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Genome and Transcriptome-Wide Analysis of WRKY Transcription Factors for *Pratylenchus coffeae* Resistance in Banana

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Abstract

Plant WRKY transcription factors (WRKY TFs) have a long evolutionary history and are involved in the regulation of various physiological processes, such as development, senescence and in-plant response to many biotic and abiotic stresses. To understand the role of WRKY TFs during a *Musa* - *Pratylenchus coffeae* (root lesion nematode) interaction, genome- and transcriptome-wide analyses were carried out. A total of 153 banana WRKY TFs (MusaWRKYs) were identified using public databases. According to their structural and phylogenetic features, they were classified into three main groups. In order to identify the role of MusaWRKY genes in the banana reaction to *P. coffeae*, the MusaWRKY expression profiles were examined from the nematode-challenged and unchallenged transcriptomic data both from a resistant cultivar 'Karthobiumthum' (ABB) and a susceptible cultivar 'Nendran' (AAB). It was observed that 121 MusaWRKYs were expressed in both the resistant and susceptible cultivar, whereas 32 MusaWRKYs were not expressed even after having been challenged with *P. coffeae*. Interestingly it was noted that more MusaWRKYs were significantly upregulated in the resistant (39.6%) than in the susceptible (28.5%) cultivar. Upregulation of more number of group IIc WRKYs (13 genes) in resistant cultivar revealed the significance of group IIc WRKYs in nematode resistant mechanism. Significant expression of MusaWRKY95only in the nematode-challenged resistant cultivar emphasizes the importance of its role in *Musa* - *P. coffeae* incompatible interaction. Thus, isolation of full length genes of *Musa*WRKY95 and *Musa*WRKY142 are in progress for understanding their function during *Musa* - *P. coffeae* interaction, particularly with regard to any role in pathogen resistance.

INTRODUCTION

Plants have evolved mechanisms to adapt to biotic and abiotic stress through activation of resistance genes (Pandey and Somssich, 2009). Eulgem (2005) reported that plant hormones and transcription factors (TFs) play an important role in transcriptional reprogramming during plant-pathogen interactions in order to control or block the associated effector proteins of pathogens. Among the ten largest TF families, the WRKY TF family is the most important for the regulation of plant defense response pathways and plays an important role in the signaling cascade of innate immunity (Pandey and Somssich, 2009).The

importance of WRKY TFs and their defense response against pathogens have been demonstrated using different molecular approaches including over-expression and gene-silencing methods, in several crops, such as cotton (Yu et al., 2012), rice (Ryu et al., 2006) and *Arabidopsis* (Xu et al.,2008).The plant-parasitic nematodes *Pratylenchus coffeae* (root lesion nematode) is a necrotrophic pathogen which causes serious damage to the banana by penetrating the root system and consequently causes loss of millions of dollars every year. But to date there has been no reports on MusaWRKY transcription factor and their role(s) against plant-parasitic nematodes in banana. In our present study, we characterized the MusaWRKYs which are available in the public domain. To understand the role of MusaWRKYs in the *Musa* - *P. coffeae* interaction, transcriptome data from resistant and susceptible cultivars challenged or not with *P. coffeae* were analyzed. The outcome of this study should enhance our knowledge on MusaWRKY TFs and lead to the understanding of their regulatory networks responsive to *P. coffeae* infestation in banana.

MATERIALS AND METHODS

Banana Root Sample Collection

Twenty suckers each of both the resistant cultivar 'Karthobiumthum' (ABB genome) and the susceptible cultivar 'Nendran' (AAB genome, Plantain subgroup) were planted in individual pots filled with fumigated potting mixture (soil and vermiculite 2:1) containing one part formaldehyde in five parts water. Active 2nd juvenile stage populations of *P. coffeae* were collected from carrot culture (Kaplan and Davis, 1990) and each 3-month-old plant was inoculated with the nematodes at the rate 2500 nematodes/plant. Root samples were collected at differing time intervals: 0, 2, 4, 6, 8 and 10 days after inoculation.

RNA Extraction, cDNA Synthesis and Real Time-Polymerase Chain Reaction

Total RNA was extracted independently from two grams of each root sample collected from nematode-challenged and unchallenged plants of resistant and susceptible cultivars, at the above-specified time intervals, using an Agilent Plant RNA isolation mini-kit (Agilent Technologies, Inc., USA). RNA integrity was checked using Agilent's Bioanalyzer 2100 (Agilent Technologies, Inc., USA). An equal amount of RNA was taken from each sample of nematode-challenged resistant cultivar and pooled for the construction of the library for challenged resistant (CR) and susceptible (CS) genotypes. The same procedure was followed in challenged root samples of susceptible cultivar (CS) and the unchallenged root samples for the construction of UR and US libraries.

Construction of cDNA Library and Illumina Deep-sequencing

The library construction (CR, CS, UR and US) and sequencing were performed by Genotypic Technology, Bangalore, The cDNA library was constructed using an mRNA-Seq assay for paired-end transcriptome sequencing and the libraries were loaded onto the channels of an Illumina HiSeq2000 instrument.

De novo Assembly, Sequence Clustering and Expression Level

De novo assembly of the clean reads was mapped to reference sequences (unigenes from the transcriptome data of the normalized cDNA library) by TOPHAT. Gene expression

levels were calculated using the Fragments per Kilobase of transcript per Million fragments mapped (FPKM) method (Kyndt et al., 2011).

Sequence Database Searches and WRKY Genes Identification

The Banana Genome Hub database (http://banana-genome.cirad.fr/) was used to download the MusaWRKYs. Further analysis was carried out in the NCBI-EST database (www.ncbi.nlm.nih.gov/dbEST) and plant transcription factor database (http://planttfdb.cbi.pku.edu.cn/).

Multiple Sequence Alignment, Phylogenetic Analysis and Motif Analysis

The neighbor-joining method was used to construct the phylogenetic tree based on amino acid sequence of WRKY domains using MEGA 5.2.1 software. The MEGA 5.2.1 analysis was carried out according to the description by Tamura et al. (2011). Analysis for conserved motifs in the WRKY proteins was carried out using the MEME database (http://meme.sdsc.edu/meme/cgi-bin/meme.cgi).

RESULTS AND DISCUSSION

A total of 153 MusaWRKYs was identified in the *Musa* genome from the CIRAD database (banana-genome.cirad.fr) All 153 MusaWRKY genes were mapped to their respective chromosomes (1 to 11) and were named from MusaWRKY1 to MusaWRKY153 based on their order on the chromosomes. The size of the WRKY family of *Musa* was compared with other species like *Arabidopsis lyrata* (79), *Arabidopsis thaliana* (74), *Glycine max* (197), *Medicago truncatula* (75), *Populus trichocarpa* (103), *Oryza sativa indica* (102), *Oryza sativa japonica* (97) and *Sorghum bicolor* (93). It was observed that *Musa* has greater number WRKY genes than other species compared here, except for *Glycine max* (Fig 1). Wu et al. (2005) observed that genome duplication events have resulted in the expansion of the WRKY genes. Based on phylogenetic analysis the MusaWRKYs were classified into three major groups (I, II and III) with five subgroups in group II (a, b, c, d and e) which is mainly based on the number of WRKY domains and the features of specific zinc finger motifs as reported in rice and *Arabidopsis* (Wu et al., 2005).

A total of 38 MusaWRKYs had three domains in their sequence whereas the remainders have two domains. This domain variation might be due to WRKY domains inserted or deleted during the evolution by sequence divergence and recombination. Among the three groups, sub groups IIc has the great number of genes (24.14%) (Table1). Among the 153 MusaWRKYs, only 81 had complete domains. Three different conserved motifs (two WRKY motifs and one zinc finger motif) in the MusaWRKYs were identified using the MEME database. Among 81 MusaWRKY genes, 80had highly conserved WRKYGQK sequence, while MusaWRKY43 of group III had one amino acid mismatch (WRKYGNK) in the sequence.

MusaWRKYs were distributed throughout all the chromosomes, with the greatest intensity on chr-7 (14.37%) followed by chr-4 (11.6%), whereas the chromosomes with the lowest number of MusaWRKYs (7) were distributed in three chromosomes namely chr-1, 2 and 11 (4.57% over the three chromosomes). Chr-7 and 4 shared a number of WRKYs through tandem and segmental duplication. Nearly 54% (82) of WRKYs were associated in

duplication events either in a tandem (20 WRKYs) and/or segmental (62 WRKYs) events. Tandem duplication was observed in chr-4, 5, 7, 9 and 10 only, whereas segmental duplication was observed in all the chromosomes except chr-1 and 11. A large number of segmental duplication appeared to have occurred in chr-4 and chr-7. No tandem/ segmental duplication events were observed for the group III and group Ia WRKY family due to the low members of WRKYs in the groups. The comparison with the MusaWRKY domains of several groups of *Arabidopsis* WRKYs resulted in a better separation of the different groups and subgroups.

The transcriptome analyses of the *P. coffeae* resistant ('Karthobiumthum') and susceptible ('Nendran') cultivars revealed that the maximum number of genes expressed in the resistant cultivar was 118 genes compared with 113 in the susceptible cultivar. A total of 32 WRKYs in the nematode-challenged and unchallenged root tissues of both the cultivars showed no expression and these WRKYs were assumed not to have a vital role in root development and/or nematode stress. It was also observed that thirteen members of group IIc were significantly upregulated (>2 fold change) in the resistant cultivar, suggesting an involvement of group IIc members in a nematode resistant mechanism. Interestingly, it was noted that eight MusaWRKYs (MusaWRKY43, 54, 95, 103, 107, 111, 126and 138) were uniquely expressed in the resistant cultivar while none of the WRKYs were uniquely expressed in the susceptible one. Similarly, it was observed that a greater number of WRKYs were significantly upregulated, with a greater than two-fold increase in the resistant (39.6%) over the susceptible (28.5%) cultivars (Fig 2). Thus significant (with a greater than two-fold change) over-expression and unique expression of WRKYs in the resistant cultivar emphasized the important role of WRKYs in *Musa* - *P. coffeae* incompatible interactions.

The duplicated events of the WRKYs revealed that both tandem and segmental duplications significantly contributed to the expansion of the WRKY gene family in *Musa*. Twenty-nine (18 out of 62) and forty percent (8 out of 20) of WRKYs were differentially expressed among their respective segmental and tandem duplications respectively. Among the segmental duplication members MusaWRKY41 and MusaWRKY98 were significantly expressed in both the genotypes under nematode challenged condition. In resistant cultivar,21 WRKYs were significantly upregulated, of which 13 MusaWRKYs are belonging to group IIc, This emphasized the important role of group IIc members of MusaWRKYs in a nematode resistance mechanism. The important role of group IIc WRKYs members in grape berry ripening and cold acclimation has been proven by Min et al. (2014). It was observed that MusaWRKY95 was significantly expressed only in the resistant cultivar. This MusaWRKY95 is an orthologue of AtWRKY50, which is involved in secondary metabolites synthesis and stress-induced lignin modification (Gao et al., 2011). MusaWRKY142 showed significant expression in the challenged resistant cultivars when compared with unchallenged resistant and challenged susceptible cultivars. This MusaWRKY142 is an orthologue of AtWRKY60 which is expressed under different biotic and abiotic infections (Hu et al., 2012 and Chen et al., 2010). Thus, isolation of full length genes of MusaWRKY95 and MusaWRKY142 are in progress for understanding their function during *Musa* - *P. coffeae* interaction, particularly with regard to any role in pathogen resistance.

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Tables

Table 1. Details of differentially expressed MusaWRKYs under *Pratylenchus coffeae* challenged and unchallenged conditions in the resistant cultivar 'Karthobiumtham' (ABB) and the susceptible cultivar 'Nendran' (AAB) cultivars.

Comparison of WRKY TFs expression among cultivars	Total no. genes expressed	Up regulated genes		Down regulated genes		Neutral genes
		Total no. of genes	Significantly expressed	Total no. of genes	Significantly expressed	
Resistant cultivar (UR vs CR)	121	53	21	17	0	30
Susceptible cultivar (US vs CS)	113	57	17	6	0	33
Both challenged Cultivars(CS vs CR)	38	4	1	9	1	23

UR – Unchallenged resistant CR – Challenged resistant
US – Challenged susceptible CS – Challenged susceptible

Figures

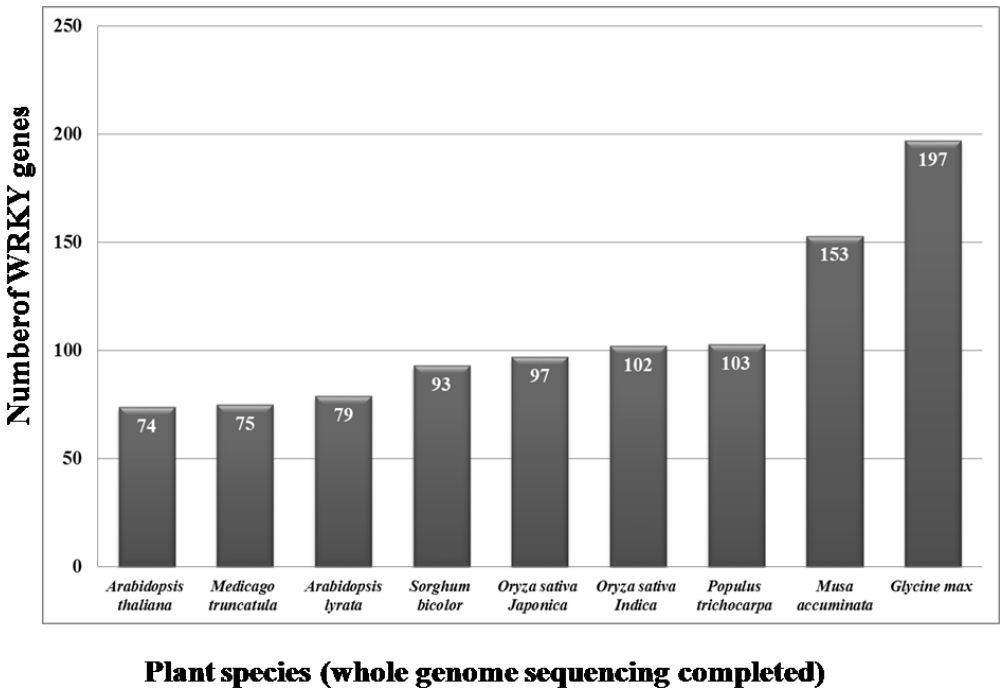

Fig. 1. Number of WRKY transcription factors present in nine plant species (http://planttfdb.cbi.pku.edu.cn/).

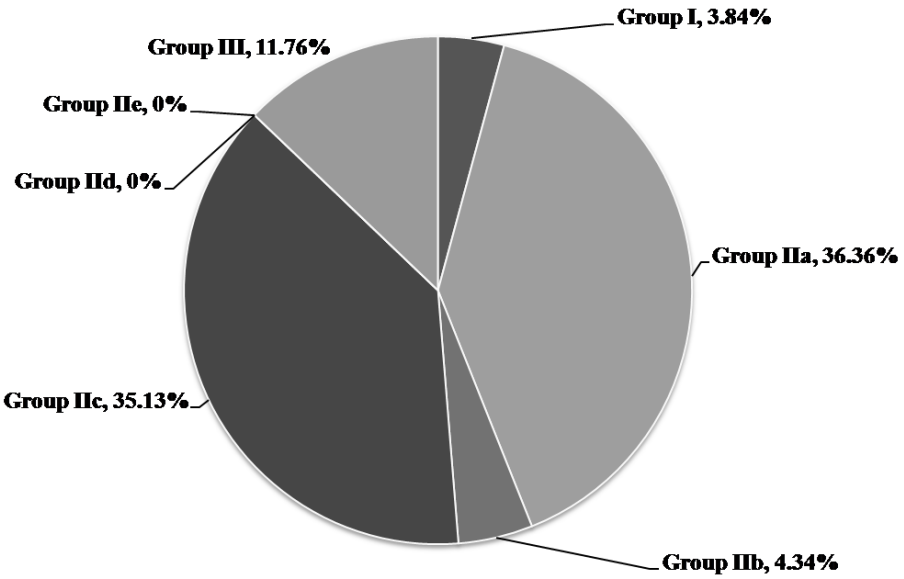

Fig. 2. Percentage of significantly over expressed WRKY groups (Eulgem et al., 2000) in *Musa* with >2-fold change in the banana resistant cultivar 'Karthobiumtham' (ABB) after being challenged by *Pratylenchus coffeae*.