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Identification of differentially expressed mRNA in black pepper (*Piper nigrum* L.) genotype for drought tolerance

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Abstract

The aim of the present study was to identify the differentially expressed, up regulated and down regulated genes by transcriptome analysis for drought tolerance. The genotypes PRS-64 (Angamali) was selected for molecular characterization among the ten genotypes through physiological characterization. The technique used to analyze the transcriptome was differentially display reverse transcriptase polymerase chain reaction (DD-RT-PCR) which allows extensive analysis of gene expression among several cell populations (sturtevant 2000) [8]. The first stand cDNA was synthesized from the RNA samples using HT11G. Each first strand cDNA was used for the second amplification with eight different arbitrary primers. The PCR product was resolved in Urea polyacrylamide gels. The upregulated, down regulated and differentially expressed cDNA fragments were retrieved from the gel and reamplified with the same set of primers. The agarose gel electrophoresis showed that the (TDFs) transcript derived fragments obtained were relatively short (400-900 bp). TDFs were cloned using pGEMT vector and the clones were sequenced. The sequence from differentially expressed TDFs showed homology to copper containing amine oxidases which have a broader range of functions including cell signaling which contributes to terminal polyamines oxidation in peroxisomes. Polyamines are involved in growth development and response to abiotic stress.

Keywords: black pepper (*Piper nigrum* L.), drought resistance, DDRT-PCR, gene expression

Introduction

The black pepper has a great export potential and the contributory factor is the presence of chemical piperine in it. In India, black pepper is generally grown as a rainfed crop. Though the average rainfall in pepper growing areas is well above 2000 mm, the distribution is not uniform. The rainfall received during December to May is very negligible. Hence, the crop suffers due to severe soil moisture shortage during these months. Ninety per cent of the area under black pepper is rainfed. These areas frequently experience severe water deficit due to uncertain and uneven rainfall distribution patterns. As India is the primary centre of diversity of black pepper, the indigenous genetic resources are reservoirs of useful genes for plant improvement programmes. Black pepper is grown mainly in Kerala, where water deficit during off season viz., December-May is a common feature. To avoid reduction in yield during water stress condition, cultivation of drought tolerant varieties is essential (Rajagopal and Balasimha, 1994) [5].

Differential display reverse transcription polymerase chain reaction (DDRT-PCR) or differential display of eukaryotic mRNA was first introduced by Liang and parde in 1992 [2]. It is used to identify and compare the differentially expressed genes. The DD technique was developed with the aim of overcoming limitations of methodologies previously used for identifying differentially expressed genes. DD has advantages over these techniques because it is based on simple and established methods, it is reproducible and sensitive, it does not require biochemical information about proteins, more than two samples can be compared simultaneously, and only a small amount of starting material is needed (Yamazaki and Saito, 2000). To generate cDNA fragments, anchored and arbitrary primers are used by reverse transcription, followed by PCR (RT-PCR). In sequencing gel the cDNA fragments are resolved and compared which reflects differences in the mRNA composition. Further, it can be eluted, cloned and sequenced. To obtain intact, DNA free RNA with DD residues then converting the mRNAs from cells into cDNAs, that differs at the last 3' non-T base using 3 individual anchored oligo-dT primers. The beginning of the poly (A) tail for any given mRNA enables the homogeneous initiation of cDNA synthesis. In the presence of a set of second primers by PCR, cDNAs are further labeled with isotopes that are short and arbitrary sequence. To maximize the number of amplified mRNA, the annealing temperature of the PCR is low

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(Liang and parde, 1992) [2]. Comparison of the cDNAs runs side-by-side on a denaturing poly acrylamide gel. The objective of the present experiment was to investigate molecular changes in Piper genotypes when subjected to water stress and probability of utilizing some of these parameters to distinguish between susceptible and tolerant types. Tolerance trait if found in any of these species can be transferred to high yielding *P. nigrum* types either through conventional or through molecular approaches.

Materials and Methods

Ten black pepper genotypes identified at Panniyur Research Station, Panniyur were selected for the study. Among the different genotypes, PRS-64 identified as most drought tolerant genotype and PRS-44 identified as susceptible genotype through different physiological parameters. Based on the physiological parameters the most drought tolerant genotype will be identified and that will be subjected to molecular analysis.

Isolation of total RNA

Young, tender leaves from the two different treatments of most drought tolerant genotype (PRS-64) i.e, Control plants and Water stress treated plants were collected early in the morning for RNA isolation. Leaves were then washed quickly with DEPC treated water. Isolation of good high quality and quantity of RNA is a pre requisite for differentially displayed reverse transcriptase PCR analyses (DD-RT-PCR). Total RNA was extracted from the treatments using TRI reagent method (Sigma- Aldrich, USA).

Quality analysis by Formaldehyde-agarose gel electrophoresis

The gel (1.2% agarose) electrophoresis was performed to check the quality of RNA, following the method as described by Sambrook *et.al.* (1989) [6].

Spectrophotometric analysis of RNA: The samples which gave three discrete bands specific to RNA on an agarose gel electrophoresis were the further analysed and quantity and quality of RNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). A ratio of 1.8 to 2.0 for OD₂₆₀/OD₂₈₀ indicated good quality RNA. Good quality total RNA from all two treatments was pooled separately.

Differential Display Reverse Transcription Polymerase Chain Reaction (DD-RT-PCR)

The method is based on using sets of anchored and arbitrary primers to generate cDNA fragments by reverse transcription, followed by PCR (RT-PCR). The cDNA fragments are resolved and compared in sequencing gels. The resulting cDNA patterns reflect differences in the mRNA composition. DD cDNAs can be isolated, cloned and sequenced.

First strand cDNA synthesis: The isolated total RNA predominantly consists of rRNA, tRNA and mRNA. The isolation of under graded mRNA is very essential for successful first strand synthesis and subsequent PCR amplification. Mature mRNA molecules carry poly (A) at their 3' termini, to which oligo dT primers anneal. Reverse Transcription enzyme can synthesize cDNA from isolated RNA under favorable conditions. The single stranded cDNA from isolated RNA under favorable conditions. The single stranded cDNA was used immediately for PCR without further purification.

Second strand c DNA synthesis: Each fraction of the single stranded cDNA was amplified by PCR with the anchored and respective arbitrary primers.

Table 1: List of Anchor (T11C) and Arbitrary primers (AP) used in DD-RT-PCR

Primer No.	Sequential information(5' to 3')	No. of bases
T11C	AAGCTTTTTTTTTTTC	16
AP1	AAGCTTGATTGCC	13
AP2	AAGCTTCGACTGT	13
AP3	AAGCTTTGGTCAG	13
AP4	AAGCTTCTCAACG	13
AP5	AAGCTTATAGGC	13
AP6	AAGCTTGCACCAT	13
AP7	AAGCTTAACGAGG	13
AP8	AAGCTTTTACCGC	13

PCR product was stored at -20°C, later electrophoresed using 6.0% PolyAcrylamide Gel Electrophoresis (PAGE) and visualized by silver staining.

Urea Poly Acrylamide Gel Electrophoresis of DD-RT-PCR fragments:

The cDNA or transcript derived fragments from the two different treatments were visualized in 6% denaturing poly acrylamide gel electrophoresis by silver staining.

Sample preparation and electrophoresis: Samples were prepared by mixing 4 µl of sample with 2 µl Loading dye (10 µl formamide, 0.5M EDTA, pH 8, 10mg of xylene cyanol and 10 mg of bromo phenol blue) Samples were then loaded into the well. Electrophoresis was conducted at 1200 volt for approximately 3 hr. After the xylene cyanol reached 2/3 portion of the gel, the electrophoresis was terminated.

Silver staining of the gel: After completion of electrophoresis, the two glass plates were carefully separated from each other. Up regulated, down regulated, differentially expressed bands and uniformly expressed bands were identified and marked on the plate. Gel was then dried overnight at room temperature.

Elution of cDNA /transcript derived fragments from Poly acryl amide gel:

The identified cDNA fragments in the gel were rehydrated for 20 min with 10 µl of distilled water, and scooped out cut from the Poly acryl amide gel with a surgical blade. Each gel slice was incubated in 40 µl of distilled water for 2 h at room temperature (Minsheng, 2006) [3]. Elute 10 µl from 40 µl and the eluent 10 µl was then PCR amplified under the same conditions as for the selective PCR reaction with modified dNTP concentration i.e, 2mM. PCR product was separated on a 1.5% agarose gel and purified with a AxyPrep DNA Gel Elution Kit (Axygen, Biosciences).

Gel elution of PCR amplified fragments: cDNA fragments was eluted using Axy Prep DNA Gel Extraction Kit (Axygen, Biosciences) as per the manufacturer's guidelines.

Cloning of cDNA/Transcript derived fragments

Screening of competent cells: The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker. The eluted and purified cDNA product was cloned in to pGEMT vector system Clone GEMT (Fermentas).

Transformation of *E.coli*: Competent cells for plasmid transformation were prepared using the competent cell preparation Kit of GeNei, Bangalore in LB agar media.

Transformation of ligated product: The aliquots of transformed cells (50, 100 and 150 µl) were plated on LB agar containing Ampicillin (5 mg/ml) plates and incubated overnight at 37°C.

Analysis of recombinant clones by Colony PCR

Preparation of stabs: Stabs were prepared for the colonies in which the presence of insert was confirmed. LB agar medium was melted, cooled to 42°C, added ampicillin and poured into storage vials aseptically in a laminar air flow. After solidification, single colony was inoculated into the medium using sterile bacterial loop. The vials were incubated at 37°C overnight and further stored at 4°C.

Sequencing of cDNA clones: The up regulated, down regulated and differentially expressed transcript derived fragments were cloned in pGEMT and sequenced using specific forward and reverse primer through SciGenome, Cochin.

In silico analysis of sequences

The sequences obtained were analyzed with the Vector screening, reverse complement of sequences, Merging of sequences, Search for Homolog online bioinformatics tools and validated.

Results and Discussion

Most notable is differential display (DD) RT-PCR, the use of which has been recently reviewed in both eukaryotes and prokaryotes. Briefly, after cDNA synthesis using reverse transcriptase and an oligo dT primer that anneals to the 3 poly A tail of mRNA, subsets of cDNA populations for comparison are PCR amplified with short, non-specific oligo nucleotide primers, in combination with oligo dT primers, and visualized on polyacrylamide gels. DD-RT-PCR was first used to isolate plant genes. DD-RT-PCR identified an *Arabidopsis* gene, *ERD15*, which was induced by drought, further demonstrating the activation of common biochemical pathways in response to different stimuli. In the present study we discuss how DDRT-PCR, genomic and bioinformatics approaches are combined to meet the challenge of identifying and characterizing transcriptional changes during water stress condition.

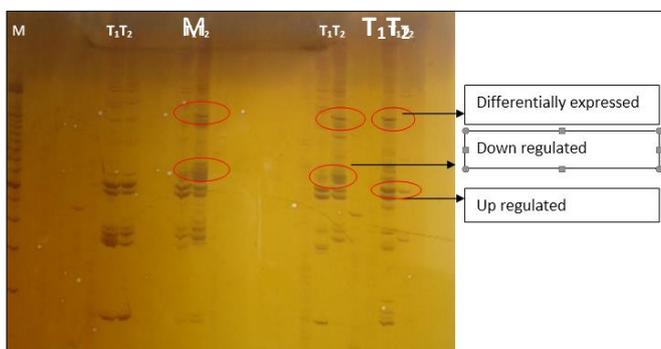


Fig 1: Differential display patterns of transcript derived fragments from two treatments in PRS-64 amplified using anchored primer H-T11 (G) and arbitrary 13-mers (HAP-1 to 4)

In the present study genes operating in ABA dependent signaling pathway, primary carbon metabolism pathway, stress signaling pathway, molecular chaperons, hypothetical

and putative proteins were identified by sequence analysis. The sequence analysis of clone 1 which was 859 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLAST n analysis showed homology to NADH dehydrogenase subunit F gene (*Piper nigrum*) which gave 100% identity with 83% of query coverage. Annotation of sequence by the BLAST x analysis showed homology to NADH dehydrogenase subunit F gene (*Piper nigrum*) 100% identity with 98% query coverage. NADH dehydrogenase or complex I (EC 1.6.99.3) is a flavoprotein that contains iron-sulfur centres. It catalyzes the transfer of electrons from NADH to coenzyme Q10 (CoQ10) and, in eukaryotes, it is located in the inner mitochondrial membrane. It is one of the "entry enzymes" of oxidative phosphorylation in the mitochondria. (Hugo gene nomenclature committee). The production of reactive oxygen species (ROS), such as O₂ and H₂O₂, is an unavoidable consequence of aerobic metabolism. In plant cells the mitochondrial electron transport chain (ETC) is a major site of ROS production. In addition to complexes I-IV, the plant mitochondrial ETC contains a non-proton-pumping alternative oxidase as well as two rotenone-insensitive, non-proton-pumping (NADPH) dehydrogenases on each side of the inner membrane: NDex on the outer surface and ND in on the inner surface. Because of their dependence on Ca²⁺, the two ND ex may be active only when the plant cell is stressed. Complex I is the main enzyme oxidizing NADH under normal conditions and is also a major site of ROS production, together with complex III. The ND in (NADH) and cytochrome oxidase function to limit mitochondrial ROS production by keeping the ETC relatively oxidized. Several enzymes are found in the matrix that, together with small antioxidants such as glutathione, helps remove ROS. The antioxidants are kept in reduced state by matrix NADPH produced by NADP -isocitrate dehydrogenase and non-proton-pumping transhydrogenase activities. When these defenses are overwhelmed, as occurs during both biotic and abiotic stress, the mitochondria are damaged by oxidative stress (Moller, 2001).

The sequence analysis of clone no 2 which was 436 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLAST n analysis showed homology to *Vicia faba* calcium-dependent protein kinase1 (CPK1) mRNA complete cds which gave 100% identity with 83% of query coverage. Annotation of sequence by the BLAST x analysis showed homology to calcium-dependent protein kinase1 (*Vicia faba*) 100% identity with 36 % query coverage. Calcium-dependent protein kinases (CPKs) are plant proteins that directly bind calcium ions before phosphorylating substrates involved in metabolism, osmosis, hormone response and stress signaling pathways. CPKs are a large multigene family of proteins that are present in all plants. Calcium (Ca²⁺) signaling is a highly integrated signaling network that plays a fundamental role in growth, development and stress responses in plants. Cytosolic Ca²⁺ concentrations change in complex spatio-temporal patterns in response to various stimuli. In plants, these altered Ca²⁺ signatures lead to specific cellular responses including stomatal movement, increased water retention, microbial detection and tip structure movement (De Falco *et al.* 2010) [1].

The sequence analysis of clone 3 which was 539 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLAST n analysis showed homology to *Piper nigrum* 5.8 ribosomal RNA genes, partial sequence: internal transcribed spacer 2 which gave 100% identity with 83% of query coverage. Annotation of sequence by the

BLAST x analysis showed homology to Hypothetical protein BVRB 017900 (*Beta vulgaris subsp. vulgaris*) 41% identity with 86% of query coverage.

The sequence analysis of clone 4 which was 649 bp was up regulated in water stressed plants. Annotation of sequence by the BLAST x analysis showed homology to hypothetical protein OsI 31800 (*Oryza sativa indica* Group) 80% identity with 61 % of query coverage. Annotation of sequence by the BLAST x analysis showed homology to hypothetical protein OsI 31800 (*Oryza sativa indica* Group) 80% identity with 61% query coverage.

The sequence analysis of clone 5 which was 706 bp was differentially expressed in water stressed plants. Annotation of sequence by the BLASTn analysis showed homology to *Vitis vinifera* copper amine oxidase1, transcript variant X2 which gave 81% identity with 36% of query coverage. Annotation of sequence by the BLASTx analysis showed homology to unknown (*Zea mays*) 88% identity with 48% of query coverage. Copper containing amine oxidases act as a disulphide-linked homodimer. In prokaryotes, the enzyme enables various amine substrates to be used as sources of carbon and nitrogen. In eukaryotes they have a broader range of functions, including cell differentiation and growth, wound healing, detoxification and cell signaling. The sequence from differentially expressed TDFs showed homology to copper containing amine oxidases which have a broader range of functions, including cell differentiation and growth, wound healing, detoxification and cell signaling. It contributes to terminal polyamines oxidation in peroxisomes. Polyamines are involved in different physiological processes such as growth development and response to abiotic stress. The other major genes identified were NADH dehydrogenase, heat shock proteins, ribosomal RNA gene, protein kinase which is all operating in major signal transduction pathways.

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