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Characterisation of Pathogenesis-Related Genes and Resistance Gene Candidates in Banana (*Musa acuminata*) and Their Expression during Host-Pathogen Interaction

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ABSTRACT

Amplified chitinase gene sequence shared 99% homology with *Musa acuminata* class III acidic chitinase and beta-1,3-glucanase gene sequence was 100% homologous to *Musa* x *paradisiaca* beta-1,3-glucanase. Three nucleotide-binding sites and the leucine-rich repeat (NBS-LRR) type of putative RGCs and one serine/threonine kinase gene were characterised at the amino acid level. Kinase-2 (LVLDDVW) and kinase-3 (GSRIIITTRD) motifs in the nucleotide-binding domain were highly conserved in RGC2 and RGC3 and these genes belong to the non-TIR-NBS class RGCs. RGC1 was also clustered into non-TIR-NBS class RGCs; however, many residue substitutions were present in the kinase-2 and kinase-3 motifs. The sub-domain IX (LTEKSDVYSFGVVL) of serine/threonine protein kinase was highly conserved in RGC5 and it shared highest homology with PTH-2 from muskmelon. RT-PCR analysis revealed the differential expression of PR and RGC genes exhibited by different banana genotypes over sampling time. Chitinase was expressed during banana-FocR4 interaction in all three banana genotypes. However, its expression was high and constant in 'Rastali Mutiara' during banana-FocR4 interaction and resulted in very low disease severity in FocR4 inoculated plants (2%) compared to 'Rastali wild-type (16%) and 'Jari Buaya' (8%) at six weeks after inoculation. This suggests that chitinase may play an important role in disease resistance against FocR4. Besides, our study also shows that 'Rastali Mutiara' can be a potential source of disease-resistant genes for molecular breeding of banana.

Keywords: Banana, *Fusarium* wilt, nucleotide-binding site, PR proteins, resistance-gene candidate, serine/threonine kinase

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INTRODUCTION

Banana is one of the important global food commodities. The commercial and subsistence production is seriously threatened by *Fusarium* wilt caused by soil-borne *Fusarium oxysporum* f. sp. *cubense* (Foc). Foc race 4 (FocR4) is considered economically important as it causes significant plantation losses in banana-producing countries predominantly in the Asia-Pacific region (Aquino *et al.*, 2013). To date, the existing control measures for this disease are not satisfactory.

Banana plants respond to attacks of pathogens by activating defence-related genes from different groups based on biological function and pattern of induction. The production of pathogenesis-related (PR) protein is a vital defence response against biological stress and pathogenic infection. Most PR proteins play a key role in plant defence in response to fungal infection. Previous studies have demonstrated that PR proteins show in vitro antifungal activity either individually or in combination (De Bolle *et al.*, 1993; Melchers *et al.*, 1993; Sela-Buurlage *et al.*, 1993; Koiwa *et al.*, 1997; Saikia *et al.*, 2005; Ye & Ng, 2005; De A Campos *et al.*, 2008; Lu *et al.*, 2012). PR proteins have already been shown to be good candidates for engineering fungal-resistant crops (Punja & Zhang, 1993; Jach *et al.*, 1995; Lin *et al.*, 1995; Tabei *et al.*, 1998; Datta *et al.*, 2001; Kalpana *et al.*, 2006). Productive interactions between chitinase and glucanase transgenes in vivo point to combinatorial expression of antimicrobial genes as an effective approach in engineering enhanced crop protection against fungal diseases (Zhu *et al.*, 1994).

On the other hand, breeding for resistance is the most appropriate approach to control the pathogen in the field. Carlier *et al.* (2000) has reported that sources of resistance to pathogens exist in germplasm across the *Musa* genus. Development of FocR4-resistant cultivars can be possibly done through introgression of Resistance (R) genes into susceptible cultivars (Miller *et al.*, 2008). Joshi *et al.* (2010) reported that more than 50 R genes have been cloned and characterised from mono- and dicotyledonous plants through map-based mapping, transposon tagging and genome homologues analysis. Most R genes identified up to now are members of the cytoplasmic nucleotide-binding and leucine-rich repeat (NBS-LRR) class. It has been reported that NBS-LRR-type R genes confer resistance to a wide variety of pathogens and pests (Dangl & Jones, 2001). According to Meyers *et al.* (1999) and Pan *et al.* (2000), the NBS-LRR-class of R genes is divided into two distinct subclasses based on the presence or absence of an N-terminal with homology to the *Drosophila* Toll and human Interleukin-1 receptors (TIR). The TIR subclass appears to be restricted to dicotyledonous species, whereas the non-TIR subclass is widely distributed in both mono- and dicotyledonous species (Meyers *et al.*, 1999; Pan *et al.*, 2000; Cannon *et al.*, 2002).

Isolation and characterisation of NBS-type sequences, called resistance gene candidates (RGCs), using PCR-based approach based on degenerate primers

have been reported in a great number of plants including banana. For instance, Pei *et al.* (2007) have isolated, characterised and analysed 12 resistance gene analogues (RGAs) in banana (*Musa* spp.). In addition, 20 fragments of RGAs have been isolated from wilt resistant Goldfinger (AAAB) banana (Sun *et al.*, 2009). Besides this, Lu *et al.* (2011) also have reported the isolation and characterisation of four RGAs in commercial banana species.

On top of that, Way (2006) has partially isolated and studied the expression of five putative RGCs from the local banana crop, 'Jari Buaya', in specific interaction of host with pathogen FocR4. However, there was lack of functional study of these RGCs. It would be most useful if we could identify potential defence genes that are involved in the banana-FocR4 interaction. With better understanding of RGCs discovered and its mechanism in disease resistance, we may contribute to disease management based upon genetic improvement in banana. Thus, this study was carried out to screen for the presence of selected defence genes in the genome of various local banana genotypes and relate their expressions during host-pathogen interaction.

MATERIALS AND METHODS

Plant materials and DNA extraction

Four cultivars of local banana (*Musa acuminata*) were used including three cultivated triploid species and one cultivated diploid species. 'Rastali wild type' (AAB) was obtained from Johor Plant Tech Sdn Bhd (Ayer Hitam, Johor). 'Rastali Mutiara' (AAB) and 'Jari Buaya' (AA) were obtained from United Plantations Berhad (Teluk Intan, Perak), while 'Rastali Transgenic' (AAB) was provided by Professor Maziah Mahmood from the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. 'Rastali Mutiara' is known to be tolerant to FocR4, while 'Jari Buaya' is known to be resistant to FocR4 (Chai *et al.*, 2004). For gene expression study, only three banana genotypes were used due to the unavailability of 'Rastali Transgenic'. Genomic DNA was extracted from the lower stem of each healthy banana cultivar (one-month old seedlings) using a Genomic Purification Kit (Fermentas, USA) following the manufacturer's instructions. The concentration of DNA samples was adjusted to 10 ng/µL before use.

Primer Sets

Primers targeted for chitinase and beta-1,3-glucanase were designed based on the conserved regions of similar sequences obtained from GenBank. Primers targeted to Resistance Gene Candidate (RGC) 1, 2 and 3 were designed based on isolated cDNA sequences which were amplified using degenerate primers (Way, 2006). Sequences of RGC1, 2 and 3 belong to NBS-LRR type of Resistance gene. Primers targeted to RGC5 were designed based on isolated cDNA sequences belonging to kinase class. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of banana was used as an internal positive control. Details for the primer sequences of each gene are illustrated in Table 1.

TABLE 1
Primer sequences and PCR annealing conditions used to amplify target genes

Gene	[z]Tm (°C)	Product size (bp)	Forward primer (5'-3') Reverse primer (5'-3')	Genbank accession number/ Reference
GAPDH	58	124	F:GCAGGTCAAGCATCTTTGATGCCA R:ATGTGGCGGATCAGGTCGATTACA	AY821550
chitinase	47	224	F:TGCTGTTATTTGCGTTCCTG R: GTTGTTCCGAGGGTCACAGT	AY525367
beta-1,3-glucanase	48	194	F:CCCTCAGGAACTCCAACATC R:GAGGATGTACTGCGCCAGAT	EU014210
RGC1	55	449	F:ATGGCGCTTCTTCTCATGTCGC R:TCAACAACGAGCTCAAGGAGAA	Way, 2006
RGC2	45	426	F:CCTGTGTCCTTTAGATATTGGGCA R:TGGTAAAATCAAAGCCAGCTTCCG	Way, 2006
RGC3	45	448	F:CCTGTGTCCTTTAGATATTGGGCA R:TCGCTCAGAAGTTGTTCAATGATGG	Way, 2006
RGC5	55	~400	F:CGTACTTCTTCAGCGAGGCGGA R:ACGTCAAGACCACCAACATCC	Way, 2006

[z] Annealing temperature for PCR amplification

PCR Amplification

PCR amplification of each gene was performed in a final volume of 25 µL. PCR-reaction mixture contained 0.2 U *Taq* DNA polymerase, 1X PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of each primer, 10 ng DNA template and milli-Q of water added up to 25 µL. The PCR reactions were performed using a Biometra T-Professional thermocycler (Goettingen, Germany) and programmed as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 45-55 °C (depending on Tm of primers) for 30 s and 72 °C for 1 min; and an additional elongation period of 10 min at 72 °C.

DNA Sequence Analysis

Amplified products were separated on 2.0% agarose gel and visualised under UV light. PCR products were purified using a Gel Extraction Kit (Qiagen, Germany). DNA sequencing was performed using 3730 x l DNA analyser (Applied Biosystems ABI, USA) by NextGene Company (Selangor, Malaysia). The identity of chitinase, beta-1,3-glucanase and RGCs was analysed by comparison of DNA and amino acid sequences with the GenBank database using BLASTX and BLASTP (Altschul *et al.*, 1990) algorithms. Multiple sequence alignment with Clustal-X (Thompson *et al.*, 1997) was then conducted along with three RGCs (RGC1, 2 and 3), five R genes previously reported in other plant species (tobacco N; *Arabidopsis* RPM1; flax M; rice XA1 and potato RGA2) and one *Musa* AAB resistance gene analogue (banana MRGL2). The same NBS-encoding R genes or RGAs were used for protein phylogenetic analysis. RGC5 was aligned using Clustal-X along with serine/threonine kinase genes of other

plants (muskmelon PTH-2; potato Pto-like; tomato Pto and Fen). The construction of a neighbour-joining tree (Saitou & Nei, 1987) was conducted using the MEGA4.0.2 software, and the reliability of tree branches was evaluated using the Bootstrap method with 1000 bootstrap iterations (Felsenstein, 1985).

Preparation of Inoculum and Inoculation

A pure isolate of FocR4 obtained from the fungal culture collection of the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, was cultured on Potato Dextrose Agar (PDA) and incubated for seven days at 26±2 °C. Spores were harvested with sterile distilled water and adjusted for a stock solution at 4 x 10^5 spores/mL. One-month old seedlings of three different banana genotypes, 'Rastali wild type', 'Rastali Mutiara' and 'Jari Buaya', were inoculated by soil drenching with 100 mL stock solution of FocR4 for each plant and maintained in the glasshouse. The plants were watered daily and fertilised monthly with NPK (15:15:15, W:W:W). Three sampling intervals were carried out at 0, 2 and 4 weeks after FocR4 inoculation (WAI).

Disease development was evaluated based on foliar symptom at weekly intervals until 6 WAI and expressed as percentage disease severity (% DS) using a scale of 0 to 5; 0 = healthy plant; 1 = lowest leaf with yellowish streaks and brown necrosis absent; 2 = less than 25-50% of the total number of leaves with yellowish streaks and brown necrosis present; 3 = more than 50-75% of the total number of leaves with yellowish streaks and brown necrosis present; 4 = 100% of total number of leaves with yellowish streaks and brown necrosis present; 5 = plant collapsed and died due to severe wilting. DS (%) of *Fusarium* wilt was calculated based on the following formula:

$$\frac{\Sigma \text{ (No. of diseased plants in each rating category x Severity rating)}}{\text{Total no. of plants assessed x Highest scale}} \times 100$$

The experiment was conducted in randomised complete block design (RCBD) with four replications, where each replicate comprised 10 seedlings. All data were analysed by ANOVA using SAS 9.0. The mean comparison was performed using least significant difference (LSD) at $p \leq 0.05$).

RNA Extraction and RT-PCR

Total RNA was extracted from the lower stem of various banana genotypes for each sampling interval using RNeasy Plant Mini Kit (Qiagen, Germany). The integrity and concentration of total RNA of each genotype were determined using UV-spectrophotometer (NanoDrop Technologies, USA) and agarose gel electrophoresis. A two-step RT-PCR amplification was performed. First strand cDNA was constructed using Omniscript Reverse Transcription Kit (Qiagen, Germany). Primers targeted to GAPDH, chitinase, beta-1,3-glucanase, RGC1, RGC2 and RGC5 genes were used in this study. Each PCR reaction was performed in a final volume of 25 µL in a PCR-

reaction mixture containing 0.2 unit *Taq* DNA polymerase, 1X PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM of each forward and reverse primers, 50 ng cDNA template and milli-Q water. The RT-PCR amplifications were performed in a Biometra T-Professional thermocycler (Goettingen, Germany) and programmed as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 45-55 °C (depending on Tm of primers) for 30 s and 72 °C for 1 min; and an additional elongation period of 10 min at 72 °C. PCR products were separated on 2.0 % agarose gel and visualised under UV light.

RESULTS AND DISCUSSION

PCR Amplification of Targetted Defence-related Genes and Sequence Analysis

PCR amplification of chitinase, beta-1,3-glucanase and RGCs resulted in a single DNA band of approximately expected size in the gel (Table 1). Identical results were produced in three replicate experiments. Sequences of banana RGCs and chitinase were submitted to the GenBank with accession numbers as follows: RGC1 (KC864792; 449 bp), RGC2 (KC864793; 409 bp), RGC3 (KC864794; 443 bp), RGC5 (KF006850; 365 bp) and chitinase (KC864795; 224 bp). However, the sequence of beta-1,3-glucanase (194 bp) was not submitted to the GenBank due to its short sequence.

DNA sequence analysis of PR genes revealed that the amplified chitinase gene sequence showed 99% homology (E value = 2e-109) to *Musa acuminata* class III acidic chitinase (AY525367). Meanwhile, beta-1, 3-glucanase gene sequence was 100% homologous (E value = 1e-95) to *Musa* x *paradisiaca* beta-1,3-glucanase (EF051254). On the other hand, the nucleotide sequences of the isolated RGC genes were translated into amino acid sequences using the ExPASy Translate Tool (http://web.expasy.org/translate/) and the amino acid sequences of banana Resistance Gene Candidates (RGCs) were compared with the protein sequences deposited in the GenBank using BLASTP algorithm (Table 2). The RGC1 showed 100% identity with NBS-type resistance protein of *Musa* sp. (ACK44409.1 and ABY75803.1), followed by more than 99% identity with NBS-LRR-type disease resistance protein of *Musa acuminata* (ABB96971.1). RGC2 showed 100% identity with NBS-type resistance protein of *Musa* ABB group (ACK44406.1) and *Musa acuminata* AAA Group (ABW96279.1). It also showed 100% identity with putative disease resistance protein of *Musa balbisiana* (CBW30194.1). RGC3 showed 100% identity with NBS resistance protein of *Musa* ABB Group (ACK44406.1), NBS-LRR disease resistance protein of *Musa* AAB Group (CAP66295.1) and NBS-LRR class resistance protein of *Musa acuminata* AAA Group (ABW96279.1). RGC5 showed 100% identity with Pto-like serine/threonine kinase of *Capsicum chinense* (AAQ82660.1) and putative Pto-like serine/threonine kinase of *Solanum sucrense* (AAK82707.1).

TABLE 2
Similarity between banana RGC sequences and GenBank accessions carried out using the BLASTP algorithm

Musa NBS	GenBank protein accession showing the highest similarity	GenBank ID	Identity[z]	E-value
RGC1	NBS resistance protein (*Musa* ABB Group)	ACK44409.1	100	5e-100
	Resistance gene candidate NBS-type protein, partial (*Musa acuminata* subsp. *malaccensis*)	ABY75803.1	100	8e-99
	NBS-LRR type disease resistance protein (*Musa acuminata*)	ABB96971.1	99	8e-100
RGC2	NBS resistance protein (*Musa* ABB Group)	ACK44406.1	100	2e-85
	NBS-LRR class resistance protein (*Musa acuminata* AAA Group)	ABW96279.1	100	1e-24
	Putative disease resistance protein (*Musa balbisiana*)	CBW30194.1	100	1e-81
RGC3	NBS resistance protein (*Musa* ABB Group)	ACK44406.1	100	4e-93
	NBS-LRR disease resistance protein (*Musa* AAB Group)	CAP66295.1	100	4e-43
	NBS-LRR class resistance protein (*Musa acuminata* AAA Group)	ABW96279.1	100	1e-28
RGC5	Pto-like serine/threonine kinase (*Capsicum chinense*)	AAQ82660.1	100	3e-60
	Putative Pto-like serine/threonine kinase (*Solanum sucrense*)	AAK82707.1	100	4e-59

[z] Amino acid identity

Sequence Analysis of Resistance Gene Candidates for Conserved Motif

The amino acid sequences of three RGCs (RGC1, 2 and 3) isolated in this investigation were compared with other known R genes from different plants using the Clustal-X multiple alignment programme. As shown in Fig.1, kinase-2 (LVLDDVW), one of the crucial motifs of the NBS domain, was highly conserved among RGC2, RGC3 and the known NBS-LRR R-proteins. However, a little diversity existed in the kinase-2 motif of RGC1 where it was substituted by VLLDDVW. The same trend has been reported in *Musa* RGA-L (Pei *et al.*, 2007). Besides this, kinase-3 motif (GSRIIITTRD) was present in all RGCs, but more residue substitutions were observed. Furthermore, all RGCs had a conserved tryptophan (W) residue at the end of the kinase-2 domain (Fig.1). Absence or presence of TIR domain is used to classify the NBS-LRR genes into two different subfamilies where subfamily I contains the TIR element while subfamily II lacks it (Meyers *et al.*, 1999; Pan *et al.*, 2000). Several reports have demonstrated that the last residue of the kinase-2 domain can be used to predict with 95 % accuracy whether an RGA belongs to the TIR-NBS or to the non-TIR-NBS family; conservation of tryptophan (W) at this location is tightly linked to non-TIR R genes (RPM1, XA1,

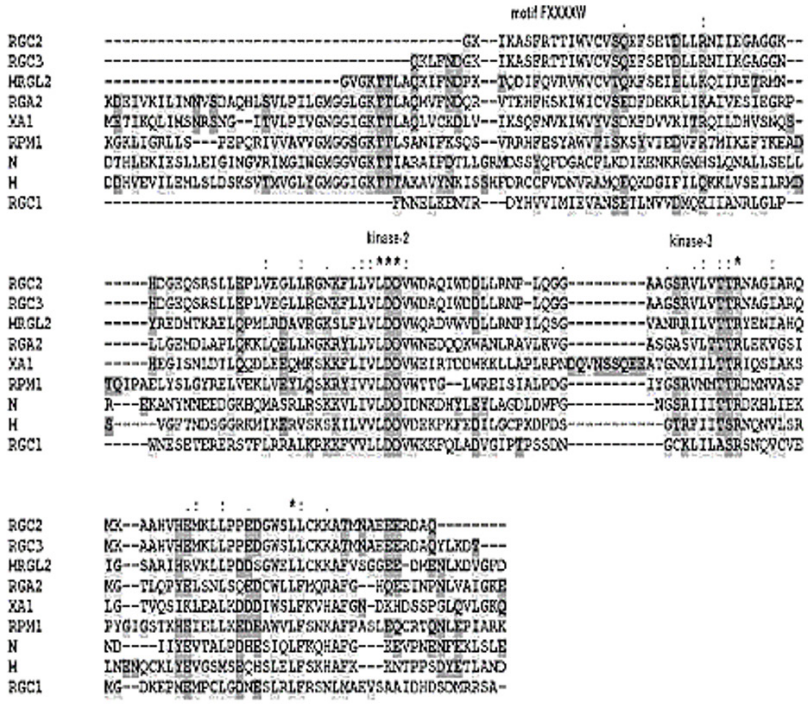

Fig.1: Multiple alignment of amino acid sequences of *Musa* RGCs with known NBS class R genes constructed with Clustal X. The R genes used were tobacco N (GenBank accession No. BAD12594.1); Arabidopsis RPM1 (GenBank accession No. NP187360.1), flax M (GenBank accession No. AAB47618.1); rice XA1 (GenBank accession No. BAA25068.1), potato RGA2 (GenBank accession No. AAP45188.2) and banana MRGL2 (GenBank accession No. HQ 199834.1). The conserved motifs identified are shown in the top panel. The presence of motif FXXXXW before kinase-2 in RGCs (2 and 3), Arabidopsis RPM1, potato RGA2, rice XA1 and banana MRGL2 indicates the non-TIR class as described by Pan *et al.* (2000).

RGA2), whereas conservation of aspartic acid (D) or its uncharged derivative aspartate (N) is characteristic of the TIR class of R genes (N and M) (Meyers *et al.*, 1999; Pan *et al.*, 2000; Jeong *et al.*, 2001; Song *et al.*, 2003). In this study, the conservation of tryptophan (W) residue at the end of the kinase-2 domain in RGC1, 2 and 3 evidently confirmed that R genes in banana were non-TIR-NBS-LRR type. This can be further confirmed with the presence of motif FXXXXW before the motif of kinase-2 in RGCs (2 and 3), RPM1, RGA2, MRGL2 and XA1, which also indicates the non-TIR class as described by Pan *et al.* (2000).

On the other hand, multiple-sequence alignment of RGC5 and known serine/threonine protein kinase genes showed significant homology to the sub-domain IX (LTEKSDVYSFGVVL) of serine/threonine protein kinase gene particularly motif DxxxxG (Fig.2). However, residue substitutes were present in the deduced amino acid of RGC5 along with the sub-domain VII through XI (except sub-domain

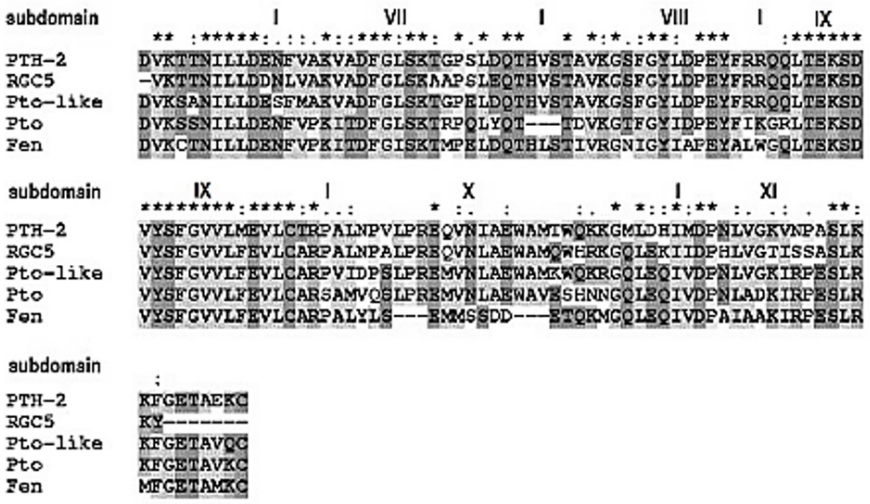

Fig.2: Multiple alignment of amino acid sequences of RGC 5 with serine/threonine kinase genes of other plants constructed with Clustal X. The serine/threonine kinase genes genes used were PTH-2 (GenBank accession No. AAL83882.1) from muskmelon, Pto-like serine/threonine kinase (GenBank accession No. AAK82715.1) from potato, Pto (GenBank accession No. AAB47421.1) and Fen (GenBank accession No. AAF76314.1) from tomato. Roman numerals identify the serine/threonine kinase subdomains as described by Vallad *et al.* (2001).

IX). This phenomenon was also represented in chestnut rose (Xu & Deng, 2010) and bean (Vallad *et al.*, 2001).

Phylogenetic Analysis of RGCs and Other Cloned R Genes

The deduced amino acid sequences of the three RGCs (RGC 1, 2 and 3) and several known NBS-LRR R-proteins from other plant species and banana plants were pooled for phylogenetic analysis. The resulting neighbour-joining phylogenetic tree (Fig.3) indicated that the known R proteins and RGCs could be classified into two groups: TIR- and non-TIR-NBS-LRR R-proteins. All RGCs isolated in this work were grouped into non-TIR-NBS-LRR type. RGC2 and 3 were significantly homologues and clustered within subclass containing non-TIR-NBS-LRR R-proteins. In addition, the amino acids encoded by these RGCs had the largest similarity with MRGL2 from *Musa* (AAB), suggesting that these genes are orthologs. As shown in Fig.3, none of the banana RGCs shared homology with the TIR-NBS-LRR R-proteins, namely N from tobacco and M from flax. The result obtained was in agreement with the hypothesis that the non-TIR subfamily was present in both mono- and dicotyledonous taxa (Pan *et al.*, 2000). On the other hand, RGC1 was divided into a separate subclass of non-TIR-NBS-LRR R-proteins from RGC2 and 3 (Fig.3). This indicated the presence of a diverse gene family coding for proteins with NBS-LRR domains in banana as previously reported by Miller

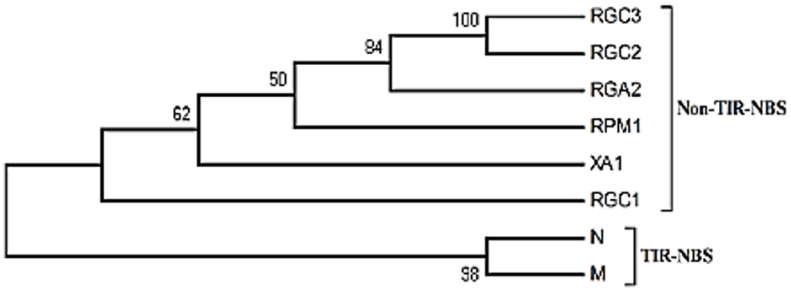

Fig.3: Phylogenetic tree of the deduced amino acid sequences of RGCs based on the neighbour-joining method. The numbers on the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The sources of known R genes were the same as shown in the note of Fig.1.

et al. (2008). The phylogenetic analysis also suggested that RGC1 is probably a new class of non-TIR RGCs in banana. Nevertheless, Pei *et al.* (2007) reported that this phenomenon was dependent on the known NBS-LRR R-proteins included in the analysis as references and requires a more comprehensive study.

Meanwhile, RGC5 was clustered together with PTH-2, a resistance gene homolog of muskmelon and Pto-like serine/threonine kinase from potato (Fig.4). However, phylogenetic analysis revealed that RGC5 had close relationship with PTH-2 from muskmelon, but not with Pto resistance gene from tomato.

Expression of Defence-associated Genes during Host-Pathogen Interaction

The GAPDH gene, two PR genes and four RGCs were detected in the genome of all four banana genotypes with the exception of beta-1,3-glucanase gene, which was not detected in 'Jari Buaya' (Fig.5).

RT-PCR analysis revealed the differential expression of PR and putative RGC genes exhibited by different banana genotypes over time (Fig.6). RGC3 was not used because the gene sequence showed an exact match with RGC2 through sequence analysis. The expression pattern of defence-associated genes in 'Rastali wild-type' (A) and 'Jari Buaya' (C) was similar, except for RGC2. As shown in Fig.6, the band intensity of chitinase was high in 'Rastali Mutiara' (B) compared to 'Rastali wild type' (A) and 'Jari Buaya' (C) before (0 WAI) and after FocR4 inoculation (2 and 4 WAI). This could be correlated with the absence of disease symptoms throughout the period of study (Fig.8). The induction and rapid accumulation of *SolChi*, a gene encoding an acidic isoform of class III chitinase upon infection with *Fusarium oxysporum* f.sp. *lycopersici* in a genotype-resistant tomato suggested its putative role in defence against fungal pathogens (Amaral *et al.*, 2012).

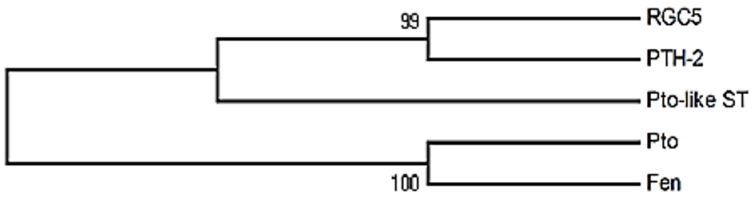

Fig.4: Phylogenetic tree of the deduced amino acid sequences of RGC5 with serine/threonine kinase genes of other plants based on the neighbour-joining method. The numbers on the branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The sources of serine/threonine kinase genes of other plants were the same as shown in the note of Fig.2.

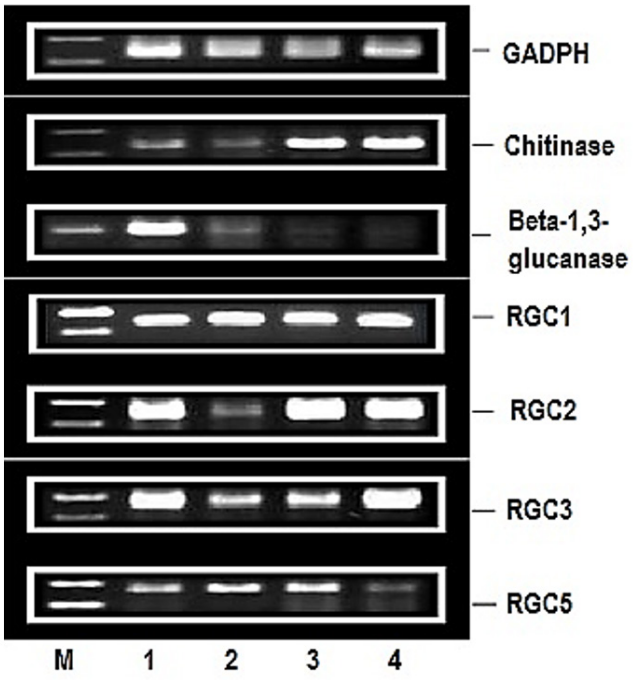

Fig.5: Detection of defence-associated genes and internal control (GAPDH) in various banana genotypes using 10 ng of genomic DNA. Lane M = 100 bp DNA ladder, 1 = 'Rastali wild type', 2 = 'Rastali Mutiara', 3 = 'Rastali Transformed', 4 = 'Jari Buaya'. The sizes of amplified products after sequencing were as follows, GADPH = 390 bp, chitinase = 224 bp, beta-1,3-glucanase = 194 bp, RGC1 = 449 bp, RGC2 = 409 bp, RGC3 = 443 bp, RGC5 = 365 bp.

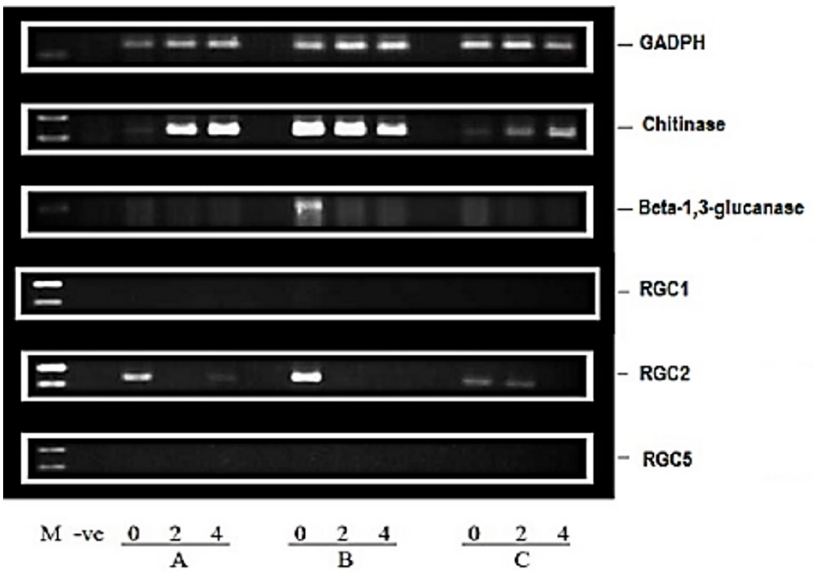

Fig.6: Expression of defence-associated genes and internal control (GAPDH) in various banana genotypes after challenge-inoculation with FocR4 at different intervals. Lane M = 100 bp DNA ladder, -ve = without templates, 0-4 = sampling weeks, A = 'Rastali wild type', B = 'Rastali Mutiara', C = 'Jari Buaya'. The sizes of amplified products after sequencing were as follows: GADPH = 124 bp, chitinase = 224 bp, beta-1,3-glucanase = 194 bp, RGC1 = 449 bp, RGC2 = 409 bp, RGC3 = 443 bp, RGC5 = 365 bp.

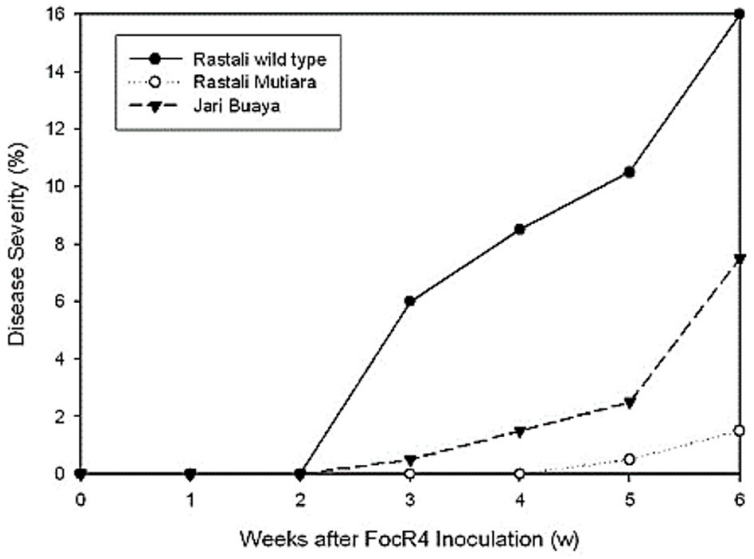

Fig.7: Disease severity of various banana genotypes according to weeks after challenge-inoculation with FocR4.

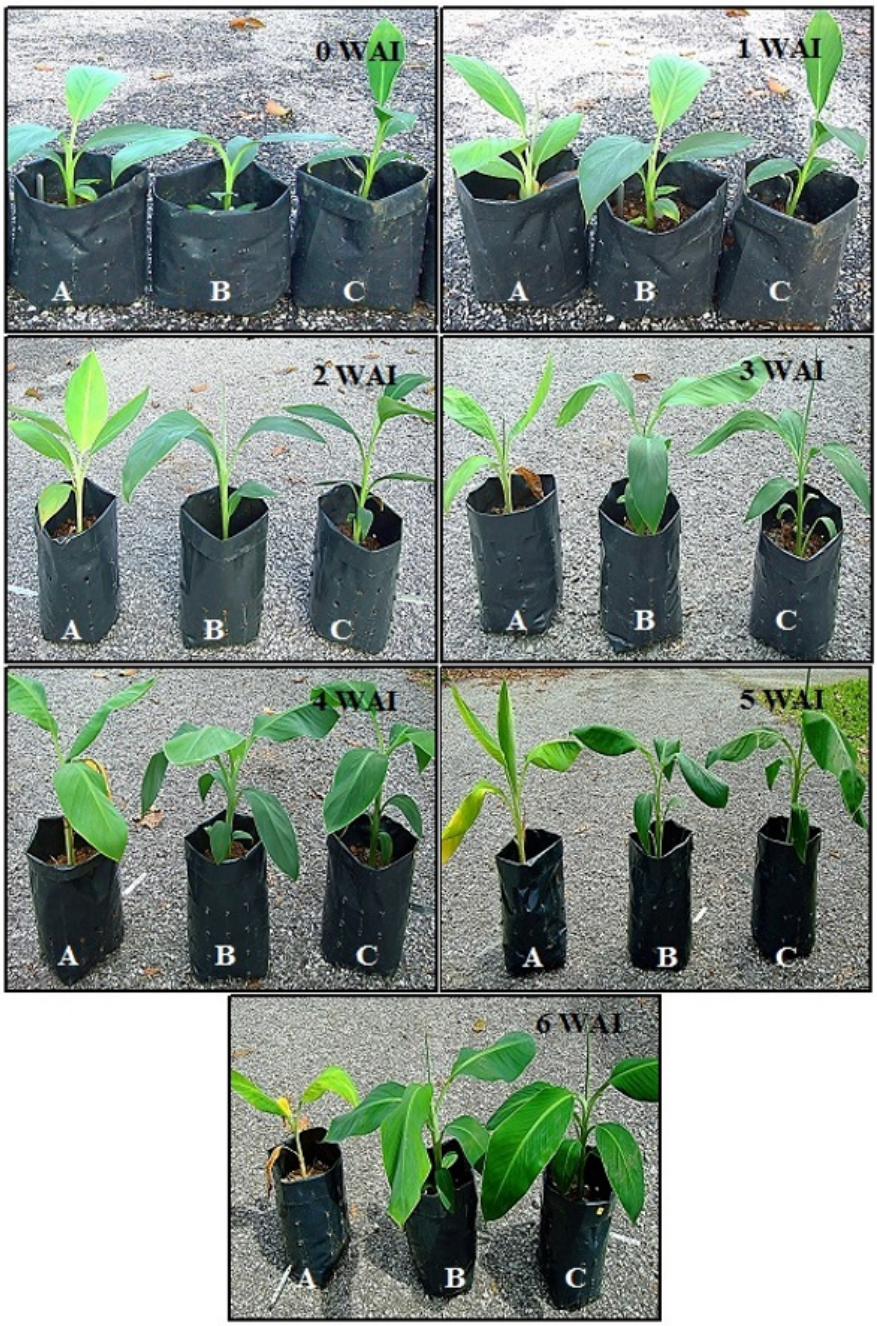

Fig.8: *Fusarium oxysporum* f. sp. *cubense* R4-inoculated banana (*Musa acuminata*) plants from three different cultivars under glasshouse conditions at 6 weeks after inoculation (WAI), A: 'Rastali' Wild-type, B: 'Rastali Mutiara', C: 'Jari Buaya'.

Likewise, Malafaia *et al.* (2013) also have associated the expression of chitinase with resistance of resistant tomato cultivar against *Fusarium* wilt. Moreover, the upregulation of chitinase in both 'Yueyoukang 1', the resistant-banana cultivar and 'Brazilian', the susceptible-banana cultivar after FocTR4 infection, has been related to plant defence in banana roots (Bai *et al.*, 2013). Hence, the higher and constant expression of chitinase against FocR4 in 'Rastali Mutiara' may possibly contribute to the lower percentage of disease severity and also explain the tolerant nature of this cultivar as claimed by the Food and Agriculture Organization (FAO) of the United Nations (Chai *et al.*, 2004). On the other hand, temporal change of chitinase expression in both 'Rastali wild type' and 'Jari Buaya' as shown in Fig.6 suggested that a defence mechanism has likely been activated in these cultivars upon FocR4 infection; however, it had negative correlation with disease resistance to *Fusarium* wilt (Fig.7).

Beta-1,3-glucanase only expressed in 'Rastali Mutiara' at 0 WAI and its expression was not detected in 'Rastali wild type' (A) and 'Jari Buaya' (C) during any period of sampling. This was inconsistent with the previous finding reported by Bai *et al.* (2013). According to Ebrahim *et al.* (2011), different clones of the same plant species can exhibit different production of beta-1,3-glucanase after pathogen inoculation. For instance, the activity of beta-1,3-glucanase enzyme increased in the tolerant clone of *Hevea brasiliensis* upon infection with *Corynespora cassiicola*, while in the susceptible clone it decreased (Philip *et al.*, 2001). Nonetheless, a more comprehensive study should be done on the expression of beta-1,3-glucanase in 'Rastali Mutiara', a tolerant variety against FocR4, to determine the factors affecting its down-regulation during host-pathogen interaction.

The expression level of RGC2 was high before inoculation in 'Rastali wild-type' (A) and 'Rastali Mutiara' (B). Conversely, its expression was down-regulated after inoculation at 2 WAI followed by slight up-regulation at 4 WAI in 'Rastali wild-type' (A). On the other hand, the expression of RGC2 was not detected after inoculation in 'Rastali Mutiara' (B) at 2 and 4 WAI. In 'Jari Buaya' (C), the expression of RGC2 was similar before inoculation and 2 WAI, but was not detected at 4 WAI. The expression of RGC2 in Rastali Mutiara (B) was negatively correlated with disease assessment and recorded a lower disease incidence in 'Rastali Mutiara' compared to 'Rastali wild-type' and 'Jari Buaya' (Fig.7). Resistance gene candidates from different plant species have been previously associated with resistance to phytopathogens. For example, expression of RGA1, RGA2, RGA5 and RGA23 was associated with resistance to *Plasmopara viticola* in grapevine (Wang *et al.*, 2013). Moreover, Peraza-Echeverria *et al.* (2007) have reported the association of RGC2 isolated from *Musa acuminata* subsp. *malaccensis* with resistance against *Fusarium oxysporum*. Although RGC2 has not been correlated with resistance to *Fusarium* wilt disease in this study, its

potential role in disease resistance could be tested in future using new technologies of the post-genomic era, such as RNA interference (RNAi) as proposed by Waterhouse and Helliwell (2003).

Furthermore, RGC1 and RGC5 were not expressed during pathogen-host interaction even though it was detected in the genome of all banana genotypes studied (Fig.5). Previous studies have shown that most of the R genes encode nucleotide binding site (NBS), leucine rich repeat (LRR) motif and serine-threonine protein kinase. However, Meyers *et al.* (1999) have reported that this type of NBS motif and serine-threonine kinase not only encoded in R genes but also encoded as important genes involved in development and signal transduction. From this result, we propose that RGC1 and RGC5 were not involved in activation of defence response against pathogen attack in *Musa acuminata*-FocR4 interaction. In future research, we will focus on cloning and sequence analysis of full-length *Musa* RGC1 and RGC5 in order to acquire a better understanding of its function in banana.

CONCLUSION

In this study, two PR proteins and four RGCs were isolated from different banana genotypes. All RGCs that were characterised at the amino acid level may provide a basis for cloning the full length of disease-resistant gene where only a couple of cases of R genes in banana have been reported so far. In addition, the expression pattern exhibited by these PR proteins and RGCs during host-pathogen interaction was also demonstrated in this study. Chitinase, with constant expression over time in all 'Rastali' genotypes studied, may play an important role in disease resistance against FocR4. Intense expression of chitinase in 'Rastali Mutiara' and occurrence of very low disease severity (2%) in FocR4 inoculated plants demonstrated that this cultivar can be a good source of resistance.

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