for novel chemistry for efficient black Sigatoka control, although alternative products, such as the succinate dehydrogenase inhibitors (SDHIs) and Qols, are also prone to resistance development (Arango Isaza et al., 2016; Scalliet et al., 2012). Therefore, disease management should, in the long run, embark on the availability of resistant banana germplasm. As this will take years, fungicide sensitivity monitoring and the strict adoption of application recommendations remain absolute necessities, irrespective of which banana cultivars dominate the export trade. A more science-driven disease management and extension practice in global banana production is the prerequisite for a continuous production of this global fruit and major staple food.

**EXPERIMENTAL PROCEDURES**

*Pseudocercospora fijiensis* isolates

A set of 25 monoascosporic *P. fijiensis* isolates from Africa, Asia and Latin America was used for fungicide sensitivity assays. Eight of the Latin American isolates were collected in Ecuador and 11 in Costa Rica (see Table 1). The larger set of Costa Rican isolates originated from four different banana plantations: Cartagena (Ca), Zent (Z), San Pablo (SP) and San Carlos (ZTSC) (see also Arango Isaza et al., 2016). The first three are frequently sprayed with fungicides, whereas the San Carlos plantation is in a plantain-growing area with low *P. fijiensis* incidence, and hence fungicides are not required for disease control. We consider the *P. fijiensis* population from this area as a wt population. Isolates were obtained from CORBANA (Costa Rica), CIBE-ESPOL (Ecuador) and the Westerdijk Fungal Biodiversity Institute (Africa and Asia).

Determination of the in vitro sensitivity to DMI fungicides

The fungicides propiconazole, cyproconazole and difenoconazole were provided by Syngenta (Syngenta Crop Protection AG, Basel, Switzerland) and epoxiconazole was obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA). All compounds were of technical grade quality and were maintained in 100× stock solutions in either methanol or dimethyl sulfoxide (DMSO). When applied to the culture medium, the final concentration of the solvents was <1% (v/v). For the initial in vitro sensitivity assays, the final concentrations tested for propiconazole were 10, 5.62, 3.16, 1.78, 1.0, 0.56 and 0.31 mg/L. Subsequently, to evaluate sensitive isolates more accurately, lower concentrations of fungicides were included in the assays (10.24, 2.56, 0.64, 0.16, 0.04, 0.016, 0.004 and 0 mg/L) and exploited to evaluate the performance of *P. fijiensis* transformants in the presence of propiconazole, difenoconazole and epoxiconazole.

The fungicide sensitivity of each isolate was determined by calculation of EC50. Quantitative analysis of fungal growth was determined by a modified 96-well microtitre plate dilution assay (Montoya et al., 2006). Fifty microlitres of a 1 × 105 mycelial parts/mL solution from each isolate were inoculated in 200 μL of potato dextrose broth (PDB) medium per well of a 96-well polystyrene, flat-bottomed, transparent plate (Coming, New York, USA; cat. # 3370). Plates were incubated at 25 °C in an incubator (Elbanton, Kerkdriel, the Netherlands) for 7 days before mycelial growth was measured. Each concentration was tested in duplicate per isolate, and per plate four blank controls were present. Individual plates were considered as one biological replicate, and tests were performed three times. Absorbance was initially measured at 620 nm in a TECAN A5082 plate reader (Männedorf, Switzerland) but, because of the variation of mycelial colours over the isolates, as well as the different colony morphologies, we eventually monitored growth at an absorbance of 690 nm in an Infinite® M200 PRO reader (TECAN), which enabled the measurement of higher sensitivities. The read design per well was settled at room temperature, leaving a border of 1000 μm, a bandwidth of 9 μm, circle-filled reads of 21 read points (5 × 5, with no corner points for circle distribution) and each read point was measured five times. Read averages were plotted against days post-inoculation (dpi) and compared with the other isolates and controls. The fungicide sensitivity of transformants and control isolates was determined in the aforementioned 96-well polystyrene plates. Sealed plates were maintained at 27 °C in an incubator (Elbanton) in the dark and fungal growth was evaluated at 10 dpi. Plates were evaluated at 690 nm, whilst covered to reduce contamination. Data were analysed using GraphPad Prism7 (GraphPad Software, La Jolla, CA, USA).

**Pfcyp51** coding domain and promoter amplification and sequencing

To amplify the *Pfcyp51* gene and the promoter region, specific primers located at the first repeat element and 22 bp upstream of the open reading frame (ORF) were used: *CYP51_Pfijien_F1* (5′-AAGTCATATGGACG-3′) and *CYP51_Pfijien_R1* (5′-GAATGTATCGTGTGACA-3′). A basic PCR mix was prepared and the PCR program consisted of 5 min of denaturation at 94 °C, followed by 34 cycles of 30 s at 94 °C, 30 s of annealing at 55 °C and 90 s of extension at 68 °C. An additional extension step of 7 min at 72 °C was performed at the end. DNA sequencing of the gene was performed at Macrogen (Seoul, Korea) and by the Genomics Facility of Wageningen University and Research (WUR), directly using the PCR products. To obtain the entire sequence of the gene and the promoter region, four primers were used in the sequencing reactions: *CYP51_Pfijien_F2* (5′-ACAGAAACATACCTTCC-3′), *CYP51_Pfijien_F3* (5′-ATTCGAATCTCTGATAG-3′), *CYP51_Pfijien_F4* (5′-CTTACCCAAGATCTCGAC-3′) and *CYP51_Pfijien_R2* (5′-GAATGTATGATAGTGTGACA-3′). The sequences obtained were assembled in contigs per isolate using CLC DNA Workbench software (CLC bio, Aarhus, Denmark) and the ORF was translated to amino acids and the protein sequences were aligned using the
**Pfcyp51 gene expression analysis**

Extraction of total RNA was carried out with mycelia of *P. fijiensis* isolates grown for 10 days in PD5 by using the Qiagen RNA extraction plus mini kit (Qiagen Inc., Valencia, CA, USA). The integrity of the RNA was checked using agarose gel electrophoresis and the concentration was determined by measurement of the absorbance at 260 nm in a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA, USA). Expression analysis was performed by qRT-PCR using the primers QRTCPY-forward: (5'-GGGCGATCCAGAGGGTTCG-3') and QRTCPY-reverse: (5'-TAAGCTTCTACAGGCCGCACG-3'), which amplify a fragment of 89 bp of the *Pfcyp51* gene, and primers QRTACT-forward: (5'-TCTGCTCTGCTCTTGAAGTCT-3') and QRTACT-reverse: (5'-TGATACGGAGATCTTGGAGAAGC-3'), which amplify a fragment of 146 bp of the *P. fijiensis* actin gene which was used to normalize the expression. qRT-PCRs were performed using 20 ng of total RNA per isolate in an Applied Biosystems ABI 7500 thermocycler.

**Analysis of promoter repeats of the Pfcyp51 gene in four Costa Rican *P. fijiensis* populations**

Genomic DNA (gDNA) samples of 225 *P. fijiensis* isolates from the four Costa Rican populations were analysed: 82 from the Cartagena population, 43 from the San Pablo population, 84 from the Zent population and 16 from the San Carlos wt population (Table S1, see Supporting Information). PCR fragments were amplified from gDNA using the specific primer pair, P._fijiensis_repeats_F (5'-TCTCTGACGATAACCCACTTGGCA-3') and P._fijiensis_repeats_R (5'-TGGTGTTAGGGGTAGCCCA-3'), which was designed to amplify the promoter region of *Pfcyp51*. PCR conditions comprised 2 min at 95 °C, 30 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 68 °C and 2 min of extension at 72 °C, with an additional extension step of 10 min at 72 °C at the end of the reaction. PCR products were visualized and evaluated on 1% agarose gels and 11 isolates were selected for sequencing and subsequent analysis of promoter and coding sequences. Different repeat elements were aligned and a weblogo consensus sequence was generated (Crooks et al., 2004) to graph nucleotide conservation within the elements.

**Promoter swapping**

We performed a promoter swapping experiment to test the effect of promoter repeats on *Pfcyp51* expression and, henceforward, on the sensitivity to several azole fungicides. The *Pfcyp51* donor promoter for homologous recombination was obtained from the resistant isolate Ca5_16. The recombination construct pPROM_CYP51_Ca5_16 comprised an upstream 2043-bp fragment (the *Pfcyp51* gene has an antisense position in the genome), obtained using primers 5'-CYP-Prom Fwd (5'-GGGGAGCACAATTTGATAGATGTCGAGGCGAA-3') and Rev (5'-GGGGAGCTCCTTCCCCACATGCCCCACGAC-3'), which were cloned in front of a cassette with the *hph* resistance gene and the GFP gene, followed by the upstream region of 1737 bp obtained with primers 3-CYP-Prom Fwd (5'-GGGGACAGCTCTTGTACAAATGTCGAGGCGAA-3') and Rev (5'-GGGGACGCACTTGTACAAATGTCGAGGCGAA-3').

**ACKNOWLEDGEMENTS**

C.D.-T. thanks the National Council of Science and Technology (CONACYT) of Mexico for PhD scholarship # 187781. P.C. is grateful to the Escuela Superior Politécnica del Litoral (ESPOL) and the National Secretary of Higher Education, Science and Technology from Ecuador (SENESCYT) and Syngenta for a PhD scholarship. R.E.A.I is grateful to the Universidad Nacional de Colombia, sede Medellin, for funding collaborative research with Wageningen University and Research and Wageningen Plant Research. Amir Mirzadi Gohari is gratefully acknowledged for technical advice on Pseudocercospora fijiensis transformation. This research was partially enabled through a grant from the Dutch Dioraphte Foundation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Text S1 Genomic sequence of Pfcyp51 in a set of 25 isolates of Pseudocercospora fijiensis from Asia, Africa and Latin America.

Table S1 Analysis of Pfcyp51 promoter repeats in 225 Pseudocercospora fijiensis isolates from Costa Rica, compared with 14 isolates from other countries.

Fig. S1 Cross-resistance between propiconazole and cyproconazole. The 50% inhibitory concentration (EC50) values were determined for both compounds on Pseudocercospora fijiensis colonies for the indicated strains at 10 days post-inoculation (results are means of three independent experiments).