THE EVALUATION OF RESISTANT GENE ANALOGUES (RGAS) ON TWO WILD *MUSA* SPECIES AGAINST FUSARIUM WILT DISEASE

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ABSTRACT

Information about the molecular aspects of genes that control the mechanism of resistance to *Fusarium oxysporium cubense* (*Foc*) wilt disease is still very limited. Most of resistance genes (R genes) encode receptor proteins. About 70% of receptor proteins of R genes contain the domain nucleotide binding site and leucine rich repeat (NBS-LRR) which control resistance to pathogens. The expression of resistant gene analogues (RGAs) on *Foc* infected banana plantlets will be evaluated in this study. The two wild *Musa* species (*Musa acuminata* ssp. *halabanensis* and *Musa balbisiana* from Nusa Tenggara Timur) used in this research are native to Indonesia. Two *Foc* isolates (TR 4 or VCG 01213/16 and race 1 or 0124/5) were used for evaluation. Four RGAs were used on semi quantitative RT-PCR of *Foc* infected root samples. Two RGAs namely MNBS16 and RGC2 were expressed on only the infected plants, while MNBS5 and MNBS15 were expressed on both infected and uninfected plants. From this study it can be concluded that MNBS16 and RGC2 were involved in the resistance mechanism of banana against *Foc*.

Keywords: RGAwild, *Musa* species, *Fusarium* wilt

1. INTRODUCTION

Plant growth is strongly influenced by climate factors. In addition to influencing production, climate also influences the development of pathogens, one of which is the *Fusarium oxysporum* f.sp. *cubense* (*Foc*) fungus. *Foc* causes wilt disease on bananas. The use of disease resistant cultivars is one of the strategies to control banana plant diseases (Rowe & Rosales, 1996). Information about the molecular aspects of genes that control the mechanism of resistance to Foc wilt disease is still very limited. Most of resistance genes (R genes) encode receptor proteins. About 70% of receptor proteins contain the domain nucleotide binding site and leucine rich repeat (NBS-LRR) which are involved in resistance mechanism to pathogens such as insect pests, fungi, bacteria, viruses, and nematodes (Dangl & Jones, 2001).

The NBS-LRR receptor protein recognizes pathogen effectors proteins and produces transduction signals that will stimulate the expression of defenses against pathogens (Caplan *et al*., 2008). NBS-LRR gene composition consists of several domains, namely N-terminal, nucleotide binding site (NBS), and C-terminal LRR. The NBS domain contains *p-loop* or kinase-1, kinase-2, kinase-3a and hydrophobic (GLPL) motifs that are conserved in various ATP- / GTP-binding proteins from various organisms (Traut, 1994).

Up to 2013, National Center for Biotechnology Information (NCBI) has registered more than 180 resistant gene analogue (RGA) gene sequences from the NBS-LRR class isolated from bananas. After being reexamined based on nucleotide and amino acid sequences, it is known that from

180 RGAs only 168 RGAs have NBS-LRR characteristics (Pei *et al*., 2007; Peraza-Echeverria *et al*., 2008; Azhar & Heslop-Harrison, 2008; Sun *et al*., 2009; Sutanto *et al*., 2014).

The variety of NBS-LRR type RGAs gives a big picture of the family of NBS on *Musa*. Peraza-Echeverria *et al*. (2008) found an RGA from wild *Musa acuminata* ssp. *malaccensis* that expresses against fusarium fungal infection of race 4. Based on this, it is estimated that there are still many resistance genes to *Foc* wilt disease found in other wild Musa. This study will evaluate the expression of RGAs on two wild Musa species after infected by *Foc* wilt disease, especially for TR 4 (VCG 01213/16) and race 1 (0124/5).

2. MATERIAL AND METHOD

2.1. Materials

The two wild bananas used in the study are native to Indonesia. These were *Musa acuminata* spp. *Halabanensis* and *Musa balbisiana* from Nusa Tenggara Timur. Meanwhile, *Foc* suspension (dH20 + *Foc*) at density 106/mL was used for inoculation of samples. The *Foc* isolates used in this study were VCG 01213/16 (TR4) and VCG 0124/5 (race 1).

2.1. Method

2.1.1. Inoculation of Foc

Seeds of the two wild species were germinated and maintained until 10 cm in height (seedling). The seedling roots were dipped in *Foc* suspension for 15 minutes. The RNA extraction was carried out at 24 and 48 hours after *Foc* inoculation.

2.1.2. Transcript expression analysis

RNA Extraction

The total RNA was extracted from the roots of *Foc* treated or untreated seedlings, using Plant Total RNA Mini Kit (Geneaid, USA) as company instruction.

Semi quantitative RT-PCR

The cDNA was synthesized from total RNA using Tetro cDNA Synthesis Kit (Bioline) based on company intructions. The primers used were the primer pairs that were designed based on:

a. MNBS5: 5′-AAAAGTTCCAGTTGGCGGAC-3′ & 5′-GTCGCTGTCATGGTTGATGG-3′ (Sutanto *et al*., 2014);
b. MNBS15: 5′-CGGAGAGTTAATTCGGTGCG-3′ & 5′-CGTGCTGCTACCTACTGCTT-3′ (Sutanto *et al*., 2014);
c. MNBS16: 5′-TGGGGCACAGATGTATGGGA-3′ & 5′-CCCAATCTCAGGCTCCTCCT-3′ (Sutanto *et al*., 2014);
d. RGC2: (5′-GGGTGTGTGTGTCTGACGAT-3′ & 5′-ATGGGGCTAACAGGCTTTCC-3′ (Peraza-Echeverria *et al*., 2008);
e. 25S rRNA (AY651067) (5′ACATTGTCAGGTGGGGAGTT-3′ and 5′-CCTTTTGTTCCACACGAGATT-3′) (Van den Berg *et al*., 2007). 25S rRNA is usually used as an endogenous internal control because this gene is a housekeeping gene with stable expression in the organism (Van den Berg *et al*., 2007).

f. PCR was carried out in a volume of 25 µL containing 20 ng cDNA, 12.5 µL MyTaq™ Red Mix (Bioline), 1 µL of each 10 µM primer, and double destilled water. The PCR reactions were 3 min. of 95°C and followed by 29 cycles of 95°C for 1 min., 53°C for 10 sec., and 72°C for 10 sec. Finally the reactions were incubated at 72°C for 10 min. PCR products were separated using agarose gel electrophoresis and visualized using a gel doc. The bands of PCR products were quantified using GelQuant.net software (http://biochemlabsolutions.com/GelQuantNET.html) in order to estimate the level of expression.

3. RESULT

Analysis of RGA expression is indicated by the abundance of RNA after wild bananas were inoculated with *Foc* suspension. The abundance of RNA can be quantified based on the thickness of the electrophoresis band. Electrophoresis results on *M. acuminata* spp. *halabanensis* (Figure 1) and *M. balbisiana* (Figure 2) were diverse. MNBS16 primer on *M. balbisiana* did not produce a band. This might have been caused by human errors that occurred during the study.

The band profile in agarose gel is the result of electrophoresis from wild banana cDNA. Normalization of real-time PCR data using control genes is needed to obtain accurate and reliable gene expression results. 25S is one of the internal control genes that will be displayed very uniformly in living organisms during various phases of development and in different environmental conditions (Jain *et al.*, 2006)

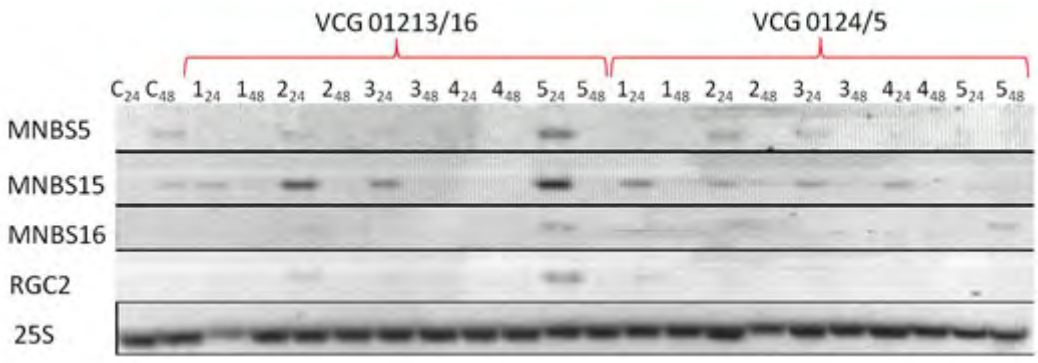

Figure 1: MNBS5, MNBS15, MNBS16, and RGC2=primers, C=without inoculation, 1-5= sample number 1...5 (*M. acuminata* ssp. *halabanensis*) were inoculated by VCG01213/16 (*Foc* TR4) and VCG0124/5 (*Foc* race 1), 24 and 48=24 and 48 hours after *Foc* inoculation.

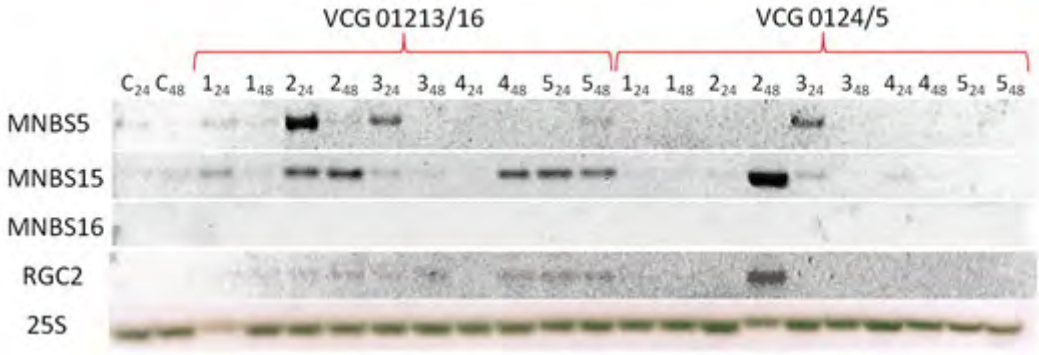

Figure 2: MNBS5, MNBS15, MNBS16, and RGC2=primers, C=without inoculation, 1-5=sample number 1...5 (*M. balbisiana*) were inoculated by VCG01213/16 (*Foc* TR4) and VCG0124/5 (*Foc* race 1), 24 and 48=24 and 48 hours after *Foc* inoculation.

In this research, the abundance of RNA was measured semi-quantitatively using Gelquant.net software. The abundance of RNA is indicated by the level of RGA expression. The presence of RGA expression indicates the activation of genes that can recognize the presence of *Foc* wilt disease (fig. 3-6= *M. acuminata* ssp. *halabanensis* with primers MNBS5, MNBS15, MNBS16, and RGC2; and fig. 7-9= *M. balbisiana* with primers MNBS5, MNBS15, and RGC2).

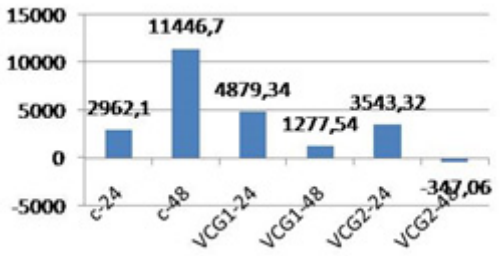

Fig. 3. RGA expression on *M. acuminata* ssp. *halabanensis* using primer MNBS5

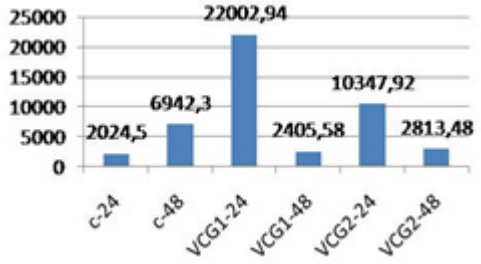

Fig. 4. RGA expression on *M. acuminata* ssp. *halabanensis* using primer MNBS15

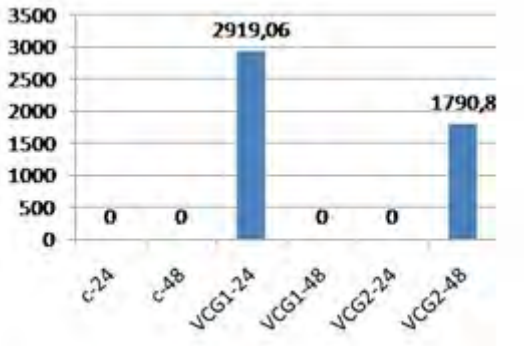

Fig. 5. RGA expression on *M. acuminata* ssp. *halabanensis* using primer MNBS16

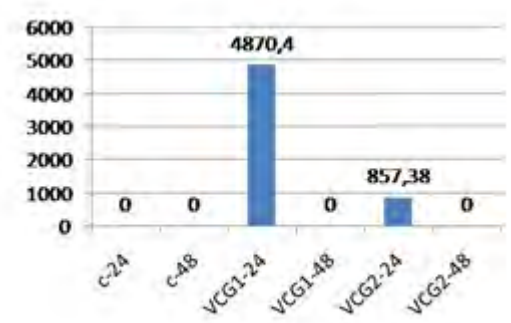

Fig. 6. RGA expression on *M. acuminata* ssp. *halabanensis* using primer RGC2

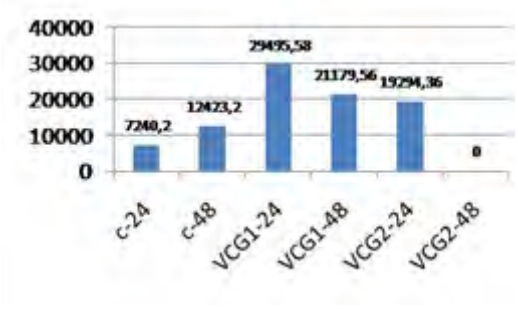

Fig. 7. RGA expression on *M. balbisiana* using primer MNBS5

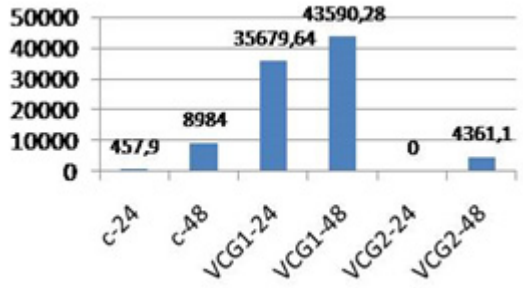

Fig. 8. RGA expression on *M. balbisiana* using primer MNBS15

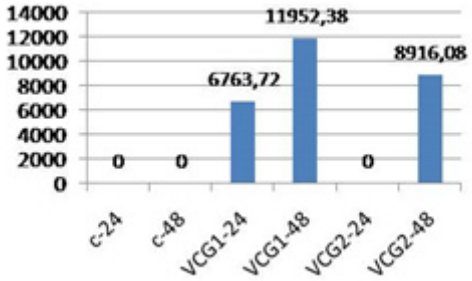

Fig. 9. RGA expression on *M. balbisiana* using primer RGC2

Based on the results of capture of RNA abundance, not all RGA expressions were related to the gene activites against *Foc* infection. In the figures 3, 4, 7, and 8, there were abundance of RNA expressions from MNBS5 and MNBS15 in the control treatment or no *Foc* infection. These data in the figures 3, 4, 7, and 8 showed that MNBS5 and MNBS15 were not involved in the resistance mechanism against *Foc*.

RGA MNBS16 was actively expressed in the roots 24 hours after being infected by *Foc* TR 4 and 48 hours after being infected by *Foc* race 1 on *M. acuminata* ssp. *halabanensis* (fig. 5). In the same wild banana, RGA RGC2 was also actively expressed 24 hours after being infected by *Foc* TR4 and race 1. RGA RGC2 was also actively expressed in the roots of *M. balbisiana* after 24 and 48 hours after infection of *Foc* TR4; and 48 hours after infection of *Foc* race 1.

4. DISCUSSION

The results of this study indicated that there were potential RGAs as candidate genes that can be obtained from Indonesian wild *Musa*, namely *Musa acuminata* ssp. *halabanensis* and *Musa balbisiana* from Nusa Tenggara Timur. The MNBS16 and RGC2 have proven to actively express against *Foc* infection both TR4 (VCG 01213/16) and race 1 (VCG 0124/5). It is likely that the data will be better if the RGA analysis using MNBS16 primer on *M. balbisiana* is repeated. Similar studies of other RGA primers and other wild Indonesian *Musa* will enrich information about RGA against *Foc* wilt disease. The next step that can be carried out is isolating the whole gene sequences. Based on the whole sequences, single-nucleotide polymorphisms can be identified for designing single nucleotide amplified polymorphism (SNAP) markers. The SNAP markers can support breeding programs for *Foc* resistance of banana.

5. CONCLUSIONS

Two RGAs namely MNBS16 and RGC2 were actively expressed in the *Foc* treated plants of *Musa acuminata* ssp. *halabanensis* and *Musa balbisiana*. It can be concluded that MNBS16 and RGC2 are potentially the R gene candidates against Foc TR4 (VCG 01213/16) and race 1 (0124/5).

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