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Short Communication

Differential expression of metallothionein type-2 homologues in leaves and roots of Black pepper (*Piper nigrum* L)

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Abstract

Black pepper (*Piper nigrum* L.), member of the family *Piperaceae* is indigenous to India and is one of the most widely used spices in the world. In this paper we report the results of our attempts to identify a set of genes differentially expressed in the leaves of *Piper nigrum*, which could facilitate targeted engineering of this valuable crop. A PCR-based Suppression Subtractive Hybridization (SSH) technique was used to generate a leaf-specific subtracted cDNA library of *Piper nigrum*. A tester population of leaf cDNA was subtracted with a root derived driver cDNA. The efficiency of subtraction was confirmed by PCR analysis using the housekeeping gene actin. On sequence analysis, almost 30% of the clones showed homology to metallothionein type-2 gene. The predominance of metallothionein transcripts in the leaf was further confirmed using Real-Time PCR analyses and Northern blot. The possible role of metallothionein type-2 homologues in the leaf is discussed along with the feasibility of using SSH technique for identification of more number of tissue-specific genes from *Piper nigrum*.

Key words: Piper nigrum, suppression subtractive hybridization, metallothionein type-2, real-time PCR analysis, Northern blotting.

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Piper nigrum (Black pepper), the most acclaimed member of the family *Piperaceae* is a perennial climbing vine grown for its berries, which are highly priced as spice and medicine all over the world. India, especially the Southern states, accounts for a major portion of black pepper production. Recently, a very efficient micropropagation strategy through somatic embryogenesis was reported from seeds of *Piper nigrum* (Nair and Gupta, 2006), which could enable genetic manipulation of this crop for specific agronomic traits including abiotic and biotic stress tolerance. Often, improvement of transgenic traits in plants is achieved by using specific promoters to accomplish high tissue-specific protein production in transgenic plants. In *Piper nigrum*, promoters with leaf and root targeted expression would ensure that the gene product will not be present in the edible portions. Moreover, native promoters are known to mimic the expression of endogenous genes more effectively and do not appear to be greatly affected by gene silencing (Song *et al.*, 2000). Having a resource of candidate genes and promoters would enable effective development of more acceptable transgenic varieties. Previously, our group has used SSH technique, which is a modified PCR based cDNA subtraction, to successfully generate a defense gene enriched subtracted cDNA library in the resistant wild pepper (*Piper colubrinum* Link) (Dicto and Manjula, 2005). In the present study, Suppression Subtractive Hybridization strategy was used to identify a set of genes preferentially expressed in the leaf tissue of *Piper nigrum* and we report here the results of the forward subtraction using leaf as the tester and root as the driver population.

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Fresh vine cuttings of *Piper nigrum* var Panniyur-I were procured from the Kerala Agricultural University, Vellayani, Trivandrum, Kerala, India and transferred to pots containing soil and sand mixture in 3:1 proportion. They were maintained in the greenhouse of Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India. Leaves and roots were collected from 6-month-old plants. Two-three leaves from very young (1st leaves) to mature fully expanded (5th and 6th) stages were pooled for the extraction of RNA and further cDNA synthesis. Root samples were collected by carefully up-rooting the plants. The tissues were surface sterilized separately in running water using detergent followed by a couple of rinses in sterile Milli Q water. They were dried in filter paper and cut into 1 cm long pieces.

For SSH, 800 mg of leaf and 1.2 g of root samples were frozen in liquid nitrogen and ground to fine powder in a mortar and pestle. Total RNA extraction was carried out using TRIzol (Invitrogen) method. Poly (A) mRNA was

purified from the total RNA using the Poly AT-tract mRNA isolation kit (Promega Inc. Madison, USA) and Suppression Subtractive Hybridization was performed using PCR-Select cDNA Subtraction kit (Clontech, USA) as instructed by the manufacturer. Two populations of cDNA were synthesized, a tester cDNA corresponding to 2 µg of mRNA was prepared from leaves and a driver cDNA was prepared from 2 µg of mRNA isolated from roots. Both the cDNA populations were digested with *RsaI* and the tester cDNA was sub-divided into two populations, and each was ligated with a different cDNA adaptor provided in the kit. Two rounds of hybridizations were performed followed by two rounds of PCR amplifications. The subtracted PCR products were checked on a 1.2% agarose/EtBr gel. For analysis of subtraction efficiency, the house keeping gene, actin from *Piper nigrum* was amplified using gene-specific primers 5' ACATCCGCTGGAAGGTGC 3' and 5' TCTG TATGGTAACATTGTGCTC 3' as forward and reverse primers respectively. PCR was performed on subtracted and unsubtracted secondary PCR products using the actin primers. The samples were analyzed after 18, 23, 28 and 33 cycles of PCR. All the PCR products were then subcloned into pGEM®-T Easy Vector System (Promega Inc. Madison, USA) and transformed in JM109 cells to construct a subtracted cDNA library. DNA sequencing was carried out using the Big Dye Terminator kit (ABI Systems) using the sequencing primers T7 (5'CTAATACGACTCACTATA GGGC 3') and Sp6 (5' CATACGATTTAGGTGACACT ATA 3'). The sequence database similarity searches were done using BLAST programme of NCBI and the sequences were submitted in dbEST (GENBANK_ACCN EY458058-EY458062). Alignment of deduced amino acid sequences of *Piper nigrum* metallothionein type-2 to other type-2 metallothionein sequences was obtained using the program CLUSTALW. To determine the full length coding sequence of metallothionein homologue, 5'and 3' RACE was performed. The cDNA was synthesized following SMART RACE cDNA Amplification Kit protocol (Clontech, USA) and PCR amplified using the 5'and 3' RACE primers provided in the kit along with gene-specific primers (Forward 5' AAGCAGCACTTCACCGAGG 3' and Reverse 5' GGAGGAACATTTTTACACACG 3') for 3' and 5' RACE respectively. The RACE products were cloned into pGEM®-T Easy Vector and sequenced.

The differential transcript abundance of metallothionein type-2 in leaf and root tissue was further quantified by Real-Time quantitative RT-PCR, using SYBR® Green PCR Master Mix kit (Applied Biosystems, Europe). The gene-specific primers (Forward 5' AAGCAGCACTTCA CCGAGG 3' and Reverse 5' GGAGGAACATTTTTACA CACG 3') designed using the Primer Premier software were used to amplify the target gene in Real-Time PCR analysis. A black pepper actin gene was used as an endogenous control in the RT-PCR reactions. Each reaction was carried out using 2 µL of the cDNA preparation in a final reaction volume of 25 µL following the manufacturer's protocol. Three replicate reactions of each tissue were maintained for the Real-Time PCR experiments. The endogenous actin gene was also analysed in triplicate. PCR was performed with two initial steps of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 60 °C for 1 min with final extension at 72 °C for 5 min. To quantify abundance of metallothionein mRNA, the data is presented as a function of the abundance of the housekeeping gene β-actin. The threshold cycle (Ct) was calculated by the 7500 Real Time PCR System (Applied Biosystems, Europe) software to indicate significant fluorescence signals rising above background during the early cycles of the exponential amplification phase. The dissociation curve of the amplified product was examined for each reaction to rule out the possibility of primer-dimers contributing to the amplification signal.

For Northern blotting, 20 µg of total RNAs isolated from leaf, root and stem of *Piper nigrum* were separated on 1.5% (w/v) agarose-formaldehyde denaturing gel, run in 1X MOPS buffer and blotted onto a nylon membrane (Hybond-N, Amersham) with 3 M Nacl/0.01 N NaOH. After UV cross-linking, the membrane was hybridized with an α^{32}P labeled probe in Church-Gilbert buffer (0.5 M sodium phosphate buffer, 7% SDS, 0.01% BSA and 1 mM EDTA at 65 °C overnight). The probe was generated by restriction digestion of the 353 bp metallothionein homologue obtained from the subtracted library and labeled by α^{32}P dCTP incorporation following the probe labeling protocol (NewEngland Biolabs, USA). The membrane was washed with 1X SSC/0.1% SDS at room temperature and at 65 °C for 10 min each, followed by a final wash with 0.5X SSC/0.1% SDS at 65 °C for 10 min The image was then captured by exposing the membrane to Molecular Imager® FX (BioRad, USA).

The quality of RNA was checked by gel electrophoresis using 2% (w/v) agarose gel stained with ethidium bromide. Absorbance was determined at wavelengths 260 and 280 nm, which gave a ratio ranging from 1.8 to 2, indicating good quality of RNA. The subtracted products on gel analysis appeared as a smear ranging in size from 400 bp to 1.5 kb, with a definite band at 500 bp (Figure 1a, lane5). The experimental control subtraction product (Figure 1a, lane 3) yielded a pattern identical to ØX174/HaeIII digest (Figure 1a, lane 1), which was identical to the control subtraction provided in the kit (Figure 1a, lane 2), confirming that the experimental control subtraction has worked. The efficiency of experimental subtraction procedure using leaf as tester and root as driver was assessed by comparing the relative abundance of the constitutively expressed gene actin before and after subtraction (Figure 1b). PCR amplification of actin revealed that the band corresponding to actin appeared after 28cycles in the unsubtracted sample (Figure 1b, lane 8) while in the subtracted sample, the amplification product was visible after 33 cycles (Figure 1b, lane 5).

Seventy-five subtracted clones were obtained and all were sequenced. The clones showed varying degrees of homologies to Alanine aminotransferase (17%), Cinnamoyl CoA reductase (10%), Leaf peroxidase (4%), Germacrene D-synthase (11%), Photosystem II (6%), SOD chloroplast precursor (3%), and proteins of unknown function (19%). One of the subtracted clones (PNMS5), which yielded a 353 bp fragment, showed 30% redundancy. Sequence analysis of the fragment exhibited 93% identity to *Citrullus lanatus* CLMT2 mRNA for metallothionein type-2 at the nucleotide level. Translated query of the same demonstrated 85% identity to *Malus x domestica* metallothionein-like protein mRNA and 62% identity to *Salix matsudana* (metallothionein type-2) mRNA. RACE analysis of metallothionein type-2 revealed a full length coding sequence of 408 bp length (GENBANK_ACCN EY458058). The identity of the RACE product was confirmed by sequencing. The deduced amino acid sequence contains Cys- rich regions typical of plant type-2 metallothioneins, including the presence of a CXC motif at the carboxyl terminus. The alignment of deduced protein to other eight plant type -2 metallothioneins is reported in Figure 2.

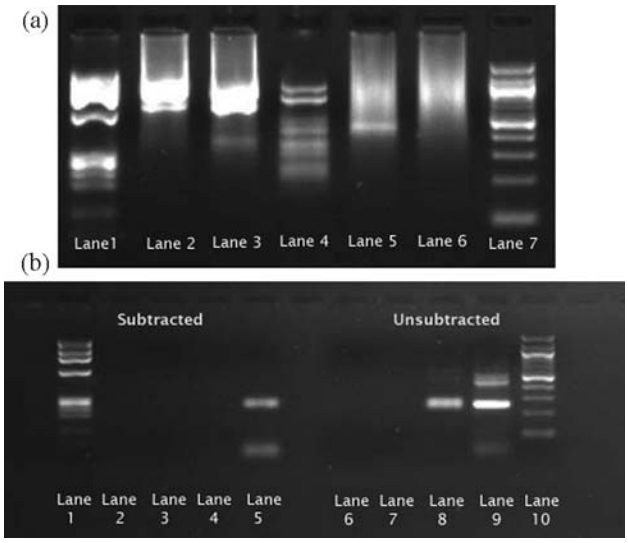

Figure 1 - (a) Secondary PCR products after subtraction. Lane 1 - ØX174/HaeIII digested marker; lane 2 - Control subtracted sample from kit; lane 3 - Experimental subtracted control cDNA; lane 4 - Experimental unsubtracted control; lane 5- Experimental subtracted sample; lane 6 - Experimental unsubtracted sample; lane 7-100 bp ladder; (b) PCR analysis of subtraction efficiency using actin primers. PCR was performed on subtracted and unsubtracted samples using equal quantity (50 ng) of cDNA from each sample. Lane 1 - ØX174/HaeIII digested marker; lanes 2 and 6-18 cycles; lane 3 and 7-23 cycles; lanes 4 and 8-28 cycles; lanes 5 and 9-33 cycles; lane 10-100 bp marker.

The relative abundance of metallothionein transcripts in leaves was further quantified by 7500 Real time PCR system analysis (Applied Biosystems, Europe) using the SDS 3.1 software (Table 1). The Ct values in Table-1 depict the average Ct of triplicates for each tissue. A comparison of the transcript level of metallothionein type-2 homologues in leaf, root and stem tissues was carried out using Northern blotting. The total RNA used for Northern analysis using the partial metallothionein fragment as the probe showed a high expression of metallothionein gene in leaves when compared to root and stem (Figure 3a) which did not show detectable level of transcript accumulation. Equal intensities of 18SrRNA from the tissue samples served as loading control (Figure 3b).

SSH technique has been efficiently used for identification of many differentially expressed plant genes (Chou *et al.*, 2006) and for generation of equalized and enriched tissue-specific cDNA library (Stahl *et al.*, 2004). One of the advantages of using SSH is the normalization step in-

Figure 2 - Multiple Sequence Alignment. Sequence alignment of metallothionein type-2 of *Piper nigrum* to type-2 metallothioneins from other plant sources. Sequences were aligned by the CLUSTALW program. Conserved CXC amino acids in the 3' domain are shown.

Table 1 - Real-Time RT-PCR analysis of metallothionein type-2 transcript. Relative quantification study was done to calculate the fold expression of metallothionein in root and leaf. Analysis was performed with cDNAs from root (avgCt = 23.592) and leaf (avgCt = 16.649). Metallothionein transcript levels are normalized to β-actin levels using the formula $dC_T = C_T$ (metallothionein) - C_T (actin) where avgCt of actin in root and leaf is (C_T = 21.570) and (C_T = 21.657) respectively.

Sample	Detector	Task	AvgCt[a]	AvgdCt[b]	dCt std error	ddCt[c]	RQ[d]
Root (calibrator)	ME	Target	23.592	2.023	0.075	0.000	1.000
Leaf	ME	Target	16.649	-5.009	0.050	-7.031	*130.816

[a]Avg Ct indicates the average of the C_T values (threshold cycles) obtained for three triplicates.
[b]AvgdCt is determined by subtracting the average actin C_T value from the average metallothionein C_T value.
[c]The calculation of ddCt involves subtracting dCt calibrator value (root) from the dCt target value (leaf).
[d]*Relative quantification is given as the fold expression change given by the equation 2^{-ddCt}.

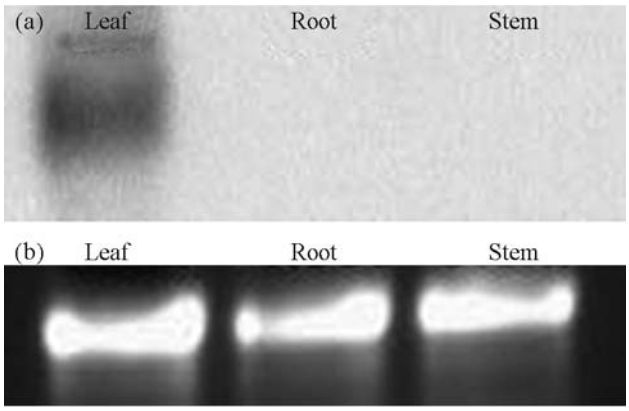

Figure 3 - (a) Northern blot analysis. Equal amount (20 µg) of RNA from leaf, root and stem tissue was loaded and the membrane was probed with α^{32}P labelled metallothionein type-2 homologue from *P. nigrum*. (b) 18SrRNA control. The equal intensity of 18SrRNA from all the three tissues serves as loading control.

volved, which is highly efficient in removing the transcripts common to driver and tester population, allowing the enrichment of differentially expressed cDNA in the subtracted population (Diatchenko *et al.*, 1996). In the present study, PCR amplification of constitutively expressed gene actin occurred after cycle 28 in the unsubtracted sample, whereas in the subtracted sample enriched for differentially expressed transcripts, an amplification product was visible only after 33 cycles. This result indicates the successful subtraction of actin and hence establishes the efficiency of the subtraction procedure. Among the subtracted clones, predominance of metallothionein type-2 homologues in the population could reflect it's overexpression in the tester (leaf) population, as those genes that are highly differentially expressed, will appear in the library a large number of times. Real Time PCR data established that the transcript level of metallothionein type-2 in leaf is 130 fold higher than in the root and the Northern blot analysis confirmed the metallothionein transcript upregulation in leaf compared to stem.

Typically, plant metallothioneins are Cys-rich proteins and further categories as types 1and 2 have been designated on the basis of predicted location of Cys residue (Scott and Albert, 2005). Metallothionein type-2 transcripts have been detected primarily in mature leaves. The high levels of expression of metallothionein type-2 in leaves, especially in trichomes is correlated to the secretory function of trichomes to exude excess heavy metals accumulating preferentially in leaves (Garcia-Hernandez *et al.*, 1998) and moreover, leaf trichomes produce secretions that are thought to provide a first line of defence against pests and pathogens (Wang *et al.*, 2002). A comparative study on accumulation of Cd, Fe, Cu, Mn and Zn in commonly used tropical spices indicated a highest concentration of these metals in the leaves and seeds of *Piper nigrum* (Ozkuthu *et al.*, 2006). Through our preliminary results, SSH has demonstrated its efficiency and we will now use it to further characterize a set of tissue specific genes and promoters for genetic improvement of black pepper.

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Internet Resources

BLAST /NCBI, www.ncbi.nlm.nih.gov/BLAST/
CLUSTALW, http://workbench.sdsc.edu.

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