Functional genetics and genomics of the banana black Sigatoka pathogen *Pseudocercospora fijiensis*

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CHAPTER 1

INTRODUCTION

Banana and Plantain

Food security is a major concern with an expected increase of the global population to nine billion people in 2050 (Godfray et al., 2010). Traditionally, the major staple foods wheat, rice, and maize are considered to be of prime importance to secure sufficient food for the future (FAOSTAT, 2015). However, reducing food waste and diversifying diets are also important drivers for food security. For instance, the consumption of vegetables is very important for proteins and vitamins, but still far below the required level in many countries (Godfray et al., 2010). In addition, other crops are of prime importance as staples for millions of people. These include crops such as cassava, millet, but also bananas and plantain. Such crops are frequently called “orphan crops” as they are far less attractive for research, hence research and development are generally lagging behind compared to the aforementioned leading staples, but ironically feed millions of people (Esfeld et al., 2013). Bananas originated in Southeast Asia, where a long domestication period of wild species derived from inter- or intra crosses between the founding seeded diploid banana species *Musa acuminata* (AA, 2n=22) and *M. balbisiana* (BB, 2n=22) resulted into a great diploid, triploid and tetraploid diversity with over 1,000 registered accessions. The edible sterile triploids, comprising AAA, AAB (plantain) and ABB (cooking banana), several tetraploids and parthenocarpic diploids are clonally propagated (Heslop-Harrison and Schwarzacher, 2007; Ortiz and Vuylsteke, 1994), which contributed to their dissemination into all tropical and subtropical areas. Roughly, local varieties are the preferred fruits and staples at domestic markets, whereas the AAA triploids such as “Cavendish” are the preferred commodity (Valmayor et al., 2000) that is produced across all tropical and sub-tropical environments with Ecuador as the prime exporter (Table 1, FAOSTAT, 2015). In short, banana is a major crop in the developing economies of Asia, Africa and Latin America for both domestic and export markets. Over 80% of the global production is locally consumed and just 17 million tonnes (18%) with a

monetary value of 29.3 billion US$, is representing the top fruit in Western supermarkets (FAOSTAT, 2015).

Table 1. Major producers, exporters and importers of banana and plantain

Producers*	Million metric tonnes	**Exporters****	(%)	**Importers****	(%)
India	26.217	Ecuador	23.3	United States	18.8
Philippines	8.687	Guatemala	10.5	Belgium	8.8
China	8.042	Costa Rica	8.5	Russia	7.1
China mainland	7.834	Belgium	8.0	Germany	7.1
Brazil	6.998	Colombia	7.8	Japan	6.6
Ecuador	6.701	Philippines	5.3	United Kingdom	5.9
Indonesia	6.005	Netherlands	3.8	China	4.2
Guatemala	2.448	Dominican Republic	3.8	Netherlands	3.7
United Republic of Tanzania	2.447	United States	3.7	Italy	3.5
Mexico	2.151	Ivory Coast	3.1	France	3.3
Others	62.675	Others	22.2	Others	31.0

Sources: FAOSTAT2015. Data from 2014*

www.worldtopexports.com. Data from 2016 **

The sterile - thus seedless - triploid edible bananas, such as the “Cavendish” varieties, are vigorous high-yielding plants that are easily propagated and have an acceptable taste. The tailored logistic chain seamlessly provides millions of bananas on a daily basis to Western consumers. Cavendish varieties dominate the export trade due to their resistance to Panama disease or *Fusarium* wilt that is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The so-called Race 1 strains of this fungus (Ordonez et al., 2015) wiped out its predecessor, the “Gros Michel” bananas in Latin America in the previous century (Ploetz, 2015, 2006). As a result, the transnational export companies were gradually changing to the less preferred, but resistant “Cavendish” clones, a process strongly intermingled with politics and societal unrest (Koeppel, 2008). Despite their resistance to Panama disease, “Cavendish” bananas are highly susceptible to black Sigatoka, a destructive foliar blight caused by the Dothideomycete *Pseudocercospora fijiensis* (previously *Mycosphaerella fijiensis* Morelet).

This disease is a major concern to the industry as the foliage has to be disease free to avoid premature ripening which turns the crop unfit for export. Since "Cavendish" bananas are very susceptible to *P. fijiensis,* fungicides are a cornerstone for global export banana production (Lepoivre P., 2003; Marín et al., 2003). In addition, other pathogens and pests challenge bananas wherever they are grown, including fungal, bacterial and viral disease as well as a range of nematode and insect pests. Nematodes are important soil-borne threats of banana, primarily the endoparasitic *Radopholus similis,* which is the most damaging species worldwide, along with other species such as *Helicotylenchus* spp., root-lesion and root knot nematodes from the genera *Pratylenchus* and *Meloidogyne,* respectively (Speijer and De Waele, 1997).

The banana weevil *Cosmopolites sordidus* is the most serious insect pest in most production environments, but thrips species cause major cosmetic damage to the fruit for the export trade (Dubois and Coyne, 2011; Gold et al., 2001). Besides, stem borers, moths, beetles, fruitflies and whiteflies are significant pests (Gold et al., 2001). The latter two excrete honeydew, facilitating foliar and fruit molds, thereby reducing the market value. Aphids often transmit viruses such as banana bunchy top virus (BBTV), which is a major constraint to production in several African countries. The disease can be prevented with regular inspections and destruction of infected plants (Kumar et al., 2015), but extension frequently fails and hence local epidemics jeopardize production. Banana streak virus (BSV) is a complex of different circular dsDNA episomal viral species that are integrated in the genomes of banana varieties with the B genome (Iskra-Caruana et al., 2014).

In the group of bacterial diseases, *Xanthomonas* wilt, caused by *Xanthomonas campestris*, Moko disease, caused by *Ralstonia solanacearum* biovar 1 race 2, and brown rot caused by *Ralstonia solanacearum* biovar 1, race 1, are major threats to bananas and plantains. They are dispersed by insects and contaminated tools worldwide, but their

incidence is remarkably irregular (Tinzaara et al., 2006). Recent advances in breeding and genetic engineering provide new options for disease control (Tripathi et al., 2014).

As mentioned before, with respect to fungal disease, "Cavendish" quenched the Panama disease epidemic (Ploetz, 2006). However, since the 1960 another Foc strain - colloquially called Tropical Race 4 (TR4) - has emerged that now develops into a pandemic thereby threatening the entire "Cavendish" production, which comprises approximately 40% of the global production with immense importance for domestic and export markets. Due to the "orphan crop" label, investment in research and development has been minimal over the past decades and hence, there is no commercially viable replacement for these varieties. In addition, TR4 also kills many local varieties. Thus "Cavendish", once a blessing, now is a vehicle for disaster. Lack of attention, unawareness of the problem and poor vision have left growers with unmanageable disease problems and therefore, new initiatives are urgently required that professionalize the development of new plant material through advanced breeding programs. Until new varieties reach the market, bluntly, exclusion is basically the only effective control strategy for the majority of banana disease and pests.

Pseudocercospora fijiensis

The black Sigatoka fungus *P. fijiensis* was originally described by Rhodes (1964) in the Sigatoka district, on Viti Levu, Fiji islands. Together with *P. musae* (previously *M. musicola*) causal agent of Sigatoka disease, and *P. eumusae* (previously *M. eumusae*) that causes Septoria leaf spot, they form the Sigatoka complex, as these pathogens can coexist on the same leaf or in the same lesion even along with other fungi of minor economic importance (Arzanlou et al., 2008). Sigatoka pathogens diminish photosynthetic capacity due to leaf necrosis (Lepoivre P., 2003; Marín et al., 2003; Okole and Schulz, 1997), induce early

ripening and reduce bunch weight up to 40% (Castelan et al., 2012) unless crop protection agents are used.

The main dispersing propagules of this bipolar heterothallic pathogen are the sexual air-borne ascospores, and the asexual conidiospores, which are continuously produced under field conditions. The disease cycle starts when spores land on the abaxial side of the youngest “cigar” leaf. Therefore, disease symptoms initially appear at the perimeter of the leaf towards the midrib. Under favorable conditions (>25°C temperature, 95% relative humidity for at least 72h) spores germinate, grow epiphytically and enter the leaf through stomata and subsequently, hyphae colonize the apoplastic space of the mesophyll and accumulate in the substomatal cavities to produce the fructifications thereby gradually forming chlorotic lesion in the foliage that eventually expand into large coalescing necrotic blotches. According to Meredith and Lawrence (1969) and Fouré (1985), disease development can be divided into six stages. The first necrotic spots appear at the abaxial leaf side, not even visible by transmittent light. When spots enlarge parallel to the veins, they become visible also at the adaxial side, which is distinctive of stage 2 and sporodochia are generated to produce and release conidia on conidiophores, which emerge through stomatal openings at both sides of the leaf. Conidia disperse typically just a few meters by wind or splash as well as by contact with other leaves. Once streaks coalesce to form larger and ticker streak lesions, stage 3 is reached. In the meantime, sporodochia develop spermagonia with spermatia that are required for the sexual reproduction. At stage 4 lesions on both leaf sides darken and at stage 5 these lesions become depressed and surrounded by a yellow halo. Eventually, lesions develop into paper-like dry and whitish/greyish colored blotches, surrounded by a dark border and the yellow halo that over time coalesce into large necrotic blotches with dry and inner gray zones bearing the perithecia that are visible as minute dark spots on both sides of the foliage. They discharge air-borne ascospores upon drastic changes in relative humidity, which disseminate over

relatively short distances (Burt et al., 1999; Rieux et al., 2014) and start a new disease cycle (Figure 1).

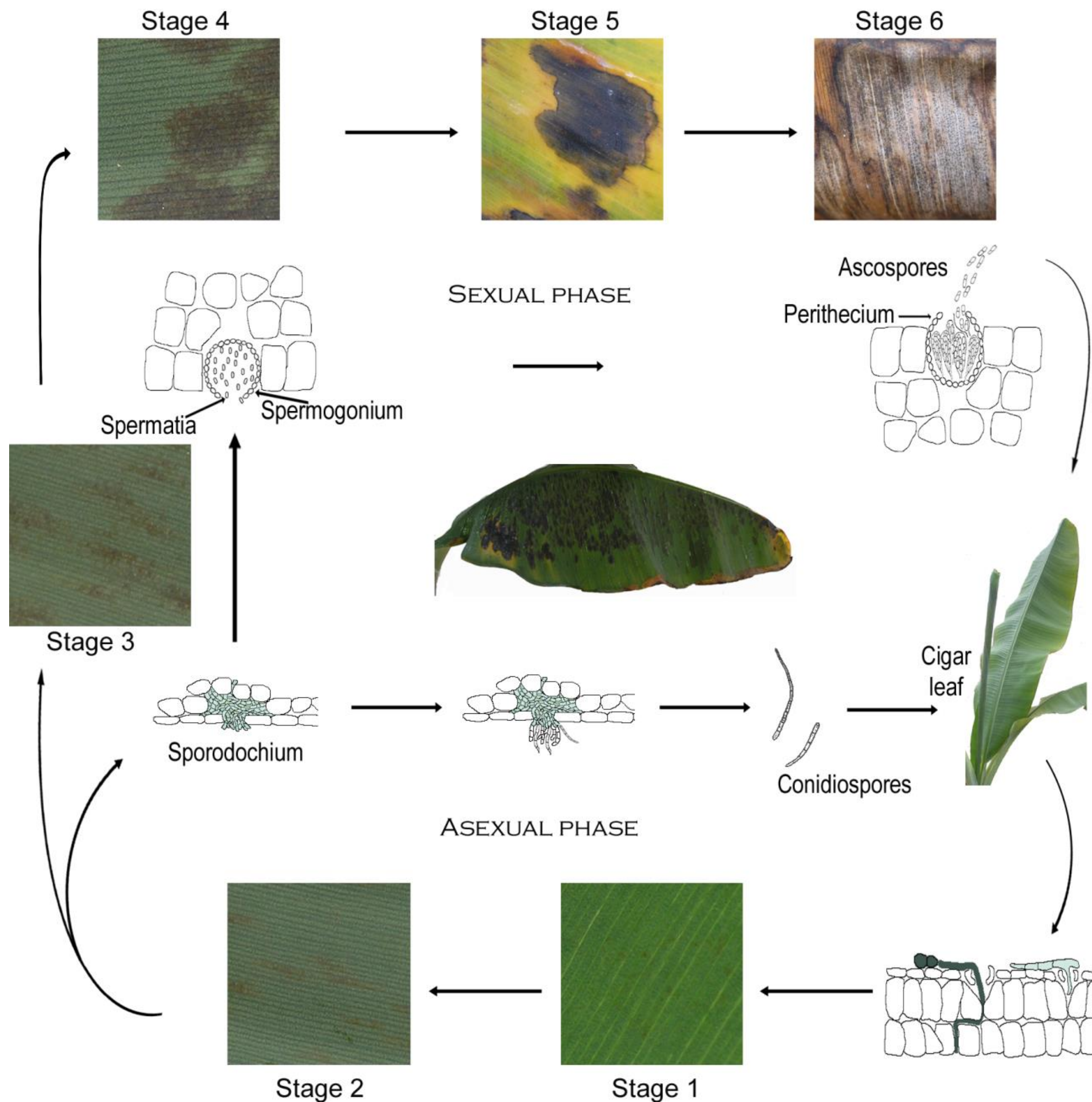

Figure 1. The black Sigatoka disease cycle.

The evolution of the Sigatoka complex results in visually undistinguishable symptoms of the individual *Pseudocercospora* species. Yellow Sigatoka spread globally but was gradually outcompeted by the more aggressive black Sigatoka disease. Presently, *P. musae* is confined to highland regions > 1000 m with a few exceptions (Amil et al., 2007), whereas *P. fijiensis* has spread from Fiji (Rhodes P.L., 1964) to almost all tropic and subtropical areas

where bananas are grown and is still migrating to new areas, for instance in Brazil where the Northeast region is still not affected due to a “buffer zone” that is already affected (Brito et al., 2015), and in Australia where it was eradicated during an intensive campaign (NSW Primary Industries, 2013). Field populations of *P. fijiensis* are genetically extremely diverse due to their bipolar heterothallic mating system (Carlier, 1994; Chong, 2016; Conde-Ferráez et al., 2007). Yet, the global population can be divided into four main groups: Southeast Asia, Pacific Islands, Africa and Latin America that all trace back to the gene center of banana in Southeast Asia (Carlier, 1994; Carlier et al., 1996). Founder effects of these populations have shown that the dissemination of *P. fijiensis* was mainly due to human transport of contaminated plant material, as often leaves are used for different activities, such as food wrapping and transport of the fruit. Thus the independent introductions in Latin America and Africa in 1972 and 1973, respectively, generated two genetically highly diverging populations. At the continental scale, stochastic spreading has been suggested by either a limited natural dispersal of ascospores or by transport of infected material (Carlier et al., 1996; Rivas et al., 2004). Most of these conclusions were derived from extensive populations genetic analyses by using a wide array of genetic markers, which comprise restriction fragment polymorphisms (RFLPs) (Carlier, 1994; Carlier et al., 1996; Hayden and Carlier, 2003), AFLP, SSRs, Microsatellites (Mueller R. et al., 1995; Neu C. et al., 1999); PCR-RFLP (Fahleson et al., 2009; Hayden and Carlier, 2003; Zapater et al., 2004), and recently also genome sequence based markers including single nucleotide polymorphisms (SNPs) (Zandjanakou-Tachin et al., 2009), mini satellites and variable numbers of tandem repeats (VNTRs) (Chong, 2016; Garcia et al., 2010). These data were used to scrutinize the structure of local populations and underscored the huge diversity at all levels, even between isolates derived from one lesion (Arzanlou et al., 2007; Müller R. et al., 1995; Müller et al., 1997),

which was also reported for the related *Zymoseptoria tritici* pathogen of wheat (Linde et al., 2002).

The third species of the Sigatoka complex, *P. eumusae* is spreading towards Asia and Africa (Carlier et al., 2000; Crous and Mourichon, 2002) and is considered to be more aggressive than *P. fijiensis* (Zandjanakou-Tachin et al., 2013). As mentioned above, these *Pseudocercospora* species trigger similar symptoms in banana and plantain leaves, are morphologically similar and often coexist in the same leaf (Carlier et al., 2000; Crous and Mourichon, 2002). *Pseudocercospora fijiensis* can be clearly differentiated from *P. musae* with genetic markers, based on the ITS1 region (Johanson and Jeger, 1993). In 2007, TaqMan markers were identified by Arzanlou et al. (2007), which quantitatively detect the individual species. Recently, Chang et al. (2016) showed that the infection biology of *P. eumusae* slightly differs from *P. fijiensis* and *P. musae*. These data, altogether, contribute to a better understanding of the Sigatoka complex and its control in banana and plantain.

Control of *Pseudocercospora fijiensis*

The management of *P. fijiensis* in banana is challenging because export bananas are typically cultivated in large monocultures. Despite the use of different control strategies, including good field practices such as early warning/forecasting systems, symptoms screening, biocontrol agents and leaf pruning, the only truly effective black Sigatoka management relies on frequent fungicide applications (Chong, 2016). Evidently, such frequent applications - usually >50 times per year - drive selection in pathogen populations, requiring even more applications that eventually spiral into unmanageable disease situations and a fall back to protectants that threaten the often precautious tropical environments and plantation workers even more and take a 30% share of the overall production costs (Chong,

2016; FRAC, 2010; Marín et al., 2003). The versatility of the fungus to maintain genetic diversity through its continuous sexual reproduction complicates disease control due to an unparalleled trend towards reduced sensitivity. Therefore, complex spraying schedules are developed to minimize this trend. These comprise a range of chemistries, including protectants such as benomyl (Stover R.H., 1990), chlorothalonil and the dithiocarbamates mancozeb (Romero and Sutton, 1997) that are considered as "low-resistance" risk products (FRAC, 2010). Besides, they include systemic fungicides, such as the demethylase inhibitors (DMIs) and strobilurins (Amil et al., 2007; Sierotzki et al., 2000). Despite the reduced sensitivity to DMIs in most production environments they are still the cornerstone of black Sigatoka disease management (Cañas-Gutiérrez et al., 2009; Marín et al., 2003). Clearly, there is a great need to better understand the mechanisms of reduced sensitivity, which is essential to develop better management strategies. The Fungicide Resistance Action Committee (FRAC) banana working group monitors and evaluates control strategies and recommends on active ingredients and application strategies to minimize sensitivity loss. Recent research described a global sensitivity map base on analyses of the *Pfcyp51* gene, which is the target for DMIs (Cañas-Gutiérrez et al., 2009; Chong, 2016). Interestingly, a recent market pull also favored alternative technologies and production areas enabling the export of organic bananas that are grown in areas which are not conducive for *P. fijiensis*, such as in Peruvian highlands, and hence do not require fungicide applications (USDA, 2008). The demand and export of such organic bananas annually increases up to 19% according to "Peru: organic banana exports grow by 19%" (2016).

Host resistance to *Pseudocercospora fijiensis*

As mentioned above, the predecessors of the commercial banana and plantain varieties are the seeded diploids *M. acuminata and M. balbisiana.* Edible banana, however, share one common denominator, which is seedlessness, either due to triploidy or parthenocarpy (Heslop-Harrison and Schwarzacher, 2007; Perrier et al., 2011; Sardos et al., 2016). Clearly, breeding bananas requires seed production and smart strategies to eventually develop seedless, edible fruits while capturing genetic diversity. Yet, low fertility, triploidy, slow propagation, space requirement and cycling time all contributed to a complex process that has not attracted manifold breeders and hence, the release of new attractive germplasm has been minimal over the last decades (Ploetz et al., 2015). Therefore, crop improvement has largely concentrated on selection within clones, for instance for productivity, fruit flavor, hardiness, height, bunch weight and shelf life. Evidently, under such conditions and circumstances, on top of the "orphan status" of banana, progress on the genetic control of fungal disease has been extremely slow, and with regard to black Sigatoka, hardly any progress has been realized. As a matter of fact, not a single gene for resistance to black Sigatoka has been identified, which is an important basis for improving resistance to this disease. Genetic analyses that have used natural inoculum for phenotyping segregating populations are essentially useless due to the known genetic diversity of natural populations and the huge effect of fluctuating environmental factors (Ortiz and Vuylsteke, 1995). In conclusion, the very basic work for understanding the banana-black Sigatoka pathosystem has still to start. The current thesis is a humble effort to begin developing the required basis for advanced (population) genetics and genomics studies of *P. fijiensis* to close existing knowledge gaps supporting the development of a more sustainable banana production.

Fullerton and Olsen (1995) were the first to challenge a differential set of banana and plantain accessions with individual *P. fijiensis* isolates from three global populations and

compared these data with previous field performance. Such data are basic, yet crucial to understand diversity. Other, previously considered notoriously difficult pathosystems such as *Leptosphaeria maculans-Brassica napus* (Balesdent et al., 2002) and *Zymoseptoria tritici*-wheat (Brading et al., 2002; Ghaffary et al., 2011; Habig et al., 2017; Kema et al., 2018; Kema et al., 2000; Mirzadi Gohari et al., 2015), are current model systems that are grounded in large host-pathogen matrices that eventually resulted in the identification of a range of resistance genes that are applied in contemporary advanced breeding programs (Goodwin, 2007; Kohli and Skovmand, 1997; Rouxel and Balesdent, 2013). Phenotyping diversity in the banana-*P. fijiensis* pathosystem is evidently more complicated, hence it would benefit from a smart, simplified selection process, for instance by using effectors. Previously, highly resistant, partially resistant or susceptible responses to *P. fijiensis* have been reported (Fouré et al., 1990; Mourichon et al., 1987), and cytological studies revealed that only the resistant host displayed an immediate response upon stomatal penetration (Beveraggi et al., 1995), suggesting a gene-for-gene relationship and partial resistance between *P. fijiensis* and "Yangambi Km5" and "Fougamou", respectively. However, the resistance of "Yangambi Km5" was eventually circumvented, even without commercial exploitation and increased selection pressure, which eventually resulted in establishing the International *Musa* testing program (ITMP). It initially comprised hybrids from the Honduras Foundation for Agricultural Research (FHIA) and a differential set of standard clones that were planted at geographically different locations in Latin America and Africa (Jones, 1994). Data analyses identified *M. acuminata* spp. *burmannicoides* "Calcutta 4" as a reference for resistance to *P. fijiensis* (Table 2; Jones and Tézenas du Montcel, 1994).

Table 2. Results of the International *Musa* testing program, Phase 1 surveying for resistance to black Sigatoka (INIBAP, 1992).

Accessions	International Transit Centre Code	Genome formula	Type	Response to Black Sigatoka
Tuu Gia	0610	AA	Edible cultivar	ER
Musa acuminata spp. *burmannicoides*	0249	AA	Wild species	ER
Musa acuminata spp. *malaccensis*	0609	AA	Wild species	ER
Pisang Lilin	0001	AA	Edible cultivar	HR
Pisang Berlin	0611	AA	Edible cultivar	R
Pisang Mas	0653	AA	Edible cultivar	R
Musa balbisiana	0405	BB	Wild species	R
SF215/NBA14	0267	AA	Edible cultivar	S
Niyarma Yik	0269	AA	Edible cultivar	HS

ER: Extremely resistant HR: Highly resistant R: Resistant S: Susceptible HS: Highly susceptible

In the pre-genomic era, attempts to identify and use phytotoxins (Molina, 1989; Okole and Schulz, 1997; Stierle et al., 1991; Strobel et al., 1992; Upadhyay et al., 1990) tried to avoid the necessary aforementioned groundwork, but did contribute to a deeper understanding of pathogenesis on resistant and susceptible banana varieties. More recently, Hoss et al. (2000) found that 2,4,8-trihydrotetralone accumulates in susceptible bananas just before severe disease symptoms develop, while the timing in resistant bananas occurred during the very early stage of infection. Finally, additional attempts for resistance screening included the use of juglone, other toxins and secondary metabolites belonging to phenylpropanoid pathway (Chuc-Uc et al., 2011; Cruz-Cruz et al., 2009; Hadrami et al., 2005; Otálvaro et al., 2007), suggesting phytoalexins to play a role. Molecular analyses have also discovered a first insight in the defensive arsenal of banana and include the generation of H_2O_2 (Beltrán-García et al., 2014; Cavalcante et al., 2011; Torres et al., 2012), peroxidase, phenylalanine amonia lyase, β-1,3-glucanase (Torres et al., 2012) and chitinases (Escobar-Tovar et al., 2015a; Torres et al.,

2012). However, as pointed out above, the major missing yet required link is a better understanding of the genetics of resistance and pathogenicity. Unveiling the genetic basis of resistance in "Calcutta 4" is still pending, which is mostly due to the poor amenability of *P. fijiensis.* The genome sequencing of *M. acuminata* ssp. *malaccensis* (D'Hont et al., 2012) and *M. balbisiana* (Davey et al., 2013) are, along with classical analyses and genome wide association studies, promising turning points for gene discovery.

Genomics, genetics and gene expression of *Pseudocercospora fijiensis*

The release of the draft *P. fijiensis* genome sequence was an immediate resource for multiple comparative analyses (Arzanlou et al., 2010; Chang et al., 2016; Chong, 2016; Churchill, 2011; Couoh-Uicab et al., 2013; de Wit et al., 2012; Escobar-Tovar et al., 2015a; Kantún-Moreno et al., 2013; Noar and Daub, 2016a, 2016b; Ohm et al., 2012; Stergiopoulos et al., 2010, 2014). The complete genome sequence (Arango Isaza et al., 2016), was until recently the largest Dothideomycete genome, which is presently exceeded by the >150Mb genome of the only mycorrhizal species *Cenococcum geophilum* (Peter et al., 2016) in this class of fungi. Genome expansion is largely due to repetitive elements in both species. It was intriguing that once the *P. fijiensis* genome was released, the first homologue of the *Cladosporium fulvum* effector *Avr4* was discovered (Stergiopoulos et al., 2010). The heterologously produced *PfAvr4* effector as well as most *Avr4* isoforms, triggered hypersensitive response on tomato leaves carrying the cognate *Cf4* resistance gene (Kombrink, 2012; Stergiopoulos et al., 2014, 2010).

Clearly, the fungal and host genome sequences helped to reveal the first glimpses of the banana-*P. fijiensis* interaction, but many questions remain unresolved. The abovementioned initial effector analysis, two fungal linkage maps for genome assembly

(Arango Isaza et al., 2016), a detailed global analysis of DMI sensitivity (Chong, 2016) and several expression analyses involving glycosyl phosphatidyl-inositol (GTI) proteins (Kantún-Moreno et al., 2013) and a putative ABC transporter - the orthologue of the *MgAtr4* in *Z. tritici* - (Couoh-Uicab et al., 2013) are resulting milestones. Even more so, once such studies are lined-up with the infection process which is accompanied by differential accumulation of melanin and H_2O_2 (Beltrán-García et al., 2014; Torres et al., 2012). Recent analysis of Escobar et al. (2015a) and Chang et al. (2016) suggest that pathogenesis is aided by an overall stealth weakening of host tissue rather than a rapid collapse, which accords with the known pathogenesis in other Dothideomycetes (Goodwin et al., 2011; Ohm et al., 2012).

With the advance of genome analyses, hence gene discovery, tools for functional analyses become indispensable. Several protocols have been published (Balint-Kurti et al., 2001; Escobar-Tovar et al., 2015b) that enable random mutagenesis, but do not facilitate functional analyses. Only recently, Onyilo et al., (2017) reported the first gene targeted by silencing in *P. fijiensis*; a very powerful transient transformation method particularly for reversible and/or incomplete gene expression prevention.

In this thesis I describe the genome sequence of *P. fijiensis,* a protocol for its transformation and the application in tool development for functional assays. Together, these should aid the advance of (functional) genetics in this important banana pathogen, thereby contributing to the overall aim of a more sustainable and diversified banana production.

Outline

Overall, at the start of my PhD thesis project I identified major bottlenecks to advance research in the banana - *P. fijiensis* pathosystem. Firstly, I considered that sequencing the genome of *P. fijiensis* would be a powerful base to increase the understanding of its (infection) biology. Secondly, I contemplated on the possibility to develop a protocol for homologous recombination, which would facilitate functional analysis of any given gene. Hence, my thesis was divided into five chapters.

Chapter 1 introduces the crop banana and plantain, presents their importance and describes the major biological threats of the crop with an emphasis on the fungal diseases to eventually focus on black Sigatoka. The biology of its causal agent *P. fijiensis* and the interaction with banana are briefly described. Furthermore, a critical analysis of the current standing in banana and black Sigatoka research is provided, thereby indicating the major bottlenecks. I considered that resolving the latter would bring banana research to another level.

Chapter 2 describes the genome sequencing of *P. fijiensis,* which resulted in a 74 Mb, genome size, mostly containing repetitive DNA and a remarkable differential GC content, which was also observed in the other Sigatoka complex constituents *P. musae* and *P. eumusae*. Characteristics of gene content, gene models, synteny, gene expression, and genome dynamics are compared with the Dothideomycete reference genome of *Zymoseptoria tritici*, as well as with other Dothideomycetes, and discussed in relation to its life style. An initial functional analysis of *PfAvr4* is included, which is indicative for gene-for-gene interaction in the banana - *P. fijiensis* pathosystem. Finally, the genome information was used to analyze strobilurin fungicide resistance dynamics in natural *P. fijiensis* populations in various banana plantations in Costa Rica.

Chapter 3 focuses on developing a protocol for *Agrobacterium*-mediated transformation (ATMT) of *P. fijiensis*. Initially, I developed a protocol for random mutagenesis and generated GFP- and Ds Red- labeled strains. Subsequently, I modified the protocol for targeted mutagenesis and used it for functional analysis of *Pfavr4* and for developing a *Pfku70* knock-out strain. The latter is expected to have an impaired non-homologous end joining pathway, which would increase homologous recombination and thereby enhance the throughput for homologous recombination and hence, developing targeted knock-out strains.

Chapter 4 describes the use of the aforementioned transformation protocol to analyze the mechanism of DMI sensitivity in *P. fijiensis.* Besides abundant mutations in the coding sequence of the target *Pfcyp51* gene, we discovered multiple repeated insertions in the *Pfcyp51* promoter region that are crucial for reduced sensitivity of *P. fijiensis* resistance to triazoles. The promoter sequences were detected in various international field strains with reduced sensitivity to propiconazole, difenoconazole and epoxiconazole as well as in field strains from Costa Rican farms, which suggests that these mutants were selected by the frequent fungicide applications in these plantations. ATMT was used to replace the promotor of a sensitive strain by the promotor of a resistant strain to show that both structural variants are required for reduced sensitivity.

Finally, **Chapter 5** provides a general and critical treatise of the achieved results and brings a wider scope for forthcoming research that is necessary to improve our understanding of the banana - *P. fijiensis* pathosystem and to develop strategies that contribute to a sustainable banana production.

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CHAPTER 2

COMBATING A GLOBAL THREAT TO A CLONAL CROP: BANANA BLACK SIGATOKA PATHOGEN *PSEUDOCERCOSPORA FIJIENSIS* (SYNONYM *MYCOSPHAERELLA FIJIENSIS*) GENOMES REVEAL CLUES FOR DISEASE CONTROL

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Abstract

Black Sigatoka or black leaf streak disease, caused by the Dothideomycete fungus *Pseudocercospora fijiensis* (previously: *Mycosphaerella fijiensis*), is the most significant foliar disease of banana worldwide. Due to the lack of effective host resistance, management of this disease requires frequent fungicide applications, which greatly increase the economic and environmental costs to produce banana. Weekly applications in most banana plantations lead to rapid evolution of fungicide-resistant strains within populations causing disease-control failures throughout the world. Given its extremely high economic importance, two strains of *P. fijiensis* were sequenced and assembled with the aid of a new genetic linkage map. The 74-Mb genome of *P. fijiensis* is massively expanded by LTR retrotransposons, making it the largest genome within the Dothideomycetes. Melting-curve assays suggest that the genomes of two closely related members of the Sigatoka disease complex, *P. eumusae* and *P. musae*, also are expanded. Electrophoretic karyotyping and analyses of molecular markers in *P. fijiensis* field populations showed chromosome-length polymorphisms and high genetic diversity, but limited gene flow. Genetic differentiation was also detected using neutral markers, suggesting strong selection with limited gene flow at the studied geographic scale. Frequencies of fungicide resistance in fungicide-treated plantations were much higher than those in untreated wild-type *P. fijiensis* populations. A homologue of the *Cladosporium fulvum Avr4* effector, *Pfavr4*, was identified in the *P. fijiensis* genome. Infiltration of the purified PfAVR4 protein into leaves of the resistant banana cultivar Calcutta 4 resulted in a hypersensitive-like response. This result suggests that Calcutta 4 could carry an unknown resistance gene recognizing *Pfavr4*. Besides adding to our understanding of the overall Dothideomycete genome structures, the *P. fijiensis* genome will aid in developing fungicide treatment schedules to combat this pathogen and in improving the efficiency of banana breeding programs.

Introduction

Black Sigatoka or black leaf streak disease (BLSD), caused by the Dothideomycete fungus *Pseudocercospora fijiensis* (previously: *Mycosphaerella fijiensis*) (Arzanlou et al., 2008), is a major threat to global banana production (Marin et al., 2003). The disease is part of the Sigatoka complex, which involves two other closely related pathogens in addition to *P. fijiensis*: *P. musae* (previously: *M. musicola*) causal agent of yellow Sigatoka disease; and *P. eumusae* (previously: *M. eumusae*) causal agent of the eumusae leaf spot disease. Among the three species, *P. fijiensis* is the most aggressive and predominant member of the Sigatoka disease complex worldwide. These pathogens occur exclusively on the foliage of bananas and plantains, with continuous sexual and asexual reproduction in nature (Arzanlou et al., 2008; Carlier et al., 2000, 1996; Crous and Mourichon, 2002).

BLSD was first reported in the Sigatoka Valley of the Fiji islands during the 1960s and has since spread to nearly all banana-producing areas worldwide. It can only be managed by intensive fungicide applications, requiring weekly interventions throughout the year in most production areas. Black Sigatoka inflicts huge costs on global banana production, surpassing US $500 million per year (Ploetz, 2004). Expenses for fungicide treatments usually represent more than 35% of total production costs (de Bellaire et al., 2010; Romero and Sutton, 1997). Infection with *P. fijiensis* also results in crop losses and massive indirect costs by inducing early ripening of the fruit, making it unsuitable for sale with concomitant effects on the export trade and the retail sector.

Export banana cultivars are sterile, triploid plants that can only be propagated clonally and are grown in huge monocultures of genetically identical individuals. The international banana trade is based solely on a few closely related clones of the Cavendish type, all of which are highly susceptible (Ploetz, 2004); disease management, therefore, relies primarily on fungicide applications with enormous environmental impacts (Churchill, 2011). Moreover,

the selection pressure on *P. fijiensis* populations continuously reduces the efficacy of fungicides resulting in control failures and unmanageable levels of disease (Marin et al., 2003; Romero and Sutton, 1997). Therefore, there is an urgent need for scientific discoveries that will lead to the development of better methods for protecting banana crops, both for export fruit production and for small holders around the world who rely on bananas as a staple food (Marin et al., 2003).

Taxonomically, *Pseudocercospora* belongs to the order Capnodiales in the class Dothideomycetes, previously known as the *Loculoascomycetes* (Eriksson and Winka, n.d.), which is the largest and most diverse class of ascomycete fungi comprising over 20,000 species. Dothideomycete fungi include endophytes and epiphytes of plants, but also saprobes degrading cellulose and other complex carbohydrates of dead plants, and plant pathogens (Schoch et al., 2006). The latter cause a range of diseases in various key food, fiber and fuel crops, including *Zymoseptoria tritici* (Septoria tritici blotch of wheat) (Kema, 1996), *Venturia inaequalis* (apple scab) (Bowen et al., 2011), and *Leptosphaeria maculans* (blackleg of Brassica crops) (Howlett et al., 2001). Therefore, genome sequences of several Dothideomycetes have been published (Ellwood et al., 2010; Hane et al., 2007; Goodwin et al., 2011; de Wit et al., 2012; Ohm et al., 2012; Condon et al., 2013; Manning et al., 2013; Dhillon et al., 2015) or are in the process of being completed (http://genome.jgi.doe.gov/dothideomycetes/dothideomycetes.info.html). The genome sequence of *Z. tritici* is the reference for all other Dothideomycetes as it is the only one that has been completely finished (Manning et al., 2013).

The poor experimental amenability of *P. fijiensis* has significantly hampered progress in understanding its basic biology (Churchill, 2011) and the development of research tools. For instance, infection assays are cumbersome due to the need for very specific environmental conditions with respect to temperature, light and relative humidity, and the slow development

of the disease that may take up to 50 days until symptoms are expressed (Abadie et al., 2008; Nfor et al., 2011). Therefore, basic information on pathogenesis is not available and almost nothing is known about the genetic basis of disease resistance in banana germplasm (Beveraggi et al., 1993; Torres et al., 2012). Hence, new tools and research methods are needed to better understand the disease and ensure continued production of the world's number one fruit, which is a staple food for millions of people in many developing countries.

A previous comparative analysis of 18 Dothideomycetes genomes (Ohm et al., 2012) included the *P. fijiensis* isolate CIRAD86 genome for a global analysis of genome organization and evolution. However, *P. fijiensis* was not the primary focus of that analysis and few specifics were discussed. Here we focus on the genome sequence of *P. fijiensis* isolate CIRAD86, describe the sequence of a second isolate, CIRAD139, and analyze in detail the species' genome structure, content and function with a goal of delivering new data that could give clues for global disease management of this devastating banana pathogen.

Results

Sequencing, assembly and annotation of the *P. fijiensis* genome

The genomes of the *P. fijiensis* isolates were sequenced using either Sanger technology (CIRAD86) or Illumina for resequencing (CIRAD139a). The final assembly size of ~74 Mb consisted of 56 scaffolds with the largest at 11.8 Mb and an N50 of 50 Kb. Inclusion of a newly made genetic map facilitated assembly of the physical genome (Table S1).

Genetic map construction involved 376 loci that segregated in the progeny of the mapping population, among which 322 (233 DArT, 86 SSR, 3 minisatellite) markers were mapped into 19 linkage groups (Figure 1). The number of loci per linkage group varied from

2 to 35 with an average of 17 and linkage groups 1, 2, 8 and 9 contained the largest numbers of markers with 35, 29, 31 and 26, respectively. Map distances between consecutive markers varied from 0 to 20.4 cM with the largest gaps between markers on linkage groups 14 and 17, of 6.1 and 20.4 cM, respectively (Figure 1).

After filtering for EST support, completeness and similarity to other species, 13,107 genes were structurally and functionally annotated. The average gene length in the version 2 assembly is 1,833 nt with 3.62 exons per gene; 88%, are complete with start and stop codon, 74% have similarity support, and 49% have Pfam domains (Table S2). Most of the gene models (96%) are located in 12 scaffolds, numbers 1-10, 12 and 19. Gene density in these 12 scaffolds varies from 151 to 229 per Mb but gene density for the remaining scaffolds larger than 0.5 Mb drops to only 2 to 94 genes per Mb (Table S1). More detailed information on the assembly, annotation and EST support data can be found in supporting Text S1.

Genome structure

The Pseudocercospora fijiensis *genome is greatly expanded*

The 74-megabase genome of *P. fijiensis* is greatly expanded relative to those of other related Capnodiales such as *Sphaerulina musiva* (previously *Septoria musiva* with teleomorph *Mycosphaerella populorum), S. populicola* (previously *Septoria populicola* with teleomorph *Mycosphaerella populicola)* and less related species such as *Dothiostroma septosporum*, *Baudoinia compniacensis*, and *Z. tritici*, but less so compared to *C. fulvum,* the closest Capnodiales relative sequenced and only other Dothideomycete with an expanded genome of 65 Mb (Figure 2). The predominant repetitive elements in the *P. fijiensis* genome belong to the long terminal repeat (LTR) retrotransposons (50%) (Figure 3), which is much higher than in *Z. tritici,* but similar to the proportion seen in *C. fulvum*. Compared to these other two

species, the genome of *P. fijiensis* contained much higher percentages of repetitive DNA and unclassified transposons, whereas that of *C. fulvum* had the highest percentage of non-LTR retrotransposons among the three species (Figure 3). The estimated number of gene models is 13,107, which is approximately 28% and 34% higher than in *S. musiva* and *S. populicola*, respectively (Table 1) and 7% smaller than *C. fulvum*, Using the 80:80 criterion (Wicker et al., 2007), i.e., 80% sequence identity across 80% alignment length, all of the *P. fijiensis* repeat families were unique. However, using a 70:70 cutoff criterion, elements from 50 *P. fijiensis* repeat families, amounting to 449 kb, were similar to those in the *C. fulvum* genome. A non-LTR repeat family from *P. fijiensis* (family 6), with an average element length of 4.9 kb, had the highest representation with 36 copies in the *C. fulvum* genome.

Analysis of repeat-induced point mutation (RIP) showed a clear CA<-> TA dinucleotide bias in the repetitive elements identified in the *P. fijiensis* genome (Figure 4). Some families also showed a CT<->TT dinucleotide bias. A similar pattern has been observed in a number of ascomycete genomes, including *Parastagonospora nodorum* (Hane and Oliver, 2008).

Repetitive elements often clustered to form blocks of AT-rich DNA. When an average DNA content of 45% or less was used to define AT-rich regions, a total of 1,865 AT blocks was identified in the *P. fijiensis* genome, ranging in length from 1 to 514 kb. These blocks account for 45 Mb (61%) of the *P. fijiensis* genome and 84% comprised repetitive sequences. A total of 482 (4%) genes were associated with the AT blocks. About 20% (96) of these genes have associated annotations and 6% (28) can potentially be secreted.

If a lower value of 40% average percent GC is used, the number of AT blocks diminished drastically to 640, amounting to a total length of 18 Mb or approximately 25% of the *P. fijiensis* genome. Repetitive sequences make up 84% and 152 (1.2%) genes were

associated with these AT-rich blocks. Approximately 22% (33) of the genes associated with AT blocks have an annotation and about 10% (15) have signal peptides.

The average RIP index was 0.2 in the AT-rich blocks as compared to a higher average RIP index of 1.37 across the rest of the genome. Plots of the RIP index were very low (indicating a high level of RIP) in the AT blocks (Figure 5A) but much higher (low RIP) in the regions of the genome with lower AT content (Figure 5B). As expected, there was a strong inverse relationship between GC content and the amount of RIP as measured by the index (Figure S1). Very few of the genes (just over 3%) in AT-poor (= GC rich) regions of the genome showed any evidence or RIP (index of 1.0 or less) compared to a little over half (53%) of those in AT-rich regions (Table 2). In contrast, all but two out of 7,674 repeats in AT-rich regions showed evidence of RIP and almost 93% of those in AT-poor regions (Table 2). Exceptions were few and minor (Figure S2).

First-derivative graphs obtained for melting profiles of *Z. tritici* showed a narrow curve with a single peak (Figure 6A); in contrast, those for *P. fijiensis* showed a broad curve with two peaks with G+C contents of 39.4 and 51.6% indicating heterogeneity (Table S3). This agreed with the GC plots of sequence reads from *P. fijiensis,* which clearly showed a double-peak phenomenon, the lower peak corresponding to transposon-rich regions (Figure 6E).

The melting profiles obtained from the DNA of both *P. eumusae* and *P. musae* also demonstrated a double-peak pattern of genomic G+C content. The G+C content pattern in *P. eumusae* was almost identical to that in *P. fijiensis* with peaks at 39.6 and 51.6%. In *P. musae*, both peaks corresponded to lower, albeit still comparable, G+C contents of 37.2% and 50.9% (Figure 6 A-D, Table S3). Plotting genome size on a phylogenetic tree of the Capnodiales

identified at least two expansions, one leading to *P. fijiensis* and the second to the biotrophic tomato pathogen *C. fulvum* (Figure 2).

To estimate the age of the transposon expansion in the *P. fijiensis* genome, approximately1600 LTR retrotransposons were identified using ltrharvest. A further search for protein domains identified 1,147 bona fide, full-length LTR retrotransposons. Of these, 529 elements (46% of the total) had LTRs that were highly similar in terms of mutations accumulated over time with a hypothetical insertion age of less than one million years (Figure 7). Many older elements also were identified (Figure 7) but these decreased with time, indicating that most of the transposon insertions occurred relatively recently.

Electrophoretic karyotyping suggests variability in genome content and/or organization among isolates

Pulsed-field gel electrophoresis of the CIRAD86 and E22 strains showed small and large chromosomes, but no chromosomes in the medium range of 1.5 to 3.9 Mb. Isolate CIRAD86 showed 11 bands representing chromosomes, four of which appeared to be composed of double, co-migrating bands (Figure 8A). Small chromosomes were in the size range of 0.83 to 1.45 Mb. Bands of 0.95 and 1.03 Mb showed approximately twice the intensity and were assumed to represent at least two chromosomes each (Figure 8A). Conditions for separation of large chromosomes showed a band of 5.2 Mb, a co-migrating chromosomal band of 4.33 and a smaller band of 4.27 Mb (Figure 8B). Strain E22 showed at least 12 bands in total, five of which likely contain co-migrating chromosomes. Small chromosomes were in the range of 0.70-1.45 Mb, and large chromosomes had estimated lengths between 4.05 and 6.80 Mb (Figure 8B). Additionally a comparison of small chromosomal bands of different strains originating from a single banana field showed that

every isolate contained between five and nine small chromosomal bands with unique length polymorphisms (Figure S3) indicating substantial variation in genome content and/or genome organization among individuals.

Synteny analysis suggests a core set of 12 chromosomes

Analysis of similarity between the genomes of different Dothideomycetes shows a high degree of conservation of genes in syntenic scaffolds. Mesosynteny was observed between *P. fijiensis* and all other Dothideomycetes analyzed including the Capnodiales *B. compniacensis, Cercospora zeae-maydis, Cladospoerium fulvum, D. septosporum* (Figure 9), *S. musiva* (Figure S4), *Zasmidium cellare* and *Z. tritici*, the Pleosporales *Cochliobolus heterostrophus, L. maculans, Pyrenophora tritici-repentis* and *P. nodorum,* and the Hysteriales species *Hysterium pulicare.* Microsyntenic blocks of up to 10 Kb were found only with the closest relatives *C. fulvum, D. septosporum* and *Z. tritici* (*S. musiva* was not tested for this analysis). No macrosynteny was observed between *P. fijiensis* and any of the presently sequenced Dothideomycetes.

Using *Z. tritici* as a reference it is clear that gene content is conserved among large blocks of chromosomes. For example, scaffold 1 of *P. fijiensis* shows synteny with chromosomes 1, 4 and 5 of *Z. tritici*, scaffold 2 with chromosomes 2, 10 and 13, whereas scaffold 6 of *P. fijiensis* shows synteny only with *Z. tritici* chromosome 6 (Figure S5). Interestingly, no significant synteny was found between any of the scaffolds of *P. fijiensis* and the dispensable chromosomes of *Z. tritici* (Figure 10), supporting the hypothesis of their independent origin, possibly by recent horizontal transfer, in the latter species (Goodwin et al., 2011).

Most of the synteny found in *P. fijiensis* with *Z. tritici* as well as with all other Dothideomycetes tested is present in scaffolds 1 through 10, 12 and 19. In addition, these

scaffolds showed the highest percent of genes with expression data (0.8% or greater), all of which suggest that these 12 scaffolds might represent the core genome. Many of the smaller scaffolds in the *P. fijiensis* genome have the physical characteristics observed for dispensable chromosomes in *Z. tritici*; they are smaller, with lower G+C contents and gene densities (Table S1). Based on these criteria, the core genome of *P. fijiensis* comprises 63.9 Mb or almost 87% of the genome, while the remaining 13% may be a dispensome.

Analysis of the synteny plots also showed some past chromosomal rearrangements. For example, approximately 22% of the gene content of the central part of scaffold 1 of *P. fijiensis* was missing from the largest scaffolds of *D. septosporum* (Figure 9) and *S. musiva* (Figure S4), and instead was found on scaffolds 8 and 7 of those species, respectively. This difference also was seen in the comparison with the more distantly related *Z. tritici* (Figure S5), although the result was not as clear and more chromosomes were involved. In a direct comparison, scaffold 1 of *D. septosporum* showed complete mesosynteny with scaffold 1 of *S. musiva* (Figure S6), suggesting that the central part of *P. fijiensis* scaffold 1 might have translocated after the divergence of all three species from an unknown common ancestor. The chromosome that likely supplied the translocation, corresponding to scaffolds 8 and 7 in *D. septosporum* and *S. musiva*, respectively, showed 1:1 mesosynteny in the direct comparison between those two species (Figure S6), but also showed mesosynteny with *P. fijiensis* scaffolds 12 and 19 (Figures 9 and S4). This suggests that scaffolds 12 and 19 of *P. fijiensis* may belong to a single chromosome that has not been assembled completely. Similar analyses identified a possible translocation or incomplete assembly involving scaffolds 3 and 8 of *P. fijiensis*, which correspond to scaffolds 5 (Figure 9) or 4 (Figure S4) of *D. septosporum* and *S. musiva*, respectively.

Re-sequencing of P. fijiensis *isolate CIRAD139A shows a 12 % difference in genome content*

Among the more than 73 million reads of paired-end sequence data obtained for isolate CIRAD139A, 60% could be aligned uniquely to the *P. fijiensis* reference genome of isolate CIRAD86 (Table S4). Another 28% of the reads aligned to multiple locations in the reference sequence, most likely due to duplications or repetitive elements in the genome. Almost 12% of the reads did not map to the reference, suggesting that some genome content present in CIRAD139A could be absent in the reference strain.

The numbers of polymorphisms varied widely among scaffolds roughly in proportion to size, and the number of SNPs was much higher than for indels on all scaffolds analyzed (Table S5). Mean SNP frequency on each scaffold calculated across a 10-kb window was more uniform, ranging from 59.2 for scaffold 13 to 84.1 for scaffold 11 (Table S5). Plotting the SNP density relative to gene density for the 21 largest scaffolds containing 99% of all gene models separated the scaffolds into two groups. One group contained most of the largest scaffolds and showed lower variability in SNP counts, while the second contained scaffolds with low gene densities and showed much more variability in SNP counts.

Genome content

Decreased numbers of pathogenicity-related genes

Enzymes that degrade plant cell walls (CWDEs) and other physical barriers including cutin are important pathogenicity factors, particularly in necrotrophic fungi. Comparison of the number of genes related to cell wall degradation in *P. fijiensis* with those in other fungi revealed a significant reduction, particularly when compared to necrotrophic Dothideomycetes. Cutinases, xylanases and chitinases are reduced three to five-fold when

compared to three fungi in the Pleosporales: *P. nodorum*, *P. tritici-repentis* and *C. heterostrophus* (Table 3). Additionally, carbohydrate-binding proteins, including those with chitin-binding and cellulose-binding modules as well as β glucosidases also are reduced (Table 3). EST support was found for four chitinases, 18 glucosidases, one cellulose binding and four chitin binding genes.

Similar to the CWDEs, the *P. fijiensis* genome shows a relatively low number of genes involved in the production of secondary metabolites, such as polyketide synthases (PKSs), with approximately half the number of genes found in the necrotrophs *P. nodorum, P. tritici-repentis* and *C. heterostrophus.* Contrary to the situation for PKSs, genes encoding non-ribosomal peptide synthetases (NRPSs) are not reduced in *P. fijiensis.* Its genome encodes 13 NRPSs and one hybrid NRPS-PKS, which is comparable to the numbers found in other Dothideomycetes (Table 3). However, EST support was found for only six of the PKS genes and four of the NRPS and the hybrid NRPS-PKS genes. This low level of EST support might be a sampling phenomenon due to the EST coverage; none of the libraries came from *in planta* conditions where these genes are more likely to be expressed.

The P. fijiensis *secretome*

Filamentous fungal pathogens are able to modulate resistance responses in the plant cell by secreting a class of proteins known as effectors. In many fungal pathosystems, effectors are important pathogenicity or virulence factors that determine the success of a fungal infection (de Wit et al., 2009; Oliva et al., 2010). The majority of described fungal effectors share many characteristics and belong to the class of small, secreted, cysteine-rich proteins (SSPs) (de Wit et al., 2009). A search of the genome with the above criteria showed that *P. fijiensis* possesses 172 genes encoding SSPs (smaller than 300 AAs in size) with four or more cysteine residues. Sixty-two percent of the *P. fijiensis* SSPs have no blast hits (107

proteins), 21% (37 proteins) have assigned GO terms and 23% have InterPro IDs other than SignalP (40 proteins). Thus, the number of potential SSP-encoding genes in *P. fijiensis* is 31% and 8% lower than in the genomes of *P. nodorum* (250 genes) and *Z. tritici* (187 genes), respectively (Figure S7). These results accord with Ohm et al. 2012 who found reduced numbers of SSPs in several Capnodiales.

Among the identified SSPs, one shows high similarity to *C. fulvum Avr4*, which is known to have a chitin-binding domain and is a well-studied effector in the *C. fulvum*-tomato interaction (van Esse et al., 2007). This *P. fijiensis* putative *Avr4* (*PfAvr4*) homolog is a 121 amino acid protein present on scaffold 4 from co-ordinates 183261-183623 and was shown to protect *Trichoderma viride* cell walls against hydrolysis by plant chitinases through chitin binding and to trigger a *Cf4*-mediated hypersensitive response (HR) in tomato (Stergiopoulos et al., 2014, 2010). Additionally, three homologs of *C. fulvum* effector *Ecp2* were found, one of which was able to induce different levels of necrosis or HR in tomato lines depending on whether they lack or contain a putative corresponding Cf-ECP2 protein (Stergiopoulos et al., 2014, 2010). It seems highly likely that at least some of these *P. fijiensis* effector proteins that are similar to known effectors in *C. fulvum* will play a role in pathogenicity or virulence of *P. fijiensis* on banana.

Genome function

Functional analysis of a putative effector protein

Infiltrations into banana and tomato leaves were performed to test the hypothesis that *PfAvr4* acts as an avirulence factor in banana. Different accessions were infiltrated with different concentrations of PfAVR4. Physical damage (small tear and occasional slight necrosis limited to the site of infiltration) caused by either the syringe or the fermentor

medium was similar among accessions (Figure 11) and was less intense in tomato plants (Figure 11B). Both large and small plants of *M. acuminata* cv. Grand Naine showed only physical damage with an occasional slight chlorotic effect at the infiltrated area (Figure 11A) after infiltrating the PfAVR4 protein; no hypersensitive response (HR) was observed at 10 days post infiltration (dpi) regardless of the protein concentration used.

In contrast, PfAVR4 triggered a clear HR-like necrosis when infiltrated into leaves of *M. acuminata* ssp. *burmannicoides* var. Calcutta IV, which has resistance against *P. fijiensis* (Figure 11A). The necrosis was already visible on large plants at 4 dpi, and was stronger by 10 dpi at both concentrations of PfAVR4. In small plants the earliest necrosis was observed at 10 dpi. Fermentor medium triggered a slight necrosis on both small and large plants of var. Calcutta IV, but this was very different from the HR-like necrosis induced by PfAVR4 and the combined effect triggered by the fermentor product (Figure 11A). Furthermore, tomato plants without a resistance gene (*Cf0* plants) showed only physical damage following infiltration, while those containing the *Cf4* resistance gene showed a HR to crude fermentor products containing PfAVR4 and to the purified PfAVR4 protein (Figure 11B).

Analyses of fungicide resistance and molecular markers within populations

In total, 621 hierarchically sampled *P. fijiensis* isolates were genotyped and partially phenotyped and showed that the commercial (sprayed) plantations were entirely or nearly fixed for quinone outside inhibitor (QoI) or strobilurin resistance (92-100%), whereas all 87 isolates sampled from the wild type, unsprayed San Carlos population were sensitive (Table S6). Subsequently, we used the genome sequence to develop primers for five Variable Number of Tandem Repeat (VNTR) loci enabling population diversity analyses that were combined with assessment of the mating type loci. We observed that the ratios between the two mating type alleles *mat1-1* and *mat1-2* are not significantly different from 1:1 in each

individual population as well as the overall total number of isolates (Table S6), and that the VNTR loci in all populations are in gametic equilibrium and hence, could be used to estimate genetic differentiation between populations, which was small but statistically significant (Table S7).

Discussion

The genome of *P. fijiensis* is one of the largest among all of the Dothideomycetes sequenced to date; it is 3.4 times larger than that of the Dothideomycete with the smallest genome, *Baudoinia compniacensis*, 1.85 times larger than that of *Z. tritici* and 1.2 times larger than that of *C. fulvum*, which is related to *P. fijiensis* and also has an expanded genome (de Wit et al., 2012). Almost all of the increased size is due to the proliferation of LTR retrotransposons, as described here and in another publication (Santana et al., 2012). The thermal denaturation results indicate that G+C content heterogeneity is not limited to *P. fijiensis* but also occurs in its close relatives within a monophyletic clade of banana pathogens. Based upon the observed similarity of DNA composition between these three banana pathogens, we predict that the genomes of both *P. eumusae* and *P. musae* also are expanded and that all three pathogens that often co-occur in nature seem to have a recent common ancestor (Carlier et al., 2000).

The only other member of the Capnodiales known to have a similarly expanded genome is the biotrophic tomato pathogen, *C. fulvum* (de Wit et al., 2012). Based on the positions of the species with expanded genomes on the phylogenetic tree (Figure 2), there appear to have been at least two independent expansions in genome size within the Capnodiales, one involving *P. fijiensis* (and most likely related banana pathogens) and another for *C. fulvum*. Lack of similarity between the transposable elements in the genomes of *P. fijiensis* and *C. fulvum* supports the hypothesis of independent expansions in each genome.

The terminal repeats of LTR retrotransposons are identical at the time of insertion and this provides a means to estimate the relative ages of transposable element insertions. This phenomenon has been very useful for estimating transposon insertion times in plants (SanMiguel et al., 1998; Wicker and Keller, 2007) but is less useful for fungi where RIP

greatly increases the rate of mutations in repetitive sequences. Using this approach in the basidiomycete *Laccaria bicolor*, three periods of transposon insertion were identified, ranging in age from 0 to 59 million years ago (Mya) (Labbé et al., 2012). Whether RIP occurs in *L. bicolor* is not known, but its genome does not contain the genes known to be required for RIP in other fungi (Smith et al., 2006) and no evidence for RIP was found among transposons in the genomes of other members of the Agaricomycotina (Horns et al., 2012) so it seems unlikely. Therefore, this approach for estimating the ages of transposon insertions is most likely valid for *L. bicolor*.

A similar analysis of LTRs of transposons in the genome of *Z. tritici* yielded hypothetical insertion ages ranging from 0 to 5.7 Mya, with the strong caveat that the times were probably vastly overestimated due to RIP (Dhillon et al., 2014). Even with the bias introduced by RIP, the estimated ages of transposon insertions in the genome of *Z. tritici* were an order of magnitude younger than those in *L. bicolor*, indicating that they must have occurred relatively recently. A more accurate approach to estimating transposon age in the presence of RIP would be to exclude the RIP-susceptible sites from analysis (Rouxel et al., 2011). Using this approach, transposon insertions in *L. maculans* and closely related species in the Pleosporales, another large order of the Dothideomycetes, mostly were relatively recent, within the past four million years (Rouxel et al., 2011). For *P. fijiensis*, the results of a similar analysis clearly suggest a recent, rapid burst of LTR retrotransposon insertions. The young age and high proliferation rate of around 46% of the LTR retrotransposons suggest that *P. fijiensis* has a highly dynamic genome. Such a recent, high level of activity of retrotransposons can have evolutionary as well as regulatory implications for gene expression that can be better understood using genomic comparisons with other closely related species.

Large genome expansions due to amplifications of repetitive elements have been observed in other plant-pathogenic fungi. The published genome sequences and analyses of

the powdery mildew fungi *Blumeria graminis* f. sp. *hordei, Erysiphe pisi* and *Golovinomyces orontii* show a marked genome expansion with a massive proliferation of non–LTR retrotransposons and a corresponding decrease in gene content (Spanu et al., 2010). The missing genes in these obligate biotrophs include enzymes for primary and secondary metabolism, carbohydrate-active enzymes, transporters, and secreted proteins such as effectors. The genome of *P. fijiensis* also shows a moderate decrease in certain gene families associated with pathogenicity such as PKSs and CWDEs when compared to necrotrophic Dothideomycetes such as *P. nodorum* or *P. tritici-repentis* (Hane et al., 2007). In a similar way, the hemibiotrophs *Z. tritici* (Goodwin et al., 2011), *S. populorum* and *S. populicola* (Dhillon et al., 2015; Ohm et al., 2012) also show a marked decrease in CWDEs [19], although not to the extent seen in the powdery mildews. This reduction in *Z. tritici* is thought to have evolved as a mechanism to evade detection by host defenses during stealth pathogenesis (Goodwin et al., 2011). This hypothesis also could fit the lifestyle of *P. fijiensis* since it has an even longer biotrophic phase of up to 28 days before necrotic symptoms start to appear so may have a greater need for stealth (Nfor et al., 2011). However, the association is not perfect because the reduction in CWDEs in *Z. tritici* is greater, particularly for β glucosidases.

Thus, within Ascomycetes there seems to be a correlation between pathogenic lifestyle (biotrophic vs. hemibiotrophic vs. necrotrophic) and diversity of certain gene families such as PKSs and CWDEs. This correlation does not hold up when extended to other fungal groups. For example, rust fungi are obligate biotrophs with greatly expanded genomes due to either the proliferation of LTR retrotransposons similar to *P. fijiensis* for the wheat stem rust pathogen *Puccinia graminis*, or to class II DNA transposons for the poplar rust fungus *Melampsora larici-populina* (Duplessis et al., 2011). However, in both rusts gene numbers were greatly expanded including those for SSPs that may be involved in interactions with

their hosts (Duplessis et al., 2011). Thus, evolution of a biotrophic lifestyle has involved very different changes in the genetic architecture of fungal genomes, from the vast reductions in gene content noted in the powdery mildew fungi to huge expansions for the rusts. Hemibiotrophs such as *P. fijiensis* fall in the middle of the continuum. The one constant is the increased genome size due to transposons, which seems much more common in biotrophic and hemobiotrophic pathogens compared to necrotrophs or saprotrophs.

The mechanisms of these transposon expansions remain mostly unknown. The two main unanswered questions revolve around the source of the invading elements and the mechanism by which they proliferate. The most obvious source would be their host plants, but so far there appears to be little evidence that transposons are being transferred from hosts to their pathogens. Biotrophic fungi should be the most suited for acquiring transposons because they are restricted to growing in a very limited ecological niche and have specialized feeding structures to retrieve nutrients from their hosts. For *P. fijiensis*, a search of the banana genome sequence revealed that transposable elements account for almost half of the *Musa* sequence with LTR retrotransposons representing the largest part (Arango et al., 2011; D'Hont et al., 2012), so the transposons might have come from banana. If not from the hosts, then they most likely have been acquired from other fungi or pests that are associated with the hosts. Horizontal transfer of genes has been shown in other fungi such as *P. nodorum* (Friesen et al., 2006) and *Fusarium oxysporum* f. sp. *lycopersici* (Ma et al., 2010) and it could occur for transposons. Horizontal transfer has the potential to broaden host range and pathogenicity of fungal pathogens or even create a new pathogen from a non/pathogenic strain (Friesen et al., 2006; Ma et al., 2010). Solving the mystery about the origin of invading transposons is important for understanding the dynamics of fungal genome expansions, and the causal agents of the Sigatoka complex on bananas represent a good model to address such a question.

A little more is known about the mechanisms for transposon expansion after they have been acquired. Almost all fungi are capable of Repeat-Induced Point (RIP) mutation, a mechanism for identifying and mutating repetitive sequences (Cambareri et al., 1989; Goyon and Faugeron, 1989; Grayburn and Selker, 1989; Rouxel et al., 2011; Selker et al., 1987). For transposons, the mutations caused by RIP prevent successful translation of the genes coding for transposon movement proteins so they become inactive and can no longer replicate. This provides a very effective defense against transposon expansions in most fungi. In the powdery mildew fungi, the genetic machinery required for RIP was missing (Spanu et al., 2010) and this likely allows unrestricted multiplication of transposons. For the rust fungi, no mechanisms for genome expansion were proposed or tested (Duplessis et al., 2011).

For *P. fijiensis*, *rid*, the only gene known to be required for RIP, is present and the reading frame appears to be intact. Repetitive sequences in the genome of *P. fijiensis* show high frequencies of the C to T transitions that are characteristic of RIP, so this phenomenon seems to be active. Because RIP is only active during meiosis, a possible explanation for fungi with extensive asexual phases could be that transposon expansion occurs during asexual reproduction and then is slowed by RIP during rare sexual reproduction. If transposons have expanded enough and RIP is not completely efficient, some intact copies of transposons could remain after meiosis to continue expanding during the next extended asexual phase. This explanation is possible in *P. fijiensis*; although it has been classically considered to be primarily reproducing by sexual mating, more recent studies suggest that asexual reproduction also plays an important role during epidemics (Rieux et al., 2013a, 2014). Transposon expansion most likely occurs episodically when RIP or other mechanisms are relaxed, but when and how these episodes occur is currently unknown.

In *Z. tritici*, a different type of genome expansion occurred through the acquisition of a large set of dispensable chromosomes, referred to as the dispensome (Goodwin et al., 2011).

The *Z. tritici* dispensome contains at least eight chromosomes with no known function that appear to have been acquired by horizontal transfer from an unknown donor more than 10,000 years ago (Stukenbrock et al., 2010). Such a large number of dispensable chromosomes with no known effects on fitness so far is unique among fungi. Many potential dispensable chromosomes were identified among the genomic scaffolds of *P. fijiensis* based on the characteristics of known dispensable chromosomes in *Z. tritici*. However, dispensability still has not been proven for *P. fijiensis*. None of the *P. fijiensis* linkage groups were missing in progeny isolates, one of the hallmarks of dispensable chromosomes in *Z. tritici* (Wittenberg et al., 2009). However, this is not surprising because none of the linkage groups corresponded to any of the putative dispensable scaffolds. If *P. fijiensis* does contain a dispensome, it is different from that in *Z. tritici* because there was almost no similarity between the dispensable chromosomes of *Z. tritici* and any of the scaffolds of *P. fijiensis*, or vice versa. This raises the intriguing possibility of separate events leading to horizontal transfer of large numbers of chromosomes between species in the Capnodiales and other fungi.

Electrophoretic karyotyping of *P. fijiensis* showed a remarkable level of variability among isolates, even those coming from the same population. This high variability in chromosome length and number was also described previously in *P. fijiensis* (Rodríguez-García et al., 2006) and in other fungi (Cooley and Caten, 1991; Iwaguchi et al., 1990; Talbot et al., 1993). The mechanisms of such variation include chromosome rearrangements during meiotic recombination and the presence of dispensable chromosomes (Zolan, 1995). From a different perspective, it has been shown that chromosomal reshuffling can drive evolution of virulence in asexual plant-pathogenic fungi (Jonge et al., 2013); thus both sexual and asexual life cycles could be a source of chromosomal variation. This could constitute a mechanism of adaptation to environmental changes such as selective pressure from chemical fungicides.

Field isolates karyotyped in this work were collected in Costa Rica in an area with a high level of fungicide applications.

Overall, *P. fijiensis* chromosomes are larger than those from other Ascomycetes, including its completely sequenced relative *Z. tritici* (Goodwin et al., 2011). Remarkably, *P. fijiensis* contains a scaffold larger than 10 Mb, which is at the limit of PFGE resolution. Chromosomes of this size have been observed in other fungi (Cox et al., 1990; Migheli et al., 1995; Orbach et al., 1988) but they are not common. Medium-sized chromosomes were not found, similar to previous records for Mexican isolates (Rodríguez-García et al., 2006). Interestingly, the seven smallest main scaffolds, including the smallest calculated core chromosome (0.61 Mb) and six of the putative dispensable chromosomes did not appear in PFGE, as CIRAD86 did not show a chromosome smaller than 0.8 Mb. The total number of chromosomes separated by PFGE is at least 11, and probably up to 15 when possible co-migrating bands are counted separately, in addition to the five unresolved largest scaffolds.

The availability of a genome sequence enables the identification of genes that might be involved in pathogenicity, including those encoding putative effector proteins. Fungal effectors are proteins that aid pathogenicity, usually by subduing host defenses. However, these same proteins affecting pathogenicity also can be recognized by the host resistance proteins, triggering a defense response and making them advantageous or disadvantageous to the pathogen depending on the host genotype. Bioinformatic analysis of the *P. fijiensis* genome identified many putative effectors, including one that appears to be a homolog of the *Avr4* effector in the related Dothideomycete, *C. fulvum* (van Esse et al., 2007). The *P. fijiensis* putative *Avr4* homolog *Pfavr4* was on scaffold 4 adjacent to repeats of 617 and 2765 bp and a 6-kb AT-rich block with a GC content of 39.3%, similar to known effectors in other fungi, which often are in AT-rich regions (Rouxel et al., 2011; Stukenbrock et al., 2010). Other genes in this region of the *P. fijiensis* genome were different from those in *Z. tritici* and *C.*

fulvum, indicating little synteny among genes around the *Pfavr4* homologs in related species. Previous research showed that PfAVR4 is a functional homolog of the CfAVR4 virulence factor in *C. fulvum*, and that, despite a low amino acid identity of only 42%, it could be recognized by the Cf4 resistance protein to stimulate an HR in tomato (Stergiopoulos et al., 2010). However, whether *PfAvr4* could be recognized by banana cultivars had not been tested until now.

The response of the banana varieties to the PfAVR4 protein strongly suggests that it acts as an avirulence factor that is recognized by a resistant banana accession where it elicits an HR-like necrosis. Most probably this protein has a function similar to that of its homolog in *C. fulvum* where it is an effector that facilitates disease in susceptible cultivars of tomato and can be recognized by the Cf-4 receptor in a resistant tomato cultivar to elicit the HR (van Esse et al., 2007). To our knowledge, this is the first fungal effector known to induce a cultivar-specific, HR-like necrosis in banana, suggesting that *M. acuminata* ssp. *burmannicoides* var. Calcutta IV most likely has a functional R gene that recognizes PFAVR4, and which appears to be a functional homologue of *Cf4* in tomato. Additional experiments are needed to thoroughly test the hypothesis that var. Calcutta IV contains an HR-inducing resistance gene effective against *P. fijiensis*. These could include analysis of progeny from controlled crosses between var. Calcutta IV and a susceptible banana to test for co-segregation of necrosis induced by PfAVR4 and resistance to *P. fijiensis*, or deletion of PfAvr4 to test whether resulting mutant becomes virulent to var. Calcutta IV. However, these experiments would be challenging due to experimental limitations in this pathosystem: crosses in banana frequently suffer from segregation distortions due to the occurrence of translocations and functional analyses in *P. fijiensis* are not routine.

The banana var. Calcutta IV has been a source of resistance against fungi, bacteria and nematodes in *Musa* breeding programs (Ortiz and Vuylsteke, 1994; Simmonds, 1953). It is

one of the most resistant accessions in field evaluations against *P. fijiensis* populations from around the world, with the exception of some isolates from the Pacific islands and Papua New Guinea (Fullerton and Olsen, 1995), which are considered as the center of origin of the disease (Carlier et al., 1996). In addition, var. Calcutta IV has shown resistance to crude extracts from *P. fijiensis* (Hernández, 1995; Okole and Schulz, 1997). The identification of *PfAvr4* as a likely avirulence factor in var. Calcutta 4 provides a major advance for banana breeding programs aiming at increasing the level of resistance against black Sigatoka. Purified effector proteins can be used to identify other resistance genes and to facilitate rapid selection of resistant progeny from segregating populations. The current selection process in resistance breeding is inadequate as it exclusively relies on field evaluations, and is slow because black Sigatoka has a latent period of a month or longer and the banana cycling time, depending on the species, is approximately 10-15 months. Similar experiences slowed down resistance breeding in wheat to *Z. tritici* until the elucidation of its mating system showed single-gene inheritance of pathogenicity factors that facilitated more precise isolate characterizations and subsequent R-gene discovery (Goodwin, 2007; Goodwin et al., 2011; Kema et al., 2000, 1996; Ponomarenko et al., 2011). Other potential genes involved in pathogenicity are discussed in supporting Text S2.

QoIs represent a class of fungicides that initially showed impressive efficacy against many plant pathogens (Gisi et al., 2002). However, resistance evolved rapidly and soon rendered the compound of little use in multiple pathosystems (Brent and Hollomon, 1995). Diagnostic primers for the mitochondrial *cytb* gene showed that *P. fijiensis* is no exception, as the three commercial and frequently sprayed plantations were nearly or completely fixed for resistance. This is a remarkable shift compared to analyses performed during 2000-2003 when only part of the population was resistant (Amil et al., 2007). Interestingly, the San Carlos population, which was not sprayed with fungicides, is still entirely sensitive. This result

suggests limited genetic exchange between these populations that are separated by about 100 km. Nevertheless, even limited gene flow could have an impact on untreated areas. Because the selection pressure exerted by strobilurin is quite strong, the resistance frequency rapidly increases from a low number of resistant individuals to widespread resistance soon after fungicides are used, particularly since these compounds do not prevent sexual reproduction (Ware, 2006). This prompted us to process the *P. fijiensis* genome sequence with a bioinformatics pipeline to develop VNTR markers for rapid PCR-based population analyses to compare with a priori neutral markers. Nonetheless, some genetic differentiation occurs between the populations as already described earlier in Costa Rica using microsatellite markers (Halkett et al., 2010). However, because populations have not yet reached mutation–drift equilibrium, gene flow could not be estimated using classical genetic models based on genetic differentiation (Halkett et al., 2010). Fortunately, new indirect (Rieux et al., 2013b) and direct (Rieux et al., 2014) methods have been recently used to provide estimates of dispersal in *P. fijiensis* that could be integrated in theoretical and spatially explicit models to predict spatial patterns of fungicide resistance evolution under different management strategies.

The availability of the CIRAD86 genome sequence and the resequence data of CIRAD139 for *P. fijiensis* will blunt its continued threat to global production by facilitating the development of resistant cultivars in banana breeding programs. The rapid development of fungicide resistance and extreme variability of the *P. fijiensis* genome among isolates coupled with a high level of sexual reproduction make this pathogen highly adaptable to changing environmental conditions. Diversifying and increasing the level of host resistance in banana may be the only way to slow the devastation caused by this fungus in the future.

Materials and Methods

Fungal culture conditions and DNA extraction

Pseudocercospora fijiensis isolate CIRAD86 (*Mat1-1* mating type, originating from Cameroon in 1988) was chosen for sequencing because it is the epitype for the species, has been the subject of intensive analyses previously and is one parent of an existing mapping population (Manzo-Sánchez et al., 2008). CIRAD139a (*Mat1-2*, originating from Colombia in 1990) was used for resequencing. CIRAD86 is maintained at the CBS-KNAW Fungal Biodiversity Centre (CBS 120258).

Mycelia for DNA extraction were grown in 1-l Erlenmeyer flasks containing 200 mL of PDB (potato dextrose broth; Becton Dickinson, NJ, USA) shaken at 120 rpm at 28°C. Mycelial mats produced during culture were filtered to remove the broth and lyophilized. Samples containing 50 mg of lyophilized mycelia were placed in 2-mL tubes and ground with a Hybaid Ribolyser (model n° FP120HY-230) for 10 s at 2500 rpm with a tungsten-carbide bead. DNA was extracted from the ground mycelia using the Wizard Magnetic DNA Purification system (Promega, Netherlands) for food according to instructions provided by the manufacturer.

Genomic sequencing, genetic mapping, assembly and annotation

Whole-genome shotgun sequencing and assembly of the *P. fijiensis* genome were done using Sanger sequencing of three different-sized libraries (3- and 8-kb plasmids, and 40-kb fosmids) as described previously for *Z. tritici* (Goodwin et al., 2011) and other species (Ohm et al., 2010). The initial version 1 assembly was improved by aligning the physical scaffolds to a genetic linkage map constructed using Joinmap V 4.0 software (Van Ooijen and Voorrips, 2001) to analyze the segregation data for 322 markers that were scored on 135

progeny of the cross between isolates CIRAD86 and CIRAD 139A (Manzo-Sánchez et al., 2008). For each molecular locus, a goodness-of-fit analysis was performed to test for deviation from the expected 1:1 segregation ratio at a 1% significance level. Linkage groups were established using a minimum LOD score of 9.0 and final mapping was achieved by combining two or more linkage groups belonging to the same chromosome. The order of the markers on each chromosome was determined using a minimum LOD score of 1.0, recombination threshold of 0.4, jump of 5.0, ripple value of 1 (default) and Haldane's mapping function as parameters. In cases of uncertainty, some markers were removed and the order was recalculated until a more stable order was achieved.

Three methods were used to identify the *P. fijiensis* repetitive sequences. Repeated sequences in the genome were identified *de novo* using RECON (Bao and Eddy, 2002) and the k-mer based method RepeatScout (Price et al., 2005). A custom set of repeats and the RepBase Update library of 234 fungal repeats (Jurka et al., 2005) were then used to mask the *P. fijiensis* genome using RepeatMasker (http://www.repeatmasker.org/) (Smit et al., 1996).

Repeat families with 10 or more elements identified by RepeatScout were annotated and classified into categories based on the presence of protein domains (BLAST (Altschul et al., 1990)). Structural features including Long Terminal Repeats (LTRs) and Terminal Inverted Repeats (TIRs) were verified using the EMBOSS (Rice et al., 2000) software package. Sequences with no known proteins or structural features were grouped into the unclassified category.

Identification and annotation of protein-coding genes were performed using the JGI Annotation Pipeline, which takes multiple inputs (scaffolds, ESTs, and known genes), runs several analytical tools for gene prediction and annotation, and deposits the results in the JGI

fungal genome portal MycoCosm (http://jgi.doe.gov/fungi) (Grigoriev et al., 2013) for further analysis and manual curation.

Several gene-prediction programs falling into three general categories were used to annotate the repeat-masked assembly as described by Ohm et al. (Ohm et al., 2012). The resulting set of putative genes was then filtered for the best models based on EST and similarity support to produce a non-redundant representative set. This representative set of filtered gene models from the automated annotation pipeline was subject to further analysis and manual curation as described by Goodwin et al. for *Z. tritici* e and by Ohm et al. (Ohm et al., 2010) for more recently sequenced species. Measures of model quality included proportions of the models complete with start and stop codons (88% of models), those that were consistent with ESTs (30% of models) and those supported by similarity with proteins from the NCBI NR database (74% of models) as summarized in Table S8.

Functional annotations for all predicted gene models were made using SignalP (Nielsen et al., 1997), TMHMM e, InterProScan (Zdobnov and Apweiler, 2001), and BLASTp (Altschul et al., 1990) against the nr, SwissProt (http://www.expasy.org/sprot/), KEGG (Kanehisa et al., 2008) and KOG (Koonin et al., 2004) databases as described by Ohm et al. (Ohm et al., 2012). Multigene families were predicted with the Markov clustering algorithm (MCL) (Enright et al., 2002) to cluster the proteins using BLASTp alignment scores between proteins as a similarity metric. Functional annotations are summarized in Table S9. Manual curation of the automated annotations was performed using the web-based interactive editing tools of the JGI Genome Portal to assess predicted gene structures, assign gene functions, and report supporting evidence. Gene models predicted by the JGI annotation pipeline were also analyzed using the program Blast2GO (Conesa et al., 2005) with an E-value of $< 10^{-6}$. Blast2GO assigns GO terms based on the BLAST definitions. Comparisons

between groups of genes for enrichment of GO terms were done by using Fisher's exact test implemented in the Blast2GO program.

Potential secreted proteins were identified with a python script made to run all gene models through SignalP 3.0 (Dyrlov Bendtsen et al., 2004) and subsequently filtered for proteins that had no transmembrane domains, no signal anchor motifs, were fewer than 300 amino acids in length and had at least 4 cysteine residues. The gene models that fulfilled these criteria were considered as potential Small Secreted Proteins (SSPs).

For re-sequencing of isolate CIRAD 139A, a paired-end library was made using the standard Illumina library prep protocol with NEB reagents. Average insert size of the library was 272 base pairs. Sequencing was done on an Illumina GAIIx in one lane of a 54-cycle paired-end run using 36-cycle version 5 SBS Kits. The flow cell was built using a version 4 paired-end cluster generation kit. Eventually, 37 million reads were obtained for a total yield of 4 gigabases. Paired reads were aligned to the *P. fijiensis* v2 Assembly reference scaffolds using GSNAP (2010-03-09 release), allowing up to 3 mismatches or 1 indel and with end trimming enabled. Uniquely aligned reads were then used to call variant sites using the Alpheus pipeline, requiring that a variant have support from at least 2 reads with an average quality of bases of at least phred 10 and at least 80% of the reads covering the site calling that variant. Nonsynonymous SNP differences were assessed against the coding regions in the *P. fijiensis* v2 FrozenGeneCatalog 20100402.

To survey the non-synonymous SNPs in the annotated protein set of CIRAD86, a simple analysis of functional bias in variant proteins was conducted using a ranking comparison approach. All genes were ranked based on their non-synonymous SNP count (normalized for coding sequence length) and two selected gene sets were compared with the whole-genome set. The two sets used for comparison were the 1500 most-variant proteins

(Set V) and the 1500 least-variant proteins (Set C), taken from the list of genes ranked by non-synonymous SNP count. The annotation files for the *P. fijiensis* v2 Frozen Gene Catalog 20100402 were used as the source of GO terms for the genes. The ranked frequency of occurrence of GO terms in the gene annotations for the whole genome was compared with those for Sets C and V.

Repetitive element analysis

For each repetitive element family, a subset of elements with lengths within 50% of the longest element was aligned using clustalX (Larkin et al., 2007). These alignments were submitted to RIPCAL (Hane and Oliver, 2008) to determine the dinucleotide bias observed in repetitive elements. RIPCAL estimates 'RIP dominance' for each dinucleotide containing a cytosine. It is the ratio of a given dinucleotide (e.g., CA) to the sum of the other three dinucleotides (CG/CC/CT).

To test for isochores in the *P. fijiensis* genome, a contiguous stretch of sequence with an arbitrarily chosen average GC content of less than 45% was categorized as an AT block. Custom python scripts were used to calculate the percent GC across the genome, to generate AT blocks and to calculate the average percent GC across the AT blocks and those fewer than 500 bp apart were merged into blocks of at least 1 kb in length, which were retained and analyzed for their composition and distribution of repetitive sequences and genes.

To estimate and compare the amount of RIP between the AT-rich blocks and the rest of the genome, a custom python script was written using a 500-bp sliding window with a step size of 100 bp. The amount of RIP was calculated as an index (CpA+TpG)/(ApC+GpT) and estimated separately for each of the AT-rich versus AT-poor regions in the genome. The RIP

index measures the depletion of the RIP targets CpA and TpG; thus, lower values of (CpA+TpG)/(ApC+GpT) are indicative of a higher degree of RIP [28].

To estimate the ages of transposon insertions, LTR retrotransposons were identified and annotated using the LTRharvest [100] and LTRdigest [101] modules in GenomeTools [102]. LTR sequences from these elements were aligned using ClustalW [99] and manually curated to estimate the numbers of mutations that had accumulated over time. All transition mutations were ignored in this analysis to remove the bias caused by RIP. Age of the LTR retrotransposons was calculated using the average rate of 1.09×10^{-9} substitutions/site/year as proposed for fungal sequences [103].

To test whether the transposons in the *P. fijiensis* genome were unique, a comparison was made to TEs in the genome of *C. fulvum*, the only other sequenced fungus in the Capnodiales with an expanded genome. RepeatMasker (Price et al., 2005) was used to mask the *C. fulvum* genome using the repeat database from the *P. fijiensis* genome. The resulting file was parsed using the 80:80 rule of Wicker et al. (Wicker et al., 2007), i.e., 80% identity across 80% length to identify repeats in common between the two genomes. Another run was done at a 70:70 cutoff to allow for greater divergence generated by RIP.

Generation and analysis of EST sequences

The CIRAD86 strain was grown in three culture media for production of cDNA libraries: yeast-glucose broth as a rich medium (10 g of yeast extract and 30 g of glucose per liter); minimal nutrient medium (1 g of KH_2PO_4, 1 g of KNO_3, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose per liter); and minimal nutrient medium without a nitrogen source (as above but without KNO_3). Fungal mycelia were grown in each medium at 25˚C for 10 days with a photoperiod of 12 hours using cool-white fluorescent light on a rotary

shaker at 100 rpm. Mycelia derived from all three *in vitro* conditions were harvested by filtration and ground in liquid nitrogen. The RNA was isolated by the trizol method with the RNeasy kit (Qiagen, Netherlands) with 2 g of starting material. RNA quality and quantity were assessed by spectrophotometer and by gel electrophoresis according to standard procedures. For cDNA library construction, first-strand cDNA synthesis was done using polyA+ RNA, reverse transcriptase (SuperScriptII (Invitrogen, CA, USA)) and an oligo dT-*Not*I primer (5' GACTAGTTCTAGATCGCGAGCGGCCGCCCT15VN 3'). Second-strand synthesis was done by *E. coli* DNA ligase, polymerase I and RNaseH before end repair with T4 DNA polymerase. The *Sal*I adaptor (5' TCGACCCACGCGTCCG and 5' CGGACGCGTGGG) was ligated to the cDNA and digested with *Not*I before selecting the size range by gel electrophoresis. Sizes were 0.6-2 kb and 2-10 kb. The cDNA of *P. fijiensis* grown in yeast-glucose medium was divided into libraries CBBT and CBHU (0.6-2kb) and CBHT (2-10kb). The cDNA from culture on minimal nutrient medium was divided into libraries CBBW and CBHX (0.6-2kb) and CBBU and CBHW (2-10kb), and for the libraries of culture on minimal nutrient medium without nitrogen source, cDNA was divided into libraries CBBX and CBHY(0.6-2kb). The size-selected inserts were cloned into the pCMVSPORT6 vector (Invitrogen) and digested with *Sal*I and *Not*I. Ligated vectors were transformed into ElectroMAX T1 DH10B cells (Invitrogen).

Sequence reads from cDNA libraries were trimmed of vector, linker, adapter, poly-A/T, and other artifact sequences with the Cross-match software. Internally developed software at the JGI-DOE identified short patterns and low-quality regions (Q15). The longest high-quality region of each read was counted as an EST. Clustering of ESTs was performed based on pairwise alignments generated using the Malign software, a modified version of the Smith–Waterman algorithm (Smith and Waterman, 1981), which was developed at the JGI for use in whole-genome shotgun assembly. ESTs sharing an alignment of at least 98% identity

with 150-bp overlap were assigned to the same cluster. For each cluster of EST sequences, a consensus sequence was generated by running the Phrap software (Ewing et al., 1998; Ewing and Green, 1998). Comparison of the relative expression of genes in the different libraries was done by mapping ESTs to the whole set of predicted gene models and comparing their occurrences between a pair of libraries using Fisher's exact test. A P value of < 0.05 was considered as statistically significant.

Functional analysis of a putative effector protein

Plantlets of *M. acuminata* ssp. *burmannicoides* var. Calcutta IV (recognized as a resistant standard for BLSD) were multiplied and rooted *in vitro*, whereas Cavendish "Grand Naine" tissue culture plants were hardened for three to four weeks in a greenhouse environment. Subsequently, all plantlets were grown for three months (small plants), and some plants of var. Calcutta IV and "Grand Naine" were grown for eight months (large plants) in a controlled-environment greenhouse compartment at 25˚C with a relative humidity of >80% and 16 hours of light per day.

Plants of tomato (*Lycopersicum esculentum*) cv. Moneymaker (MM), which has no known *e* resistance genes (*Cf0*), or an isogenic line previously transformed with the *Cf4* resistance gene were grown under greenhouse conditions as described by Stergiopoulos et al. 2010 (Stergiopoulos et al., 2010) during 3-4 weeks.

The mature protein from the *P. fijiensis* putative effector gene *PfAvr4* was produced heterologously by culturing *Pichia pastoris* isolate GS115 in a fermentor as described previously (Rooney et al., 2005, p. 2). Following production in the fermentor, the protein was further purified from excess liquid medium and smaller proteins by filtration through a 3-kDa membrane (Amicon Ultra-15 Centrifugal filter unit, Millipore, USA).

Infiltration of the complete fermentor product or purified PfAVR4 protein into banana and tomato leaves was done by injection with a 1-ml syringe with no needle. Infiltrations on banana leaves were done at the original concentration and a six-fold higher concentration. For tomato, all infiltration materials were diluted fifty or seventy-five times prior to infiltration. Samples of the fermentor medium and water were infiltrated separately as negative controls for all plants. At each infiltration point, the observed water soaking of tissue was marked with a permanent marker. Observations were recorded with an Olympus C-8080 digital camera at 4 and 10 days post infiltration (dpi) on banana leaves, and at 6 dpi for tomato plants. Protein preparation and controls were infiltrated in at least two banana leaves from each genotype in small and large sizes, with at least 3 repetitions per leaf. Infiltrations in tomato plants were performed in at least 4 leaves with one repetition.

Thermal denaturation assays

The thermal denaturation method of Marmur and Doty (Marmur and Doty, 1962), performed basically as described by Smith et al. (Smith et al., 1995), was used to estimate G+C contents of DNA from *P. fijiensis* isolate CIRAD86 (CBS120258) plus that from the closely related banana pathogens *P. musae* (isolate UQ430; CBS121371) and *P. eumusae* (isolate CBS122457) as well as the previously sequenced *Z. tritici* isolate IPO323 (CBS115943) (Goodwin et al., 2011). Genomic DNA was isolated from cultures grown in PD broth at 25°C on a rotary shaker (150 rpm) following the procedure described by Raeder and Broda (Raeder and Broda, 1985) and was dissolved in 0.1X SSC. Melting curves were obtained on a Perkin-Elmer λ25 spectrophotometer equipped with a thermal programmer. The G+C contents were calculated from the T_m values (melting/transition temperature) derived from the peaks of the first derivatives of the melting curves (Owen et al., 1969). DNA from *Candida parapsilosis* isolate CBS604 (T_m in 0.1 x SSC, 70.6°C) (Crous and Mourichon,

2002) was used as a calibration control. Determinations were performed at least twice for each isolate.

Phylogenetic analysis

Phylogenetic analysis showing the placement of species within the Capnodiales with expanded genomes was done using Internal Transcribed Spacer regions (ITS). DNA sequences were downloaded from GenBank with the following accession numbers: AF181692 for *Z. tritici*, EU514233 for *P. eumusae*, EU514265 for *P. musae* and EU514248 for *P. fijiensis* or obtained from genome data available at the Fungal Genome portal at JGI. Sequence alignment was done using MUSCLE (Edgar, 2004) and the phylogenetic tree was generated with MEGA 6.0 (Tamura et al., 2013) using a Maximum Likelihood statistical method and the Tamura 3-parameter substitution model. Support for the nodes of the tree was estimated by bootstrapping with 1000 replications.

Electrophoretic karyotyping

Isolates of *P. fijiensis* grown for 3 weeks in PD broth at 28°C, 150 rpm, were blended and grown for 48 hrs in the same medium at 20% strength amended with 1 µM tricyclazole. Decanted culture was washed with 1 M sorbitol, and added to 40 mL of OM buffer (1.2 M $MgSO_4$, 10 mM K phosphate, pH 5.8 with 700 mg of glucanase (Sigma, Germany), 256 mg of yatalase (Takara, Japan), 7500 U of β-glucuronidase (Sigma) and 0.8 g of driselase (Sigma)) in a ratio of ~1:3 (mycelium:buffer). The enzymatic treatment was incubated at 33 °C and shaken at 50 rpm for 4.5 hrs.

Protoplasts were filtered through a plastic mesh of 30 µm and washed 3 times with 1 M sorbitol in sterile conditions. When the concentration was at least 1×10^8 per mL, protoplasts were embedded in low-melting point (SeaKem® Gold) agarose at a final

concentration of 0.5%. Agarose plugs were treated with proteinase K as described previously (Mehrabi et al., 2007), washed with cold 50 mM EDTA, and kept in the same solution at 4°C until used.

Chromosomes of *P. fijiensis* were separated in a CHEF DR-II system (Bio-Rad, Netherlands). Small chromosomal bands were discriminated as described before (Rodríguez-García et al., 2006) using the chromosomes of *Saccharomyces cerevisiae* (Bio-Rad) as a high molecular weight (HMW) standard. Large chromosomes were separated in a 0.8% low-melting point (SeaKem® Gold) agarose gel, with 0.5% TBE buffer at 11°C, and 50 V for 195 hrs with switching times from 4800 to 1800 sec, and 24 hrs from 1800 to 1300 sec, followed by 20 hrs at 60 V from 1300 to 800 sec, and finally 27 hrs of 800 to 600 sec at 80 V. HMW standards were *Schizosaccharomyces pombe* and *Hansenula wingei* chromosomes (BioRad). Agarose gels were stained with SYBRGold (Invitrogen) and destained in water for 30 and 20 min, respectively, observed under a UV transilluminator and recorded with an Eagle Eye II (Stratagene) still video system.

Whole-genome comparisons and synteny analyses

Two tools, Circos (Krzywinski et al., 2009) and MUMmer (Kurtz et al., 2004), were used for structural analysis of the *P. fijiensis* genome. A nucleotide-based similarity search was done between the masked *P. fijiensis* and *Z. tritici* genomes and visualized using Circos (Krzywinski et al., 2009), whereas protein comparisons between the masked genomes were done using Promer (Kurtz et al., 2004). Proteins with greater than 60% identity were reported.

Proteins in *P. fijiensis* and *Z. tritici* with at least 50% amino-acid identity and match length were grouped as orthologs using OrthoMCL (Li et al., 2003) and synteny blocks were determined using Orthocluster (Vergara and Chen, 2009). The *Z. tritici* protein dataset also

was compared to two other phylogenetically distant Dothideomycetes in the order Pleosporales, *P. nodorum* and *P. tritici-repentis*.

Fungicide sensitivity and population analyses

To analyze the frequencies of molecular marker alleles and fungicide resistance within populations, four farms in Costa Rica were sampled during 2008 (Figure S8). San Pablo, Zent, and Cartagena are located in Limón province, where bananas are grown at high density on large plantations and diseases are controlled by using chemical fungicides. These farms are located in the main Costa Rica banana production area with approximate sizes of 285, 342 and 64 ha, respectively. A fourth farm, San Carlos (0.5 ha), is located in Alajuela province and is isolated geographically from the principal banana-production area. Leaf tissue was collected from ten banana plants from each farm. Ascospores were discharged from the pseudothecia onto water agar ("FRAC Methods for Monitoring Fungicide Resistance," 1991) and single ascospores were transferred immediately to 15 x 100-mm petri dishes filled with potato dextrose agar (PDA). Between eight to ten ascospores from each sample point were placed on each dish of PDA. After 4 days colonies were transferred to Mycophil agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated for 15 days at 25°C under continuous fluorescent light for colony growth and conidial production. Eventually, 649 isolates were collected and analyzed for phenotypic and molecular variability.

To obtain DNA for population genetics analyses, mycelia of 190 isolates from each of the three commercial plantations and of 95 isolates from the San Carlos population were harvested and lyophilized for 24 hours. Genomic DNA was extracted using the Wizard Magnetic DNA Purification System for Food Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and 2 μL per sample were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Mating type

(*Mat*) PCR assays (Conde-Ferráez et al., 2007) were performed in a 50-µL total volume containing 50 ng of template genomic DNA, 2 mM $MgCl_2$, 600 µM dNTPs, 5 µM of each primer, and 0.4 U of Taq DNA polymerase (Roche, Mannhein, Germany). Temperature cycling was carried out with the following program: 94°C for 2 min, 40 cycles of 94°C for 1 min, 70°C for 30 s and 72°C for 1 min, and a final elongation period of 10 min at 72°C.

Analysis of VNTR markers was done as reported previously (Garcia et al., 2010). For genotyping strobilurin resistance, primers were developed on the basis of the G143A mutation in the *cytb* gene (Sierotzki et al., 2000) to identify sensitive and resistant *P. fijiensis* field strains (Table S10) in 20-µL aliquots containing 50 ng of template genomic DNA, 2 mM $MgCl_2$, 600 µM dNTPs, 5 µM of each primer and 0.4 U of Taq DNA polymerase (Roche, Mannhein, Germany). Temperature cycling was conducted with the following program: 94°C for 2 min, 40 cycles of 94°C for 1 min, 70°C for 30 s and 72°C for 1 min, and a final elongation period of 10 min at 72°C. Amplicons were separated by electrophoresis using 1.0% (for *mat* and *cytb* assays) or 3.0% (for VNTRs) agarose gels containing 0.3 µg/mL ethidium bromide, in 0.5× TBE buffer at 120 V for approximately 1 h (for *mat* and *cytb* assays) or 5 h (for VNTRs) and were visualized and photographed using a UV transilluminator and Eagle Eye II (Stratagene) still video system.

To analyze the data, frequencies of the two mating types within each population and in the overall sample were tested for deviation from a 1:1 ratio with χ^2 tests. A molecular multilocus haplotype was constructed for each isolate by combining the allelic data at all five VNTR loci. Gene diversity within each population (H_S) in total and by locus was calculated using GenAlEx 6.4 (Peakall and Smouse, 2006). Total diversity over the entire sample (H_T), mean gene diversity within populations (H_S), genetic differentiation among populations (G_{ST}) and the corrected, standardized measure of genetic differentiation (G''_{ST}) were calculated using GENODIVE Beta version 2.0 (Meirmans and Van Tienderen, 2004). In all cases, H_T

and H_S refer to the unbiased estimates as developed by Nei (Nei, 1987). Pairwise estimates of G''_{ST} and of Jost's differentiation (D) (Jost, 2008) also were calculated with GENODIVE. Multilocus haplotype diversity was calculated with multilocus (http://www.bio.ic.ac.uk/evolve/software/multilocus/#what).

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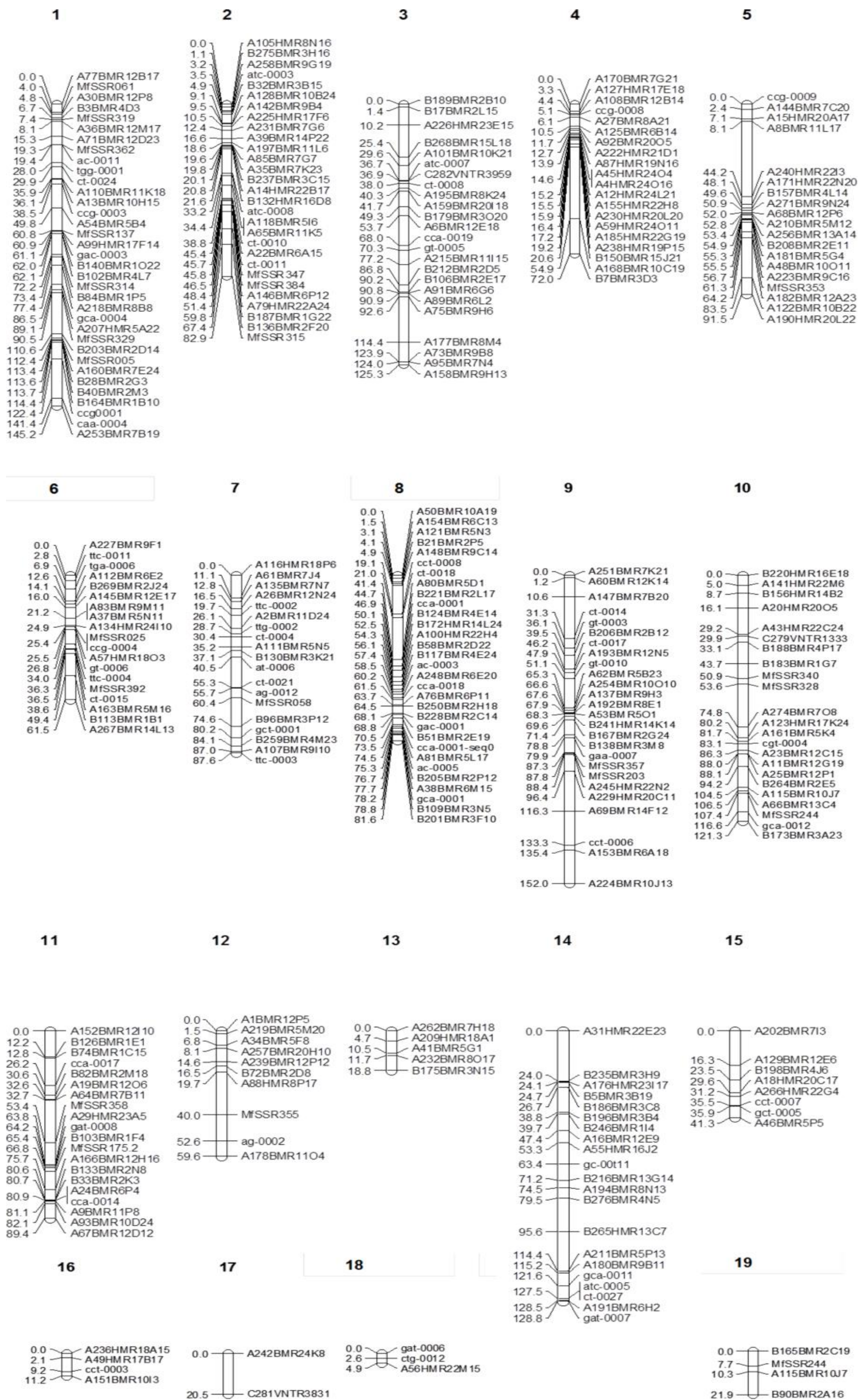

Figure 1. Genetic linkage map of *Pseudocercospora fijiensis* constructed from segregation data at 322 loci (233 DArT, 86 SSR and 3 minisatellite markers) among 135 individuals of a cross between the sequenced isolates CIRAD86 and CIRAD139A. The Diversity Arrays Technology (DArT) markers were named according to the output of proprietary DArT analysis software. For each of the 19 linkage groups (listed on top) the cumulative map distances (cM) as calculated using the Haldane mapping function are shown to the left.

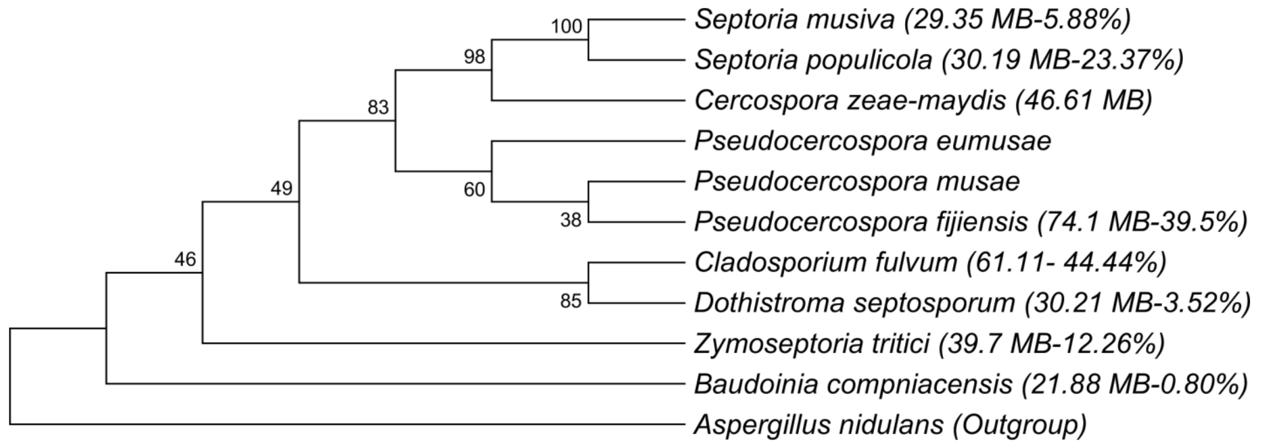

Figure 2. Phylogenetic analysis showing the placement of Dothideomycete species within the Capnodiales with expanded genomes. At least two genome expansions may have taken place; one leading to the banana pathogen *Pseudocercospora fijiensis* and one that contributed to its close relative the tomato pathogen *Cladosporium fulvum*. Genome sizes and percentages of the genome containing repeat elements are indicated in parentheses.

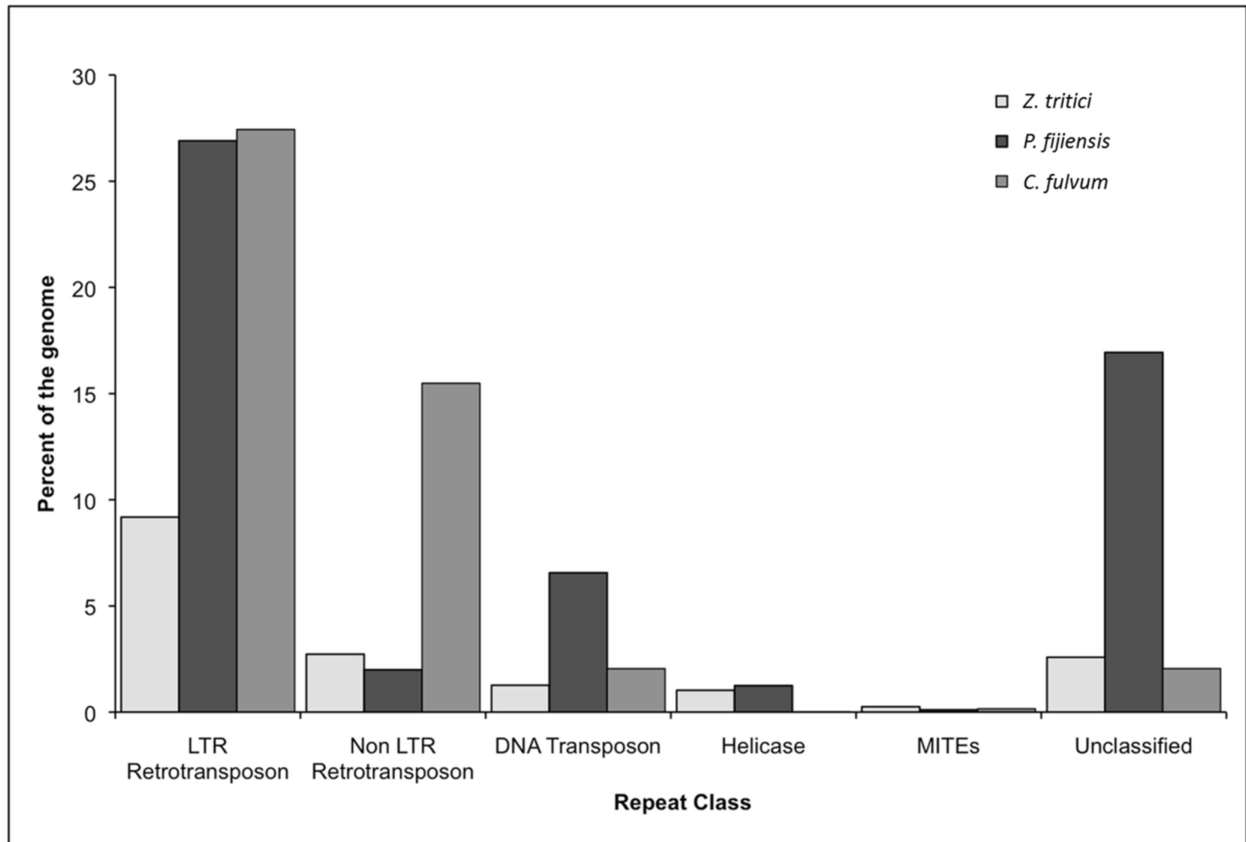

Figure 3. Comparison of repeat classes among *Zymoseptoria tritici*, the only Dothideomycete with a completely sequenced genome, *Pseudocercospora fijiensis* and *Cladosporium fulvum*, the only other Dothideomycete known to have a transposon-expanded genome.

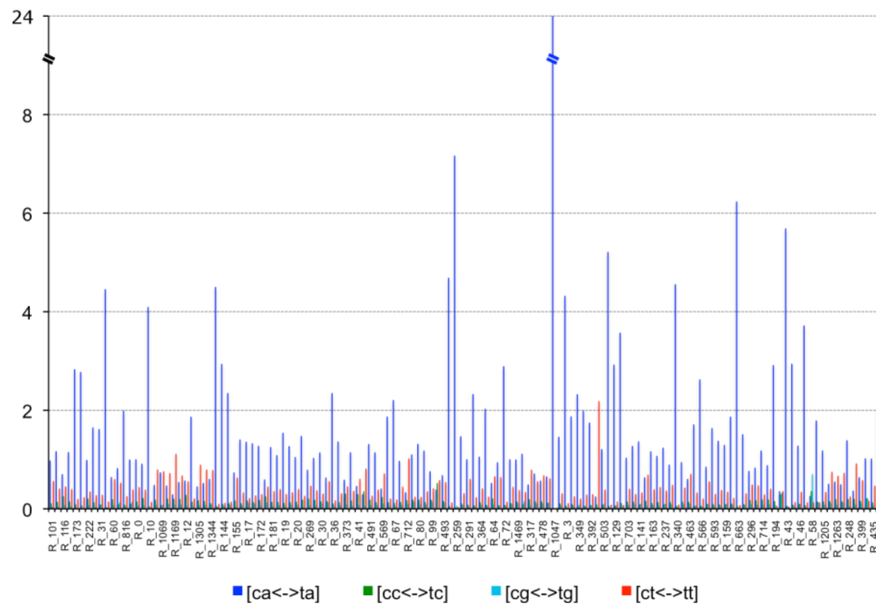

Figure 4. Repeat-induced point mutation (RIP) dinucleotide bias in *Pseudocercospora fijiensis* genome. A clear CA <-> TA dinucleotide bias is observed in *P. fijiensis* repetitive families, indicating that RIP likely occurs and mutates CA nucleotide pairs to CT.

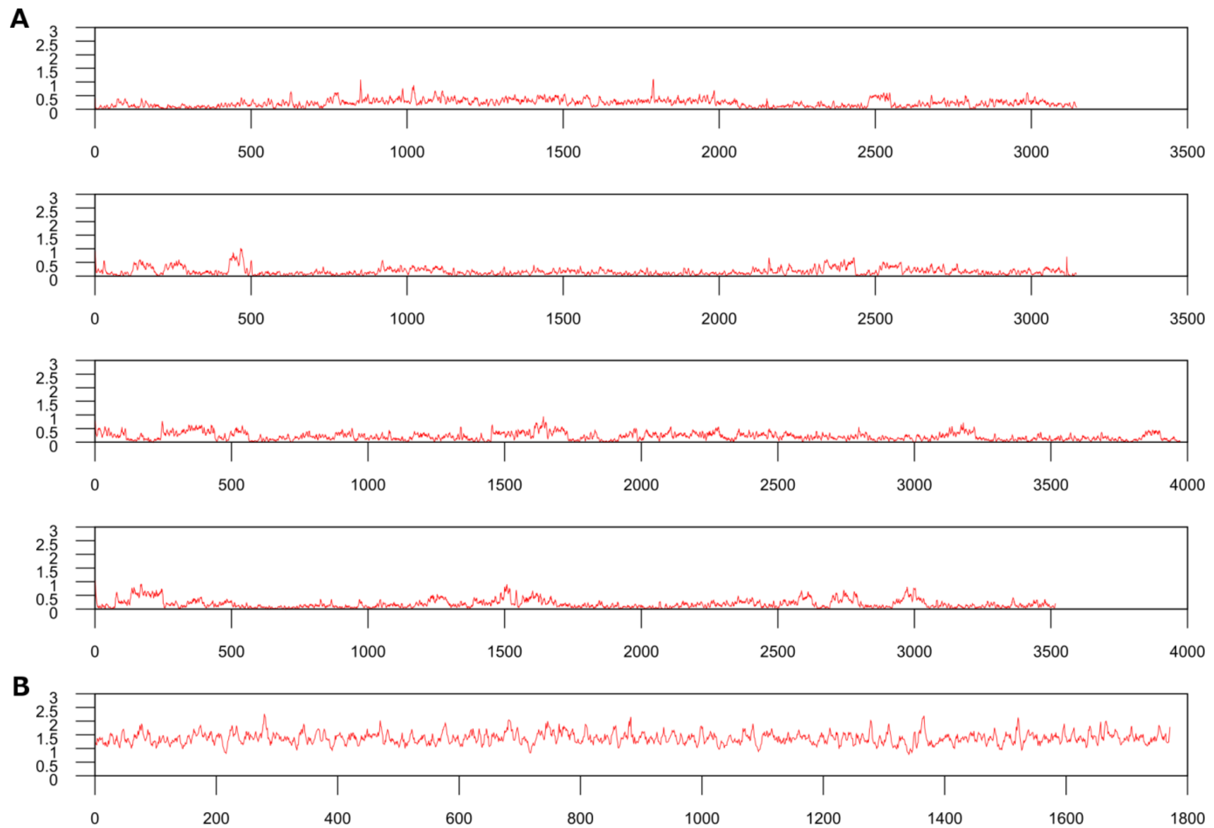

Figure 5. Comparison of the amount of repeat-induced point mutation (RIP) between AT-rich blocks and more GC-rich regions of the *Pseudocercospora fijiensis* genome as measured by the RIP index (CpA+TpG)/(ApC+GpT). (A) AT-rich blocks have a lower RIP index indicating a depletion of RIP-susceptible sites due to a higher frequency of RIP compared to (B) an AT-poor region (higher GC) of the genome, which has a higher RIP index reflecting very little RIP. Four AT-rich blocks are shown along with one AT-poor region for comparison. Length of each block in kilobases is shown along the x-axis and the RIP index (CpA+TpG)/(ApC+GpT) is shown on the y-axis.

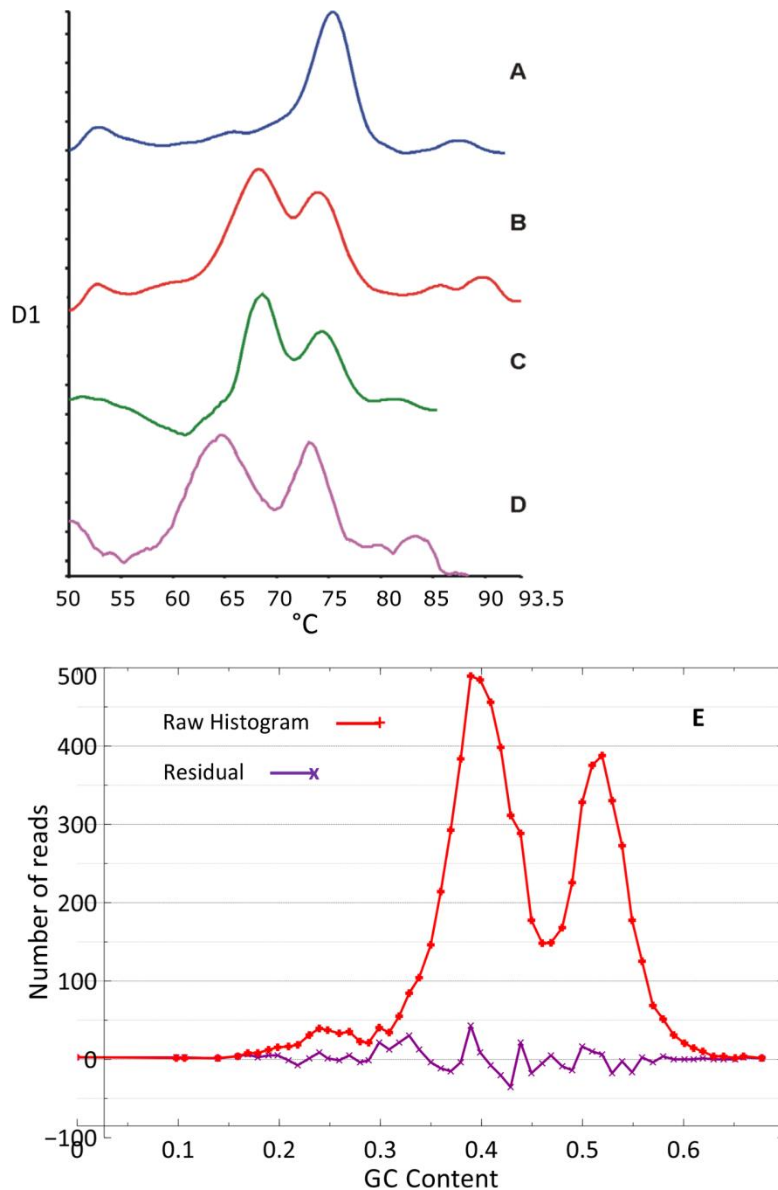

Figure 6. First-derivative graphs of melting curves of four different Dothideomycetes. Examples of first-derivative graphs of melting curves obtained for *Zymoseptoria tritici* (A), *Pseudocercospora fijiensis* (B), *P. eumusae* (C) and *P. musae* (D). E: A plot of G+C contents from sequence reads of *P. fijiensis*. This graph is very similar to the melting-curve analyses showing the difference in G+C content between the genomes of *P. fijiensis* and the other banana pathogens versus the *Z. tritici* genome.

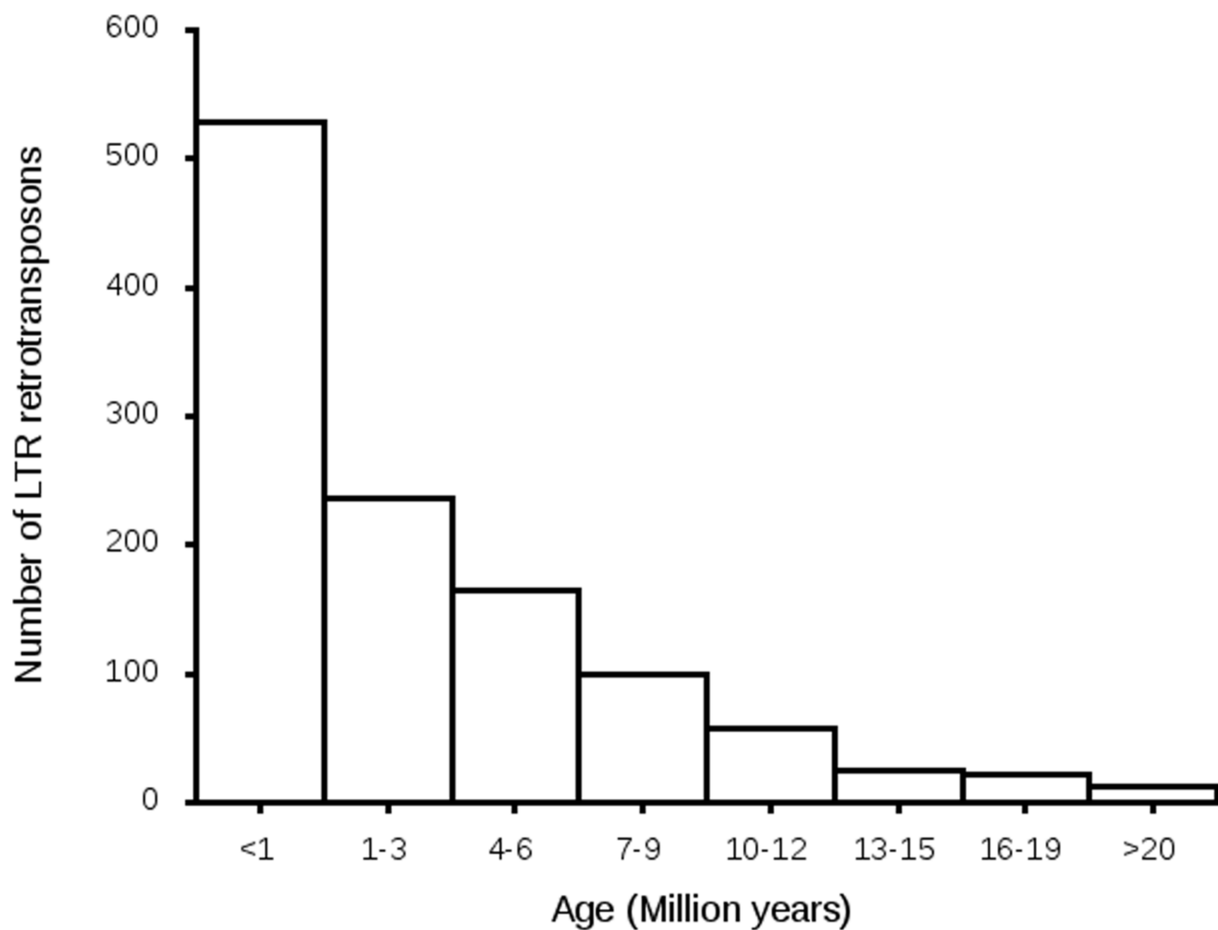

Figure 7. The numbers of long terminal repeat (LTR) retrotransposons in hypothetical age bins from less than one to more than 20 million years. Estimated age of each transposon was calculated using the number of differences between its left and right repeats. These are considered identical at the time of insertion so all changes are likely due to mutations that occurred after transposition. All transition mutations were excluded to minimize the effects of repeat-induced point mutation.

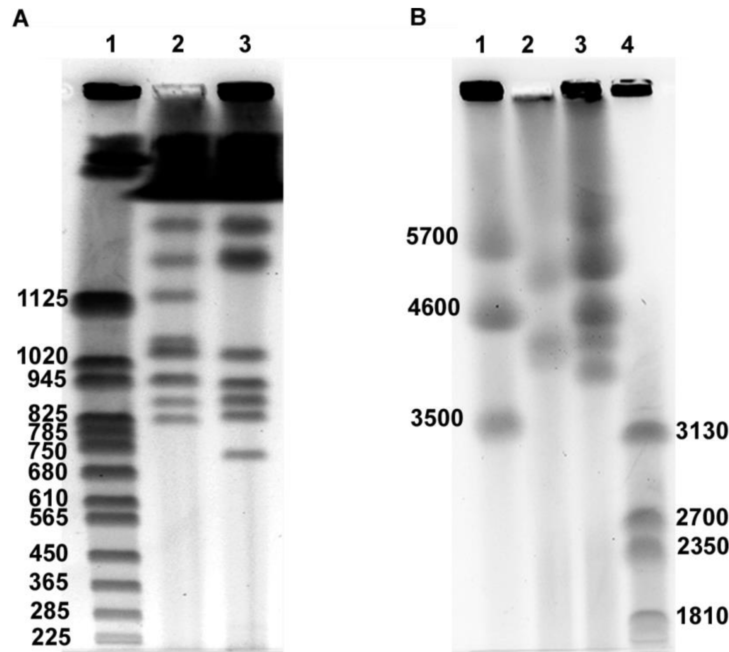

Figure 8. Electrophoretic karyotypes of two strains of *Pseudocercospora fijiensis*. A) Bands separated with conditions for small chromosomes. Lane 1, chromosomes from *Saccharomyces cerevisiae* as high-molecular-weight (HMW) marker; lane 2, strain CIRAD86; lane 3, strain E22. B) Bands separated under conditions to resolve medium and large chromosomes. Lane 1, chromosomes from *Schizosaccharomyces pombe* as HMW marker for large chromosomes; lane 2, strain CIRAD86; lane 3, strain E22; lane 4, chromosomes from *Hansenula wingei* as HMW marker for medium chromosomes in size. Marker sizes are in Kb.

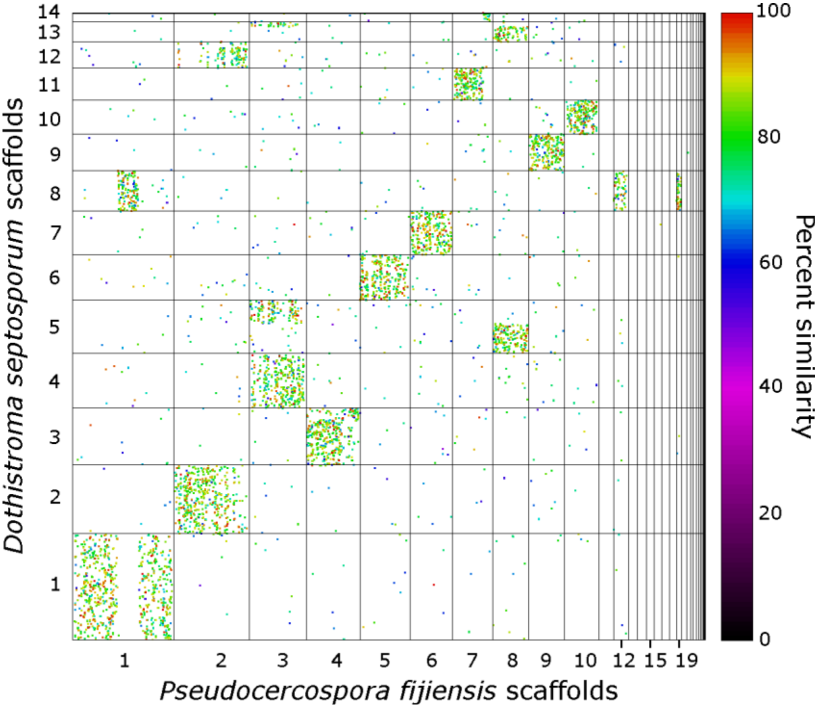

Figure 9. Dot plot showing mesosynteny between the scaffolds of *Pseudocercospora fijiensis* and *Dothistroma septosporum*.

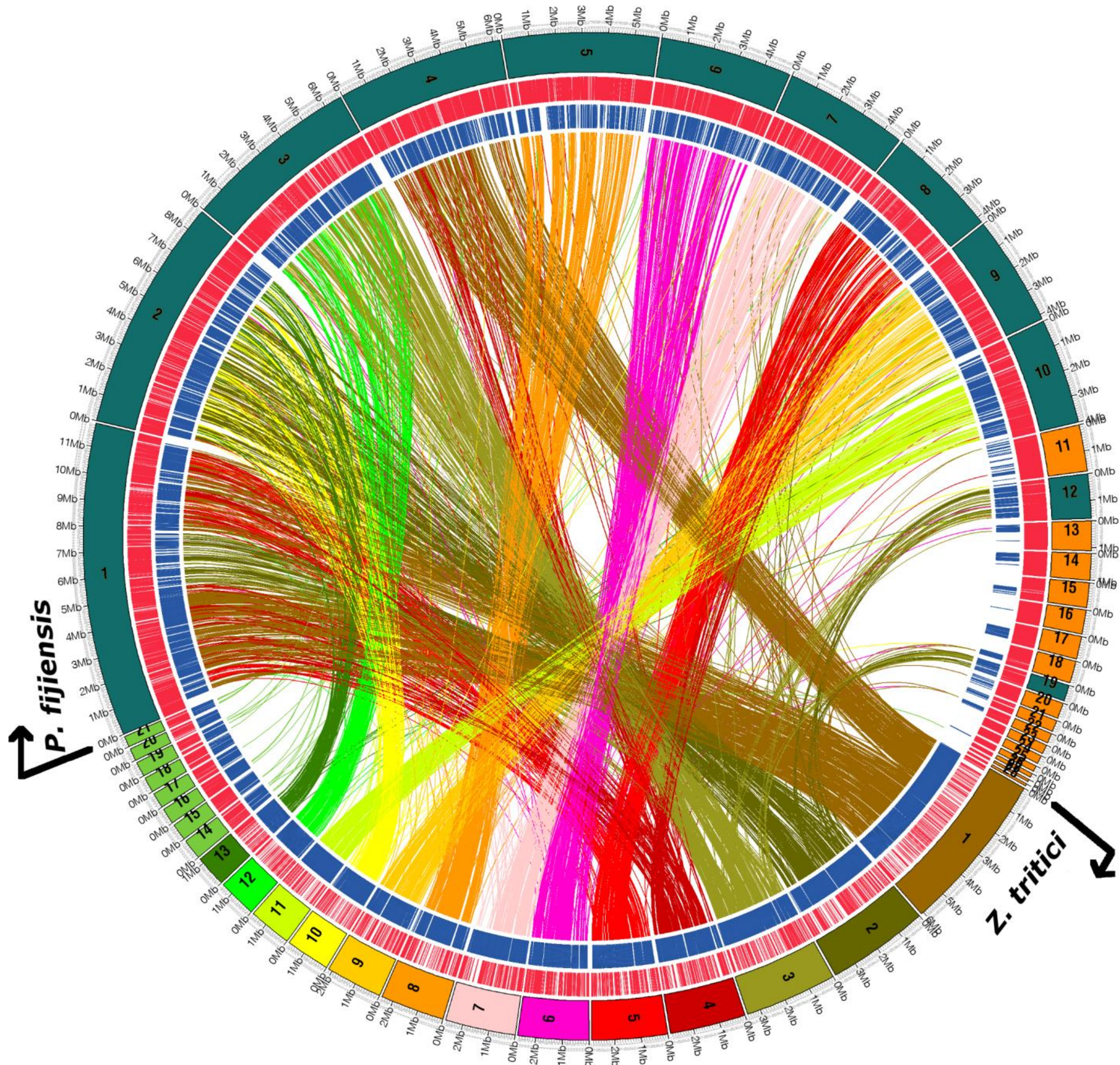

Figure 10. Genome-wide nucleotide comparison between *Zymoseptoria tritici* (lower half of the circle) and *Pseudocercospora fijiensis* (upper half of the circle). The longest 28 scaffolds from *P. fijiensis* are shown. Gene content is conserved but is scattered among different chromosomes between these two fungi. There were no significant hits to dispensable chromosomes of *Z. tritici* (14–21). The 12 major scaffolds of *P. fijiensis* showing synteny are labeled in dark blue-green and the other 16 scaffolds are labeled in orange.

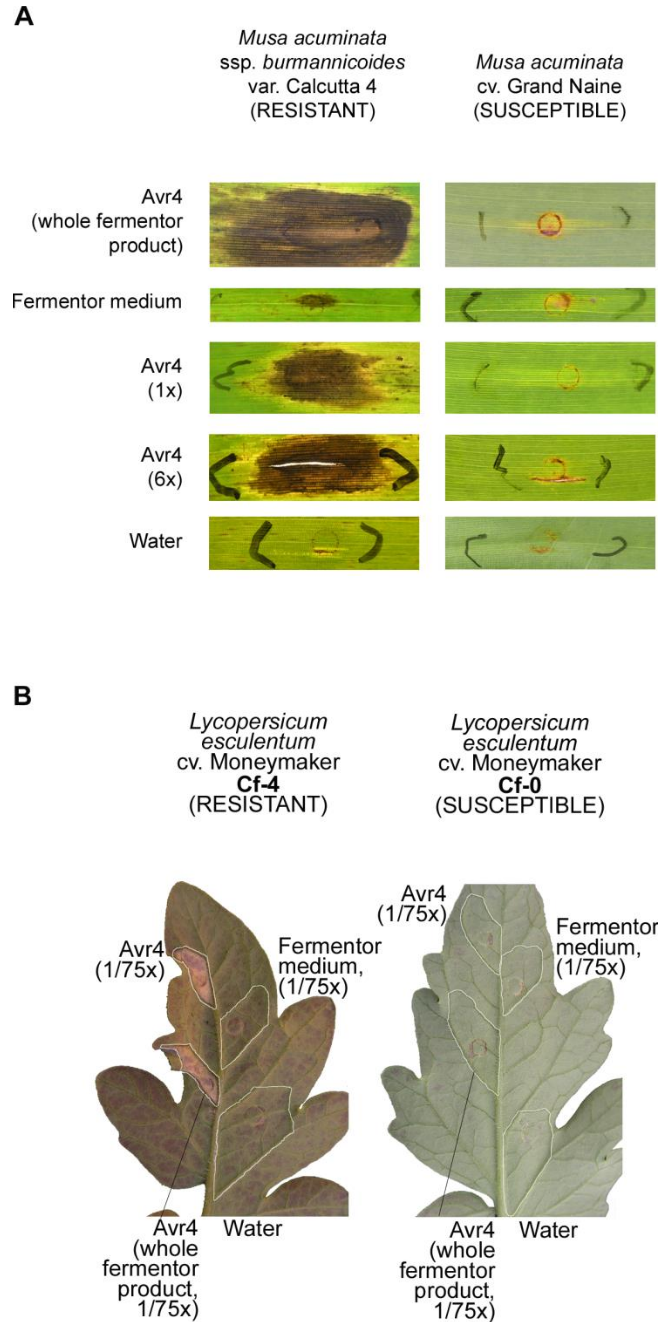

Figure 11. Infiltration of purified protein of the putative effector gene *PfAvr4* from *Pseudocercospora fijiensis* into leaves of banana and tomato. A: Infiltrations into leaves of resistant and susceptible banana varieties. B: Infiltrations into leaves of tomato with or without the *Cf4* resistance gene known to interact with PfAVR4. Experiments were done with crude fermentor product and concentrated or diluted product. Fermentor medium alone and water were used as controls.

Tables

Table 1. Comparative genome statistics of the version 2 assembly of *Pseudocercospora fijiensis*, and several other sequenced fungi in the order Capnodiales.

Genome statistic	*P. fijiensis* V 2.0	*C. fulvum* V 1.0	*Z. tritici* V 2.0	*B. compniacensis* V1.0	*D. septosporum* V1.0	*S. populicola* V 1.0	*S. musiva* V 1.0
Genome size	74 MB	61.11 MB	40 MB	21.88 MB	30.21 MB	33.19 MB	29.35 MB
Scaffolds	56	4865	21	19	20	502	72
Scaffolds > 50 Kb	28	N.A*	21	17	14	141	13
Largest scaffold	11.8 MB	0.53 MB	6.0 MB	2.03 MB	5.1 MB	1.06 MB	5.11 MB
Percent in scaffolds > 50 KB	99.8	N.A	100	N.A	N.A	N.A	N.A
Gene models	13,107	14,127	10,952	10,513	12,580	9,739	10,233
Coverage	6.9×	N.A	8.9×	43x	34x	18x	35x

*N.A. Data not available at respective genome site.

Table 2. The repeat-induced point mutation (RIP) index calculated as (CpA+TpG)/(ApC+GpT) for genes[a] and repeats[a] in AT-poor and–rich regions of the *Pseudocercospora fijiensis* genome.

	Number of genes in		Number of repeats in	
RIP index	AT-poor blocks	AT-rich blocks	AT-poor blocks	AT-rich blocks
0.0	0	0	1	3
0.5	80	134	343	7,062
1.0	291	37	62	607
1.5	8,699	128	30	2
2.0	1,937	22	1	0
2.5	18	0	1	0
3.0	1	0	0	0
Total	11,026	321	438	7,674

[a] The minimum sequence cutoff length for this analysis was 500 bp. A lower RIP index indicates a higher frequency of RIP mutations and vice versa.

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Table 3. Comparison of selected gene families with potential roles in pathogenicity among five Dothideomycete fungi and the saprotrophic Sordariomycete *Neurospora crassa*.

	Pseudocercospora fijiensis	*Zymoseptoria tritici*	*Parastagonospora nodorum*	*Pyrenophora tritici-repentis*	*Cochliobolus heterostrophus*	*Neurospora crassa*
Peptidases	189	187	381	265	248	168
Cutinases	7	6	0	8	13	0
Beta Glucosidase activity	9	2	6	13	13	3
Chitinases	5	3	36	17	21	8
Chitin binding	5	2	35	13	16	3
Cellulose binding	4	0	18	46	55	26
Xylanases	7	5	31	21	31	5
NRPS	13	11	18	13	11	5
Polyketide synthases	11	12	21	28	21	4
Map kinases	5	5	12	5	4	4
Peroxidases	29	22	32	26	28	10
Carbohydrate Metabolic process	127	77	231	204	209	129
O-glycosyl hydrolase activity	77	51	129	97	106	77

doi:10.1371/journal.pgen.1005876.t003

Supplementary data

Text S1. Additional information on genome sequencing, assembly and EST support.

Genome sequencing and assembly

Pseudocercospora fijiensis isolate CIRAD86 was sequenced using Sanger sequencing on ABI 3730XL capillary machines. Three libraries with different sized inserts (3- and 8-kb plasmids, and 40-kb fosmids) were used as templates for the plasmid subclone sequencing process and both ends were sequenced. After trimming sequences for vector sequences and eliminating those with low quality, a total of 867,068 reads was assembled into 382 main genome scaffolds using a modified version of Arachne (Jaffe et al., 2003). The resulting *P. fijiensis* v1.0 draft assembly totaled 73.6 Mb at an average read depth coverage of 7.11×. Almost 84% of the genome was contained in 10 scaffolds that were each at least 4.0 Mb in length (Table S1).

Inclusion of the genetic map data to facilitate assembly of the physical genome sequence involved sequencing 288 markers and aligning them to the version 1 draft assembly. Among the 288 markers sequenced, 262 were mapped to the version 1 genome assembly while 26 markers did not map. The improved version of the genome sequence has an estimated size of 74 Mb, assembled into 56 scaffolds covering more than 99% of the genome. The largest scaffold is 11.8 Mb in length and 28 scaffolds (99.8.%) are larger than 50 Kb. Only 0.6% of sequence bases are estimated to be in gaps as compared to 8.9% in version 1.0.

Annotation of the v1.0 assembly using a variety of similarity-based and *ab initio* gene predictors (Ohm et al., 2012) yielded 10,316 genes. Annotation of the v2.0 assembly was with the same annotation pipeline but with additional filtering by mapping of the v1.0 gene catalog along with its manual curations. After filtering for EST support completeness and similarity to

other species, 13,107 genes were structurally and functionally annotated. The average gene length in the version 2 assembly is 1,833 nt with 3.62 exons per gene; 88%, are complete with start and stop codon, 74% have similarity support, and 49% have Pfam domains (Table S2). Most of the gene models (96%) are located in 12 scaffolds, numbers 1-10, 12 and 19. Gene density in these 12 scaffolds varies from 151 to 229 per Mb and drops from 2.2 to 94 genes per Mb for the remaining scaffolds larger than 0.5 Mb (Table S1).

EST support

Sequencing of nine cDNA libraries from three *in vitro* conditions generated 32,394 ESTs: 10,733 from growth in rich medium (library MFEST-3); 11,685 from growth in minimal medium with nitrogen (library MFEST-4); and 9,976 from growth in minimal medium without nitrogen (library MFEST-5). Average read length was 711.8 bp and percent GC was just over 53%, corresponding to the high-GC peak seen in thermal-denaturation assays.

Clustering of ESTs from all libraries using Cap3 yielded 3,306 contigs and 3,164 singletons. Mapping of the ESTs to the version 2 genome assembly provided support for 5,663 of the predicted gene models; 99% of these are located in 13 scaffolds. Some scaffolds such as 11 and 14 had a very low density of genes and mapped ESTs (Table S1).

Differences in the relative abundance of EST sequences among the three libraries provide some clues about gene expression under each culture condition. Under rich conditions (library MFEST-3), genes that were more abundant included those with GO terms related to metabolic processes such as carbohydrate catabolic processes (GO:0046365, GO:0019320), lipid metabolic processes (GO:0006629 GO:0045834), dicarboxylic acid metabolic process (GO:0043648) and small-molecule biosynthetic process (GO:0044281), among others. Some interesting genes expressed exclusively under rich conditions in library 3 include a polyketide

synthase, a D-3-phosphoglycerate dehydrogenase related to cytochrome p450 of *Neurospora crassa*, a Ctr copper transporter family protein (*Aspergillus clavatus* NRRL 1), and an ABC multidrug transporter mdr2 protein.

Genes with more abundance in minimal medium in the presence of nitrogen (library MFEST-4) had GO terms related to membrane fraction type proteins (GO:0005624), alcohol metabolic process (GO:0006066), binding (GO:0005488) and biosynthetic processes (GO:0009058, GO:0046364, GO:0019319). Interesting genes in MFEST-4 include a homolog of hydrophobin 1, which is the highest-expressed gene in this library, an alcohol oxidase and an extracellular cell wall glucanase. Several highly expressed genes in library MFEST-4 also were expressed in library MFEST-5 including the hydrophobin 1 and the alcohol oxidase. Enriched GO terms in library MFEST-5 included, among others, drug transmembrane transporter activity (GO:0015238 GO:0015893), ion transmembrane transporter activity (GO:0015075, GO:0006812, GO:0006811 and others) and several terms related to nucleotide binding activities (GO:0001882, GO:0032553).

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Ohm RA, Feau N, Henrissat B, Schoch CL, Horwitz BA, Barry KW, et al. 2012. Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. *PLoS Pathog*. 8: e1003037. doi:10.1371/journal.ppat.1003037

Text S2. Additional potential pathogenicity-related genes present in the *P. fijiensis* genome.

Other potential genes involved in pathogenicity could be identified from EST support under different conditions. Several interesting genes were expressed in the libraries collected in minimal medium with and without nitrogen, which resemble the conditions that the fungus might face in a natural environment, including a hydrophobin 1 and an alcohol oxidase. An alcohol oxidase is a pathogenicity factor in *Cladosporium fulvum* (Segers et al., 2001), and the class I hydrophobin, MPG1, is involved in the formation and attachment of appressoria in the rice pathogen *Magnaporthe oryzae* (Talbot et al., 1993). Hydrophobins allow fungi to escape their aqueous environment and mediate attachment of hyphae to hydrophobic surfaces, which is important during the initial steps of fungal pathogenesis, where the fungus must attach to the hydrophobic surface of the host before penetration and infection (Hektor and Scholtmeijer , 2005). Because *P. fijiensis* does not penetrate directly it must grow on the surface of a leaf until it finds a stoma. The hydrophobin protein possibly could be involved in attachment to the host during its epiphytic phase until it can locate a suitable stoma for penetration.

Additional potential pathogenicity-related genes were identified through analysis of the pathways for melanin biosynthesis. Melanin is essential for successful host penetration by several plant-pathogenic fungi (Kubo et al., 1985; Rasmussen and Hanau, 1989; Wolkow et al., 1983). Although there are different types of fungal melanin, many pathogenic fungi, e.g., *Magnaporthe oryzae*, *Verticillium dahliae*, *Blastomyces dermatitidis* and *Sporothrix schenckii*, synthesize their pigments from acetate through the 1,8-dihydroxynaphthalene (DHN)-melanin pathway (Bell and Wheeler, 1986). Both *Z. tritici* and *P. fijiensis* may use this pathway for melanin biosynthesis because the necessary genes are present in their genomes. However, in *Z. tritici* melanin does not appear to be important for pathogenicity

since melanin-deficient mutants were still pathogenic (Choi and Goodwin, 2011a), while mutants with increased melanin production showed reduced pathogenicity (Choi and Goodwin, 2011b). The importance of melanin for pathogenicity of *P. fijiensis* is not known.

The DHN-melanin pathway may be important for *P. fijiensis* even if melanin is not involved in its pathogenicity. In addition to melanin, this pathway is used for the synthesis of juglone and 2,4,8-tetrahydroxytetralone, both of which are produced by *P. fijiensis* in large amounts (Stierle et al., 1991; Host et al., 2000) and are phytotoxic to the banana plant, most probably by acting on chloroplasts (Busogoro et al., 2004). Knowing the sequences of all of the genes involved in the synthesis of these metabolites will open the way for functional analyses, allowing tests of whether they have a role in pathogenesis. The only knowledge in this respect is the virulence test of two pigment-deficient isolates (Donzelli and Churchill 200 [15]. Both isolates were capable of penetrating, but only one of them was virulent.

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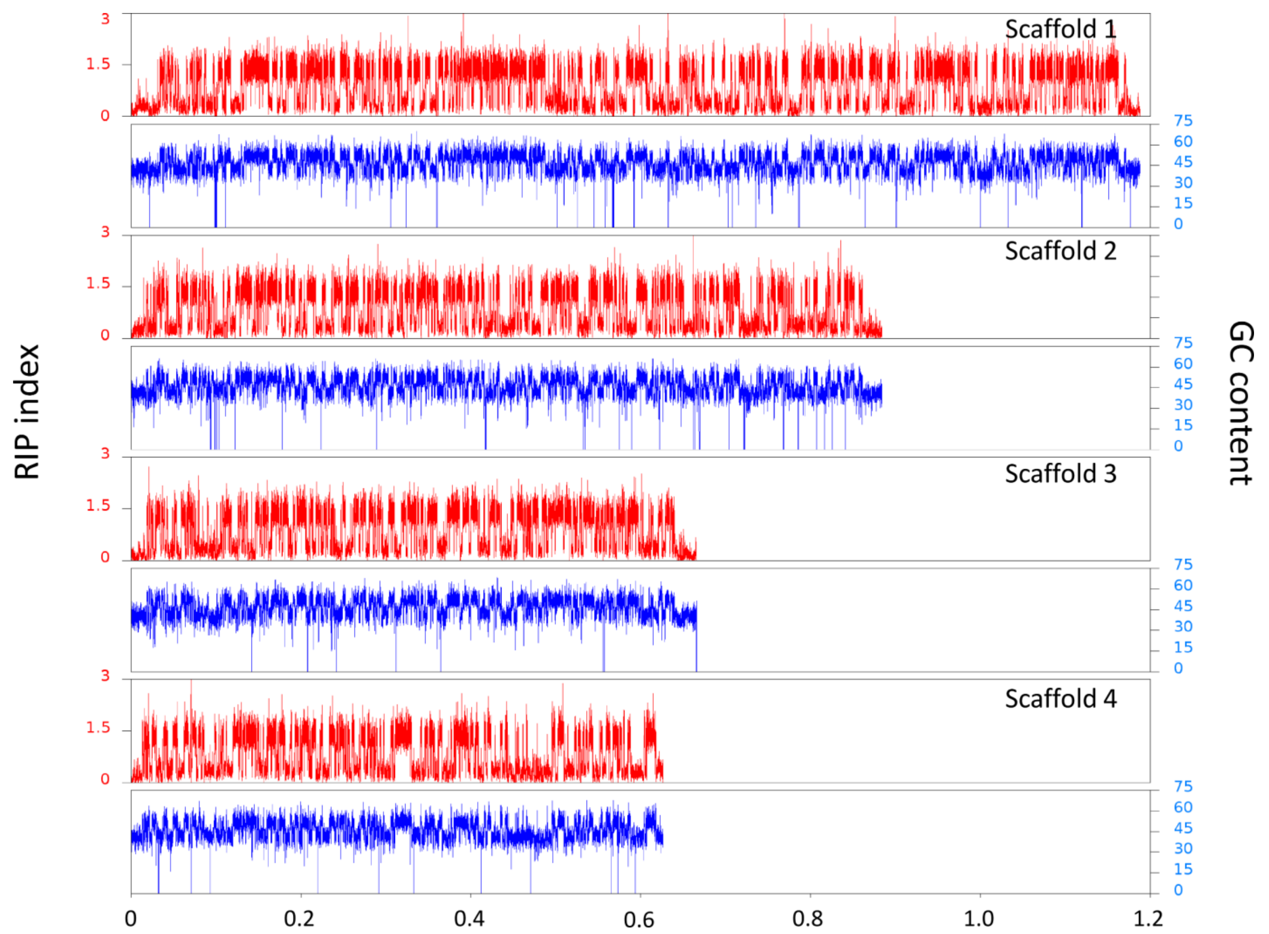

Figure S1. The strong positive association between RIP index (where a high index value indicates low RIP) and GC content shows that RIP in *P. fijiensis* is mostly restricted to repetitive elements rather than genes.

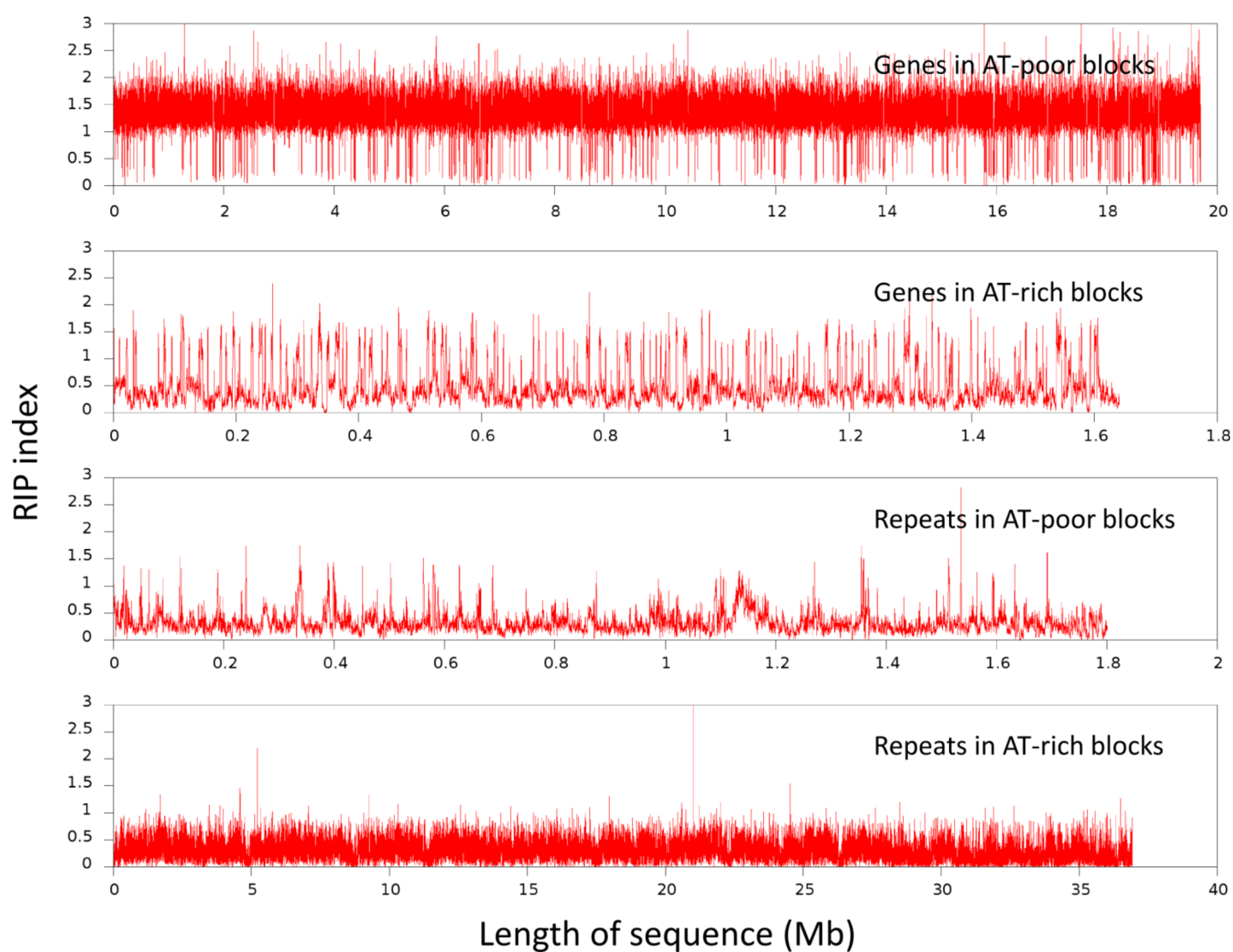

Figure S2. The RIP index in genes and repeats in AT-rich and –poor regions of the *P. fijiensis* genome. RIP is mostly absent from the genes but highly prevalent among the repeated elements of the genome.

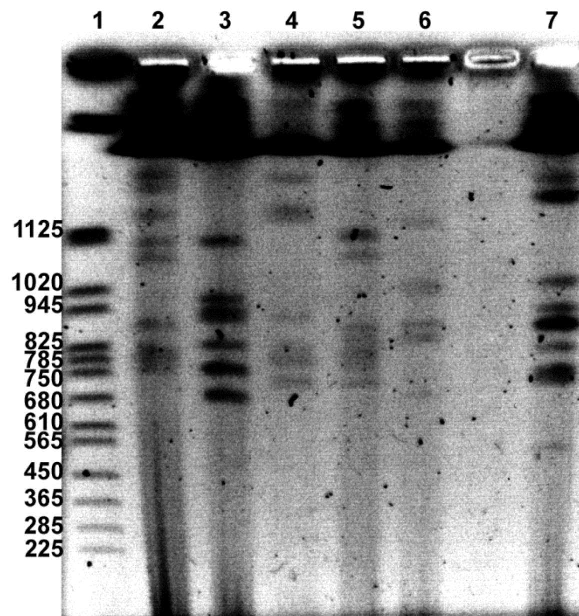

Figure S3. Separation of small size chromosomal bands by electrophoretic karyotyping of five field isolates and the CIRAD139A strain of *Pseudocercospora fijiensis*. Lane 1, chromosomes from *Saccharomyces cerevisiae* as high-molecular-weight (HMW) marker; lanes 2 to 6, different field isolates from the Cartagena farm; lane 7, the CIRAD139A strain. Marker sizes are in Kb.

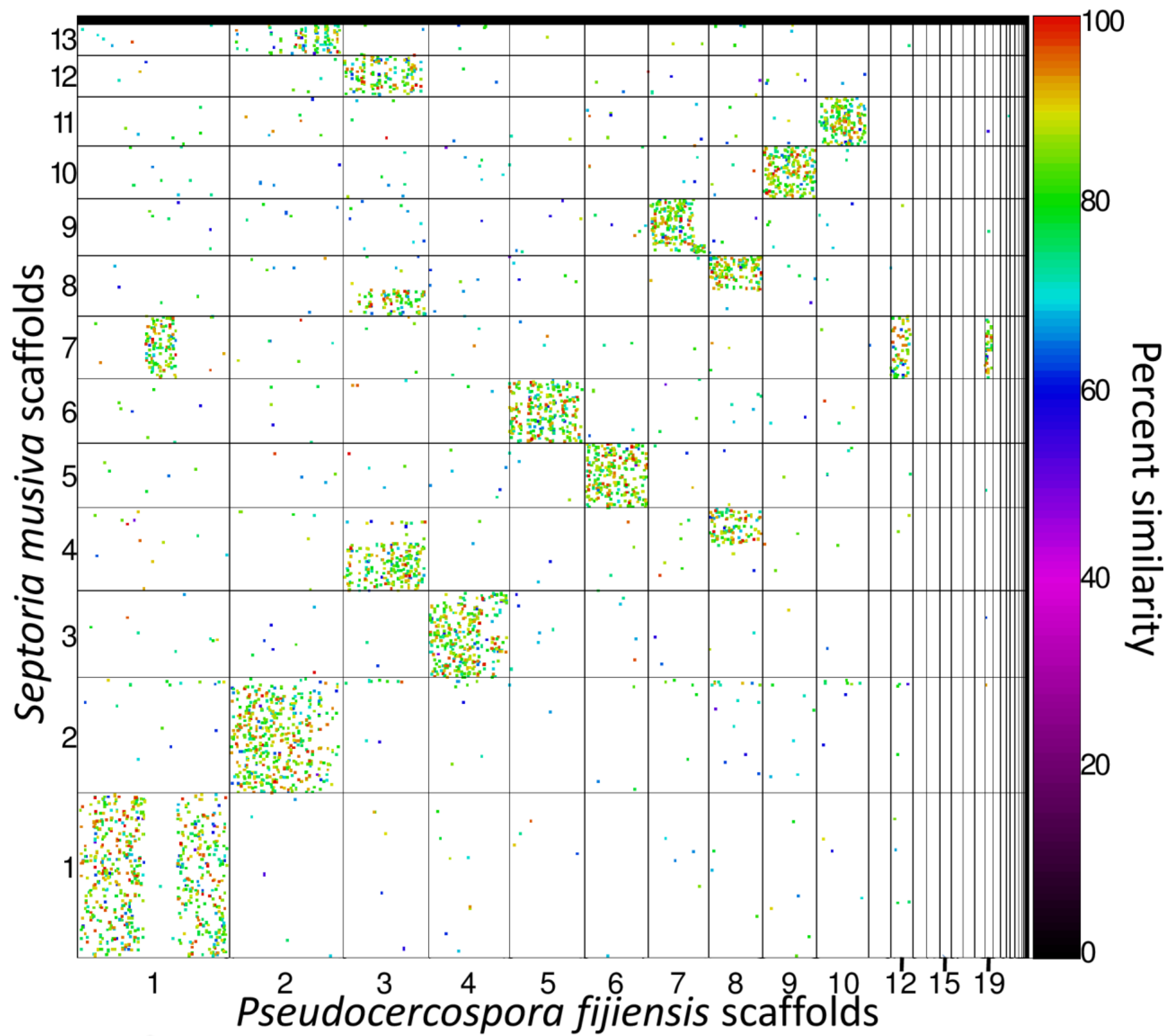

Figure S4. Dot plot showing mesosynteny between the scaffolds of *Pseudocercospora fijiensis* and *Septoria musiva.*

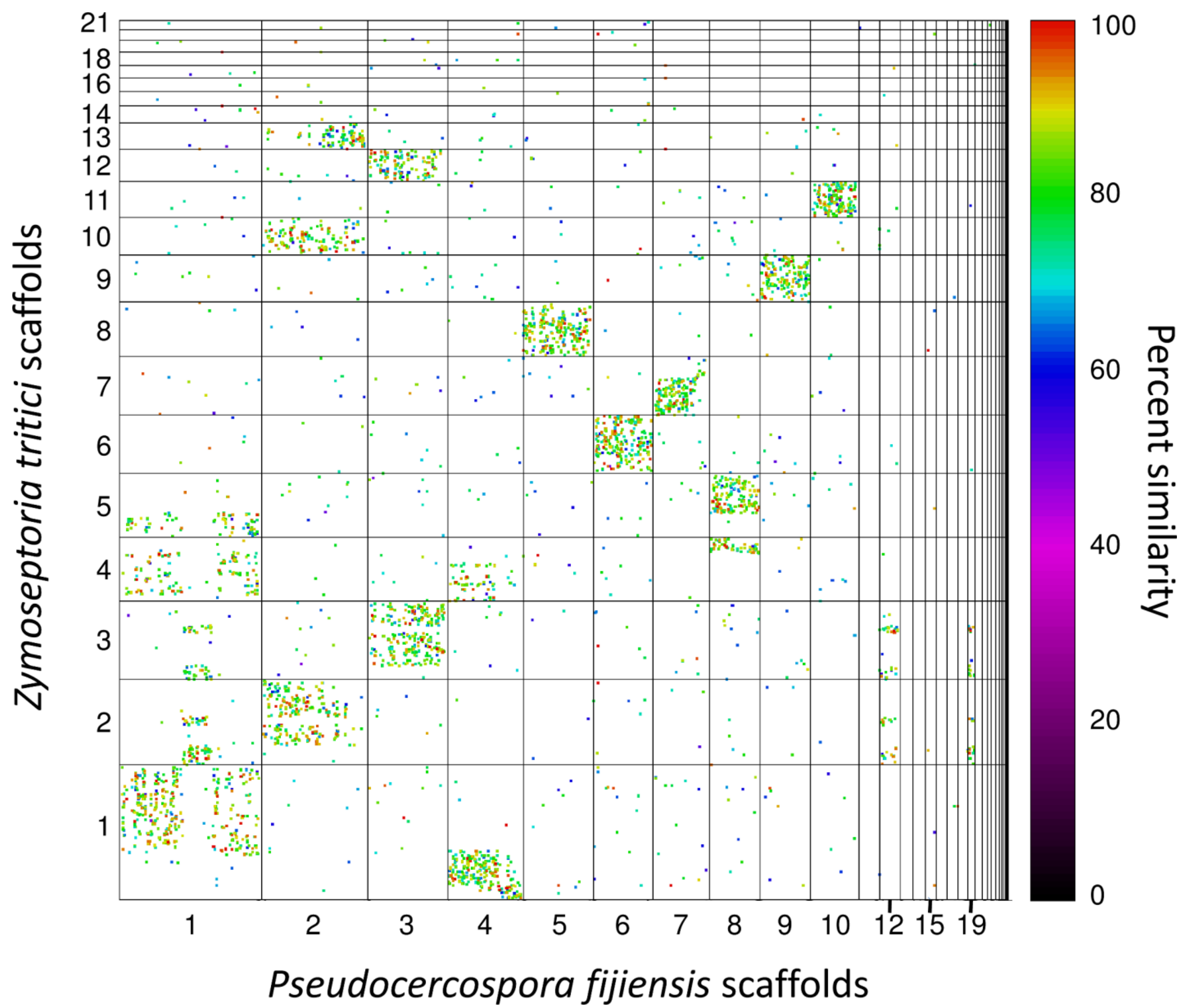

Figure S5. Dot plot showing mesosynteny between the scaffolds of *Pseudocercospora fijiensis* and *Zymoseptoria tritici*.

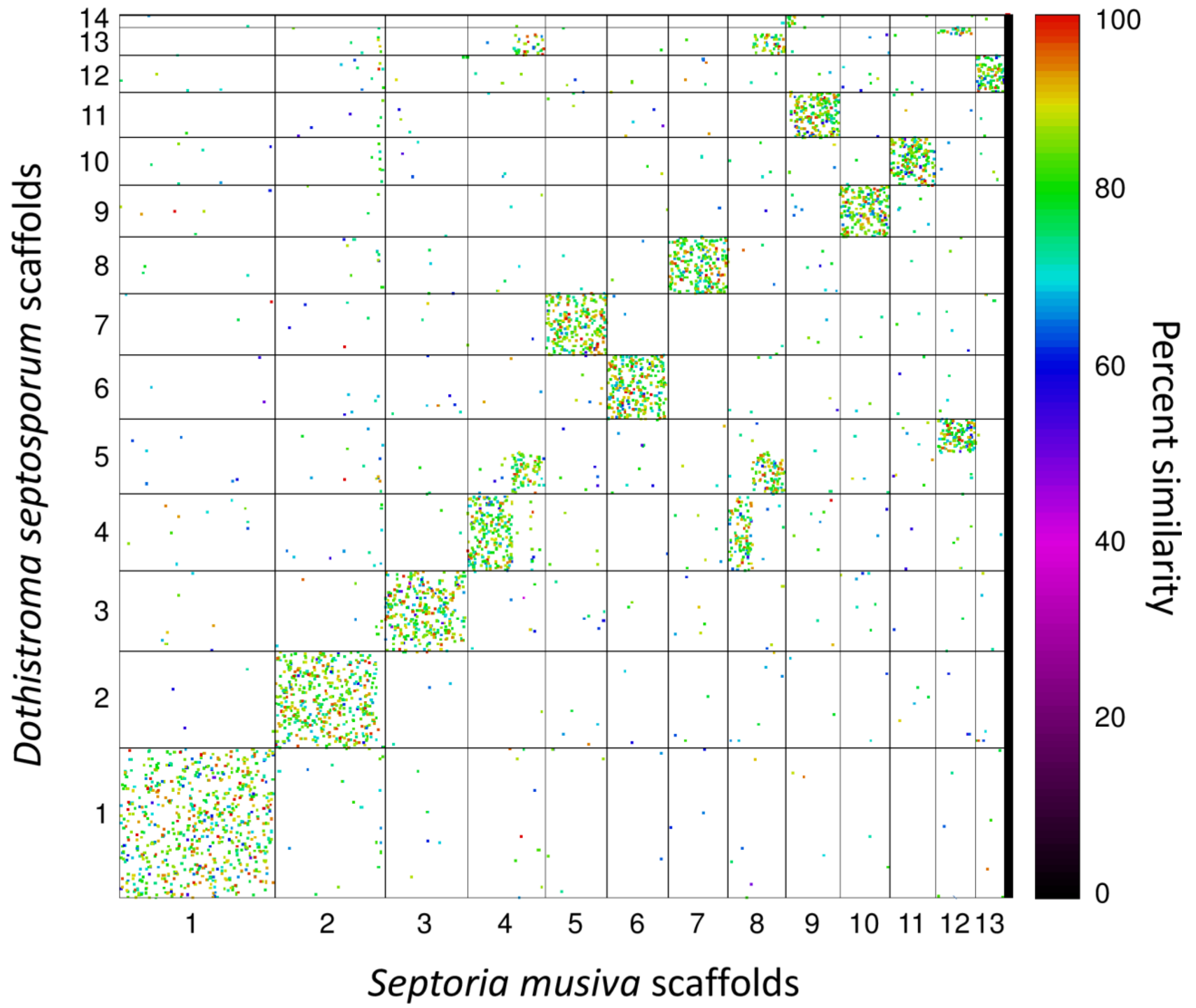

Figure S6. Dot plot showing mesosynteny between the scaffolds of *Septoria musiva* and *Dothistroma septosporum.*

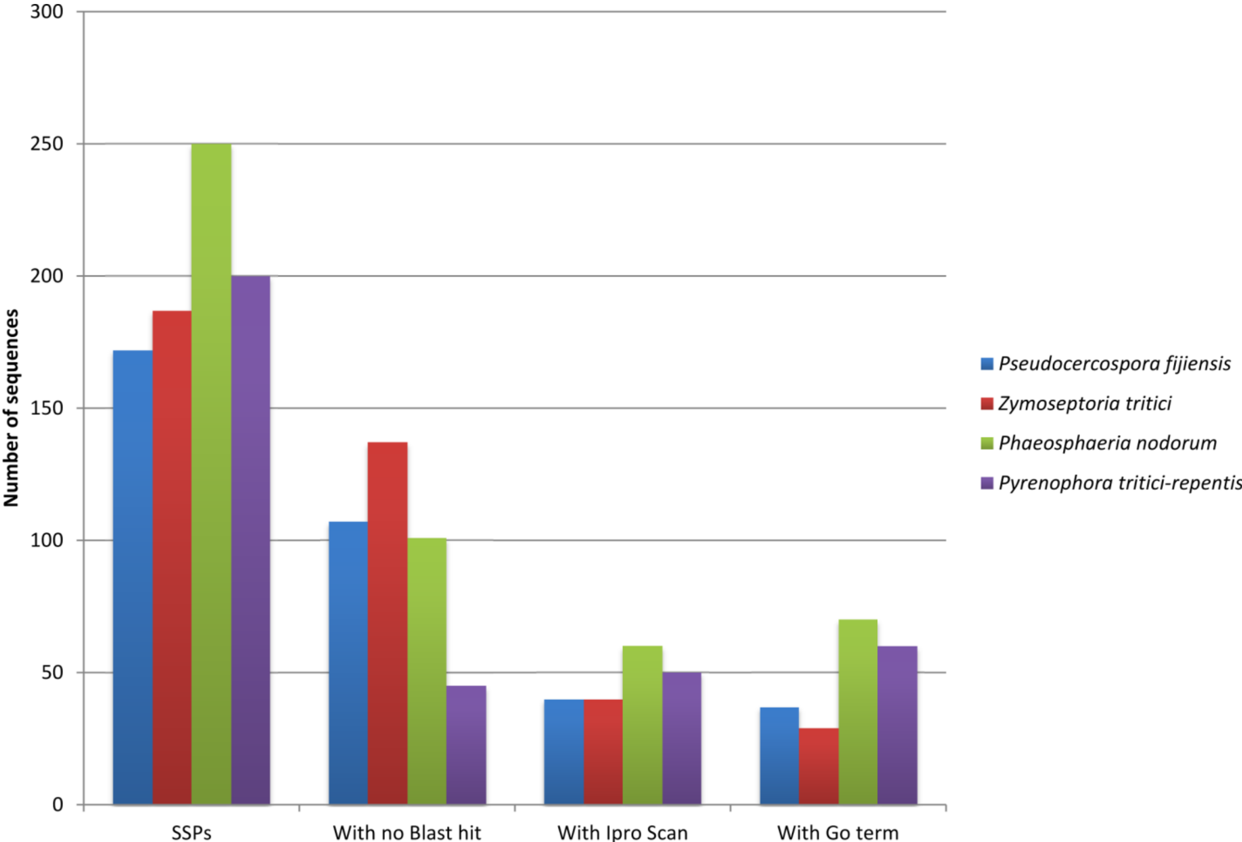

Figure S7. Small secreted proteins in the genome of *Pseudocercospora fijiensis* compared to those in the genomes of three other Dothideomycetes.

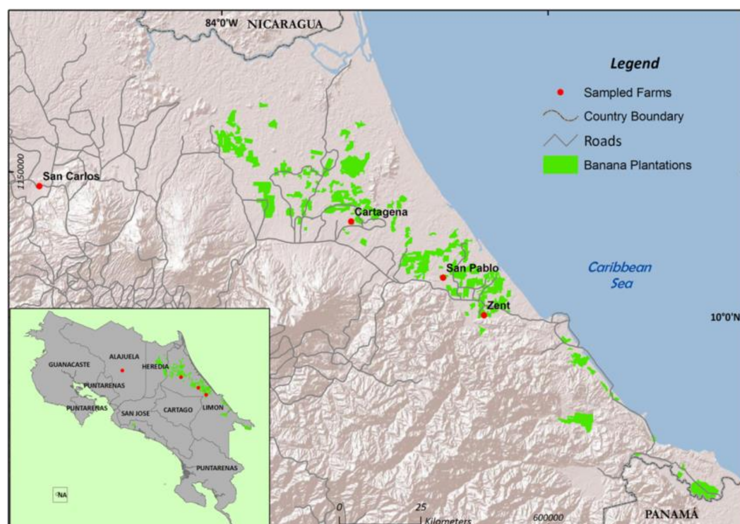

Figure S8. Locations of farms in Costa Rica that were sampled to obtain isolates of *Pseudocercospora fijiensis* for analyses of mating type, fungicide resistance and population genetics. Farms Cartagena, San Pablo and Zent are in a major banana-production area and are sprayed heavily with fungicides; the San Carlos farm is in an area of plantain production (mostly resistant to *P. fijiensis*) and is not sprayed with fungicides.

Table S1. Scaffold sizes, and identification of potential dispensable chromosomes in the genome of *Pseudocercospora fijiensis* by comparison with the characteristics of those known from *Zymoseptoria tritici*.

Scaffold	Size (bp)	G+C, %	Genes	Genes/Mb	Genes, %	Genes with PFAM, %	No. of ESTs	ESTs, %	Repeats, %
1	11880248	46.8	2676	226.8	20.4	54.7	7277	23.00	32.6
2	8841024	46.2	1881	214.4	14.4	52.2	4555	14.40	33.7
3	6657679	46.5	1526	229.8	11.6	51.0	3437	10.86	31.2
4	6264405	45.8	1207	193.3	9.2	47.1	2725	8.61	37.0
5	5901819	44.8	975	166.0	6.7	49.6	2149	6.79	45.2
6	4991523	45.0	863	174.0	6.6	49.0	2413	7.63	41.7
7	4695110	45.7	854	182.3	6.5	44.3	1953	6.17	37.6
8	4236865	46.2	886	210.0	6.8	54.6	2030	6.42	37.4
9	4185348	45.2	741	178.1	5.7	52.4	1297	4.10	40.1
10	4009308	45.4	703	177.1	5.4	45.5	2842	8.98	42.1
11*	1762310	40.2	62	35.3	0.5	3.2	13	0.04	66.0
12	1674337	44.2	253	151.4	1.9	47.0	539	1.70	43.6
13*	1121713	41.0	51	45.5	0.4	0.0			63.1
14*	1006785	39.7	37	36.9	0.3	0.0	6	0.02	60.1
15*	928877	41.6	42	45.4	0.3	4.8	13	0.04	60.8
16*	905553	39.1	2	2.2	0.0	0.0			76.0
17*	851953	42.1	65	77.5	0.5	4.6	30	0.09	32.0
18*	830772	41.7	49	59.2	0.4	2.0	38	0.12	51.5
19	609425	46.2	130	213.8	1.0	45.4	255	0.81	41.4
20*	594030	41.1	29	49.1	0.2	0.0	22	0.07	41.9
21*	427043	43.6	40	94.1	0.3	2.5	21	0.07	49.4
22*	366261	41.7	30	82.3	0.2	3.3			53.1
23*	339557	37.5	0	0.0	0.0	0.0			66.8
24*	325668	38.1	0	0.0	0.0	0.0			55.2
25*	230844	42.1	1	4.4	0.0	0.0			12.5

*Potential dispensable chromosomes are characterized by lower gene densities and higher proportions of repetitive sequences.

Table S2. Comparison of annotated genes in the version 1 and 2 assemblies of the *Pseudocercospora fijiensis* genome.

Annotation parameter	v1.0 Assembly	v2.0 Assembly
Number of gene models	10,316	13,107
Gene density per Mb DNA	140.5	187.6
Average gene length	1629 nt	1833 nt
Average protein length	436 aa	427 aa
Average exon frequency [a]	2.45	3.62
Average exon length	578 nt	395 nt
Average intron length	148 nt	154 nt
Percent complete gene models [b]	82	88
Percent of genes with homology support	70	74
Percent of genes with Pfam domains	48	49
Percent of genes with EST support		30

[a] Number of exons per gene

[b] With start and stop codons.

Table S3. Summary of G+C contents derived from the melting-curve analyses of DNA extracted from isolates of *Pseudocercospora fijiensis, P. eumusae, P. musae* and *Zymoseptoria tritici*.

Species	Isolate	Percent G+C		Number of
		Peak 1	Peak 2	replications
P. fijiensis	CIRAD86	39.4 ± 0.80	51.6 ± 0.90	18
P. eumusae	CBS122457	39.6 ± 0.03	51.6 ± 0.08	2
P. musae	UQ430	37.2 ± 1.30	50.9 ± 0.50	6
Z. tritici	IPO323	—	53.1 ± 0.50	6

Table S4. Next-generation resequencing information for *Pseudocercospora fijiensis* isolate CIRAD139A and numbers of Single-Nucleotide Polymorphisms (SNPs) compared with the reference genome of isolate CIRAD86 V2.0.

Parameter	Number (%)
Total reads	73,185,656
Uniquely aligned	43,622,808 (59.6%)
Ambiguously mapped	20,982,018 (28.7%)
Unmapped	8,580,830 (11.7%)
Genome coverage (bp)	65,956,770 (89%)
Average depth of coverage	35.6 x
Total variants	514,953
Total SNPs	509,749 (99%)
Total indels	5,204 (1%)
Variants in coding sequences	95,335 (18.5%)
Non-synonymous variants	46,295 (9%)

Table S5. Analysis of polymorphism type [Single-Nucleotide Polymorphisms (SNPs) and insertion/deletion (indels)] and frequency among the 21 largest scaffolds of the sequenced *Pseudocercospora fijiensis* isolate CIRAD86 and the re-sequenced isolate CIRAD139A.

Scaffold	Length (10^4 bp)	Total variants	Substitutions	Indels	Mean frequency (per 10kb)
1	1188	71940	70994	946	60.6
2	885	58469	57793	676	66.1
3	667	44949	44352	597	67.4
4	627	45844	45299	545	73.1
5	591	41410	41033	377	70.1
6	500	37467	37088	379	74.9
7	470	36909	36542	367	78.5
8	425	28845	28473	372	67.9
9	420	28315	28021	294	67.4
10	402	28985	28707	278	72.1
11	177	14887	14833	54	84.1
12	168	12317	12204	113	73.3
13	113	6695	6691	4	59.2
14	102	8049	8027	22	78.9
15	94	6700	6668	32	71.3
16	91	6292	6277	15	69.1
17	86	6301	6281	20	73.3
18	84	6155	6130	25	73.3
19	62	3781	3743	38	61.0
20	60	4235	4234	1	70.6
21	44	3517	3492	25	79.9

Table S6. Genotyping strobilurin resistance and mating type in four Costa Rican populations of *Pseudocercospora fijiensis*.

Population	Sample size[a]	Fungicide sensitivity			Mating type		
		Sensitive	Resistant	Percent resistant	Ratio	χ^2	P[b]
Cartagena	189	0	178	100.0	90:85	0.14	0.705
San Carlos	91	87	0	0.0	42:45	0.10	0.748
San Pablo	179	0	180	100.0	84:82	0.02	0.877
Zent	190	14	162	92.0	77:96	2.09	0.149
Total or mean	649	101	520	83.7	293:308	0.37	0.541

[a] Total number of isolates with molecular marker data for at least one locus. Sample sizes for each statistic varied depending on the number of isolates scored successfully.

[b] Probability that the observed mating type ratios are not significantly different from 1:1.

Table S7. Estimates of genetic differentiation[a] (Jost's D above the diagonal and the corrected, standardized genetic differentiation G''_{ST} below the diagonal) between pairs of populations of *Pseudocercospora fijiensis* sampled from four banana plantations in Costa Rica[b].

Population	Cartagena	San Carlos	San Pablo	Zent
Cartagena	—[c]	0.067	0.052	0.089
San Carlos	0.067	—	0.192	0.126
San Pablo	0.116	0.097	—	0.065
Zent	0.190	0.243	0.146	—

[a] *D* and G''_{ST} were calculated with GenoDive (Meirmans and van Tienderen, 2004) as described by Jost (2008) and Meirmans and Hedrick (2011), respectively.

[b] All values for each statistic were significantly different at $P = 0.001$.

[c] Not applicable to self comparisons.

Table S8. Percent of gene models supported by different kinds of evidence in the initial automated annotation of the genome of *Pseudocercospora fijiensis*.

Annotation parameter	Number or percent of genes
Number of gene models	13,107
Percent complete (with start and stop codons)	88%
Percent of genes with homology support	74%
Percent of genes with Pfam domains	49%
Percent of genes with EST support	30%

Table S9. Automated assignment of the 13,107 genes in the genome of *Pseudocercospora fijiensis* to broad functional categories.

Category	Number of genes (Percent)
Proteins assigned to a KOG	6676 (51%)
KOG categories genome-wide	3247
Proteins assigned a GO term	5655 (43%)
GO terms genome-wide	1393
Proteins assigned an EC number	2666 (20%)
EC numbers genome-wide	798
Proteins assigned a Pfam domain	6603 (50%)
Pfam domains genome wide	2446

Table S10. Primers used for population genetic diversity and fungicide sensitivity assays in four *Pseudocercospora fijiensis* populations in Costa Rica.

Locus	Forward primer[a]	Reverse primer[a]	Expected size
Fungicide sensitivity			
cytb	CTCAAATACTGCCTCAGC	CCGTAATGTGGTTCATC	285
	CTCAAATACTGCCTCAGC	GTTATAACTGTAGCTCC	198
Variable Number of Tandem repeats (VNTR) loci			
1333	GAGTGAAGTACTGCGGAGGC	AGTTGGAGAAAGGCGAAAGG	259
3959	GCGCGAGGCTTTCTATCTC	ACCCCGATTAGGGAAGGTC	184
3786	GCAGCGGAGTGCTAGTAACC	CGCGCTTTTGACTCTTCTTC	260
0252	TAGAGGCTACCCTGCCGTC	GTATACTTCCGACCTCGGGC	132
0705	ATAGGATGCGGCAGACACTC	CGTCGCGATTTGAAGTGCC	214

[a] Primer sequences written 5’ to 3’.

CHAPTER 3
TARGETED AND RANDOM GENETIC MODIFITATION OF THE BLACK SIGATOKA PATHOGEN *PSEUDOCERCOSPORA FIJIENSIS* BY *AGROBACTERIUM TUMEFACIENS* - MEDIATED TRANSFORMATION

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Abstract

Pseudocercospora fijiensis (syn. *Mycosphaerella fijiensis*) is the fungal pathogen that causes black leaf streak disease or black Sigatoka in banana and plantain crops, which is a threat to global banana production, and unfortunately the experimental amenability of the fungus is low as it develops very slowly, hardly produces conidia and molecular tools for functional genetics are barely available. Here, we report successful *Agrobacterium tumefaciens*-mediated transformation and the generation of various genetically modified *P. fijiensi*s strains. By random insertion we developed green fluorescent protein (GFP) and red fluorescent protein (DsRed) labelled strains. Through homologous recombination we also generated the first *P. fijiensis Pfavr4 and Pfku70* knock-out mutants. The former is the first avirulence gene in this pathosystem recognized by the cognate tomato resistance gene *Cf4*. The latter gene encodes a protein that triggers non-homologous end joining. Likewise, we generated various *P. fijiensis* strains with the *Pfcyp51* promoter replaced. This gene encodes CYP51, and its encoded protein is the target for lanosterol 14α-demethylase inhibitors (DMIs; azoles), a major class of functional fungicides for black Sigatoka control in banana and also applied against many other fungal plant, animal and human pathogens. With the targeted strains obtained here, we developed tools to further analyze the *P. fijiensis*-banana interaction, and to better understand reduced sensitivity to azole fungicides.

Introduction

Banana and plantain are major fruit and staple crops (FAO, 2015). Bananas are the global top export fruit, with an average of 114 million tons/year, dominated by Cavendish clones that take a major share (90%), but which are also increasingly important for local markets (FAO, 2003; Food and Agriculture Organization of the United Nations, 2015). Cooking bananas and plantains are major staple foods in tropical and subtropical countries in central Africa, South East Asia and Latin America (Mohan and Swennen, 2004; Ortiz and Swennen, 2014). Many banana varieties are highly susceptible to black Sigatoka or black leaf streak disease (Fullerton and Olsen, 1995). Disease management in this perennial crop largely relies on frequent fungicide applications. The frequency varies over environments but is commonly over 50 times per year (Chong, 2016; De Lepeyre De Bellaire 2010; FRAC, 2010) and commonly represents up to 40% of the total production costs (Chong, 2016; Martínez-Bolaños et al., 2012). Albeit that banana production provides manifold jobs and supports the livelihoods of millions of people, the dependency on fungicides evidently threatens occupational health as well as tropical fragile environments (Panganiban et al., 2004; Penagos, 2002).

Pseudocercospora fijiensis (Morelet, 1969) Deighton (1976), previously known as *Mycosphaerella fijiensis* (Morelet 1969) is the causal agent of black Sigatoka and is a hemibiotrophic ascomycete fungus from the class of the Dothideomycetes (Schoch et al., 2006), which has spread to almost all tropical and subtropical regions (Carlier et al., 1996; Hayden and Carlier, 2003; Rivas et al., 2004). The life cycle of the pathogen includes a biotrophic and a necrotrophic stage, that eventually results in the production of clonal conidia and genetically diverse sexual ascospores that drive epidemics (Burt, 1994). Once germinated, germ tubes of conidia and ascospores penetrate the leaf through the stomata and develop into mycelia that colonize the mesophyll tissue until fructification. During this process lesions of

affected foliar tissue become gradually visible and eventually coalesce in large necrotic blotches along the rim of the leaf towards the midrib. Clearly, leaf necrosis reduces the photosynthetic capacity, but also causes a metabolic switch to premature fruit ripening, which greatly affects the export market value of the crop (Beltrán-García et al., 2014; Okole and Schulz, 1997).

The genome analysis of *P. fijiensis* (Arango Isaza et al., 2016) generated a range of genetic studies (Chang et al., 2016; Chong, 2016; Churchill, 2011; Couoh-Uicab et al., 2013; de Wit et al., 2012; Kantún-Moreno et al., 2013; Noar and Daub, 2016a, 2016b; Ohm et al., 2012; Stergiopoulos et al., 2014, 2010), which turned the fungus from a recalcitrant pathogen into an interesting pathogen with unique characteristics. It has for instance one of the largest genomes in the Dothideomycetes due to a huge transposon driven genome expansion (Ohm et al., 2012). Hence, *P. fijiensis* is not only important as a plant pathogen, but is also attractive for genome wide comparative analyses (Chang et al., 2016). Nevertheless, the genetic amenability of the fungus is limited. It has a slow *in vitro* growth, poor sporulation that largely depends on isolate and environmental conditions, can only complete a sexual cycle *in planta*, and foremost, lacked a transformation protocol for a long time, which hampered any functional analysis of genes (Arango et al., 2016; Churchill, 2011).

Agrobacterium tumefaciens mediated transformation (ATMT) has been successfully used in many filamentous fungi (Abello et al., 2008; Chen et al., 2000; Combier et al., 2003; de Boer et al., 2013; Ding et al., 2011; Mirzadi Gohari et al., 2014; Nyilasi et al., 2005; Weld et al., 2006; Xue et al., 2013; Zheng et al., 2011; Zwiers and De Waard, 2001). Homologous recombination frequencies vary between 1 and 10% (Kück and Hoff, 2010), depending on the fungus and the targeted genes. Nevertheless, knocking out *ku70* genes, that encode a protein involved the DNA non-homologous end joining (NHEJ) for double-strand DNA break repair, has greatly contributed to enhanced recombination in various fungi (Bowler, et al., 2010;

Feng, et al., 2012; He et al., 2013; Nayak et al., 2006; Ninomiya, et al.,2004).

Here, we report the *Agrobacterium tumefaciens* mediated transformation (ATMT) of *P. fijiensis.* Because the *in vitro* production of *P. fijiensis* conidia is challenging (Churchill, 2011; Cruz-Martín et al., 2011.; Donzelli and Churchill, 2007), our approach involves mycelium for ATMT and this resulted in the production of several transformed *P. fijiensis* strains with ectopic integrations as well as homologous recombinations that can be used for detailed pathological and functional analyses.

Results

Random mutagenesis: generation and characterization of gfp and DsRed Pseudocercospora fijiensis mutants

Growth inhibition tests revealed that a hygromycin concentration of 50 µg·mL-^{1} enabled the differentiation of transformed from untransformed colonies of the CIRAD86 and CIRAD139a wt *P. fiji*ensis recipient strains (data not shown). Random integration of the generated *gfp* or *DsRed* constructs in the *P. fijiensis* recipient strains (Figure 1) was routinely checked through fluorescence microscopy. In three independent transformation experiments we obtained an average of 9.125 transformants per membrane by starting with a concentration of 1.5x10^{6} hyphal pieces per mL and eventually obtained each combination of fluorescent marker and recipient strain viz. strains CIRAD86::*gfp*, CIRAD86::*DsRed*, CIRAD139a::*gfp* and CIRAD139a::*DsRed*, with transformation efficiency of 0.00066%, 0.0011%, 0.0002%, and 0.00006%, respectively (Table 1); and whose genetic identities were confirmed using a genetic profile of repetitive DNA at different loci by using the Variable Number of Tandem Repeat markers (VNTR1333; Garcia et al., 2010) (Figure 2). We did not observe *in vitro* obvious morphological differences between the wt and mutant strains on solid growth medium.

Targeted mutagenesis: generation and identification of** Pfavr4, Pfku70* **knock-out strains and *Pfcyp51* promoter replacement

The homolog of *Cladosporium fulvum Avr4* in *P. fijiensis* is *Pfavr4* (Stergiopoulos et al., 2010, 2014) and is located (ID: 87167) on position 183210-183796 of scaffold 4. For the knock-out construct, we selected the upstream region 180469-183065 and the downstream region 183458-185053 (Figure 1). The *Pfku70* gene was identified from the *P. fijiensis* genome database (Arango Isaza et al., 2016) and is located (ID: 65414) on scaffold 3, position 603346-605427. This includes the start codon 21 bp upstream of the automatically predicted transcript. Note that the transcript was extended at the N-terminus without addition of introns. The resulting protein model of 702 aa is in agreement with KU70 homologs from other fungi, including *Sphaerulina musiva* (EMF13337.1), *P. musae* (KXT17510.1), and *Zymoseptoria brevis* (KJY00058.1) which have similar extensions. Aligning the KU70 proteins revealed that the *P. fijiensis* protein is the closest related to the Dothideomycete fungi *S. musiva* (74.2 %) and *Z. tritici* (69.9 %) (Figure 2), whereas the identity with the more distantly related basidiomycete *Rhodosporium toruloides* (AIA21643.1) was significantly lower (39.6 %) (Figure 3; Table 2). Domain analysis confirmed the presence of three domains, similar to other eukaryotic KU70 proteins (Figure 3): the N-terminus with a Ku_N terminal α/β domain (also called von Willebrand A [vWA] domain), in the center a KU core domain including a DNA-binding β-barrel domain, and finally the C-terminus comprising a SAP domain (Fell and Schild-Poulter, 2015) (Figure 3A).

Using LBA1100 *Agrobacterium tumefaciens* cells, we obtained 90 and 102 GFP fluorescent transformants for *Pfavr4* and *Pfku70,* respectively, which were tested by PCR for homologous recombination. Ectopic transformants showed the same PCR fragment as the CIRAD86 wt strains, whereas replacement mutants lacking this amplicon were positive for the strain specific variable number tandem repeat marker VNTR1333. Moreover, the presence

of the replacement cassette was confirmed by amplification of a diagnostic amplicon with a primer located within the replacement cassette and a unique sequence of the *P. fijiensis* genome outside the construct (Figures 5 and 6). Overall, these rounds of transformations resulted in the identification of three knock-out mutants: *Pfavr4 #10, Pfku70 #33* and *Pfku70 #45.*

By using the *A. tumefaciens* AGL1 strain in combination with the modifications in the protocol after the initially random mutagenesis, we improved the transformation efficiency with at least one order of magnitude for *Pfku70* (two knock out strains out of 102 tested hygromycin transformants, derived from $4.1x10^5$ hyphal fragments per mL). This resulted in an overall transformation efficiency of 0.025% and homologous recombination in 1.96% across all transformants. The knock-out efficiency for *PfAvr4,* using the same conditions, was 0.022 % and homologous recombinations occurred in 1.1% of the transformed strains (Table 1).

The third sequence we targeted using this method was the *Pfcyp51* gene promoter (Chong et al., 2016). With a transformation efficiency of 0.025%, we replaced the promoter of the azole sensitive strain E22 by the promoter of the azole resistant strain Ca5_16, which carried no and multiple repetitive elements in their promoters, respectively. Additional to promoter length, a diagnostic amplicon discerned two homologous recombinants resulting in a recombination efficiency of 0.8 % (Table 1).

Fitness comparisons between the wt strain CIRAD86 and the *Pfku70#33* and *Pfku70#45* strains showed that the knock-out strains were compromised in growth (-25-38%) (Figure 7). However, functional characterization of the *Pfcyp51* promoter replacement strains did not show significant fitness penalties potentially resulting from the transformation process (Díaz-Trujillo et al., 2018).

Discussion

Food security requires continuous crop improvement to meet future demands. The regular and worrisome disease threats of major crops necessitate analyses of the widest genetic resources to identify new genes to increase genetic diversity for all traits contributing to increase productivity. Therefore, it is essential to uncover the potential of such resources by using genetic and phenotypic characterization technologies during abiotic and biotic challenges. Understanding host-pathogen interactions and dynamics is a fundamental basis for sustainable crop health (De Wit, 2016; Kema et al., 2018; 2016; Michelmore et al., 2017; Mirzadi et al., 2015; FRAC, 2010; van den Bosh et al., 2014). For many crops, tools have been developed for the analysis of plant-pathogen interactions, but these are in their infancy for under-investigated crops. Banana is one of these crops, albeit a major staple food for millions of people and despite its status as the top global fruit (FAO, 2015). Disease control is mostly accomplished by extensive fungicide applications due to the vulnerability of the global Cavendish banana monoculture (Diaz-Trujillo et al., 2018). Here, we report the first *Agrobacterium*-mediated transformation of *P. fijiensis,* which is an important step to aid mining the genome of this major plant pathogen (Arango et al., 2016). We initially applied ATMT for ectopic integration of the *DsRed* or *gfp* gene, respectively, and then accomplished targeted mutagenesis by generating knock-out strains of *Pfku70, Pfavr4* and replacing the *Pfcyp51* promoter. In many fungi Δku70 strains have shown a significant increase of homologous recombination (Bowler et al., 2010; Catalano et al., 2010; He et al., 2013; Koh et al., 2014; Näätsaari et al., 2014; Ninomiya et al., 2004), and hence we considered it important to develop the Δ*Pfku70 P. fijiensis* strain to facilitate further functional analyses of *P. fijiensis* genes. We disrupted *Pfavr4*, the first effector described in this pathogen, as a starting point to further unravel the banana – *P. fijiensis* interaction (Arango et al. 2016). Recently, we also

engineered *P. fijiensis* strains with modification of *Pfcyp51* promoters, to identify a new mechanism of fungicide sensitivity (Diaz-Trujillo et al., 2018).

Frequently, conidiospores are reported as the ideal starting material for ATMT (Liu et al., 2009; Nizam et al., 2010; Zwiers and De Waard, 2001), but others have used other tissues (Eckert et al., 2005), and reported higher transformation efficiencies with mycelial pieces (Abello et al., 2008) or even fructifications (Chen et al., 2000). In the case of *Hebeloma cylindrosporum* (Combier et al., 2003) and *Muscodor albus* (Ezra et al., 2010), transformation was exclusively possible with mycelial starting material. In *P. fijiensis*, efficient and constant production of conidiospores is challenging. It is strongly influenced by light conditions (Sepúlveda et al., 2009), temperature (Churchill, 2011; Sepúlveda et al., 2009), isolate origin (Peraza-Echeverría et al., 2008), harvesting intervals (Peraza-Echeverría et al., 2008), and the number of subcultures (Cruz-Martín et al., 2011). Therefore, Donzelli et al. (2007) successfully used mycelial fragments instead of conidia to study the banana- *P. fijiensis* interaction. Therefore, we eventually used hyphal material as starting material for ATMT and showed it can be used for functional analysis of at least three genes in this recalcitrant fungus.

The constructed replacement cassette was functional in *P. fijiensis.* The *hph* gene, that confers resistance to hygromycin B, in combination with the trpC terminator, has been widely used in Ascomycetes and other fungi as selection marker (Fitzgerald et al., 2003; Krappmann et al., 2006; Mirzadi Gohari et al., 2014). Binary vectors for fungal transformation as pPm43GW have been developed based on the pCAMBIA vector series and others mainly for plant transformation (Frandsen, 2011). Rather than the classic laborious method to construct the binary vector for gene replacement, we used the simplified and high throughput Gateway cloning system according to Shafran et al. (2008), essential to allow screening of the large number of identified sequences that are potentially involved in pathogenicity, fungal growth and resistance to fungicides (Amil et al., 2007; Arango Isaza et al., 2016; Beltrán-García et

al., 2014; Cañas-Gutiérrez et al., 2006; Chang et al., 2016; Couoh-Uicab et al., 2013; Escobar-Tovar et al., 2015; Kantún-Moreno et al., 2013; Noar and Daub, 2016a, 2016b).

Gene knock-out efficiencies depend largely on the length of homologous sequence and is usually optimal at around 1 kb (Michielse et al., 2005). Low homologous transformation efficiency necessitates the screening of a large number of transformants to identify those with correct integration. A strategy to increase homologous recombination efficiency in fungi is based on the stimulated expression of genes involved in recombination although this is also prone to decreased genomic stability (Natsume et al., 2004). Alternatively, disruption of genes involved in NHEJ was shown to increase the homologous recombination frequency in several filamentous fungi (Bowler et al., 2010; Koh et al., 2014; Krappmann et al., 2006; Ninomiya et al., 2004). For *Venturia inaequalis* mycelium the observed efficiency was only 0.002% (Fitzgerald et al., 2003) and it was 0.0026 % for chopped *M. albus* mycelia (Ezra et al., 2010). For *P. fijiensis* using small young hyphal fragments, we achieved around 0.02% transformation efficiency. Previously, the transformation efficiencies of *P. fijiensis* were 0.5-4 (Balint-Kurti et al., 2001) or 2.5-3 (Portal et al., 2012) transformants per µg plasmid DNA in a restriction enzyme-mediated integration approach with 80 generated transformants (Portal et al., 2012). It is difficult to compare the efficiency of ATMT with previous *P. fijiensis* transformations, as frequencies are presented in terms of mycelial pieces or µg of plasmid DNA, respectively. More recently, Escobar et al. (2015) obtained 0.12% transformation efficiency generating random integration mutants using shock waves on *P. fijiensis* conidiospores and by RNAi silenced *Pfhog1* (Onyilo et al., 2017), which adds another tool for functional assays in this fungus. Whereas homologous recombination efficiency is highly variable from <1% to >80% depending on the fungal species, isolate, tissue, target gene, and its position on the chromosome and chromatin structure (Kück and Hoff, 2010; Weyda et al., 2017), we obtained a recombination frequency of 1.96% in *P. fijiensis* by using mycelial

fragments. Both ectopic transformation and homologous recombination efficiencies in *P. fijiensis* are thus within the same range –although at a low level- as reported for other fungi. This promises good opportunities for future research, even though fine tuning is necessary. Experience learned however, that such efficiencies can be significantly improved once more people start working on a system. Initial ATMT transformation efficiency in *Z. tritici* yeast-like spores was 0.00007% (Zwiers and De Waard, 2001) and variable homologous recombination efficiency could be as low as 0.35% (Cousin et al., 2006). However, *Z. tritici* is currently considered as a model with constant efficiencies that are two orders of magnitude higher around 0.001%, (Mirzadi Gohari et al., 2014) and homologous recombination as high as 85% (Bowler et al., 2010). Moreover, the generated *ΔPfku70* strains might be used for optimizing targeted mutagenesis since this is a key component in the NHEJ machinery. Currently CRISPR/Cas9 for targeted mutagenesis is increasingly being applied to filamentous fungi (Liu et al., 2015; Nødvig et al., 2015; Wenderoth et al., 2017; Weyda et al., 2017) and this also relies on homologous recombination. Consequently the *ΔPfku70* strains might be valuable starting material for application in *P. fijiensis*, as in other organisms (Chu et al., 2015).

The *Pfku70* gene was identified in the sequenced strain CIRAD86 (Arango Isaza et al., 2016) and is highly conserved when compared to orthologues from other organisms. The encoded protein shared a high identity with KU70 proteins from other *Dothideomycetes*, indicating a functional conservation and the involvement in NHEJ. A growth reduction was observed in the *Pfku70* strains and its function should be accessed in further studies. This was not due to the transformation process since other generated knock-out strains were not affected in fitness and so these were used as a basis for swapping promoter domains of the *Pfcyp51* gene (Diaz-Trujillo et al., 2018). In *Penicillium chrysogenum,* Hoff (2010) showed that genes related to stress response were upregulated in *ku70* disruptant strains. In several

fungi such as *Botrytis cinerea* (Choquer et al., 2008), *Parastagonospora nodorum* (Feng et al., 2012), *P. decumbes* (Z.-H. Li et al., 2010), *M. ruber* (He et al., 2013) and *Aspergillus parasiticus* (Chang, 2008) growth and morphology was not related to the lack of *ku70*. Slightly retarded growth and upregulated telomere action was detected in *Ku70* knock-out strains of *Candida albicans* (Chico et al., 2011). Bowler et al., (2010) did not detect any defects in *ku70* mutants of *Z. tritici,* whereas recently Wu et al., (2017) related NHEJ to transposon insertions as cause of intron mobility. A higher sensitivity to DNA damage was reported in *Neurospora crassa* (Ninomiya et al., 2004), *Pichia pastoris* (Näätsaari et al., 2012) and *R. toruloides* (Koh et al., 2014) Δ*ku70* strains. In all these cases higher homologous recombination frequencies were observed by using of Δ*ku70* strains for transformation.

In summary, these data provide a starting point for upscaling functional analyses in *P. fijiensis.* This is important for increased understanding of the *P. fijiensis*-banana pathosystem and eventually for contributing to improved disease control and the identification of crucial genes for its biology and those that drive pathogenesis.

Materials and Methods

Strains

The sequenced *P. fijiensis* strains CIRAD86 and CIRAD139a (Arango Isaza et al., 2016) were used as wild-type (wt) recipient strains throughout this study. They were grown on potato dextrose agar (PDA) plates amended with streptomycin 100 $\mu g \cdot mL^{-1}$ for 12-15 days and were maintained in an incubator (Elbanton, Kerkdriel, Netherlands) at 27˚C.

Identification and sequence analysis of *Pseudocercospora fijiensis* genes

The *C. fulvum Avr4* homologue *Pfavr4* gene was previously identified by Stergiopoulos et al. (2010, 2014). The *ku70* orthologues from *N. crassa* (Ninomiya et al., 2004), *R. toruloides* (Koh et al., 2014) and the Dothideomycetes *Z. tritici* (Bowler et al., 2010), *P. nodorum* (Feng et al., 2012), *A. alternata* (Wang et al., 2011) and *S. musiva* (http://genome.jgi.doe.gov/Sepmu1/Sepmu1.home.html) were used as query with the BLASTN search engine at the *P. fijiensis* genome website (http://genome.jgi-psf.org/Mycfi2/Mycfi2.home.html). Protein structure was inferred with the NCBI Conserved domains analysis tool. The *Pfcyp51* was detected in the *P. fijiensis* genome strain and amplified in other strains (Diaz-Trujillo et al., 2018).

Constructs for insertion and gene deletion

Vectors pSC001 or pSC002 were applied for random insertion (Mirzadi Gohari et al., 2015; Zhao et al., 2011). They contain the marker genes *eGFP* or *DsRed*, respectively, under control of the pToxA promoter from *Pyrenophora tritici-repentis* and the terminator Tnos in combination with the hygromycin B phosphotransferase gene (*hph*) driven by the PtrpC promoter from *A. nidulans*. These plasmids were transformed into *A. tumefaciens* strain LBA1100 by electroporation, as described (Zwiers and De Waard (2001).

Deletion constructs were generated with three DNA cassettes targeted for the knockout of a gene that was amplified using specific primers, including the attB1 and attB2 sites for Gateway® cloning (Invitrogen). These fragments were then cloned into the pDONR™221 vector, using the Multisite Gateway ® Three-fragment Vector Construction kit (Invitrogen, California, USA; Cat No. 12537-023, version D). The cassette with *hph* was constructed based on the pSC001 plasmid (Figure 1).

Upstream and downstream fragments for each gene (Figure 1) were identified and amplified using 50 ng genomic DNA from *P. fijiensis* CIRAD86, with 1 µM of corresponding primers, 0.2 mM dNTPs, 1x Pfu DNA polymerase 10X with MgSO4 buffer (Promega, Madison, USA), 0.4 units Pfu DNA Polymerase (Promega), 4.1 units TaqDNA polymerase (Roche, Penzberg, Germany), following this program: 5 minutes at 95 °C, followed by 30 cycles of 35 seconds at 95 °C, annealing temperature for 1 minute, then 1 minute and 50 seconds at 72 °C, followed by a final elongation at 72 °C for 5 minutes. Annealing temperatures were: 72 and 64 °C for upstream and downstream fragments of *Pfavr4* respectively; likewise 72 and 66 °C for upstream and downstream fragments of *Pfku70*.

Amplicons were purified by either QIAquick gel extraction or QIAquick PCR purification kits (Qiagen, Venlo, Netherlands) according to the manufacturer's conditions. Upstream and downstream purified fragments were BP-cloned into vectors plasmid pDONR™ P4-P1R, or pDONR™ P2R-P3, respectively, while the replacing cassette was BP-cloned into pDONR™221, from the Multisite Gateway® Three-fragment Vector Construction kit according to the manufacturer's suggestions and transformed into *E. coli* One Shot(R) TOP10 competent cells (Invitrogen, Carlsbad, USA). Plasmids holding the desired construct were used for the subsequent LR reaction for 15 hrs, according to the

manufacturer's recommendations, using the plasmid pPm43GW (VIB, Ghent, Belgium) as destination vector and transformed into *E coli*.

Two µl of a pPm43GW plasmid with the desired construct were electroporated (BioRad) into 50 µl of *A. tumefaciens* strain AGL1 competent cells at 12.5kV/cm, 25µF, 400 Ohm, 9 msecond in a 1 mm cuvette (CE-001-50, Eurogentec, Seraing, Belgium). Surviving cells were recovered for 4 hours in 500 µL LB mannitol (10 $mg \cdot mL^{-1}$) at 28 ºC, centrifuged at 150 rpm and then plated on LB medium amended with mannitol (10 $\mu g \cdot mL\text{-}^{1}$) (Cat M1902 Sigma, Steinheim, Germany), 25 $\mu g \cdot mL\text{-}^{1}$ rifampicin (Cat R3501 Sigma) and 100 $\mu g \cdot mL\text{-}^{1}$ spectinomycin (Cat S4014 Sigma).

Preparation of fungal hyphae for transformation

A mycelial *P. fijiensis* colony on a PDA plate with a diameter of approximately 1 cm or ~1 month old, was transferred into 1.5 mL of sterile water, amended with 0.05 % Tween20 in a 10 mL plastic tube (Cat 164161, Greiner Bio-One, Alphen aan de Rijn, Netherlands) and strongly smashed with the straight tip of a plastic Lazy-L-shaped spreader, before vortexing for 40 seconds at the highest speed. This suspension with disaggregated mycelial parts was adjusted up to seven ml using the same water/Tween20 mixture. Approximately 0.5 ml of this suspension was transferred onto PDA plates amended with streptomycin (100 $\mu g \cdot mL^{-1}$). After two to three weeks and one day before transformation, hyphae were rescued from these plates using a brush with plastic firm hairs, in the presence of Induction Medium (IM) (Per liter: 2 g K_2HPO_4, 1.45 g KH_2PO_4, 0.6 g $MgSO_4$ $7H_20$, 0.3 g NaCl, 0.01 g $CaCl_2$ $2H_2O$, 0.001 g $FeSO_4$, 0.5 mg $ZnSO_4$ $7H_2O$, 0.5 mg $CuSO_4$ $5H_2O$, 0.5 mg H_3BO_3, 0.5 mg $MnSO_4H_2O$, 0.5 mg Na_2MoO_4 $2H_2O$, 0.5 g NH_4NO_3, 2 g glucose, 0.2 mL 50 % glycerol and 80 µl IM MES pH 5.3). Hyphal fragments were then filtered through two layers of sterile gauze swabs (Cat. 708505, Noba, Wetter, Germany) and adjusted to 1.10^{6} $fragments \cdot mL^{-1}$. These were washed

thrice with IM and taken up in 10 ml IM in a 50 ml tube (Cat 210261, Greiner Bio-One), before an overnight incubation in a rotary shaker (Innova 4430, New Brunswick Scientific, The Netherlands) adjusted at 27°C and 150 rpm.

Preparation of *Agrobacterium* cells and *Pseudocercospora fijiensis* transformation

Five days before transformation of *P. fijiensis*, *A. tumefaciens* LBA1100 cells with the constructs for random mutagenesis were plated in LB medium amended with mannitol (10 $mg{\cdot}mL^{-1}$), 10 µg·mL-[1] gentamycin and 250 $\mu g{\cdot}mL^{-1}$ spectinomycin. After growing for two days, single colonies were inoculated into 20 mL LB liquid medium with same antibiotics and incubated overnight in an orbital shaker adjusted at 27 °C and 150 rpm. Cells were resuspended into 20 ml of minimal medium (MM) (same as IM, but without glycerol and MES), and 100 µl of this suspension were transferred into 20 ml of fresh MM with 10 µg·mL-[1] gentamycin and 250 $\mu g{\cdot}mL^{-1}$ spectinomycin and acetosyringone (0.1% of 200 mM) in a 50 ml tube. After approximately 24 hrs, the OD_{600} was adjusted to 0.15 with freshly prepared IM (with 10 $\mu g{\cdot}mL^{-1}$ gentamycin and 250 $\mu g{\cdot}mL^{-1}$ spectinomycin), and incubated for three hrs until an OD_{600} of 0.25. Subsequently, hyphal fragments ($1.10^{6}{\cdot}mL^{-1}$) and *A. tumefaciens* were mixed in a 1:1 ratio. Aliquots of 1.5 mL were spread on a 100 cm^2 nylon membrane (Amersham Hybond™-N, GE Healthcare, Buckinghamshire, UK) layer on IM with 1.5 % agar amended with 10 µg·mL-[1] gentamycin and 250 $\mu g{\cdot}mL^{-1}$ spectinomycin and acetosyringone (0.1% of 200 mM) (Cat 134406, Sigma-Aldrich) and incubated in the dark for 48 hrs at 22 °C. Membranes were then transferred onto PDA amended with cefotaxime (200 mM) (Cat C7039, Sigma) and hygromycin B (50 $mg{\cdot}mL^{-1}$) (Cat H0654, Sigma).

For transformation of *P. fijiensis* with the replacement cassette cloned into *A. tumefaciens* strain AGL1 essentially the same protocol was followed with some modifications. A single *A. tumefaciens* colony was transferred to a fresh LB agar plate

containing rifampicin (100 μg·mL^{-1}), spectinomycin (100 μg·mL^{-1}) and mannitol (10 mg·mL^{-1}) and allowed to grow for 36-40 hrs in 20 ml LB with the same antibiotics in an orbital shaker at 27 ºC and 150 rpm. AGL1 cells were resuspended into 20 ml of MM. Immediately 120 μl from this suspension were then transferred in 20 ml fresh MM with antibiotics and incubated again at 27 ºC and 150 rpm in an orbital shaker for approximately 40 hrs. Hyphae were rescued from 12 to 15-day-old fungal culture plates in the presence of IM amended with 0.1% of 200 mM acetosyringone. After filtering hyphae through two layers of gauze swabs (Noba), concentrations were adjusted to 10^6 fragments·mL^{-1} and washed with IM once by centrifugation at 2,000 rpm for 12 minutes, and incubated overnight in a rotatory shaker at 27 ºC and 150 rpm before transformation. During co-cultivation of hyphae with AGL1 cells in a 1:1 ratio on a 100 cm^2 nylon membrane (Amersham Hybond^{TM}-N) layer on IM with 1.5 % agar amended with rifampicin (100 μg·mL^{-1}), spectinomycin (100 μg·mL^{-1}) and acetosyringone (0.1% of 200 mM) for 48 hrs, the temperature was maintained at 27 ºC, and selection medium was amended with cefotaxime (200 mM) and hygromycin B (50 μg·mL^{-1}).

Screening of fluorescent transformants

After 15 days incubation, developing colonies on the membranes were monitored for fluorescence under a fluorescence stereoscope microscope (Leica, Wetzlar, Germany). GFP or DsRed fluorescence was detected with 488 or 563 nm excitation, and 509 or 581 nm emission settings, respectively. Images were captured with an Axiocam MRc5 camera using Axioplan 2.0 software (Zeiss, Göttingen, Germany).

Confirmation of gene replacement

Fluorescent mycelial colonies were rescued with a sterile tooth pick or pipet tip and transferred to a fresh PDA plate amended with 300 μg·mL^{-1} cefatoxime and 50 μg·mL^{-1} hygromycin. After approximately three weeks, mycelium was collected and lyophilized for

DNA isolation using the Sbeadex (R) Maxi Plant kit (Agowa, Germany) in a KingFisher Robot (Thermo Scientific, Hudson, USA). DNA of transformants was used to amplify the replaced *Pfavr4* or *Pfku70* with the primers Avr4_F1 + Avr4_R1, or KU70_F1 + KU70_R1, respectively (Table 3). The amplification mixture comprised 20 ng genomic DNA, with 0.75 μM of corresponding primers, 60 μM dNTPs (Roche), 1x DNA polymerase with $MgSO_4$ buffer and 0.8 units Taq DNA polymerase (Roche). *Pfavr4* was amplified in a thermocycler (PTC200 Peltier, BioRad, Watertown, USA) programmed at 5 min at 95 °C, 30 cycles of 30 sec. at 94 °C, 30 sec. at 58 °C and 60 sec. at 72 °C, followed by a final elongation for 5 min. at 72 °C. The amplification program for *Pfku70* was 5 min. at 95 °C followed by 30 cycles of 30 sec. at 95 °C, 45 sec. at 58 °C and 60 sec. at 72 °C and a final elongation for 2 min. at 72 °C. The expected amplicons sizes were 364 bp (*Pfavr4*) and 1,261 bp (*Pfku70*), respectively. Transformants that lacked a PCR product, but whose DNA amplified with the control variable number tandem repeat VNTR1333 marker according to García et al. (2010), were subjected to an additional PCR with a primer located in the replacing construct (Avr4_HR_F or KU70_HR_F, respectively) and one primer in the UTR region, based on the *P. fijiensis* CIRAD86 genome sequence (Avr4_HR_R or KU70_HR_R, respectively) (Table 3, Figure 1), using an amplification program of 5 min. at 95 °C followed by 30 cycles of 30 sec. at 95 °C, 60 sec. at 62 °C, 180 sec. at 72 °C and a final elongation for eight min. at 72 °C. The expected amplicon sizes for knock-out genes were 2,849 bp for *ΔPfavr4* mutants or of 2,953 bp for *ΔPfku70* mutants.

Transformation efficiency and homologous recombination

Efficiencies of random and directed mutagenesis were calculated as the percentage of transformed colonies among the number of hyphal pieces used for transformation. The

percentage of transformants with proven homologous recombination by PCR, among the number of transformed colonies, was defined as the frequency of homologous recombination.

Phenotypic characterization of knock-out *Pseudocercospora fijiensis* strains

In vitro growth of Ku70 mutants. The fitness of *Pfku70* knockout strains was analyzed by generating hyphal fragments from one-month-old colonies of *ΔPfku70#33* and *ΔPfku70#45* and the *P. fijiensis* CIRAD86 wt. For each sample 50 µl of $3.5 \cdot 10^5$ mycelial fragments·mL^{-1} were inoculated in 200 µl potato dextrose broth medium in eight wells of a 96-well, flat bottom, transparent, polystyrol plate (Cat 3370, Corning, USA) for a final concentration of 7.10^4 mycelial fragments·mL^{-1} in a total of 250 µl medium. Each sample used a column of eight wells and a column with only medium was left adjacent to it. Plates were sealed and maintained in an incubator (Elbanton, Kerkdriel, Netherlands) at 27 ºC in darkness. Mycelial growth was monitored at 0, 4, 6, 8, 10, and 12 days post inoculation (dpi) in two biological and two technical replicates, using an Infinite® M200 PRO machine (TEKAN, Männedorf, Switzerland). The reads from the control wells with only growth medium were subtracted from those with fungal inoculum and fungal growth was measured by increased absorbance values that were averaged, plotted against dpis and compared with the wt strain. Pictures of wells were recorded with an Olympus Camedia C-8080 (Olympus, Hamburg, Germany).

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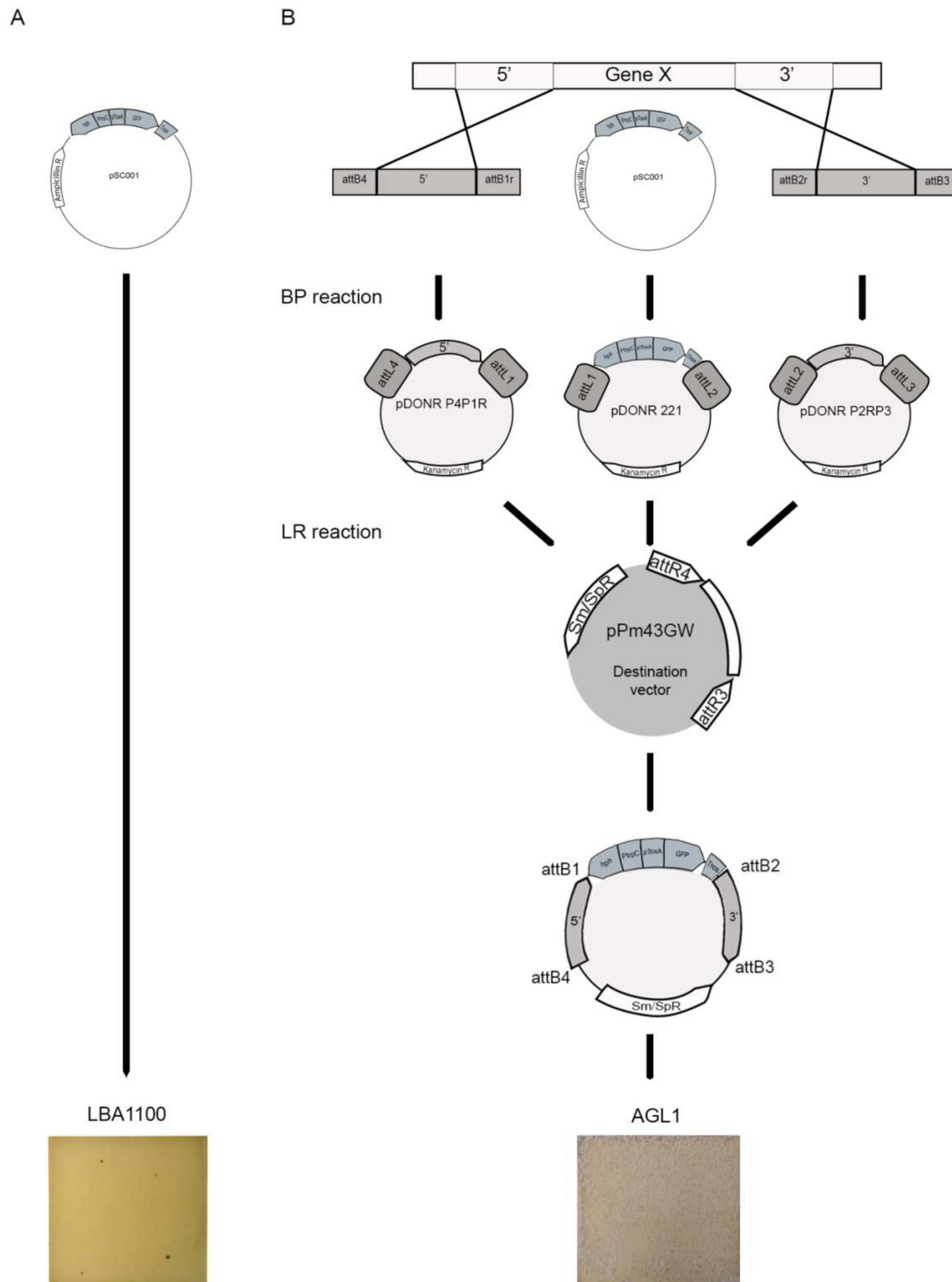

Figure 1. Cloning strategy for *Agrobacterium*-mediated mutagenesis of *Pseudocercospora fijiensis* and examples of membranes with the number of obtained transformant colonies. A) Strategy for cloning the construct with *eGFP/DsRed* and *hph* coding genes into the LBA1100 *Agrobacterium tumefaciens* strain for random mutagenesis. B) Gateway-based strategy for cloning the construct carrying an *eGFP* and *hph* coding genes into the AGL1 *A. tumefaciens* strain to knock out genes *Pfavr4* and *Pfku70*.

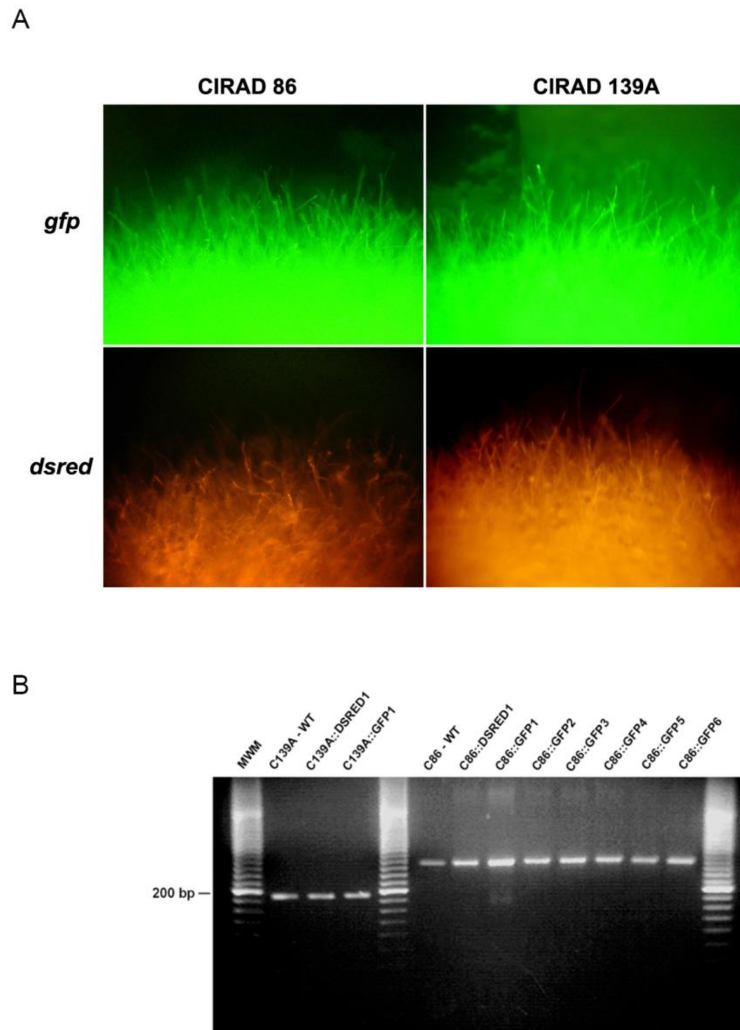

Figure 2 Transformants of *Pseudocercospora fijiensis* isolates CIRAD86 and CIRAD139A. A) Both *P. fijiensis* CIRAD86 and CIRAD139A expressing either GFP or DsRed. B) Genotyping transformants using the VNTR1333 marker. CIRAD139A contains the VNTR200 bp allele whereas CIRAD86 contains the 280 bp allele.

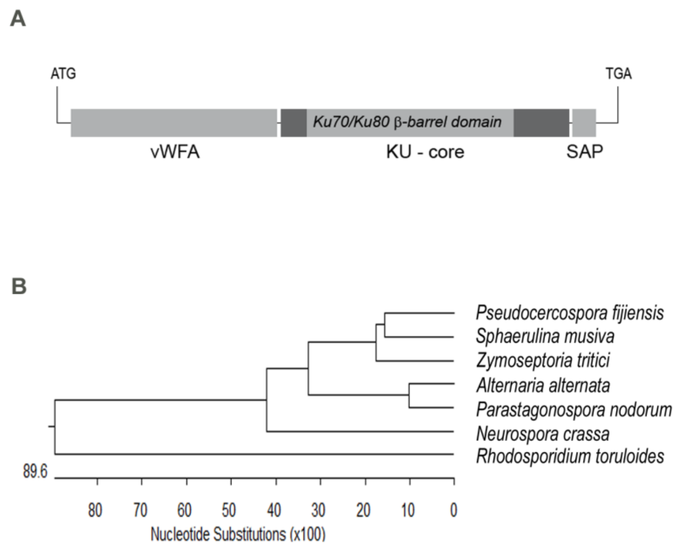

Figure 3. **The *Pseudocercospora fijiensis* KU70 (PfKU70) protein.** A) PfKU70 structure. The fused vWA domain and N terminus span from 36 to 266 aa. The KU-core domain spans 286 to 568 aa and covers the Ku70/Ku80 β-barrel domain from 293 to 491 aa. The SAP domain is located from 631 till 665 aa. B) Phylogenetic tree of Ku70 proteins of the Dothideomycetes *Sphaerulina musiva, Zymoseptoria tritici, Alternaria alternata, Parastagonospora nodorum*, *Neurospora crassa* and the basidiomycete *Rhodosporidium toruloides*.

Figure 4. Clustal alignment of KU70 protein from *Pseudocercospora fijiensis, Rhodosporium toruloides, Neurospora crassa, Alternaria alternata, Parastagonospora nodorum, Zymoseptoria tritici* and *Sphaerulina musiva.* Conserved domains are labelled with arrows and their respective names.

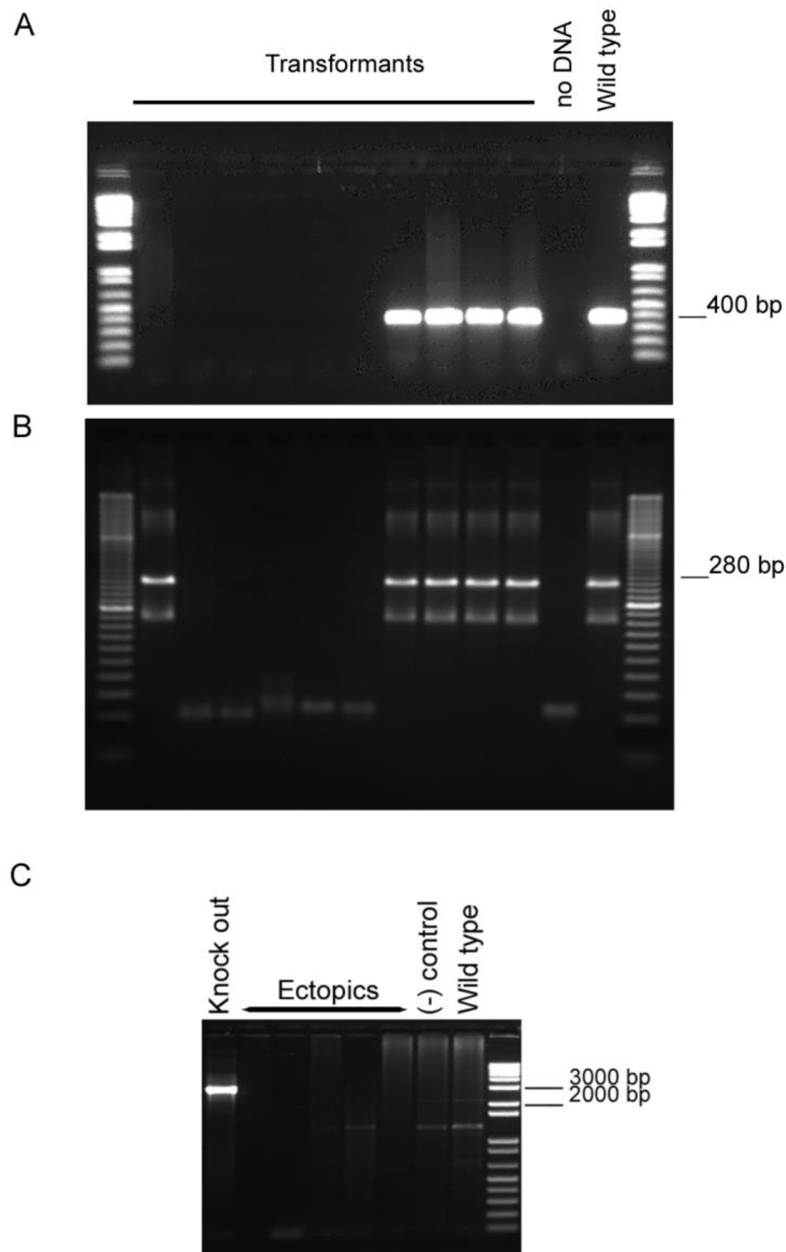

Figure 5. Disruption of *Pfavr4* in *Pseudocercospora fijiensis* CIRAD86. A) Example of transformants with or without amplification of the *Pfavr4* region of 364 bp, which is not amplified in knock-out strains. B) Only one knock-out candidate amplified the same VNTR1333 allele as the wt CIRAD86 strain, and produced the 2,849 bp amplicons representing the single homologous recombinant *PfAvr4* strain (C).

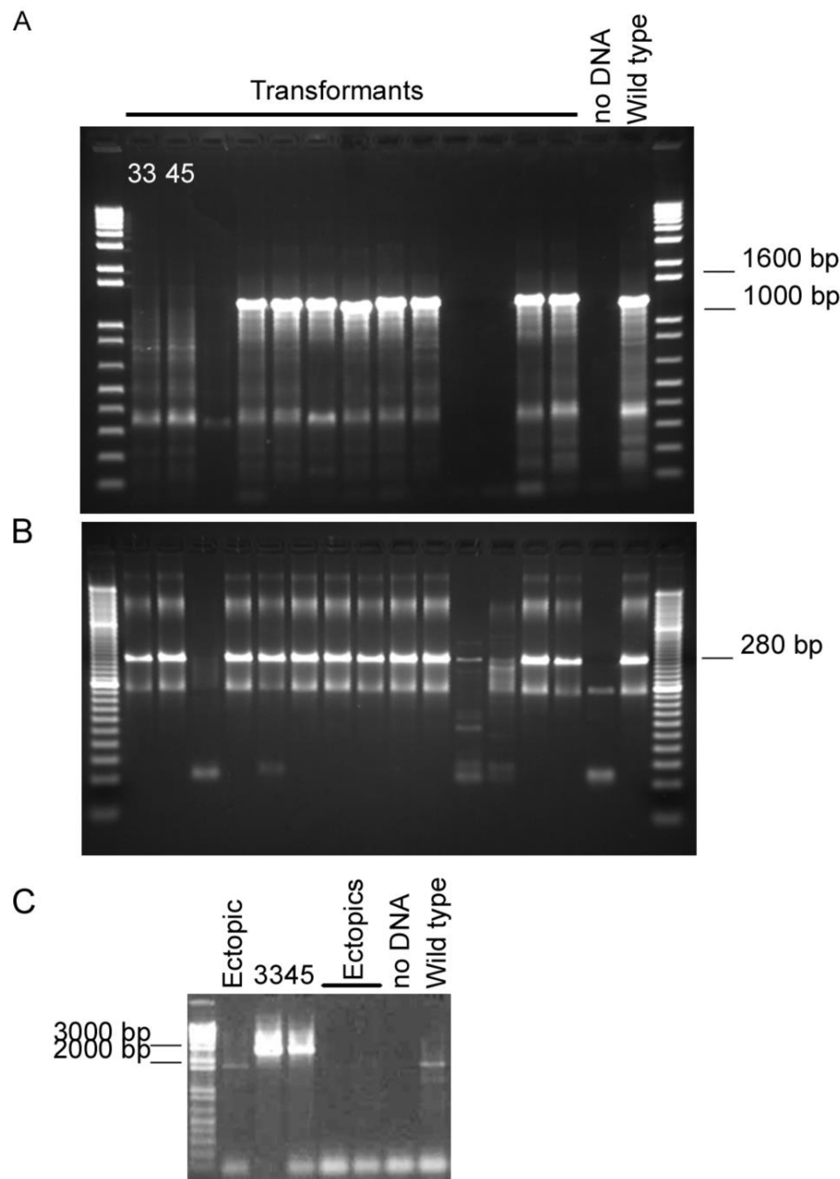

Figure 6. Disruption of *Pfku70* in *Pseudocercospora fijiensis* CIRAD86. A) Selection of transformants by selective PCR resulting in amplification of the *Pfku70* region of 1,261 bp in ectopic transformants. No amplicon was produced in potential transformants. B) Transformants 33 and 45 also amplified the same VNTR1333 allele as the wt, representing the only obtained knock-out strains. C) Candidate transformants 33 and 45 were confirmed as knock out since PCR analyses resulted in the expected and diagnostic 2,853 bp amplicon.

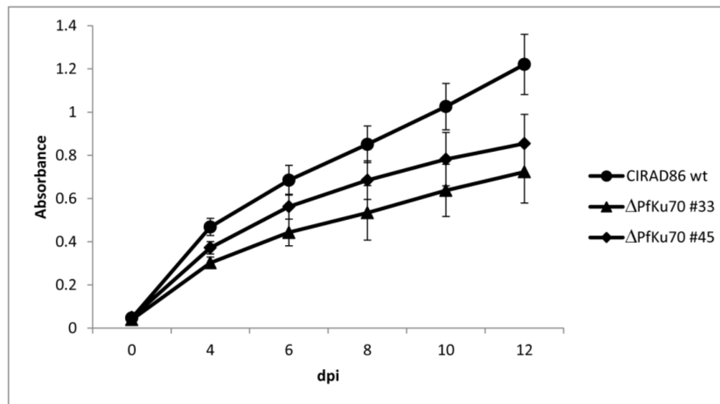

Figure 7. **Comparison of mycelial growth of the *Pseudocercospora fijiensis* wt CIRAD86 strain and the knock-out strains *ΔPfku70 #33* and *ΔPfku70 #45*.** Absorbance (690nm) reads of cultures in PDB medium were measured over time, averages of five reads over 21 positions in each of two biological and technical replicates per strain.

Table 1. Transformation efficiency of *Pseudocercospora fijiensis* for random and targeted integration using *Pfavr4, Pfku70* and the *Pfcyp51*promoter.

Gene / sequence	Mutagenesis	Marker gene	Strain	Transformants*	Transformation efficiency (%)	Hom. Rec.	Hom. Rec. efficiency (%)	Reference
eGFP	Random	*hph*	CIRAD86	20	0,0006	n.a.	n.a.	This study
eGFP	Random	*hph*	CIRAD139a	6	0,0002	n.a.	n.a.	This study
DsRed express	Random	*hph*	CIRAD86	34	0,0011	n.a.	n.a.	This study
DsRed express	Random	*hph*	CIRAD139a	2	0,00006	n.a.	n.a.	This study
Pfavr4	Targeted	*hph*	CIRAD86	90	0,022	1	1,11	This study
Pfku70	Targeted	*hph*	CIRAD86	102	0,025	2	1,96	This study
Pfcyp51 promoter	Targeted	*hph*	E22	250	0,025	2	0,80	Diaz-Trujillo *et al.*, 2018

* Random transformants number correspond to the average from two experiments
n.a. : not applicable

Table 2. Comparison of protein identity of PfKU70 with other fungal Ku70 proteins.

Specie	Class	Accession	ID (%)	Score	Reference
Neurospora crassa	Sordariomycete	BAP16622.1	49.9	2.3 E-144	Ninomiya et al., 2004
Rhodosporidium toruloides	Mycrobotryomycete	AIA21643.1	39.6	3.84 E042	Koh et al., 2014
Zymoseptoria tritici	Dothideomycete	EGP88672.1	69.4	0	Bowler et al., 2010
Alternaria alternata	Dothideomycete	ADQ73897	57.2	0	Wang et al., 2011
Parastagonospora nodorum	Dothideomycete	EAT79812.2	56.6	0	Feng et al., 2012
Sphaerulina musiva	Dothideomycete	EMF13337	74.2	0	Unpubished

Table 3. Primers used in this study.

Primer name	Sequence	Reference
Avr4_F1	CATCATGCTTTCAACTACGAG	This work
Avr4_R1	GTTGTCGCATCCTGTTTC	This work
Avr4_5´_fwd	GGGGACAACTTTGTATAGAAAAGTTGGCCTTTTGACAGTATTCTCGTCAGCC	This work
Avr4_5´_rev	GGGGACTGCTTTTTTGTACAAACTTGCCCCTAGCGTTGCCGAATACGA	This work
Avr4_3´_fwd	GGGGACAGCTTTCTTGTACAAAGTGGCCAGCGGACGCTTCTTATAC	This work
Avr4_3´_rev	GGGGACAACTTTGTATAATAAAGTTTACCGAAGGCGTAGGTTGTC	This work
Avr4-HR-F	CAGGGGGTGAAGAATGTGAG	This work
Avr4-HR-R	ACTGTCGGGCGTACACAAAT	This work
KU70_F1	GAGCAAATTCCAGGAGCAAG	This work
KU70_R1	GTCAAACAGACCTCGGCAAT	This work
KU70_5´_fwd	GGGGACAACTTTGTATAGAAAAGTTGTGCATGGAGTGGAGTCAG	This work
KU70_5´_rev	GGGGACTGCTTTTTTGTACAAACTTGTCGTTTATGGAGGCGTAGT	This work
KU70_3´_fwd	GGGGACAGCTTTCTTGTACAAAGTGGTCTCGTCGAACTGGTCAC	This work
KU70_3´_rev	GGGGACAACTTTGTATAATAAAGTTGTCCAGGCTGATCGTTATA	This work
KU70-HR-F	TCGTTCAAACATTTGGCAATAA	This work
KU70-HR-R	AAATGCAGAGGCGTGGTATC	This work
VNTR1333-F	GAGTGAAGTACTGCGGAGGC	García et al., 2010
VNTR1333-R	AGTTGGAGAAAGGCGAAAGG	García et al., 2010
M13-F (-21)	TGTAAAACGACGGCCAGT	Common known
M13-R	CAGGAAACAGCTATGACC	Common known

CHAPTER 4

A NEW MECHANISM FOR REDUCED SENSITIVITY TO DEMETHYLATION-INHIBITOR FUNGICIDES IN THE FUNGAL BANANA BLACK SIGATOKA PATHOGEN *PSEUDOCERCOSPORA FIJIENSIS*

Diaz-Trujillo, C., Chong, P., Stergiopoulos, I., Cordovez, V., Guzman, M., De Wit, P.J.G.M., Meijer, H.J.G., Scalliet, G., Sierotzki, H., Lilia Peralta, E., Arango Isaza, R.E., Kema, G.H.J., 2018. A new mechanism for reduced sensitivity to demethylation-inhibitor fungicides in the fungal banana black Sigatoka pathogen *Pseudocercospora fijiensis*. *Mol. Plant Pathol*. https://doi.org/10.1111/mpp.12637

Abstract

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.

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

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*.

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 (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**

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* 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 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 controls (0 $mg \cdot L^{-1}$). Wt strain Ca10_13 grew up to 2.56 $mg \cdot L^{-1}$ of difenoconazole or epoxiconazole, and 10.24 $mg \cdot L^{-1}$ 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 $mg \cdot L^{-1}$ of difenoconazole and 0.04 $mg \cdot L^{-1}$ 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, 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 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* (Snelders et al., 2012; Verweij et al., 2007), 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 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 with 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 (chapter 3), 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 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 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.

Material and methods

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 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 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**

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 *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 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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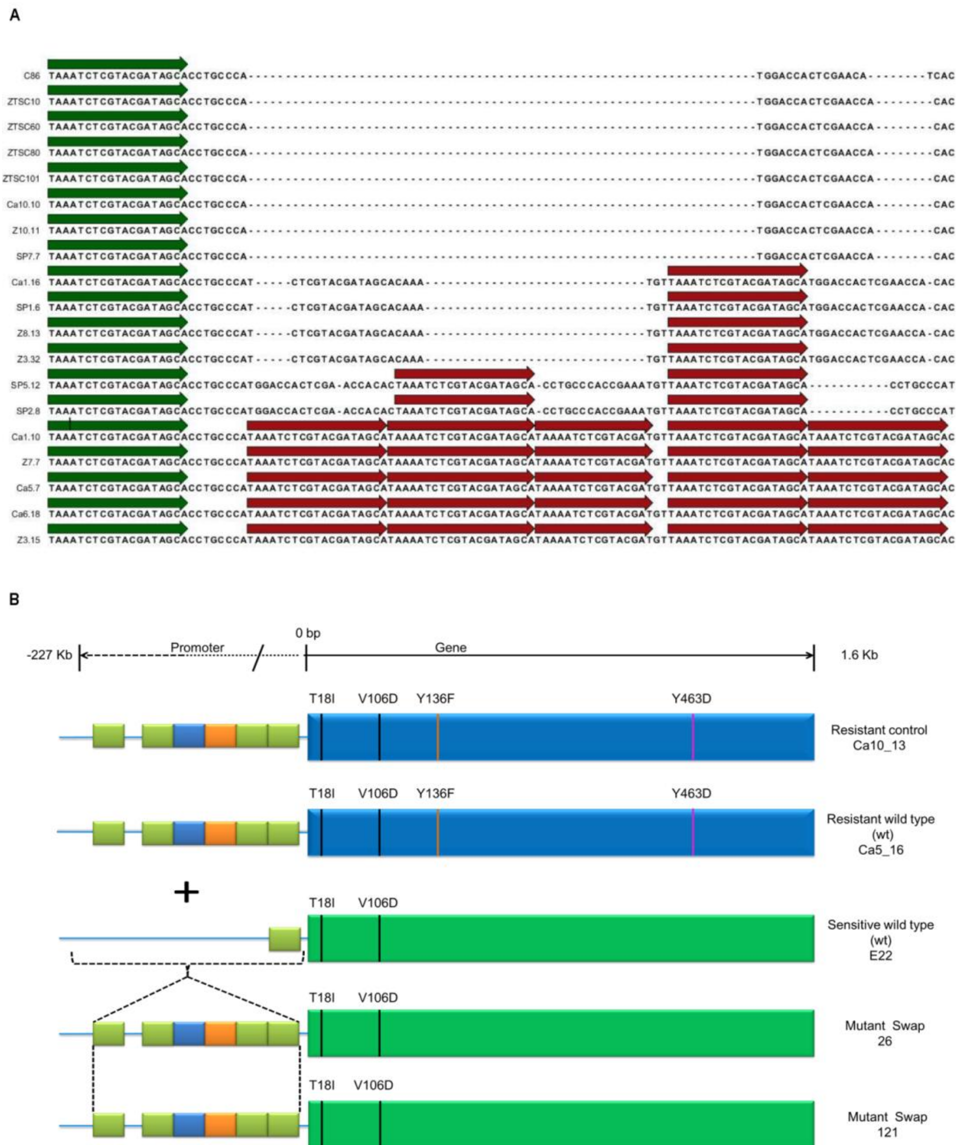


Figure 1. The *Pfcyp51* structure. A) Alignment of the promoter regions of the *Pfcyp51* gene of *Pseudocercospora fijiensis* isolates collected from the Zent (Z), Cartagena (Ca), San Pablo (SP) and the wt San Carlos (ZTSC) banana plantations in Costa Rica, isolate CIRAD86 (C86) is the reference wt isolate, 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.

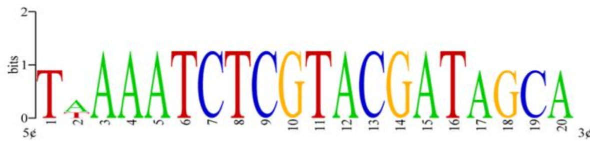


Figure 2. **Sequence logo of the Pfcyp51 promoter repeat element**. Sequences of all repeat elements were aligned and used to generate the consensus sequence. The logo displays the frequency of the nucleotides within the repeated elements of 16, 19 or 20 bp that were observed in the promoter of *Pfcyp51*

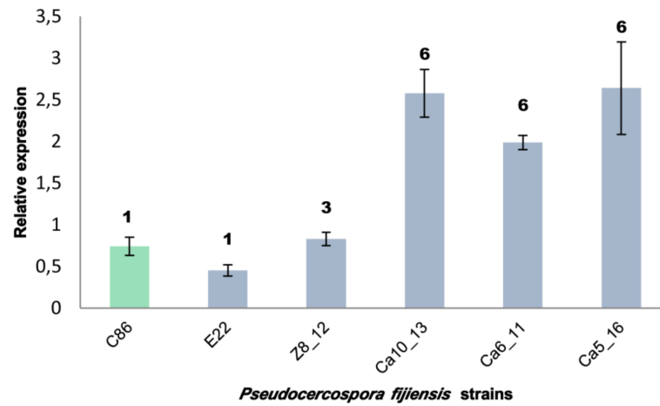


Figure 3. Relative expression of *Pfcyp51* (normalized to the *Pseudocercospora fijiensis* actin gene) in six *P. fijiensis* isolates carrying different numbers of promoter inserts (indicated on the top of each bar). Reference isolate CIRAD86 (C86) is shown in green. Data represent the averages of three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

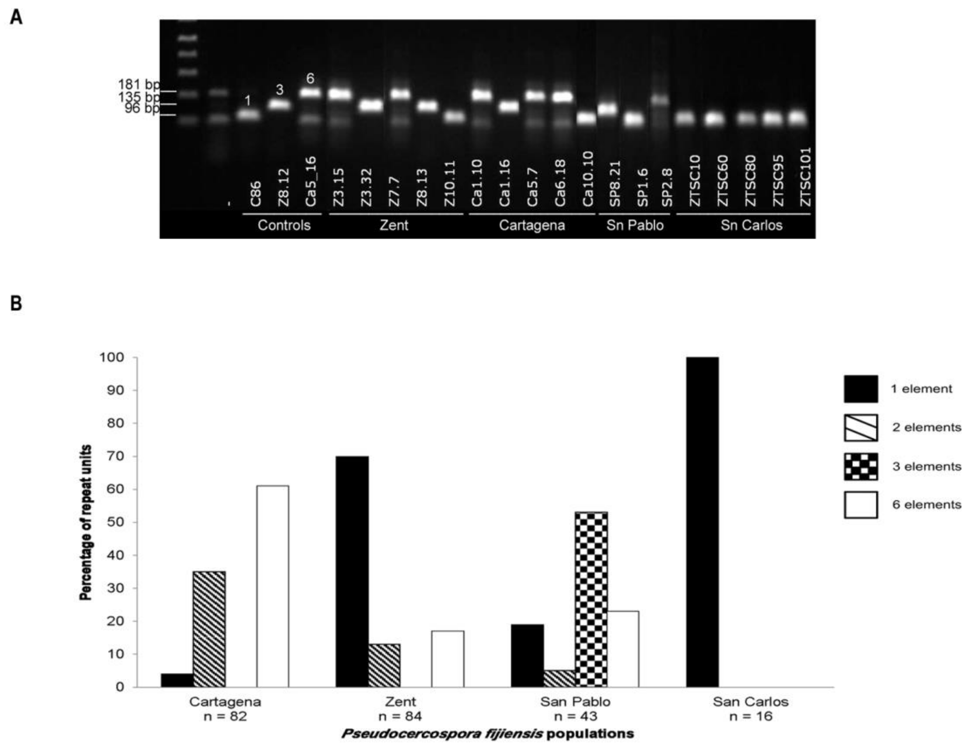


Figure 4. Quantification of the number of *Pfcyp51* promoter repeats in *Pseudocercospora fijiensis* isolates from four banana plantations in Costa Rica. (A) Example of polymerase chain reaction (PCR) amplification of the *Pfcyp51* promoter in isolates from different populations. Isolate CIRAD86 (C86) was used as a control for the presence of one repeat element, Z8.12 as a control with three repeat elements 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.

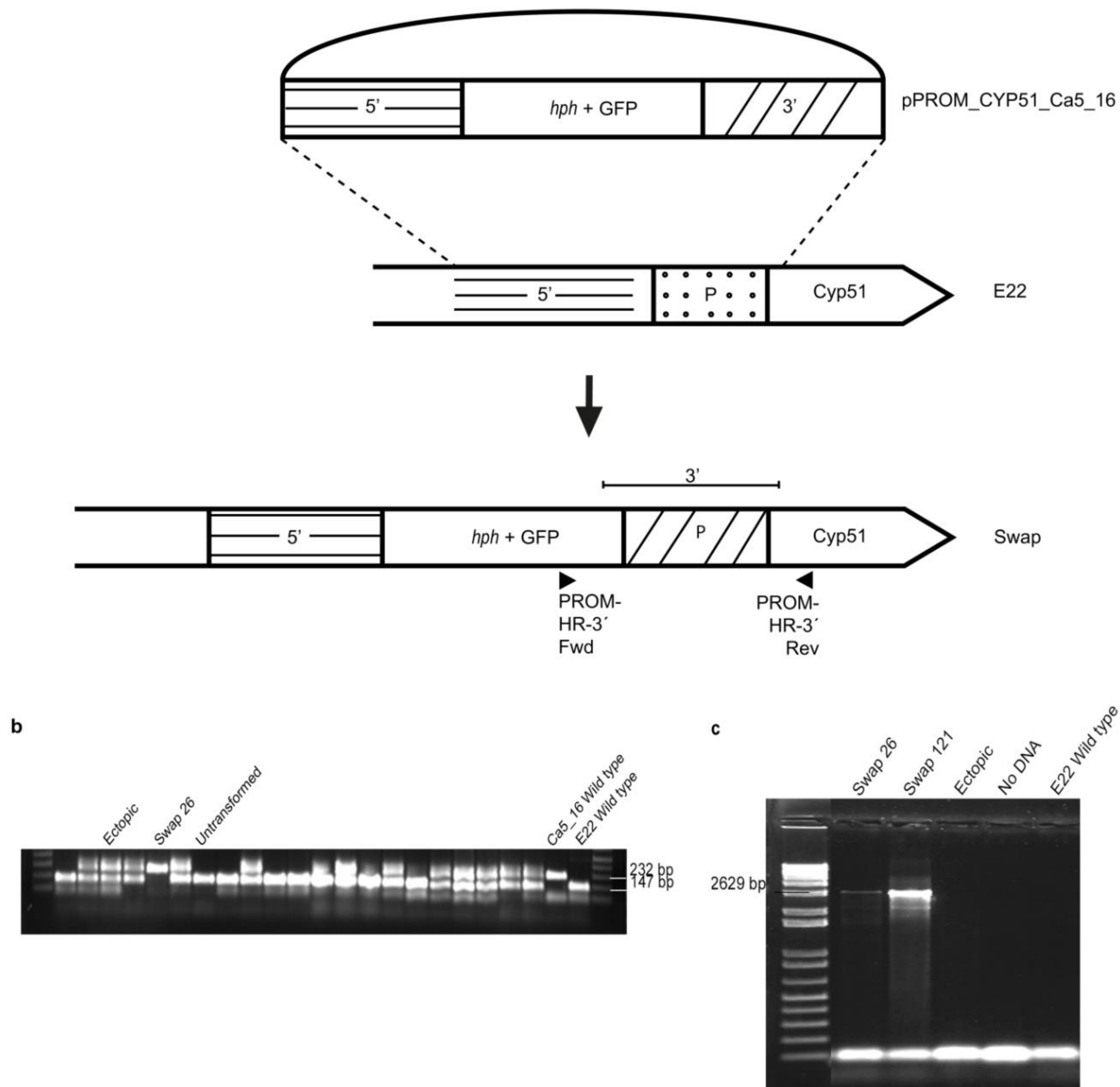
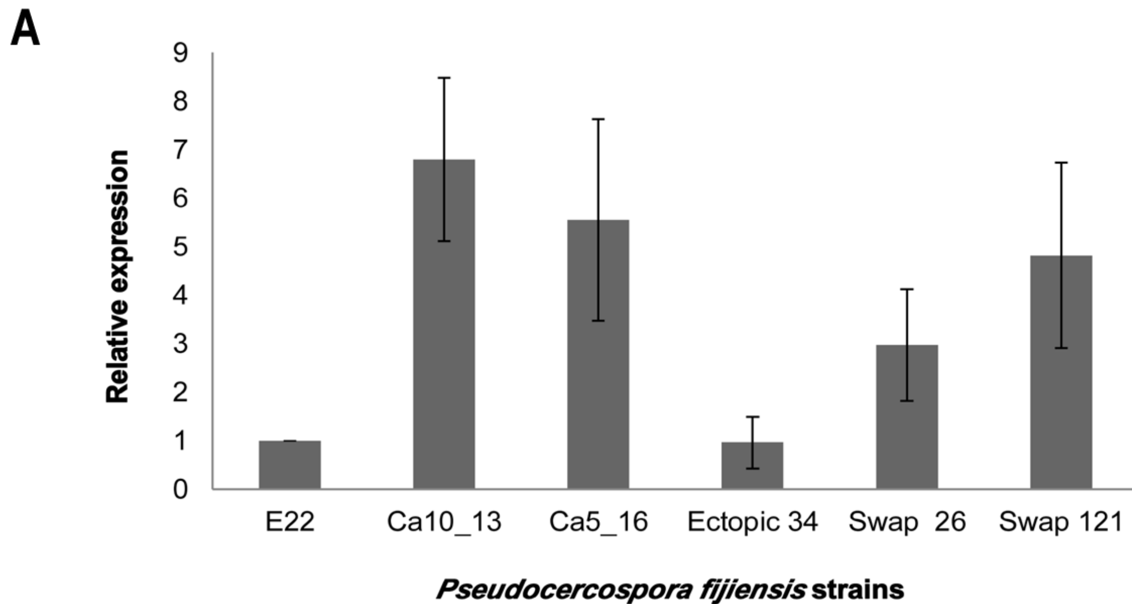


Figure 5. Transformation design to swap Pfcyp51 promoters of *Pseudocercospora fijiensis* isolates. (A) Isolate Ca5_16 is the *Pfcyp51* promoter donor with six repeat elements (slashed area part with the cross lines). The 3´and 5´recombination fragments (crossed out area part with the horizontal lines) were amplified with CYP-Prom primers and ligated to a cassette with the hph and green fluorescent protein (GFP) markers into construct pPROM_CYP51_Ca5_16. The *P. fijiensis* E22 sensitive isolate with one 19-bp promoter element (dotted area) was transformed with this construct. (B) The promoter lengths of positive GFP-tagged transformants were amplified and compared with the donor and wild-type (wt) recipient isolate. Transformant Swap 26 is shown as an example of a promoter replacement transformant, with a similar amplicon to the donor isolate. Ectopic transformats 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 promoter swapped transformants were performed by amplification of the 2629-bp cassette between the homologous recombination sites and the *Pfcyp51* coding region using primers PROM-HR-3´on GFP fluorescent transformants with a promoter amplicon similar to the donor isolate.

A



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

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 EC_{50} values ($mg \cdot L^{-1}$) of the *Pseudocercospora fijiensis* promotor swapped transformants Swap26 and Swap121 and various control isolates to three azole fungicides.

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

Supplementary data

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

>C86
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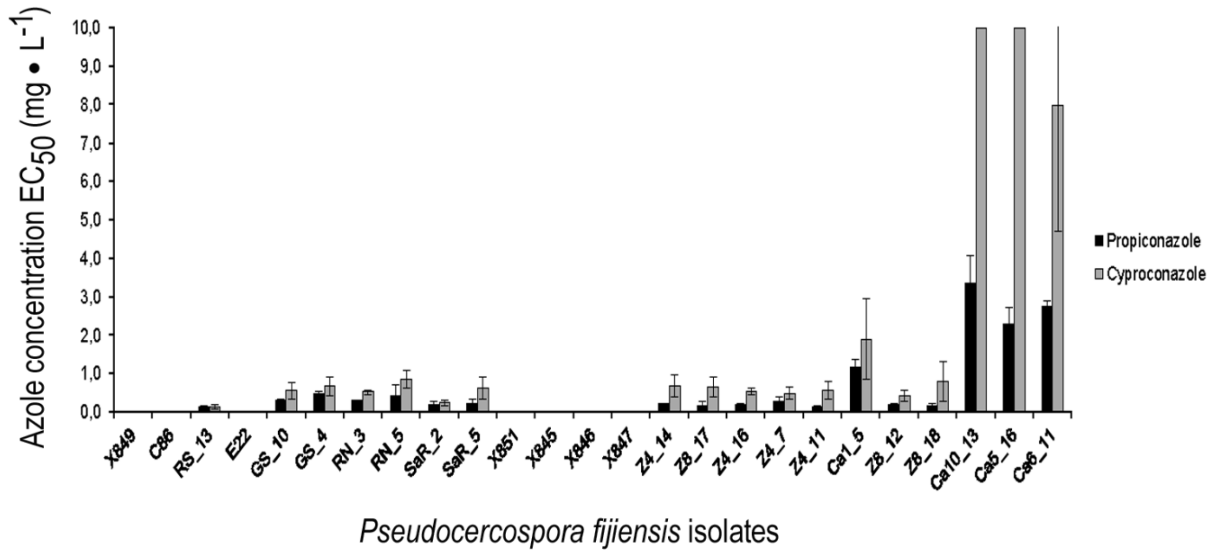

Figure S1. Cross-resistance between propiconazole and cyproconazole. The EC_{50} values were determined for both compounds on *Pseudocercospora fijiensis* colonies for the indicates strains at 10 days post inoculation (results are means of three independent experiments).

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

No.	Isolate	Origin	Plantation/Location	Repeat units
1	Ca1.1	Costa Rica	Cartagena	2
2	Ca1.5	Costa Rica	Cartagena	2
3	Ca1.7	Costa Rica	Cartagena	6
4	Ca1.10	Costa Rica	Cartagena	6
5	Ca1.16	Costa Rica	Cartagena	2
6	Ca1.20	Costa Rica	Cartagena	2
7	Ca1.24	Costa Rica	Cartagena	6
8	Ca2.1	Costa Rica	Cartagena	5
9	Ca2.5	Costa Rica	Cartagena	1
10	Ca2.11	Costa Rica	Cartagena	1
11	Ca2.13	Costa Rica	Cartagena	2
12	Ca2.15	Costa Rica	Cartagena	2
13	Ca2.16	Costa Rica	Cartagena	2
14	Ca2.17	Costa Rica	Cartagena	6
15	Ca2.19	Costa Rica	Cartagena	6
16	Ca3.1	Costa Rica	Cartagena	2
17	Ca3.3	Costa Rica	Cartagena	6
18	Ca3.5	Costa Rica	Cartagena	6
19	Ca3.7	Costa Rica	Cartagena	6
20	Ca3.10	Costa Rica	Cartagena	2
21	Ca3.14	Costa Rica	Cartagena	6
22	Ca3.20	Costa Rica	Cartagena	2
23	Ca3.22	Costa Rica	Cartagena	6
24	Ca3.24	Costa Rica	Cartagena	2
25	Ca5.1	Costa Rica	Cartagena	6
26	Ca5.5	Costa Rica	Cartagena	2
27	Ca5.7	Costa Rica	Cartagena	6
28	Ca5.10	Costa Rica	Cartagena	2
29	Ca5.12	Costa Rica	Cartagena	6
30	Ca5.13	Costa Rica	Cartagena	6
31	Ca5.15	Costa Rica	Cartagena	6
32	Ca5.16	Costa Rica	Cartagena	6
33	Ca5.17	Costa Rica	Cartagena	6
34	Ca5.19	Costa Rica	Cartagena	6
35	Ca6.1	Costa Rica	Cartagena	6
36	Ca6.3	Costa Rica	Cartagena	6
37	Ca6.5	Costa Rica	Cartagena	6
38	Ca6.7	Costa Rica	Cartagena	6
39	Ca6.9	Costa Rica	Cartagena	6
40	Ca6.11	Costa Rica	Cartagena	6
41	Ca6.12	Costa Rica	Cartagena	6
42	Ca6.15	Costa Rica	Cartagena	6

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

No.	Isolate	Origin	Plantation/Location	Repeat units
43	Ca6.18	Costa Rica	Cartagena	6
44	Ca7.1	Costa Rica	Cartagena	2
45	Ca7.5	Costa Rica	Cartagena	6
46	Ca7.9	Costa Rica	Cartagena	2
47	Ca7.15	Costa Rica	Cartagena	6
48	Ca7.18	Costa Rica	Cartagena	6
49	Ca7.20	Costa Rica	Cartagena	2
50	Ca7.23	Costa Rica	Cartagena	2
51	Ca7.28	Costa Rica	Cartagena	2
52	Ca8.2	Costa Rica	Cartagena	6
53	Ca8.4	Costa Rica	Cartagena	2
54	Ca8.8	Costa Rica	Cartagena	6
55	Ca8.11	Costa Rica	Cartagena	6
56	Ca8.13	Costa Rica	Cartagena	6
57	Ca8.16	Costa Rica	Cartagena	6
58	Ca8.20	Costa Rica	Cartagena	6
59	Ca8.23	Costa Rica	Cartagena	6
60	Ca8.26	Costa Rica	Cartagena	6
61	Ca8.28	Costa Rica	Cartagena	2
62	Ca8.29	Costa Rica	Cartagena	6
63	Ca9.1	Costa Rica	Cartagena	6
64	Ca9.3	Costa Rica	Cartagena	2
65	Ca9.5	Costa Rica	Cartagena	2
66	Ca9.8	Costa Rica	Cartagena	2
67	Ca9.10	Costa Rica	Cartagena	2
68	Ca9.12	Costa Rica	Cartagena	2
69	Ca9.14	Costa Rica	Cartagena	2
70	Ca9.17	Costa Rica	Cartagena	2
71	Ca9.19	Costa Rica	Cartagena	6
72	Ca9.22	Costa Rica	Cartagena	2
73	Ca10.3	Costa Rica	Cartagena	6
74	Ca10.5	Costa Rica	Cartagena	6
75	Ca10.7	Costa Rica	Cartagena	6
76	Ca10.10	Costa Rica	Cartagena	1
77	Ca10.13	Costa Rica	Cartagena	6
78	Ca10.23	Costa Rica	Cartagena	6
79	Ca10.25	Costa Rica	Cartagena	6
80	Ca10.27	Costa Rica	Cartagena	6
81	Ca5.18	Costa Rica	Cartagena	2
82	Ca2.3	Costa Rica	Cartagena	6
83	SP1.1	Costa Rica	San Pablo	3
84	SP1.3	Costa Rica	San Pablo	1

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

No.	Isolate	Origin	Plantation/Location	Repeat units
85	SP1.4	Costa Rica	San Pablo	3
86	SP1.5	Costa Rica	San Pablo	3
87	SP1.6	Costa Rica	San Pablo	2
88	SP1.7	Costa Rica	San Pablo	1
89	SP1.8	Costa Rica	San Pablo	3
90	SP2.32	Costa Rica	San Pablo	6
91	SP3.4	Costa Rica	San Pablo	3
92	SP3.5	Costa Rica	San Pablo	3
93	SP3.8	Costa Rica	San Pablo	2
94	SP5.2	Costa Rica	San Pablo	6
95	SP5.4	Costa Rica	San Pablo	6
96	SP5.6	Costa Rica	San Pablo	6
97	SP5.7	Costa Rica	San Pablo	3
98	SP5.10	Costa Rica	San Pablo	3
99	SP5.12	Costa Rica	San Pablo	3
100	SP5.13	Costa Rica	San Pablo	3
101	SP5.14	Costa Rica	San Pablo	6
102	SP5.16	Costa Rica	San Pablo	6
103	SP6.4	Costa Rica	San Pablo	3
104	SP6.12	Costa Rica	San Pablo	3
105	SP7.7	Costa Rica	San Pablo	1
106	SP7.10	Costa Rica	San Pablo	3
107	SP7.18	Costa Rica	San Pablo	1
108	SP7.30	Costa Rica	San Pablo	1
109	SP8.18	Costa Rica	San Pablo	3
110	SP8.21	Costa Rica	San Pablo	6
111	SP8.27	Costa Rica	San Pablo	6
112	SP9.1	Costa Rica	San Pablo	3
113	SP9.7	Costa Rica	San Pablo	1
114	SP9.19	Costa Rica	San Pablo	3
115	SP9.24	Costa Rica	San Pablo	3
116	SP10.1	Costa Rica	San Pablo	1
117	SP10.15	Costa Rica	San Pablo	1
118	SP10.17	Costa Rica	San Pablo	3
119	SP6.6	Costa Rica	San Pablo	3
120	SP6.7	Costa Rica	San Pablo	3
121	SP6.9	Costa Rica	San Pablo	3
122	SP6.10	Costa Rica	San Pablo	3
123	SP6.11	Costa Rica	San Pablo	3
124	SP6.12	Costa Rica	San Pablo	6
125	SP7.1	Costa Rica	San Pablo	6
126	Z1.3	Costa Rica	Zent	1

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

No.	Isolate	Origin	Plantation/Location	Repeat units
127	Z.1.5	Costa Rica	Zent	1
128	Z1.8	Costa Rica	Zent	1
129	Z1.10	Costa Rica	Zent	1
130	Z1.12	Costa Rica	Zent	1
131	Z1.14	Costa Rica	Zent	1
132	Z1.18	Costa Rica	Zent	1
133	Z1.20	Costa Rica	Zent	1
134	Z1.21	Costa Rica	Zent	1
135	Z1.24	Costa Rica	Zent	1
136	Z2.1	Costa Rica	Zent	1
137	Z2.2	Costa Rica	Zent	1
138	Z2.3	Costa Rica	Zent	1
139	Z2.5	Costa Rica	Zent	1
140	Z2.7	Costa Rica	Zent	1
141	Z2.8	Costa Rica	Zent	1
142	Z2.9	Costa Rica	Zent	1
143	Z2.11	Costa Rica	Zent	1
144	Z2.13	Costa Rica	Zent	1
145	Z2.14	Costa Rica	Zent	1
146	Z3.5	Costa Rica	Zent	2
147	Z3.6	Costa Rica	Zent	2
148	Z3.11	Costa Rica	Zent	2
149	Z3.15	Costa Rica	Zent	6
150	Z3.17	Costa Rica	Zent	6
151	Z3.32	Costa Rica	Zent	2
152	Z3.34	Costa Rica	Zent	6
153	Z4.2	Costa Rica	Zent	1
154	Z4.7	Costa Rica	Zent	1
155	Z4.11	Costa Rica	Zent	1
156	Z4.12	Costa Rica	Zent	1
157	Z4.14	Costa Rica	Zent	1
158	Z4.16	Costa Rica	Zent	1
159	Z4.17	Costa Rica	Zent	1
160	Z4.19	Costa Rica	Zent	1
161	Z4.22	Costa Rica	Zent	1
162	Z4.26	Costa Rica	Zent	1
163	Z4.29	Costa Rica	Zent	1
164	Z5.1	Costa Rica	Zent	6
165	Z5.4	Costa Rica	Zent	6
166	Z5.6	Costa Rica	Zent	6
167	Z5.12	Costa Rica	Zent	1
168	Z5.13	Costa Rica	Zent	6

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

No.	Isolate	Origin	Plantation/Location	Repeat units
169	Z5.15	Costa Rica	Zent	6
170	Z5.18	Costa Rica	Zent	1
171	Z5.21	Costa Rica	Zent	1
172	Z5.25	Costa Rica	Zent	1
173	Z5.32	Costa Rica	Zent	1
174	Z6.2	Costa Rica	Zent	1
175	Z6.3	Costa Rica	Zent	1
176	Z6.5	Costa Rica	Zent	1
177	Z6.7	Costa Rica	Zent	1
178	Z6.9	Costa Rica	Zent	1
179	Z6.11	Costa Rica	Zent	2
180	Z6.13	Costa Rica	Zent	1
181	Z6.15	Costa Rica	Zent	2
182	Z6.17	Costa Rica	Zent	1
183	Z7.1	Costa Rica	Zent	1
184	Z7.7	Costa Rica	Zent	6
185	Z7.9	Costa Rica	Zent	6
186	Z7.14	Costa Rica	Zent	1
187	Z7.18	Costa Rica	Zent	6
188	Z7.28	Costa Rica	Zent	6
189	Z7.31	Costa Rica	Zent	6
190	Z8.1	Costa Rica	Zent	2
191	Z8.8	Costa Rica	Zent	1
192	Z8.11	Costa Rica	Zent	1
193	Z8.12	Costa Rica	Zent	2
194	Z8.13	Costa Rica	Zent	2
195	Z8.17	Costa Rica	Zent	1
196	Z8.18	Costa Rica	Zent	2
197	Z8.20	Costa Rica	Zent	1
198	Z8.25	Costa Rica	Zent	1
199	Z8.27	Costa Rica	Zent	2
200	Z8.35	Costa Rica	Zent	1
201	Z10.1	Costa Rica	Zent	1
202	Z10.3	Costa Rica	Zent	1
203	Z10.4	Costa Rica	Zent	1
204	Z10.6	Costa Rica	Zent	6
205	Z10.7	Costa Rica	Zent	1
206	Z10.8	Costa Rica	Zent	1
207	Z10.9	Costa Rica	Zent	1
208	Z10.10	Costa Rica	Zent	1
209	Z10.11	Costa Rica	Zent	1
210	ZTSC2	Costa Rica	San Carlos	1

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

No.	Isolate	Origin	Plantation/Location	Repeat units
211	ZTSC10	Costa Rica	San Carlos	1
212	ZTSC15	Costa Rica	San Carlos	1
213	ZTSC40	Costa Rica	San Carlos	1
214	ZTSC50	Costa Rica	San Carlos	1
215	ZTSC55	Costa Rica	San Carlos	1
216	ZTSC60	Costa Rica	San Carlos	1
217	ZTSC65	Costa Rica	San Carlos	1
218	ZTSC75	Costa Rica	San Carlos	1
219	ZTSC80	Costa Rica	San Carlos	1
220	ZTSC84	Costa Rica	San Carlos	1
221	ZTSC90	Costa Rica	San Carlos	1
222	ZTSC95	Costa Rica	San Carlos	1
223	ZTSC100	Costa Rica	San Carlos	1
224	ZTSC101	Costa Rica	San Carlos	1
225	ZTSC77	Costa Rica	San Carlos	1
226	E22	Ecuador	a	1
227	GS.4	Ecuador	a	1
228	GS.10	Ecuador	a	1
229	RN.3	Ecuador	a	1
230	RN.5	Ecuador	a	1
231	RS.13	Ecuador	a	1
232	SaR.2	Ecuador	a	1
233	SaR.5	Ecuador	a	1
234	X845	Indonesia	a	1
235	X846	Phillipines	a	1
236	X847	Taiwan	a	1
237	X849	Burundi	a	1
238	X851	Gabon	a	1
239	C86	Cameroon	a	1

CHAPTER 5

GENERAL DISCUSSION

Unraveling a pathosystem is hard work. In this thesis we have begun to scratch the surface of the Sigatoka – banana complex, with a focus on the black Sigatoka pathogen *Pseudocercospora fijiensis.* The ecological context, however, is that banana encounters the Sigatoka complex, worldwide. Previous analyses unveiled that this complex contains at least 16 fungal species interacting on banana leaves (Arzanlou et al., 2008). We know that *P. musae* has been largely replaced by *P. fijiensis* (Carlier et al., 2000; Zandjanakou-Tachin et al., 2013). Recently, it has been suggested that *P. eumusae* is even more aggressive, but hard data underpinning this suggestion are largely missing. Nevertheless, it is important to realize that *P. fijiensis* is part of a complex ecological reality (Carlier et al., 2000; Chang et al., 2016; Zandjanakou-Tachin et al., 2013). Black Sigatoka is barely a problem in household farming and seems to be the plague of banana plantations, which are among the last remnants of old-fashioned monoculture agriculture. Nearly all export bananas are of the "Cavendish" type, which is susceptible to black Sigatoka (**chapter 1**). Chong (2016) has shown the consequence of this agricultural model; a highly susceptible globally dispersed host clone leaves virtually only one option for disease management. This is massive usage of fungicides, which leads to an ever-reducing sensitivity to these compounds of the sexually reproducing fungus, eventually leading to unmanageable disease situations. The only way to escape from black Sigatoka is to move, to new less disease prone areas that even allow the production of biological bananas. However, such areas are sparse and usually located in remote regions, at high altitudes where *P. fijiensis* does not thrive due to non-conducive weather conditions. Those areas, however, can also not supply the global demand. Thus, escaping to these new sub-optimal environments is not the way to go. Instead, we need the dive deep and do all the necessary research to (re-)build the system bottom-up, aiming at a radical change in the production systems. This may require expansion of the applied genetic diversity in the field by releasing new banana varieties that meet consumer demands, but also avoiding

monocultures and invest in multi-cropping by for instance integrating banana production and forestry. As *P. fijiensis* is light sensitive, similar to many other Dothideomycetes, shading might already be a factor that affects epidemics. Evidently, large monocultures do not provide shade, while agroforestry systems do. Thus, ecological embedding of a host-pathosystem is important and the banana - *P. fijiensis* pathosystem is not an exception. In this thesis, we have started to unravel the banana – *P. fijiensis* pathosystem as one of the building blocks of a solid foundation for future banana production.

Pseudocercospora fijiensis is a bipolar heterothallic Dothideomycete plant pathogenic fungus (Arzanlou et al., 2010; Conde-Ferráez et al., 2007). It is part of a group of major fungal plant pathogens affecting manifold crops and its life strategy is characterized by abundant sex. These two characteristics are important leads for improved understanding of this pathosystem. In many ways, *P. fijiensis* resembles the wheat foliar blight *Zymoseptoria tritici.* Twenty-five years ago, the interaction between this fungus and wheat was enigmatic and believed to be merely driven by quantitative variation for resistance - not a single gene for resistance was identified – and pathogenicity. By steady descriptive and explorative research, it is now one of the leading model systems (Brading et al., 2002; Goodwin and Kema, 2014; Kema et al., 2018; Mirzadi Gohari, 2015; Tabib Ghaffary et al., 2011). Many resistance genes (*Stb* genes) have been identified (Brading et al., 2002; Tabib Ghaffary et al., 2011; Kema et al., 2000) by classical genetics and are being used in commercial breeding programs around the world (Torriani et al., 2015). Recently, the first resistance gene, *Stb6,* and the first avirulence gene *AvrStb6* were cloned (Kema et al., 2018; Saintenac et al., 2018; Zhong et al., 2017). Moreover, Kema et al. (2018) discovered a sexual peculiarity of *Z. tritici* that most likely applies to many other Dothideomycetes, including *P. fijiensis*; exclusive paternal parenthood (EPP). Incorporation of EPP in epidemiological models explains extended longevity of wheat cultivars as well as the rapid dissemination of strobilurin resistance in *Z.*

tritici population across Europe. It is very likely that the rapid dissemination and fixation of strobilurin resistance in Costa Rican *P. fijiensis* populations is also due to EPP (**chapter 2**; Amil et al., 2007). Hence, sequencing the *P. fijiensis* genome was the way ahead. It generates interest, attracts researchers, widens the community, involves the industry and leads to a better understanding of genetic diversity. Before publication of the genome sequence (**chapter 2**), Stergiopoulos et al. (2010, 2014) already showed that the effector *Pfavr4* is recognized by the tomato *Cf4* cognate. We indeed showed that *Musa acuminata* ssp. *burmannicoides* var. Calcutta 4, which is iconic for its resistance to black Sigatoka, specifically responded to injections of crude protein extracts of *PfAVR4* (**chapter 2**). This is a first indication that resistance genes in banana for black Sigatoka can be identified and mapped. I speculate that banana contains cognate receptor genes, analogous of *Cf4*. Surprisingly, such studies are limited (Ortiz and Swennen, 2014; Ortiz and Vuylsteke, 1994) and not a single gene for resistance has been identified to *P. fijiensis.* In retrospect, this is explainable as mapping genes by exposing segregating populations to natural *P. fijiensis* populations in the outside environment can only lead to erroneous conclusions. Genetic studies will lead nowhere by placing segregating populations to an anonymous, segregating, highly diverse population of a pathogen. Thus far, one study used individual isolates of *P. fijiensis* on a range of banana accessions (Fullerton and Olsen, 1995). This is analogous to the studies in *Z. tritici* and an absolute requirement for understanding host-pathogen interactions (Kema et al., 2000; Kema and van Silfhout, 1997; Kema et al., 1996b, 1996a, 2018; Tabib Ghaffary et al., 2011; Ware, 2006). Once *P. fijiensis* isolates are characterized they can be used individually for genetic analyses. Given the stature of banana plants, such studies require a greenhouse setting, which also precludes any contamination with natural *P. fijiensis* populations. One of the benefits of the *P. fijiensis* genome sequence is that we now can work towards finishing the genome, resequencing other strains and develop an effector portfolio

from a global population analysis. This contributes to effector-based screening methods and can build on a detailed analysis of the population diversity (Pais et al., 2017). The development of the *Agrobacterium*-mediated transformation of *P. fijiensis* is an important achievement allowing functional analysis of important genes. The method was employed to replace the *Pfcyp51* promoter to prove that repeat elements in the promoter of this gene contribute to azole fungicides resistance. Further onwards , several results emerging from this thesis will be discussed. Firstly, the implications of the genomic structures such as high repetitive DNA content and transposons on the biology of *P. fijiensis* and its interaction with the host plant. Secondly, how banana breeders can benefit from the hypothesized *Pf4* and other resistance genes in banana for *P. fijiensis* resistance. Finally, how the biology of *P. fijiensis* can explain its interaction with and survival under azole fungicide pressure.

The *Pseudocercospora fijiensis* genome and its biological implications

We sequenced two *P. fijiensis* strains and assembled the genome by using a genetic linkage map. As discussed above, sexual reproduction is crucial for *P. fijiensis* and hence, it is a great achievement that routine crosses between isolates now can be accomplished under controlled conditions in a greenhouse after identifying the compatible mating types (*mat1-1* and *mat1-2*) of the partners (Chong, 2016). One of the first observations was the massive genetic diversity in natural populations. Electrophoretic karyotyping and analyses of molecular markers in *P. fijiensis* field populations showed chromosome-length polymorphisms and high genetic diversity. Genetic differentiation was also detected using neutral markers, suggesting strong selection with limited gene flow at the studied geographic scale. We studied five samples from the Costa Rican population “Cartagena” (**chapter 2**), and our data were truly different from the analysis of Mexican strains (Rodríguez-García et al., 2006), which is not surprising due to the high polymorphisms of isolates that was found previously at plant and lesion level (Müller et al., 1997; Rivas et al., 2004). The second

highlight was the huge genome expansion. Compared to the Dothideomycete reference *Z. tritici*, the *P. fijiensis* genome is, with 74Mb, almost twice as large (Goodwin et al., 2011). The genome contains 49% repetitive DNA, responsible for the genome expansion, similar to the genomes of the other Sigatoka complex pathogens *P. musae* (51.5 Mb, 62.2% repetitive content and unassembled sequences) and *P. eumusae* (19.2 Mb, 35.7% repetitive content and unassembled sequences) (Chang et al., 2016) and *C. fulvum* (61.1 Mb, 41% repetitive content) (de Wit et al., 2012). In *P. fijiensis*, LTR retrotransposons account for no less than 50% of the expansion, largely exceeding the 2% in *Z. tritici*, and other sequenced Dothideomycetes (Dhillon et al., 2014; Goodwin et al., 2011; Ohm et al., 2012) and similar to other unrelated fungal pathogens such as *Blumeria graminis* f. sp. *tritici* (180 Mb, 64% LTRs) (Spanu et al., 2010; Wicker et al., 2013). Until recently, Sigatoka complex pathogens had the largest Dothideomycete genomes, but the current championing genome sizes are those of *Cenococcum geophilum*, the only ectomycorrhizal symbiont that evolved within the Dothideomycetes, with a genome size of 178 Mb (Peter et al., 2016), and *Zopfia rhizophila,* an asparagus root rot fungus, with a genome size of 153 Mb (https://genome.jgi.doe.gov/Zoprh1/Zoprh1.home.html). Our melting-curve assays already indicated expanded genomes in *P. eumusae* and *P. musae* (**chapter 2**), which was recently confirmed (Chang et al., 2016). In all these fungi, transposons are the major drivers for genome evolution, similarly to processes in other plant pathogens such as *Verticillium dahliae* (Faino et al., 2016), *Leptosphaeria maculans* (Grandaubert et al., 2014) and *Pyrenophora tritici repentis* (Manning et al., 2013). In many cases, such repeat rich areas, which can be lineage specific, harbor effector genes that are crucial for orchestrating the outcome of host-pathogen interactions, like the effector *Pfavr4* on scaffold 4 (Stergiopoulos et al., 2010) . As mentioned above, the banana - *P. fijiensis* pathosystem is at a very early stage with respect to unravelling specificity. According to the zig-zag model of plant immunity (Jones and Dangl,

2006), pathogens deliver microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) that after recognition by host receptors elicit PAMP-triggered immunity (PTI). Successful pathogens, however, deliver effectors interfering with PTI and resulting in effector-triggered susceptibility (ETS). *Pfavr4* is recognized by NB-LRR proteins, which then activate effector-triggered immunity (ETI), resulting in a hypersensitive response (HR). A preliminary scheme for the *P. fijiensis* – banana interaction is shown in Figure 1.

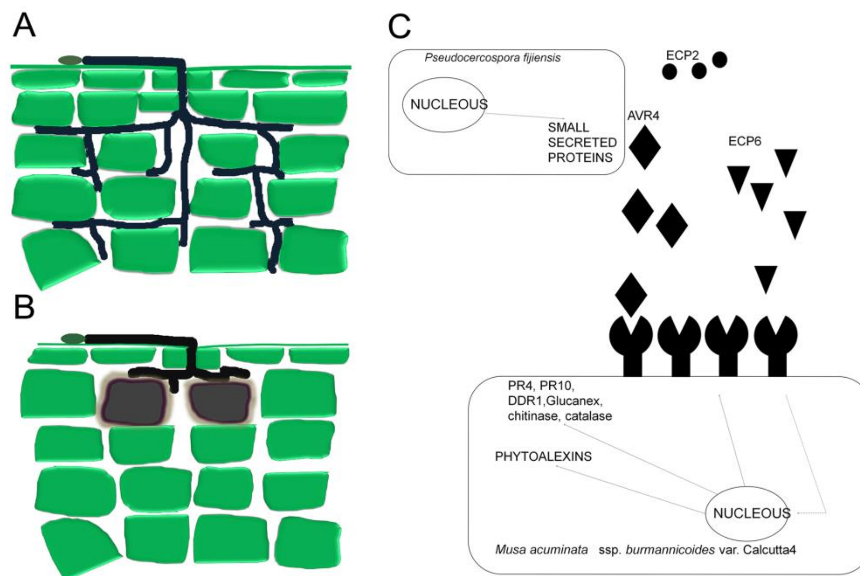

Figure 1. Interaction of *Pseudocercospora fijiensis* with banana leaf tissue. A) Scheme of a compatible interaction B) Scheme of an incompatible interaction C) Small secreted proteins including PFAVR4, ECP2 and ECP6-like, are expressed by *P. fijiensis* during the first days of infection and recognized by the resistant accession *Musa acuminata* ssp. *burmannicoides* var. Calcutta 4 along with substantial melanin production. The banana host responds with peroxidase production to release reactive oxygen species and starts the production of phytoalexins (Beltrán-García et al., 2014; Cavalcante et al., 2011; Escobar-Tovar et al., 2015; Rodriguez et al., 2016; Torres et al., 2012). At the same time the downstream resistance signaling pathway is initiated after recognition of PFAVR4 by the cognate hypothetical PF4 resistance protein, a process that in turn can be manipulated by ECP6-like effector proteins.

Pfavr4 was identified because it was the first effector that showed homology with *Avr4* of *C. fulvum* (Stergiopoulus et al., 2010). In this thesis we showed for the first time that infiltration of the crude *Pfavr4* protein in “Calcutta 4” and “Grand Naine” results in a remarkable differential host response. A HR-like response which is characteristic of gene-for-

gene interactions was observed in “Calcutta 4” but not in “Grand Naine”. This suggests that a homologue of the tomato *Cf4* resistance gene is present in the banana genome. It is therefore likely that we are close to identifying a first black Sigatoka resistance gene. Once such gene has been identified, more effectors and their cognate receptors will be identified, and their frequency can then be monitored in natural populations. Stergiopoulos et al. (2014) initiated such a comparative analysis and concluded that *Pfavr4* has at least 17 allelic forms of which some are geographically isolated, suggesting the potential for regional gene deployment as a strategy for disease management. As homologues of *Avr4* are present in a number of fungi, it is an interesting gene for comparative functional studies. However, for *P. fijiensis* the emphasis should lay on identifying more effectors by using bioinformatics on the subset of 172 small secreted proteins (<300 bp; **chapter 2**) as well as by classical mapping studies taking the *Z. tritici* – wheat pathosystem as example (Kema et al., 2018; Mirzadi Gohari, 2015; Zhong et al., 2017) and possibly by using proteomics of infected banana leaves to detect additional putative effectors (Escobar-Tovar et al. 2015; Noar and Daub, 2016b). Finally, proteomic analyses of apoplastic fluids has been a very efficient approach to find effectors of *C. fulvum* (Bolton et al., 2008; Joosten et al., 1997; Stergiopoulos and de Wit, 2009) and *Fusarium oxysporum* f. sp. *lycopersici* (Gawehns et al., 2014; Houterman et al., 2009; Ma et al., 2015). Similar strategies could be used for the banana - *P. fijiensis* pathosystem, but initial analyses have resulted in practical problems due to the high latex content of apoplastic fluids. Coagulation and oxidation of this latex complicates HPLC analysis. Therefore, the preference lies in generating sequence data to detect more effectors such as in *Bipolaris cookie*, even without a genome reference (Zaccaron and Bluhm, 2017). Along with precise phenotyping, such data enable genome wide association studies to identify effectors (Zhong et al., 2017). Therefore, developing efficient phenotyping assays should have the highest priority as working with individual *P. fijiensis* isolates is possible, but still rather

complicated due to the limited experimental amenity of the fungus: it grows slow, has limited conidia production and requires very specific environmental conditions and bio-assays take up to seven weeks. Effector-based phenotyping assays would be ideal for rapid identification of resistance sources, and would be a great support for breeding. Recent studies on the role of proteins such as chitinases and β-1,3-glucanase (Escobar-Tovar et al., 2015; Portal et al., 2011; Rodriguez et al., 2016; Torres et al., 2012), of peroxidases, and the enzyme phenylalamine ammonia-lyase (PAL), required for the production of precursor phytoalexins (Beltrán-García et al., 2014; Cavalcante et al., 2011; Escobar-Tovar et al., 2015) broaden our view of the interaction, but are beyond the horizon of breeders. This also holds for complicated *in vitro* assays such as reported by Kovács et al. (2013), who showed that susceptible banana plants transformed with constitutively expressed rice chitinase are resistant to artificially inoculated *P. fijiensis* isolates. First of all, we have to deal with the acceptance of genetically modified crops (Kikulwe et al., 2011; Lucht 2015); and secondly, perform these tests with diverse panel of *P. fijiensis* isolates. Only one isolate was tested, but we now know that *P. fijiensis* has at least 200 effectors and hence the efficacy of resistance has to be tested to the widest possible diversity of virulence factors. Moreover, given the dynamics and diversity of natural *P. fijiensis* populations, it is really questionable whether genetically modified crops are the solution for black Sigatoka in banana (Khanna et al., 2007; Kovács et al., 2013; Mlalazi et al., 2012). The validity of this approach for clonal pathogens such as *Fusarium oxysporum* f.sp. *cubense* (Foc) is entirely different. Recently, transgenic "Cavendish" plants harboring a resistance gene from the wild diploid *Musa acuminata* var. *malaccensis* were shown to be resistant to the devastating tropical race 4, which is a great proof of principle (Dale et al., 2017). Moreover, this was the first ever identified resistance gene in banana, despite the fact that resistance of "Cavendish" bananas to race 1 strains of Foc already upholds the industry for decades. Albeit that genetically modified bananas may solve

a problem in current banana cultivation, the technology does not address the underlying cause and that is genetic uniformity. Hence, particularly with an eye on black Sigatoka, diversification is urgently required and therefore, this study is an important basis for future research into the banana - *P. fijiensis* pathosystem.

The supposedly rapidly evolving *P. fijiensis* genome could potentially challenge the durability of resistance. TE-rich regions seem instrumental for providing genome plasticity and often harbor effector genes (Raffaele and Kamoun, 2012), as in *C. fulvum* and *D. septosporum* (de Wit et al., 2012), *Z. tritici* (Goodwin et al., 2011), *P. nodorum* (Hane et al., 2007), *M. oryzae* (Orbach et al., 2000; Yoshida et al., 2016), and *Phytophthora infestans* (Raffaele et al., 2010). In *L. maculans* repeat-induced point mutations (RIP) caused mutations in *AvrLm1* and *AvrLm4* to circumvent resistance to *Lm1* and *Lm4*, respectively (Fudal et al., 2007; Gout et al., 2006; Grandaubert et al., 2014; Rouxel et al., 2011). Also *Pfavr4* is next to a transposon that was potentially RIPPed and coincidently showed diversification due to non-silent mutations (Stergiopoulous et al., 2014). Together with positive selection and intragenic recombination as evolution mechanisms they facilitate evading the banana host immune system. However, until now not a single resistance gene to *P. fijiensis* was neither identified, nor mapped let alone cloned. Hence, it is important to identify as many resistance genes as possible and to develop the technologies to efficiently accomplish that goal. Recently, a series of resistance genes analogues has been explored (Capdeville et al., 2009; Pei et al., 2007; Sánchez Timm et al., 2016; Wiame et al., 2000), but none could be related to resistance to *P. fijiensis*. Genomic and cDNA libraries of *M. acuminata* Colla (Cheung and Town, 2007; Vilarinhos et al., 2003) *M. acuminata* spp. *burmannicoides* var. Calcutta 4 (Santos et al., 2005), var. TuuGia (Ortiz-Vázquez et al., 2005), var. Pahang (Arango et al., 2011), will also aid gene discovery. However, understanding the biology remains a sound basis for any gene discovery and deployment as exemplified by the aforementioned EPP mechanism, which

could be operational in the banana - *P. fijiensis* pathosystem and has a buffering effect of the longevity of resistance (Kema et al., 2018).

The developed ATMT protocol is an important tool for any functional analysis in *P. fijiensis* (**chapter 3**). The Ku70 protein is essential for the non-homologous end joining (NHEJ) pathway of DNA repair. The generation of a *ΔPfku70* strain in this study might aid higher homologous recombination rates during transformations. Similarly, CRISPR-Cas9 might be a potential tool for functional analyses in this recalcitrant fungus as it is more precise and less prone to unwanted mutations (Liu et al., 2015). We used ATMT to generate *ΔPfavr4* strains, which await further characterization, as well as promotor swaps of the *cyp51* gene that is the target of the demethylase inhibitor fungicides (DMIs); the work horse of black Sigatoka disease management around the world (**chapters 3** and **4**).

Potential of horizontal gene transfer among *Pseudocercospora* pathogens in the Sigatoka complex

Mechanisms for horizontal transfer in fungi have been discussed (Manning et al., 2013; Mehrabi et al., 2011, 2017), and the genome of *P. fijiensis* that is enriched with retrotransposons suggests that it is prone to horizontal gene transfer. *P. fijiensis, P. musae* and *P. eumusae* seem to have a common ancestor and often co-occur in nature (Carlier et al., 2000; Crous and Mourichon, 2002). The genome expansion observed in **chapter 2** and by Chang et al. (2016), which is due the high repetitive content in these genomes is suggestive for the potential of horizontal gene transfer. Similar processes were described for the host-selective toxin gene *ToxA*, which was transferred from *Parastagonospora nodorum* to *Pyrenophora tritici-repentis* (Friesen et al., 2006) and the elegant work of Ma et al. (2010) who showed chromosome transfer facilitating pathogenicity to previously non-pathogenic *F. oxysporum* strains on tomato. The Sigatoka complex likely extends beyond the

aforementioned three species to potentially 16 fungal species (Arzanlou et al., 2008) and therefore provides an excellent system to study the potential and processes involved in horizontal gene transfer. Chang et al. (2016) compared the *P. fijiensis* genomic data from **chapter 2** with the genome sequences of *P. musae* and *P. eumusae* and found reasonably distinct patterns for genome structure and metabolic processes. Goodwin et al. (2011) discerned horizontal gene transfer and RIP degeneration as a potential origin of dispensable chromosomes in *Z. tritici*, thereby constituting a mechanism for a rapid evolution (Croll et al., 2013). In other fungi such as *N. haematoccoca* supernumerary chromosomes carry virulence genes (Coleman et al., 2009). In *M. oryzae*, *Avr-Pita* is located on a dispensable chromosome (Chuma et al., 2011). Therefore these accessory chromosomes may also be part of a genome wide adaptation mechanism. For now, however, we do not know whether the *P. fijiensis* genome contains accessory chromosomes, but a more detailed analysis would be worthwhile given the physically close proximity of the species in the Sigatoka complex on banana foliage and their potential to engage in such an evolutionary process. It would also cast light on the rapid turn-over of the yellow Sigatoka fungus *P. musae* by the black Sigatoka fungus *P.fijiensis,* which is much more aggressive and therefore very competitive and still expanding its colonization into new areas (Brito et al., 2015; Rieux et al., 2013). A similar situation could arise with respect to *P. eumusae,* which is now restricted to Southern and Southeast Asia and some areas in Africa, and could compete with *P. fijiensis* due to other and complementary strategies for host attack (Chang 2016).

Sensitivity of *P. fijiensis* to azoles

Due to the lack of breeding in banana, despite its importance as a staple food crop and top global fruit commodity, disease control entirely relies on fungicide applications. Chong et al

(2016) showed that reduced sensitivity in *P. fijiensis* is a true concern as DMIs are the presently the cornerstone for disease control for black Sigatoka worldwide. As long as there are no commercially viable alternatives, these compounds will remain very important. Therefore, also from this perspective diversification and innovation is required. Fall back scenarios to broad range protective compounds such as chlorothalonil and mineral oils are not the favored strategies for sustainability. It is much better to invest into developing new compounds and alternative products with good efficacy until new resistant varieties reach the markets. In our work we have shown that mutations resulting in amino acid changes in the catalytic domain of CYP51 and promotor insertions affecting gene expression drive reduced sensitivity to DMIs. However, the monitoring of sensitivity in *P. fijiensis* populations should be professionalized and can be based on our latest data. Quick PCR scans for *cyp51* promotor length variation is a first indication of reduced sensitivity and should lead to alternative spraying schedules. Additionally, new products should be evaluated for their efficacy on preventing sexual development. Kema et al. (2018) recently showed that products such as strobilurins, which were very popular and embraced as new active ingredients for black Sigatoka control in banana, do not stop sexual reproduction. This explains why the efficacy of these products rapidly went down once resistant isolates were around (Amil et al., 2007; **chapter 2**). Finally, black Sigatoka is a burden on the shoulders of those who are interested in growing and consuming organically grown bananas. Without black Sigatoka disease control, producing bananas is virtually impossible unless the entire production zone is moved to new less disease prone areas. Even leaf pruning cannot stop the disease and hence, also this area calls for innovation in the banana production chain. The huge input of fungicides should mobilize consumers to step-up to retailers and all those that dominate the industry. The message should be loud and clear: we want something better than bananas that only survive

due to environmentally threatening production methods and that are driven only by business as usual strategies. We need truly forward thinking in banana land.

Perspectives

One of the first things that now need to be performed is developing a reference DNA sequence of *P. fijiensis.* The basis for that analysis is laid in this thesis and a few more rounds of PacBio, or Minion sequencing should be sufficient to finish the genome. This will aid comparative studies across *P. fijiensis* populations and provide a strong support for bioinformatic analyses. One of the first goals would be to determine whether *P. fijiensis* has an accessory set of chromosomes. This is important for all species within the Dothideomycetes, including those with large genomes that carry a highly repetitive DNA (Noar and Daub, 2016b, 2016a). Furthermore, we need to improve the toolbox for *P. fijiensis* research. The basic ingredients are now available with a sequenced genome (**chapter 2**), silencing (Onyilo et al., 2017) and transformation protocols (**chapter 3**). However, these can and will need to be fine-tuned and improved. As mentioned above, harvesting the *P. fijiensis* genome for effector genes with a strong emphasis on their targets and the development of rapid screening protocols is a necessity to attract new students and scientists into this area and to boost innovation in this orphan crop.

Trending topics

CRISPR-Cas9 is for many systems the current light at the end of the tunnel. The prospects are enormous and the potential of the technology in nearly any organism is exhilarating (Teboul et al., 2017). The first papers sketch a bright future for banana improvement (Kaur et al.,

2018), but for the screaming disease threats of banana, we first need to identify and map the target genes. From that perspective, banana research is way behind as we do not even know any resistance gene except one (Dale et al., 2017). Hence, the trending topic for future banana research is to focus on the genetics of the interaction between banana and its fungal threats. Without fungicides, western supermarkets will be rapidly depleted from cheap bananas. Hence, all stakeholders of the production and logistic chain need to get together to develop a plan. That plan should envisage a durable and sustainable banana production that supports local economies without jeopardizing the environment and meet consumer demands. Basic science is required and the sector should abandon secretive, repetitive local research aiming at maximizing production. Transparency and critical mass are essential to make progress and hence publishing research is key to lay the foundation for the future. Genomic and genetics will be at the basis for innovative banana improvement and diversity and hence sound production and marketing strategies. I have done my share. Who is next?

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Summary

The hemibiotroph *Pseudocercospora fijiensis* is the causal agent of the black Sigatoka disease on bananas; present worldwide being the reason of large economical losses and subject to chemical control as the best control method nowadays. Nonetheless, this fungus has shown being able to become resistant to different chemical fungicides and thus a successful global threat to bananas. The biology of this fungus is largely unknown due to the missing –omics tools applied to this pathosystem. The only manner to counteract this fungus is to understand its biology of survival and pathogenicity.

Chapter 1. Is the introduction to the fungus, it constitutes an update to the knowledge of its biology and epidemiology. It demonstrates the importance of generating more data and tools to understand this powerful pathogen.

Chapter 2. Presents the *P. fijiensis* genome sequence, the analysis of its main characteristics compared with other close fungi. A new genetic map is included counting putative core and dispensable scaffolds, whose high polymorphism was observed by electrophoretic karyotyping within isolates from the same field population. It is shown the massive genome expansion mainly due to repetitive DNA, particularly by LTR-retrotransposons, and how this can affect close sequences. The effect of RIP on the genome is analyzed and compared to that on other closely related fungi. The location close to repetitive sequence from the effector *Pfavr4* was discussed under the scope of its epidemiology and the protein employed helped to elucidate the first putative resistant cognate gene in the resistant banana cultivar Calcutta 4. Further analysis of strains originated from populations with and without fungicide selection pressure provided estimates of dispersal of strains and genetic flow that will help to predict spatial patterns of fungicide evolution under different management strategies.

Chapter 3. Describes the protocol for *Agrobacterium*-mediated transformation of *P. fijiensis* for both random and targeted mutagenesis. This method was successfully applied to the gene *Pfavr4* and the *Pfku70*. The former is the first effector described in *P. fijiensis*, and the latter is the gene codifier of the KU70 protein, the main point of the non-homologous end joining (NHEJ) pathway that has been related to an increase on homologous recombination in several fungi and other eukaryotes, providing

important tools for further use. This method was additionally employed to swap the *Pfcyp51* gene promoter that helped to decipher biology of azole resistance in *P. fijiensis*, as described in chapter 4.

Chapter 4. The appearance of resistant strains to azoles has been mostly correlated with non-synonymous point mutations in the coding sequence *Pfcyp51* gene. In this chapter, we identified a 19 base pairs (bp) repeat element in the promoter region of this gene, by a simple PCR analysis, showed that copy number correlates positively with increased resistance to azoles, as well as the exposure to azole fungicides. We swapped the promoter of a resistant strain into a susceptible strain, and thus demonstrated that presence of the repeat element proportionally upregulates *Pfcyp51* expression as well as tolerance to azoles. Besides the knowledge on genetic mechanism for azole resistance in *P. fijiensis*, the present study might offer another tool for optimizing the use of azoles in the control of black Sigatoka.

Chapter 5. A general discussion of the results obtained in this thesis is offered with a broader point of view. Implications in ecology, pathology and further expectations on the control of this fungus, together with insights on trending topics of molecular tools for future research are included.

Resumen

El hemibiotrófico *Pseudocercospora fijiensis* es el agente causal de la enfermedad de la Sigatoka negra en banana; presente alrededor del mundo siendo la razón de grandes pérdidas económicas y sujeto a control químico como el mejor método de control hoy en día. Sin embargo, este hongo ha mostrado ser capaz de ser resistente a diferentes fungicidas químicos y así ser una exitosa amenaza mundial para el banano. La biología de este hongo es ampliamente desconocida debido a la carencia de herramientas ómicas aplicadas a este patosistema. La única manera de contrarrestar este hongo es entendiendo su biología de supervivencia y patogenicidad.

Capítulo 1. Es la introducción al hongo, constituye una actualización en el conocimiento de su biología y epidemiología. Demuestra la importancia de generar más datos y herramientas para entender este poderoso patógeno.

Capítulo 2. Presenta la secuencia genómica de *P. fijiensis*, el análisis de sus características principales comparadas con otros hongos filogenéticamente cercanos. Se incluye un nuevo mapa genético contando con putativos “scaffolds” principales y dispensables, cuyo alto grado de polimorfismo fue observado por cariotipo electroforético en aislados de una misma población. Se muestra también la expansión masiva del genoma debida principalmente al ADN repetitivo, particularmente por transposones tipo LTR y cómo éstos afectan secuencias vecinas. El efecto del RIP en el genoma es analizado y comparado con el de otros hongos filogenéticamente cercanos. La ubicación del efector *Pfavr4* cercana a secuencias repetitivas fue discutida bajo el punto de vista de su epidemiología y la proteína empleada para elucidar el primer gen putativo cognado de resistencia en el cultivar de banana Calcuta 4. Análisis posteriores de líneas originarias de poblaciones diferentes con y sin selección de fungicida proporcionó la estimación de dispersión de éstas y flujo genético que ayudará a predecir patrones espaciales de evolución de resistencia a fungicidas bajo diferentes estrategias de manejo.

Capítulo 3. Describe el protocolo de transformación de *P. fijiensis* mediada por *Agrobacterium* para mutagénesis al azar y de genes blanco. Este método fue satisfactoriamente aplicado a los genes *Pfavr4* y *Pfku70.* El primero es el primer efector descrito en *P. fijiensis* y el segundo es el gen codificador

para la proteína KU70, que es el punto principal de la ruta de unión de secuencias no homólogas (NHEJ, por sus siglas en inglés) que ha sido relacionada con un incremento en la recombinación homóloga en diversos hongos y otros eucariontes, proporcionando herramientas importantes para su uso posterior. Este método fue adicionalmente empleado para intercambiar el promotor del gen *Pfcyp51* que ayudó a descifrar la biología de la resistencia a azoles en *P. fijiensis*, descrita en el capítulo 4.

Capítulo 4. La aparente resistencia de líneas del patógeno a los azoles ha sido correlacionada principalmente con mutaciones no sinónimas en la región codificante del gene *Pfcyp51*. En éste capítulo, nosotros identificamos un elemento repetitivo de 19 pares de bases (pb) en la región promotora de este gen, por un simple análisis de PCR, demostramos que el número de copias está correlacionado positivamente con un incremento en la resistencia a azoles, así como a la exposición a éstos. Intercambiamos el promotor de una línea resistente a una línea sensible y así demostramos que la presencia del elemento repetitivo regula proporcionalmente la expresión de *Pfcyp51,* así como la tolerancia a azoles. Además del conocimiento en el mecanismo genético de resistencia a azoles en *P. fijiensis*, el presente estudio podría ofrecer otra herramienta para optimizar el uso de azoles en el control de la Sigatoka negra.

Capítulo 5. Ofrece una discusión general de los resultados obtenidos en esta tesis dentro de un punto de vista más amplio. Se incluyen implicaciones en ecología, patogenicidad y expectativas a futuro en el control de este hongo, y una visión en tópicos actuales de herramientas moleculares para futuras investigaciones.

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About the author

Caucasella Díaz Trujillo was born on November 19th, 1978 in Minatitlan, Mexico. She graduated as biologist at the Autonomous University of Yucatan (UADY) in 2003, working on the *P. fijiensis* – banana pathosystem, under the guidance of Drs. Dieter Kämmer and Andrew James for her BSc thesis entitled "Obtaining microsatellite markers for Mexican lines of *Mycosphaerella fijiensis*", at the Center of Scientific Research of Yucatan (CICY). In 2004 she participated in the characterization of genomic library from a resistant banana, and started an awareness study for *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in local banana farms. In 2005 she obtained a scholarship of the National Council for Science and Technology of Mexico (CONACyT), and started her MSc studies in Plant Sciences at Wageningen University and Research (WUR). She focused, together with Dr. Sarah Ware, on *Zymoseptoria tritici* in wheat in the group of Prof. Gert H.J. Kema at Wageningen Plant Research. Her thesis "Biology of competition in *Mycosphaerella graminicola*: A molecular approach", described the determination of fungal biomass of *Z. tritici* during competition on wheat varieties with different resistance levels and under fungicide selection pressure. During her internship entitled "Functional validation of promoters in transgenic bananas" she started a survey of promoters of putative banana resistance genes at the Catholic University of Leuven in Belgium, in the group of Prof. Rony Swennen and under the guidance of Dr. Serge Remy. In 2007, Caucasella started her PhD on the interaction between *P. fijiensis* and banana with an extension of her CONACyT scholarship and in 2012-2013 she was involved as guest researcher, supported by MusaRadix B.V., in the initiation of the Panama disease program at WUR. Now, returned to Mexico, she is currently Phytopathology and Genetics Researcher at AgroStevia S.A.P.I. de CV.

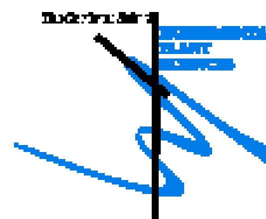
Publications

- Arango, R.E., **Díaz-Trujillo, C.,** Dhillon, B., Aerts, A., Carlier, J., Crane, C.F., Jong, T.V. de, Vries, I. de, Dietrich, R., Farmer, A.D., Fortes Fereira, C., Garcia, S., Guzmán, M., Hamelin, R.C., Lindquist, E.A., Mehrabi, R., Quiros, O., Schmutz, J., Shapiro, H., Reynolds, E., Scalliet, G., Souza Jr, M., Stergiopoulos, I., Van der Lee, T.A.J., Wit, P.J.G.M. de, Zapater, M.-F., Zwiers, L.-H., Grigoriev, I.V., Goodwin, S.B., Kema, G.H.J., 2016. Combating a global threat to a clonal crop: the banana black Sigatoka pathogen *Pseudocercospora fijiensis* (synonym *Mycosphaerella fijiensis*) genomes reveals clues for disease control. *PLoS Genetics* 12(8): e1005876. doi: 10.1371/journal.pgen.1005876.

- **Diaz-Trujillo, C**., Chong, P., Stergiopoulos, I., Cordovez, V., Guzman, M., De Wit, P.J.G.M., Meijer, H.J.G., Scalliet, G., Sierotzki, H., Lilia Peralta, E., Arango Isaza, R.E., Kema, G.H.J., 2018. A new mechanism for reduced sensitivity to demethylation-inhibitor fungicides in the fungal banana black Sigatoka pathogen *Pseudocercospora fijiensis*. *Molecular Plant Pathol*ogy. https://doi.org/10.1111/mpp.12637

- Kema, G.H.J., Mirzadi Gohari, A., Aouini, L., Gibriel, H.A.Y., Ware, S.B., van den Bosch, F., Manning-Smith, R., Alonso-Chavez, V., Helps, J., Ben M'Barek, S., Mehrabi, R., **Diaz-Trujillo, C.,** Zamani, E., Schouten, H.J., Van Der Lee, T., Waalwijk, C., De Waard, M.A., De Wit, P.J.G.M., Verstappen, E.C., Thomma, B.P.H.J., Meijer, H.J.G., Seidl, M.F., 2018. Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance | *Nature Genetics.* In press

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to:	Caucasella Díaz Trujillo
Date:	6 June 2018
Group:	Laboratorium of Phytopathology & BU Biointeractions
University:	Wageningen University & Research

1) Start-up phase	date
► **First presentation of your project**	
Title: Genomic diversity of Mycosphaerella and expression analysis of its interaction with banana	22 Jun 2007
► **Writing or rewriting a project proposal**	
Title: Genomic diversity of Mycosphaerella and expression analysis of its interaction with banana	26 Feb 2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Utrecht, NL	01 Jun 2010
► **EPS theme symposia**	
EPS theme 2 symposium "Interactions between Plants and Biotic Agents", together with Willie Commelin Scholten Day, Utrecht, NL	22 Jan 2009
EPS theme 2 symposium "Interactions between Plants and Biotic Agents", together with Willie Commeling Scholten Day, Wageningen, NL	12 Feb 2012
EPS theme 3 symposium "Metabolism and Adapatation", Wageningen, NL	14 Mar 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Platform molecular genetics annual meeting (NWO), Lunteren, NL	15-16 Oct 2009
► **Seminars (series), workshops and symposia**	
Wageningen Phytopathology symposium, Wageningen, NL	22 Jun 2007
Microarray workshop, Leiden, NL	21 Sep 2007
Mini-symposium Embrapa-PRI-CBS projects on Mycosphaerella/banana, Wageningen, NL	06 Oct 2007
Seminar Richard Olivier, "Genomics of Stagonospora nodorum: genews, genomes and growers"	16 Oct 2008
KNPV Fast forward Sping meeting, Wageningen, NL	25 May 2009
Flying Seminar Ricardo Oliva	17 Sep 2009
Seminar Series Plant Sciences at Plant Sciences Group, Wageningen, NL	13 Oct 2009
KNPV Fast forward Sping meeting, Wageningen, NL	06 Apr 2010
Seminar Paul Birch, "Trying to understand susceptibility and exploit resistance in potato"	20 May 2010
Seminar "CLC Genomics"	09 Mar 2011
INRA/WUR/Bioplante Collaborative workshop on wheat Septoria	07 Jun 2011
Seminar and master class Duncan Greig "Sexual selection in yeast"	21 Jun 2012
Seminar Gabino Sánchez Pérez	12 Mar 2013
Seminar Brian Staskawicz, "Effector-Targeted Breeding for Durable Disease Control of Xanthomonas dieases in tomato and cassava"	21 May 2013
Seminar Michael Freitag, "Chromatin structure controls centromeres and secondary metabolism in filamentous fungi"	21 Oct 2014
Banana Wageningen Day and Master class workshop, Wageningen, NL	20 Nov 2014
► **Seminar plus**	
Michael Freitag	21 Oct 2014
► **International symposia and congresses**	
9th European conference of Fungal Genetics, Edinburgh, Scotland	27-30 Mar 2008
7th International Mycosphaerella and Stagonospora Symposium, Ascona, Switzerland	18-22 Aug 2008
10th European conference of Fungal Genetics, Noordwijkerhout, NL	29 Mar-01 Apr 2010
► **Presentations**	
Talk: CBS/PRI/Wageningen Phytopathology symposium	22 Jun 2007
Poster: 9th European conference of Fungal Genetics, Edinburgh, Scotland	27-30 Mar 2008
Poster: 7th International Mycosphaerella and Stagonospora Symposium, Ascona, Switzerland	18-22 Aug 2008
Talk: KNPV Fast Forward Spring meeting	25 May 2009
Poster: Autum School "Host-Microbe Interactomics"	01-03 Nov 2011
Talk: Nutritionist Day at University Del Valle Mexico, Merida, Mexico "An approach to nutrigenomics"	12 Feb 2014
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	02 Dec 2009
► **Excursions**	
Bioplante Field for breeding breeding for resistance to Septoria and Fusarium, Florimond Desprez, Lille, France	2008
Catholic University of Leuven, Lab. of Tropical Crop Improvement, Belgium	15 Sep 2009
Subtotal Scientific Exposure	*16.1 credits**

CONTINUED ON NEXT PAGE

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Advanced course "Confocal ligth microscopy", Amsterdam, NL	02-06 Jun 2008
Summer school "On the evolution of plant pathogen interactions: from principles to practice", Wageningen, NL	18-20 Jun 2008
Advanced course "Systems biology: Statistics of ~omics data analysis", Wageningen, NL	08-11 Dec 2008
Autum School "Host-Microbe Interactomics", Wageningen, NL	01-03 Nov 2011
Advanced course 'Bio-energy Production from Crop Plants and Algae', Wageningen, NL	17-19 Nov 2014
► **Journal club**	
Member of a literature discussion group at phytopathology lab	2007-2011
► **Individual research training**	
Subtotal In-Depth Studies	*6.6 credits**

4) Personal development	date
► **Skill training courses**	
Scientific Writing	Sep-Nov 2009
Advanced Course "Guide to Scientific Artwork", Wageningen, NL	07-08 May 2012
► **Organisation of PhD students day, course or conference**	
Visit of Fresh Studio Innovations Asia & Bioversity International (Asia) to Banana group research and facilities	13 & 25 Sep 2011
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	38,0

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* *A credit represents a normative study load of 28 hours of study.*

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