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Identification of *Mycosphaerella eumusae* responsive unique genes/transcripts from a resistant banana cultivar

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Abstract

Next generation sequencing was performed using an Illumina Hiseq platform in leaf cDNA libraries of unchallenged and *Mycosphaerella eumusae* challenged plants of banana cultivars with contrasting reactions to the leaf fungus, resistant 'Manoranjitham' (AAA genome, unique) and susceptible 'Grand Naine' (AAA genome, Cavendish subgroup). Thirty-six million reads obtained from each library were assembled with the *Musa acuminata* genome sequence as a reference, using TOPHAT2 and Cufflinks software. Approximately 45,000 unigenes were obtained from each library and annotated against *Musa* and *Viridae plantae* databases. A comparison of the expression pattern from these four libraries was made based on digital gene expression (DGE) profiles. Major transitional shifts in gene expression were noticed among these four libraries. It was observed that nearly 4658 and 3261 transcripts were over-expressed in challenged libraries of the resistant and susceptible cultivar, respectively. Interestingly, the number of over-expressed transcripts was higher in the challenged resistant cultivar than in the challenged susceptible one. A total of 46 and 8 unique transcripts, which were significantly over- and under-expressed, respectively, in the challenged resistant cultivar, were identified. The *M. eumusae*-challenged resistant cultivar exhibited 36 pathogenesis-related gene families and 10 transcription factors. These findings are expected to lead towards the identification of candidate genes responsible for *M. eumusae* resistance in banana. This study revealed that pathogen resistance mechanisms in banana are part of a complex network of defense mechanisms through signal transduction involving ROS, lignin biosynthesis, hormonal pathways, detoxification and production of secondary metabolites. Apart from helping to elucidate resistance mechanisms, the transcriptome data are also being used for identifying functional markers for further use in breeding programs for pathogen resistance.

Keywords: defense, differential gene expression, *Musa*, transcriptome, eumusae leaf spot disease

INTRODUCTION

Eumusae leaf spot (caused by the fungus *Mycosphaerella eumusae*) is one of the three closely related fungi (*Mycosphaerella eumusae*, *Mycosphaerella fijiensis*, *Mycosphaerella musicola*) that causes devastating leaf spot disease on banana. Due to the disease, necrotic patches develop on the leaves leading to extensive defoliation and resulting in delayed flowering, and reduction in number of hands and fingers. The disease can reduce the crop yield by up to 80% (Sanchez and Zapata, 2002), and fungicidal sprays can increase the production costs by about 40% (Ngongo, 2002). Cultivation of leaf spot-resistant cultivars is one of the key strategies to reduce the production cost. Knowledge regarding genes responsible for disease resistance is the one of the basic requirements to develop a disease-resistant cultivar either through marker-assisted breeding or genetic transformation. Hence, this study is at identifying the genes involved in eumusae leaf spot resistance. Transcriptome sequencing was carried out in banana cultivars with contrasting reactions to *M. eumusae* under challenged and unchallenged conditions.

MATERIALS AND METHODS

Sample collection

Forty plants of secondary hardened tissue-cultured plants of each of eumusae leaf spot-resistant 'Manoranjitham' (AAA genome, unique) and susceptible 'Grand Naine' (AAA genome, Cavendish subgroup) (Anonymous, 2001-2002) were acclimatized under controlled conditions with 95% relative humidity and 22°C prior to infection. *Mycosphaerella eumusae* fungal spores were suspended in 150 mL of sterile distilled water, at a concentration of 10^8 spores mL^{-1}, and sprayed on both leaf surfaces of the 'challenged' samples, and the same volume of sterile distilled water (without inoculum) was sprayed on both leaf surfaces of the 'unchallenged' samples of both contrasting genotypes. The third leaf was collected from each plant at specific time intervals from 0 to 260 h post inoculation (hpi) (regular intervals of every 24 h from day 1 to 5 and followed by 36 h from day 6 to 10) from *M. eumusae* challenged and unchallenged plants, of each cultivar independently. Collected samples were frozen with liquid nitrogen and stored at -80°C.

Pre-processing of samples and sequencing

Total RNA was extracted from each leaf sample (2 g) using the Agilent Plant RNA isolation mini kit (Agilent Technologies, Inc, US). RNA was quantified using Nanodrop and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent Technologies, Inc, US). About 5 µg of total RNA was taken from each sample of the challenged resistant cultivar (CR), pooled together and a transcriptome library constructed according to Illumina TruSeq RNA library protocol outlined in "TruSeq RNA sample preparation guide" (Part # 15008136. Rev. A, Nov. 2010). Briefly, 5 µg of total RNA by qubit was enriched for Poly-A using RNA purification beads provided with the kit. The enriched RNA was fragmented for 4 min at elevated temperature (94°C) in the presence of divalent cations and reverse transcribed with Superscript II Reverse transcriptase by priming with random hexamers. Second strand cDNA was synthesized in the presence of DNA Polymerase I and RNaseH. The cDNA was cleaned up using AgencourtAmpure XP SPRI beads (Beckman Coulter). Illumina Adapters were ligated to the cDNA molecules after end repair and addition of "A"- base. SPRI cleanup was performed after ligation. The library was amplified using 16 cycles of PCR for the enrichment of adapter ligated fragments. The prepared library was quantified using Nanodrop and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent). In the same way, RNAs of the unchallenged resistant cultivar (UR), the challenged susceptible cultivar (CS) and the unchallenged susceptible cultivar (US) were prepared to construct respective transcriptome libraries and all libraries were sequenced using Illumina Hiseq2000 (Genotypic Technology, Pvt. Ltd., India).

Downstream processing of sequencing data

Raw reads quality was checked using SeqQC-V2.0 (Genotypic technology, Pvt. Ltd.) for filtering high-quality reads and vector-contaminated reads, trimming of adapters, low-quality reads and B-trimming. The Bowtie-2.0.9 tool was used to align the reads gap (Langmead and Salzberg, 2012). Splice junction discovery was conducted with RNA seq data using TOPHAT 2.0. The resulting alignment files were provided to Cufflinks v2.0.1 for assembly of the reads with the *Musa acuminata* genome sequence (D'Hont et al., 2012) as reference. The assembled reads were then merged together using the Cuffmerge utility (which is part of the Cufflinks package). The merged assembly then provided a uniform basis for calculating gene and transcript expression in each condition. The reads and the merged assembly were fed to Cuffdiff, to calculate expression levels and test the statistical significance of observed changes. Cuffdiff also allowed performing an additional layer of differential analysis. This allowed grouping transcripts into biologically meaningful groups (such as transcripts that share the same transcription start site (TSS)), and identifying genes that are differentially regulated at the transcriptional or post-transcriptional level. Cufflinks and Cuffdiff were used to implement a linear statistical model to estimate an assignment of abundance to each transcript in order to explain the observed reads with maximum

likelihood (Trapnell et al., 2012). Contigs were annotated using BLASTX against *Musa* and *Viridae plantae* database. Gene Ontology (GO) terms were assigned for the annotated sequences by describing their role either in the biological process or the molecular function or the cellular component. The Swiss-Prot BLAST results were imported into the GO database, (http://www.geneontology.org). These contigs were further mapped into KEGG database.

RESULTS AND DISCUSSION

Deep-sequencing of UR, CR, US and CS cDNA libraries produced at an average of 73.9 and 59.5 million raw reads with maximum length of 101 bp (2× paired-end), which corresponded to an average of 8.71and 7.25 Gbp of raw data in resistant and susceptible cultivars, respectively. These reads were assembled, and contigs generated were 45,609, 45,135, 46,032 and 35,796 genes in CR, UR, CS, and US, respectively, with an average length of 969.5 bp (Table 1).

Table 1. Details of contigs assembled using the *Musa acuminata* genome as a reference from reads generated from challenged and unchallenged plants of the resistant banana cultivar 'Manoranjitham' and the susceptible cultivar 'Grand Naine' against *Mycosphaerella eumusae*.

Contigs details	cDNA libraries used for Illumina sequencing			
	CR	UR	CS	US
Contigs generated	45609	45135	46032	35796
Maximum contig length	11760	5838	13964	5906
Minimum contig length	52	53	61	53
Average contig length	1152.5	705.7	1156.4	863.3
Total contigs length	52562871	31853840	53231284	30902005
Total number of non-ATGC characters	382645	332334	375796	257017
Percentage of non-ATGC characters	0.728	1.043	0.706	0.832
Contigs>= 100 bp	45597	45123	46022	35788
Contigs>= 200 bp	43679	40103	43976	34752
Contigs>= 500 bp	32235	22828	31978	23499
Contigs>= 1 kbp	20234	10248	20007	10998
Contigs>= 10 kbp	4	0	5	0

GO annotation was classified into three categories, namely the cellular component, the molecular function and the biological process. In the resistant and susceptible cultivars, a total of 18, 20,282, and 2, 22, 49 GO terms were assigned, respectively. In the resistant cultivar, majority of the GO annotations (76,640 (42%) distinct genes sequences) were categorized into the molecular function, followed by assignment of 59,987 (33%) distinct gene sequences to the biological process and 45,455 (25%) distinct gene sequences to the cellular component category. After GO annotation, the genes obtained from each library were mapped using the KEGG pathway. These results revealed that only 2,988 genes were mapped into 120 pathways and 804 genes were not mapped into any pathway in CR. With respect to CS, about 2,715 genes were mapped into 119 pathways and 546 genes were found to be not mapped. The Digital Gene Expression (DGE) analysis revealed that, in resistant cultivar about 4,658 genes were significantly upregulated with more than twofold change compared to challenged susceptible cultivar (Figure 1). It was also observed that 46 genes were expressed only in CR but not in others (Table 2).

This present study focused mainly on the identification and elucidation of the role of resistance genes in incompatible interactions between banana cultivars and *M. eumusae*. Over-expression of genes in resistant cultivars like horcolin gene might be involved in signaling (Grunwald et al., 2007) and a chitinase isotope – a well-known PR protein, is known to be involved in the first layer of the defense response in restricting pathogen entry. Additionally, induction of flavin containing monoxygenase in the resistant cultivar under

challenged condition revealed that it may act as one of the basal resistance mechanisms by detoxification of virulence factors produced by the fungus (Koch et al., 2006).

Table 2. Unique gene details of the resistant banana cultivar 'Manoranjitham', challenged with *Mycosphaerella eumusae*.

Sl no	Unique gene ID	Protein names
PTI		
1.	GSMUA_Achr9G10720_001	Horcolin
2.	GSMUA_Achr9G16770_001	Chitinase 10 (Pathogenesis related)
Basal resistance		
3.	GSMUA_Achr6G11710_001	Probable flavin-containing monooxygenase 1
ROS and scavengers		
4.	GSMUA_Achr7G03450_001	Polyphenol oxidase, chloroplastic
5.	GSMUA_Achr5G15800_001	Peroxidase 5
6.	GSMUA_Achr7G07720_001	Calmodulin-like protein 8
7.	GSMUA_Achr5G07710_001	Probable glutathione S-transferase
8.	GSMUA_Achr1G11390_001	Hexokinase-3
Lignin pathway		
9.	GSMUA_Achr2G04820_001	Caffeic acid 3-O-methyltransferase
10.	GSMUA_Achr2G04380_001	Tricetin 3',4',5'-O-trimethyltransferase
11.	GSMUA_Achr8G15700_001	Dirigent protein 11
12.	GSMUA_Achr7G20390_001	caffeine synthase
Phenylpropanoid pathway		
13.	GSMUA_Achr2G18040_001	Probable 4-coumarate--CoA ligase 2
14.	GSMUA_Achr1G00770_001	Naringenin,2-oxoglutarate 3-dioxygenase
15.	GSMUA_Achr9G14110_001	Trans-cinnamate 4-monooxygenase
Alkaloid production		
16.	GSMUA_Achr1G24050_001	Reticuline oxidase
17.	GSMUA_Achr9G23620_001	Thebaine 6-O-demethylase
18.	GSMUA_Achr11G04520_001	S-norcoclaurine synthase 1 (CjNCS1)
Membrane transporter		
19.	GSMUA_Achr1G08050_001	ABC transporter G family member 39
ABA biosynthesis		
20.	GSMUA_Achr6G07190_001	Zeaxanthin epoxidase, chloroplastic
21.	GSMUA_Achr7G07200_001	Abscisic acid 8'-hydroxylase 1
22.	GSMUA_Achr4G03370_001	indole-3-acetic acid-amidosynthetase GH3.8
Ethylene biosynthesis		
23.	GSMUA_Achr4G24930_001	1-aminocyclopropane-1-carboxylate synthase
Protease inhibitor		
24.	GSMUA_Achr8G33360_001	Putative germin-like protein 2-1
25.	GSMUA_Achr11G15750_001	Flowering-promoting factor 1-like protein 2
Others		
26.	GSMUA_Achr4G13610_001	Inhibitor of trypsin and hageman factor
27.	GSMUA_Achr6G31880_001	S-type anion channel SLAH3
28.	GSMUA_Achr10G30620_001	Alpha-humulene synthase)
29.	GSMUA_Achr6G14500_001	Epidermis-specific secreted glycoprotein EP1
30.	GSMUA_Achr10G03970_001	Very-long-chain 3-oxoacyl-CoA reductase 1
31.	GSMUA_Achr7G20640_001	Probable L-type lectin-domain containing receptor kinase
32.	GSMUA_Achr6G01170_001	Ubiquinoloxidase 1a, mitochondrial
33.	GSMUA_Achr3G17950_001	Peptide-N4-asparagine amidase A
34.	GSMUA_Achr9G19910_001	Alpha-dioxygenase
35.	GSMUA_Achr2G16600_001	6-phosphofructokinase
Transcription factors		
36.	GSMUA_Achr5G01720_001	Zinc finger protein 1
37.	GSMUA_Achr11G00410_001	Myb-related protein Myb4
38.	GSMUA_Achr1G20550_001	Dof zinc finger protein DOF5.6
39.	GSMUA_Achr11G21130_001	CBS domain-containing protein CBSX5
40.	GSMUA_Achr3G03090_001	WRKY transcription factor
41.	GSMUA_Achr8G08000_001	Ethylene-responsive transcription factor ERF110
42.	GSMUA_Achr3G21060_001	Transcription factor MYB21
43.	GSMUA_Achr11G19350_001	MADS-box transcription factor 26

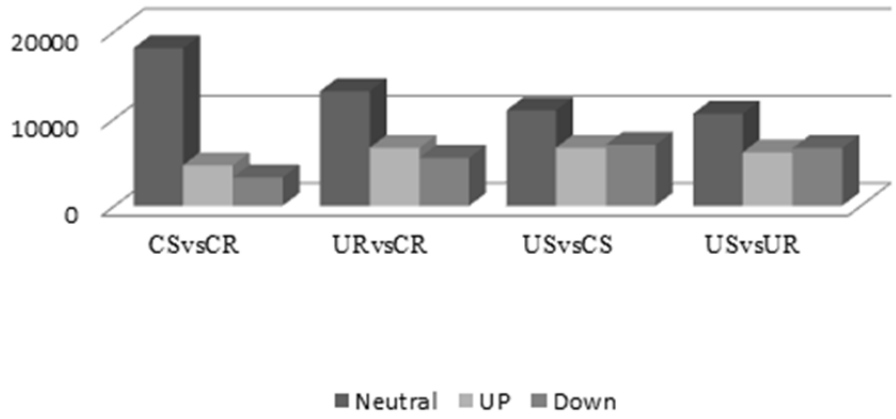

Figure 1. Transcripts expressed during *Musa* and *Mycosphaerella eumusae* interactions hit details from challenged and unchallenged plants of the resistant banana cultivar 'Manoranjitham' and the susceptible cultivar 'Grand Naine' based on DGE results.

Expression of polyphenol oxidase (PPO) and hexokinase-3 only in the resistant cultivar suggested that these might be involved in the production of reactive oxygen species (ROS) (Mayer, 2006; Kim et al., 2006). Apel and Hirt (2004) reported that ROS play a major role in resisting pathogen spread through the production of a hydrogen peroxide, hypersensitive reaction and redox, which ultimately leads to programmed cell death. Similarly, expression of calmodulin proteins in the resistant cultivar activates MAPK and leads to the production of phenols (Kurusu et al., 2005). At the same time, the expression of scavenging enzymes, namely glutathione S-transferase and peroxidase in the challenged eumusae-resistant cultivar, revealed that it would neutralize the negative effect of ROS (Dean et al., 2005; Davletova et al., 2005).

Enzymes involved in the phenylpropanoid pathway like probable 4-coumarate-CoA ligase 2 (4CL 2), Trans-cinnamate 4-monooxygenase, Naringenin2-oxoglutarate 3-dioxygenase (Flavanone-3-hydroxylase) (F3H), caffeic acid 3-O-methyltransferase (COMT), tricetin 3',4',5'-O-trimethyltransferase (TaOMT2) and dirigent protein 11 (AtDIR11) were also expressed in resistant cultivar after *M. eumusae* infection. These genes were involved in the production of lignin via a phenylpropanoid synthesis pathway for cell wall strengthening (Betz et al., 2001). Some genes like COMT and F3H in lignin production were also involved in plant defense reactions by promoting flavonoid production, which is one of the potent antifungal agents, that acts by combining with CoA esters (Burlatet al., 2001; Ma and Xu, 2008) and naringenin synthesis (Gebhardt et al., 2007); an intermediate in the flavanoid pathway from the phenylpropanoid pathway, respectively.

Expression of reticulin oxidase and thebaine 6-O-demethylase, involved in alkaloid and secondary metabolites production (Dittrich and Kutchan, 1991; Carter and Thornburg, 2004) was observed only in resistant cultivar. Similarly, ABC transporters were also found to be expressed in the resistant cultivar, indicating that they might be involved in transporting these metabolites, which are involved in this defense mechanism (Yazaki, 2006).

The expression of ABA synthesizing genes like zeaxanthin epoxidase and abscisic acid 8'-hydroxylase in *M. eumusae* challenged resistant cultivar indicated the important role of hormones in the eumusae leaf spot resistance mechanism. Mauch-Mani and Mauch (2005) reported that ABA biosynthesis might play a major role in restricting pathogen spread by contributing positively to disease resistance through modulation of callose deposition and stomatal closure.

CONCLUSIONS

This study revealed that resistance mechanisms in banana are part of a complex network of defense mechanisms through signal transduction involving ROS, lignin biosynthesis, hormonal pathways, detoxification and production of secondary metabolites.

Apart from helping to elucidate resistance mechanisms, the transcriptome data are also being used for identifying functional markers for further use in breeding programs.

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