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Differentially expressed transcripts of wild and cultivated jute (*Corchorus* spp.) varieties upon fungal (*Macrophomina phaseolina*) infection

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ABSTRACT

Macrophomina phaseolina can cause disease to most of the commercially grown jute (Corchorus spp.) species. This fungus is one of the major growth limiting factors of two of the most widely cultivated species-Corchorus olitorius and C. capsularis. Resistance to this fungus is present in a wild type species C. trilocularis. In this study, the differential display approach was applied to identify the genes that are expressed differentially upon fungal infection between the resistant species C. trilocularis and susceptible counterpart C. olitorius var. O-72. Differential display identified three transcripts showing different banding patterns. Cloning and sequencing revealed that one of the transcripts has homology with disease resistance gene of other plants, one with non-LTR retrotransposon and the third one has no significant homology with any reported sequence in the nucleotide database. Semi-quantitative reverse transcription PCR was performed to study expression profile under different stress conditions.

Key words: Differential display, Jute, Disease resistance, Bioinformatics analysis, RT-PCR.

INTRODUCTION

Jute, a fiber crop grown in the Indian subcontinent for centuries, has immense economic importance [1, 2]. Like all other commercially important plants, one of the major bottlenecks of jute production is its constant exposure to different biotic and abiotic stresses, causing a major threat in crop production. *Macrophomina phaseolina*, the causal agent of stem-rot disease in

hundreds of plant species spanning a wide geographic distribution [3], is one of the most devastating pathogens to which the cultivated species of jute (*C. olitorius* and *C. capsularis*) are susceptible [4].

Current agricultural practice to deal with this problem is the use of fungicides which, in turn, raises concerns about environmental safety and the possibility of developing fungicide resistance [5]. A wild jute species (*C. trilocularis*) is known to be resistant to *Macrophomina phaseolina* [6]. Thus it is possible that the genes for fungus resistance are present in this jute species. In our endeavor to identify disease resistance genes, a differential display [7] approach for differential gene expression in both the sensitive and tolerant variety was applied to understand the mechanism of disease resistance at the transcript level. Three differentially expressed transcripts were identified from both the varieties.

Disease resistance genes are expressed differently in different plants [8, 9, 10, 11] and subtle variation in their expression level upon stress induction can be observed from semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). So expression profiling of the identified sequences were conducted to confirm their variation at the expression level.

MATERIALS AND METHODS

Plant materials and stress treatment

Seeds of both the sensitive and tolerant varieties were germinated onto moist blot paper in petri dishes at around 25°C for 3 days. *M. phaseolina* fungal culture was prepared in PDA media [12] and fungal suspension was made using distilled water before being infected on samples of both the varieties. Seedlings from the infected samples of both the species were collected 15 and 24 hours after spraying fungal suspension. To analyze differential gene expression after fungal infection, one set of uninfected seedlings of both the varieties were kept as control. The collected seedlings were frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

RNA isolation and first strand c-DNA synthesis

Total RNA from resistant and susceptible jute seedlings, under both normal and infected conditions, were isolated at different time intervals by Chomczynski's protocol [13] and the purity and integrity of the isolated RNA was checked by gel electrophoresis while it's concentration was determined using a Nanodrop (ND-1000). 4.5 µg of poly (A+) RNA were reverse transcribed by SuperScript™ First-Strand Synthesis system for RT-PCR (Invitrogen, USA) according to the manufacturer's protocol using thermal cycler (Eppendorf Mastercycler Personal). Single base anchored primers ($T_{12}A$, $T_{12}C$ and $T_{12}G$) were used which divided the total mRNA into three groups [14].

Differential display PCR

The Differential display PCR was carried out as defined by Liang and Pardee in 1992 [7] using the first strand c-DNA as template, while 13-mer Arbitrary primer ARB-04 and three anchor primers ($T_{12}A$,$T_{12}C$ and $T_{12}G$) which formed three primer pairs, were used in the cycling condition that had a denaturing step of 3 min at 94°C, 40 cycles with 94°C for 50 seconds, 38°C for 50 seconds, 72°C for 1 min 20 seconds and a final extension period of 5 min at 72°C. Then the amplified PCR products were subjected to polyacrylamide gel electrophoresis at 60V for 3

hours and visualized by silver staining [15]. Differentially expressed bands were excised and incubated overnight in a 1.5 mL tube containing elution buffer (0.5M EDTA pH 8.0, 1M ammonium acetate). Then ethanol precipitation allowed the recovery of the DNA. Finally, the recovered DNA was reamplified by the same conditions of differential display PCR with the exception of the final extension period of 20 minutes at 72°C.

Cloning and sequencing of the differentially expressed transcripts

PCR fragments were cloned into pCR2.1 vector of TA cloning kit (Invitrogen). Ligated DNA was transformed by heat shock into competent *Escherichia coli* strain DH5α cells. Colonies were grown overnight at 37°C on antibiotic containing Luria-Bertani agar plates. X-gal and isopropyl-β-D-thiogalactopyranoside were used for blue-white screening to obtain the positive colonies that contained the inserted sequences. Next plasmid was isolated, followed by PCR with vector specific M13 forward and M13 reverse primers. After agarose gel electrophoresis, bands were extracted using QIAGEN MinElute Gel Extraction Kit and finally sequencing was done by the molecular services provided by 1st Base Malaysia.

Bioinformatics analysis

Once the sequences were obtained comparative bioinformatics analysis was done online by the NCBI and Expasy websites. BLAST [16] search was used to find homology with the sequences present in the database. The protein sequences were generated by ESTScan 2 webserver [17, 18]. Gene specific primers were designed using Primer-BLAST. Domain prediction was done by InterProScan.

DNA isolation

DNA was isolated according to the protocol described by Haque and his colleagues [19].

Table 1. List of primers (with their sequences) used in this study

Primer name	Primer type	Sequence (5′-> 3′)
$T_{12}A$	Anchor primer	TTT TTT TTT TTT A
$T_{12}C$	Anchor primer	TTT TTT TTT TTT C
$T_{12}G$	Anchor primer	TTT TTT TTT TTT G
ARB-04	Arbitrary primer	AAG CTT GAT TGC C
Clone-02 For	Gene specific primer	GCG CCT AAG TGC TGA GAT TCG C
Clone-02 Rev	Gene specific primer	TGC ACG CCA GGC TGA CTC TG
Clone-03 For	Gene specific primer	TGC CTC AGA ATG CCC TGC CT
Clone-03 Rev	Gene specific primer	TGG GAG AAA GCA ATG GCA CCT TTC
Clone-04 For	Gene specific primer	TGA TTG CCC AAA ACT TCC AGC TGA T
Clone-04 Rev	Gene specific primer	GGC CAT TCT TGC TAC CAC ACC GA

Expression profile analysis

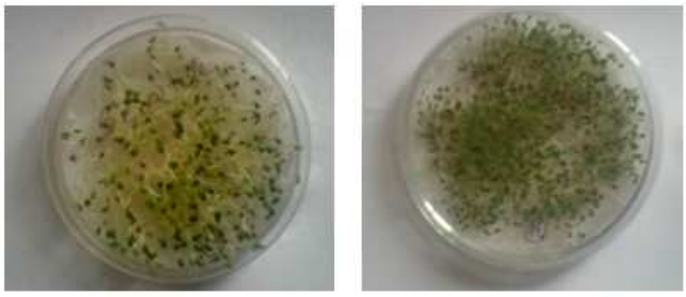
The expression profiles were implemented by one-step RT-PCR reactions with equal amount of total RNA from each sample, and semi-quantitative one-step RT-PCR was done according to the manufacturer's instructions (Invitrogen, USA). To normalize the semi-quantitative RT-PCR event

the house keeping gene β-actin was used as the internal control. The PCR products were then run on 1.5% agarose gel for 40 minutes at 80 V.

RESULTS AND DISCUSSION

Phenotypic changes in the seedlings

Visible changes in the *C. olitorius* seedlings were observed upon fungus solution spraying. These changes were prominent particularly in the root with gradual yellowing over time. On the contrary, *C. trilocularis* seedlings were unaffected (figure 1).



(a) *C. olitorius* (b) *C. trilocularis*

Figure 1. Effect of *M. phaseolina* on seedlings of sensitive & tolerant species

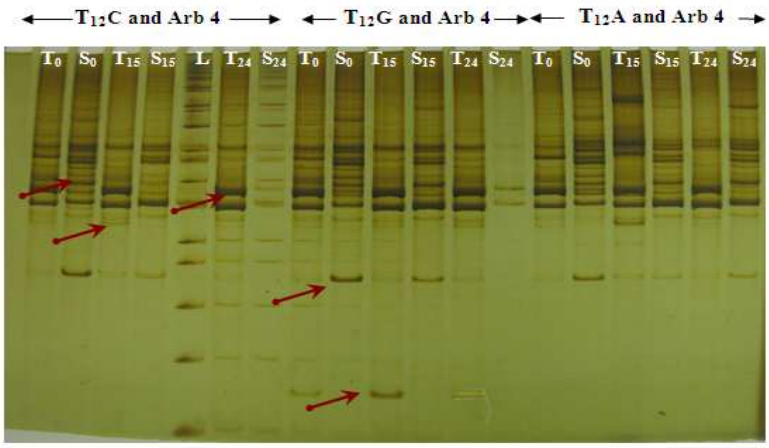


Figure 2. Polyacrylamide gel electrophoresis after DD-PCR

Here, 'S_0' and 'T_0' indicates uninfected samples from susceptible and resistant jute species; 'S_{15}', 'T_{15}', 'S_{24}' and 'T_{24}' indicates samples from both the spices after 15 and 24 hours of infection respectively.

Differential expression of fungal stress responsive genes

The mRNA differential display was used to screen the fungal stress responsive transcripts. An arbitrary (ARB-04) and three anchored ($T_{12}A$, $T_{12}C$ and $T_{12}G$) primers were used in three different primer pair combinations to generate the transcripts. Differential display produced five bands (figure 2) showing difference between the two species under consideration. Three of them were successfully cloned in TA cloning vector and then sequenced. The sequences have been submitted to NCBI with GenBank accession numbers HO214332-34.

Bioinformatics analysis

BLAST analysis revealed that the first transcript with GenBank accession no. HO214332 had no significant homology with any other sequence in the database both at the nucleotide and protein levels. The second transcript with GenBank accession no. HO214333 had homology with non-LTR retrotransposons of other plants. The third transcript (GenBank accession no. HO214334) showed sequence homology to *Populus* and *Ricinus* disease resistance genes at the protein level. Interestingly, nucleotide BLAST failed to show significant match to any of the known genes. This may be due to the fact that many of the disease resistance genes evolve through adaptive evolution and therefore, no significant homology is found in their nucleotide sequences [20]. Domain prediction of the non-LTR retrotransposon like transcript revealed the presence of reverse transcriptase domain (InterProScan id IPR000477) while the disease resistance homologue contains iron-sulphur binding domain (IPR006058) and a thiolase active site (IPR020610) as shown in figure 3.

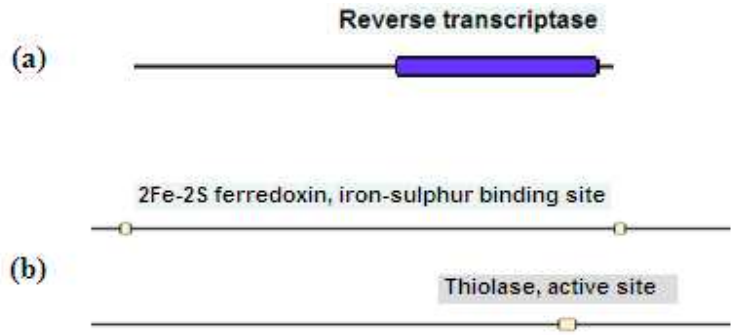


Figure 3. InterProScan predicted domains of (a) the non-LTR retrotransposon like transcript and (b) disease resistance homologue

Based on the gene ontology prediction and InterProScan domain annotation result, the disease resistant homologue is involved in the biological process of metabolism (GO: 0008152) and the molecular functions are transferase activity (GO: 0016747) and electron carrier activity (GO: 0009055). Gene Ontology of the non-LTR retrotransposon like gene suggested that it is involved in the biological process of RNA-dependent DNA replication (GO: 0006278) and the molecular functions are RNA binding (GO: 0003723) and RNA-directed DNA polymerase activity (GO: 0003964). These features are required for retrotransposon gene replication [21].

Confirmation of the presence of the transcripts in jute genome

Sequences of differential display fragments were used to the design gene specific PCR primer pairs. These primers were used to verify that the transcripts found by differential display were actually from jute genome by performing PCR from genomic DNA. All the three fragments gave distinct bands (figure 4) when PCR with gene specific primers were done which, thereby, confirms their presence in the genomic sequence.

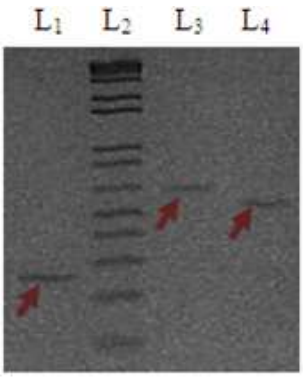


Figure 4. Bands confirming the presence of the transcripts in jute g-DNA

Expression profile by semi-quantitative RT-PCR

Semiquantitative RT-PCR was done to confirm the differential expression profile of the transcripts in both the jute varieties upon fungal infection. The same amount of RNA was used prior reverse transcription, and β-actin was used as an internal control for determining equal loading (figure 5 a). The transcript of unknown function showed higher level of expression in the uninfected sample compared to the infected samples of susceptible species. The band intensity in susceptible species slightly decreases after fungal infection, while there is no variation in both the samples of the tolerant variety (figure 5 b). Similar expression pattern was also observed during the initial polyacrylamide gel electrophoresis. However the second transcript, a putative non-LTR retrotransposon, failed to show any significant variation in expression pattern in both the jute species (figure 5 c). This could be due to the presence of multiple bands in the excised polyacrylamide gel and the band that actually showed the differential expression pattern, was not cloned. Gene specific primers designed from the putative disease resistance homologue produced a band of desired size in the tolerant variety *C. trilocularis*, but was absent in the sensitive counterpart *C. olitorius* (figure 5 d). The expression level of the transcript in the tolerant variety appeared to be constitutive.

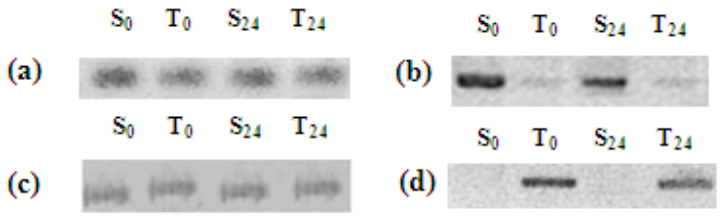


Figure 5. Semi quantitative RT-PCR using gene specific primers

The transcripts identified during this study represent valuable resources for the development of markers for molecular breeding and development of resistance gene analogs for jute as well. The disease resistance homologue identified in the wild type variety (*C. trilocularis*) can be transformed to the susceptible variety in future once the full length sequence is deduced. Such an example of successful disease resistance gene transfer is the introduction of RCT1 gene from *M. truncatula* into alfalfa cultivars for anthracnose resistance [10].

It is known that transposable elements are associated with some resistance gene clusters, and may generate further variation at these complexes [22]. The identified non-LTR retrotransposon did not show any variation in its expression level when semi-quantitative RT-PCR was done. Nevertheless, the possibility of the transposable element being embedded between a disease resistance gene cluster cannot be ruled out. Some recent studies reported that chimeric transcripts comprising retrotransposons and disease resistance genes may function in plant disease resistance [23].

CONCLUSION

In spite of having of immense potential, studies on molecular mechanism of disease resistance in jute have been very insignificant. Although it is necessary to obtain the full length cDNA and further experimental verification to characterize their function, this study provides valuable insight to understand some of the fungal stress responsive gene transcripts in jute. A better understanding of the genetic diversity present within the cultivated species and its wild relatives is critical for improving disease resistance characteristics. The study is not able to draw any definite conclusion. Nevertheless, it paves the way for future development of disease resistant jute varieties.

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