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GENOME-WIDE IDENTIFICATION AND EXPRESSION ANALYSIS OF BANANA RBOH GENES IN RESPONSE TO *Fusarium oxysporum* f. sp. cubense Tropical Race 4

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Abstract

Respiratory burst oxidase homologs (RBOH) is the key enzyme responsible for the production of reactive oxygen species which act as important signal during plant responses to abiotic and biotic stresses. However, RBOH homologs have not been characterized in banana. In this study, we have identified twelve *Rboh* genes distributed on eight chromosomes of *Musa acuminata* subsp. *malaccensis* (DH Pahang) through a genome-wide analysis. *MaRbohs* exist as sibling paralogs with variable exon-intron structures and highly conserved functional domain. Phylogenetic analysis clustered MaRBOH into four distinct subgroups (I, II, III and IV). The expression of *MaRbohs* following 24 hours of inoculation with *Fusarium oxysporium* f. sp. *cubense* Tropical Race 4 revealed diverse patterns in root tissues. *MaRbohH1* and *MaRbohA2* were strongly upregulated and downregulated, respectively in response to the root-dip inoculation. This is the first report on genome-wide characterization of *Rboh* genes from banana and their expression in response to the fungal pathogen TR4. This research provides a basis for exploration of the role of NADPH oxidase in banana defence against the root pathogen.

INTRODUCTION

Pathogen infection triggers production of reactive oxygen species (ROS) in plant tissues as a timely response to a successful pathogen recognition which is known as 'oxidative burst'. There are several mechanisms reported to be involved in generating cellular sources of ROS in plant, but membrane-localized NADPH oxidase is recognized as the major source [1,2]. In plants, NADPH oxidase-encoding gene is recognized as respiratory burst oxidase homologs (*Rbohs*) [3]. Since the discovery of the first *Rboh* gene in rice [3], a number of *Rbohs* were identified not only in the model plant *Arabidopsis thaliana* (*Arabidopsis*) [4] but also in other species including mustard, jatropha, tobacco and cotton [5,6,7,8].

The *Rbohs* are present in multiple copies in a plant genome and organized into a multigenic family [9]. The genome of *Arabidopsis* encodes ten *Rboh* homologues (*AtrbohA–J*) with different expression profiles and function in plant morphogenesis, development and stress responses [4]. In rice, a total of nine *Rboh* homologues have been identified (*OsrbohA-I*; [10]). The expansion of *Rboh* gene family and variation in the gene number within plant species could be attributed to segmental duplications and structural diversity within the gene family [5]. RBOH proteins contain NADPH- and FAD-binding domains in their core C-terminal region [11]. However, unlike their animal counterparts, the plant proteins have a cytosolic N-terminus consisting of two

calcium-binding EF-hand motifs and six transmembrane domains [12].

There are two layers of plant defence system, the pathogen-associated molecular patterns (PAMPs)-triggered immunity and the effector-triggered immunity (ETI; [13]). In *Arabidopsis*, ROS production is predominantly dependent on RBOHD in both PTI and ETI [14] which is regulated by phosphorylation and ubiquitination [15]. An *Arabidopsis atrbohd* mutant was compromised in ROS production in response to infection by ETI-inducing pathogen or elicitor treatment [16]. The *Rboh*-dependent ROS production in response to pathogen infection was also shown in other plant species, including rice and tobacco [17,18]. Due to the structural diversity and complexity of *Rboh* genes in plant, pathogen-induced ROS production is not unique to *RbohD*. In plant species such as rice, potato and tomato, *RbohB* was implicated with the pathogen-induced ROS production [19].

The Musa genus includes both bananas and plantains. Being the most exported fruits in the world, banana and plantains are valuable market commodity. They are also recognized as major sources of staple food in many developing countries particularly in Latin America, Africa, Asia and Caribbean [20]. In Malaysia, *Musa acuminata* cv. Berangan (AAA genome) is known as the most popular local cultivar [21] but at the same time highly susceptible to Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4; [22]). The situation necessitates a clear deciphering of stress response mechanisms underpinning banana-TR4 interaction. However, as of today, the functions of banana *Rboh* family genes as essential players of responses to stress and their regulatory mechanisms are still largely unknown. In this study, in-silico characterization and quantitative analysis of gene expression was conducted to identify the banana *Rboh* genes and study their functions specifically in response to TR4. The outcome of this study will undoubtedly enhance our knowledge of the function and regulation of *Rboh* especially in non-model crop.

MATERIALS AND METHODS

Plant Material and Inoculation

2-month-old micropropagated TR4-susceptible Berangan seedlings were purchased from Green Global Sdn. Bhd. (Klang, Selangor). The seedlings were placed in a greenhouse with a surrounding temperature of 29-32°C and watered daily. Pure culture of TR4 strain 9888 was obtained from Fusarium Culture Collection, School of Biological Science, Universiti Sains Malaysia and subcultured on potato dextrose agar (PDA). A root dip inoculation method was used to inoculate Berangan plants with TR4 as described in [23]. Briefly, the conidial suspension of TR4 were collected by rinsing the culture on PDA with distilled water and filtered with sterile Mira cloth. The final spore suspension concentration was adjusted to 10^6 conidia/mL. The plants were removed from the polythene bags and the roots were washed with tap water to remove soil. During this process, the plants were kept with adequate water level to avoid stress. The root tips were cut, and the roots were left dipping inside the suspension for 2 hours after which the plants were removed from the suspension and replanted in the greenhouse. The roots were collected at 24 hours post inoculation (hpi) during which TR4 is expected to accumulate in the vascular tissue. The experiment was conducted in a completely randomized design (CRD) with three biological replicates for each treatment at each time point.

Identification of Banana Respiratory Burst Oxidase Homolog (*Marboh*) Genes

For the identification of *Rboh* family members in banana, Hidden Markov models (HMMs) profile of RBOH domains namely Ferric_reduct (PF01794), NADPH_Ox (PF08414), NAD_binding_6 (PF08030) and FAD_binding_8 (PF08022), were retrieved from the Pfam database (http://pfam.xfam.org/). The HMMs were used as queries in BLASTP program (E-value = 1×10^{-10}) to scan *Musa acuminata* AA Group (taxid:4641) in the National Center for Biotechnology Information (NCBI) database. Different transcripts sequences that mapped to a single gene were removed. Protein sequences were collected and the online softwares SMART (http://smart.embl-heidelberg/), NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and MOTIF (https://www.genome.jp/tools/motif/) were used to ratify the reliability of the RBOH domains. Annotation of the candidate *Rboh* genes was cross-checked with the reference genome of *Musa acuminata* DH Pahang from the Banana Genome Hub - South Green (genome-hub.southgreen.fr).

Phylogenetic Tree Construction and Sequence Analysis

The predicted amino acid sequences of the RBOH homologs of *Musa acuminata, Oryza sativa, Triticum aestivum, Hordeum vulgare* and *Arabidopis thaliana* were aligned using MUSCLE in Mega X [24]. Phylogenetic trees were constructed in MEGA X using the maximum likelihood method with the Jones-Taylor-Thornton matrix-based model [25]. Estimation of the best model was done using MEGA X. Initial trees for the heuristic search were obtained automatically. A bootstrap analysis with 1000 replicates was used to validate the tree topology. The structures of *Marbohs* were analysed using GSDS server (http://gsds.cbi.pku.edu.cn/) and the protein motifs were analyzed using MEME (http://meme-suite.org/tools/meme) with default parameters. Prediction of subcellular location and putative signal peptides were carried out via the SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and ProtComp

9.0(http://www.softberry.com/berry.phtml?topic=protcompl&group=programs&subgroup=proloc). Annotation of predicted transmembrane regions was done using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Quantitative Analysis of *Marboh* Expression during Compatible Interaction with TR4

Extraction of total RNA from banana root samples was done using CTAB method [26]. Following quality and integrity assessment, the total RNA (1 µg) was reverse-transcribed using the Tetro cDNA Synthesis Kit (Bioline, USA) according to the manufacturer's instructions. The expression level of *MaRboh* genes were determined by quantitative RT-PCR that was performed using KOD SYBR® qPCR Mix (Toyobo, Japan) in triplicate of each sample. The following program was used on the iQ5 real-time PCR system (Bio-Rad, USA): 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 30 s. A melting-curve analysis was included to verify primer specificity. The amplification efficiency of each primer pair was evaluated by the LinRegPCR program [27]. The relative expression level was analyzed using Pffafl method [28] using ribosomal protein L2 (*L2*) as an internal control [29]. Primer pairs for *MaRboh* and *L2* genes are listed in Table 1. All *Rboh*-specific primers were designed using Primer-Blast in NCBI and synthesized by Apical Scientific Sdn Bhd. All data are presented as means ± standard deviation (SD).

Table 1. Primers for quantitative RT-PCR.

Gene name	Forward primers (5' to 3')	Reverse primers (5' to 3')
MaRbohA1	AGCACCAAGTTCGACTTCCA	GCGGTTGTGTTCATCTACGC
MaRbohA2	TGCTACGACCACAACCAGAG	TGCTCATCAGCATCCGTTGG
MaRbohA3	TGGCGCAGGAGCTTAACAAG	ACCGTGTGGATAAGTAATGCGA
MaRbohB1	ACCGACACTGGTGAAAGAGC	TACTATTGGCGTGCGTTTGC
MaRbohB2	AGCTAGCCACGGATTTCTCG	AAAAGGCAGGAAGGTGGTGT
MaRbohC	CCAGCTGGCTCAGGATTTCA	AGCGAGTGGTGTGTCTAAGAG
MaRbohE1	CAACACGCTTCCATTTCCACA	TGCTTGTGTAGATTGATTATGCAGG
MaRbohE2	AAGAGCTGAGGAAACTGTCGC	TTGTCTTGCTGTACCGTATCCATT
MaRbohH1	ATCAACGCGTCGGAGTCTTT	AGAATAGCTGACAGCATCAGAAGT
MaRbohH2	GCGCCACTCGTTTCGATTTC	GCACCGGAACATCAACCAAC
MaRbohH3	ATTCAGCCATGACACCAGCA	CAAGACTTTTCTAATGGTTCCCGA
MaRbohH4	GGACCTCCCACACTCACAAA	ACGACCATGTTTTGGCTACC
L2	AGGGTTCATAGCCACACCAC	CCGAACTGAGAAGCCCCTAC

RESULTS AND DISCUSSION

ROS flux and redox regulation have been established to be the key regulator of fungal disease development and host plant-resistant responses, principally by means of ROS generation via NADPH oxidase [30]. In plants, the enzyme is coded by a multi-gene family of *Rbohs*. A search was performed using a hidden markov model (HMM) to screen *Rboh* gene family in the genome of *Musa acuminata* subsp. *Malaccensis* (DH Pahang, AA). From the search, twelve non-redundant putative protein sequences with four conserved domains typical of NADPH oxidase proteins; namely NADPH_Ox, FAD_binding_8, Ferric_reduct and NAD_binding_6 domain were retrieved from NCBI. The genes were then named *MaRbohA1, A2, A3, B1, B2, C, E1, E2, H1, H2,* H3 and *H4* according to multiple sequence alignment with rice and *Arabidopsis.*

The presence of duplicate genes in the *Rboh* gene family of banana is anticipated since the A genome of banana is known to contain a high number of multigene families with abundant paralogs [31]. The redundancy is caused by three whole genome duplications (WGDs) underwent by the ancestral monocot genome probably as an adaptation to altered environments. All twelve genes were mapped to a specific chromosome (2, 3, 5, 6, 8, 9, 10 and 12) of DH Pahang genome with uneven distribution where chromosome-6 contained the topmost number of three genes (Table 2). High redundancy of *MaRbohs* in chromosome 6 might suggests the significant function of this chromosome in plant stress responses in banana.

MaRbohH2 displayed the shortest predicted gene length (3922 bp) while the longest is exhibited by *MaRbohE2* (12,335 bp). Their predicted protein lengths were 833 and 946 amino acids, respectively. The molecular weight of MaRBOH proteins ranged between 93.9 to 107.5 kDa, while their isoelectric points (pI) varied from 8.95 (MaRBOHB1) to 9.67 (MaRBOHE1). All MaRBOHs were predicted to localize in the plasma membrane like other plant homologs, depicting their major function in numerous oxidation-reduction associated processes. At this stage, it can be concluded that twelve *MaRboh* genes with four conserved domains typical of NADPH oxidase proteins were identified from the DH Pahang genome. A few of the genes were present as duplicate copies.

Table 2. List of *MaRboh* genes identified in current study.

Gene name	Gene ID		Position	Gene size (bp)	AA	MW (kDa)	pI
	NCBI	Banana Genome Hub					
MaRbohA1	104000638	Ma10_g12530	Chr 10	5588	940	106.0	9.08
MaRbohA2	103989616	Ma06_g33550	Chr 6	5347	941	106.5	9.39
MaRbohA3	103997017	Ma09_g05320	Chr 9	6000	950	107.5	9.22
MaRbohB1	103976777	Ma02_g23200	Chr 2	4650	909	102.0	8.95
MaRbohB2	103987964	Ma06_g17780	Chr 6	4866	938	103.7	9.11
MaRbohC	103987581	Ma06_g13290	Chr 6	4809	938	105.9	9.33
MaRbohE1	103995190	Ma08_g24400	Chr 8	8586	954	107.3	9.67
MaRbohE2	103977436	Ma03_g05770	Chr 3	12335	946	106.3	9.34
MaRbohH1	103986290	Ma05_g29360	Chr 5	4171	891	101.0	9.40
MaRbohH2	103999897	Ma10_g06240	Chr 10	3922	833	93.9	9.24
MaRbohH3	103972261	Ma11_g22240	Chr 11	4566	848	95.9	9.49
MaRbohH4	103986089	Ma05_g31620	Chr 5	6708	863	97.8	9.25

Based on the unrooted phylogenetic tree, *MaRboh* genes were separated into four distinct clades as sibling paralogs: *MaRbohA1, Bs, C* and *H1* in the first clade, *MaRbohA2* and *A3* in the second clade, *MaRbohEs* in the third clade and the rest of the *MaRbohHs* in the fourth clade (Fig 1A). Members of the same clade might share a common evolutionary history. As shown in Fig 1B, the gene structures differ among the *MaRbohs* with similar exon numbers observed in each clade. Members of the first clade contain the least number of exon. The order of the exons and the length of introns also mostly varied. *MaRbohE2* is the only member with an upstream region.

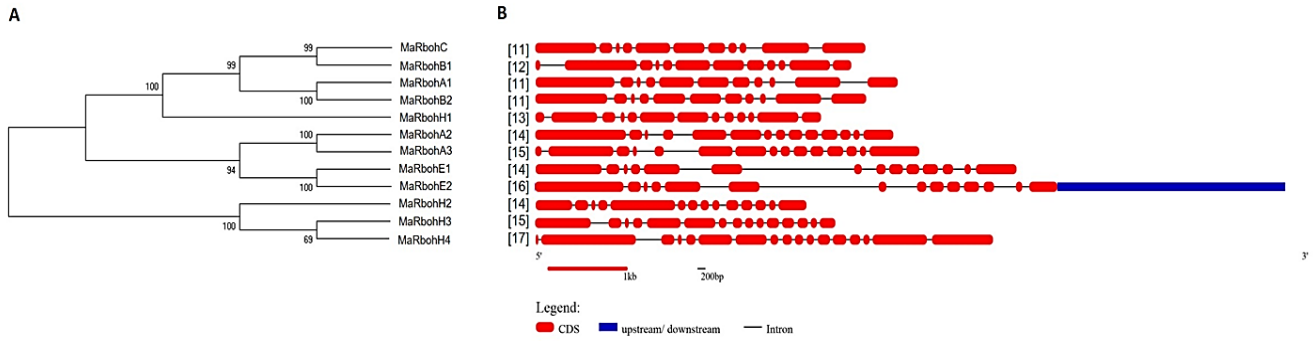

Figure 1. Phylogenetic tree and exon–intron structures of *MaRboh*. (A) The unrooted maximum-likelihood phylogenetic tree of *MaRboh* family members. Numbers above or below the nodes represent bootstrap values from 1,000 replications. (B) Exons, introns, and downstream regions are indicated by red boxes, black horizontal lines, and blue boxes, respectively. The scale bars for exons and intron represent 1 kb and 200 bp, respectively. Numbers in square brackets [ ] indicate exon number.

In general, the RBOH family consists of four characteristic motifs which include NADPH oxidase, ferric reductase as well as FAD- and NAD-binding domains. In addition to major functional domains in NADPH proteins, all twelve MaRBOH proteins contained EF-hand domain that is deemed critical for the activation process of RBOH proteins (Fig 2) [14,17]. Most plant RBOH proteins including banana contain one or more EF-hands motifs to bind Ca^{2+}. As reported in other plants, NADPH oxidase domain is positioned at the N-terminus before EF-hand domain. Despite the structural variation among *MaRboh* genes, no difference could be detected in their domain organization indicating similar and potentially redundant function. Functional redundancy of duplicated genes greatly contributes to the sturdiness of biological systems [32]. A classic example in the case of *Rboh* is the redundancy of *AtrbohD* and *F* [33]. Based on the *in-silico* characterization,

MaRbohs are structurally varied in terms of gene structure but the functional domains are highly conserved.

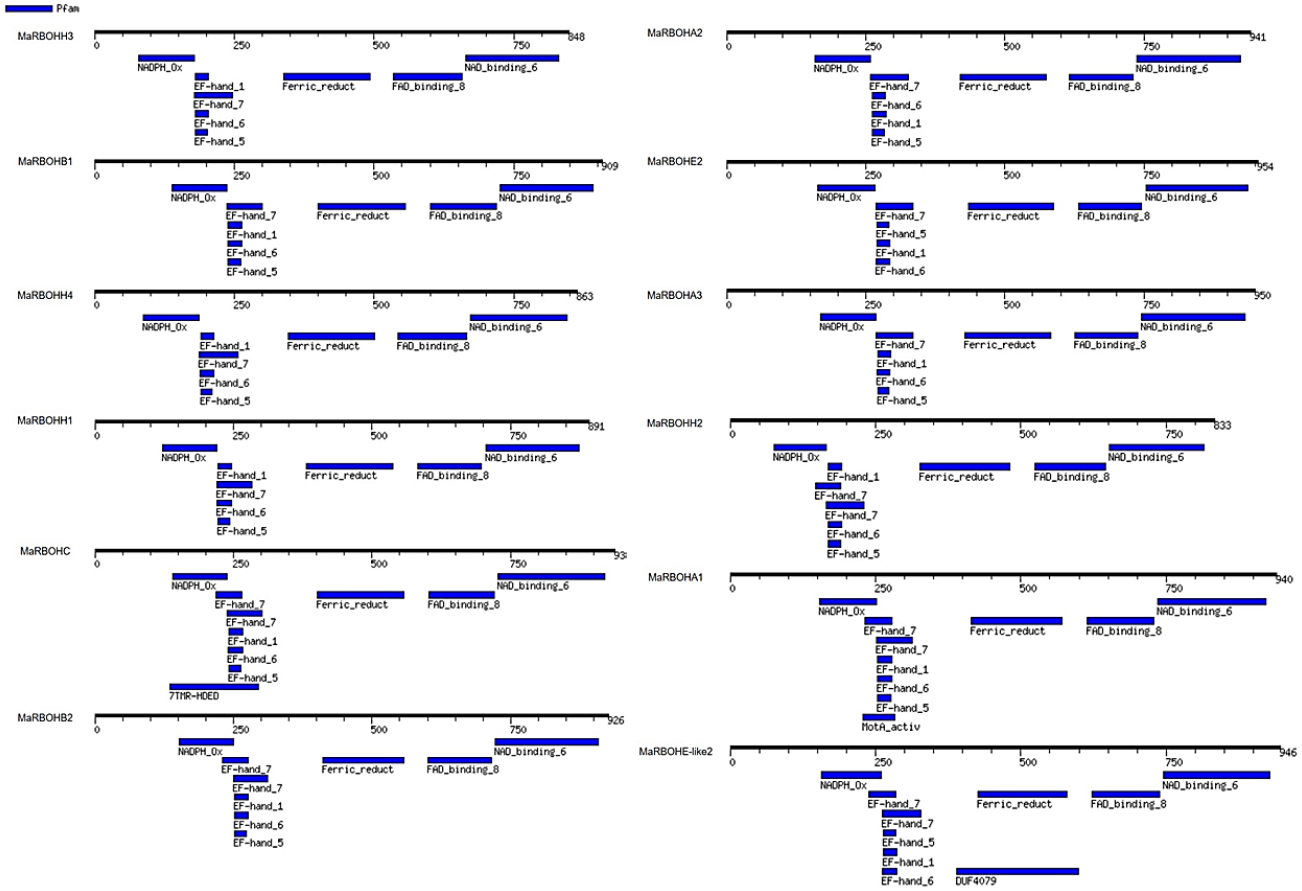

Figure 2. Domain structure of MaRBOH proteins. Four major domains typical of NADPH oxidase proteins namely NADPH_Ox, Ferric_reduct, FAD_binding_8, and NAD_binding_6 including Ef-hand analyzed using MOTIF.

To reveal the evolutionary relationship of *MaRboh* gene family with other plant species, a total of 42 RBOH homologs were selected from banana, wheat, barley, rice and *Arabidopsis*. The latter two represent the model monocot or eudicot, respectively. The aligned protein sequences of the selected RBOH homologs were used to build an unrooted phylogenetic tree using MEGA X program. As observed with the unrooted tree previously, the inferred ML tree classified MaRBOH proteins into four distinct groups (I, II, III and IV), each supported by high bootstrap values. MaRBOHs present in the same group are denoted as sisters of the same clade and are expected to share similar gene structure and possibly involved in similar functions. This was reported in an evolutionary analysis of 134 RBOH homologs in plants by [6] where comparable gene structure and protein motifs were evident within each subgroup. Each subgroup were represented by at least two members of MaRBOH proteins with a maximum number of 5 proteins in subgroup IV. RBOH proteins with diverse functions in plants are scattered in all four subgroups. In this study, members of the subgroup IV are of particular interest since they are mostly involved in plant immunity [19,34].

Hypothetical roles of *MaRbohs* could be deduced from their expression in response to a specific inducer, in this case, TR4. The fungus is a hemi-biotrophic root pathogen and the differential expression pattern of *MaRbohs* in the roots gives a clue about their possible direct involvement in ROS production during TR4-banana interaction. To study the expression profile of *MaRbohs* in response to TR4, analysis was carried out using qRT-PCR at 24 hpi in a local susceptible variety, cv. Berangan. *MaRboh* genes showed considerable change in expression in infected plants compared with the uninoculated control plants; 9 were upregulated and 3 were downregulated (Fig 4). Due to the profound role of ROS as signalling molecules in response to biotic and abiotic stresses, *Rboh* genes always show differential responses to pathogens as observed in previous studies. Interestingly, both members of group III were downregulated and all the members of group IV were upregulated. The highest upregulation was shown by *MaRbohH1* followed by *MaRbohH4* and *MaRbohH2*. The

elevated level of expression of three duplicate copies of *MaRbohHs* might be advantageous for banana especially in stressing environment such as pathogen infection. Two of them, *MaRbohH1* and *4* are located on the same chromosome (Chr 5), hinting a positional regulation [35].

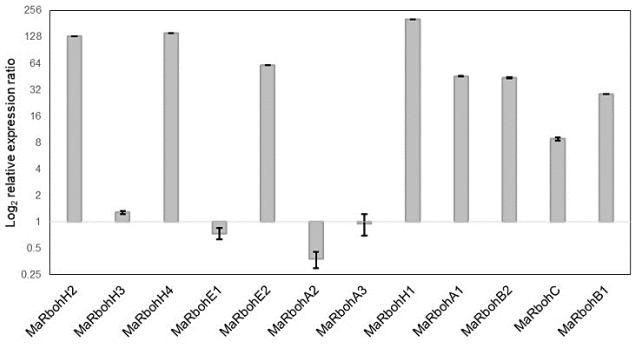

Figure 4. Modulation of *MaRbohs* expression by TR4. Log_2 relative expression ratio of *MaRbohs* genes in susceptible local banana at 24 hpi. The expression rate above and below 0 indicate an increase and decrease in gene expression (in relation to uninfected control). All data are presented as means ± standard deviation (SD).

On the other hand, *MaRbohA2* which is closely related to *AtRbohF* was strongly downregulated. Mutation in *AtRbohD* and *AtRbohF* resulted in a reduced amount of H_2O_2 thus increased the susceptibility in the mutant plants compared to wild-type [10]. Hence, *MaRbohHA2* could be the target for TR4 to disrupt ROS production as part of its infection strategy since RBOH-derived ROS possesses concentration-dependent roles and that disease development or pathogen elimination is determined by swiftness and efficacy of the oxidative burst orchestrated by plants [36].

CONCLUSION

In the present study, twelve *MaRboh* genes with conserved functional domains were identified from the DH Pahang genome and characterized using in-silico and qRT-PCR experiment for their inducible expression in response to TR4. This information contributes to increasing knowledge of NADPH oxidases in plants. Phylogenetic analysis helped to identify and establish the evolutionary relationship among RBOH family members in banana and other plants, suggesting the possible functional roles for *MaRbohs*, specifically for *MaRbohH1* and *MaRbohA2* in plant defence. However, further study is warranted to establish their individual role in banana reactions towards TR4 attack.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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