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ORIGINAL ARTICLE

Identification of expression profiles of tapping panel dryness (TPD) associated genes from the latex of rubber tree (*Hevea brasiliensis* Muell. Arg.)

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Abstract Tapping panel dryness (TPD) occurrence in high latex yielding rubber tree (*Hevea brasiliensis*) is characterized by the partial or complete cessation of latex flow upon tapping leading to severe loss in natural rubber production around the world. The goal of this study was to identify genes whose mRNA transcript levels are differentially regulated in rubber tree during the onset of TPD. To isolate TPD responsive genes, two cDNA libraries (forward and reverse) from total RNA isolated from latex of healthy and TPD trees were constructed using suppression subtractive hybridization (SSH) method. In total, 1,079 EST clones were obtained from two cDNA libraries and screened by reverse Northern blot analysis. Screening results revealed that about 352 clones were differentially regulated and they were selected for sequencing. Based on the nucleotide sequence data, the putative functions of cDNA clones were predicted by BLASTX/BLASTN analysis. Among these, 64 were genes whose function had been previously identified while the remaining clones were genes with either unknown protein function or insignificant similarity to other protein/DNA/EST sequences in existing databases. RT-PCR analysis was carried out to validate the up-regulated genes from both the libraries. Among them, two genes were strongly down-regulated in TPD trees. The level of mRNA transcripts of these two genes was further examined by conventional Northern and RT-PCR analysis. Results indicated that the expression level of two genes was significantly lower in TPD trees compared to healthy trees. Many TPD associated genes were also up-regulated in TPD trees suggesting that they may be involved in triggering programmed cell death (PCD) during the onset of TPD syndrome. The results presented here demonstrate that SSH technique provides a powerful complementary approach for the identification of TPD related genes from rubber tree.

Keywords *Hevea brasiliensis* · High latex yielding clone · Subtracted cDNA library · Myb transcription factor · Translationally controlled tumor protein gene · Tapping

Abbreviations

TPD	Tapping panel dryness
SSH	Suppression subtractive hybridization
PCD	Programmed cell death
EST	Expressed sequenced tags
RT-PCR	Reverse transcription polymerase chain reaction
TCTP	Translationally controlled tumor protein
ROS	Reactive oxygen species

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Introduction

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important perennial crop that produces natural rubber which is a cis 1,4-polyisoprene. Although over 2,000 species of higher plants are recognized for producing latex with polyisoprenes, only the rubber tree (*H. brasiliensis*) has been established as a key industrial rubber source due to its high yield

and excellent commercial properties of the natural rubber products. Rubber molecules are produced, aggregated and packaged in latex vessels (laticiferous cells) of the rubber tree. When the bark of the rubber tree is systematically wounded the cytoplasmic contents of these laticifers are expelled in the form of latex. Latex, the milky substance, upon coagulation and processing yields natural rubber (Adiwilaga and Kush 1996). Nearly, 9.5 million hectares of cultivated rubber trees produce more than 8.7 million tons of dry rubber each year world-wide (Anonymous 2006). However, latex producers still face serious economic losses due to tapping panel dryness (TPD) syndrome. About 12–50% of productive trees are being affected by TPD in almost every rubber-growing region. It was estimated that TPD contributes to 12–14% loss of the annual rubber production (Chen et al. 2003).

The tapping panel dryness syndrome of rubber tree is characterized by the partial or ultimately complete stoppage of latex flow upon tapping. Biochemical studies indicate that TPD syndrome is a physiological disorder resulting from wounding and over exploitation (over tapping and over stimulation by ethephon treatment) (Faridah et al. 1996). It was previously shown that a non-compensated oxidative stress may be involved in the onset of TPD (Faridah et al. 1996). The TPD syndrome is associated with exhaustion of major nutrients and reduction in protein, nucleic acids, thiols and ascorbic acid levels (Fan and Yang 1994). Membrane destabilization leading to bursting of the lutoids and consecutive in situ latex coagulation due to over-stimulation has been proposed to be associated with the occurrence of an uncompensated oxidative stress within the latex cells (Chrestin 1989). Even though the surrounding tissues of tapping panel are not affected much (no necrotic symptoms) they show some changes in their metabolite profile (Yusof et al. 1995; Krishnakumar et al. 1999), accumulate proline (Wickremasinghe et al. 1987), decrease cytokinins (Krishnakumar et al. 1997), increase inorganic phosphorous and bark respiration (Krishnakumar et al. 2001a, b). There were several attempts to identify protein markers for TPD wherein, the protein pattern of healthy and TPD tree latex cells was compared by SDS-PAGE (Sookmark et al. 2002; Darussamin et al. 1995; Dian et al. 1995; Lacrotte et al. 1995). This led to identification of several putative protein markers that are associated with TPD, but the nature and exact function of these proteins remain unknown.

Molecular mechanism underlying TPD is not known and currently there is no effective prevention or treatment for this syndrome. It is reported that different *Hevea* clones showed different levels of TPD severity in the field tests (Yan and Fan 1995). This indicates the tolerance of rubber tree to TPD is apparently genetically determined but genes associated with TPD have yet to be identified (Chen et al. 2003). mRNA differential display has been attempted to identify genes associated with TPD in which the decreased expression of a Myb transcription factor is likely to be associated with TPD (Chen et al. 2003). At present, it is not clear whether the latex tapping stress induces expression of genes or the activation of pre-existing proteins or both are responsible for the development of TPD syndrome. The physiological significance of the association of TPD related genes with the onset of TPD is not studied at the molecular level. Many physiological changes associated with TPD are controlled at the transcriptional level. As yet, no comparative analysis of gene expression between healthy and TPD trees of *Hevea* have been performed. Therefore, identification of genes associated with TPD syndrome is one of the prerequisites to understand the molecular mechanisms of this complicated physiological disorder. In order to dissect the molecular changes occur during TPD development in a comprehensive manner at the genomic level, and to identify novel genes involved in this process, it is necessary to survey global gene expression pattern following the onset of TPD syndrome in *Hevea*. To obtain further information about the differences between the mRNA expression profiles of the healthy and TPD trees, we used the PCR based suppression subtractive hybridization (SSH) method. The SSH is a powerful technique that enables one to specifically clone ESTs representing genes that are differentially expressed in different mRNA populations. The SSH method which combines normalization and subtraction in a single step, is based on suppression PCR. The normalization step equalizes the abundance of cDNAs with the tester population and the subtraction step excludes the common molecules between the tester and driver populations and achieves a greater than 1,000-fold enrichment of differentially expressed cDNAs (Diantchenko et al. 1999). The technique has already been applied in many studies to isolate genes involved in responses to biotic and abiotic stresses (Mahalingam et al. 2003; Degenhardt et al. 2005; Zhang et al. 2005).

In order to study the genes associated with TPD, we selected latex samples which represents cytoplasmic content of laticiferous cells for RNA isolation. In *Hevea* tree, the latex biosynthesis is greatly affected during the onset of TPD syndrome, therefore, it is more appropriate to study the gene expression in latex. Here, we report the global changes in gene expression in latex during the development of TPD syndrome. Two cDNA libraries were prepared, one containing cDNA fragments corresponding to mRNAs whose levels increased in healthy tree (forward library) and the other containing cDNA fragments corresponding to mRNAs whose abundance was enhanced in the TPD tree (reverse library). This study also resulted in identification of candidate genes that could be used as markers in monitoring TPD development.

Materials and methods

Plant material

The elite clones (RRII 105) of high latex yielding rubber trees of 18-year age from experimental field at Rubber Research Institute of India (RRII) were selected for the present experiment. These trees are being regularly tapped [tapping is a controlled shaving of thin bark section (about 2 mm) with a special kind of tapping knife on alternate days (d/2 tapping system)] for latex collection for the past 13 years. Some of these trees developed TPD syndrome and they continued to be tapped along with healthy trees to maintain uniform bark wounding stress (conditions) before sample collection for SSH experiments. The fresh latex samples were collected from five healthy and TPD affected trees and pooled before total RNA extraction.

RNA isolation

Total RNA was extracted from latex samples of healthy and TPD affected trees according to the method of Venkatachalam et al. (1999). Poly(A)$^+$ mRNA was purified from total RNA using mRNA isolation system (Promega, Madison, WI, USA). The mRNA quantity and quality were determined by spectrophotometrically at 260 and 280 nm.

Construction of suppression subtractive hybridization cDNA libraries

Suppression subtractive hybridization (SSH; Diantchenko et al. 1999) was performed to create forward and reverse subtracted cDNA libraries using the PCR-Select cDNA subtraction kit (Clontech, CA, USA). For forward subtraction, mRNA from healthy trees was used as a "tester" and TPD tree mRNA was used as a "driver". A reverse subtracted cDNA pool was made with healthy tree mRNA as "driver" and TPD tree mRNA as "tester". The subtracted and PCR amplified products were cloned into the TOPO-pCR 2 plasmid vector by using the TA cloning kit (Invitrogen, CA, USA). The ligated products were then transferred into chemically competent *E. coli* (TOPO 10F) cells to generate SSH libraries. The library was plated onto LB agar plates containing 100 μg/ml kanamycin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indolyl b d-galactopyranoside (X-Gal) for blue-white screening. White colonies were picked and cultured at 37°C in LB broth containing kanamycin. The glycerol stocks of bacterial cultures were frozen in liquid nitrogen and stored at −80°C.

Amplification of cDNA inserts

For differential screening of transcripts each recombinant clone was picked and grown on LB agar plate containing 100 μg/ml kanamycin. An aliquot of the colony was used to amplify inserts by PCR using M13 forward and reverse sequencing primers. PCR reactions (20 μl) contained 13 μl of DD H_2O, 1 μl of each M13 forward and reverse primers (250 μM), 2 μl of 10× buffer, 2 μl of dNTPs (2.5 mM), 1 μl of Taq DNA polymerase and bacterial colony as DNA template. A MJ research DNA engine was programmed as follows: 94°C for 4 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1.30 min and extension at 72°C for 2 min; final extension at 72°C for 7 min was used. The samples were then electrophoresed on a 1.2% (w/v) agarose gel and the clones yielding a single PCR product were selected for further investigation.

Differential screening of subtracted cDNA libraries by reverse Northern analysis

To identify differentially expressed genes, reverse Northern blot analysis was performed with radio labeled (^{32}P) cDNA probes. The tester and driver cDNAs from subtracted and unsubtracted library were used in separate labeling reactions. Aliquots of cDNA (2 μl) were added to a mixture containing 2 μl of the polymerase provided with the Smart Kit (Clontech Inc., CA, USA), 2 μl of 5′ PCR primer IIA (10 μM), 2 μl of dNTPs (10 μM dATP, dTTP, dGTP and 0.05 μM dCTP) and 5 μl of [α-^{32}P]dCTP (specific activity >3,000 Ci/mol, 10 μCi/μl). The labeled cDNA probe was obtained and purified on Sephadex G50 columns. cDNA fragments of all colonies were amplified with M13 primers from forward and reverse subtraction libraries. They were denatured with 0.6 N NaOH (1:1 ratio) and 2 μl of each fragment was spotted onto nylon membranes. Two identical membranes with cDNA inserts (≈10 ng) spotted in duplicate were prepared. Blots were hybridized and washed according to standard procedures (Sambrook et al. 1989). Afterwards, the membranes were exposed on X-ray film (X-Omat, Kodak) at −80°C for 24 h. The fragments that hybridized only with the labeled tester cDNA or showed at least threefold higher signals on these membranes compared to the signals on the membrane hybridized with the labeled driver cDNA were selected for sequencing.

DNA sequencing, sequence analysis and accession numbers

Plasmids containing cDNA fragments that were differentially expressed were sequenced using universal M13 forward and reverse primers homologous to vector sequence. The sequencing was performed at Purdue University

Genome Core Facility. EST identities and potential functions were determined by sequence comparison to the non-redundant GenBank database by BLASTN (http://www.ncbi.nlm.hih.gov) with default parameters. Further searches of the protein database were also conducted by BLASTX and sequence homology information was used to assign putative identities. The similarity scores between the cDNA clones and known sequences were represented by the BLAST probability *E* values. The cDNAs were classified according to homologous sequences in the database and cDNAs with BLAST scores <45 bits (no homologous stretch >50 bp) were designated as having "no similarity". The sequences of the *Hevea* cDNA clones identified in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank) and their accession numbers are listed in Tables 1 and 2.

Semi-quantitative determination of transcripts levels by RT-PCR

Total RNA was isolated as described previously by Venkatachalam et al. (1999), treated with DNaseI and subsequently used for RT-PCR. Two microgram total RNA of each sample was used for first-strand cDNA synthesis in 20 µl reaction containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 5 mM dNTPs, 200 U MuMLV reverse transcriptase (Promega Inc., Madison, WI, USA) and 50 pmol oligonucleotides T15. Reverse transcription was performed at 42°C for 60 min with a final denaturation at 70°C for 15 min. The cDNA (1 µl) was used directly for PCR with 1 U of Taq polymerase (Promega Inc., Madison, WI, USA) in the presence of dNTPs at 250 µM and the appropriate primers. Initially primers corresponding to seven and six target genes from forward and reverse libraries, respectively, along with one control (18S ribosomal RNA) were synthesized based on EST sequences. The list of primers is provided in Table 3. Further gene specific RT-PCR primers for two differentially expressed genes which were cloned from cDNA library of *Hevea* clone RRII 105 (Accession Nos. MYB-DQ323739 and TCTP-DQ323740) through PCR amplification were designed. The following primer pairs were used to amplify full-length cDNAs; Myb transcription factor MybF (5′-ACCATGGATCGGGGAATTGA-3′) and MybR (5′-CCT AAATCCCCCAAACTTA-3′) and for translationally controlled tumor protein gene TCTPF (5′-GCACGAGCGTAC TTGTTGCTGCT-3′) and TCTPR (5′-CAAAATAGAAG GCACGCGGCACC-3′). RT-PCR was done using total RNA samples isolated from pooled latex of three trees with the following thermal cycling parameters: 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 50–63°C for 1.30 min and 72°C for 2 min. The final extension was performed at 72°C for 7 min. A 530 bp 18S rRNA gene fragment was amplified as positive control using the primer pair 5′-GGTCGCAAGGCTGAAACT-3′ and 5′-ACGGG CGGTGTGTACAAA-3′. The RT-PCR products of full-length cDNA for Myb and TCTP genes were sequenced to verify the specificity of PCR amplification. For quantification, amplification products were immediately separated by electrophoresis in an 1.5% (w/v) agarose gel and the integrated intensity of the PCR bands was corrected with the corresponding housekeeping gene data and the normalized data were used for quantification analysis with the Fuji film Image Guage 3.12 version software package (Fujifilm Inc., Japan).

Northern blot analysis

The probe DNA fragment was amplified by PCR amplification from a plasmid containing the Myb transcription factor and TCTP genes with gene specific primers (full-length cDNA) as given above. A cDNA library prepared from mRNA isolated from elite rubber clone (RRII105) was used as template. Amplification products were analyzed on 1.5% (w/v) agarose gel and then purified for labeling. The purified DNA fragments (25 ng) were labeled with [α-^{32}P] dCTP by utilizing the random labeling kit (Ambion, USA), according to the manufacturer's protocol, followed by purification with Sephadex G-50 spin columns. Aliquots (10 µg) of total RNAs from samples representing healthy and TPD trees were denatured at 65°C for 15 min in a buffer containing formamide, formaldehyde, 10× MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and ethidium bromide (10 mg/ml). RNA samples were fractionated in a formaldehyde–agarose gel (1.2% w/v) at 65 V for 3 h in 1× MOPS buffer, then transferred to nylon membranes by capillary blotting and fixed by UV irradiation. Membranes were pre-hybridized at 42°C for 2 h in pre-hybridization buffer (50% Formamide, 6× SSPE, 5× Denhardt's, 0.5% SDS, 0.2 mg/ml salmon sperm DNA) and hybridized in the same buffer containing the labeled probe at 42°C overnight. The membranes were washed and exposed to X-ray film (X-Omat, Kodak) for signal detection at −80°C for 24–48 h. Total RNA was isolated from the latex collected from three trees and used for Northern analysis. This experiment was repeated thrice. The intensity of each band was normalized with the corresponding rRNA band and quantified as mentioned above.

Results

We have undertaken a gene discovery project in rubber tree aimed at identifying ESTs representing genes expressed during the onset of TPD syndrome after latex cells tapping. The TPD appears to be a result of physiological disorders in

Table 1 Identification of non-redundant clones (unigenes) down-regulated in TPD trees from the forward SSH library

SSH clone ID	cDNA insert size (bp)	GenBank accession no. [a]	BLAST sequence homology [b]	*E*-value
Stress/defense response				
SSH-1	667	DQ306730	REF-like stress related protein 1 (RLP) mRNA	0
SSH-2	520	DQ306731	Translationally controlled tumour protein (TCTP) mRNA	0
SSH-3	706	DQ306732	Latex Pl 3.5 protein Hev b5 mRNA	0
SSH-4	346	DQ306737	Putative low temperature and salt responsive protein gene	$1e^{-21}$
SSH-5	668	DQ306738	Heavy metal associated domain containing protein copper chaperone (CCH) related gene	$2e^{-49}$
SSH-6	327	DQ306739	Thioredoxin H-type (TRX-H) mRNA	$3e^{-29}$
Cellular metabolism/pathway regulation				
SSH-7	338	DQ306741	Glyoxylate pathway regulator gene mRNA	0
SSH-8	382	DQ306742	Putative fatty acid hydroxylase gene	$2e^{-13}$
SSH-9	534	DQ306783	*C. sinensis* Valencene synthase mRNA	$9e^{-17}$
Cell growth, division and development				
SSH-10	260	DQ306743	Glycine-rich RNA binding protein gene mRNA	$2e^{-68}$
DNA/RNA binding				
SSH-11	253	DQ306744	Histone H2A like protein mRNA cds	$1e^{-30}$
SSH-12	612	DQ306745	Myb1 transcription factor (Myb) mRNA	$5e^{-06}$
SSH-13	477	DQ306748	Putative small nuclear ribonucleoprotein F (snRNP-F) gene	$8e^{-38}$
Protein synthesis, process and transport				
SSH-14	526	DQ306749	26S proteasome subunit 7 mRNA	$1e^{-121}$
SSH-15	387	DQ306750	Translation initiation factor 5 mRNA	$8e^{-14}$
SSH-16	503	DQ306751	60S ribosomal protein L12 gene	$5e^{-72}$
SSH-17	308	DQ306752	Putative 60S acidic ribosomal protein gene	$5e^{-46}$
SSH-18	296	DQ306753	Vacuolar sorting receptor protein gene	$6e^{-53}$
Structural				
SSH-19	670	DQ306754	Actin depolymerizing factor mRNA	$1e^{-132}$
Amino acid biosynthesis				
SSH-20	653	DQ306755	Alanine-glyoxylate aminotransferase/AGT gene	$5e^{-32}$
SSH-21	763	DQ306756	b-Keto acyl reductase gene	$7e^{-55}$
Protein degradation				
SSH-22	413	DQ306757	Ubiquitin-conjugating enzyme E2 (UBIE2) mRNA	$2e^{-40}$
Cellular communication and signaling				
SSH-23	456	DQ306758	Calcium-binding protein (PCA18) mRNA	$1e^{-08}$
Unknown function				
SSH-24	376	DQ306763	Acylphosphatase family	$5e^{-25}$
SSH-25	635	DQ306764	Hypothetical protein	$1e^{-52}$
SSH-26	700	DQ306765	*A. thaliana* clone 17598 mRNA complete sequence	$2e^{-12}$
SSH-27	346	DQ306766	*O. sativa* cDNA clone J023071K01 full insert sequence	$6e^{-11}$
SSH-28	382	DQ306767	*A. thaliana* cDNA sequence from clone GSLTFB89ZE07 of flower and buds of strain col-0	$2e^{-22}$
SSH-29	262	DQ306768	*A. thaliana* complete cDNA sequence from clone GSLTSIL77ZB04 of silique of strain col-0	$1e^{-11}$
SSH-30	190	DQ306769	*M. truncatula* clone mth2-1817 complete sequence	$9e^{-27}$
SSH-31	421	DQ306770	*Z. mays* PCO 139688 mRNA sequence	$6e^{-13}$
SSH-32	477	DQ306771	*Z. mays* PCO 146768 mRNA sequence	$1e^{-60}$
SSH-33	454	DQ306772	*A. thaliana* unknown protein (At5g06560) mRNA complete sequence	$1e^{-31}$

Table 1 continued

SSH clone ID	cDNA insert size (bp)	GenBank accession no. [a]	BLAST sequence homology[b]	*E*-value
SSH-34	668	DQ306773	*L. corniculatus* Genomic DNA TM0366 complete sequence	$7e^{-49}$
SSH-35	308	DQ306774	*A. thaliana* Genomic complete sequence BAC F3L24	$6e^{-66}$
SSH-36	341	DQ306775	*N. benthamiana* 9-079 unknown mRNA	$6e^{-07}$
SSH-37	635	DQ306776	*A. thaliana* Genomic complete sequence BAC F9P8	$3e^{-07}$
SSH-38	327	DQ306777	*A. thaliana* mRNA clone 22166 complete sequence	$1e^{-49}$
SSH-39	501	DQ306778	*A. thaliana* mRNA clone 94584 complete sequence	$2e^{-12}$
SSH-40	653	DQ306779	*A. thaliana* complete cDNA sequence from clone GSLTSIL32ZC12 of silique of strain col-0	$2e^{-08}$
SSH-41	537	DQ306780	*A. thaliana* Genomic complete sequence BAC T16O11	$3e^{-10}$
SSH-42	296	DQ306781	*Cucurbita* mRNA for PV72 complete cds	$3e^{-34}$
SSH-43	853	DQ306782	*A. thaliana* Kelch repeat-containing F box family protein	$2e^{-34}$

[a] The GenBank accession numbers of the nucleotide sequences are given

[b] BLASTX was used to determine homologous genes and putative functions of genes. BLASTN was used in case of failure to return any hits by BLASTX. Clones that did not share significant homology with known sequences are not shown and correspond to: SSH-44 (218 bp; DQ306784), SSH-45 (617 bp; DQ306785), SSH-46 (670 bp; DQ306786) and SSH-47 (121 bp; DQ306787)

the bark tissue of high latex yielding *Hevea* clones (Fig. 1a). It has been proved that high yielding rubber clones are more prone to TPD syndrome (Anonymous 2002). In order to maximize our chances of identifying TPD associated genes in *Hevea*, we generated subtracted and normalized cDNA libraries from mRNA isolated from latex cells cytoplasm of healthy and TPD trees. One of the objectives of this study was to isolate genes up-regulated during the onset of TPD in high latex yielding *Hevea* RRII 105 clone. This led to the identification of cDNAs representing low abundance genes or those expressed in response to latex cells tapping (Fig. 1b). It is quite clear that bark cells from tapping panel of TPD tree are being dried (cell death) and rubber biosynthesis (latex production) was ultimately inhibited partially (Fig. 1c) or ceased completely after tapping (Fig. 1d) while no such symptom was noticed in healthy tree in the subsequent tapping (Fig. 1b). To capture a wide spectrum of differentially expressed genes, latex samples were collected and pooled from different trees for mRNA isolation and cDNA library construction. A total of 1,079 vector-containing bacterial colonies were obtained from two libraries and assayed for the presence of cDNA insert by PCR. Analysis of the PCR products showed that the cDNA inserts ranged from 200 bp to 1.2 kb (data not shown). The resulting subtractive clones were then used to identify differentially expressed genes by reverse Northern blot analysis.

Identification and differential expression analysis of cDNAs by SSH

The 'forward' subtraction set lead to identification of the clones down-regulated in TPD trees compared to healthy trees after tapping, while the 'reverse' set enabled identification of clones up-regulated in TPD trees compared to healthy trees. To increase the specificity of healthy tree- and TPD tree-specific cDNAs prior to sequencing, differential screening steps (Diantchenko et al. 1999) were performed. PCR amplified products of 1,079 cDNA clones from the subtractive library were spotted onto nylon membranes. The differentially expressed genes were first identified by successive screening with unsubtracted tester and driver cDNAs as probes separately. The forward SSH library blots showed strong hybridization signals for many clones with the probe from healthy tree (Fig. 2a), whereas most of them did not hybridize to the cDNA probe from TPD tree and those clones were considered as differentially regulated cDNAs specific to healthy trees (Fig. 2b). On SSH blot from reverse subtraction library, the cDNA probe from TPD tree was strongly hybridized to many clones that were considered as specific cDNAs up-regulated by TPD syndrome (Fig. 2c), whereas the healthy tree probe did not hybridize with a large number of clones or hybridized weakly with few (Fig. 2d). Some clones hybridized to both cDNA probes (healthy and TPD trees) indicating that they were not differentially expressed between healthy and TPD trees. Similarly, secondary screening was also performed with subtracted probes (data not shown). This screening step made it possible to rapidly eliminate a number of false positive clones. On the forward SSH library, 310 clones were identified following differential screening and 112 clones among them displaying differentially expressed signals specific to healthy tree were selected for sequencing. In the case of reverse SSH library, a total of 769 clones were obtained and about 240 clones showing differential expression specific to TPD tree were sequenced. Sequence analysis revealed that 134 clones represent non-redundant cDNA inserts. Further bioinformatics analysis lead to the identifi-

Table 2 Identification of non-redundant clones (unigenes) up-regulated in TPD trees from the reverse SSH library

SSH clone ID	cDNA insert size (bp)	GenBank accession no.[a]	BLAST sequence homology[b]	*E*-value
Stress/defense response				
SSH-1	106	DQ306788	Chitinase gene mRNA cds	$2e^{-52}$
SSH-2	853	DQ306789	Pseudohevein gene mRNA cds	0
SSH-3	606	DQ306790	Latex allergen mRNA (1,444 bp)	0
SSH-4	293	DQ306791	Putative senescence-associated protein	$1e^{-40}$
SSH-5	297	DQ306792	Universal stress protein (USP) family protein	$7e^{-08}$
SSH-6	536	DQ306793	Phosphatidic acid phosphatase—related PAP2 gene	$2e^{-25}$
SSH-7	462	DQ306794	Pathogenesis-related protein Osmotin precursor (NP24) gene	0
SSH-8	563	DQ306795	Ethylene biosynthesis gene mRNA cds	0
SSH-9	323	DQ306796	Heat-shock protein (HSP) 19 class I mRNA	$5e^{-14}$
SSH-10	390	DQ306797	Desiccation protectant protein (LEA1) mRNA	$7e^{-20}$
SSH-11	676	DQ306798	Violaxanthin de-epoxidase precursor mRNA (TVDE1)	$1e^{-136}$
SSH-12	129	DQ306799	Hev b3 (922) protein mRNA	$1e^{-64}$
SSH-13	711	DQ306800	Pro-hevein precursor (Major hevein contains Hevein allergen Hev b6) win-like protein	$1e^{-72}$
SSH-14	323	DQ306801	17.3 KDa class heat shock protein (HSP17.3)	$2e^{-12}$
SSH-15	387	DQ306802	Late embryogenesis abundant (LEA3) family protein	0
SSH-16	603	DQ306841	*L. esculentum* beta-carotene hydroxylase (CrtR-2 gene) mRNA	0
Cellular metabolism/pathway regulation				
SSH-17	271	DQ306803	Glyceraldehyde-3-phosphate dehydrogenase (GapC1) mRNA	$2e^{-62}$
SSH-18	516	DQ306804	Acid invertase gene mRNA	$1e^{-157}$
SSH-19	403	DQ306805	CTP synthase/UTP ammonia ligase mRNA	$9e^{-25}$
Cell growth, division and development				
SSH-20	456	DQ306806	Cell wall protein mRNA	$1e^{-122}$
SSH-21	269	DQ306807	Cyclophilin gene mRNA	$6e^{-32}$
SSH-22	400	DQ306808	Membrane related protein CP5 mRNA	$1e^{-60}$
SSH-23	422	DQ306842	*A. thaliana* inosine-uridine nucleoside hydrolase protein	$1e^{-15}$
DNA/RNA binding				
SSH-24	346	DQ306809	Zinc Finger family protein	$5e^{-09}$
Protein synthesis, process and transport				
SSH-25	451	DQ306810	Cysteine protease like mRNA	$1e^{-06}$
SSH-26	488	DQ306811	Polyubiquitin gene	$1e^{-23}$
SSH-27	359	DQ306812	Translation elongation factor eEF−1beta chain	$5e^{-27}$
SSH-28	512	DQ306813	DnaJ protein mRNA complete cds	0
SSH-29	449	DQ306814	80S ribosomal protein L31 mRNA complete cds	$5e^{-37}$
SSH-30	267	DQ306815	60S ribosomal protein L37 a (RL37a) mRNA complete cds	$2e^{-16}$
SSH-31	287	DQ306816	28S ribosomal RNA gene	$1e^{-100}$
Structural genes				
SSH-32	568	DQ306817	Actin (ACT1) mRNA complete cds	0
Cellular communication and signaling				
SSH-33	512	DQ306818	ASR-like protein 2 (ASRLP2) mRNA	$2e^{-13}$
Rubber biosynthesis				
SSH-34	213	DQ306819	HMG-CoA synthase 2 mRNA	$1e^{-106}$
Unknown function				
SSH-35	590	DQ306820	*A. thaliana* expressed protein gene	$8e^{-59}$

Table 2 continued

SSH clone ID	cDNA insert size (bp)	GenBank accession no.[a]	BLAST sequence homology[b]	*E*-value
SSH-36	455	DQ306821	*A. thaliana* hypothetical protein gene	$3e^{-21}$
SSH-37	390	DQ306822	*A. thaliana* nodulin-related protein gene	$3e^{-06}$
SSH-38	296	DQ306823	*Mus musculus* forming binding protein 2 mRNA	0
SSH-39	293	DQ306824	*A. thaliana* At3g41970 mRNA sequence	$1e^{-155}$
SSH-40	198	DQ306825	*H. sapiens* DC 48 mRNA complete cds	$2e^{-05}$
SSH-41	297	DQ306826	*Z. mays* PCO144363 mRNA sequence	$1e^{-08}$
SSH-42	536	DQ306827	*A. thaliana* At5g03080 gene complete cds	$6e^{-12}$
SSH-43	455	DQ306828	*O. sativa* genomic DNA PACP0030G02 complete sequence	$9e^{-07}$
SSH-44	748	DQ306829	*A. thaliana* full-length cDNA complete sequence from GSLTPGH1ZE12of hormone treated callus of strain Col-0	$1e^{-73}$
SSH-45	646	DQ306830	*A. thaliana* unknown protein (At4g37680) mRNA complete cds	$1e^{-58}$
SSH-46	329	DQ306831	*C. vulgaris* C-27 chloroplast DNA complete cds	$2e^{-04}$
SSH-47	331	DQ306832	*O. sativa* clone 001-124-F02 full insert sequence	$9e^{-13}$
SSH-48	590	DQ306833	*A. thaliana* complete sequence GSL TSIL72ZF07 of silique of strain col-0	$2e^{-15}$
SSH-49	341	DQ306834	*A. thaliana* AtPH1 mRNA complete cds	$2e^{-16}$
SSH-50	351	DQ306835	*A. thaliana* complete cDNA clone GSLTSIL95ZE04 of silique of strain col-0	$3e^{-09}$
SSH-51	330	DQ306836	*O. sativa* cDNA clone J013059F24 full insert sequence	$3e^{-12}$
SSH-52	283	DQ306837	*Z. mays* CL2022-1 mRNA sequence	$3e^{-09}$
SSH-53	559	DQ306838	*H. sapens* Genomic DNA clone CMB9-88B16 complete sequence	0
SSH-54	443	DQ306839	*M. truncatula* clone mth2-53p19 complete sequence	$7e^{-17}$
SSH-55	364	DQ306840	*O. sativa* cDNA clone J033107E20 full insert sequence	$6e^{-14}$
SSH-56	516	DQ306843	*L. esculentum* minor allergen beta-fructofuranosidase precursor	$5e^{-42}$
SSH-57	493	DQ306844	*G. max* HMG1/2-like protein (SB11 protein)	$7e^{-07}$
SSH-58	330	DQ306845	*A. thaliana* ring-box protein gene	$3e^{-04}$
SSH-59	751	DQ306846	Annexin-like protein RJ4	$1e^{-22}$
SSH-60	658	DQ306847	*A. thaliana* NC domain containing protein	$2e^{-12}$
SSH-61	479	DQ306848	*A. thaliana* putative protein	$7e^{-37}$
SSH-62	180	DQ306849	*Thermococcus sp.* AL662 bglT gene	$1e^{-13}$

[a] The GenBank accession numbers of the nucleotide sequences are given

[b] BLASTX was used to determine homologous genes and putative functions of genes. BLASTN was used in case of failure to return any hits by BLASTX. Clones that did not share significant homology with known sequences are not shown and correspond to: SSH63-87 (DQ306850-306874)

cation of 47 clones from forward SSH library (Table 1) and 87 clones from reverse SSH library (Table 2) as consistently up-regulated genes.

Functional classification of differentially expressed genes

Differentially expressed genes were classified into 10 functional categories according to the putative function of their homologous genes in the databases generated by BLAST analysis. Data presented in Tables 1 and 2 show the functional classification of the differentially expressed genes identified from forward and reverse SSH libraries. Based on BLAST analysis results, putative functions were assigned to 134 clones in which (1) 57 genes were with known functions, (2) 48 ESTs had matches with genes in GenBank database of unknown functions, and (3) 29 had no matches (uncharacterized). The sequences generated in this study have been deposited in the GenBank database and can be viewed on the NCBI website (http://www.ncbi.nlm.nih.gov) under the accession numbers given in Tables 1 and 2. Sequences that yielded no significant homology are not included. There were differences in the distribution of expressed genes among functional classes between the healthy and TPD trees (Fig. 3a, b).

Stress/defense-related genes

A total of six up-regulated clones identified in healthy tree, had homology to known stress/defense related genes (Table 1). This included a member with homology to REF-

Table 3 List of primer sequences used for semi-quantitative RT-PCR analysis

Name of the genes	Primer sequences	
	Forward (5′–3′)	Reverse (5′–3′)
MYB	GGAAAGGCGGGTCCTT	GCCCTTAGCGTGGTCC
TCTP	ACAAACTCGTTGGGTC	GCTTCTTCTCTCCTTT'
CBP	CGGCCGAGGTGCCCTTAG	GGAAATTCACACCCCCAT
TRX-H	ACTTTTCCAAAAACAG	TGCCGTTTCATTAGTC
RLP1	CTCAATCGCCGCCGAC	ACATCAAAGAAAGGAT
F-box	GGCTGCCAACTTAGGGTT	GACCGACCGGACTATCCC
REF	AACTTTTTACCGGCCT	AATACTGTTGAGAGTA
CP	CGTCATCATGCAGAACTC	CCCCTATCTTGGATCGGA
PRO	GTACACATGAGATGTAAC	TCGGAGGATGTAACAACC
EB	CGCGGCCGAGGTACTTAT	CTCGACGAGCAATGGATC
ALP	AGCTCTCAAGACCAACAC	CAACCCAGACTGGGAGAT
PAP	ATACGGTGCCGCAGGTGT	TGTAGAGATCCTGCGAGT
ASR-2	TGCACAACAGAACCACCA	CCACACCACCACAAGGAA

MYB Myb transcription factor gene, *TCTP* Translationally controlled tumour protein gene, *CBP* Calcium-binding protein, *TRX-H* Thioredoxin H-type gene, *RLP1* REF-like stress related protein 1, *F-box* F box family protein, *REF* Rubber elongation factor protein gene, *CP* Cysteine protease like mRNA, *PRO* PR-Osmotin precursor gene, *EB* Ethylene biosynthesis gene, *ALP* Annexin-like protein RJ4, *PAP* Phosphatidic acid phosphatase-related gene, and *ASR-2* ASR like protein 2

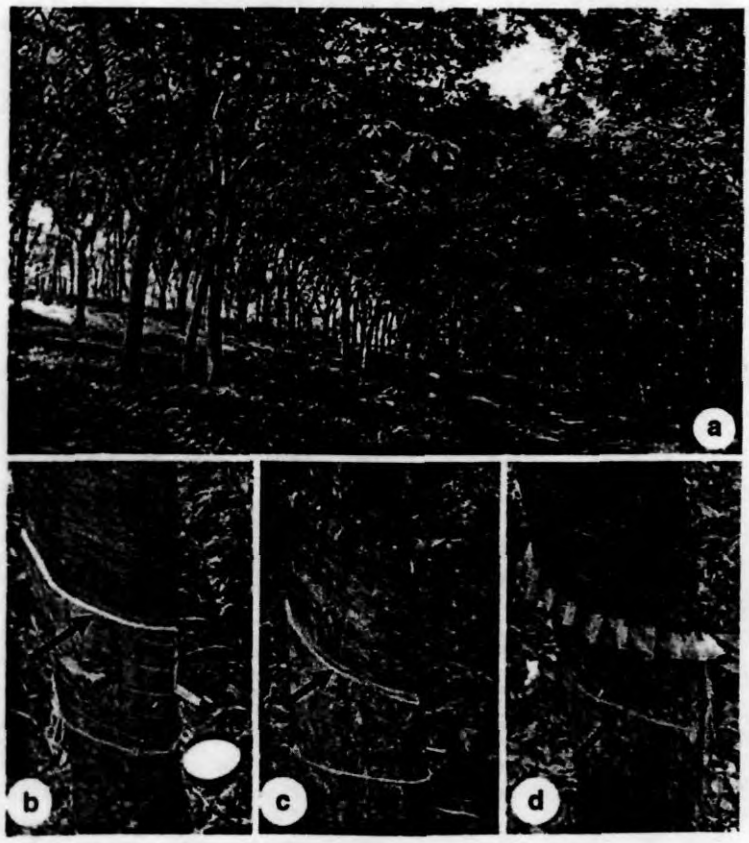

Fig. 1 **a** An overview of a high yielding *Hevea* rubber tree (clone RRII 105) plantation at RRII. **b** A healthy tree with normal latex flow (indicated by *arrow*) through out the tapping panel and flow of latex to the cup. **c** A rubber tree partially affected by TPD in which latex flow is observed in patches (indicated by *arrow*) at the cut surface of tapping panel. **d** A rubber tree fully affected by TPD syndrome with no latex flow (indicated by *arrow*) and completely dried tapping panel

like stress related protein-1 (RLP-1). Another clone from healthy plant had homology to translationally controlled tumour protein (TCTP), a highly conserved cytosolic calcium binding protein found in various organisms including plants. Along the same line, we isolated a gene from healthy tree that has sequence homology to a Thioredoxin H type (TRX-H) protein that is believed to interact with ROS-detoxifying enzymes such as ascorbate peroxidase,

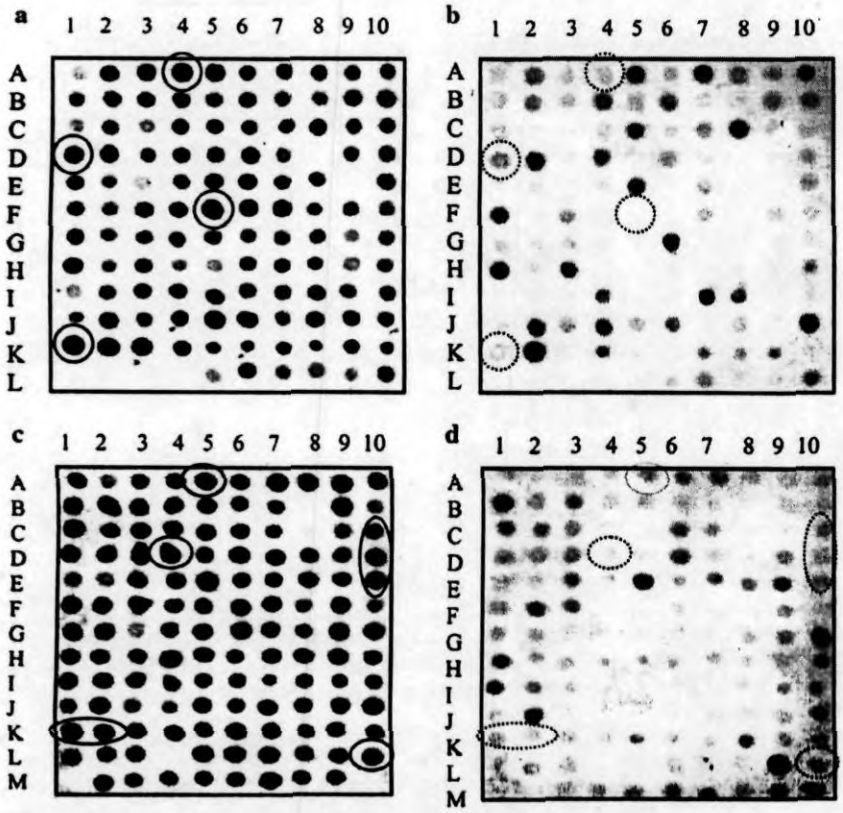

Fig. 2 Differential screening of cDNA clones from the *Hevea* SSH libraries (forward-healthy tree specific library and reverse-TPD tree specific library). Duplicate dot-blots were prepared and the membranes were hybridized with radiolabeled probes. The spots differing in their intensity between membranes were classified as either up- (circled by *continuous line*) or down-regulated (circled by *dotted line*). Two cDNAs, F5 (Myb transcription factor gene), and K1 (TCTP gene) specific to healthy tree are indicated by circle. **a** Clones from forward SSH library hybridized with unsubtracted labeled tester cDNA (healthy tree). **b** Clones from forward SSH library hybridized with unsubtracted labeled driver cDNA (TPD tree). **c** Clones from reverse SSH library hybridized with unsubtracted labeled tester cDNA (TPD tree). **d** Clones from reverse SSH library hybridized with unsubtracted labeled driver cDNA (healthy tree)

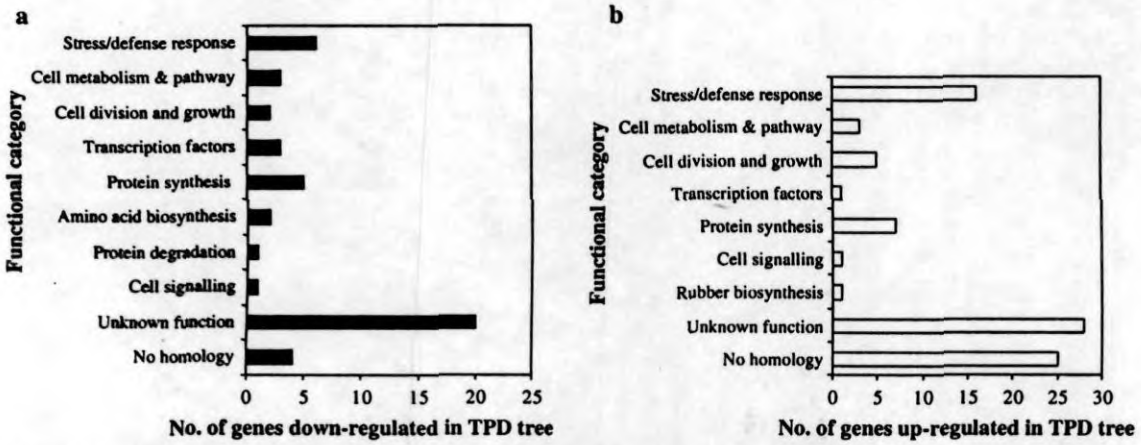

Fig. 3 Functional categories of TPD responsive genes identified from *Hevea* SSH libraries. Sequences having homology to unknown protein sequences from BLAST comparison were classified as unknown functional genes. Sequences with no similarity to known DNA/gene/protein sequences in the database were classified as no homology. The *bars* represent the number of gene transcripts: **a** down-regulated in TPD trees (forward SSH library); **b** up-regulated in TPD trees (reverse SSH library)

catalase, glutathione peroxidase, peroxiredoxins and superoxide dismutase. A sequence similar to copper chaperone related gene was also induced in healthy tree. The principal function of Cu chaperones is to deliver Cu to copper-dependent enzymes such as Cu–Zn–dependent superoxide dismutase, cytochrome oxidase and tyrosinase. This strengthens

an earlier report that SOD enzyme activity was significantly higher in healthy tree as compared to that in the TPD tree (Xi and Xiao 1988).

Most of the stress/defense related genes were isolated from TPD affected trees (Table 2). The osmotin-precurser-like gene was up-regulated in TPD tree and it was confirmed by RT-PCR analysis (Fig. 5a). Since the expression of osmotin-like genes is correlated with cell death at the time of suberization in bark tissues of cork oak (Pla et al. 1998), a similar type of response may be occurring in TPD affected bark tissues in rubber trees. Also among the clones isolated from TPD tree, there was a homologue of heat-shock proteins (HSPs) 19 class I and HSP 17.3. This suggests that HSPs have chaperone-like activity in refolding of damaged proteins in TPD affected trees. We also identified LEA1, 3 and chitinase genes, which are shown to be regulated by ABA and play an important role in protecting macromolecules and membranes from oxidative stress (Shinozaki and Yamaguchi-Shinozaki 1999). Also from the TPD library, we identified a gene with homology to a putative ethylene biosynthesis gene which has been involved in systemically induced stress defense response. A putative-senescence associated gene that could predispose tissue to senescence and cell death was identified in TPD tree. In addition, three clones for a hevein-like and pseudohevein-like protein genes were identified in TPD tree. A clone from TPD tree, that has sequence homology to a violaxanthin de-epoxidase precursor mRNA that is believed to act as an ABA precursor in wound stressed tree. Another clone from TPD had homology to phosphatidic acid phosphatase related gene also involved in wound-induced lipid hydrolysis (Wang et al. 2000; Park et al. 2004). In addition an universal stress protein (USP), which is a member of a family of stress-regulated (ABA, drought, salt and cold) genes of unknown functions was found to be induced in TPD affected trees.

Protein synthesis, degradation and amino acid biosynthesis related genes

In healthy tree, we found up-regulation of five genes associated with protein synthesis, one for protein degradation and two for amino acid biosynthesis. Several transcripts, homologues to ribosomal proteins and a gene encoding translation initiation factor were also up-regulated in healthy tree. We also identified a clone of 26S proteosome subunit 7 mRNA from healthy tree and it is believed to be associated with translation initiation complex during various environmental stresses. Another gene encoding vacuolar sorting receptor protein (VSR) was identified in healthy tree and this protein is implicated in sorting and compartmentation of proteins within the endomembrane system of plant cells (Paris et al. 1997). In addition, two up regulated genes in healthy tree represent Alanine:glyoxylate amino transferase gene (AGT) and b-keto acyl reductase genes which are believed to be involved in amino acid biosynthesis. Also identified in healthy trees was a clone with homology to an ubiquitin-conjugating enzyme E2 (UBIE2), which is involved in protein degradation (Hellmann and Estelle 2002).

In TPD tree, several up-regulated genes involved in protein synthesis, process and transport were also identified. We identified a homologue of cysteine protease like mRNA from TPD tree and it is believed to be involved in nuclear degradation. In general, proteases are thought to be involved in a range of processes including senescence and defense resistance. In TPD tree, a polyubiqutin gene was induced and its homolog is known to play important role in various cellular functions. Ubiqutin-mediated proteolysis is a fundamental process regulating diverse cellular events, including plant responses to environmental stresses controlled by jasmonate, ethylene and abscisic acid (Sullivan et al. 2003). DnaJ protein mRNA, identified in this study as up-regulated in TPD tree may be functioning as a molecular chaperone. Also we isolated a clone encoding translation elongation factor (eEF-1) beta chain protein mRNA from TPD library and it is presumed to play a role in translation initiation. It was also observed that three ribosomal protein mRNAs (80S, 60S, and 28S) were up-regulated under TPD conditions. Though, the up-regulation of ribosomal genes during the onset of TPD is not clear, it can be correlated with the changes occur in protein metabolism to adjust nutritional imbalance between healthy and TPD trees.

Cellular metabolism and signaling genes

In healthy tree, there were three up-regulated genes with predicted functions in cellular metabolism or signaling. Among them, a clone homologous to fatty acid hydroxylases that is thought to play a role in synthesis of cuticles and signaling molecules derived from fatty acids was identified. A clone from healthy tree with homology to calcium binding protein mRNA was isolated. Three genes potentially functioning in cellular metabolic pathway were found to be up-regulated in response to TPD. They include glyceraldehyde-3-phosphate dehydrogenase, acid invertase and CTP synthase/UTP ammonia ligase. The invertase is assumed to increase internal oxygen levels and improve sucrose metabolism in plant tissues. CTP synthase/UTP ammonia ligase plays an essential role in synthesis of cell membrane phopholipids in eukaryotic cells. Also among the clones isolated from TPD tree was a homologue of ASR like protein 2 predicted to be involved in abiotic stress signaling.

Cell structure, division and growth related genes

This functional category included two clones (glycine-rich RNA binding proteins (GR-RNPs) and actin depolymeriz-

ing factor mRNA) isolated form healthy tree library. The genes encoding GR-RNPs respond often to environmental stresses suggesting a stress-related role for these proteins. Glycine-rich proteins possessing RNA-binding domains are suggested to have regulatory function in addition to their role as cell wall components. Actin depolymerizing factor (ADF) is required for a number of essential cellular processes, including cell division and cell expansion (Kost et al. 1999). In TPD tree, a clone homologus to actin gene was isolated and it is thought to be involved in abiotic strees response. In addition a homolog of cell wall protein mRNA presumed to be involved in wound healing and plant defense was also induced in TPD tree along with a cyclophilin gene, which is likely to be involved in protein folding.

Genes involved in transcriptional regulation

In this category, we identified four transcription factors and only one of them is differentially regulated in healthy trees. This is homologous to a Myb transcription factor that appears to be induced due to tapping of laticiferous tissue in healthy tree (Chen et al. 2003). Also in healthy tree, one clone corresponding to histone H2A like protein was up-regulated and it is well known that histone genes are expressed during S-phase specifically in proliferating cells. Up-regulation of histone genes during cell cycle indicates that normal cell proliferation and growth of bark takes place in the tapping panel region of healthy trees. The last identified gene induced in healthy trees under this category was a putative small nuclear ribonucleoprotein F (SnRNP-F) a member of splicesome binding specific proteins. These proteins are implicated in basic and alternative splicing of nuclear pre-mRNAs which are crucial for regulating gene expression and in controlling development and differentiation.

Rubber biosynthetic genes

Interestingly, one of the rubber biosynthetic genes is up-regulated (HMGS-CoA) in TPD tree. The importance of up-regulation of the HMGS-CoA gene in TPD tree is not clear. However, extended latex dripping in rubber tree is an early physiological indicator for the onset of TPD syndrome. The extended latex synthesis in TPD prone trees can be correlated with up-regulation of HMGS-CoA gene.

Gene products of unknown function

Though we could classify many genes under several functional categories, still there are several genes identified in both forward and reverse SSH libraries that have no significant homologies to cDNAs with assigned functions for the products. In healthy tree library, there were 20 genes up-regulated and two of these genes were previously reported to be induced in rubber trees. Acylphosphatase protein is one of the previously described members of the c-terminal domain (CTD) proteins identified in rubber. Not much is known about the role of acylphosphatase and its impact on gene expression in plants. Another gene homologous to Kelch repeat-containing F-box family protein was also induced in healthy trees. A total of 28 genes with no known function were up-regulated in TPD tree.

Validation of differential expression

We selected seven genes (from forward library) involved in signaling (1), rubber biosynthesis (1), unknown (1), stress/defense (3) and a MYB transcription factor and six genes (from reverse library) representing stress/defense (3), protein metabolism (1), signaling (1) and unknown (1) to validate their differential expression. The MYB transcription factor chosen for Northern analysis has been shown to be up-regulated in healthy trees (Chen et al. 2003). Other genes were either down-regulated in TPD trees and are involved in important processes associated with the onset of TPD. Initially RT-PCR was performed with them to verify that the corresponding genes were differentially regulated. It was found that all the seven genes [(Myb transcription factor gene (MYB), translationally controlled tumor protein gene (TCTP), calcium-binding protein (CBP), rubber elongation factor protein gene (REF), thioredoxin H-type gene (TRX-H), F box family protein (F-box) and REF-like stress related protein I (RLP1)] were significantly up-regulated in healthy trees (Fig. 4a, b). Further, it is interesting to note that mRNA transcript accumulation for all the six genes [cysteine protease like mRNA (CP), PR-osmotin precursor gene (PRO), ethylene biosynthesis-related gene (EB), annexin-like protein RJ4 (ALP), phosphatidic acid phosphatase-related gene (PAP), ASR like protein 2 (ASR-2)] was found to be significantly higher in TPD affected trees (Fig. 5a, b). Subsequently, two unigenes MYB and TCTP expressed in healthy trees were selected for Northern analysis. The size of Myb and TCTP genes was 970 and 670 bp, respectively. The sequences were similar to the Myb and the TCTP genes reported earlier (Chen et al. 2003; Han et al. 2000). As predicted from the SSH results and RT-PCR (Fig. 6a), the levels of the Myb and TCTP mRNA transcripts were higher in healthy trees (Fig. 6b). The semi-quantitative RT-PCR analysis showed that the mRNA levels of Myb and TCTP genes increased by nearly four and three-folds, respectively in healthy trees (Fig. 6c). However analysis of intensity of radioactive signals in Northern blots indicated that Myb and TCTP were induced by nearly 15 and 12 folds, respectively in healthy trees (Fig. 6d).

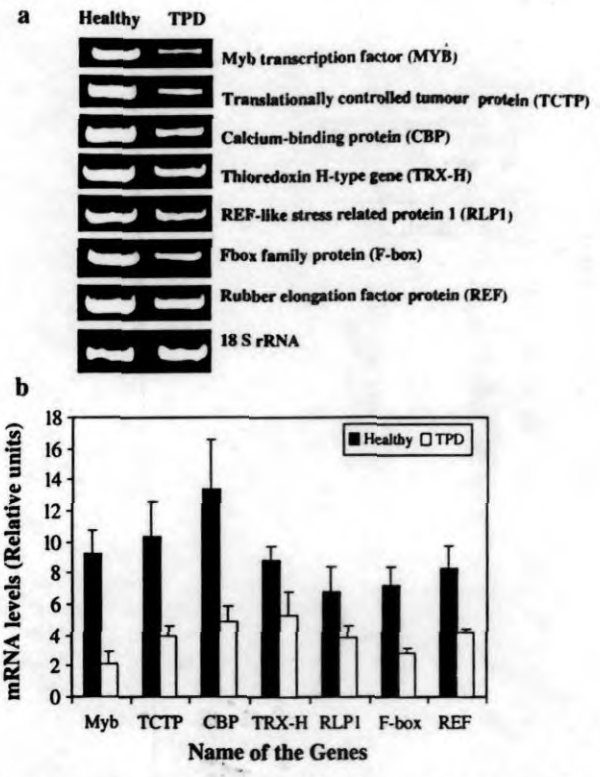

Fig. 4 a RT-PCR analysis of up-regulated genes in healthy tree. Total RNA extracted from latex cells of healthy and TPD trees of a high yielding *Hevea* clone RRII 105 was used for RT-PCR and the 18S rRNA served as constitutive control gene. Specific primers were used for the amplification of indicated genes. b Semi-quantitative analysis of mRNA transcript accumulation levels (Relative units) of various genes using Fujifilm ImageGauge version 3.12 software and normalized to account for loading differences in corresponding lanes

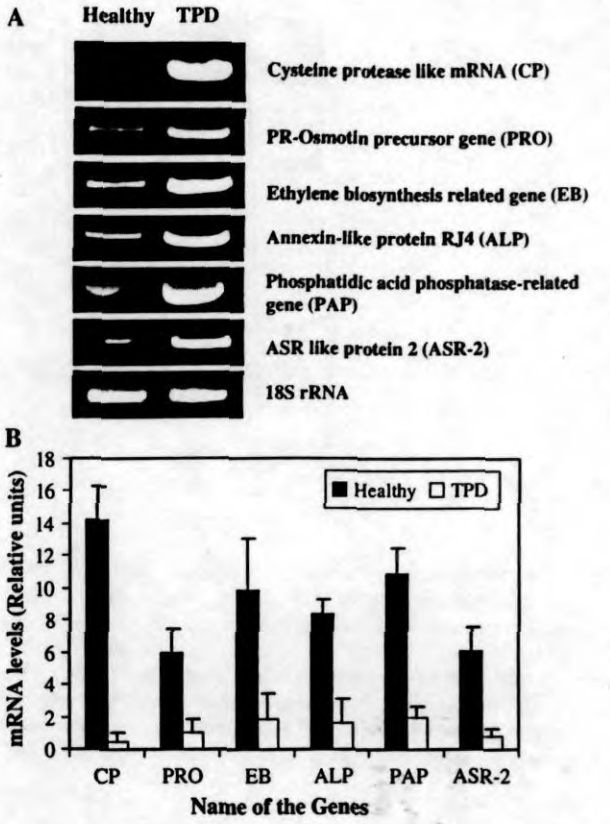

Fig. 5 a RT-PCR analysis of up-regulated genes in TPD tree. Total RNA extracted from latex cells of healthy and TPD trees of a high yielding *Hevea* clone RRII 105 was used for RT-PCR and the 18S rRNA served as constitutive control gene. Specific primers were used for the amplification of indicated genes. b Semi-quantitative analysis of mRNA transcript accumulation levels of various genes using Fujifilm ImageGauge version 3.12 software and normalized to account for loading differences in corresponding lanes

Discussion

The goal of this research was to identify and characterize genes that control/associated with the onset of TPD syndrome by which the latex biosynthesis (rubber) is affected significantly or stopped completely due to cell death (panel dryness) in rubber tree. With the advent of genomic methodology that permits the monitoring of expression of large number of genes simultaneously, it is now possible to identify sets of genes that are active during a specific developmental process. Moreover, the existing technology allows the assignment of functions to genes based on similarity to others in the database. Identification of these gene pools is an important and necessary first step towards understanding TPD developmental processes. In this study, the transcription profiles of healthy and TPD trees were compared by SSH analysis. This is the first report of global analysis of the transcriptional responses of *Hevea* trees during the onset of TPD syndrome.

The most noticeable difference in gene expression between healthy and TPD trees was the induction of large number of stress related genes in TPD affected tree. RT-PCR result indicates that the accumulation of mRNA transcripts for ethylene biosynthesis gene was significantly high in TPD trees compared to healthy one (Fig. 5a). It is well documented that ethylene biosynthesis gene is playing an important role in the regulation of stress responses in plants (Leon et al. 2002). Ethylene exerts its action through a complex regulation of its own biosynthesis, perception and signal transduction, leading to changes in gene expression (Chang and Shockey 1999). Earlier study in *Arabidopsis showed that genes responsive to reactive oxygen species* (ROS) and ethylene were among the earliest to be induced suggesting that both oxidative burst and production of ethylene played a role in the activation of cell death (Gechev et al. 2004). We also found that phosphatidic acid phosphatase, a major enzyme thought to be involved in many signal transduction pathway was up-regulated in TPD tree,

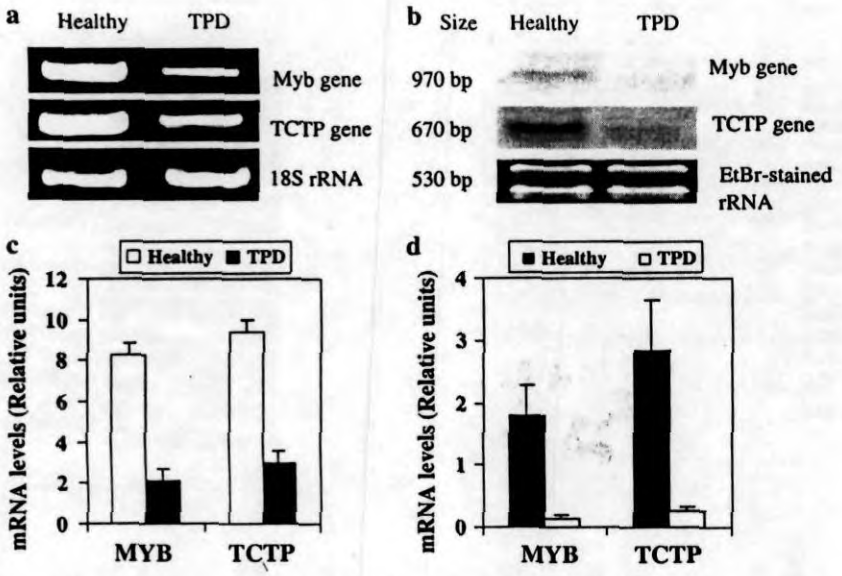

Fig. 6 Verification of differential expression pattern of the selected genes using either a RT-PCR or Northern blot analysis with RNA extracted from laticiferous cells of healthy and TPD trees of a high yielding *Hevea* clone RRII 105. **a** RT-PCR analysis of gene expression. The length of the RT-PCR products is indicated. The 18S rRNA was used as constitutive control. Specific primers were used for amplification of MYB, TCTP and 18S rRNA. PCR products were sequenced to confirm the identity of amplified gene. **b** Northern analysis of MYB and TCTP transcripts. Total RNA (15 μg) was separated on a 1.2% formamide denaturing agarose gel, blotted onto nylon membrane and hybridized with gene specific probes labeled with ^{32}P. EtBr stained gel is shown as loading control. **c** and **d** Semi-quantitative analysis of mRNA transcript levels of MYB and TCTP genes using Fujifilm ImageGauge version 3.12 software and normalized to account for loading differences in corresponding lanes

whereas it was not induced in healthy tree. Up-regulation of this gene in TPD affected tree was further confirmed by RT-PCR analysis (Fig. 5a). An earlier study reports that PA may be an important regulator of ROS generation and the cell death during various stress and defense responses of plants (Park et al. 2004). Actin is another gene induced in TPD trees. Actin structures and phosphatidic acid (PA) levels change during many defense and wound responses in plants (Lee et al. 2003). This result implicates that the up-regulation of PA gene may influence the induction of ROS leading to cell death (bark cell dryness) in the tapping panel of the TPD tree. Interestingly RT-PCR results indicate that cysteine protease mRNA transcript was accumulated significantly in TPD tree but not in healthy tree (Fig. 5a). It is well documented that cysteine protease is involved in programmed cell death in plants (Bozhkov et al. 2005). It has been shown that in soybean cells, oxidative stress induced an active cell death program that required biosynthesis of a set of cysteine proteases (Solomon et al. 1999).

Further, TPD trees exhibit abnormally high level of NAD(P)H oxidase activities which leads to the release of toxic O_2^- form of oxygen (ROS) (Jacob et al. 1994), and scavenging of such toxic oxygen species by SOD enzyme provides protection against lutoid membrane damage in rubber tree (Chrestin 1989). The fact that expression of above mentioned genes in TPD trees strongly suggests that they may be involved in protecting damaged plant tissues from further oxidative burst (lutoid membrane burst) by inhibiting latex biosynthesis and other associated metabolisms. Perhaps the most pertinent finding is that the up-regulation of most of the genes in TPD tree is associated with the regulation of the PCD process. Also, the TPD tree exhibits various stages of the programmed cell death (PCD) phenomenon known in plants (del Pozo and Lam 1998; Heath 2000). As discussed above, the differentially expressed genes identified in this study showed a significant correlation between PCD and TPD development. Interestingly, none of these genes were induced in healthy tree which produces latex in the bark tissue upon tapping for over 25 years. It is possible that the up-regulation of Cu chaperone related gene in healthy tree leads to increased SOD activity by transporting more Cu ions resulting in reduced ROS. Eventually this could promote normal cellular metabolism and rubber biosynthesis without TPD syndrome (cell death) in healthy rubber trees.

Induction of heat-shock proteins (HSPs) in TPD tree further highlight the stress endured by them and HSPs are known to play important roles in protecting plants against stresses. The smHSPs can act as molecular chaperone that bind to partially folded or denatured substrate proteins and thereby prevent irreversible aggregation or promote proper folding to protect cells from stress damage (Sun et al.

2002). Also, significant number of genes such as senescence-associated genes, chitinases, hevein, prohevein, pathogenesis related osmotin, universal stress protein, LEA proteins are induced as late stress response genes. Hevein like proteins play a role in plant defense mechanisms which are crucial to protect bark tissue against infection after tapping. Further, accumulation of mRNA transcripts for PR-osmotin precursor in TPD trees was also confirmed by RT-PCR (Fig. 5a). According to the RT-PCR results, ASR-2 gene expression was significantly up-regulated in TPD trees as compared to healthy one.

In the present study, a number of genes that are likely be involved in the control of cell division and growth regulation were identified as being specifically up-regulated in healthy tree. For example, genes encoding glycine-rich membrane related protein and cyclophilin were induced. Formation of lignin and suberin in the cells surrounding the tapping site is seen as a mechanism for resistance to degradation by microorganisms and as a barrier to moisture loss. Earlier study suggests that NT16 gene encoding a novel glycine-rich protein was developmentally regulated and induced by mechanical wounding (Yasuda et al. 1997). Our study showed that in healthy tree the glycine rich protein transcript level was induced to a greater level than that in TPD tree. The up-regulation of this protein may contribute to healing at tapping site and thus keep the tree in healthy condition. We also found that a glyoxylate pathway regulator gene, representing a major regulatory enzyme in lipid metabolism, was up-regulated in healthy tree. The glyoxylate cycle links lipid and carbohydrate metabolism via succinate produced from the condensation of two acetyl-CoA molecules in the glyoxysome (Eastmond and Graham 2001) providing carbon intermediates during tapping to promote biosynthesis of polyisoprene molecules (rubber molecules). Up-regulation of calcium binding protein gene transcripts was recorded in healthy tree compared to the TPD one (Fig. 4a). Induction of calcium binding protein gene was also noticed in healthy tree suggesting the potential involvement of Ca^{2+} signal transduction during regeneration of bark following rubber tapping. Rubber biosynthesis is a very active process involving changes in protein synthesis and activation. This was reflected in up-regulation of ribosomal proteins and others (translation initiation factor or elongation factor) that are likely be involved in translation. Genes that are predicted to function in the ubiquitin/26S proteasome pathway including ubiquitin, polyubiquitin, 26S proteasome, DnaJ and vacuolar sorting receptor proteins were all induced in healthy tree. Although the significance of modulation of the ribosomal gene expression in response to TPD development is not obvious and or but it can be linked to the changes in protein composition between healthy and TPD trees.

One of the growth related genes, TCTP was up-regulated in healthy tree and this correlates with normal cell division taking place at tapped site of healthy tree. Further, a significant accumulation of TCTP mRNA transcripts in healthy tree was confirmed by both RT-PCR and Northern analysis (Fig. 6a, b). The TCTP is one of the recently identified meristematic cell growth-related proteins that are actively expressed in rapidly dividing cells (Kang et al. 2005). In plants, TCTP transcription is also regulated by environmental and stress stimuli. The TCTP proteins have been isolated from cancerous tissues in animals and rapidly growing plant parts, suggesting a regulatory role in cell proliferation and differentiation. Two novel TCTP genes were also found to be preferentially up-regulated under oxidative stress caused by aluminum in the root tips of Al-tolerant soybean cultivars but not in Al-sensitive ones (Ermolayev et al. 2003). It is interesting to note that this gene was down-regulated in TPD tree (Fig. 6a, b). This may be one of the causes for lack of vascular differentiation of cells at tapping site which undergo severe necrosis (bark dryness) leading to TPD (cell death) and partial or complete cessation of latex biosynthesis.

It is well characterized that the immediate effect of tapping is shrinkage in the outermost cells at the site of injury and replacement of the lost tissues by bark regeneration. The regeneration of bark is of high practical value in *Hevea* rubber tree, since the renewed bark is also exploited commercially for latex production in the usual collection cycle (Premakumari and Panikkar 1992; Vinoth et al. 1995). In the course of development, vascular cambial activity was enhanced and bark regeneration takes place normally in the healthy tree. The TPD activated response appears to be directed to healing of the damaged tissues and to the activation of defense mechanism to prevent further damage. Proteins encoded by those genes expressed in TPD trees may play one of the following functions: (a) repairing of damaged plant tissue, (b) producing substances that inhibit growth of the microbes, (c) participating in the activation of wound defense signaling pathways and (d) adjusting plant metabolism to the imposed nutritional demands.

Many regulatory protein genes such as snRNP-F, histones and transcription factors were up-regulated in healthy tree. Accumulation of mRNA transcripts for Myb transcription factor was significantly higher in healthy tree as compared to TPD trees and it seems to be involved in the control of the cell cycle in plants and other eukaryotes. In this context lack of Myb gene expression in TPD trees may be correlated with TPD syndrome development. Furthermore, Myb transcription factors are crucial to the control of proliferation and differentiations in a number of cell types (Dietrich et al. 1997; Weston 1998, Mayda et al. 1999). The tapping panel dryness in TPD trees is likely due to lack of active cell division or proliferation as revealed by

decreased expression of Myb and TCTP genes. It is possible that the increased expression of Myb transcription factor may regulate TCTP gene that can lead to normal cell division and growth of bark tissues at tapping panel of healthy tree.

Results of this study provide the first comprehensive and global analysis of gene expression associated with TPD syndrome for *H. brasiliensis*. Genes identified in this study are likely to provide a link between TPD syndrome and PCD. There is a potential for using Myb transcription factor and TCTP as molecular markers in monitoring the development of TPD syndrome in rubber trees.

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26.03.07

Submitted:

Sub: regarding the forwarding of application for participation in the training programme of BARC

Ref: 2/15/2007 Res. Dt. 6.03.2007

According to the above reference, the application forms of myself and Dr.Geetha for the participation in the forth coming training programme on "Safety aspects in the Research applications of Ionizing Radiation (RA)" to be conducted during 9th April to 17th April 2007, have been forwarded to BARC on 6.03.07. When enquired over phone regarding the confirmation of our participation, it was learnt that our application has not been received there. The officer concerned informed us to send an official letter regarding our participation by fax so that our name can be included in the final list of participants.

In this context, it is required to send an official communication regarding our application at the earliest. The copy of the application and DD for Rs. 6000/- can be taken along with and submitted after arrival there. Hence it is requested to send an official communication to BARC by fax at the earliest.

M.B. MOHAMED SATHIK
Scientist S3

Dy. Director (in-charge)

DS (Res)