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Development of normalized cDNA library from *Fusarium* wilt infected roots of a tolerant banana genotype 'Calcutta-4' *Musa acuminata* ssp. *burmannicoides*

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ABSTRACT

Management of the most devasting disease, *Fusarium* wilt of banana, caused by the fungus *Fusarium oxysporum* f. sp., *cubense,* is a challenge to the plant pathologist and the banana grower. Currently, genomics is providing the way for understanding plant defense mechanism, having acquired an important place in crop improvement. To identify the relevant genes and to understand the defense mechanism induced during *Fusarium* wilt infection, a normalized cDNA library was constructed from infected root samples of a tolerant banana genotype, *Musa acuminata* spp. *burmannicoides* 'Calcutta-4', by duplex specific nuclease (DSN) based normalization, using the SMART (switching mechanism at 5' end of RNA transcript) full-length cDNA construction method. Sequencing and analysis of 600 clones revealed 392 non-redundant clones. In all, of 88% of the sequences were annotated using *Musa* genome database, and the remaining 12% were identified as novel loci not annotated. We observed several resistance genes, ROS scavenging genes and genes involved in ubiquitin-proteosome pathway in this study. These genes may have a possible role against *Foc* infection. These sequences would enrich the EST data developed against specific stress, which is an indispensable tool for predicting functional genes and understanding the defense mechanism.

Key words: *Fusarium oxysporum* f. sp. *cubense*, banana, cDNA library, defense response, normalization

INTRODUCTION

Banana is an economically and nutritionally important fruit crop cultivated worldwide. But *Fusarium* wilt, a vascular disease of the banana caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *Cubense,* is causes major devastatation. In spite of the poor history of banana production since 1876 due to *Fusarium* wilt having destroyed thousands of hectares (Ploetz 2000), uptil now a comprehensive solution has not been identified to control the disease. Change in climatic factors and an increasing population may lead to a great demand for banana production in future. Under these conditions, necessary steps should be taken to understand and overcome production constraints.

Identification of the defense genes and an understanding of plant-pathogen interaction may lead to development of new strategies to control this plant disease. When the pathogen attacks the host, in general, the initial response of the host should be an efficient recognition of the pathogen (either to hinder or limit the entry of the pathogen) with the help of resistance genes (R-genes) and by cell wall strengthening. Recognition leads to activation of various defense signaling pathways like ROS, salicylic acid, jasmonic acid, ABA, auxin, nitric oxide and calcium. These signal pathways modulate each other through a complex network of regulatory mechanisms based on host-pathogen interactions (Swarupa *et al*, 2014). Other parallel changes such as altered regulation of ROS scavenging enzymes that maintain ROS homeostasis, including various biochemical and physiological changes, also occur in the host to debilitate the pathogen.

ESTs and other cDNA sequences are among the most reliable evidences for understanding molecular mechanisms operating at the cellular level and for identifying gene-rich regions in a genome species (Garg *et al*, 2011). Generation of large-scale ESTs is a very useful approach to accelerate research on non-model plants like the banana. ESTs generated from a whole cDNA library should represent all the genes expressed in the tissue used for constructing the library. However, abundance of mRNA is different for different genes which makes it difficult to fish out rare mRNAs from cDNA libraries. Also, it increases redundant

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sequencing of clones, leading to decreased efficiency and cost-effectiveness of the EST approach (Bonaldo *et al*, 1996). To overcome problems of redundancy in large-scale cDNA sequencing projects, normalization of cDNAs is done for construction of the library. The normalization process generally utilizes second-order reaction kinetics for reassociation of denatured DNA, so that relative transcript concentrations within the residual single-stranded (ss) cDNA fraction are equalized to a considerable extent (Young and Anderson, 1985).

Duplex-specific nuclease (DSN) based normalization has been extensively used in animal tissues successfully; however, it has rarely been used in plant systems. Therefore, we have made an attempt to develop DSN based normalized cDNA library to identify a possible set of genes expressed during *Fusarium* wilt infection.

MATERIAL AND METHODS

Plant material

Healthy suckers of the tolerant genotype *Musa acuminata* ssp.*burmannicoides*, 'Calcutta-4' were planted in cement pots of 12 inch diameter filled with sterilized soil, after disinfection (using Bavistin 2%). Routine cultural practices were followed for two months to establish the plants. Susceptible cultivar 'Kadali' was used as a (control plant to check for symptom development. After the plants established, small pieces of *Fusarium oxysporum* f. sp. *cubense (Foc)* infected suckers were chopped and used an inoculum to induce the disease in both genotypes. Two months after infection, we observed disease symptoms in the susceptible cultivar 'Kadali'. Then, the roots of 'Calcutta-4', a tolerant, genotype were collected, frozen in liquid nitrogen and stored until future use.

Construction of normalized cDNA library

Total RNA was extracted from *Foc* inoculated (two months after inoculation) root tissues of 'Calcutta-4' as per Liu *et al* (1998). cDNA library was constructed using CLONTECH Creator SMART cDNA Library Construction Kit (CLONTECH, USA. Cat. No. 634903) following the manufacturer's protocol, with a modification, for normalization. Purification of long distance (LD) PCR products was done using AxyPrep PCR cleanup kit (AXYGEN, Cat. No. AP-PCR-250). Then, normalization was done for the amplified cDNA using duplex-specific nuclease enzyme (Evrogen Biotech, Cat. No. EA003) as per the protocol described by Zhulidov *et al* (2004). DSN untreated sample was maintained as the Control. DSN treated and untreated samples were loaded onto 1.1% agarose gel to check the quality of normalization. 2 µl of normalized single-stranded transcript mixture was used in the remaining steps from LD PCR for library construction. The final fractionated PCR products, after pooling, were ligated with the vector provided in the kit, and transformed using electrocompetent *E.coli* (DH5α) cells. Individual colonies were picked up and amplified in fresh chloramphenicol LB agar plates (30µg/ml). Plasmid isolation was done using the Plasmid Isolation Kit (Sigma Co.; Cat. No. NA0160) and, in all, 600 clones were sequenced. Homology search for all the sequence data was done on Banana Genome Hub (Droc *et al*, 2013) using Blastn.

RESULTS AND DISCUSSION

Despite the economic importance of banana, fundamental molecular mechanisms underlying its defense mechanism against *Fusarium* wilt is poorly understood. As host-expressed molecules provide insights into the underlying defense mechanism, a normalized cDNA library, corresponding to genes expressed during *Foc* infection from root tissues of the tolerant genotype 'Calcutta-4', was constructed.

In this study, we observed that *Foc* inoculated plants of the tolerant genotype 'Calcutta-4' showed negligible symptoms of discolouration of corm portions and were generally healthy. Uninoculated plants were also healthy. Infected 'Kadali' showed both discoloration of corms and symptoms of wilting. Zhulidov *et al* (2004) reported that DSN-based normalization can be used successfully for normalization of cDNA from different sources before sequencing the expressed sequence tags (ESTs). Purified LD PCR products obtained from 'Calcutta-4' were used for normalization using the DSN method (to enrich rare transcripts prior to sequencing). The low intensity and downward shift of normalized cDNA smear on agarose/ EtBr gel (Fig. 1) compared to non-normalized cDNA depicts efficiency of the normalization by DSN. Only high molecular weight and bright cDNA fractions obtained at the final step were pooled for use in sequencing after cloning into the plasmid vector.

From sequence data of the 600 clones obtained, we removed low quality, short sequences. 392 clone sequences were finally selected for further analysis (Table 1). Functional characterization of the sequences was performed by homology-comparison using Banana Genome Hub database and the sequences were classified into seven categories (Fig. 2). A major functional category of the genes

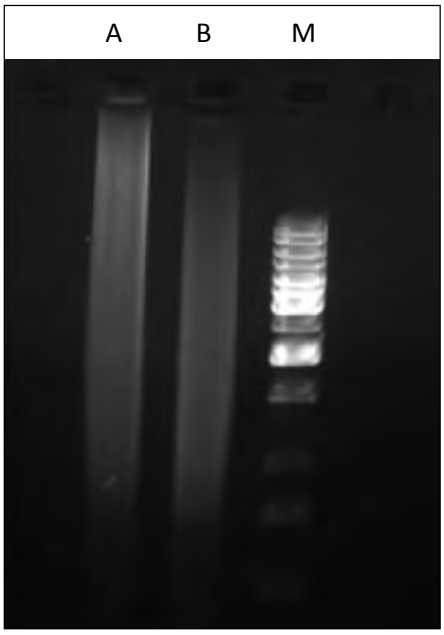

Fig 1. DSN treatement for normalization of cDNA library Lane A - Untreated cDNA; Lane B - DSN treated cDNA; M - 1Kb ladder

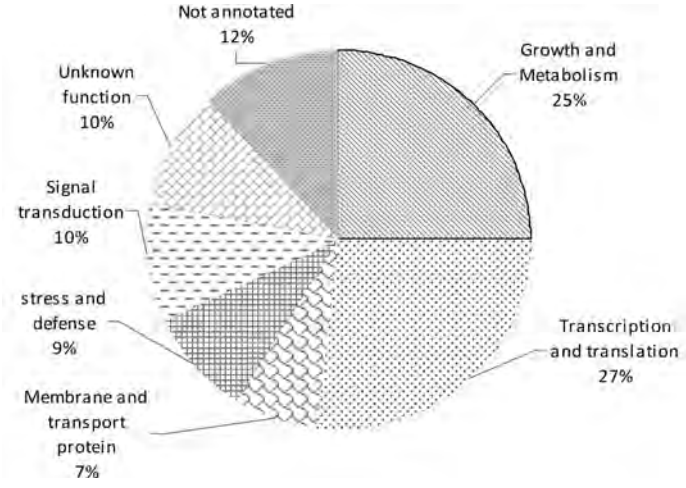

Fig 2. Pie chart showing functional categorization of expressed sequence tags (ESTs) developed from normalized cDNA library: Seven functional categories of non-redundant ESTs were identified in 'Calcutta-4' (*Musa acuminata* ssp. *burmannicoides*) in response to *Fusarium oxysporum* f. sp. *cubense* inoculation.

corresponded to genes involved in transcription and translation (27%), followed by those involved in growth and metabolism (25%). Defense genes comprised 9% while, signal transduction genes represented 10%. A significant fraction of the defense genes was involved in cell-wall orientation, strengthening and detoxification. A total of 369 ESTs were submitted to NCBI, GenBank (Accession number JZ349661 to JZ350029).

Table 1. Defense related genes identified against *Fusarium* wilt resistance, developed using infected root samples of the tolerant genotype, 'Calcutta-4', by normalized cDNA library

GenBank Accn.	Functional annotation of sequences using Blastn homology search (Banana Genome Hub)	Length (no. of base pairs)	E-value
JZ349796	Wound-induced basic protein~ PR4	329	4E-11
JZ349806	Putative Peroxidase 52~ PER52	265	2E-12
JZ349835	Peroxiredoxin, putative	311	4E-20
JZ349669	Chitinase-like protein 1~ CTL1	318	8E-28
JZ349864	Peroxiredoxin-2C~ PRXIIC	371	0
JZ349886	Putative Probable LRR receptor-like serine/threonine-protein kinase	204	4E-16
JZ349887	Wound induced protein	294	3E-21
JZ349880	Pathogenesis-related protein 1~ PR1	231	1E-120
JZ349683	Heat shock protein DnaJ, putative~ dnaJ	204	2E-27
JZ349962	Putative Stress-associated endoplasmic reticulum protein 2~ Serp2	136	2E-69
JZ349729	Heat shock cognate 70 kDa protein~ HSP70	310	2E-16
JZ349923	Mitogen-activated protein kinase kinase kinase 1~ MEKK1	204	3E-17
JZ349666	NBS-LRR disease resistance protein, putative~ RGA1	351	4E-11
JZ349733	bZIP family transcription factor~ RF2a	389	1E-20
JZ349696	SNF1-related protein kinase catalytic subunit alpha KIN10	204	4E-22
JZ349768	Lipoxygenase A~ LOX1	141	3E-62
JZ350024	Powdery mildew resistant protein 5 ~ MJ1458	351	4E-11
JZ349893	Actin-depolymerizing factor 11~ ADF11~	358	1E-23
JZ349668	Tubulin alpha-3 chain~ TUBA3	379	8E-50
JZ349918	Profilin-1~ PRO1	338	4E-11
JZ349814	Calreticulin~ CRT2	409	3E-74
JZ349842	Calmodulin~ CAM	305	3E-92
JZ349756	C2 domain containing protein ~ PP16-2	388	8E-19
JZ349792	MYB family transcription factor~ MYB86	351	6E-41
JZ349717	Serine/threonine-protein kinase AFC2	420	4E-27
JZ349951	Protein kinase family protein~ CTR1	204	2E-15
JZ349944	Small ubiquitin-related modifier 2~ SUMO2	296	7E-28
JZ349969	Ubiquitin-conjugating enzyme E2 28~ UBC28	358	4E-36
JZ349846	E3 ubiquitin-protein ligase At4g11680	204	2E-14

From this study, we profiled over 30 prominent genes known to be involved directly or indirectly in defense against the pathogen. Expression of genes which help in perception of the pathogen, viz., disease resistance protein RGA2 and NBS-LRR disease resistance protein, RGA1, various protein kinases like serine-threonine protein kinases, Mitogen-activated protein kinase kinase kinase 1(*MEKK1*), transcription factors like bZIP family transcription factor, MYB family transcription factor (*MYB86*), were all found in our study. Li *et al* (2013) reported induction of various defense related genes in *Foc* infected genotype and not in Control plants. These genes include PR-proteins (thaumatin like protein, endochitinase, etc.); transcription factors (Myb4, WRKY40); and, kinases [Mitogen-activated protein kinase kinase 2, Wall-associated receptor kinase (WAK 1)]. This suggests that the above-stated genes are involved in defense response in the banana.

Genes that pertain to cell wall re-orientation trait, namely profilin, actin depolymerizing factor, tubulin and alpha-3 chain, were also identified. Expression of host cell-wall thickening genes, fungal cell-wall degrading enymes, and, counteracting inhibitor proteins like polygalacturonase inhibitor protein (PGIP) against fungal proteins (Ravishankar *et al*, 2011) during fungal attack are considered important for hindering initial pathogen-entry into the host cell. Schmelzer (2002) reported that cell wall thickening also involves re-orientation of actin filaments as a defense response. Expression of these genes in the present study suggests cell-wall reorientation as an important part of the plant defense mechanism.

β-1,3 glucan and chitin are major components of the fungal cell wall. Chitinases have been known as direct defense enzymes, with a capability for attacking fungal cell-walls (Thakker *et al*, 2009). Hence, the expression of chitinases, P*R1* and *PR4*, in 'Calcutta-4' suggests that these may help prevent *Foc* multiplication in the host. Similar pattern was observed in a study by Van den Berg *et al* (2007) describing involvement of endochitinase (*PR3*) and and *PR1* in antifungal activity against *Foc* in this tolerant genotype of banana.

Following successful pathogen recognition, production of reactive oxygen species (ROS) called the oxidative burst, is one of the earliest cellular responses (Torres *et al*, 2006). Here, expression of various ROS scavenging enzymes like peroxidase, peroxiredoxin and peroxiredoxin 2C suggests that these genes act as indicators of ROS production. ROS controls several processes in plants. These act as signaling molecules for eliciting other defense responses; but, being toxic molecules, these are also capable of causing injury to cells (Mittler *et al*, 2004). Hence, in cells, ROS quantity needs to be strictly regulated; studies have proved that ROS scavenging enzymes fine-tune the ROS metabolism. Also, induction of various defense response genes in tolerant genotypes and not in susceptible genotypes has been represented by various transcriptome and proteomic studies (Bai *et al*, 2013; Li *et al*, 2013b). In our previous study we have observed the earlier and increased expression of antioxidant enzymes like peroxidase and glutaredoxin during *Foc* infection in the tolerant genotype (Swarupa *et al*, 2013). Abundance of transcripts for genes involved in ROS detoxification: phenylalanine ammonia lyase (that helps in biosynthesis of antimicrobial compounds) and 4-coumarate-CoA ligase (important in biosynthesis of flavonoids), were observed during *Mycosphaerella musicola- Musa acuminata* ('Calcutta-4') interaction (Passos *et al*, 2013). Hence, our study suggests that ROS could play a possible role in activating other downstream defense responses and which is fine-tuned by the above mentioned scavenging enzymes, to maintain ROS homeostasis.

We ascertained upregulation of various Ca^{2+} signaling genes in our previous study (Swarupa *et al*, 2013). Various calcium-binding proteins like calmodulin, calreticulin and C2 domain containing protein, and lipoxygenase, were found in our present study too. Activation of cyclic nucleotide gated channels (CNGCs)-Ca^{2+} influx (that influences calcium signaling and induction of lipoxygenase involved in JA signaling during *Fusarium* wilt infection) in a tolerant genotype of banana has been reported by Li *et al* (2012). Hence, expression of these specified genes supports their role in defense against *Foc* in banana. Involvement of Ubiquitin–proteasome system (UPS) in plant immune signaling which generally helps in post-translational mechanisms has been reported in various studies (Marino *et al*, 2012; Craig *et al*, 2009; Dhawan *et al*, 2009). Expression of various genes of the ubiquitination pathway in 'Calcutta-4' suggests their active role in *Fusarium* wilt infected plants.

Use of DSN based normalization for construction of cDNA library from root tissues against *Fusarium* wilt has diminished redundancy and enriched the identification of novel genes. Seventy eight per cent of the transcripts were annotated with known functions of DH Pahang genome, a

banana genotype also resistant to *Fusarium* wilt. Data generated in this study would be useful in studying defense response against the causative fungus, for selection of elite genotypes for various traits, and for crop improvement. Even though banana genome sequence is available, short studies like these are necessary to characterize and attribute functions to genes involved in various biotic and abiotic stresses. Further, this would also help identify and study the extent of gene expression during stress. Further, detailed analysis of genes identified in this study may help reveal the mechanism of plant defense response.

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