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Full Length Research Paper

Polymorphism at selected defence gene analogs (DGAs) of *Musa* accessions in Mauritius

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One of the major diseases affecting banana is Sigatoka or leaf spot disease that comprises three species, *Mycosphaerella fijiensis*, *Mycosphaerella musicola and Mycosphaerella eumusae*. Plants have a large number of defence related genes which trigger a cascade of defense responses that halt the spread of pathogens. Knowledge of the diversity present in genes related to the defense against Sigatoka disease will be useful in developing disease resistant banana cultivars. The defence genes of all sterile commercial banana cultivars (AAA genomes) are considered to have arisen from a similar gene pool belonging to the *Musa acuminata* complex. The objectives of this study were, (i) to assess the disease response of twelve banana cultivars to *M. eumusae*, (ii) to assess the level of polymorphisms in selected genes associated with defence against Sigatoka in banana, and (iii) ascertain if this polymorphism was related to levels of resistance to *M. eumusae.* Defence genes reported to act in response to *M. fijiensis* were selected and related to the response of *M. eumusae*. The genetic diversity of selected defence gene analogs (DGA) was assessed using degenerate primers designed from conserved motifs in the aligned amino acid sequences from known resistance genes. Highly polymorphic amplicon profiles for DGAs were selected for comparison. Cluster analysis was used to differentiate to some extent, cultivars considered as resistant/susceptible to *M. eumusae*. Specific amplicons from the profiles of phenylalanine ammonia-lyase (PAL), iron superoxide dismutase (FeSOD) and ascorbate peroxidase (APX) were unique to a group of resistant cultivars and could act as markers for resistance to *M. eumusae*.

Key words: Banana, defence gene analogs, polymorphism.

INTRODUCTION

Banana (*Musa* sp.) is the developing world's fourth most important food crop after rice, wheat and maize (Arias et al., 2003). Bananas and plantains are attacked by several pathogens that cause severe crop losses (Pillay et al., 2002). Most commercial banana cultivars with an AAA genome composition were derived from wild diploids of the *Musa acuminata* complex that is native to the Malay Peninsula (Simmonds and Dodds, 1949). Continuous domestication of preferred genotypes together with vegetative propagation have eroded the genetic base of the crop, making most of the cultivars equally susceptible to major disease outbreaks. This was the case of the commercial cultivar 'Gros Michel' (AAA) which became susceptible to race 1 of *Fusarium oxysporum* f.sp. *cubense* (Molina et al., 2008). A more recent example is that of banana *Xanthomonas* wilt that is destroying a range of different banana cultivars in many farmers' fields in Eastern Africa (Biruma et al., 2007).

One of major diseases of bananas worldwide is the Sigatoka disease complex that is due to three species *Mycosphaerella fijiensis*, *Mycosphaerella musicola and Mycosphaerella eumusae* which are the causal agents of black Sigatoka, yellow Sigatoka and *Septoria* leaf spot, respectively. Sigatoka and *Septoria* do not kill the plant immediately, but crop losses increases gradually as the plants get older. The quantity and quality of the yield is

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Abbreviations: DGAs, Defence gene analogs; **PAL,** phenylalanine ammonia-lyase; **FeSOD,** iron superoxide dismutase; **APX,** ascorbate peroxidase.

Table 1. Banana cultivars, their genomes and their known reactions to *M. fijiensis* used in this study.

cultivars	Genome	Resistance level	Source
Petite Naine (AAA)	AAA	Susceptible	Johanson, 1997
French Clair (plantain)	AAB	Susceptible	Pefoura et al., 2001
Williams (Cavendish)	AAA	Highly susceptible	Reyes-Borja et al., 2007
FHIA21	AAAB	Partial resistance	Tirado and Zapata, 2003
Pisang Ceylan (Mysore))	AAB	Partial resistance	Mourichon et al., 1997
Pisang Mas	AA	Partial resistance or Susceptible	Mourichon et al., 1997; Pefoura et al., 2001
FHIA17	AAAA	Hypersensitive type resistance	Mourichon et al., 1997
Yangambi km5	AAA	Hypersensitive type resistance	Mourichon et al., 1997
GCTCV-119 (ex-Taiwan Cavendish)	AAA	Highly tolerant	Nakyanzi, 2002
FHIA18	AAAB	Highly tolerant	Nakyanzi, 2002
Gingeli 1	Unknown	Unknown	

reduced drastically in extreme cases. Commercial plantations producing bananas for export rely on a costly fungicide spray program to control black Sigatoka (Sagi et al., 1998). Genetic resistance is regarded as the most suitable strategy to control the many pathogens that affect banana. However, breeding for resistance in banana is a slow process due to the long life cycle, triploidy and sterility of most cultivars (Pillay et al., 2002; Pillay and Tripathi 2007).

Molecular breeding for genetic control of resistance is being recognized as the most economical and environmentally efficient solution for disease management (Dracatos et al., 2008). Plants defend themselves against attack by pathogens with a large number of diverse defence related genes that are involved in detection of pathogens, signalling, pathogen attack, and defence or cell death (Dreher and Callis, 2007). For efficient utilization of these genes, it is necessary to characterize them. All plant species contain families of disease related genes that have conserved domains. Polymerase chain reaction (PCR) amplification with degenerate primers designed from key conserved motifs has been used to amplify resistant gene analogues (RGAs) of many plant species including banana (Pei et al., 2007; Peraza-Echeverria, 2007, Azhar and Heslop-Harrison, 2008). An understanding of the variation pattern in disease resistance (R) genes is essential for its use in breeding programs aimed at neutralizing the threat of pathogens (Ding et al., 2007).

The objectives of this study were; (i) to assess the disease response of twelve banana cultivars to *M. eumusae*, (ii) to assess the level of polymorphisms in selected genes associated with defence against Sigatoka in banana, and (iii) ascertain if this polymorphism was related to levels of resistance to *M. eumusae. M. fijiensis* is not found in Mauritius. Consequently defence genes reported to act in response to *M. fijiensis* were selected and related to the response of *M. eumusae*. The selected genes included iron superoxide dismutase (FeSOD), copper-zinc superoxide dismutase (CuZnSOD), ascorbate peroxidase (APX), catalase, peroxidase, and ferredoxin NADPH oxidoreductase (FNR) associated with the antioxidant cycle, key genes of the phenylpropanoid pathway including phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), and chalcone synthase (CHS). Osmotin, a pathogenesis related protein PR-5 (Selitrennikoff, 2001), the transcription factors WRKY and R2R3Myb, two chaperones HSP70 and HSP90 and an NBS-LRR type R-gene were also included. The phenylpropanoid pathway has been linked to resistance to diseases such as *Erwinia amylovora* (fire blight) in apple (Pontais et al., 2004) and *Phytophthora infestans* in potato (Trognitz et al., 2001). Previous studies showed that *P. infestans* induces the accumulation of osmotin-like proteins, while WRKY transcription factors control resistance responses in diseased potato (Lyon, 1997).

MATERIALS AND METHODS

The plant material used in this study (Table 1) was obtained from The Agricultural Research and Extension Unit (AREU) and The Food and Agricultural Research Council (FARC) in Mauritius. The plants were selected on the basis of their resistance or susceptibility to *M. fijiensis*. In addition, the study also included two common monocots maize and rice and two closely related members of banana, *Ravenala madagascariensis* and *Heliconia* sp.

Determining resistance levels for *M. eumusae*

Mycosphaerella fijiensis is not present in Mauritius. However, the closely related disease *M. eumusae* with similar symptoms as *M. fijiensis* has been identified in the country and affects locally grown bananas. A strain of *M. eumusae* obtained from Mauritius was used as inoculum in this study. The conidia were isolated and identified morphologically with a microscope as described by Faerraez et al. (2008). The study was conducted in the University fields, Reduit, Mauritius. Mature plants over 1 m in height were inoculated twice on the same day, once in the morning and once in the afternoon. The lower surfaces of the youngest two leaves of the plants were inoculated by spraying with conidia (2×10^5 conidia per ml). Two plants per cultivar were inoculated (four replicates). As a control, the third youngest leaf of each replicate was inoculated with distilled

water containing 1% gelatine. The average daily temperature at Reduit, Mauritius, during the time of inoculation was 22°C. Disease symptoms were recorded after every four days from inoculation. Data was taken 13 times for each infected plant until day 52.

Six stages of infection are recognised in symptom development for *M. fijiensis*. The same stages were used to describe the infection by *M. eumusae* since the two diseases are considered to produce similar symptoms (Carlier et al., 2000). These stages are as follows:

Stage 1: A small whitish or yellow coloured spot appears.
Stage 2: A stripe appears, generally brown in colour and visible on the underside of the leaf. Stage 3: The stripes become longer, wider and can reach a length of 2 or 3 cm.
Stage 4: Brown spots appear on the underside while black spots appear on upper side of the leaf.
Stage 5: Elliptical spots that are totally black appear and spread to the underside of the leaf. The elliptical spots are then surrounded by a yellow halo with the centre that begins to flatten out. Stage 6: The centre of the spot dries out, turns light grey, and is surrounded by a well-defined black ring, which is, in turn, surrounded by a bright yellow halo.

A modification of the Fullerton and Olsen (1995) system was used to assess the resistance reaction of the cultivars. The assessment is defined by the most advanced stage of symptom observed at specific points in time. The different stages of symptom development were then associated with specific grades as follows: grade 1 was defined as small necrotic spots from day zero to day 52 and response is hypersensitive type. Grades two, three, four and five are and defined by stages one, two, three and four at 52 days, respectively. Grades six, seven and eight are and defined as stage five, early stage six and late stage six (complete necrosis) at 52 days, respectively.

DNA extraction

Approximately 1 g of young leaf material was ground in liquid nitrogen with a mortar and pestle. and placed in 7.5 ml extraction buffer (1.5% sodium dodecyl sulphate (SDS), 0.3% β-mercaptoethanol, 0.7 M NaCl and 50 mM ethylene diamine tetra-acetic acid (EDTA), 50 mM Tris-HCl, pH 8 and 3% (V/V) PVP (Mr: 70,000) and incubated at 45°C for 2 h.

The samples were extracted with 15 ml of chloroform: isoamyl alcohol (24:1, v/v) and centrifuged at 6000 rpm for 5 min. The upper aqueous phase was transferred to a new tube and extracted as before with chloroform: isoamyl alcohol. The DNA was precipitated by adding two-thirds volume of ice-cold isopropanol and 0.1 M NaCl and recovered by centrifugation at 6000 rpm for 5 min. After centrifugation, the pellet was washed with 95% ethanol dried and dissolved in distilled water.

Primer design

Sequences of the following genes from NCBI were aligned using MultAlin (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988).

PAL. |29691920|emb|AJ555536.1|,
|21264489|sp|P45724|PAL2_ARATH, |534893|emb|CAA57056.1| ,
|14326459|gb|AAK60275.1|AF383152_1,
|7208616|gb|AAF40224.1|AF237955_1,
|7208616|gb|AAF40224.1|AF237955_1,
|23451811|gb|AAN32867.1|AF460204_1,
|1709563|sp|P53443|PAL2_ORYSA,
|3024361|sp|Q42858|PAL2_IPOBA, |44889624|gb|AAS48415.1|,
|62736192|gb|AAX97448.1|, |29691921|emb|CAD88242.1|,
|1483612|emb|CAA68064.1|;

CuZnSOD. |113367099|gb|DQ866814.1|SOD1,
|2645997|gb|AAB87572.1|, |2305111|gb|AAD05576.1|,
|2305109|gb|AAB66812.1|,
|21542454|sp|O78310|SODCP_ARATH,|134682|sp|P14831|SODCP_LYCES,|47117142|sp|Q7M1R5|SODC_SOYBN,
|586004|sp|Q07796|SODC_IPOBA,
|134595|sp|P28756|SODC1_ORYSA,
|12744890|gb|AAK06837.1|AF328859_1,
|1204052|emb|CAA65041.1|, |1204050|emb|CAA65043.1|,
|854248|emb|CAA60826.1|, |39840779|emb|CAD21706.2|,
|46810649:1-643, |2997702|gb|AAC08581.1|,
|27449246|gb|AAO14117.1|AF457209_1;

WRKY. |52631990|gb|AY655725.1|, |51854273|gb|AAU10654.1|,
|50843954|gb|AAT84155.1|, |46394352|tpg|DAA05114.1|,
|54290337|dbj|BAD61141.1|,
|23305051|gb|AAN16970.1|AF459793_1,
|49388020|dbj|BAD25136.1,
|29839675|sp|Q9SI37|WRKY1_ARATH,
|20978796|sp|Q9XI90|WRKY4_ARATH,
|29839696|sp|Q9ZUU0|WRK44_ARATH,
|29839654|sp|Q9M8M6|WRK66_ARATH,
|29839580|sp|Q8VWQ5|WRK50_ARATH,
|20978784|sp|Q9CAR4|WRK36_ARATH,
|15991724|gb|AAL13039.1|AF418308_1,
|50938955|ref|XP_479005.1|;

Ascorbate peroxidase. |5758111|gb|AAD50682.1|,
|2586150|gb|AF001529.1|AF001529, |34394032|dbj|BAC84063.1|,
|50725765|dbj|BAD33296.1|, |50909385|ref|XP_466181.1|,
|42407723|dbj|BAD08870.1|, |50947721|ref|XP_483388.1|,
|217833|dbj|BAA03334.1|, |17066705|gb|AAL35365.1|AF442387_1,
|39939493|gb|AAR32786.1|, |68342454|gb|AAY90125.1|,
|6066418|emb|CAB58361.1|, |217833|dbj|BAA03334.1|;

Osmotin. |19783|emb|CAA46623.1|, |1196835|gb|AAB67852.1|,
|887390|emb|CAA61411.1|,
|14165167|gb|AAK55411.1|AF376058_1,
|2854101|gb|AAC02549.1|;

CHI. |145359723|ref|NP_201423.2|, |73696207|gb|AAZ80911.1|,
|166398|gb|AAB41524.1|, |20982|emb|CAA78763.1|,
|27530707|dbj|BAC54038.1|,
|21327031|gb|AAM48130.1|AF509335_1;

FeSOD. |74229683|gb|ABA00456.1|, |74483452|gb|ABA10481.1|,
|74483450|gb|ABA10480.1|, |74483448|gb|ABA10479.1|,
|74483448|gb|ABA10479.1|, |33439120|gb|AAQ18699.1|,
|3719455|gb|AAC63378.1|, |37654895|gb|AAQ13492.1|,
|6840824|gb|AAF28773.1|AF077224_1,
|27762607|gb|AAO20086.1|, |27543371|gb|AAO16563.1|,
|16974682|gb|AAL32441.1|AF377344_1;

Catalase. |109390456|gb|ABG33767.1|, |20192|emb|CAA43814.1|,
|18394888|ref|NP_564120.1| CAT3, |22234|emb|CAA38588.1|,
|6006609|emb|CAB56850.1|, |90818818|emb|CAI43950.1|,
|12002676|gb|AAG43363.1|AF151368_1,
|6980084|gb|AAF34718.1|AF227952_1.

The most up- and down-stream conserved consensus sequences of at least seven amino acids long were retained. The codon usage table for *M. acuminata* from Kasuza (http://www.kazusa.or.jp/codon/) (Nakamura et al., 2000) was used to translate the Amino acid sequences (Figure 1) using Entelechon

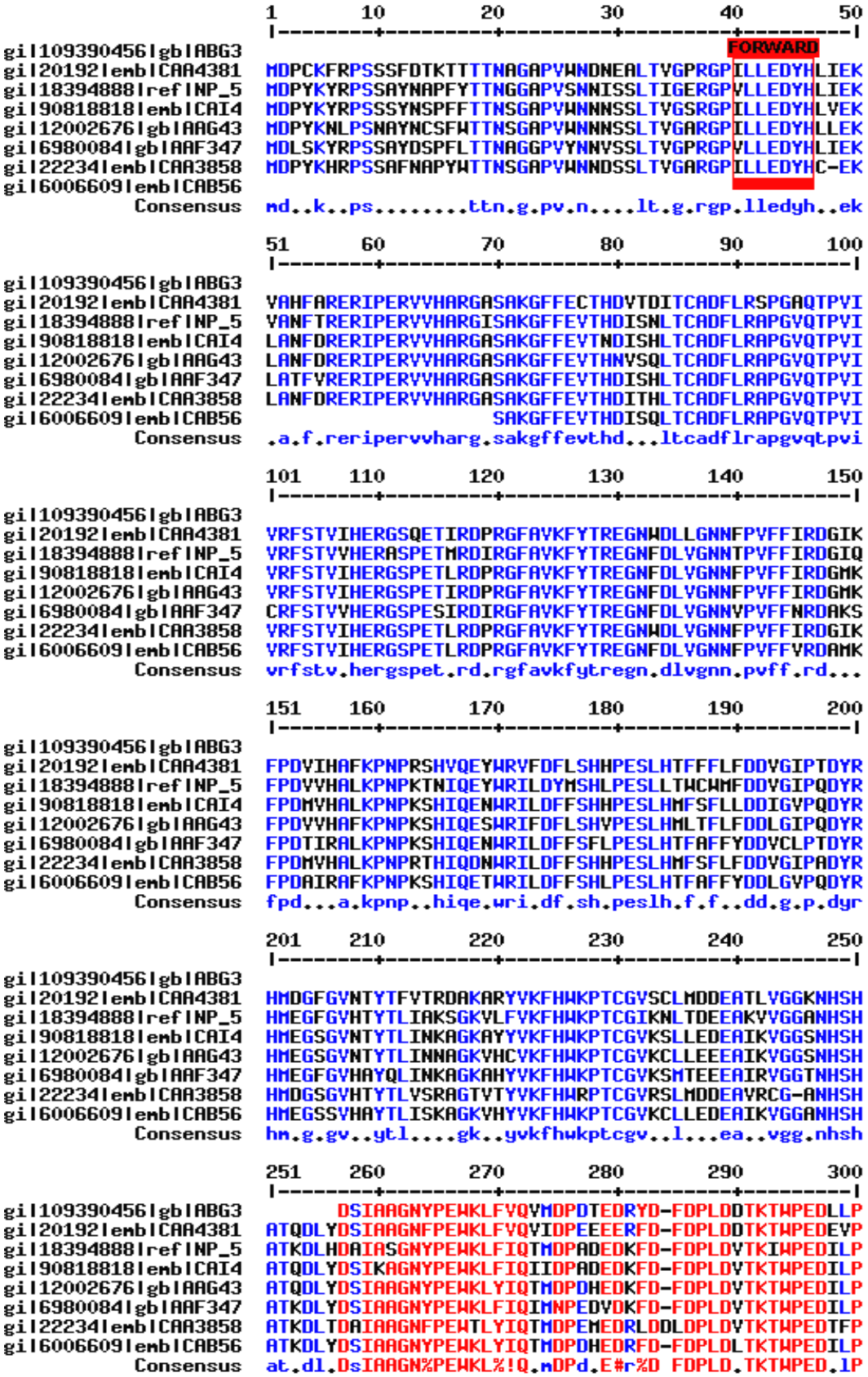

Figure 1. Amino acid sequence alignment on MultAlin from different accessions for the catalase gene. The same primers design procedure was repeated for all the genes selected in this study.

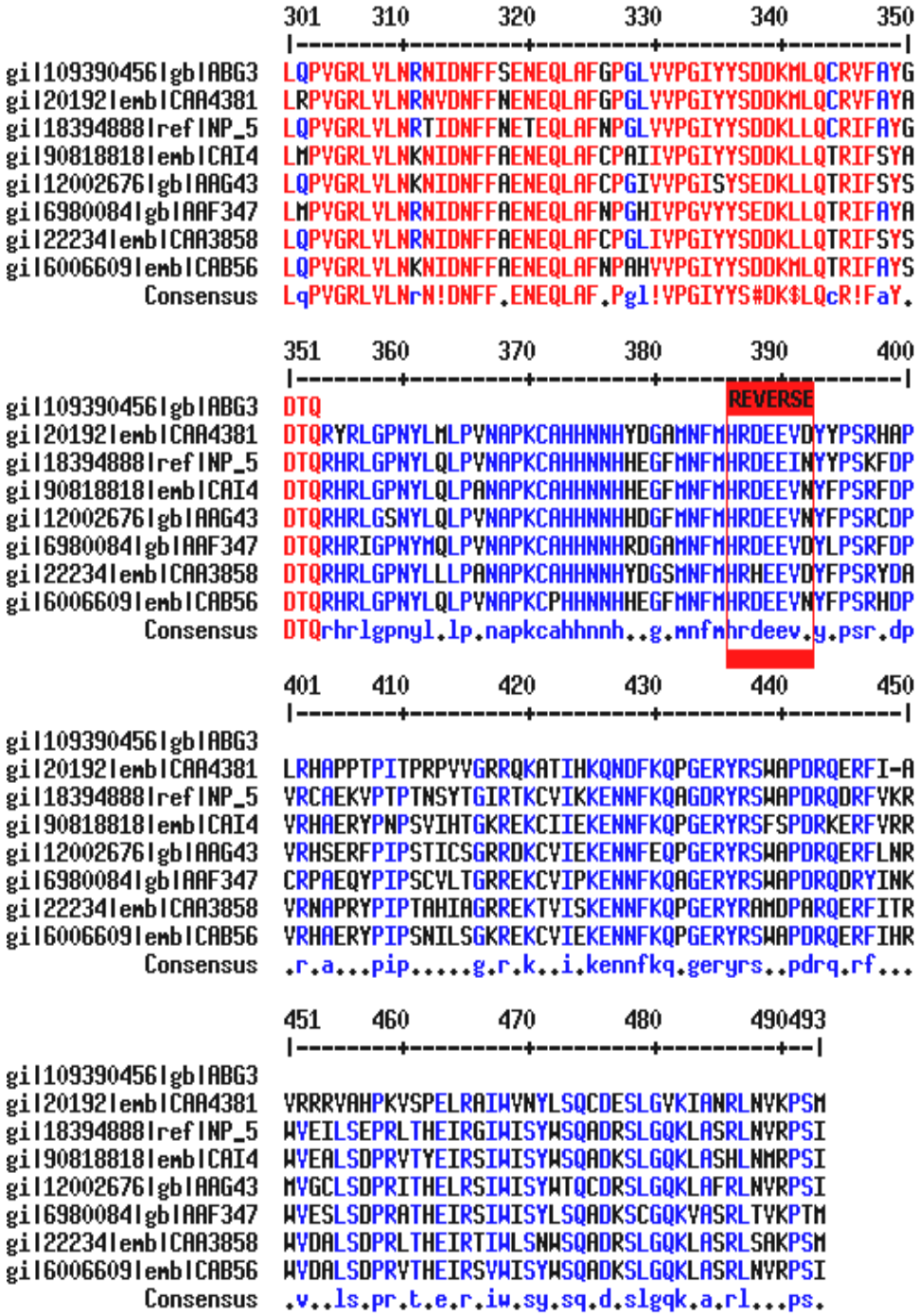

Figure 1. Contd.

(http://www.entelechon.com/backtranslation) (Markus, 2006). The program Primer3 (http://fokker.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000) was used to determine the best possible reverse and forward primers. Entelechon (http://www.entelechon.com/backtranslation) (Markus, 2006) was used to design degenerate primers since it provides several possible DNA consensus sequences from one single amino acid consensus sequence based on the codon usage bias of banana. The results from Entelechon were aligned to the primers obtained from Primer3 (http://fokker.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000). In cases where several possible DNA bases are present in the alignment are degenerate, such degenerate positions were converted into IUB code. Nucleotide positions which were degenerate were then replaced by either the rice or *Zantedeschia aethiopica* base at the aligned position to obtain specific primers. In addition, specific primers were designed from complete gene sequences of the catalase gene from *Z. aethiopica* (Arum Lily) and *Oryza sativa* (rice). All the primers were synthesized at Inqaba Biotech, Pretoria, South Africa.

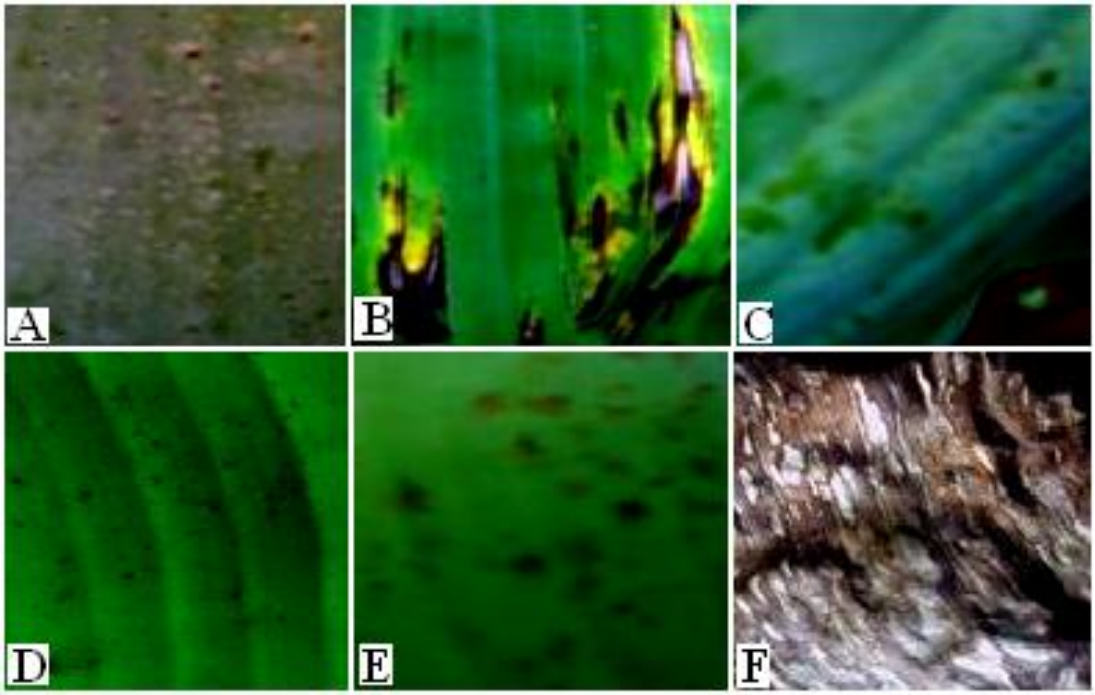

Figure 2. Typical symptoms of *M. eumusae* observed in different banana cultivars (some not used in this study). A, Small whitish spots not visible in transmitted light and present only on the lower leaf surface at 20 days (stage 1 symptoms) on 'Gingeli'; B, upper leaf of 'Gingeli' at 52 days with stage three symptoms; C, light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days on lower leaf of 'Ginzli Savanne' (stage two symptoms); D, small necrotic specks at 20 days on lower leaf of 'Yangambi km5' (hypersensitive response); E, necrotic specks at 52 days on lower leaf of 'Yangambi km5' (hypersensitive response); F, complete necrosis and presence of grey spots at 52 days on upper leaf of Petite Naine (stage six symptoms).

PCR protocols

The primers were used in standard PCR amplification reactions of 25 µL, each containing 200 ng DNA, 1x Buffer, 2.0 mM $MgCl_2$, 30 pmol primer, 0.2 mM dNTP, and 1.25 U Taq polymerase (Bioline, London, UK). For long PCR, the long PCR enzyme mix from Fermentas (#K0181) was used in 25 µL reactions consisting of: buffer with $MgCl_2$: 1X + 1.5 mM Mg^{2+}, 30 pmol primer, 0.2 mM dNTP, 2.5 units Taq polymerase, 200 ng DNA. The PCR conditions were: 95°C for 5 min, 45 cycles of 95°C for 30 s, X°C for 30 s and 72°C for 2 min. The final cycle was 72°C for 10 min. 'X' is the annealing temperature. The touchdown PCR conditions included: 95°C for 5 min, 10 cycles starting at T_a: X°C decreasing by 0.6°C for each cycle followed by 35 cycles at 95°C for 30 s, X-6°C for 30 s and 72°C for 2 min. The final cycle was 72°C for 10 min. 'X' is the annealing temperature. Furthermore, the Expand™ Taq PCR conditions were: 95°C for 5 min, 45 cycles of 95°C for 30 s, X°C for 30 s and 68°C for 4 or 5 min. The final cycle was 68°C for 15 min. 'X' is the annealing temperature. In each case, three PCR replicates were performed with three DNA extractions from three different plants of the same cultivar- two that were inoculated and one uninoculated.

Clustering of defence gene analogs using Winclada

DNA bands were scored as present (1) and absent (0). A matrix of 81 informative characters (binary state – non additive Fitch) was constructed in WinDada [from the Winclada (Nixon, 2002) suite of programs], with only polymorphic data across cultivars from PCR amplicon profiles and Southern blots and analysed on Nona (Nixon, 2002) with heuristics. The bootstrap was set at 1500 replications.

RESULTS

Resistance response of banana cultivars to *M. eumusae*

The disease response of the different accessions used in this study to *M. eumusae* is listed in Table 3. A modification of the Fullerton and Olsen (1995) grade system was used for resistance reaction assessment. The assessment is defined by the most advanced stage of symptom observed at specific points in time. The different stages of symptom development are then associated with specific grades. Grade one was defined as small necrotic spots from day zero to day 52 and response is hypersensitive type. Grades two, three, four and five are resistant and defined by stages one, two, three and four at 52 days respectively. Grades six, seven and eight are susceptible and defined as stage five, early stage six and late stage six (complete necrosis) at 52 days, respectively. Typical disease symptoms are shown in Figure 2.

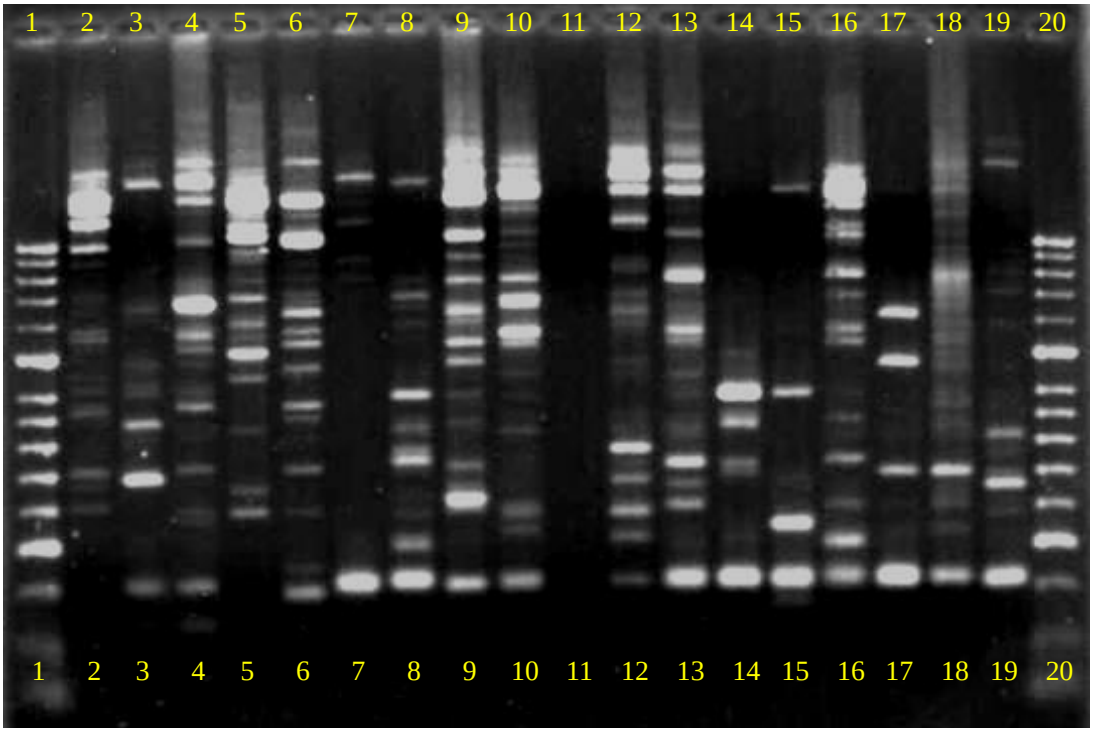

Figure 3. Banding profiles obtained with degenerate primers of APX T4. The cultivars and lanes are: *Heliconia*; 2, *Ravenala*; 3, Ravenala; 4, French Clair; 5, Enano Currare; 6, FHIA21; 7, FHIA18; 8, FHIA17; 9, Pisang Ceylan; 10, Pisang Mas; 11, -ve control; 12, Gingeli 2; 13, Gingeli 1; 14, Yangambi Km5; 15, Williams; 16, Petite Naine; 17, GCTCV119; 18, maize; 19, rice. Lanes 1 and 20 are the Hyperladder II marker (Bioline). APX, ascorbate peroxidase.

Amplicon profiles

The amplicon profiles obtained for the different banana cultivars and *Ravenala madagascariensis*, *Heliconia*, maize and rice for ascorbate peroxidase (T4 primers); iron superoxide dismutase (T1 primers) and phenylalanine ammonia-lyase (T6 primers) are shown in Figures 3, 4 and 5, respectively.

Individual amplicons occurring only in resistant cultivars

Despite being the most polymorphic amplicon profile obtained in all the sets tested, five amplicons from ascorbate peroxidase T4 primer set (shown with red arrows in Figure 6) are common in several resistant cultivars but absent in several susceptible onces as shown in Figure 6.

Dendrogram showing the clustering of cultivars based on defence gene analogs (DGA) amplicons and Southern blots

A dendrogram was constructed using only those PCR and hybridisation profiles with the highest polymorphism. These were obtained from FeSOD, CuZnSOD, APX, PAL, CHI and osmotin. The dendrogram (Figure 7) was constructed using WinClada.

For the combination of defence genes assessed, the three susceptible cultivars (Williams, GCTCV119 and Petite Naine) are in clades different from the resistant ones. Out of three susceptible cultivars, two are within the same clade (Williams and GCTCV119) and two highly tolerant cultivars are within the same clade (FHIA18 and Pisang Mas). Petite Naine is in a clade separate from all other *Musa* cultivars. *Heliconia* is the outgroup of all *Musa* cultivars. The cultivars in the same clade do not necessarily have the same ploidy level.

DISCUSSION

Disease response of cultivars to *M. eumusae*

Mycosphaerella eumusae is the most recently identified species in the Sigatoka disease complex which also includes *M. fijiensis* and *M. musicola* (Carlier et al., 2000). It was identified in the mid 1990s. The three *Mycosphaerella* species have been reported to produce very similar symptoms in susceptible banana cultivars (Carlier et al., 2000).

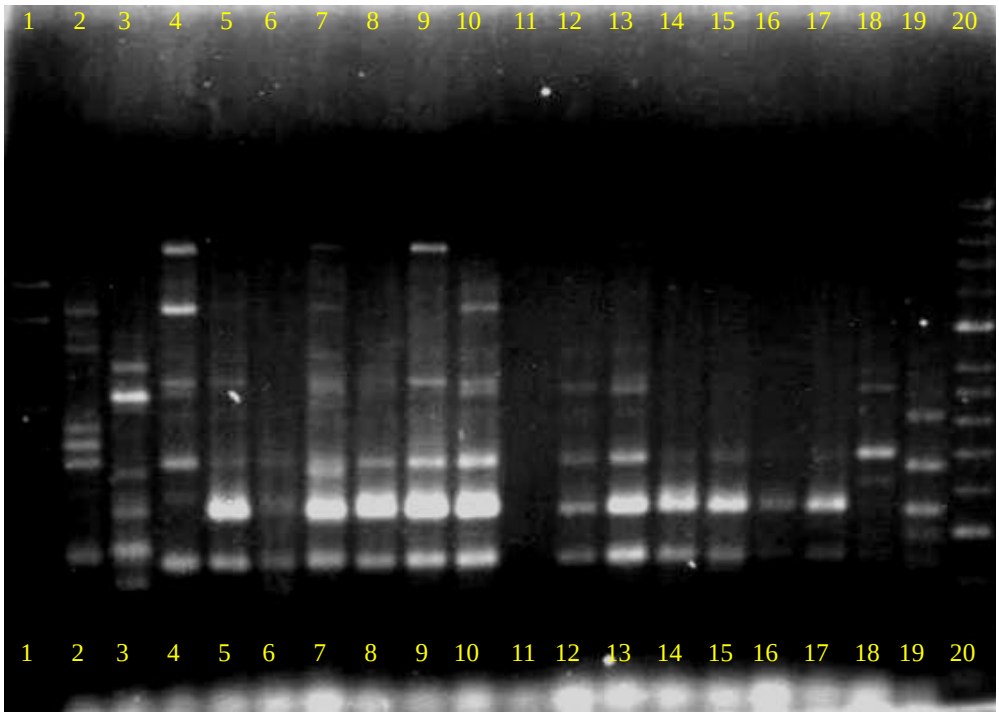

Figure 4. Amplification pattern of RGAs with primer pair T1. Accessions and lanes are: 2, *Heliconia*; 3, *Ravenala*; 4, French Clair; 5, Enano Currare; 6, FHIA21; 7, FHIA18; 8, FHIA17; 9, Pisang Ceylan; 10, Pisang Mas; 11, -ve control; 12, Gingeli 2; 13, Gingeli 1; 14, Yangambi Km5; 15, Williams; 16, Petite Naine; 17, GCTCV119; 18, maize; 19, rice. Lanes 1 and 20 represent the hyperladder II molecular weight marker (Bioline).

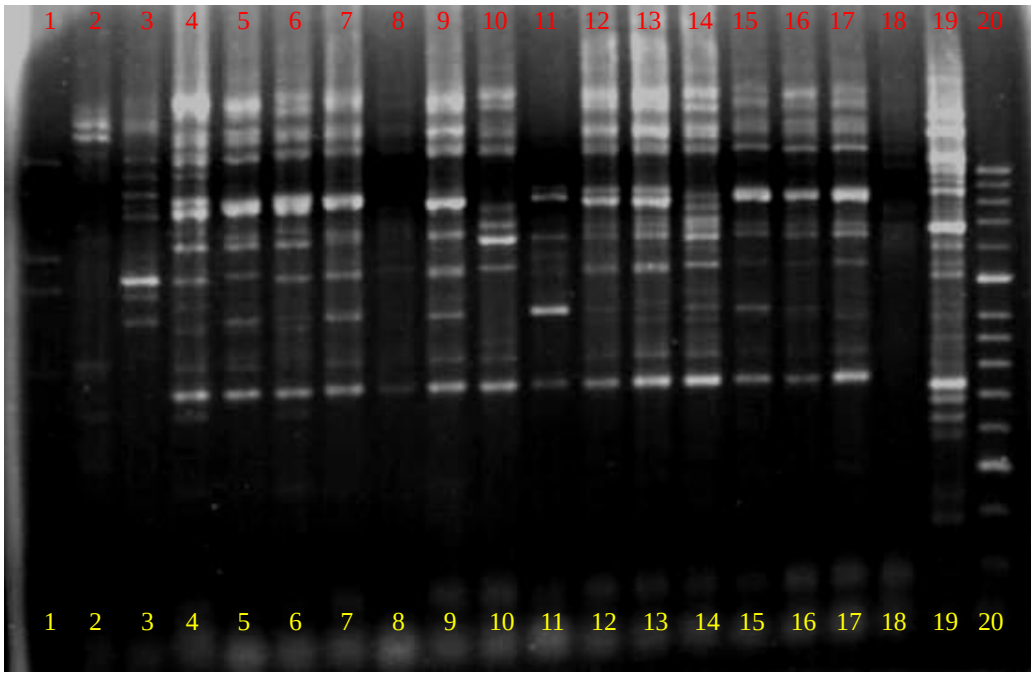

Figure 5. Amplicons obtained with PAL T6 primer with Expand (Fermentas #K0181). Accessions and lanes are: 1, Roche DIG-labelled Marker 6; 2, *Heliconia*, 3, *Ravenala*; 4, French Clair; 5, Enano Currare; 6, FHIA21; 7, FHIA18; 8, FHIA17; 9, Pisang Ceylan; 10, Pisang Mas; 11, Gingeli 3; 12, Gingeli 2; 13, Gingeli 1; 14, Yangambi Km5; 15, Williams; 16, Petite Naine; 17, GCTCV119. PAL, Phenylalanine ammonia-lyase.

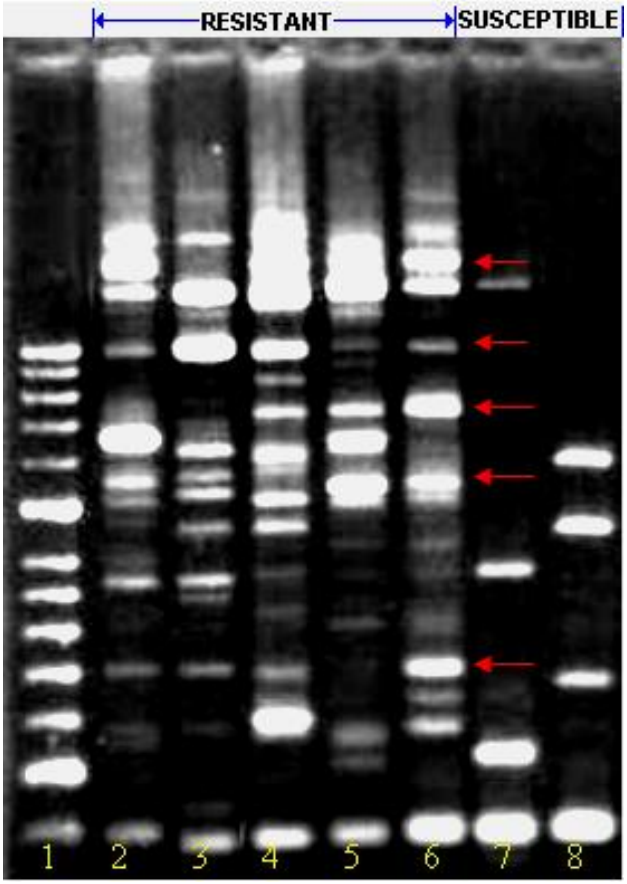

Figure 6. Some unique DNA fragments present in resistant cultivars (lanes 2 to 6) after PCR with primer APX T4. The resistant cultivars and lanes are: 2, French Clair; 3, FHIA21; 4, Pisang Ceylan; 5, Pisang Mas; 6, Gingeli 1; while the susceptible ones are: 7, Williams; 8, GCTCV119. APX, Ascorbate peroxidise.

Therefore it is believed that although *M. eumusae* was present for some time, its identity as a separate species was not easily recognizable. The disease has been detected in Nigeria, India, Malaysia, Thailand Sri Lanka, Vietnam, Mauritius and Reunion (Jones, 2003). Accessions affected by *M. eumusae* include the plantains (AAB), 'Grande naine' (AAA), 'Pisang kapas' (AA or AAB), 'Kluia hom thong' (AAA), 'Kluia lep munang' (AA), Cavendish (AAA), 'Anamala' (AAA, syn. 'Gros Michel'), 'Pisang Mas' (AA), 'Mysore', and 'Sucrier' (AA) (Jones, 2003). Udugama (2002) reported that the disease was also present in Sri Lanka and affected the cultivars, 'Embul' (AAB-Mysore group), 'Pooalu' (AAB-Pome group), 'Anamalu'and 'Bimkehel' (AAA- Cavendish subgroup). There is a single report showing that a banana cultivar resistant to *M. fijiensis* and *M. musicola* was found to be susceptible to *M. eumusae* (PaDIL Plant Biosecurity Toolbox- accessed 23/10/10). This is one of the first studies to assess the effect of *M. eumusae* in a range of banana cultivars. The study found that with the exception of 'French Claire' ('Obina L'ewai') and 'GCTCV-119', the resistance/susceptibility levels of the banana cultivars to *M. eumusae* are similar to that of *M. fijiensis*. 'French Claire' and 'GCTCV-119' were reported to be susceptible and highly tolerant to *M. fijiensis,* respectively (Pefoura et al., 2001; Nakyanzi, 2002).

However, this study found that 'French Claire' was resistant, while 'GCTCV-119' was susceptible to *M. eumusae*. The response of 'Pisang Mas' to *M. fijiensis* has been reported to vary considerably. The cultivar's response ranged from being highly resistant to susceptible against different strains of *M. fijiensis* (Fullerton and Olsen 1995) and partially resistant (Mourichon et al., 1997) or susceptible to *M. fijiensis* (Pefoura et al., 2001). This study found that 'Pisang Mas' was highly resistant to *M. eumusae.* Similarly, 'FHIA18' previously classified as partially resistant to *M. fijiensis* (Nakyanzi, 2002) was found to be susceptible to *M. eumusae*. To date, there are very few studies or reports about the effects of *M. eumusae* in different banana

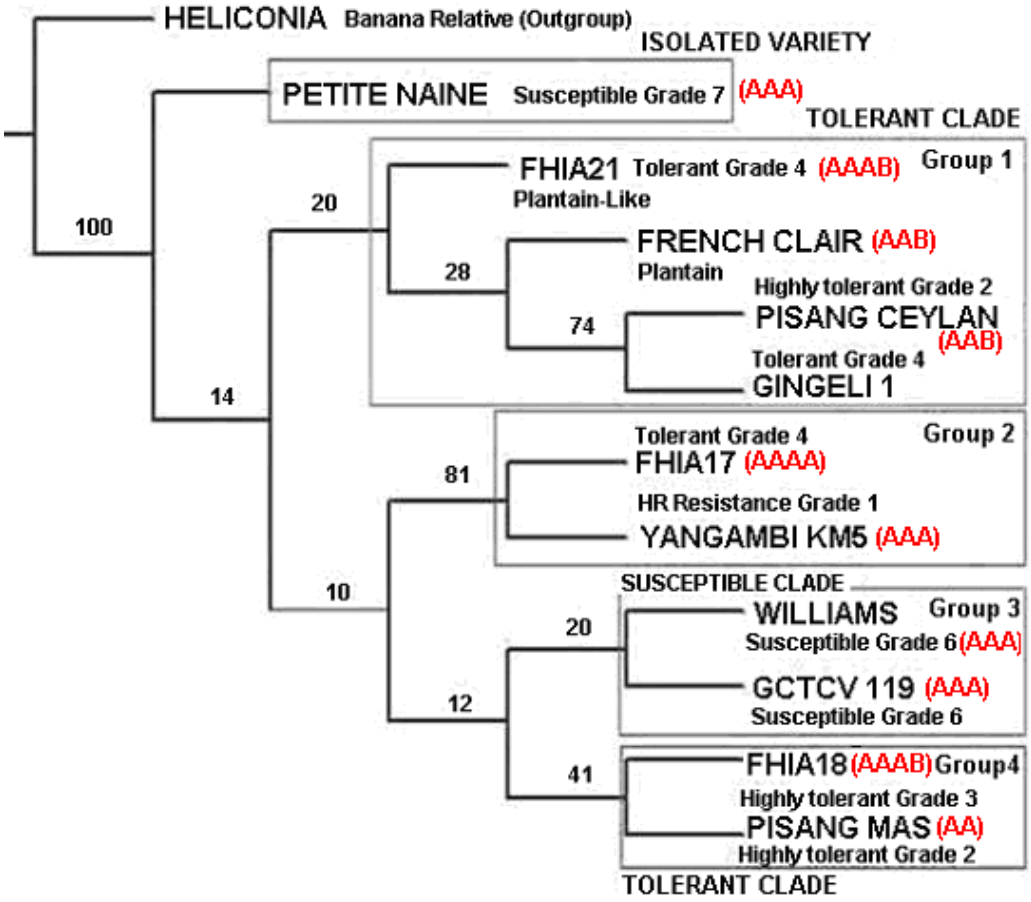

Figure 7. Clustering of cultivars based on defence gene analog amplicons. Branch lengths are proportional. A bootstrap value of 1500 was used. The resistance/susceptible grades were experimentally determined for each cultivar in the dendrogram. The resistance grade and level against *Mycosphaerella eumusae* was also included alongside each cultivar.

cultivars post artificial inoculation. *M. fijiensis* has several biological characteristics that make it more competitive than *M. musicola* (Romero, 2002). The results of this study suggest that *M. eumusae* may be even more pathogenic that *M. fijiensis* since some highly tolerant cultivars to the latter disease has been found to be susceptible to the former. However, more studies are necessary to validate this assumption. Jones (2003) stated that “the apparent dominance of *M. eumusae* in parts of Asia suggest that it was established before the introduction of *M. fijiensis*” and was able to resist the invasion by the latter.

Polymorphisms in resistant genes

In this study, 20 primer pairs developed from protein sequences representing 15 defense genes and/or transcription factors were used to amplify analogous genes in 12 banana cultivars and four monocotyledonous species (*Ravenala madagascariensis* , *Heliconia* sp., maize and rice). Nine of the primers (Table 2) produced highly polymorphic banding patterns among the samples, while 11 of the primers were not as informative. Primers that were highly polymorphic were linked to the six genes coding for APX (Figure 3), FeSOD (Figure 4), CuZnSOD, PAL (Figure 5), CHI and osmotin. The primers from the nine genes coding for catalase, peroxidase, FNR, chalcone synthase, R2R3Myb, WRKY, HSP70, HSP90 and NBS-LRR type R-gene were less informative. Consequently variation in only the former six genes will be considered for further discussion. The APX gene analogs showed the most significant polymorphism among the accessions even when the annealing temperature was set as high as 62°C. APX, superoxide dismutase and catalase function in the ascorbate-glutathione cycle to scavenge for active oxygen species that are produced during biotic and abiotic stressed plants (Mittler et al., 2004; Edreva, 2005). Antioxidants have been implicated in resistance against diseases in plants.

There are seven members of the chalcone synthase gene family in rice and only one member in maize (Dixon et al., 2002). Five bands were observed in the case of all

Table 2. Genes, primer code, forward and reverse primers and annealing temperatures used in this study.

Gene	Primer code	Forward primer (5'→3')	Reverse primer (5'→3')	Temperature (°C)
FeSOD	T2	AGCTGAAGCCHCCHCCHTACN	CAGGAAACAAGCTTATCCAT	53
CuZnSOD	T3	GGGAAAGCACCACAGAACC	NACCCTSCCTCCGGCGTTS	57 - 51*
Ascorbate peroxidase	T4	CTSTCCCACGGMGCCAACN	NGTTTGSWSGTSAGGAARG	62Ⅱ
Ascorbate peroxidase	T5	AAGAAGCAGAGGAGCATGGA	AGGTTGAGGCCTGGAGGAT	62
PAL	T6	ACGAGGTCAAGAGGATGGTGN	NGCAGATWGGCAGWGGGGCGCCGTTCC	57Ⅱ
PAL	T7	CAACAACCTCCACAACAACG	TGCCGAGCTCCTCGCTCACG	62
CHI	T8	VGGVGCCGGSAAAN	NACACKCCGTGCTCKCC	52 - 46*
PR-5 (osmotin)	T9	NSCTAYACSGTSTGGAAGCN	NGCAVGTGAAVGTGGAVGT	53
Catalase	G3	ATCCTGCTGGAGGACTACCA	GTTCACCTCCTCGTCGCTGT	53
Peroxidase	P1	TTYCACGACTGCTTYGTGN	NRATSTBGCCCATCTTVAYCA	54 - 48*
Peroxidase	P2	TTCCACGACTGCTTCGTG	CTACAGCGGGTAGAAATTGT	50
Ferredoxin NADPH oxidoreductase (FNR)	F1	AATGATAGCCACGGGAACAG	AGACCTCAACGTTCCACTGC	50
WRKY1	G1	TGGTAGTCCGCGACCGA	CCCGAACGGGAACAGCAC	50
WRKY2	G2	TTTGGGGGACAGGCCCGG	GAACGGGAGCATCCACCA	50
R2R3Myb	R1	RTGYGGCAAGTCCTGYAGRN	NGCGCRTTCCAGTARTTCTTGAT	58 - 52*
HSP70	H1	GCCATYGGCATYGACCTGN	NCCTGGTACATCTTGGCRATRA	59 - 53*
NBS-LRR type R-gene	N1?	GGRGGVGTGGGSAAGACSACSN	NSARGGCSARTGGSARGCC	55

*Denotes reactions using touchdown PCR temperatures; Ⅱdenotes reactions with Expand (Fermentas) PCR. PAL, Phenylalanine ammonia-lyase; FeSOD, iron superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase; PCR, polymerase chain reaction.

banana cultivars in this study (data not shown). No polymorphism was found among 'FHIA 21', 'FHIA 18', 'Williams', 'GCTCV119' and 'Petite Naine' for this gene family. In contrast, *Heliconia* produced three polymorphic bands while sugarcane produced four polymorphic bands (data not shown). Significant polymorphism was observed among the banana cultivars for the chalcone synthase gene with a maximum of twelve bands appearing in the case of 'Pisang ceylan'. Rice has only one copy of CHI gene while maize has two (Dixon et al., 2002). The polymorphism that exists among the banana cultivars for the enzymes of the antioxidant cycle could perhaps indicate the differential ability of the cultivars to resist the attack from *M. eumusae*. Similar finding were reported for *M. fijiensis* (Busogoro et al., 2004a, 2004b) and should be further explored. The banding patterns observed with primers for ascorbate peroxidase, phenylalanine ammonia lyase and iron superoxide dismutase revealed that several similar sized amplicons were present only in resistant cultivars and absent in the susceptible ones within each gene.

Meanwhile, one of the most interesting insights of this study was that the polymorphic banding patterns in the APX gene were able to identify resistant from susceptible cultivars (Figure 7) for *M. eumusae*. Five unique fragments (500 bp, 1.1 kb, 1.5 kb, 2.0 kb, 2.5 kb) appeared in the resistant cultivars 'French Clair', 'FHIA21', 'Pisang Ceylan', 'Pisang Mas' and 'Gingeli 1'. Previous studies on variation patterns in R genes point to two contrasting scenarios. Many R genes are hypervariable, among populations, such as *Rpp13* in Arabidopsis (Rose et al., 2004) and *L* in flax (Ellis et al., 1999). A low level of nucleotide diversity was observed in many conservative R genes, such as *Rpm1* and *Pi-ta* (Bergelson et al., 2001; Jia et al., 2003, Shen et al., 2006). It has been suggested that plants should theoretically have a large number of R genes to withstand the attack of the many pathogens and fast evolving avirulence genes (Dangl and Jones, 2001). The number of the most prevalent R genes in rice and *Arabidopsis* were estimated to be 149 and 480, respectively (Meyers et al., 2003; Zhou et al., 2004). This number is considered to be too small to confer resistance to the multitude of pathogens that a plant species is likely to encounter (Dangl and Jones, 2001). The large variation seen in the RDA genes in this study is perhaps one way of explaining the need for a large number of virulence genes in plants.

Table 3. Response of banana accessions to *M. eumusae* in this study.

Cultivar	Genome	Response to *M. fijiensis* from literature source	Observed symptom	Grade	Response to *M. eumusae* (this study)
1. Petite Naine	AAA	Susceptible[1]	Centre spot dries, turns grey & surrounded by a well-defined black ring, surrounded by a bright yellow halo at 52 days	7	Susceptible
2. French Clair (plantain)	AAB	Susceptible[2]	Elliptical spot totally black & spread to underside of leaf. Surrounded by yellow halo with centre beginning to flatten out at 52 days	6	Susceptible
3. Williams (Cavendish)	AAA	Highly susceptible [3]	Elliptical spot totally black & spread to underside of leaf. Surrounded by yellow halo with centre beginning to flatten out at 52 days	6	Susceptible
4. FHIA21	AAAB	Partial resistance[4]	Light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days at 52 days.	3	Resistant
5. Pisang Ceylan (Mysore))	AAB	Partial resistance[5]	Light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days at 52 days.	3	Resistant
6. Pisang Mas	AA	Partial resistance[6] or susceptibile[7]	Small whitish spots not visible in transmitted light and present only on the lower leaf surface at 20 days at 52 days.	2	Resistant
7. FHIA17	AAAA	Hypersensitive type resistance[8]	Light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days at 52 days.	3	Resistant
8. Yangambi km5	AA	Hypersensitive type resistance[9]	Small necrotic spots from day zero to day 52.	1	Hypersensitive response
9. GCTCV-119	AAA	Highly tolerant[10]	Elliptical spot totally black & spread to underside of leaf. Surrounded by yellow halo with centre beginning to flatten out at 52 days.	6	Susceptible
10. FHIA18	AAAB	Highly tolerant [11]	Light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days at 52 days.	3	Susceptible
11. Gingeli	Unknown	Unknown	Light brown flecks and an almost inconspicuous collapse of epidermal cells with pale-green ghost spotting at 20 days at 52 days	3	Resistant

[1]Johanson, 1997; [2,7]Mouliom-Pefoura et al., 2001; [3]Reyes-Borja et al., 2007; [4]Tirado and Zapata, 2003; [5,6,8,9]Mourichon et al., 1997; [10,11]Nakyanzi, 2002.

It is essential to assess the genetic diversity among accessions and relate to the possible relevance to resistance to diseases. The results presented here could be used as a baseline for further studies in finding markers that can address the problem of disease susceptibility. This study

has provided baseline information on the genomic structure of some resistance genes in banana. Sequence polymorphism has also been reported in NBS-LRR type of R-genes in *Musa* sp. (Azhar and Heslop-Harrison, 2008) as well as for catalase (Khoyratty et al., 2008). A high degree of diversity and considerable sequence variation was also described among RGAs in the banana genome. Sequences within a single family shared an identity ranging from 81 to 97%, while those from different families shared an identity of 24 to 70% depending on gene that was considered (Pei et al., 2007).

Clustering of cultivars based on banding patterns of defence related genes

With the exception of 'Petite Naine' that was alone in a single clade, the other cultivars were grouped in four clearly identifiable groups (Figure 7). Although 'Petite Naine' was found to be highly susceptible to *M. eumusae,* it does not cluster with the other susceptible cultivars ('Williams' and 'GCTCV119'). The dendrogram was able to separate the resistant and susceptible cultivars for *M. eumusae.* Group 1 was composed of the cultivars 'FHIA 21, French Claire, Pisang Mas and Gingeli 1 that were tolerant or highly tolerant to *M. eumusae.* Group 2 consisted of a tolerant and highly resistant cultivar. The two susceptible cultivars clustered together in group three while group four was composed of two highly tolerant cultivars. To a certain extent the clustering was affected by different grades of resistance. For example, group one included cultivars that were tolerant and highly tolerant to *M. eumusae*, while group three included the susceptible cultivars, Williams and GCYCV. The susceptible cultivar 'Petite Naine', however, did not cluster with the group three cultivars. The clustering was not affected by ploidy or genome composition of the cultivars.

It is well known that gene expression is affected by allele copy number present in polyploid genomes. However, banana cultivars with multiple copies of the A genome (AAA, AAAA) may not contain multiple identical alleles. The A genome of banana appear to be different since bananas with AAA genomes such as the East African Highland banana are quite different from the AAA desert bananas. Cultivars with a B genome all appeared to be tolerant or highly tolerant to *M. eumusae.* It has been hypothesized that the B genome does confer hardiness and disease resistance to banana cultivars (Robinson, 1996). It may be possible that the B genome carries different resistance genes than the A genome. However, Pei et al. (2007) reported that RGAs may not be identical in all banana cultivars. Their study showed that RGA *Mu-I* appeared only in the cultivars 'Zhongshandajiao' (ABB) and 'Gongjiao' (AA), RGA *Mu-K* was found only in 'Zhongshandajiao' (ABB), while 10 RGAs were present in all five of the banana cultivars that were tested, irrespective of the genomes.

This study showed that the amplicon profiles for most of the defense genes were shared among the banana cultivars and its close relatives *Heliconia* sp. and *R. madagascariensis*, suggesting that the genes are highly conserved in the order Zingerberales. However, sufficient variation was observed to separate the two genera from the banana cultivars (Figure 7). Polymorphisms in the defense genes could be used to phylogenetically separate *Heliconia* and *R. madagascariensis* from *Musa.*

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