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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 share for sterol demethylation-inhibitors (DMIs). The continued use of DMIs puts a 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 19bp element in the wild type (wt) *Pfcyp51* promoter that concatenates in strains with reduced DMI sensitivity. A 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 wild type 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 upregulation 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 reduced DMI sensitivity of *P. fijiensis.* These results bring 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: Fungicide, DMI, *Pfcyp51* promoter.

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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 et al., 2016; Conde-Ferráez 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 et al., 2016). This situation has contributed to a grave increase in the number of fungicide applications, which can tally up to over 50 applications per year (maximally 10 applications with sterol 14α-demethylation inhibitors, DMIs) in most banana export countries (Chong, 2016; De Lapeyre De Bellaire et al., 2010; FRAC, 2010; Martínez-Bolaños et al., 2012), thereby frequently comprising a 30% share of the production costs (Marín et al., 2003). This practice poses a threat on 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 became widely used since 1991 when propiconazole, one of the major contemporary DMIs, was introduced in the market (Chong, 2016; Romero and Sutton, 1997). Currently, several DMIs, such as difenoconazole, bitertanol, and epoxiconazole are used in disease management programs, either alone or in mixes with other fungicides with different modes of action. DMIs inhibit the activity of the CYP51 enzyme that is involved in the 14α-demethylation of

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the ergosterol precursor eburicol (24-methylene-24, 25-dihydrolanosterol). Ergosterol regulates cellular membranes 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 (Arango et al., 2016; Amil et al., 2007; Cañas-Gutiérrez et al., 2009, 2006; Romero and Sutton, 1997). Previous studies on *P. fijiensis* revealed the correlation between reduced efficacy of propiconazole and point mutations in the coding domain of the *Pfcyp51* gene, which caused non-synonymous amino acid (aa) substitutions surrounding the Substrate Recognition Sites (SRS) at positions Y136, A313, Y461 and Y463 (Cañas-Gutiérrez et al., 2009; Chong, 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* among *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*.

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Results

In vitro sensitivity to propiconazole

The *P. fijiensis* isolates that were tested for sensitivity to propiconazole were classified in three groups; sensitive isolates with (1) EC_{50} values of ≤0.10 $mg.L^{-1}$; (2) moderately resistant isolates with EC_{50} values between 0.10 to 1.0 $mg.L^{-1}$ and (3) resistant isolates with EC_{50} values >1.0 $mg.L^{-1}$ (Table 1). Among the 25 isolates tested for sensitivity to propiconazole, seven were sensitive, 14 moderately resistant and four were resistant. Clear cross-resistance between propiconazole and cyproconazole was observed, since the majority of isolates showed similar EC_{50} values (Table 1, Figure S1).

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 possess an insertion in the promoter at 103bp 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 comprise repeats of 19bp elements "TAAATCTCGTACGATAGCA" present once in the *Pfcyp51* promoter 122bp upstream from start codon, at scaffold 7:2121794 – 2121813 of the CIRAD86 reference (*Pseudocercospora fijiensis* v2.0, JGI) (Figures 1 and 2).

Some isolates contain part of the element in their insertions, while others have a modified element due to a few additional nucleotides. Additional to the 19bp element, a slightly modified 16bp (TAAAATCTCGTACGAT) and a 20bp

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(TAAAATCTCGTACGATAGCA) were also present in the *Pfcyp51* promoter. For example, in resistant isolates Ca1_5, Ca5_16, Ca6_11, and Ca10_13 (Table 1; S Text) the basic 19bp element is 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 contain identical mutations in the coding region of the *Pfcyp51* gene, and that the overall length of the *Pfcyp51* promoter inserts accumulates to 100bp (Table 1).

Repetitive elements in the promoter of *Pfcyp51* upregulate 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 RT-PCR, normalized to the expression of the actin gene (*Pfact*) as compared to wild type (wt) controls. *P. 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 as compared to control isolate E22, and a smaller difference to the other control strain CIRAD86 that only have the basic 19bp element (Figure 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 addition of propiconazole in 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**

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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 were previously studied (Arango 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 (Figure 4).

Isolates containing six repeat elements dominated (50 out of 82) the Cartagena population, followed by isolates with two copies (29 out of 82), whereas isolates with merely the original 19bp element were scarce (3 out of 82). In contrast, the Zent population was dominated by isolates with only the 19bp element in the *Pfcyp51* promoter (59 out of 84), but isolates containing two and six promoter repeats were also found (11 and 14 out of 84, respectively). The San Pablo population was dominated (23 out of 43) by a genotype with three promoter repeats that was not observed in the other populations in addition to strains with one (8 out of 23) and two (2 out 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 19bp element in the *Pfcyp51* promoter (Figure 4).

Sequence analyses revealed that the accumulated promoter repeat elements varied from 42bp (two elements), 59bp (three elements) up to 100bp (six elements). All repeat elements are inserted exactly 103bp upstream of the start codon of *Pfcyp51* and are either 20bp (TAAAATCTCGTACGATAGCA), 19bp (TAAATCTCGTACGATAGCA) or 16bp (TAAAATCTCGTACGAT) in length and concatenate in tandem or are separated by a few nucleotides. Elements of 20bp and 19bp only differ by one extra adenine, whereas the 16bp element represents a shorter version of the 19bp insert (Figure 1). The 19bp element was found in isolates with one, two and three copies, whereas in isolates with six *Pfcyp51*

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promoter inserts the 19bp element was always accompanied by single inserts of the 16bp and 20bp units. Hence, the 19bp element is the commonest insertion across all isolates analysed (Figure 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 aa changes Y136F, A313G, Y463D/H/N that were previously reported and associated with reduced sensitivity to propiconazole (Cañas-Gutiérrez et al., 2009). Here, we identified nine new aa 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 aa substitutions A313G and Y463N/D/S/H were observed in 11 and 16 out 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, Y463D/N/S, which were found in combination with two, three or six copies of the *Pfcyp51* repeat element. Strains with the T18I, V106D, Y136F and Y463D *Pfcyp51* modifications showed the least sensitivity to the tested fungicides. In addition, several other combinations of aa substitutions were observed in the analysed cohort of *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 *Pfcyp51* promoter insertions

We discovered a range of promoter insertions in *P. fijiensis* isolates from banana plantations that were treated with fungicides. These promoter insertions, in particular the six

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repeat inserts, conferred enhanced expression of *Pfcyp51*. The isolates carrying these insertions also displayed reduced sensitivity to DMI fungicides, but also carried *Pfcyp51* mutations in the coding sequence, which is the hitherto only explanatory mechanism for reduced DMI sensitivity. To disentangle the relation between mutations in the coding sequence and the promoter insertions, we introduced the *Pfcyp51* promoter from the resistant *P. fijiensis* isolate Ca5_16 with six repeat elements into the sensitive wt E22 isolate from Ecuador (Table 1; Figure 5).

Transformation of wt *P. fijiensis* isolate E22 resulted in 250 green fluorescent protein (GFP) and hygromycine (*hgh*) positive transformants. The transformants were characterized by PCR to differentiate isolates with six repeats in the *Pfcyp51* promoter at the correct integration site from ectopic transformants (Figure 5). Two independent transformants, Swap26 and Swap121, showing the Ca5_16 promoter amplicon and positive for the correct integration site were selected for further analyses (Figure 5). Subsequently, we performed qRT-PCR analyses on Swap26 and Swap121 along with the *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 express *Pfcyp51* at a higher level than the wt E22 recipient isolate. Moreover, the expression of *Pfcyp51* was significantly increased in both Swap26 and Swap121 compared to wt strain E22 and the ectopic isolate, and not significantly different from the resistant donor isolate Ca5_16 (Figure 6). Hence, these results prove that replacing the *Pfcyp51* promoter from a sensitive *P. fijiensis* isolate by the promoter from a resistant strain results in over expression of *Pfcyp51*.

To determine whether the observed effect was independent of azole fungicides we challenged the transformants with difenoconazole, epoxiconazole and propiconazole in 96-well plates and calculated the EC_{50} values. A consistent growth pattern was observed for all

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controls (0 mg·L^{-1}). Wt strain Ca10_13 grew up to 2.56 mg·L^{-1} of difenoconazole or epoxiconazole, and 10.24 mg·L^{-1} of propiconazole (wt isolate Ca5_16 was removed due to contamination). The sensitive wt isolate E22 and the ectopic transformant only grew up to 0.016 mg·L^{-1} of difenoconazole and 0.04 mg·L^{-1} of epoxiconazole or propiconazole. The Swap26 and Swap121 transformants grew on DMI concentrations that were at least fourfold higher than those of the sensitive wt control isolate E22. For difenoconazole, transformants Swap26 and Swap121 displayed a twofold and over fourfold (4,25) increment of EC_{50} compared to the sensitive wt check E22, respectively (Figure 6). For epoxiconazole, Swap26 displayed a 4.48-fold reduction in sensitivity, while Swap121 displayed a slightly higher 8.36-fold reduction. Finally, the EC_{50} for propiconazole of the wt strain E22 was 4.65-fold and 5.23-fold lower compared to Swap26 and Swap121, respectively. The ectopic transformant, displayed a similar sensitivity as wt E22 regardless of the fungicide used (Figure 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). Due to 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 prophylaxis measures. Despite the use of decision support systems accompanied with 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,

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2015). Consequently, the selection pressure on the pathogen has been enormous that 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 previously related 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 19bp element, whose concatenation upregulates *Pfcyp51* expression and confers reduced DMI sensitivity. Our data represent the first report of targeted genetic modification of *P. fijiensis* to demonstrate a new mechanism for DMI sensitivity modulation in this organism.

PfCYP51 substitutions Y136F, A313G, A381G, Y461D, Y463D, Y463H and Y463N were found in the present study in accordance to what has been previously described for *P. fijiensis* for propiconazole (Cañas-Gutiérrez et al., 2009) as well as 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 100bp 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 have usually two, three or more copies of this element (Chong, 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

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retrotransposon in *Blumeriella jaappi* (Ma et al., 2006), a MITE-like transposon named PdMLE1 in *Penicillium digitatum* (Sun et al., 2013), or a larger transposon of 1.8 kb in *A. fumigatus* (Albarrag et al., 2011; Verweij et al., 2013), or transcription factor binding sites in *Venturia inaequalis* (Villani et al., 2016). More detailed studies would be required in *P. fijiensis* to decipher whether the repeat elements that we observed 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 199bp to 5.6 kbp-sequence transposons in *V. inaequalis* (Schnabel and Jones, 2001; Villani et al., 2016), the *Pfcyp51* promoter insertion merely comprise 19bp elements, or minor 16bp and 20bp variants, which accumulate up to 100bp in length, shorter than insertions in *V. inaequalis*, and *Z. tritici* (Cools et al., 2012), where no transposons were reported. Thus the insertions in the *Pfcyp51* promoter are shorter than any promoter insertions reported in *A. fumigatus* (Verweij et al., 2007; Snelders et al., 2012), and *Pyrenopeziza brassicae* (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 “MCPtacs” (Li et al., 2012). In this way, a higher number of mutations in the coding region could be controlled, which would compromise the activity of the enzyme and hence to reduced sensitivity (Cools et al. 2012; Leroux & 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 19bp (TAAATCTCGTACGATAGCA) repeat element is the commonest 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

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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 19bp element. Using a targeted reverse genetics approach in *P. fijiensis* we, for the first time, could validate that the presence of six copies of this element in the promoter increases the expression of *Pfcyp51* at least three-fold compared to wt isolates and others 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 either a non-existent or unimportant mechanism in this fungus. However, this was likely due to the use of fewer isolates that showed a limited reduction of 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.

Even though *P. fijiensis* is a difficult fungus to transform (Díaz-Trujillo et al. unpublished data), and despite that 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 three azole fungicides, as a result of the promoter replacement. The Swap26 and Swap121 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 of 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

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coding domain, but multiple promoter inserts, which we have never encountered in nature, should result in increased sensitivity. However, discovery of additional mechanisms for DMI sensitivity require genetic studies, either genome wide associations or mapping analyses (Chong, 2016). We expect, however, that the combination of overexpression conferred by promoter insertions and *Pfcyp51* target site mutations explain most DMI sensitivity modulations.

DMIs are and will likely remain a cornerstone for global black Sigatoka disease management. However, the risks of bad practices or excessive applications exert a significant selection pressure on *P. fijiensis* populations, turning these increasingly insensitive. Hence, DMI applications may lose their competitive advantage compared to 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. Albeit that we focus here on *P. fijiensis,* DMIs are evidently under pressure due to overall reduced sensitivity issues (Chen et al., 2016; Hayashi et al., 2002; Leroux and Walker, 2011; Liu et al., 2015; Mullins et al., 2011; Sun et al., 2014, 2013; Villani et al., 2016) and are, therefore, increasingly 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; Maier et al., 2016; Nikou et al., 2009; Rallos 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 QoIs, are also prone to resistance development (Arango et al., 2016; Scalliet et al., 2012). Therefore, disease management should on 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

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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 top 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 isolates 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 et al., 2016). The former three are frequently sprayed with fungicides, whereas the San Carlos plantation is in a plantain growing area with low *P. fijiensis* incidence, 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).

Determining 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, Missouri, USA). All compounds were technical grade quality and were maintained in 100x stock solutions, either in methanol or DMSO. When applied to the culture medium the final concentration of the solvents was <1% (v/v). For the

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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^{-1}. 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, 0 mg·L^{-1}) and exploited to evaluate the performance of *P. fijiensis* transformants in the presence of propiconazole, difenoconazole and epoxiconazole.

Fungicide sensitivity of each isolate was determined by calculating the 50% inhibitory concentration (EC_{50}). Quantitative analysis of fungal growth, was determined by a modified 96 -well microtiter plate dilution assay (Montoya et al., 2006). Fifty microliters of a $1x10^5$ mycelial parts·mL^{-1} solution from each isolate were inoculated in 200 µl potato dextrose broth (PDB) medium per well of a 96-well polystyrene, flat bottom, transparent, plate (Corning, USA; cat. # 3370). Plates were incubated at 25ºC in an incubator (Elbanton, Kerkdriel, Netherlands) for seven 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 thrice. Absorbance was initially measured at 620 nm in a TECAN A5082 plate reader (Männedorf, Switzerland), but due to 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, Männedorf, Switzerland), which enabled measuring higher sensitivities. The read design per well was settled at room temperature, leaving a border of 1,000 µm, a bandwidth of 9 µm, circle-filled reads of 21 read points (5x5, with no corner points for circle distribution), and each read point was measured five times. Read averages were plotted against days after 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 ºC in an incubator (Elbanton, Kerkdriel, Netherlands) in

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darkness and fungal growth was evaluated 10 dpi. Plates were evaluated at 690 nm, while covered to reduce contamination. Data were analysed using GraphPad Prism7 (GraphPad Software, La Jolla, 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 22bp upstream of the open reading frame (ORF) were used: *CYP51_Pfijien*_F1 (5'-AAGGTCATATCGCAGG-3') and *CYP51_Pfijien*_R1 (5'-GAATGTTATCGTGTGACA-3'). A basic PCR mix was prepared and the PCR program consisted of five min. of denaturation at 94 °C followed by 34 cycles of 30 sec. at 94 °C, 30 sec. of annealing at 55 °C and 90 sec. of extension at 68 °C. An additional extension step of seven 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'-ACAGAAACATCACCTCC-3'), CYP51_Pfijien_F3 (5'-ATTGCTTCACTTTCATCC-3'), *CYP51_Pfijien*_F4 (5'-CTCTACCACGATCTCGAC-3') and *CYP51_Pfijien*_R2 (5'-GATATGGATATAGTTGTC-3'). The obtained sequences were assembled in contigs per isolate using CLC DNA Workbench software (CLC bio, Aarhus, Denmark) and the ORF was translated to aa and the protein sequences were aligned using the ClustalW plug in. The sequence alignments allowed the identification of mutations.

***Pfcyp51* gene expression analysis**

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Extraction of total RNA was carried out with mycelia of *P. fijiensis* isolates grown for 10 days in PDB using the Qiagen RNA extraction plus mini kit (QIAGEN Inc., Valencia, USA). The integrity of the RNA was checked using agarose gel electrophoresis and the concentration was determined by measuring absorbance at 260 nm in a Nanodrop spectrophotometer (Thermo scientific, Wilmington, USA). Expression analysis was performed by quantitative real time -PCR (qRT-PCR) using primers QRTCYP-forward: (5'-CGCCAGTATTCGGCACAGATGTCG-3') and QRTCYP-reverse: (5'-TAACGTAGGACTGGAGGGCGGA-3'), which amplify a fragment of 89bp of the *Pfcyp51* gene and primers QRTACT-forward: (5'-TCCGTCCTTGGTCTCGAATCTGGT-3') and QRTACT-reverse: (5'-TGCATACGGTCGGAGATACCTGGA-3'), which amplify a fragment 146bp of the *P. fijiensis* actin gene that was used to normalize the expression. Quantitative RT-PCR reactions were performed using 20 ng of total RNA per isolate in an Applied Biosystems ABI 7500 thermocycler (Waltham, USA) using the Applied Biosystems Power SYBR® Green RNA-to-CT™ 1-Step Kit, according to the manufactures instructions. The delta-delta Ct method was used - with the actin gene as the endogenous control - to determine the level of *Pfcyp51* gene expression (Livak and Schmittgen, 2001).

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

Genomic DNA (gDNA) of 225 *P. fijiensis* isolates from the four Costa Rican populations was 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). PCR fragments were amplified from gDNA using the specific primer pair, *P._fijiensis_repeats*_F (5'-TCTCGTACGATAGCACCTGCCCA-3') and

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*P._fijiensis_repeats*_R (5’-TGTTGGTGTAGGGGGTTAGGCCA-3’) that was designed to amplify the promoter region of *Pfcyp51*. PCR conditions comprised two min. at 95 °C, 30 cycles of 30 sec. denaturation at 95 °C, 30 sec. of annealing at 68 °C, and two 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 eleven 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 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 2,024bp fragment (the *PfCyp51* gene has an antisense position in the genome), obtained by using primers 5-CYP-Prom Fwd (5´-GGGGACAACTTTGTATAGAAAAGTTGAGGATATCAAGCACGCAC-3´) and Rev (5´-GGGGACTGCTTTTTTGTACAAACTTGGAAGAGAAACGGACTCCA-3´), which was cloned in front of a cassette with the *hph* resistance gene and the GFP gene, followed by the upstream region of 1,737bp obtained with primers 3-CYP-Prom Fwd (5´-GGGGACAGCTTTCTTGTACAAAGTGGGAATGAGCATTTGAGAGC-3´) and Rev (5´-GGGGACAACTTTGTATAATAAAGTTAATACTAGCGGAGGTTCG-3´), containing the promoter region of isolate Ca5_16, which has six promoter repeats. Transformations were performed by *Agrobacterium tumefaciens* mediated transformation (Díaz-Trujillo et al. unpublished data) using the sensitive wt *P. fijiensis* isolate E22, with a single repeat element

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and no mutations in the coding region. The promoter length of 250 GFP labelled transformants was compared with the promoter length of the resistant donor Ca5_16 and the sensitive recipient isolate E22. Transformants with a Ca5_16 sized promoter are considered to be homologous recombinants, hence promoter swapped transformants, which were subsequently analysed for the integration site using PCR of a 2,629bp amplicon using primers PROM-HR-3´ Fwd (5´-TGAGCATTTGAGAGC-3´) and Rev (5´-TTATGATCGCCTCCAAGC-3´) located in the cassette and the *Pfcyp51* ORF, respectively.

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Supporting information legends

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.

Figure S1. Cross-resistance between propiconazole and cyproconazole. The EC_{50} values were determined for both compounds on *Pseudocercospora fijiensis* colonies for the indicates strains at 10 days post inoculation (results are means of three independent experiments).

Figure legends

Figure 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 wt San Carlos (ZTSC) banana plantations in Costa Rica, isolate CIRAD86 (C86) is the reference wt isolate, the repeat element present in all isolate at position -122 bp is shown in green arrows and additional repeated elements identified in various P. fijiensis isolates are shown as red arrows (see for origin of isolates Table 1). B) Configuration of the Pfcyp51 promotor and coding domains of the wt Pseudocercospora fijiensis isolates used to generate transformants. The promoter region is shown at the left as a blue line with different coloured boxes: green, blue and orange boxes represent the 19 bp, 20 bp, or 16 bp promoter repeat elements; rectangular boxes at the right represent the coding regions of the Pfcyp51 gene in these isolates: green represent the sensitive wt and blue the resistant donor (resistant wt) coding region. Vertical lines in the coding regions represent amino acid substitutions.

Figure 2. Sequence logo of the Pfcyp51 promoter repeat element. Sequences of all repeat elements were aligned and used to generate the consensus sequence. The logo displays the

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frequency of the nucleotides within the repeated elements of 16, 19 or 20 bp that we observed in the promoter of Pfcyp51.

Figure 3. Relative expression of Pfcyp51 (normalized with the P. fijiensis actin gene) in six Pseudocercospora fijiensis isolates carrying different numbers of promoter inserts (indicated on top of each bar). Reference isolate CIRAD86 (C86) is shown in green. Data represent averages of three biological repetitions with each at least three technical replicates (error bars indicate standard variations).

Figure 4. Quantification of the number of Pfcyp51 promoter repeats in Pseudocercospora fijiensis isolates from four banana plantations in Costa Rica. A) Example of PCR amplification of the Pfcyp51 promoter in isolates from different populations. Isolate CIRAD86 (C86) was used as a control for the presence of one repeat element, Z8.12 as a control with three element repeats and Ca5_16 as a control with six repeat elements. The number of repeat elements in each control sample is indicated above the corresponding amplicon. The other isolates originated from banana plantations extensively treated (or not) with azole fungicides and contain varying numbers of repeat elements in the Pfcyp51 promoter. B) Distribution of repeat elements in the Pfcyp51 promoter within Costa Rican populations of P. fijiensis, based on 225 PCR amplifications.

Figure 5. Transformation design to swap Pfcyp51 promoters of Pseudocercospora fijiensis isolates. A) Isolate Ca5_16 is the Pfcyp51 promotor donor with six repeat elements (slashed area). The 3´ and 5´ recombination fragments (crossed out area) were amplified with CYP-Prom primers and ligated to a cassette with the hph and GFP markers into construct pPROM_CYP51_Ca5_16. The P. fijiensis E22 sensitive isolate with one 19bp promoter element (dotted area) was transformed with this construct. B) The promoter lengths of positive GFP tagged transformants was amplified and compared with the donor and the wt recipient isolate. Transformant Swap26 is shown as an example of a promotor replacement

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transformant, with a similar amplicon as the donor isolate. Ectopic transformants possess the promoter fragment of both the donor and the recipient isolate, whereas untransformed isolates only show the wt-sized amplicon. C) Detection and characterization of promotor swapped transformants were performed by amplification of the 2,629 bp cassette between the homologous recombination sites and the Pfcyp51coding region using primers PROM-HR-3´ on GFP fluorescent transformants with promoter amplicon similar to donor isolate.

Figure 6. In vitro sensitivity of Pseudocercospora fijiensis transformants Swap26 and Swap121 with swapped Pfcyp51 promoters vs. various control isolates. (A) The relative expression (normalized with the expression in wt sensitive donor isolate E_22) of Pfcyp51 in Swap26 and Swap121, the wt E22 and the resistant isolate (Ca10_13) with identical promoter and coding region as donor isolate (Ca5_16) as well as the ectopic control isolate (Ectopic 34). Data represent the averages of three replications. (B) Table with means of EC50 values (mg · L-1) of the Pseudocercospora fijiensis promotor swapped transformants Swap26 and Swap121 and various control isolates to three azole fungicides.

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Table 1. Origin and characteristics of the *Pfcyp51* gene and its promoter in 25 *Pseudocercospora fijiensis* isolates used in this study, including their sensitivity to propiconazole and cyproconazole (EC_{50}).

Origin	Isolate	Promoter insertion	Repetitive units	CYP51 modulations												Propiconazole		Cyproconazole	
																EC50 (mg ·L-1)	SD	EC50 (mg ·L-1)	SD
Burundi	X849	WT	1			V106D										0,004	0,002	0,006	0,001
Cameroon	C_86	WT	1													<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										0,011	0,009	<0,001*	
Ecuador	GS_10	WT	1	T18I		V106D				A313G					Y463N	0,291	0,050	0,552	0,208
Ecuador	GS_4	WT	1	T18I		V106D				A313G					Y463N	0,481	0,047	0,666	0,253
Ecuador	RN_3	WT	1	T18I		V106D				A313G					Y463H	0,284	0,011	0,514	0,059
Ecuador	RN_5	WT	1	T18I		V106D				A313G					Y463H	0,420	0,285	0,843	0,242
Ecuador	SaR_2	WT	1	T18I		V106D				A313G			Y461D			0,197	0,085	0,231	0,067
Ecuador	SaR_5	WT	1	T18I		V106D				A313G					Y463N	0,214	0,121	0,611	0,284
Gabon	X851	WT	1			V106D										<0,001*	-	0,010	0,002
Indonesia	X845	WT	1	T18I	Y58F	V106D										0,009	0,011	0,003	0,001
Philipines	X846	WT	1	T18I		V106D	V116L									0,007	0,006	0,002	0,001
Taiwan	X847	WT	1			V106D			K171R			A446S				<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					A381G			G462A		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	Ca1_5	CTCGTACGATAGCACAAATGTTAAATCTCGTACGATAGC	3	T18I		V106D		Y136F			A381G				Y463D	1,144	0,209	1,883	1,055
Costa Rica	Z8_12	CTCGTACGATAGCACAAATGTTAAATCTCGTACGATAGC	3	T18I		V106D		Y136F							Y463D	0,188	0,040	0,403	0,146
Costa Rica	Z8_18	CTCGTACGATAGCACAAATGTTAAATCTCGTACGATAGC	3	T18I		V106D		Y136F							Y463D	0,153	0,056	0,784	0,514
Costa Rica	Ca10_13	AAATCTCGTACGATAGCATAAAATCTCGTACGATAGCATAA AATCTCGTACGATGTTAAATCTCGTACGATAGCATAAATCT CGTACGATAGCACCTGCC	6	T18I		V106D		Y136F							Y463D	3,346	0,725	>10*	-
Costa Rica	Ca5_16	AAATCTCGTACGATAGCATAAAATCTCGTACGATAGCATAA AATCTCGTACGATGTTAAATCTCGTACGATAGCATAAATCT CGTACGATAGCACCTGCC	6	T18I		V106D		Y136F							Y463D	2,292	0,420	>10*	-
Costa Rica	Ca6_11	AAATCTCGTACGATAGCATAAAATCTCGTACGATAGCATAA AATCTCGTACGATGTTAAATCTCGTACGATAGCATAAATCT CGTACGATAGCACCTGCC	6	T18I		V106D		Y136F							Y463D	2,75	0,13	7,979	3,293

*Out of dose range for calculations
SD = Standard deviation

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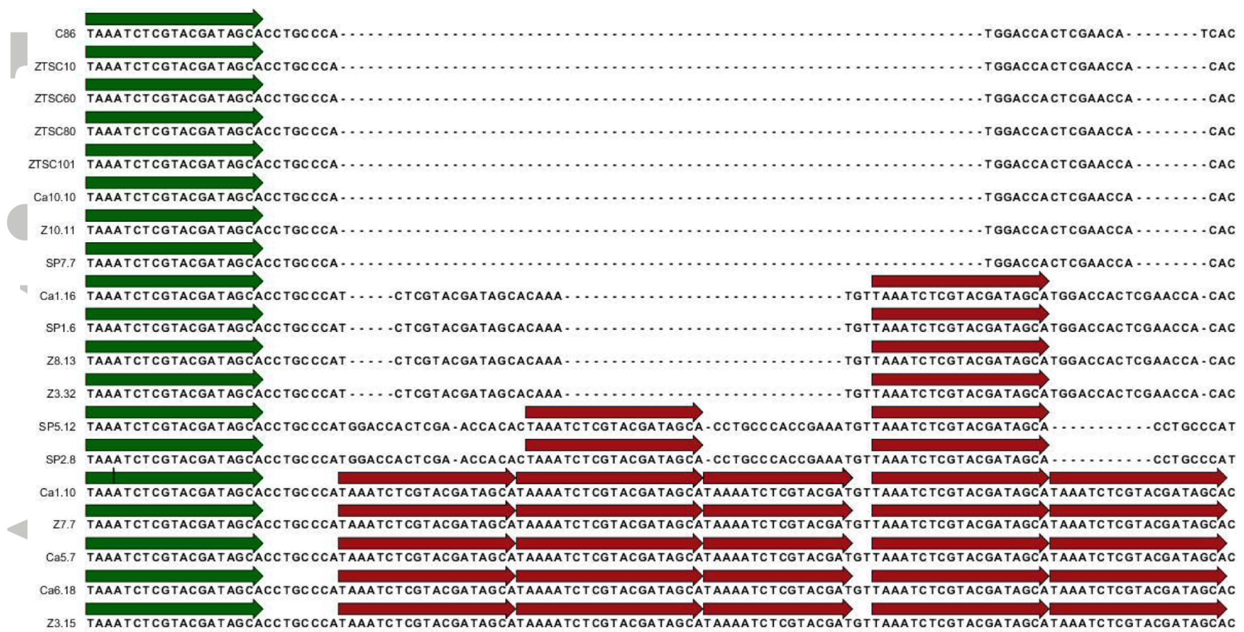

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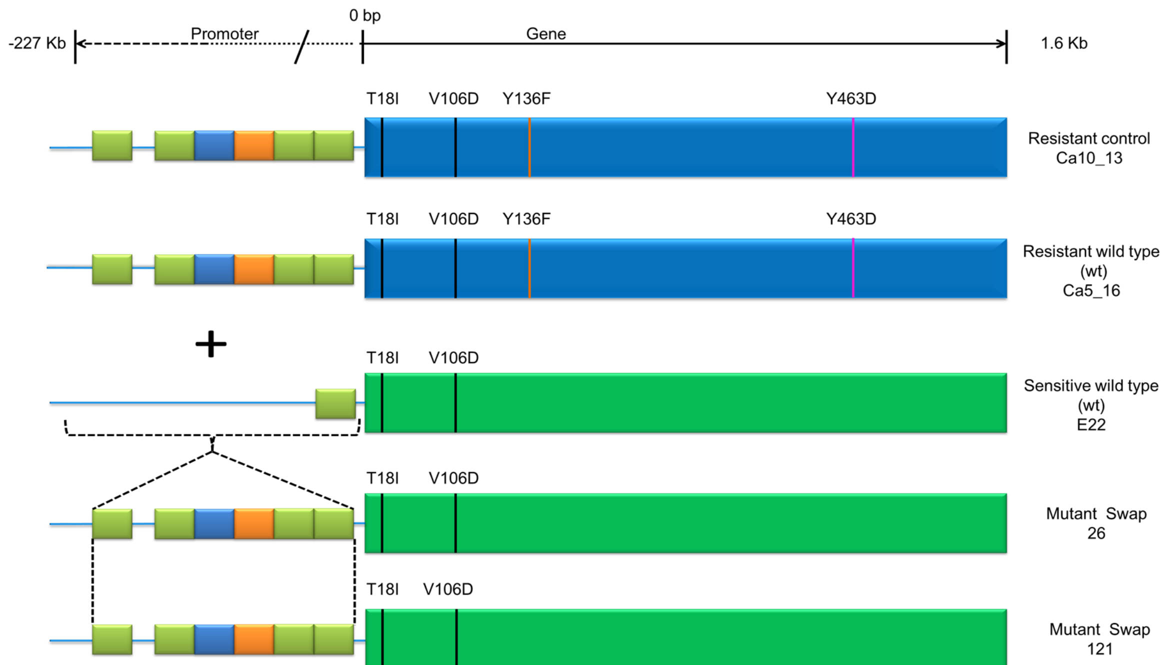

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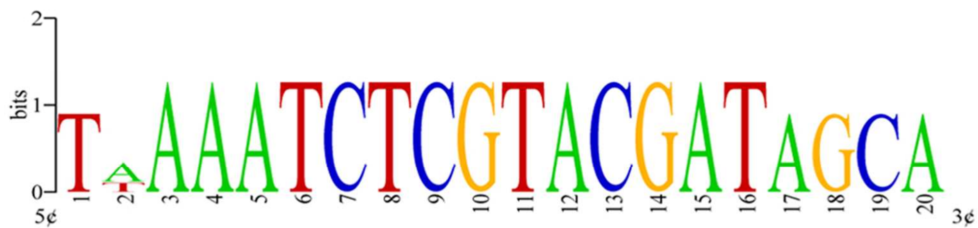

Figure 2. Sequence logo of the Pfcyp51 promoter repeat element. Sequences of all repeat elements were aligned and used to generate the consensus sequence. The logo displays the frequency of the nucleotides within the repeated elements of 16, 19 or 20 bp that we observed in the promoter of Pfcyp51.

80x22mm (300 x 300 DPI)

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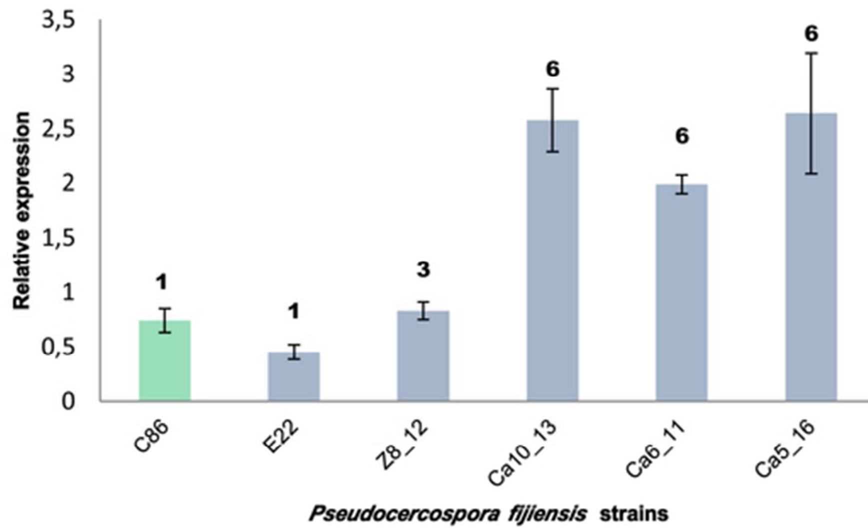

Figure 3. Relative expression of Pfcyp51 (normalized with the P. fijiensis actin gene) in six Pseudocercospora fijiensis isolates carrying different numbers of promoter inserts (indicated on top of each bar). Reference isolate CIRAD86 (C86) is shown in green. Data represent averages of three biological repetitions with each at least three technical replicates (error bars indicate standard variations).

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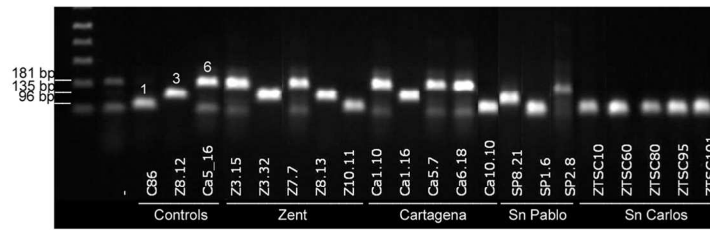

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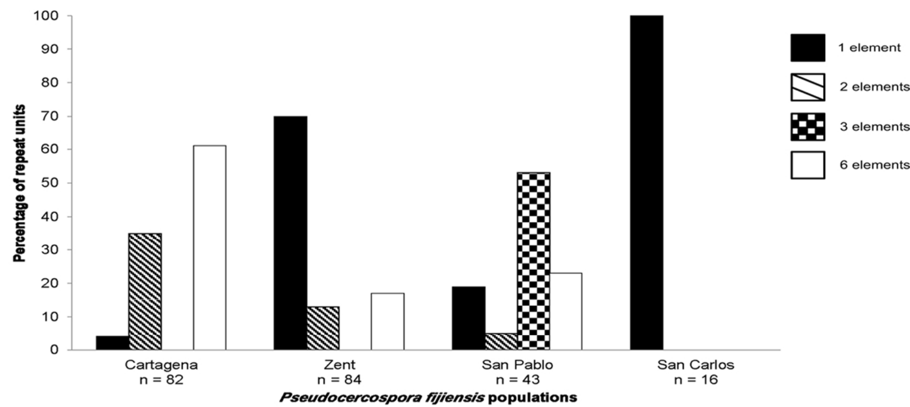

Figure 4. Quantification of the number of Pfcyp51 promoter repeats in Pseudocercospora fijiensis isolates from four banana plantations in Costa Rica. A) Example of PCR amplification of the Pfcyp51 promoter in isolates from different populations. Isolate CIRAD86 (C86) was used as a control for the presence of one repeat element, Z8.12 as a control with three element repeats and Ca5_16 as a control with six repeat elements. The number of repeat elements in each control sample is indicated above the corresponding amplicon. The other isolates originated from banana plantations extensively treated (or not) with azole fungicides and contain varying numbers of repeat elements in the Pfcyp51 promoter. B) Distribution of repeat elements in the Pfcyp51 promoter within Costa Rican populations of P. fijiensis, based on 225 PCR amplifications.

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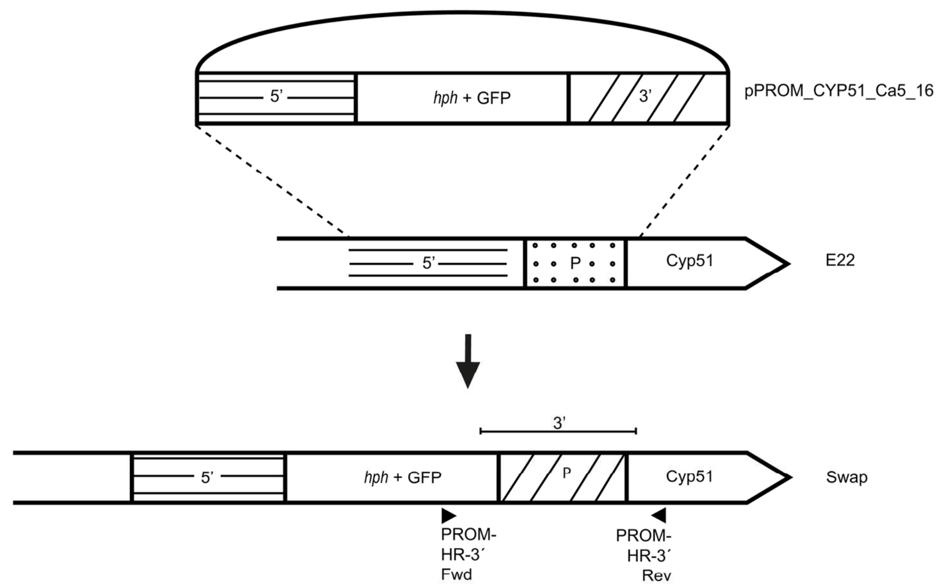

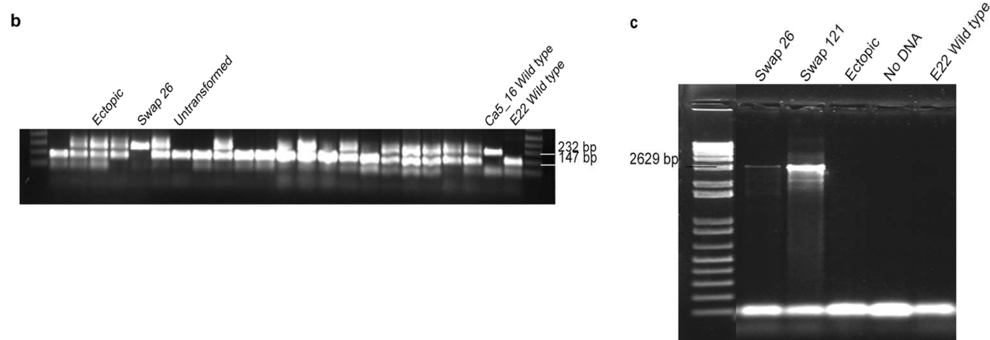

Figure 5. Transformation design to swap Pfcyp51 promoters of Pseudocercospora fijiensis isolates. A) Isolate Ca5_16 is the Pfcyp51 promotor donor with six repeat elements (slashed area). The 3´ and 5´ recombination fragments (crossed out area) were amplified with CYP-Prom primers and ligated to a cassette with the hph and GFP markers into construct pPROM_CYP51_Ca5_16. The P. fijiensis E22 sensitive isolate with one 19bp promoter element (dotted area) was transformed with this construct. B) The promoter lengths of positive GFP tagged transformants was amplified and compared with the donor and the wt recipient isolate. Transformant Swap26 is shown as an example of a promotor replacement transformant, with a similar amplicon as the donor isolate. Ectopic transformants possess the promoter fragment of both the donor and the recipient isolate, whereas untransformed isolates only show the wt-sized amplicon. C) Detection and characterization of promotor swapped transformants were performed by amplification of the 2,629 bp cassette between the homologous recombination sites and the Pfcyp51coding region using primers PROM-HR-3´ on GFP fluorescent transformants with promoter amplicon similar to donor isolate.

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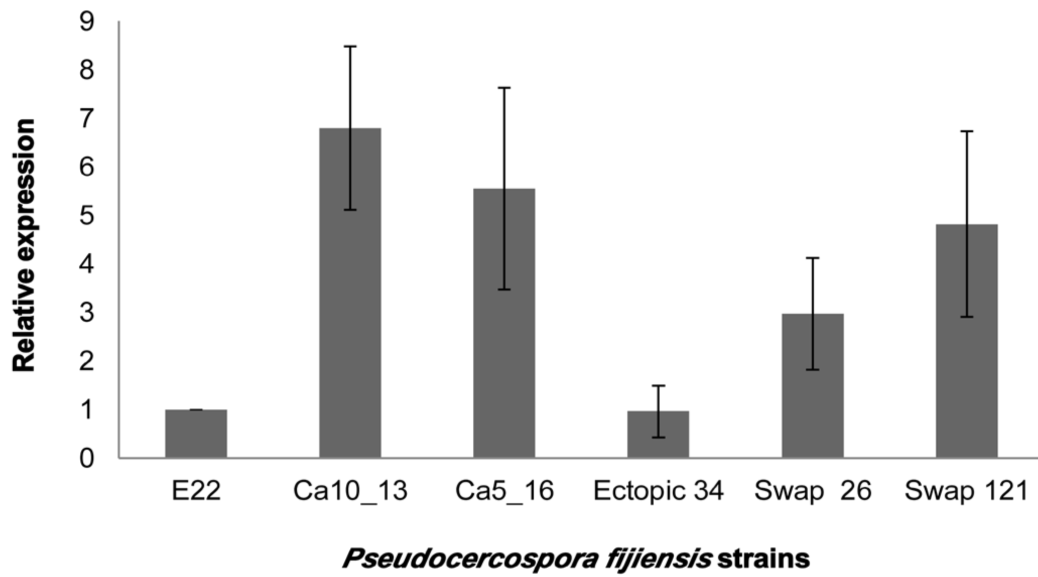

B

Sample	Difenoconazole	Epoxiconazole	Propiconazole
Ca10_13 (Resistant)	5,629 ± 0,1789	4,646 ± 0,1818	5,653 ± 0,1905
E22 (Sensitive)	0,008 ± 0,0009	0,025 ± 0,0014	0,026 ± 0,0012
Swap 26	0,016 ± 0,0062	0,112 ± 0,0205	0,121 ± 0,0228
Swap 121	0,034 ± 0,0010	0,209 ± 0,0450	0,136 ± 0,0370
Ectopic	0,003 ± 0,0001	0,023 ± 0,0011	0,014 ± 0,0016

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