RESEARCH ARTICLE

Molecular Investigation on Expression Analysis of Tapping Panel Dryness (TPD) Syndrome Associated Genes in Rubber Tree (*Hevea brasiliensis* Muell. Arg.)

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Abstract

Rubber clones, especially high-yielding clones (RRII 105, PCK2, PB 217, and GT 1), are considered to be susceptible to tapping panel dryness (TPD) syndrome. The main objective of this study was to find the significance of TPD-associated genes in rubber tree and their expression of mRNA transcript level by RT-real-time PCR and semi-quantitative RT-PCR. RT-real-time PCR screened a total of 15 TPD-associated candidate genes. Among these, eight candidate genes were analyzed by semi-quantitative RT-PCR. RT-real-time PCR result revealed that among 15 genes screened, the mRNA transcript levels of 3 genes (TOM 20, TCTP, and Myb 1) were downregulated significantly in various TPD-affected clones of the rubber tree compared to healthy, and their expression pattern was altered in different rubber clones due to various degrees of TPD tolerance. Semi-quantitative RT-PCR results proved a down-regulation of five genes (TOM 20, TCTP, Myb 1, ABC, and PT2) in the TPD tree in comparison to the healthy tree. The findings of this study confirmed that the mRNA transcript levels of the TOM 20, TCTP, and Myb 1 genes were significantly lower in various rubber clones that had been affected by TPD and also suggested that these genes may be crucial for the development of TPD, which results in impaired latex biosynthesis in rubber trees.

Keywords: *Hevea brasiliensis*, Tapping panel dryness, Translocase of the outer mitochondrial membrane, Translationally controlled tumor protein, MYB transcription factor.

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Introduction

The rubber tree (*Hevea brasiliensis* Muell. Arg.), which comes under the Euphorbiaceae family, is a perennial tropical tree for the synthesis of natural rubber (NR). Moreover, natural rubber cannot be replaced or challenged by any other synthetic rubber or botanical source due to its ascendancy. The yield of rubber has been significantly increased over the past decades by the cultivation of high-yielded clones (RRII 105, PCK2, PB 217, and GT 1). Rubber trees produce more than 8.7 million tons of dry rubber from 9.5 million hectares of cultivated plantations every year worldwide. TPD affects about 12–50% of productive trees in almost every rubber-growing region, and the estimated percentage of losses in rubber production due to TPD is 15–20% of the annual rubber production (Chen *et al.,* 2003; Huang *et al.,* 2023). Natural rubber is a very long chain cis-1, 4-polyisoprene polymer, a secondary metabolite, and plays an important and irreplaceable role in the world economy. One of the most serious threats to natural rubber is tapping panel dryness syndrome (TPD). It causes serious economic losses in latex production, especially in high-yielding clones. The TPD of rubber trees is characterized by the partial or permanently complete stoppage of latex flow upon tapping. TPD syndrome is associated with reduced protein, nucleic acids, thiols, and ascorbic acid levels and exhaustion of major nutrients (Fan and Yang, 1984). In TPD, cell membrane destabilization owing to the bursting of the plutoids and following in situ latex coagulation due to over-stimulation has been proposed to be associated with the occurrence of uncompensated oxidative stress within the latex cells, resulting in TPD (Chrestin *et al.,* 1989). The first symptom of TPD is the appearance of partial dry zones along with the tapping panel, which may even become fully dry. In addition to this, the occurrence of thickening, browning, and flaking of bark are the other symptoms of TPD (Sookmark *et al.,* 2002). Trunk phloem necrosis (TPN) is an irreversible bark disease that spreads from the collar to the tapping panel of the rubber tree causing necrosis of the internal phloem and the latex vessels, thus stopping latex production (Pellegrin *et al.,* 2007). The molecular mechanism behind TPD remains poorly understood. The Semi-Quantitative RT-PCR and RT-real-time PCR methods have been used to obtain further information about the mRNA expression profiles between healthy and TPD trees. Thus, analyzing the molecular changes that occur during TPD development at the genomic level and identifying genes involved in the onset of TPD syndrome in *Hevea* is more important.

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The literature mentioned ten functional categories of genes associated with TPD, such as stress/defense response (Sivasubramaniam *et al.,* 1995; Chye & Cheung, 1995), signal transduction, metabolism and energy, transporter, etc. Among the genes involved in rubber biosynthesis, the rubber elongation factor (*REF*) gene is highly expressed in laticifers (Goyvaerts *et al.,* 1991). Other rubber synthesis-related genes, namely small rubber particle protein (SRPP) (Oh *et al.,* 1999) and HMGs-CoA reductase (Chye *et al.,* 1992), are also highly expressed in the latex. According to Han *et al.* (2000), as a step toward understanding rubber biosynthesis at the molecular level, they identified several genes expressed in the latex, some of which are latex-specific. An earlier study reports that among the transcripts, *REF* was the most abundant (6.1%), followed by small rubber particle protein (SRPP) (3.7%), *Sdh* 524 (3.7%), Sdh 525 (1.6%), Hevein (2.5%), and *HbLAR* (*Hevea brasiliensis* latex- abundant mRNA) (2.9%). Ten ESTs (Expressed Sequence Tags) showed latex-specific gene expression, and the genes expressed abundantly (71%) in the latex included stress-induced cysteine proteinase, abscisic acid-activated gene, SRPP, *eIF-4A*, CROC-1 (DNA binding) protein, *HbLAR*, *Sdh* 524, *DnaJ* protein and *TCTP*. A total of 134 genes associated with TPD in rubber trees were identified by Venkatachalam *et al.* (2007) through the SSH (Suppression et al.) method. Besides, they analyzed the expression patterns of partial genes and discussed the differentially expressed genes in TPD. A gene *HbTOM20* (*Hevea brasiliensis* Translocase of the Outer Mitochondrial Membrane) associated with TPD was identified by mRNA differential display. *HbTOM20* might play an important role in the alteration of mitochondrial metabolism, which finally tends to be impaired latex biosynthesis (Venkatachalam *et al.,* 2009). Li *et al.* (2010) identified only 21 genes due to the use of different stages of TPD, Hevea clones, and tapping systems. Compared with the functional classification of TPD-related genes from Venkatachalam *et al.* (2007), one obvious similarity between the two studies was that many stress/defense response genes were up-regulated in the TPD tree. The upregulation of these genes might destroy the normal cellular metabolism and lead to the occurrence of TPD in rubber trees. Venkatachalam *et al.* (2007) and Li *et al.* (2010) indicated that translationally controlled tumor protein (TCTP) transcripts were found to be higher in healthy trees than in TPD ones. Venkatachalam *et al.* (2007) further suggested that down-regulation of TCTP transcripts in TPD trees might cause severe necrosis (bark dryness) due to lack of vascular cell differentiation at tapping sites and leading to TPD (cell death) and partial or complete cessation of latex biosynthesis. TCTP gene (HbTCTP) was cloned in a rubber tree by Liang *et al.* (2009), and observed that HbTCTP transcripts were constitutively expressed in the latex, leaves, and bark. Besides, HbTCTP was induced by ethylene, suggesting that HbTCTP may be involved in ethylene signaling. Few genes like Myb transcription factor (Myb) and TCTP have been reported as TPD-associated in rubber trees (Chen *et al.,* 2003; Venkatachalam *et al.,* 2007). Few protein markers have also been previously reported in the latex cytosol of TPD-affected trees (Sookmark *et al.,* 2002). Therefore, the isolation of more TPD-associated genes from Hevea is essential to dissect the molecular regulation of gene expression during the onset of TPD syndrome. Although a number of studies have been undertaken to analyze the expression pattern of TPD-associated genes in the recent study, detailed information is not available on genes associated with TPD. So, the main objective of this study is to identify and analyze the expression of major genes involved/associated with the TPD in rubber trees.

Materials And Methods

Plant Material

Elite rubber clones, namely RRII 105, RRII 285, PCK2, PB 235, PB 5/51, PB 217, and GT 1 from M/s Harrisons Malayalam Pvt. Ltd., Punalur, Kerala, were identified for TPD incidence and selected for the present experiments. These trees have been regularly tapped on alternate days for latex collection for the past 20 years. TPD syndrome plants were also tapped to mimic the healthy control plants to maintain uniform wound stress before sample collection for an experiment. The occurrence of TPD was also confirmed earlier by physiological and biochemical studies. The fresh latex and bark tissue samples were collected from seven healthy and TPD-affected trees and pooled for RNA isolation and subsequent analysis.

Total RNA Extraction

Total RNA was isolated from both laticifer cells and barks tissue of healthy and TPD-affected rubber clones, as described by Venkatachalam *et al.* (2009). RNA (20 µg) was treated with 10U of RNase-free DNase for 30 min at 37°C to digest the remaining genomic DNA contamination. Then it was treated with DNaseI inactivation reagent for 5 minutes, followed by centrifugation for 3 minutes at 13,000 rpm, and transferred to a fresh tube. The quantity of isolated RNA was measured spectrophotometrically at 260 and 280 nm, and the quality was checked by using formaldehyde agarose gel electrophoresis.

RNA Isolation and cDNA Synthesis from Laticiferous Cells

Using total RNA as a template, First strand cDNA synthesis was performed from 1 µg of RNA using the cDNA synthesis kit (Takara et al.) according to the manufacturer's instructions. In each real-time RT-PCR reaction, the 18S rRNA gene was used as the internal control, and the gene-specific primers were used. Before qRT-PCR analysis, each cDNA was diluted 10 times with nuclease-free water.

Quantitative Real-time PCR

All the Quantitative RT-PCR reactions were performed using SYBR green detection chemistry in a StepOne plus real-time machine (Applied Biosystems, USA). A reaction cocktail of 20 µL was constituted of 10 µL SYBR Green PCR Master Mix (Applied Biosystems, USA), 0.4 µL each of the forward and reverse primers, and 2 µL diluted cDNA. The list of primes used for the present experiment is given in Table 1. PCR cycling was carried out at an initial denaturation for 30 seconds at 95°C, followed by 40 repeated cycles, each consisting of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 30 seconds. To check amplicon specificity, dissociation curve analysis was carried out by the constant increase of temperature between 60°C to 95°C. All qRT-PCR experiments were carried out in at least three biological replicates with three technical replicates each time, and the values were presented as mean ± SD. The statistical

analysis was performed using SPSS software, the experimental data were computed using one-way ANOVA, and the mean differences were compared for pair analysis between healthy and TPD-affected trees by using Student-Newman Keul's Test at p <0.05% level. The value of p <0.05 was considered to be a significant difference.

Table 1: List of Primers for TPD-associated genes used for qPCR analysis

S.No	Gene	Primer Sequence (5'-3')	Tm	Product Size (bp)
1	Annexin-like protein RJ4	ALPRJ4 F: 5'-GGATCACAGGAGAACTGCAAAG-3'	63	96
		ALPRJ4 R: 5'-GTTTATCCGGTGCCCATGTT-3'		
2	ASR-like protein 1	ASR F:5'GAATGAAGAAGGTCATGGAAAGAAG3'	62	106
		ASR R: 5'-CCCAACTCGATCCAACTGATAG-3'		
3	Calcium binding protein	CBP F: 5'-CAAAGCTACGGGTTCTACCTAC-3'	62	99
		CBP R: 5'-GCGAACTCCTCCAGATTGAT-3'		
4	Cysteine protease	CP F: 5'-ACCCTTATCTTGCTCGTGATG-3'	62	109
		CP R: 5'-TGTAGTGCCGCCTCATTATTC-3'		
5	Ethylene biosynthesis protein	EBP F: 5'-TTCGTGTCCCGTCTGTTTG-3'	62	107
		EBP R: 5'-GCGCCACTCTATTGTGGTTA-3'		
6	TOM20	TOM20 F: 5'- GGAGGAGGCTTTGGTGATAAA-3'	62	93
		TOM20 R: 5'- CTCAGCAAGGTCAGAAGGAAA-3'		
7	Type II metacaspase	MET F: 5'-GACTATGGTGACAGTGGCTATG-3'	62	120
		MET R: 5'-AAGTGTTGGTCTCAGCTTCC-3'		
8	F-box family protein	FBOX F: 5' CACTCAGAAGCGTCTGTTTCTA-3'	62	108
		FBOX R: 5'-CTAATCTCCAGCCACACAATCT-3'		
9	Heat shock protein	HSP F: 5'- GAGTTAAGGAAGAGGGCAGAAG-3'	62	97
		HSP R: 5'- TGAAGCCAGAGGTTAGGAGA-3'		
10	MYB transcription factor (MYB)	MYB F: 5'-GTGACCACTAGAACACCAACTC-3'	62	97
		MYB R: 5'-GGATGCTGGACCTTCTCTTATC-3'		
11	REF-like stress related protein 1 (RLP1)	RLP F: 5'-GCTAGTGCTGTGGCTTCTTAT-3'	62	102
		RLP R: 5'-CAACTGGAAGCAATTGGTGAAG-3'		
12	Thioredoxin h	THR F: 5'- AGCTAAGTCAAGAGTGGTGAAG-3'	62	117
		THR R: 5'- GGGATACACCATGAAGCAGTAA-3'		
13	Translationally Controlled Tumor Protein (TCTP)	TCTP F:5'-GGCAGTCTTGTGTTTGCTTAC-3'	62	109
		TCTP R: 5'- AGCTAGCTATCCTCCCTTCTT-3'		
14	Phosphatidic acid	PA F: 5'-GTTTGCTGGTGCTACTTTGG-3'	62	104
		PA R: 5'-CGAACCTACTCTCCTCAATCAC-3'		
15	Osmotin precursor	OSM F: 5'-ATCAGTACTTGCAGGCATCTC-3'	62	100
		OSM R: 5'- ATTTACTTGCCCTGGTGGTAG-3'		
	18sRNA	5'GGTCGCAAGGCTGAAACT3'	62	95
		5'ACGGGCGGTGTGTACAAA3'		

Gene Expression Analysis by Semi-Quantitative RT-PCR

Total RNA was isolated from bark samples according to the procedure of Venkatachalam *et al.* (2009), treated with DNaseI, and subsequently, reverse transcription of total RNA was carried out as per the manufacturer protocol. The cDNA (2 µL) was then used for PCR amplification in a solution with 0.5 µM of gene-specific primers designated for each cDNA, 2.5 µL 10 × buffer, 2.5 µL dNTPs (2.5 mM), 2.5 µL $MgCl_2$ (1.5 mM), and 1-U Taq DNA polymerase (Promega). PCR amplification was carried out for 4 minutes at 94°C, followed by 25 cycles of 94°C for 30 sec, 50-55°C for 1 minute, and 72°C for 2 minutes. The final extension was performed at 72°C for 7 minutes. The primer sequences and optimal PCR annealing temperatures are depicted in Table 1. At least three replications were conducted using independently isolated total RNAs. cDNA samples were separated by electrophoresis on 1.5% (w/v) agarose gel, and the images of the ethidium bromide-stained bands were obtained with the Gel Documentation Imaging system. The integrated density of each band was corrected with the corresponding housekeeping gene data, and normalized data were used for quantification analysis. A constitutively expressed 18S rRNA gene (forward:5'-GGTCGCAAGGCTGAAACT-3' and reverse:5'-ACGGGCGGTGTGTACAAA-3') was used as an internal control for RT-PCR analysis according to the protocol of Zhu and Altmann, (2005).

Statistical Analysis

All the data were analyzed with SPSS software, and the differences between healthy and TPD-affected trees were analyzed by one-way ANOVA and Student-Newman Keul's

Test at p <0.05%. The value of p <0.05 was considered to be a significant difference. Each value was the average of two biological replicates tested in triplicate.

Results And Discussion

Semi-quantitative RT-PCR results clearly showed that Among the nine candidate genes, the expression level of HBT, SOD, Myb1, TCTP1 ABC, PT2, and REF1 genes was more abundant in a healthy tree and down-regulated in TPD-affected clones. The accumulation of ASR and VHA gene mRNA transcripts was up-regulated in TPD-affected trees than in healthy clones (Figs. 1 and 2). A quantitative real-time RT-PCR analysis was also performed to verify the expression pattern of eight putative candidate genes in different high-yielding (RRII 105, PCK2, PB 217, and GT 1) and low-yielding clones (PB 5/51, PB 235, and PB 285) of healthy and TPD rubber trees. The genes were selected from a range of functional categories, with the most prominent classes being metabolism, cell signaling, stress, and cell defense. All primers were used to amplify the cDNA of healthy and TPD rubber trees. Melting curve analysis indicated that each primer pair amplified a single major product (Fig. 3). The mRNA transcripts levels of eight candidate genes in healthy and TPD clones were investigated. Most genes' relative expression patterns were similar in both high- and low-yielding clones. Among the mRNA transcripts, ASR and VHA genes were up-regulated in the TPD condition. In contrast, the remaining six candidate genes were downregulated in TPD-affected clones as compared to the healthy trees (Fig. 4).

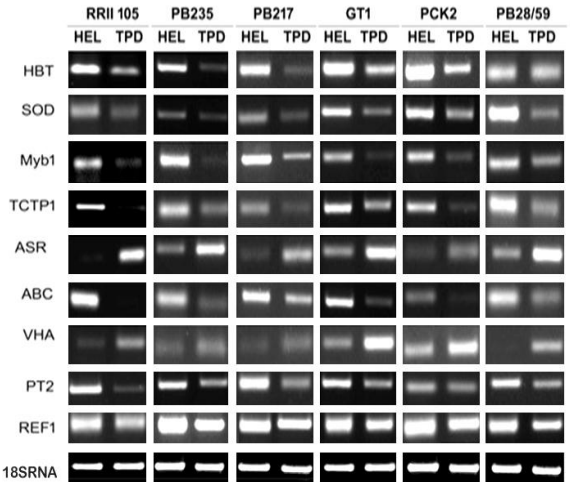

Fig. 1: Semi-quantitative RT-PCR analysis of 9 candidate gene expression patterns in high and low-yielding rubber clones. The values are the means of three independent experiments ± SE

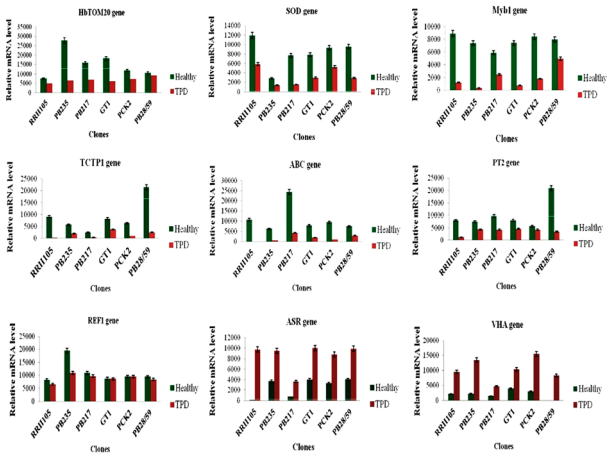

Fig. 2: Accumulation level of mRNA transcripts of 9 candidate genes in high and low-yielding rubber clones. The values are the means of three independent experiments ± SE

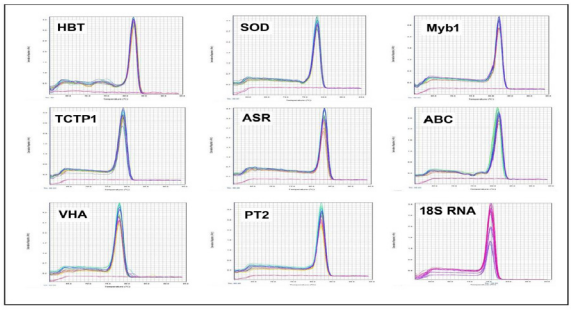

Fig. 3: Melt curves with a single peak generated for each of 8 candidate target genes in high and low-yielding rubber clones

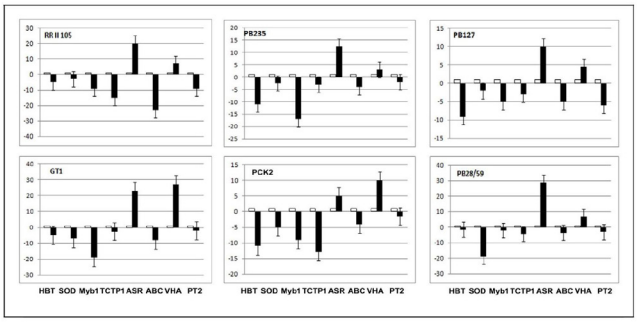

Fig. 4: Validation of differentially expressed (Up-regulation/Down-regulation) genes using RT-qPCR in different high-yield and low-yielding clones of healthy and TPD-affected rubber trees. The values are the means of three independent experiments ± SE

HBT- *Hevea brasiliensis* TOM-20 (Translocase of the Outer Mitochondrial Membrane)
SOD- Super oxide dismutase
MYB1- MYB transcription factor
TCTP1- Translationally controlled tumor protein
ASR- ASR like protein
ABC-ABC transporter
VHA - Vacuolar H+ATPase subunit E
PT2 - Phosphate transporter 2
REF1 -REF, like stress-related protein

Fifteen differentially expressed candidate genes were selected from SSH (Suppression et al. technique) to analyze their expression pattern under TPD conditions in various rubber clones (RRII 105, RRII 285, PCK2, PB 235, PB 5/51, PB 217, and GT 1). The selected genes were Annexin-like protein RJ4 (ALP), ASR-like protein 1 (ASR-1), Calcium-binding protein(CBP), Cysteine protease(CP), Ethylene biosynthesis protein (EBP), Translocase outer mitochondrial membrane protein TOM20, Typell metacaspase (T2M), F-box family protein (FBP), Heat shock protein(HSP), MYB transcription factor (MYB), EF-like

stress related protein1(RLP1), Thioredoxin (THRD), Translationally controlled tumor protein (TCTP), Phosphatidic acid (PHD), Osmotin precursor (OSP). The fold change in the expression of genes in six rubber clones was summarized in Table 2. Among the clones, PB235 rubber clones affected with TPD showed up-regulation of all candidate gene expression except TOM20, MYB, and TCTP genes. Among the genes tested, Ph.D., OSP, and ASR-1 showed approximately a seven-fold increase compared to the healthy clones (Fig. 5A). In contrast, TPD affected PB 217 clone showed no induction or downregulation of selected genes as compared to the control plants (Fig. 5C). In PB 285 TPD clones only three genes, i.e., ALP, ASR-1, and CP displayed significant induction. These genes exposed about two-fold increases over the healthy plants whereas other genes were not significantly induced except TOM20, MYB, and TCTP were down-regulated (Fig. 5B). The high-yielding clone RRII 105 showed mixed result among fifteen genes, three genes CP, EMB, and HSP were up-regulated over the control sample, whereas ALP, CPB, T2M, FPB, RLP1, MYB, PHD, TCTP, TOM20, and OSP were down-regulated and reaming genes ASR-1and THRD did not show any significant difference (Fig. 5D). The clone PB5/57 result almost mimicked as RRII105; the genes such as CP, EMB, HSP, OSP, and THRD were up regulated when compared to the control healthy plants and remaining genes were down regulated (Fig. 5E). In case of PCKT2 clone, no gene showed significant induction except THRD which is about 1.9 folds. Genes, namely ALP, CBP, TOM20, T2M, FBP, MYB, Ph.D., TCTP, and OSP, were down-regulated, whereas ASR-1, CP, HSP, and RLP1 showed no significant induction over the control healthy plants (Fig. 5F).

Annexins is a plant multigene family with several members, eight in *Arabidopsis thaliana* (Cantero *et al.,* 2006) and nine in *Oryza sativa* (Moss and Morgan, 2004). In a more direct way, plant annexins could respond to oxidative stress at the membrane. By lowering the lipid packing order inside the membrane, oxidative stress in the form of ROS affected the structural integrity of phospholipid membranes. (Megli and Sabatini, 2003; Megli *et al.,* 2005). It is an intriguing thought that plant annexins may function in this process of membrane resealing and up-regulate in plants (Draeger *et al.,* 2011), and there is general agreement about the protective involvement of plant annexins in oxidative stress response and post-translational modification (Konopka-Postupolska *et al.,* 2011) Plant annexins are involved in oxidative stress responses (Richards *et al.,* 2014; Deng *et al.,* 2016) and abiotic stress responses (Huh *et al.,* 2010). Their involvement in membrane organization, vesicle trafficking, and signaling is basic to cellular processes such as growth, differentiation, secretion, and repair (Clark *et al.,* 2012). Their expression is regulated developmentally and by a number of different environmental stimuli, such as biotic and abiotic stresses. Proteomic studies have found that annexins are up-regulated by salinity in a wide variety of plant species (Laohavisit *et al.,* 2009; Zhang *et al.,* 2012). Transcript levels of annexin genes in Arabidopsis vary depending on age and tissue type, suggesting a specific role at different developmental stages of the plant (Clark *et al.,* 2005). Annexin expression increases during fruit ripening, revealing hormonal control in plants (Vandeputte *et al.,* 2007), and AnxAt7 gene expression in Arabidopsis is also up-regulated (Mortimer *et al.,* 2008).

In different species, *ASR* genes are expressed in different organs under different conditions and expression patterns (Vaidyanathan *et al.,* 1999; Maskin *et al.,* 2001). In response to abiotic stresses, ASR genes are involved in plant development

Table 2: Summary of gene fold change in expression in various rubber clones affected by TPD syndrome. The data were collected from the real-time PCR analyses and shown as averages ± SE. The expression level of each gene in the healthy tree was defined as 1.0 fold

Sno	Gene name		Rubber Clones					
			PB235	RRII 285	PB217	RRII 105	PB5/57	PCKT2
1	Annexin-like protein RJ4	*ALP*	↑2.714	↑2.703	→1.156	↓0.923	↓0.961	↓0.386
2	ASR like protein 1	*ASR-1*	↑7.665	↑1.953	→1.009	→1.004	↓0.914	→1.576
3	Calcium-binding protein	*CBP*	↑5.977	→1.519	↓0.654	↓0.245	↓0.517	↓0.471
4	Cysteine protease	*CP*	↑3.175	↑2.013	↓0.784	↑3.692	↑2.607	→1.805
5	Ethylene biosynthesis protein	*EBP*	↑3.39	→1.752	↓0.218	↑2.748	↑5.664	→1.642
6	TOM20	*TOM20*	↓0.24	↓0.553	↓0.597	↓0.38	↓0.488	↓0.549
7	TypeII metacaspase	*T2M*	↑3.5	→1.288	↓0.989	↓0.948	↓0.916	↓0.633
8	F-box family protein	*FBP*	↑2.704	→1.4	→1.556	↓0.45	→1.513	↓0.544
9	Heat shock protein	*HSP*	↑4.758	→1.255	↓0.483	↑3.72	↑3.292	→1.082
10	MYB transcription factor	*MYB*	↓0.328	↓0.551	↓0.664	↓0.297	↓0.496	↓0.404
11	REF-like stress relateed protein1	*RLP1*	↑5.281	→1.225	↓0.797	↓0.723	↓0.724	→1.559
12	Thioredoxin	*THRD*	↑5.724	→1.761	↓0.56	→1.186	↑4.116	↑1.937
13	Translationally controlled tumor protein	*TCTP*	↓0.287	↓0.288	↓0.653	↓0.272	↓0.42	↓0.266
14	Phosphatidicacid	*PHD*	↑6.407	→1.251	↓0.879	↓0.861	↓0.728	↓0.467
15	Osmotin precursor	*OSP*	↑6.364	→1.695	↓0.365	↓0.5	↑5.078	↓0.238

↑ Up-word arrow indicate up regulation; ↓ down word arrow indicated down regulatior

→ horizontal arrow indicated no significant induction

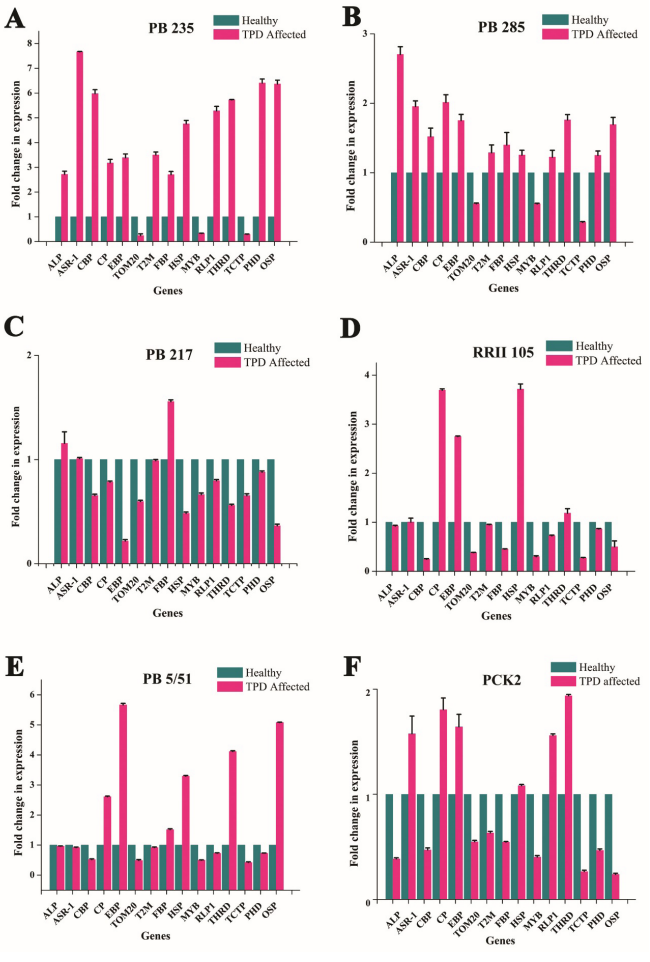

Fig. 5: Expression analysis of differentially expressed genes in TPD affected rubber clones. Total RNA was isolated from latex cells of healthy and TPD trees of various rubber clones and was used for qRT-PCR analysis, and 18S rRNA served as an internal control. The data were collected from the real-time PCR analyses and shown as averages ± SE. The expression level of each gene in the healthy tree was defined as 1.0 fold

processes (Jeanneau *et al.,* 2002; Çakir *et al.,* 2003). Both TCTP and its homologs have been reported from many eukaryotic organisms; Few references are available for plant species even though sequences of several plant TCTP homologs have been deposited in public databases. A first plant TCTP homolog was reported by Sage-Ono *et al.* (1998) from a short-day plant Pharbitis nil cv. Violet. Its latex-specific expression suggests that it has some biological functions associated with latex. The rubber elongation factor (REF) is an enzyme involved in rubber biosynthesis and is highly expressed in laticifers (Goyvaerts *et al.,* 1991). The expression levels of other rubber synthesis-associated genes, such as small rubber particle protein (SRPP) (Oh *et al.,* 1999) and HMG-CoA reductase (Chye *et al.,* 1992), are also high in the latex (Han *et al.,* 2000). A homolog of the antifungal protein osmotic was isolated from rubber clones which are less susceptible to fungal diseases. Osmotin is an abundant cationic multifunctional protein discovered in cells of tobacco. Besides its role as an osmoregulator, it provides plants protection from pathogens. The osmotin-induced proline accumulation has been reported to confer tolerance against both biotic and abiotic stresses in plants overexpressing the osmotin gene. osmotic could be regulating these plant responses through its involvement either as a transcription factor, cell signal pathway modulator, or both in plants. Osmotin is a stress-responsive multifunctional protein involved in plants' osmotolerance (Zhu *et al.,* 1995). The Osmotin gene showed higher proline content in transformed plants under stress and normal conditions.

The protein HbTOM20 has 32% and 27% similarity to *Populus* TOM20 and *Solanum* TOM20, respectively. In this current study, both RT-real-time PCR and Semi-Quantitative RT-PCR results revealed that the HbTOM20 expression was significantly up-regulated in healthy trees compared to TPD-affected ones. These results suggest that the down-regulation of HbTOM20 in TPD-affected trees may play an important role in the alteration of mitochondrial metabolism resulting in impaired latex biosynthesis. The multiple sequence alignments of HbTOM20 with other known plant TOM20 genes revealed that the HbTOM20 exhibits 32% with *Populus trichocarpa* (EF144334), 27% similarity with *Solanum tuberosum* TOM20 (X92491) & *Elaeis guineensis* (EU285004), 26% with *Arabidopsis thaliana* TOM20 (AJ296024), and 24% with *Picea sitchensis* (EF081837). RT-PCR results indicated that there is a 5-fold increase of HbTOM20 gene transcripts in the healthy tree compared to TPD-affected one. Both RT-real-time PCR and Semi-Quantitative RT-PCR showed that the accumulation of mRNA transcript of HbTOM20 was decreased by 2–5 folds in TPD affected tree, compared to a healthy one. Interestingly, the percentage of TPD occurrence in different clones is proportional to the accumulation of HbTOM20 mRNA transcript level of these clones. HbTOM20 is a member of the TOM20 family with a distinct TOM20 domain located at the N terminus of the deduced polypeptide (Werhahn *et al.,* 2001). These results suggest that the development of TPD is associated with the suppression of HbTOM20 expression. Mitochondrial function has been impacted in TPD-affected trees by a decrease in HbTOM20 translocase that could interrupt the import of nuclear protein from cytosol to mitochondria (Venkatachalam *et al.,* 2009). Biochemical evidence has further supported the hypothesis that the down-regulation of TOM20 in TPD trees may be associated with a low yield of latex biosynthesis or flow. Down-regulated expression of HbTOM20 showed a positive correlation with the TPD development in rubber trees (Venkatachalam *et al.,* 2009). Cloning of a TOM20-like protein gene (Chen *et al.,* 2003) and a Myb transcription factor HbMyb1 implicated that PCD (Programmed Cell Death) in bark cells and/or dysfunction of mitochondrial metabolism possibly results in TPD occurrence (Venkatachalam *et al.,* 2009; Liu *et al.,* 2015). HbMyb1 maybe suppress stress-induced cell death in rubber trees. HbMyb1 was expressed more strongly in healthy rubber trees' latex than TPD trees in all test clones. The expression levels of HbMyb1 in different clones were varied in accordance with the TPD tolerance of the respective clones (Chen *et al.,* 2003). In the regulation of xylem, phloem, and endosperm differentiation in the plant through PCD processes, Myb transcription factors play an important role (Demura & Fukuda, 2007; Peng *et al.,* 2011). *HbMyb1* is expressed in leaves, barks, and latex of rubber trees, but its expression is significantly decreased in the barks of TPD trees. Earlier reports suggest that the function of HbMyb1 is associated with the integrity of bark tissue of rubber trees due to the expression of *HbMyb1* is likely associated with TPD.

HbMyb1 showed considerable homology to other plant-my-related transcription factors, suggesting that HbMyb1 may act as the transcription factor involved in TPD. Researchers propose that HbMyb1 acts as a negative regulator for PCD-induced genes; the intense tapping and ethylene stimulation result in decreased expression of *HbMyb1* and, thus increased expression of genes required to manifest programmed cell death in bark cells (Chen *et al.,* 2003). MYB family proteins control a variety of cellular metabolism in plants as well as expression of HbMyb1 (Chen *et al.,* 2003) and HbTOM20 were significantly decreased in TPD trees (Venkatachalam *et al.,* 2007; Venkatachalam *et al.,* 2009). Few references on MYB genes in rubber trees suggested that MYB genes might be involved in regulating plant development and/or latex biosynthesis, and its expression was significantly decreased in TPD rubber trees (Chen *et al.,* 2003). In plants, the over-expression of Hsp genes and other stress-related genes during stress conditions cause depressing other multiple metabolic genes also and thus affects plant normal growth and development (Fusco *et al.,* 2005; Wang & Bughrara, 2007; Tang *et al.,* 2013). Venkatachalam *et al.* (2007) also reported that two members of the heat shock proteins were up-regulated in the TPD tree, and it was found that heat shock protein protected cells from apoptosis (Li *et al.,* 2010). F-box proteins are reported to be involved in the regulation of various developmental processes in plants, such as leaf senescence, flowering, branching, phytochrome and phytohormone signaling, circadian rhythms, and self-incompatibility (Rahman *et al.,* 2013). The studies indicated that F-box proteins are involved in the important biological processes of plant growth and development, and their results revealed that most of the F-box protein genes showed a low-level expression compared with four housekeeping genes in various tissues. F-box genes organize the largest multigene superfamilies and regulate many important biological functions. plant F-box genes have appeared genetically to regulate many crucial processes, such as hormonal responses, pathogen resistance, seedling development, senescence, floral organogenesis, and embryogenesis (Xu *et al.,* 2009).

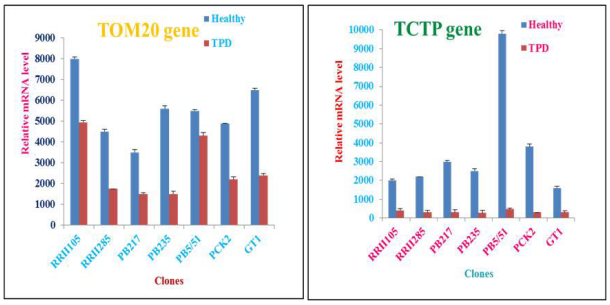

Fig. 6: Accumulation of mRNA transcripts level of TOM20 and TCTP genes in bark tissues of healthy and TPD affected various rubber clones. The values are the means of three independent experiments ± SE

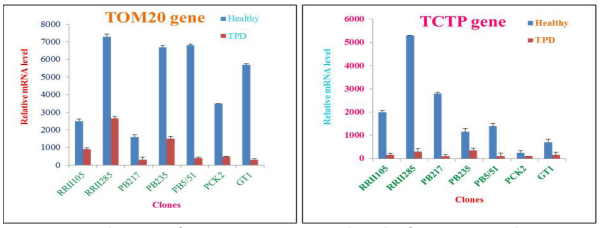

Fig. 7: Accumulation of mRNA transcripts level of TOM20 and TCTP genes in laticiferous cells of healthy and TPD-affected various rubber clones. The values are the means of three independent experiments ± SE.

Among the differentially expressed genes analyzed in TPD-affected clones, the accumulation of mRNA transcript levels of TOM20, TCTP, and MYB genes on RT-real-time PCR was found to be high in high-yielding clones (RRII 105, PCK2, PB 217, and GT 1) compared to the low yielders (PB 5/51, PB 235, and PB 285) in healthy rubber tree. In addition, among the high-yielding clones, the abundance of mRNA transcript levels was more in RRII105, followed by other clones (Fig. 5). An accumulation of mRNA transcript levels of TOM20 and TCTP genes was found to be high in bark tissues of healthy clones compared to the TPD affected rubber clones (Fig. 6). Among the healthy rubber clones, the mRNA transcript level of TOM20 gene was more abundant in RRII105 followed by other clones, while in the case of TPD trees, the gene expression level was substantially down-regulated almost in all the clones used. The level of mRNA transcripts of the TCTP gene was more in healthy clones, whereas the expression level was downregulated, and only base-level expression was noticed in almost all the TPD-affected clones tested (Fig. 6).

RT-real-time PCR results clearly further support and suggest that the level of TOM20 and TCTP gene expression was found to be high in laticiferous cells of healthy clones compared to the TPD-affected rubber clones in general (Fig. 7). The abundance of mRNA transcripts level of the TOM20 gene was more in RRII285, followed by other healthy clones. In contrast, in the case of TPD trees, a decreased level of gene expression was noticed in all the clones used compared to the healthy clones. The abundance of TCTP gene transcripts was more in healthy clones, while a downregulated mRNA transcript level was noticed in almost all the TPD-affected clones tested, and base-level expression was observed in RRII105 and PCK2 clones (Fig. 7).

Conclusions

The study revealed that the expression of genes associated with TPD syndrome is differentially regulated during the onset of TPD syndrome, and the mRNA transcript level of genes varied, and it depends on the TPD condition and genetic stability of the rubber clones. Among the eight candidate genes, five genes, viz., TOM 20, TCTP, Myb 1, ABC, and PT2, were down-regulated in the TPD tree compared to the healthy tree, according to Semi-Quantitative RT-PCR analysis. RT-real-time PCR studies revealed that among 15 genes screened, the mRNA transcript level of 3 genes (TOM 20, TCTP, and Myb 1) was down-regulated significantly in various TPD-affected clones of rubber tree compared to healthy ones. The current molecular investigation unequivocally concluded that the three genes TOM 20, TCTP, and Myb 1 play a crucial role as molecular markers in screening the development of TPD in rubber trees and preventing the loss of latex biosynthesis caused by TPD syndrome.

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Author Contributions

The following author's contributions are Indra Arulselvi Padikasan and Geetha Natesan: Who conceived and designed the experiments. Ganesh Babu Irulappan: Performed the experiments. Indra Arulselvi Padikasan: analyzed the data. Ganesh Babu Irulappan: wrote the paper.

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