Interaction between sugarcane and *Colletotrichum falcatum* causing red rot: Understanding disease resistance at transcription level

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**Abstract** Detailed studies were taken up on host pathogen interaction between sugarcane and *Colletotrichum falcatum* causing red rot at the transcription level with a set of sugarcane varieties varying in disease resistance. A set of gene specific primers were designed to detect transcripts of resistance gene analogues (RGA’s), defense-related genes, transcription factors and signaling pathway genes induced during the host-pathogen interaction. mRNA extracted from pathogen inoculated canes at different time intervals were screened with 50 primers. Many of the transcripts were found to be expressed from the time of inoculation till 48h. However, differential gene expression was found only for chitinase, metallothionein, R30 (RGA), receptor protein kinase, reversibly glycosylated protein and signal sequence hydrophobic region (SSH) between resistant and susceptible varieties. Additionally differences in transcript size were noticed for some of the screened primers. We have standardized differential display (DD)-RT-PCR protocol with silver staining method to identify differential transcripts. Work on transcriptional variation in *C. falcatum-*sugarcane interaction has been performed for the first time and this promises a new approach to identify gene(s) involved in red rot resistance in sugarcane.

**Keywords** Gene specific primers, expressed sequence tags, resistance gene analogue, differential display RT-PCR, differential expression.

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**Introduction**

The single largest and most significant contribution in sugarcane transcriptomics was done by the Brazilian Organization for Nucleotide Sequencing and Analysis (ONSA) consortium (Simpson and Perez, 1998). Better known as Sugarcane EST Project, (SUCEST) they have developed database containing approximately 2,38,000 ESTs from 26 cDNA libraries derived from at least 12 sugarcane cultivars at various stages of development. The SUCEST resource has since been used to identify genes associated with the response of sugarcane to cold (Nogueira et al., 2003) and oxidative stress (Kurama et al., 2002); to identify genes for protease inhibitors associated with resistance to insect pests (Soares-Costa et al., 2002; Falco and Silva-Filho, 2003; Mello et al., 2003) and to characterize disease resistance gene analogues (Rossi et al., 2003). A survey of ESTs derived from the apex, leaves and stem of sugarcane after floral induction (Ma et al., 2004) have added 17,135 EST collections followed by the Australian group primarily focused on the function of the sugarcane stem during vegetative growth in internodes actively accumulating sucrose (Casu et al., 2001, 2003, 2004) representing an additional resource of ESTs, resulting in a combined total of 2,63,633 ESTs derived from sugarcane. The analysis of ESTs and microarray-derived expression data has been used to investigate other stem functions that accompany stem maturation and sugar accumulation (Casu et al., 2004).

However with such a great pool of resources in terms of EST sequences available in the public domain, little is known about the molecular background of the interaction between pathogen and host. Only a few reports have been published describing molecular basis of disease resistance in sugarcane. The cDNA-AFLP analysis of differential gene expression during the interaction between sugarcane and *Ustilago*
scitaminea causing smut (Borrás-Hidalgo et al., 2005) and sugarcane and Puccinia melanocephala causing common rust (Carmona et al., 2004) have been reported, wherein genes involved in recognition, signaling, defense and general response were identified.

Red rot of sugarcane is caused by the fungus Colletotrichum falcatum Went (Perfect state; Glomerella tucumanensis (Speg). Arx & Muller) infects the cane stalks and causes symptoms of tissue discoloration, invasion of succrose due to production of pathogen induced invertases and drying of cane stalks. The disease remains a major constraint to sugarcane production in India, Pakistan, Bangladesh, Thailand, Vietnam, USA, Sudan etc. Although the disease is effectively managed by releasing disease resistant varieties (Viswanathan and Samiyappan, 1999), the resistance in the varieties is not static due to the emergence of new pathogenic strains which cause breakdown of resistance in the popular sugarcane varieties, also the mechanism governing red rot resistance has not been studied in detail. The differential induction of chitinases and thaumatin-like proteins (TLP’s) in sugarcane in response to infection by C. falcatum has been demonstrated using biochemical and Western blot studies (Viswanathan et al., 2005). However, the molecular tools have yet to be fully explored to characterize the genes activated during pathogen invasion.

One approach to study genes expressed at the transcript or mRNA level of an organism is to isolate cDNA copies of the mRNA in specified tissues and undertake DNA sequence analysis of these copies. Sequences obtained in this manner are termed expressed sequence tags (ESTs) and represent the genes functioning in the tissues at the time of sampling. However, to capture all expressed genes very large EST collections must be obtained from multiple tissues and treatments. In order to study the expression pattern of a particular gene or group of genes large scale study is not suitable. Hence we have designed gene specific primers (GSP’s) for resistance gene analogues (RGA’s), defense-related genes, transcription factors and signaling pathway genes from consensus sequences obtained from gene sequences of similar crops including sugarcane available at the National Center for Biotechnology Information (NCBI) database to identify the transcripts induced in response to C. falcatum infection in a set of resistant and susceptible varieties. Additionally differential display RT-PCR was standardized to identify novel transcripts expressed in our host-pathogen interaction.

Materials and methods
Pathogen inoculation and sampling

Two sugarcane varieties namely Co 93009 (resistant to red rot) and CoC 671 (highly susceptible to red rot) were used in this study. The sets of these varieties were planted in the field during February 2006 and normal package of practices were followed to raise the crop. During July 2006, the pathogen inoculum multiplied on oatmeal agar (cf671 pathotype isolated from sugarcane cv. CoC 671; 10³ spores/ml) was inoculated on to the canes (Chona, 1954; Viswanathan et al., 1998). An puncture was made in the second or third internode above the ground level using red rot inoculator. The inoculum was placed in the bore hole using Pasteur pipette and the removed tissue core was replaced. The injured portion was sealed by pressing a small pellet of china clay. Usually the inoculation period coincides with active monsoon period at Coimbatore, which favours disease development. The relative humidity was between 75 and 90% during the incubation period (upto 48hr after inoculation). The minimum and maximum temperatures, during the incubation period, ranged from 22-26°C and 31-34°C, respectively.

Cane samples were collected from the field at different time intervals after pathogen inoculation i.e. 0, 3, 6, 12, 24 and 48 h. The injury control samples were taken from 6 and 12 h, respectively after pathogen inoculation and an uninjured control was kept for the both varieties. At least three canes of inoculated or injured canes constituted a sample. Pathogen inoculated/injured internode was cut along with one internode above and one internode below. The samples were immediately frozen in liquid nitrogen and stored at -80°C until processing.

RNA Extraction

Tissue samples of 5 g were scooped around the pathogen inoculated or injured bore hole using scalpel and ground to a fine powder in liquid nitrogen. The total RNA extraction was carried out using TRIZOL™ reagent (Ambion®, Canada) following the manufacturer’s instruction with certain modifications. Two ml of TRIZOL reagent was used per gram of cane tissue due to the presence of low phenolics and high