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Characterization of disease resistance gene candidates of the nucleotide binding site (NBS) type from banana and correlation of a transcriptional polymorphism with resistance to *Fusarium oxysporum* f.sp. *cubense* race 4

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Abstract Most plant disease resistance (R) genes encode proteins with a nucleotide binding site and leucine-rich repeat structure (NBS-LRR). In this study, degenerate primers were used to amplify genomic NBS-type sequences from wild banana (*Musa acuminata* ssp. *malaccensis*) plants resistant to the fungal pathogen *Fusarium oxysporum* formae specialis (f. sp.) *cubense* (FOC) race 4. Five different classes of NBS-type sequences were identified and designated as resistance gene candidates (RGCs). The deduced amino acid sequences of the RGCs revealed the presence of motifs characteristic of the majority of known plant NBS-LRR resistance genes. Structural and phylogenetic analyses grouped the banana RGCs within the non-TIR (homology to Toll/interleukin-1 receptors) subclass of NBS sequences. Southern hybridization showed that each banana RGC is present in low copy number. The expression of the RGCs was assessed by RT-PCR in leaf and root tissues of plants resistant or susceptible to FOC race 4. RGC1, 3 and 5 showed a constitutive expression profile in both resistant and susceptible plants whereas no expression was detected for RGC4. Interestingly, RGC2 expression was found to be associated only to FOC race 4 resistant lines. This finding could assist in the identification of a FOC race 4 resistance gene.

Keywords Banana · Disease · *Fusarium* · NBS · Resistance genes

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Introduction

Banana (*Musa* spp.) is one of the most important fruit crops in the world, serving as both a staple food in many developing countries and an export commodity for numerous agricultural-based economies. Diseases are a major constraint to banana production in most growing regions with one of the most serious threats being Fusarium wilt or Panama disease. Panama disease is caused by the soil-borne fungus *Fusarium oxysporum formae specialis* (f. sp.) *cubense* (FOC)

which results in a lethal vascular wilt for which there are no effective chemical control measures (Ploetz and Pegg 2000). FOC has been classified into four physiological races based on pathogenicity to host cultivars in the field. Of these races, FOC race 4 is considered the most important because it affects cultivars that produce more than 80% of the world's bananas, including the important Cavendish subgroups (Ploetz 2005). Race 4 has caused serious losses in subtropical regions of many countries including Australia, South Africa, the Canary Islands and Taiwan. A genetically distinct form of race 4 which infects Cavendish cultivars in the tropics (known as "tropical" race 4), has been recently reported and poses a significant threat to banana production in the Americas and Africa (Ploetz 2005).

Although natural sources of resistance against Panama disease have been reported in wild bananas (Ploetz and Pegg 2000), the introgression of this resistance into edible cultivars by conventional breeding has been hampered by problems associated with triploidy and low fertility (Roux et al. 2004). The development of a reliable and efficient genetic transformation system for banana (Becker et al. 2000; Khanna et al. 2004) provides the opportunity for the generation of disease-resistant bananas using a molecular breeding approach. To date, however, no R gene/s capable of conferring resistance to FOC race 4 has been reported. One potential source of *Fusarium* R genes is the wild diploid banana, *Musa acuminata* ssp. *malaccensis*, which shows resistance to FOC race 4 (Ploetz and Pegg 2000). In field trials using a population of healthy *Musa acuminata* ssp. *malaccensis* originating from a heavily FOC tropical race 4-infected site on Sumatra, the population segregated for resistance in an Australian FOC subtropical race 4-infected site in a Mendelian ratio of 3:1 suggesting a single dominant gene was involved in conferring resistance to the fungal pathogen (Smith and Hamill 1999). As such, this population represents a promising source of potential R genes for use in genetic transformation of banana.

The majority of plant R genes encode proteins with cytoplasmic nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains that confer resistance to a wide variety of pathogens and pests including viruses, bacteria, fungi, nematodes and insects (Dangl and Jones 2001). The NBS-LRR proteins are thought to recognize pathogens and respond by activating signal transduction pathways leading to disease resistance (Belkhadir et al. 2004). The C-terminal LRR region has been considered the candidate pathogen recognition domain while the N-terminal region, including the NBS, is thought to be involved in signalling (Belkhadir et al. 2004). The LRR domain is the most variable region in closely related NBS-LRR proteins and is under diversifying selection (Michelmore and Meyers 1998; Richter and Ronald 2000). NBS-LRR genes are abundant in plant genomes with 149 and 480 isolated from *Arabidopsis* and rice, respectively (Meyers et al. 2003; Zhou et al. 2004) mostly organized in clusters (Hulbert et al. 2001). The NBS-LRR class of R genes is divided into two distinct subclasses based on the presence or absence of an N-terminal domain that shows similarity to the *Drosophila* Toll and the human Interleukin-1 receptor (TIR) (Meyers et al. 1999; Pan et al. 2000). Widely distributed in both monocotyledonous (monocot) and dicotyledonous (dicot) species, the non-TIR subclass commonly has an N-terminal region comprising a predicted coiled-coil structure, sometimes in the form of a leucine zipper (Meyers et al. 1999; Pan et al. 2000; Hulbert et al. 2001). The TIR subclass, however, appears restricted to dicot species.

Genes that confer resistance to *Fusarium* have been isolated from tomato (Simons et al. 1998) and melon (Joobeur et al. 2004) by mapping approaches. Both the tomato *I2* and melon *Fom-2* genes were shown to encode non-TIR-NBS-LRR type R genes suggesting that targeting this class of genes may facilitate the isolation of genes from wild banana species that confer resistance to *Fusarium.* The highly conserved motifs in the NBS domain have been targeted to isolate NBS-type sequences by PCR-based strategies using degenerate primers. Using this approach, resistance gene candidates (RGCs) have been isolated from soybean (Kanazin et al. 1996; Yu et al. 1996; Graham et al. 2000), potato (Leister et al. 1996), lettuce (Shen et al. 1998), rice and barley (Leister et al. 1998), wheat (Seah et al. 2000), sunflower (Ayele-Gedil et al. 2001), common bean (Rivkin et al. 1999; López et al. 2003), strawberry (Martínez-Zamora et al. 2004), apple (Calenge et al. 2005) and recently from banana (Pei et al. 2007). The PCR approach has also been useful in isolating resistance gene candidates of the Pto-type from banana (Peraza-Echeverria et al. 2007).

This study presents the PCR-based amplification and subsequent characterization of five RGCs of the

NBS-type from the FOC race 4 resistant wild banana, *M. acuminata* ssp. *malaccensis*. Further, we show that the expression of one of these RGC sequences correlates with resistance to FOC race 4. The availability of these sequences opens the possibility of applying different strategies for their functional analysis and for developing disease resistance in this crop.

Methods

Plant material

Musa acuminata ssp. *malaccensis* (AA) plantlets which were resistant (accessions 850 and 852) or susceptible (accessions 845 and 846) to FOC subtropical race 4 (Smith et al. 1998) were obtained from Dr Mike Smith, DPIF, Nambour, Australia, and were maintained in a phytotron at 28°C. Leaves and roots were harvested from 4-month-old plants, frozen in liquid nitrogen and stored at −80°C until use.

PCR and cloning

DNA was extracted from plant tissue as described by Dellaporta et al. (1983). A pair of degenerate primers [forward 5′-GGiGGiGTiGGiAAiACiAC-3′; reverse 5′-A(A/G)iGCTA(A/G)iGGiA(A/G)iCC-3′] (i = inosine) was used to amplify a ~530 bp fragment of the NBS domain of R genes spanning the GGVGKTT kinase motif to the GLPLA motif (Kanazin et al., 1996; Aarts et al. 1998). PCR mixtures (50 µl) contained 300 µM of each dNTP, 2 µM of each degenerate primer, ~200 ng DNA, 1× PCR buffer and 1 U of Taq DNA polymerase (Roche). Cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 45°C for 30 s and 72°C for 1 min, and a final incubation at 72°C for 10 min. Gel-purified amplicons were cloned into the plasmid pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells as per the manufacturer's instructions. Plasmids were extracted using the High Pure Plasmid Isolation Kit (Roche).

RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

RNA was extracted from plant tissue as described by Schuler and Zielinski (1989). The isolation of the 5′ cDNA ends of RGC1, RGC2, RGC3 and RGC5 was achieved using the GeneRacer™ Kit (Invitrogen™ Life Technologies) according to the manufacturer's protocol. Briefly, RNA was treated with alkaline phosphatase and tobacco acid pyrophosphatase (TAP), then the GeneRacer™ RNA oligo was ligated to the 5′ end of the mRNA using RNA ligase followed by the synthesis of cDNA with reverse transcriptase. The 5′ RACE was performed using the reverse specific primers GSP1 (5′-CAAGTCTTGTC GAATCGAAC-3′) for RGC1, GSP2 (5′-ACGCTTC CCCGTCCTCCGGCATC-3′) for RGC2, GSP3 (5′-A CCCGCGATTACCATGTGG-3′) for RGC3, and GSP5 (5′-TCCACCTTGGTAGCAGACTC-3′) for RGC5 in combination with the GeneRacer™ 5′ primer, respectively. Amplicons were subjected to a second round of PCR using the nested specific primers nGSP1 (5′-CTTCGCATCGAATGTTCGATTCG-3′) for RGC1, nGSP2 (5′-ACACACACCCACATTCT CAATGG-3′) for RGC2, nGSP3 (5′-CTGCATATCG ACCACGTTGAGCG-3′) for RGC3, and nGSP5 (5′-TCTTTGGTCAGTCTCTTCAC-3′) for RGC5 in combination with the GeneRacer™ nested 5′ primer, respectively. The PCR products were cloned into pGEM-T Easy and sequenced.

Genome walking by PCR

The sequence of the 5′ genomic region of RGC4 and the sequence of the 3′ genomic region (corresponding to the GLPLA motif) of all banana RGCs were isolated by genome walking (Siebert et al. 1995). Briefly, genomic DNA from *M. acuminata* ssp. *malaccensis* was incubated with *Pvu*II and *Eco*RV and GenomeWalker adapters were ligated to the digested DNA. PCR reactions were carried out on each GenomeWalker library using the adapter primer (AP1) and a reverse gene specific primer (5′-GT TGGACTTCATGGATGTG-3′) for RGC4, and forward specific primers for RGC1 (5′-CAAGTCTT GTCGAATCGAAC-3′), RGC2 (5′-AGCCTGTTAG CCCCATTAGATGC-3′), RGC3 (5′-ACCCGCGAT TACCATGTGG-3′), RGC4 (5′-GCCGTGTCACAA TCTTACAAGG-3′) and RGC5 (5′-CTGCTACCAA GGTGGAACAATC-3′). PCR products were subjected to a second round of PCR, using the nested adapter primer (AP2) and nested reverse gene-specific primer for RGC4 (5′-TACCTCTTGTCC TGGAGATGG-3′), and nested forward gene specific

primers for RGC1 (5′-AGCTTCGACATCAGAAG AGAGGC-3′), RGC2 (5′-GTCACCGGCACGATG GAGCCATAC-3′), RGC3 (5′-CCTCGCGGTCGA ACCAGGTGTGC-3′), RGC4 (5′-TCATAGGCCAT CTCCAGGACAAGAG-3′), and RGC5 (5′-ACTCG AGACACAAAGATTGCCAGC-3′). The amplicons were cloned into pGEM-T Easy and sequenced.

Southern hybridization

Genomic DNA (5 μg) was digested independently with *Eco*RI, *Eco*RV, *Hin*dIII, *Bgl*II, and *Sac*I. Digests were electrophoresed through a 1.2% agarose gel, capillary-blotted onto a nylon membrane (Roche) and baked for 2 h at 80°C. Prior to hybridization, the membrane was blocked for 1 h at 42°C with DIG Easy Hyb (Roche). DIG-labelled probes were PCR-amplified for each RGC using a mixture of DIG-labelled and standard dNTPs (1:3 ratio). The membrane was hybridized with DIG-labelled probes for at least 12 h at 42°C followed by two washes at room temperature (10 min) in 2× SSC/0.1% SDS and two washes at 65°C (15 min) in 0.1× SSC/0.1% SDS. Detection of the hybridized probe was done using CDP-STAR (Roche) according to the manufacturer's instructions.

Sequence analysis

All sequence reactions were carried out with the BigDye terminator sequencing kit version 3.1 and separated on an ABI 3730 automatic sequencer (Applied Biosystems) at the Australian Genome Research Facility (www.agrf.org.au). All sequences were assembled and edited using the Lasergene software package version 4.03 (DNASTAR, Madison, WI). Similarity searches were performed with the BLAST program (Altschul et al. 1997) (www.ncbi.nlm.nih.gov) using the default settings. Percentages of identity between sequences were determined by the MEGALIGN program of the Lasergene software package and potential coiled-coil structures were predicted by the COILS program (Lupas 1996) using the default settings. Predicted protein sequences were aligned using the ClustalX program version 1.81 with the defaults settings (Thompson et al. 1997). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software package version 2.1 (Kumar et al. 2001) with the Poisson correction. Bootstrapping (1000 replicates) was used to evaluate the degree of support for particular grouping patterns in the trees.

Transcript expression analysis

Total RNA was extracted from leaf and root tissue as described above and was treated with RNase-free DNase (Promega) as per the manufacturer's protocol. Synthesis of cDNA was carried out using 200 U of Superscript II (Invitrogen) according to the manufacturer's instructions. PCR mixtures (50 μl) contained 300 μM of each dNTP, 0.2 μM of each forward and reverse primer, 5 μl of diluted cDNA (1:20), 1X PCR buffer and 1 U of Taq DNA polymerase (Roche). The following combinations of forward and reverse primers were used in the reactions: for RGC1, 5′-CAAGTCTTGTCGAATCGAAC-3′ and 5′-TCGTCGGCATGCCAGAATAC-3′; for RGC2, 5′-CCATTGAGAATGTGGGTGTG-3′ and 5′-ACTCCTCGAGAACGTATGG-3′; for RGC3, 5′-ACCCGCGATTACCATGTGG-3′ and 5′-GCGCTTCTTCTCATGTCGC-3′; for RGC4, 5′-GCCGTGTCACAATCTTACAAGG-3′ and 5′-GTTGGACTTCATGGATGTG-3′; for RGC5, 5′-CTGCTACCAAGGTGGAACAATC-3′ and 5′-GCACAATTCTTGAACAGCTCC-3′. Cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 50–55°C for 30 s and 72°C for 1 min, followed by a final incubation for 10 min at 72°C. Gel-purified PCR products were cloned into pGEM-T Easy and sequenced. In RT-PCR experiments, primers specific for the banana *actin 1* gene, and spanning an intron (~100 bp), were included as a control to detect any genomic DNA contamination (Hermann et al. 2001).

Results

Amplification and cloning of RGCs of the NBS-type from banana

Degenerate primers were designed to amplify a ~530 bp fragment between the P-loop and GLPLA motifs of the NBS region of the NBS-LRR class of R genes. When used in a PCR with genomic DNA extracted from FOC race 4-resistant banana accession 850, a band of the expected size was amplified; the amplicons were cloned and a total of 88 clones were

sequenced. Analysis of the deduced amino acid sequences of all clones revealed the presence of the typical NBS motifs of R genes in the spatially correct locations. Based on a >80% amino acid sequence identity threshold criterion, the 88 sequences were grouped into three classes, designated RGC1 (77 clones), RGC2 (four clones) and RGC3 (seven clones), with sequences of an individual class showing at least 97% identity. A representative clone of each class was chosen for further analysis. These sequences presented uninterrupted ORFs indicating the absence of introns in this region.

Two distinct classes of RGCs have been previously isolated from the related banana *M. acuminata* ssp. *burmannicoides* in our laboratory using a similar PCR-based strategy (Taylor 2005). Using specific primers designed from the sequences of the *M. a. burmannicoides* genes, two further classes of RGCs were amplified from the genome of *M. a. malaccensis* accession 850 and were designated RGC4 and RGC5. In total, therefore, five different classes of NBS sequences containing continuous ORFs were identified in the genome of *M. a. malaccensis*. To further characterize the RGCs, the 5′ ends of RGC1, RGC2, RGC3 and RGC5 were amplified from leaf tissue using 5′ RACE, while the 5′ end of RGC4 and the sequences corresponding to the GLPLA motif of all the RGCs were obtained using PCR genome walking. The putative amino acid sequences of the N-terminal regions of the *M. a. malaccensis* RGCs 1–5 have been lodged in GenBank with the accession numbers EU239819-EU239823, respectively. The LRR-domains of the putative banana R-genes were not isolated in this study.

Sequence analysis of banana RGCs

Analysis of the putative amino acid sequences of the banana RGCs revealed the presence of appropriately located consensus P-loop/kinase-1a (GVGKTT), kinase-2 (LLVLDDVW), RNBS-B (GSRIIITTRD) and GLPLA motifs characteristic of the NBS domain of R genes (Meyers et al. 1999) (Fig. 1). The non-TIR (nT) motif (WVxxIRELAYDIEDIVDxY) (Bai et al. 2002) which is associated with the non-TIR subclass of NBS sequences, was present in four (RGC1-3 and 5) of the N-terminal region of the *M. a. malaccensis* sequences (Fig. 2). The N-terminal regions of the banana RGCs showed no sequence similarity to the TIR domain suggesting the RGCs of this species belong to the non-TIR-NBS-LRR type like other monocot R gene and RGC sequences. RGC2, 3 and 5 also contained a potential coiled-coil (CC) structure in the non-TIR domain. Interestingly, the non-TIR domain spanning residues 1 to 182 of the RGC4 sequence (designated as RGC4-N-ter in Fig. 1) appeared to be a partial duplication of the NBS region. Although the duplicated region shared 47% amino acid identity to the RGC4 NBS region, the motifs were not conserved suggesting the duplicated N-terminal region would not bind nucleotides.

Sequence comparisons of the RGCs were done using the region spanning the P-loop and GLPLA motifs (~170 amino acids) of the NBS domain. The N-terminal domain of the banana RGCs was not used for analyses because sequences in this region share little similarity and their use in comparisons has been questioned (Meyers et al. 2003). When the deduced amino acid sequences of *M. a. malaccensis* RGC sequences were compared, the levels of identity ranged from 16.9% (between RGC2 and RGC3) to 48% (between RGC2 and RGC5) (Table 1). These identities were equivalent to those observed between the RGCs or the NBS-LRR resistance genes of other plant species (Shen et al. 1998; Ayele-Gedil et al. 2001; López et al. 2003). When the sequences were compared to the twelve classes of RGC sequences recently isolated by Pei et al. (2007), RGC1 and RGC3 shared 99.4% and 96% sequence similarity, respectively, with the *Musa* sequences MuRGA-D and MuRGA-L (Pei et al. 2007) and, thus, can be considered in the same class. Furthermore, similarity searches revealed that three of the isolated *M. a. malaccensis* RGCs were identical or highly similar to partial NBS sequences of *Musa acuminata* already in GenBank. *Musa* sequences in entries AAM97903 (100% identity) and AAM97904 (=AAM97905 and AAM97906) can be considered in the same class as RGC1/MuRGA-D. Similarly, *Musa* sequences from entries ABB96971 and CAD58967 are in the same class as RGC3/MuRGA-L while RGC5 shared 93% sequence identity to entry AAM97908. Three partial *Musa* NBS sequences spanning the region between the P-loop and RNBS-B motifs (~100 amino acids) appear in GenBank (AAM97909, AAM97910 and AAM97911) and were considered as three novel classes of *Musa* NBS-type sequences since they have no counterparts to any reported *Musa* sequences (Table 1). Therefore, 18 distinctive NBS-type

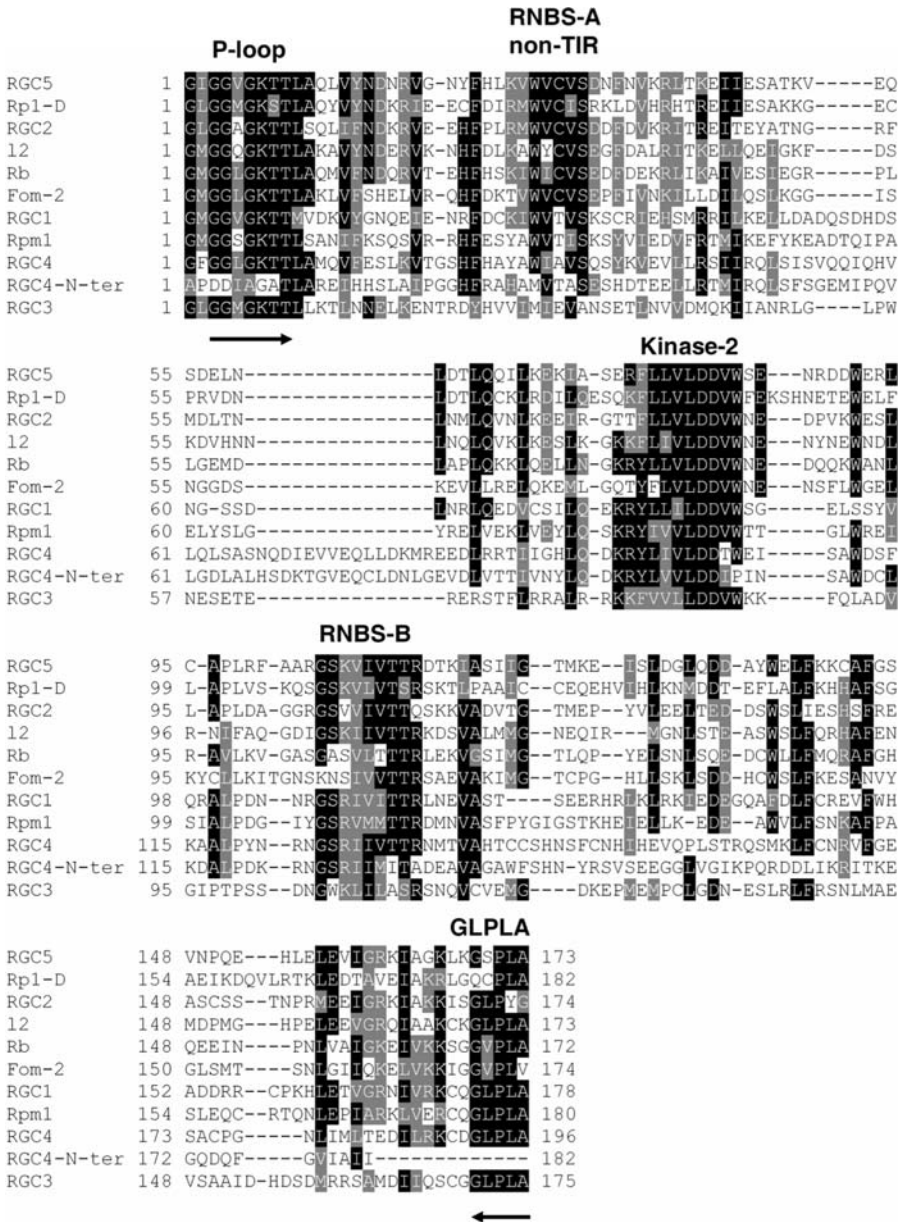

Fig. 1 Alignment of the deduced amino acid sequences of the NBS regions of the five classes (RGC1 to RGC5) of *M. acuminata* ssp. *malaccensis* NBS sequences and selected non-TIR-NBS-LRR R genes known to confer disease resistance. The duplicated NBS sequence of RGC4 located in the non-TIR region was included in the alignment as RGC4-N-ter. Rp1-D (AAD47197) confers resistance to *Puccinia sorghi* in *Zea mays*, I2 (AAD27815) and Fom-2 (AAS80152) confer resistance to *Fusarium oxysporum* in *Lycopersicon esculentum* and *Cucumis melo*, respectively, Rb (Q7XBQ9) confers resistance to *Phytophthora infestans* in *Solanum bulbocastanum*, RPM1 (A57072) confers resistance to *Pseudomonas syringae* in *Arabidopsis thaliana*. Conserved NBS motifs are indicated above the alignment while identical amino acids are shaded in black and conservative substitutions are shaded in grey. The arrows indicate the position and orientation of the degenerate primers used in this work (Kanazin et al. 1996). The 5′ region corresponding to the P-loop motif of RGC1, RGC2, RGC3 and RGC5 was obtained using 5′ RACE, while the P-loop of RGC4 and the region corresponding to the GLPLA motif of all *M. acuminata* ssp. *malaccensis* NBS sequences were obtained using genome walking PCR

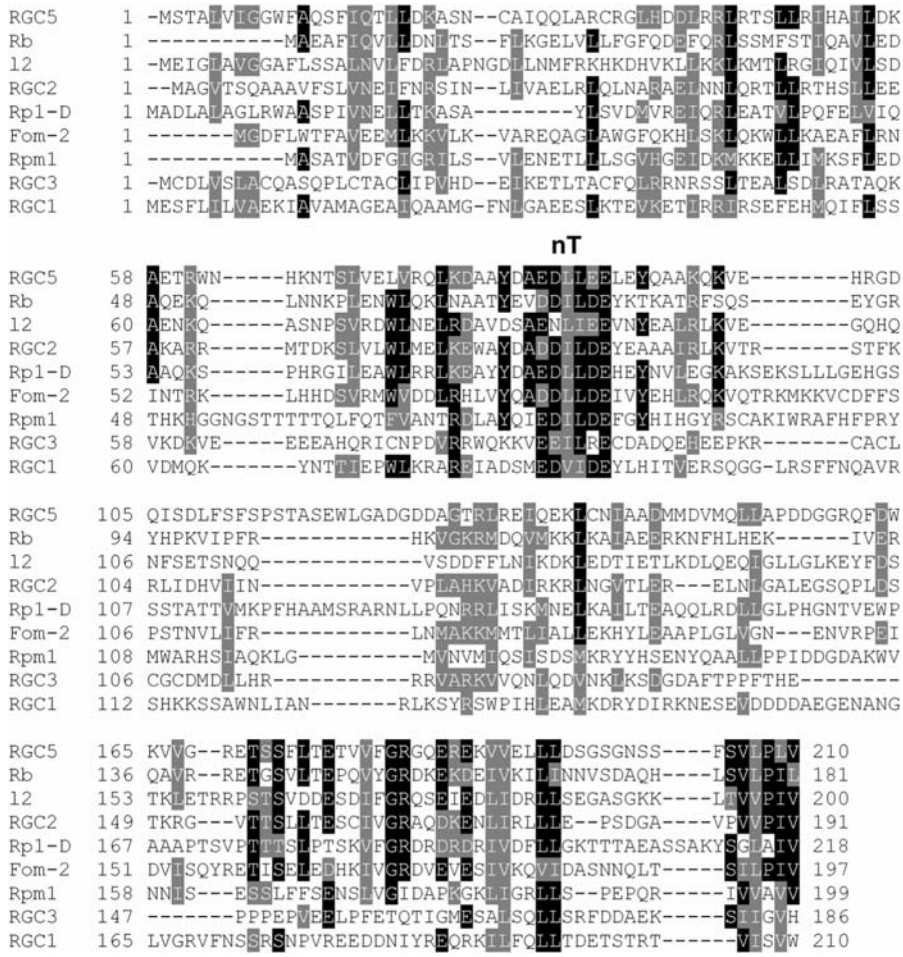

Fig. 2 Alignment of the deduced amino acid sequences of the non-TIR domain of *M. acuminata* ssp. *malaccensis* RGC1 to RGC5 and the corresponding domain of selected non-TIR-NBS-LRR R genes. See legend to Fig. 1 for an explanation of species and genes selected. The non-TIR (nT) motif is indicated above the alignment while identical amino acids are shaded in black and conservative substitutions are shaded in grey

sequences that share less than 80% amino acid identity have been isolated from banana to date.

Southern analysis performed under conditions of high stringency revealed hybridization patterns comprising one to three bands for each gene (Fig. 3), suggesting that the banana RGCs occur as a single copy or possibly as a few highly, similar copies in the diploid genome of *M. a. malaccensis*. The hybridisation patterns were consistent with the number of genes isolated from genomic DNA in this study, from the results of Pei et al. (2007) and from sequences in the GenBank database. For example, the banding pattern for RGC1 is supported by the finding of three banana sequences of >98% identity (RGC1, MuRGA-D and AAM97903). RGC2 showed a banding pattern consistent with two copies present in the genome. Although no homologous sequences appear in GenBank, a second RGC2 gene was indeed isolated in this study during cloning of the full-length cDNA of RGC2 (data not shown). The second RGC2 gene shared 98% sequence similarity to the first RGC2 across a limited region but was not characterised further due to the presence of a two base deletion at nucleotides 1736 and 1737 resulting in a frameshift mutation leading to a premature stop signal at codon 600. In order to examine whether banana RGCs typically occurred as single genes or in very small gene families, gene probes were constructed for other banana RGC sequences available in GenBank specifically to target the entries AAM97909 (NBD05), AAM97910 (NBD09), and AAM97911 (NBD25). While the gene encoding entry AAM97910 showed hybridization patterns consistent with a small gene family of one or two members, the genes encoding entries AAM97909 and AAM97911 showed patterns comprising ten or more bands suggesting the latter

Table 1 Percentage identity derived from pairwise comparisons between isolated banana resistance gene candidates (GenBank and Pei et al. 2007) and the NBS domain of NBS-LRR resistance genes

Class	RGC1	RGC2	RGC3	RGC4	RGC5	MuRGA A	MuRGA B	MuRGA C	MuRGA D	MuRGA E	MuRGA F	MuRGA G	MuRGA H	MuRGA I	MuRGA J	MuRGA K	MuRGA L	AAM97909	AAM97910	AAM97911	Rx	HERO
RGC1		22.1	17.3	29.5	26.3	29.5	26.9	28.0	99.4	23.8	21.2	26.5	25.1	23.8	25.6	23.8	17.9	33.0	34.0	22.4	28.2	31.1
RGC2	.....		16.9	20.3	48.0	44.2	43.9	39.9	21.5	27.4	26.5	30.6	26.3	26.8	22.7	22.0	18.0	49.5	22.7	26.2	22.1	26.9
RGC3	.....	.....		20.2	17.0	18.5	18.7	20.2	17.3	20.8	20.0	20.6	18.6	17.9	19.1	19.0	96.0	23.9	20.6	20.6	19.0	19.8
RGC4	.....	.....	.....		22.2	27.2	23.4	25.6	29.0	23.8	24.7	24.1	21.6	24.4	24.4	22.6	21.4	26.6	32.0	21.5	30.7	31.7
RGC5	.....	.....	.....	.....		42.1	47.4	58.9	25.7	29.8	34.1	31.8	29.9	29.8	27.5	25.0	17.5	49.5	28.9	31.8	25.8	29.9
MuRGA A	.....	.....	.....	.....	.....		43.9	41.1	28.9	33.9	38.2	37.1	33.5	31.0	28.3	28.6	20.2	44.0	30.9	29.0	28.2	31.7
MuRGA B	.....	.....	.....	.....	.....	.....		45.8	26.3	31.5	40.0	32.9	32.9	30.4	31.0	29.2	19.3	46.8	27.8	35.5	27.0	30.5
MuRGA C	.....	.....	.....	.....	.....	.....	.....		27.4	28.0	33.9	30.4	34.1	29.2	27.4	25.6	20.8	47.7	29.9	30.8	25.2	29.3
MuRGA D	.....	.....	.....	.....	.....	.....	.....	.....		23.2	20.6	25.9	24.6	23.2	25.0	23.2	17.9	32.1	33.0	21.5	28.2	31.1
MuRGA E	.....	.....	.....	.....	.....	.....	.....	.....	.....		56.0	47.6	40.1	39.9	34.5	42.9	21.4	33.9	26.8	48.6	25.8	26.9
MuRGA F	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		48.8	44.3	41.1	37.1	38.7	20.6	33.9	28.9	43.9	25.8	26.9
MuRGA G	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		41.3	39.3	38.2	39.3	21.8	38.5	25.8	45.8	27.6	26.9
MuRGA H	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		55.1	49.1	53.9	19.2	27.5	28.9	57.9	23.3	28.1
MuRGA I	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		49.4	50.6	19.6	27.5	22.7	54.2	27.0	26.9
MuRGA J	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		47.0	21.4	27.5	33.0	52.3	27.0	25.7
MuRGA K	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		19.6	27.5	23.7	78.5	24.5	26.3
MuRGA L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		24.8	21.6	21.5	18.4	19.8
AAM97909	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		28.9	29.0	24.8	29.4
AAM97910	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		24.7	22.7	33.0
AAM97911	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		21.5	29.0
Rx	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		38.0
HERO	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	

Rx (CAB50786) confers resistance to *Potato virus X* in *Solanum tuberosum* and HERO (CAD29729) confers resistance to *Globodera rostochiensis* in *Lycopersicon esculentum*

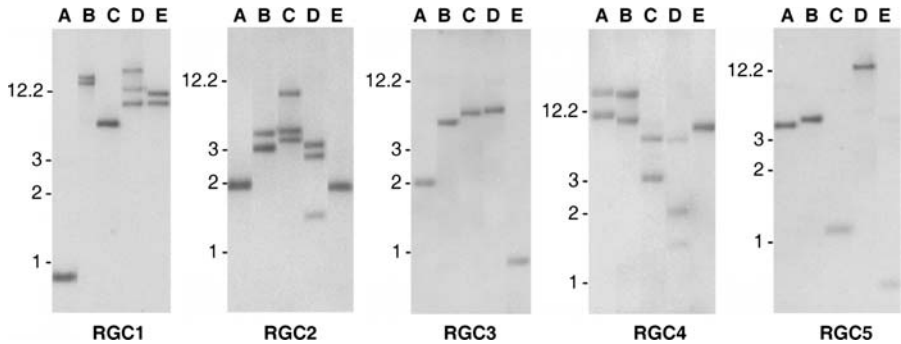

Fig. 3 Southern blot analysis of RGC1 to RGC5 from *M. acuminata* ssp. *malaccensis* under high conditions of stringency. The restriction enzymes used are: (A) *Eco*RI, (B) *Eco*RV, (C) *Hin*dIII, (D) *Bgl*II and (E) *Sac*I. Molecular weight markers (kb) are indicated on the left of each blot

banana RGCs are members of sizeable multigene families (data not shown).

Similarity searches of the protein databases also revealed that each *M. a. malaccensis* RGC showed significant similarity to RGCs isolated from other monocots such as *Oryza sativa*, *Saccharum officinarum* and *Avena sativa* and also to known non-TIR-NBS-LRR resistance genes. RGC1 showed approximately 38% sequence identity to RXO1 of *Zea* and RPM1 of *Arabidopsis*; both of which confer resistance to bacterial infection. When conservative substitutions are taken into account, the level of sequence similarity increases to 55–63%. RGC2 showed approximately 47% sequence identity and 63% similarity at the amino acid level to RPG1-B of *Glycine* and the I2 protein which confers resistance to *Fusarium* in *Lycopersicon*. RGC3 shares approximately 37% sequence identity and 56% sequence similarity to the RPS2 and RPS5 proteins from *Arabidopsis* which confer resistance to *Pseudomonas* infection. RGC4 shares 34% sequence identity and 56% sequence similarity with the fungal resistance genes MLA1 and MLA10 from *Hordeum*. RGC5 shares 45% identity and 65% sequence similarity with the bacterial blight resistance Xa1 protein from *Oryza* and the tomato I2 protein.

Phylogenetic relationships of the banana RGCs

Phylogenetic analysis was performed using the region comprising only the NBS domain (P-loop to GLPLA) as it is present in both the TIR and non-TIR-NBS-LRR proteins and contains numerous conserved motifs that assist proper alignment. Sequences AAM97909, AAM97910 and AAM97911 were not considered in the phylogenetic tree construction as they are truncated. Statistical support for many of the branching patterns was low in the tree due to the short length of sequence used coupled with the high degree of sequence divergence of the different protein sequences (Fig. 4). Nonetheless, the banana RGCs clustered with the non-TIR subclass of NBS sequences as described by Meyers et al. (1999) which supports previous indications that the banana RGCs lack a TIR domain at the N-terminal region. The distribution of the banana RGCs in different branches of the non-TIR-NBS cluster reflects a high level of sequence divergence for these sequences. RGC5 and MuRGA-C formed a monophyletic clade, while RGC5/MuRGA-C, RGC2, MuRGA-A and MuRG-B formed a cluster with resistance genes of known function including *Oryza* Xa1, *Glycine* Rpg1-b, *Solanum* Rb, tomato I2 and melon Fom-2. MuRGAs E-K clustered as a discrete clade distant from that containing RGCs 2 and 5. RGC4, RGC1/MuRGA-D and RGC3/MuRGA-L resolved into different clades throughout the tree. Overall, the banana RGCs were more closely related to NBS sequences from other species than each other. Further, the clustering with non-TIR-NBS-LRR resistance proteins of known function suggests the banana RGCs may encode resistance gene products of as yet unknown specificity.

Expression profiles of the banana RGCs

To examine the transcript expression profiles of the banana RGCs, mRNA was extracted from healthy plants of *M. a. malaccensis* either resistant or susceptible to FOC race 4 and used as template for RT-PCR

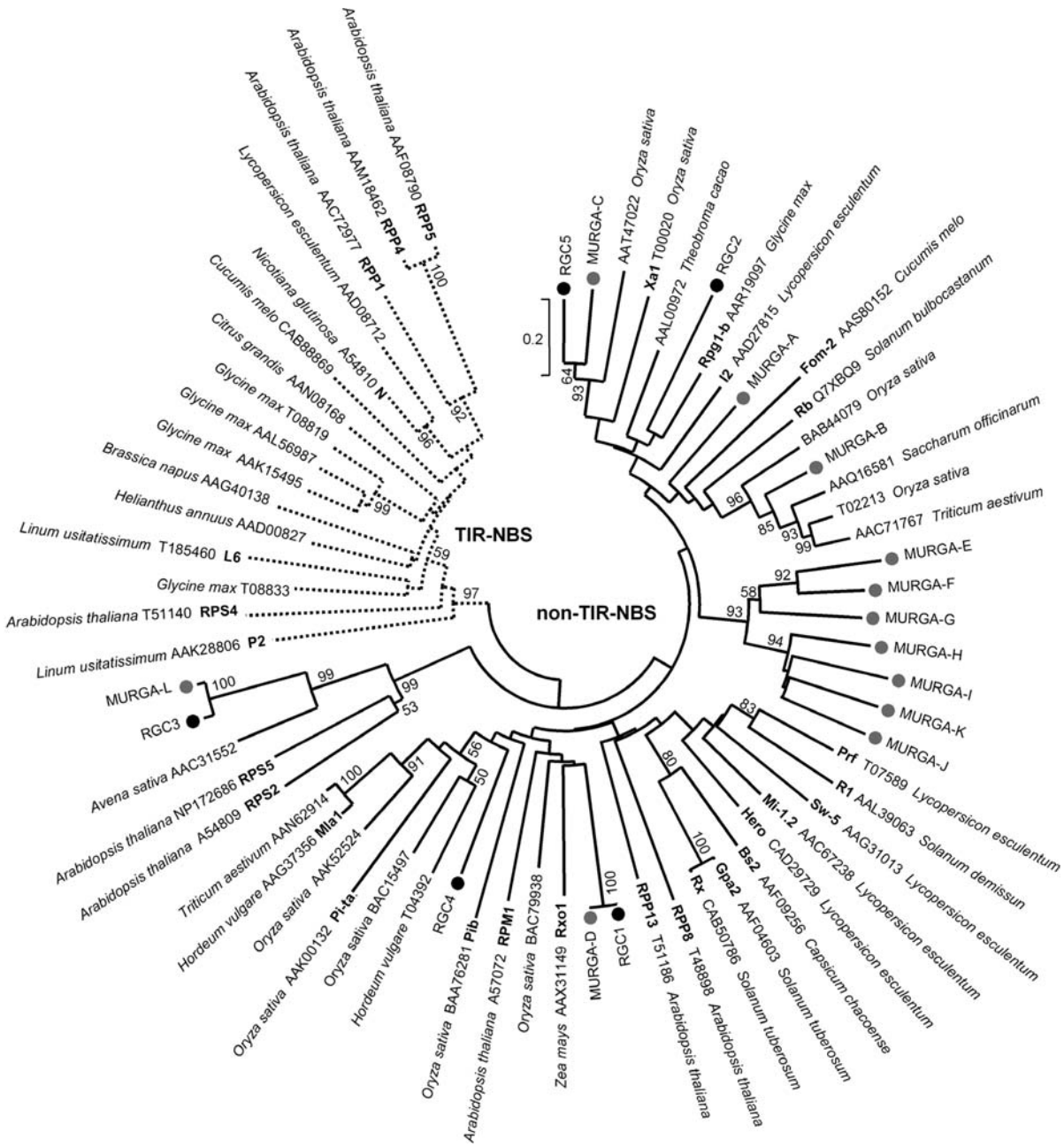

Fig. 4 Neighbor-joining phylogenetic tree of resistance gene candidate sequences from *M. acuminata* ssp. *malaccensis* (black circles), other banana genotypes (Pei et al. 2007) (grey circles), other plant species and the NBS of known TIR- and non-TIR-NBS-LRR resistance proteins (in bold). Numbers on the branches indicate the percentage of 1000 bootstrap replications supporting the particular nodes and only those with >50% support are shown. The dotted branches indicate the TIR-NBS cluster

using RGC-specific primers (Fig. 5). Amplification products were detected in leaf and root tissue from both resistant and susceptible accessions using RGC1-, RGC3- and RGC5-specific primers, while no products were amplified from any plants using RGC4-specific primers. Interestingly, an mRNA encoding RGC2 was detected in the plants resistant to FOC race 4 (accession 850) but not in plants

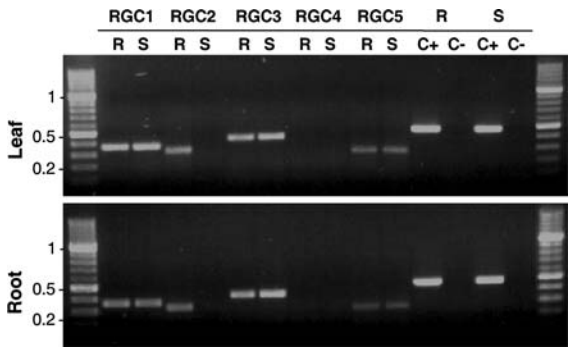

Fig. 5 RT-PCR analysis of the five resistance gene candidates in leaf and root tissue of *M. acuminata* spp. *malaccensis* plants resistant (R) or susceptible (S) to *Fusarium* wilt race 4. C+ lanes denote the positive control and show the expected ~480 bp fragment amplified from banana *actin 1* cDNA. C− lanes denote the negative control where no reverse transcriptase was added to the mRNA prior to PCR and show no banana *actin 1* cDNA amplification. Molecular weight markers (kb) are indicated on the left of each gel

susceptible to the fungal pathogen (accession 845). Identical results were obtained for other banana accessions; an RGC2 transcript was detected in the resistant accession 852 but not in susceptible accession 846 (data not shown). These results suggested that at least three of the five RGCs were expressed constitutively and that a transcriptional polymorphism may confer resistance to FOC race 4. As a control to ensure that products were not amplified from contaminating DNA, PCRs were also done using primers targeting the gene encoding banana *actin 1* and spanning an intron of approximately 100 bp. Only the expected cDNA fragment of ~480 bp was amplified indicating that cDNA preparations were not contaminated with genomic DNA.

Discussion

We have isolated and characterized five different classes of RGCs of the NBS-type from the wild banana, *Musa acuminata* ssp. *malaccensis*. To date, there is evidence for at least 18 distinct classes of NBS-LRR sequences in the banana genome. Pei et al. (2007) isolated 12 classes of banana RGCs from three cultivated triploid and two wild diploid species of banana, while the GenBank database contains three novel putative NBS-LRR-type sequences isolated from *Musa acuminata* that show little similarity to the sequences presented in the former studies or to each other.

Several features of the banana putative RGCs isolated in this study suggest they are non-TIR-NBS-LRR disease resistance genes. For example, the characteristic motifs of the NBS domain of known resistance genes described by Meyers et al. (1999) and Pan et al. (2000) are present in each banana RGC at similar positions. One of these motifs, the highly conserved P-loop, has been shown to bind ATP in the NBS-LRR resistance proteins I2 and Mi from tomato (Tameling et al. 2002) suggesting the banana RGC proteins may also bind ATP. The non-TIR (nT) motif (Bai et al. 2002), which is associated only with the non-TIR subclass of NBS sequences, is found in the N-terminal region of the banana RGCs and none of the motifs associated with the TIR subclass are found in the corresponding region of the banana RGCs. Moreover, the banana RGC1, RGC2 and RGC5 showed the presence of a putative coiled-coil (CC) structure in the non-TIR domain, which is another common feature of this region (Pan et al. 2000). In the rice genome, for example, 174 of the 535 NBS sequences contained a CC motif in the non-TIR domain (Zhou et al. 2004). The lengths of the non-TIR domain of the banana RGCs are also similar to the lengths of the non-TIR domain of monocot and dicot R genes which range from 200–250 amino acids from the start of the coding region to the beginning of the NBS domain (P-loop) (Bai et al. 2002; Meyers et al. 2003). Furthermore, the C-terminal domain of *M. a. malaccensis* RGC2 and RGC5 has been found to comprise LRRs (data not shown). Taylor (2005) has also shown that the *M. a. burmannicoides* homologues of RGCs 1 to 5 also comprise LRR domains. The finding of non-TIR-NBS-LRR genes in banana is consistent with the structure of R genes isolated from other monocots where the TIR domain appears to be absent (Meyers et al. 1999; Pan et al. 2000; Bai et al. 2002; Zhou et al. 2004). Based on an analysis of R genes isolated from several dicot species and a limited distribution of monocot species (i.e., grasses), Meyers et al. (1999) noted that the absence of the TIR domain in R genes could be predicted by the presence of the motif RNBS-A-non-TIR near to the P-loop and also by the presence of a tryptophan residue (W) at the end of the kinase-2 motif. The *Musa* sequences suggest that the predictive nature of these motifs and residues applies across the monocotyledonous plants as *Musa* are the most distant of monocot species from the grasses.

RGC4 showed a duplicated NBS-type sequence in the N-terminal region. Whole genome analysis has revealed the presence of duplicated N-terminal domains in NBS-LRR proteins in rice (Monosi et al. 2004; Zhou et al. 2004) and *Arabidopsis* (Meyers et al. 2002), while other NBS-NBS-LRR gene have been deposited in GenBank from *Populus* (DQ513232) and *Ipomoea* (ABO15685). The number of genes reported with N-terminal duplications remains small; four or five out of 480 in rice and four out of 149 in *Arabidopsis*. The nature and size of the duplicated regions are diverse involving fusions of additional TIR and/or NBS domains (Meyers et al. 2002). In rice, one of these fusion NBS-NBS-LRR genes may be a pseudogene (Monosi et al. 2004). Although no constitutively-expressed transcript was found for banana RGC4, the ORF present in RGC4 contained no mutations that might render the gene inactive suggesting the gene may be inducible.

Phylogenetic analysis supports the classification of the banana RGCs into the non-TIR subclass since they all cluster with other NBS sequences of the non-TIR subclass. The TIR domain has not been found in the structure of monocot NBS-LRR R genes even in the complete rice genome sequence (Bai et al. 2002; Monosi et al. 2004; Zhou et al. 2004). It has been hypothesised that the loss of the TIR domain from the NBS-LRR genes in monocot plants may have occurred during the divergence of the monocots and dicots (Pan et al. 2000). As banana shares a common evolutionary origin with other monocot plants, it is likely that this domain is also absent from the structure of all banana NBS-LRR resistance genes. Therefore, the fact that only NBS sequences of the non-TIR subclass were isolated in this study is unlikely to be an artefact of PCR but instead the result from the absence of the TIR domain in the *Musaceae* family. Although Pei et al. (2007) did not report the sequence of the N-terminal region of their banana genes, the presence of the RNBS-A-non TIR motif and a Trp at the end of the kinase 2 motif would suggest that these banana sequences also represent non-TIR-NBS-LRR resistance genes.

All *M. a. malaccensis* RGC sequences hybridised to a relatively small number of restriction fragments (one to three) indicating that the RGCs are present within the banana genome as singletons or as members of a small gene family. Single copy RGCs also exist in other plants such as soybean (Kanazin et al. 1996), potato (Leister et al. 1996), *Arabidopsis* (*Arabidopsis* Genome Initiative 2000) and rice (Bai et al. 2002; Ramalingam et al. 2003). The majority of R genes and RGCs, however, have been found as multicopy, clustered sequences (Leister et al. 1998; Hulbert et al. 2001; Bai et al. 2002; Monosi et al. 2004; Zhou et al. 2004). For example, the *Fusarium I2* resistance gene from tomato is within a 90 kb cluster of seven paralogues (Simons et al. 1998). Although the banana RGCs isolated in this study are present in low copy number, the copy number of other banana RGCs appears to be considerably higher (data not shown). It also remains to be demonstrated whether the banana RGC sequences are organized in gene clusters.

Previous reports have shown that NBS-LRR resistance genes are not inducible but are expressed in a constitutive manner. Northern or RT-PCR analyses on different R genes such as *RPM1* (Grant et al. 1995), *Prf* (Salmeron et al. 1996), *RPP5* (Parker et al. 1997), *Mi* (Milligan et al. 1998), *I2* (Mes et al. 2000) and others have revealed the presence of low levels of transcripts in plants that have not been infected with a pathogen. These findings are in agreement with the postulated role of NBS-LRR proteins acting as preformed receptors that recognize a pathogen in a gene-for-gene correlation for disease and resistance in plants (Van Der Biezen and Jones 1998). The lack of a circulatory system in plants might be compensated for by a capacity to express each R protein constitutively in every cell that potentially could be attacked (Van Der Biezen and Jones 1998). The presence of RGC1, RGC2, RGC3 and RGC5 transcripts in both leaf and root tissue of healthy banana plants suggests these genes are constitutively expressed. The apparent absence of RGC4 transcripts in both tissues suggests a non-functional promoter precedes this gene. Alternatively, the gene could be expressed in other tissues or be inducible by an unknown stimulus. Interestingly, the transcriptional polymorphism shown by RGC2 correlated with resistance to FOC race 4. The presence of RGC2 mRNA in two resistant banana accessions and absence in two susceptible accessions suggests a possible role for this RGC in resistance to FOC race 4. A similar expression profile was shown by the *Fusarium I2* resistance gene from tomato where the expression of the *I2* gene was only present in both leaf and root tissue of plants resistant to *Fusarium*

oxysporum f.sp *lycopersici* race 2 and absent in susceptible plants (Mes et al. 2000). The correlation of a transcriptional polymorphism of RGC2 with resistance to FOC race 4 and the observation that both RGC2 and RGC5 cluster with *Fusarium I2* resistance gene from tomato has led to the further characterization of *M. a. malaccensis* RGC2 and RGC5. The full-length cDNAs of both genes have been cloned into *Agrobacterium*-based binary vectors under the control of promoters of different strength and the constructs transformed into several agronomically important, but susceptible, banana cultivars. The ability of these RGCs to confer resistance to FOC race 4 will be tested in field trials of the transgenic plants.

Although the other banana RGCs have not been correlated with resistance to a particular banana disease, their potential role in disease resistance could be tested using new technologies of the post-genomic era, such as RNA interference (RNAi) (Waterhouse and Helliwell 2003). This technology could be used to test the function of multiple banana RGCs by silencing their corresponding targets in disease resistant genotypes. Those disease resistant plants transformed with RGC-RNAi constructs that become susceptible after the infection with a particular banana pathogen would assist in the identification of a particular R gene. The RNAi approach has recently been successfully used to determine the function of genes involved in disease resistance in barley epidermal cells (Douchkov et al. 2005; Dong et al. 2006). Another promising strategy to facilitate the identification of multiple R genes in banana is the use of Binary Bacterial Artificial Chromosome (BIBAC) library technology. A BIBAC vector can be used to transfer large DNA fragments (up to 120 kb) into the plant genome via *Agrobacterium tumefaciens* (He et al. 2003). A BIBAC library is now available for the cultivar 'Tuu Gia' (*Musa acuminata*) which is resistant to the most serious diseases of banana (Ortiz-Vázquez et al. 2005). This library could be screened, using the banana RGCs as probes, to identify and retrieve BIBAC clones harbouring RGCs organized either as singletons or clusters. These could be used to transform existing disease-susceptible banana cultivars via the highly efficient *Agrobacterium*-mediated transformation method (Khanna et al. 2004), thus generating RGC-BIBAC transgenic lines ready to be screened for disease resistance. Other resources that could be used to screen for potential R genes are BAC libraries from the wild bananas *Musa acuminata* ssp. *burmannicoides* or *Musa balbisiana* (Vilarinhos et al. 2003; Safar et al. 2004). The application of these technologies promises to unravel the function of RGCs and assist in the development of cisgenic disease resistant banana cultivars (Jacobsen and Schouten 2007).

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