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Tapping panel dryness in *Hevea brasiliensis*: Investigations on the gene transcripts from latex by suppression subtractive hybridization

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Hevea brasiliensis, the major source of natural rubber, contributes 99% of the world's natural rubber production. The yield of rubber is influenced by various factors such as climate, nutrient availability, system of tapping, genetic nature of the clone, etc. (Jacob *et al*., 1999). Rubber trees are susceptible to biotic (fungal, bacterial, etc.) as well as abiotic (drought, cold, wintering and wounding) stress factors (Jacob *et al*., 1999) that affect the crop yield significantly. Apart from these factors, tapping panel dryness (TPD) is a common phenomenon in high yielding clones of *Hevea* that limits crop productivity. This phenomenon occurs in about 15-20 % of rubber trees in a plantation and the loss in dry rubber due to TPD is 12-20 % of annual rubber production (Chen *et al*., 2002). At the initial stage of the symptom, tapping panel shows patches of intermittent dry regions without any latex oozing from it. Subsequently, the dryness spreads to the whole panel resulting in complete cessation of latex flow, thus reducing crop yield.

Previous investigations on TPD indicate that it is a physiological disorder resulting from the over-exploitation of trees such as higher tapping intensity and over-stimulation with ethylene releasing compounds (Fan and Yang, 1995). It is reported that when harvesting of latex exceeds the inherent capacity of a particular genotype, the tree succumbs to TPD (Jacob *et al*., 1994). The altered levels of hormones such as auxins and cytokinins (that are required for differentiation of cambium into laticifers) due to stimulation is also a factor suspected to induce TPD (Nataraja *et al*., 2006). Recent gene expression studies indicated the expression of Myb transcription factor (HbMyb1) at higher levels in leaves, barks and latex of normal trees than in the TPD affected trees (Chen *et al*., 2002; Ko *et al*., 2003; Venkatachalam *et al*., 2007). Genes involved in apoptosis, senescence and metabolic activities and genes corresponding to transport proteins, cytoskeleton maintenance proteins, pathogen response proteins (Sathik *et al*., 2006b), signal transduction, stress/pathogen response proteins, TCTP (a calcium binding protein) and Myb transcription factor (Venkatachalam *et al*., 2006) were found differentially expressed in TPD trees. However, not much information is available on the genes that might play a role in inducing TPD.

In this report, in order to characterize the genes associated with the development of TPD in *Hevea*, mRNA isolated from latex of healthy and TPD affected trees were subjected to subtractive hybridization. The subtracted clones were sequenced and subjected to BLAST analysis for gene identification and annotation. The possible role of such annotated transcripts in TPD is discussed.

Latex samples were collected from healthy and TPD affected trees (n=5) of clone RRII 105 from RRII campus as described previously (Sathik *et al*., 2006a). The trees were 18 years old under S/2(RG) 6d/7 system of tapping for 11 years. Isolation of mRNA from latex samples was optimized using magnetic beads (mRNA isolation kit, Promega). Equal quantities of high quality mRNA isolated from five samples of each healthy and TPD trees were pooled together separately. The mRNA samples were used for SSH using PCR-select cDNA subtraction kit (Clontech, USA). In the forward reaction, the mRNA isolated from TPD and healthy trees were

used as 'tester' and 'driver', respectively and vice-versa for the reverse reaction. Subtraction was performed as per the manufacturer's recommendations. The subtracted products were cloned into PCR-TRAP vector (GenHunter, USA) and transformed into *E. coli* cells. The transformed cells were selected by PCR using vector specific primers and were sequenced by M/s. Macrogen, Korea. Homology of the ESTs and their potential function were determined by blasting with NCBI database (http://www.ncbi.nlm.nih.gov). The EST data have been submitted in the GenBank database (Accession No. GR305128-GR305315).

A total of 180 and 25 ESTs were obtained from forward and reverse libraries, respectively. After removal of redundant clones, the forward library had a total of 92 clones among which 53 transcripts showed homology with known proteins, 15 transcripts showed homology with unknown proteins and 23 transcripts showed no homology to known genes (Table 1). There were a total of 22 clones in the reverse library among which 13 transcripts showed homology with known genes, five transcripts had homology with unknown protein and four transcripts with no homology (Table 2). Fifteen

Table 1. Putative identity of clones specifically expressed in latex of TPD affected trees

Sl. No.	Putative gene identity	E-value	score
SSH34	*D. rerio* mRNA for Cu/Zn-superoxide dismutase	0.005	48.1
SSH91	*Flaveria trinervia* TAF10 mRNA for TATA box-binding protein	9.00E-14	85.7
SSH177	*Hevea brasiliensis* latex abundant protein 1 (LAP1) mRNA	0.00E+00	710
SSH146	*Prunus armeniaca* 40S ribosomal protein S4 (RPS4) mRNA	3.00E-39	170
SSH39	*Siniperca chuatsi* transposase mRNA	0.001	52
SSH115	unknown protein, *Triticum aestivum* clone		
SSH163	*Vitis vinifera*, unknown protein	2.00E-25	125
SSH4	60S ribosomal protein L21 [*Oryza sativa*]	1.00E-49	197
SSH173	*A. thaliana*, peroxisomal small heat shock protein Hsp15.7 mRNA	2.00E-05	58
SSH61	AB074308.1 *Hevea brasiliensis*, mRNA for rubber elongation factor	0.00E+00	648
SSH38	AF193438.1 *Hevea brasiliensis* ubiquitin precursor (ubi) mRNA	0	700
SSH55	*Arabidopsis thaliana* cytochrome-c oxidase (AT2G47380) mRNA	1.00E-09	71.9
SSH58	*Arabidopsis thaliana* mRNA for magnesium transporter protein	7.00E-05	56
SSH23	*Arabidopsis thaliana* unknown protein	2.00E-25	125
SSH136	*Arabidopsis thaliana* unknown protein (AT3G62010) mRNA	5.00E-23	117
SSH143	*Arabidopsis thaliana* unknown protein (AT3G62010) mRNA	2.00E-25	125
SSH175	ATP dependent helicase, EIF4A-2, Eukaryotic Initiation Factor 4A-2	1.00E-44	188
SSH47	AY221985.1 *Hevea brasiliensis* protease inhibitor protein 1 (PI1) mRNA	1.00E-92	347
SSH86	AY275680.1 *Hevea brasiliensis* ubiquitin mRNA	7.00E-37	163
SSH5	*Capsicum annuum* histone H3-like protein mRNA	2.00E-34	155
SSH176	*Citrus sinensis* hypoxia-responsive family protein mRNA	2.00E-31	145
SSH25	*Ctenopharyngodon idella* glucose phosphate isomerase mRNA	2.00E-04	54
SSH33	*Cucumis sativus* mRNA for isocitrate dehydrogenase	9.00E-39	168
SSH87	DQ306739.1 *Hevea brasiliensis* isolate SSH10 mRNA		
SSH30	DQ306813.1 *Hevea brasiliensis* isolate SSH84 mRNA	7.00E-94	351
SSH95	DQ306847.1 *Hevea brasiliensis* isolate SSH118 mRNA sequence	3.00E-171	609
SSH24	*Eucalyptus globulus* partial mRNA for putative ribosomal protein	8.00E-04	52
SSH31	*Flaveria trinervia* TAF10, TATA box-binding protein associated factor 10	6.00E-12	79.8
SSH43	globulus partial mRNA for putative ribosomal protein L2	8.00E-04	52
SSH12	Glycine max ribosomal protein S6 (RPS6) mRNA	2.00E-40	174
SSH70	*Hevea brasiliensis* latex abundant protein 1 (LAP1) mRNA	0	710
SSH174	*Hevea brasiliensis* protease inhibitor protein 1 (PI1) mRNA	9.00E-141	507
SSH15	*Hevea brasiliensis* REF-like stress related protein 2 (RLP2) mRNA	3.00E-52	212
SSH45	*Hevea brasiliensis* ubiquitin precursor (ubi) mRNA	0	700
SSH28	*Hevea brasiliensis*, small rubber particle protein (SRPP) mRNA	0	682
SSH36	*Lycopersicon esculentum* clone 114241R	1.00E-41	178
SSH125	*Manihot esculenta* aspartic protease mRNA	2.00E-29	137
SSH11	*Medicago truncatula* thioredoxin h1 mRNA	2.00E-15	91.7
SSH113	*Musa acuminata* MA-EIL4 mRNA for ethylene transcription factor	0.001	48.1

SSH85	*N. tabacum* (cv.Samsun NN) L19 mRNA for ribosomal protein L19	4.00E-56	226
SSH134	NC_008474.1 *Populus trichocarpa* linkage group VIII	1.00E-36	161
SSH54	NM_116066.2 *Arabidopsis thaliana* unknown protein	1.00E-26	115
SSH3	*Oryza sativa* 60S ribosomal protein L21 (RPL21) mRNA	2.00E-31	145
SSH6	*Picea abies* dehydrin 1 (Dhn) mRNA /Cold inducible mRNA	0.008	50.1
SSH10	*Populus nigra* PnOgg1 mRNA for 8-oxoguanine DNA glycosylase	3.00E-10	73.8
SSH26	*Prunus armeniaca* 40S ribosomal protein S4 (RPS4)	6.00E-39	170
SSH100	ribosomal protein S6 (RPS6) mRNA	4.00E-41	176
SSH8	*Silene latifolia* SlSS mRNA for strictosidine synthase family protein	3.00E-04	54
SSH42	*Siniperca chuatsi* transposase mRNA	0.001	52
SSH48	*Tragopogon dubius*, putative polyubiquitin mRNA	3.00E-48	200
SSH9	*Triticum aestivum* clone wlsu2.pk0001.h3:fis,	0.003	90.2
SSH20	*Triticum aestivum* clone wpi1s.pk008.k5:fis	7.00E-04	94.2
SSH145	*Triticum aestivum* ribosomal protein L7 mRNA	5.00E-29	137
SSH68	Unknown	0.013	50.1
SSH13	unknown protein	0.005	48.1
SSH123	unknown protein	0.19	44.1
SSH41	unknown protein	0.007	50.1
SSH2	unknown protein [Oryza sativa]	2.00E-08	60.8
SSH101	unknown protein, *Hevea brasiliensis*	4.00E-13	83.8
SSH172	unknown protein, *Hevea brasiliensis*	3.00E-171	609
SSH111	unknown protein, *Hevea brasiliensis*	7.00E-91	341
SSH129	unknown protein, *Vitis vinifera*	8.00E-11	75.8
SSH158	unknown protein, *Vitis vinifera*	5.00E-12	79.8
SSH133	unknown protein, *Vitis vinifera*	6.00E-62	246
SSH142	unknown protein, *Vitis vinifera*	3.00E-14	87.7
SSH169	unknown protein, *Vitis vinifera*	2.00E-08	67.9
SSH139	unknown protein, *Vitis vinifera*	1.00E-26	129
SSH74	unknown protein, *Vitis vinifera*	1.00E-17	115
SSH44	*Zea mays* ribosomal protein s6 RPS6-2 (rps6-2) mRNA	4.00E-41	176
	23 clones had no similarity		

Table 2. Putative identity of clones specifically expressed in latex of healthy trees

Sl no.	Size	Putative gene identity	E value	score
SSH181	767	no similarity		
SSH182	200	RNA dependent RNA polymerase [*Medicago truncatula*]	2.00E-19	97.8
SSH183	433	putative TPR-repeat protein [*Arabidopsis thaliana*]	4.00E-39	162
SSH184	387	electron transport flavoprotein mRNA, *A. thaliana*	1.00E-10	75.8
SSH185	648	amino acid carrier mRNA, *Ricinus communis*	6.00E-97	363
SSH187	723	acyl-CoA-binding protein (ACBP) mRNA, *Jatropha curcas*	2.00E-84	321
SSH188	282	Zinc finger, BED-type predicted [*Medicago truncatula*]	3.00E-06	53.5
SSH190	609	unknown protein, *Arabidopsis thaliana*	9.00E-26	70.9
SSH193	511	aquaporin, plasma memb intrin protein 2-1 (PIP2-1)	2.00E-28	135
SSH194	764	no similarity		
SSH195	761	unknown protein, *Arabidopsis thaliana*	3.00E-20	102
SSH196	1334	*H. brasiliensis* hevein (HEV1) mRNA	0.00E+00	1043
SSH197	213	*Hevea brasiliensis* thioredoxin	7.00E-29	135
SSH199	690	translation initiation factor [*Arabidopsis thaliana*]	2.00E-20	102
SSH201	764	transcription factor, *Vitis riparia*	1.00E-17	93.2
SSH202	765	no similarity		
SSH203	726	SAM-depnt methyltransferase or caffeoyl-CoA O-methyltransferase	2.00E-86	322
SSH204	476	40S ribosomal protein S15a-1, *A. Thaliana*	1.00E-64	247
SSH205	738	no similarity		

transcripts related to protein synthesis were found expressed in the latex of TPD affected trees. They were mainly ribosomal protein (40S, 60S, S6, L2, L7, L19 and L21), a putative ribosomal protein, ubiquitin precursor protein and ubiquitin.

Expression of ubiquitin (which is a small regulatory protein involved in protein recycling) in TPD plants indicate the recycling process of various protein molecules. There were about nine stress related genes expressed in the latex of TPD affected trees. Expression of Cu/Zn-superoxide dismutase in TPD trees suggests the possible existence of higher levels of ROS in TPD trees and the triggered ROS scavenging mechanism in the latex. A clone homologous to peroxisomal small heat shock protein, Hsp15.7 and protease inhibitor protein (PI1) were also found in the TPD plants. Rubber elongation factor-like stress related protein 2 (REF RLP2, function unknown), ethylene transcription factor (known to express in response to wounding or pathogen attack) have also been found in TPD trees. A transcript similar to cold inducible, aspartic protease which has a variety of roles in connection with programmed cell death (PCD) processes and hevein (involved in coagulation of latex) were also found in TPD trees.

Genes coding for cytochrome-c oxidase and glucose phosphate isomerase were present in the latex of TPD trees. The isocitrate dehydrogenase which has an antioxidant role was found in TPD trees. Gene coding for thioredoxin h1 protein which interacts with ROS-detoxifying enzymes was also found in TPD affected plants as reported earlier (Venkatachalam *et al*., 2007). Strictosidine synthase which is involved in the biosynthesis of terpenoid indole alkaloids and induced by fungal elicitors was also expressed in TPD samples. The genes coding for flavoprotein in the electron transport chain, thioredoxins (involved in the redox potential) and S-adenosyl methionine-dependent methyl transferase were found only in healthy plants indicating their possible involvement in maintaining the normal metabolic activities of the healthy plants.

Genes associated with regulation of various gene expressions such as TATA binding protein (TBP), TATA box binding protein associated factor 10 (TAF10), eukaryotic initiation factor 4A-2 (EIF4A-2), transposase, histone protein, TAF10 and transcription factor IIB (TFIIB) were differentially expressed in TPD affected trees. The healthy plants expressed genes similar to RNA dependent RNA polymerase (RDRP, involved in siRNA production), zinc finger (involved in gene transcription, translation, mRNA trafficking, cytoskeleton organization), BED-type predicted protein, putative TPR repeat protein, translation initiation factor and transcription factor.

TPD plants also contained transcripts such as latex abundant protein 1 (LAP 1), rubber elongation factor (REF), small rubber particle protein (SRPP) and 8-oxoguanine DNA glycosylase (involved in repairing the damage caused by oxidative stress to DNA). There were also transcripts similar to protein with unknown identity, similar to the sequences identified in *Triticum aestivum, Vitis vinifera, Arabidopsis thaliana, Hevea brasiliensis* (SSH10, SSH84, SSH118) and *Lycopersicon esculentum* (clone 11421R). A transcript similar to acyl CoA binding protein (ABCP) which binds with fatty acyl-CoA to facilitate transport across membranes was expressed only in the healthy plants. Similarly, a transcript for magnesium transporter protein was found in TPD samples. An amino acid carrier and aquaporins (plasma membrane intrinsic protein, PIP) which is selectively involved in conducting water molecules across membranes were found in healthy trees indicating the existence of normal transport across cells.

Though the differentially expressed transcripts can be separated using this technique, it is also possible that some genes may escape the subtraction and get represented in both the groups. Interestingly, the generated data did not show many common genes between both groups. The number of clones obtained in the healthy plants is less than expected and needs further investigation. In the present study, many transcripts belonging to stress/defense related pathways have been found in TPD samples indicating that TPD affected trees are under severe stress. Further, investigations based on more number of subtractions followed by qPCR validation of candidate genes could explain the association of such genes with TPD.

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