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Characterization of tolerance to *Fusarium oxysporum* f.sp., *cubense* infection in banana using suppression subtractive hybridization and gene expression analysis

V. Swarupa [a], K.V. Ravishankar [a,*], A. Rekha [b]

[a] Division of Biotechnology, Indian Institute of Horticultural Research, Hesaraghatta Lake Post, Bangalore 560089, India
[b] Division of Fruit Crops, Indian Institute of Horticultural Research, Hesaraghatta Lake Post, Bangalore 560089, India

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ABSTRACT

Identification of defense response genes in the host is one of the most essential steps in understanding disease resistance mechanisms in plants. In this study, suppression subtractive hybridization (SSH) library was constructed to study the genes involved in response to fusarium wilt disease in banana. Here cDNAs from a tolerant genotype *Musa acuminata* spp. *burmannicoides* 'Calcutta-4' infected by *Fusarium oxysporum* f.sp., *cubense* were used as tester and cDNAs from uninfected 'Calcutta-4' as driver population. After hybridization and cloning, EST library of 83 non-redundant clones were obtained. Based on sequence analysis and homology search in NCBI database the clones were assigned to different functional categories. The expression pattern of selected eight defense related genes namely peroxidase, glutaredoxin, polyphenol oxidase, glutamate synthase, S-adenosyl methionine synthetase, 14-3-3, heat shock protein, mannose binding lectin were analyzed through real-time PCR in contrasting genotypes. It was observed that the expression of these genes during initial progression of disease was found to be higher in tolerant genotype 'Calcutta-4' than in susceptible genotype 'Kadali'.

1. Introduction

Banana, one of the most important fruit in many countries, it has a worldwide economic and nutritive importance. It stands second, in production in India [1]. During crop life period, banana plants are challenged with various biotic and abiotic stresses. Of these, soil born disease fusarium wilt, caused by fungal pathogen *Fusarium oxysporum* f.sp., *cubense (Foc)* is a predominant biotic constraint that affects the growth, development, and yield potential. It is least controllable disease because once the soil is infected, the pathogen persists in the soil by colonizing on non-susceptible hosts and produces chlamydospores [2].

The development of new cultivars with better yields under adverse conditions is the basic objective of crop improvement. However, improving tolerance to biotic stresses has proved to be a complex task. In this regard, advances in plant physiology and genomics in the last 20 years has helped in understanding, how plants respond to stress and mechanisms responsible for range of tolerance observed in nature. Therefore gene expression study constitutes an important tool to understand mechanisms involved in tolerance. Gene expression profiling has allowed the identification of many genes induced when plants were exposed to stress [3–6]. ESTs provide a direct approach for discovering genes associated with stress response. This has been demonstrated in several plant groups [7–11]. Plants respond to pathogen attack by regulating specific and overlapping set of genes with healing and protective functions. The responses involve both gene activation and repression. Both of these processes appear to be important in coordinating the signaling pathways and downstream effectors to minimize the effect of biotic stress. Thus this study has envisaged identifying the genes that are upregulated during fusarium wilt infection in banana using contrasting genotypes.

2. Materials and method

2.1. Plant materials and inoculation

Micropropagated banana plantlets of tolerant wild species *Musa acuminata* ssp. *burmannicoides* 'Calcutta-4' and susceptible cultivar 'Kadali' both belonging to AA genomic group were planted in sterilized plastic pots of 2″ diameter containing 1:1 ratio of sterilized peat and soil mixture. They were maintained in a greenhouse

* Corresponding author. Tel.: +91 9480050523; fax: +91 80 28466291.
E-mail addresses: kv_ravishankar@yahoo.co.in, kvravi@iihr.ernet.in (K.V. Ravishankar).

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for hardening. After 1 month, plants were transferred to 6-inch diameter pots. Each pot contained two plantlets of both the tolerant 'Calcutta-4' and the susceptible 'Kadali' maintained together in sterile conditions. After the establishment of plants, the roots of each plantlet were injured using a sterile needle just prior to inoculation. *Fusarium oxysporum* f.sp., *cubense* inoculum (race 1) was multiplied on sterilized sorghum grains [12]. Approximately 50 g of these inoculated sorghum seeds were mixed with potting mixture for infecting the plantlets. The experiments were conducted in three replications. Uninfected plants were maintained as control.

2.2. Sampling

Root samples were collected at different stages, just before inoculation (0), 3, 6 and 10 days post inoculation (DPI). Three plants were sampled at each time point. Tissues were frozen using liquid nitrogen and stored until use. One set of pots, both of control and infected plants were maintained up to 2 months for taking observation on visual symptoms of infection.

2.3. PCR subtractive hybridization

Total RNA was isolated from root samples of all stages following a modified Liu method [13]. mRNA from total RNA of root (at 10 DPI) samples were isolated using Oligotex mRNA minikit (Qiagen, Catalogue No. 70022). The forward subtracted cDNA library was constructed using PCR Select Subtraction kit (Clontech, TAKARA, Catalogue No. 637401) from tolerant genotype 'Calcutta-4' to identify the upregulated genes against fusarium wilt infection. Here, mRNA from non-infected and infected roots was used as a driver and tester, respectively [14]. PCR Products from the final step were purified using AxyPrep™ PCR Cleanup Kit (Axygen Scientific, USA, Catalogue No. AP-PCR-50), followed by cloning using the INSTA T/A cloning kit (Fermentas., Catalogue No. K1214). The transformed *Escherichia coli* DH5α cells were plated onto LB plates containing 100 μg/ml Ampicillin. The recombinant colonies with single inserts were selected for sequencing. Later homology search for all sequence data was done using Blastx software of NCBI.

2.4. Real-time RT-PCR

RNA samples of all the stages (0, 3, 6, 10 DPI) were DNase treated using TURBO DNA-free Kit (Ambion, Catalogue No. AM1907) to remove genomic DNA contamination. 2 μg of total RNA was used as template to synthesize cDNA from all the samples employing RevertAid™ H Minus First Strand cDNA synthesis kit (MBI Fermentas, Catalogue No. k1631). Real-time RT-PCR of Taqman assay for 7 genes (Table 1) and SYBR green assay for peroxidase gene was performed using a LightCycler 480 instrument (Roche Diagnostics). DyNAmo Probe qPCR mix (Finnzymes, F450L) for Taqman assay and DyNAmo SYBR Green qPCR mix (Finnzymes, F410L) for SYBR green assay were used for real-time PCR, using the first strand cDNAs as template. Two genes namely Glutaredoxin and Mannose binding lectin protein were selected from our own normalized cDNA library constructed against fusarium wilt in tolerant genotype 'Calcutta-4' for expression studies whereas other genes were selected from subtracted library. Primers were designed using Primer Express software (Applied Biosystems) (Table 2). A 20 μl reaction volume for PCR amplification contained 10 μl DyNAmo Probe qPCR mix, 1 μl of forward and reverse primer, (4 pM) each, 2 μl cDNA template and 6 μl PCR grade water (Roche). Control treatments contained water instead of cDNA template. All PCR reactions were performed in triplicate. The cycling conditions were as follows: pre-incubation for 15 min at 94 °C, followed by 40 cycles, each consisting of 30 s denaturing at 94 °C, 15 s annealing at 60 °C, 15 s primer extension at 72 °C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control gene. Melting curve amplification was also kept for SYBR green assay to examine the amplification specificity. At the same time, amplification efficiency of primer pairs was determined by a standard curve derived from five serial dilutions of cDNA and the comparative (Ct) method was used to determine the expression level of analyzed genes.

Table 1
Expressed sequence tags (ESTs) selected for real-time RT PCR analysis. Accession number and results of Swissprot search database using NCBI tool of clones selected for expression analysis are indicated.

S. no.	Accession no.	Blastx search result/Swissprot	*E*-value
1	JZ198038	Peroxidase 1	2e-27
2	JZ198039	Glutaredoxin	2e-42
3	JZ198040	Heat shock cognate 70 KDa protein	7e-51
4	JZ198041	S-adenosyl methionine synthetase	8e-69
5	JZ198042	14-3-3 protein A	1e-50
6	JZ198043	Polyphenol oxidase	2e-10
7	JZ198044	Mannose binding lectin	8e-05
8	JZ198045	Glutamate synthase	7e-43

3. Results

3.1. Pathogenicity test

Among *Foc* inoculated plants, 'Kadali' a susceptible genotype, developed yellowing of leaves and wilting symptoms after 6 weeks. However 'Calcutta-4' a tolerant genotype did not show any wilting symptoms and maintained green leaves (Fig. 1A). Nearly forty percent of roots were dead in infected 'Kadali' plants but minor symptoms were observed in 'Calcutta-4' (Fig. 1B) and control (uninfected) plants were healthy (Fig. 1C). Transverse section of corm portions of infected 'Kadali' showed higher vascular discoloration due to cell death (Fig. 1D), than in 'Calcutta-4', where vascular discoloration was negligible (Fig. 1E).

3.2. Sequence analysis of subtracted library

In the present study, SSH library was constructed using cDNAs from inoculated samples (10 days post inoculation) of tolerant genotype 'Calcutta-4'. Nearly 164 recombinant clones were sequenced. The homology search results revealed that out of the 164 positive clones, 83 represented unique genes. Functional characterization of these unique sequences was performed by homology comparison with sequences in the NCBI database and was classified into six categories (Fig. 2). Genes whose products were homologous to unknown proteins or showed no hits, were collectively designated 'unclassified'. The largest number of genes (30%) were assigned to growth and metabolism group, while genes involved in energy metabolism constituted the smallest group (7.0%). Genes involved in stress and cell defense formed the second largest group (17%). Genes involved in signal transduction constitute 15 percent. The functional distribution of the EST library showed that genes involved in growth and metabolism, signal transduction and cell defense are relatively high in number.

3.3. Real-time RT-PCR

We performed quantitative real-time RT-PCR to ascertain the expression pattern of 8 putative genes which are likely to be involved in defense response against fusarium wilt disease in contrasting genotypes. Initially primers for 9 genes were examined