Identification of a differentially expressed thymidine kinase gene related to tapping panel dryness syndrome in the rubber tree (*Hevea brasiliensis* Muell. Arg.) by random amplified polymorphic DNA screening

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Abstract

Tapping panel dryness (TPD) syndrome is one of the latex yield affecting factors in the rubber tree (*Hevea brasiliensis* Mull. Arg.). Therefore, identification of a DNA marker will be highly useful for screening progenies in breeding programs. The major goal of this study was to detect genetic variations and/or identification of gene fragments among 37 *Hevea* clones by the random amplified polymorphic DNA "fingerprinting" technique. Different levels of DNA polymorphism were detected with various primers and a distinct polymorphic band (2.0 kb) was obtained with OPA-17 primer. It was cloned into a plasmid vector for further sequence characterization and the nucleotide sequence shows homology with a novel putative plant thymidine kinase (TK) gene, designated as *HbTK* (*Hevea brasiliensis* thymidine kinase; GenBank accession number AY130829). The protein HbTK has 67%, 65%, 64%, and 63% similarity to TK genes of *Medicago, Oryza, Arabidopsis*, and *Lycopersicon*, respectively, and it was highly conserved in all species analyzed. The predicted amino acid sequence contained conserved domains of TK proteins in the C-terminal half. Southern blot analysis indicated that *HbTK* is one of the members of a small gene family. Northern blot results revealed that the expression of the *HbTK* gene was up-regulated in mature bark tissues of the healthy tree while it was down-regulated in the TPD-affected one. These results suggest that this gene may play important roles in maintaining active nucleotide metabolism during cell division at the tapped site of bark tissues in the healthy tree under stress (tapping) conditions for normal latex biosynthesis.

Introduction

Natural rubber (cis-1,4-polyisoprene) is produced in latex at the expense of high energy cost, and is considered as a secondary metabolite with no known function in plant cells. Latex is the cytoplasmic fluid of laticiferous cells that contain the usual organelles of plant cells such as nucleus, mitochondria, vacuoles, ribosomes, Golgi apparatus, and endoplasmic reticulum.[1] Rubber is an important raw material for many industrial uses requiring elasticity, flexibility, and resilience. Although over 2000 species of higher plants are recognized for producing latex,[2] only the rubber tree (*Hevea brasiliensis* Muell. Arg. 2n=36) has been established as a key commercial rubber source owing to its good yield of rubber and the excellent physical properties of the rubber products.[2] Apart from its latex, the rubber tree has also been harnessed for its wood for making furniture and the seeds for para-rubber seed oil used for manufacturing soap, paint, varnishes, fertilizer, and animal feeds. Exploitation of these other components of the rubber tree added further value to the planting of rubber trees. The rubber is harvested by tapping into the pipe-like network of latex-containing laticifers that run beneath the bark; a labor-intensive procedure. The expense of tapping and tropical growth requirements of the tree makes *Hevea brasiliensis* unsuitable in the United States and other cold countries.

The rubber tree is cultivated on a large commercial scale in several tropical countries including India, amounting to 9.5 million hectares, producing about 8.7 million tons of dry rubber each year worldwide.[3] The diminishing acreage of rubber plantations and various stresses to *Hevea* coupled with an increasing demand have triggered research interests in the study of the development of molecular markers for biotic and abiotic stresses.[4] In the rubber tree, tapping panel dryness (TPD) is one of the important syndromes that greatly affect the latex yield. Once TPD occurs, the tapping panel region of the rubber tree is partly or entirely dried and the latex biosynthesis is decreased significantly or stops completely. The incidence of TPD occurs in 20-50% of the trees in almost every rubber planting country. It is estimated that the loss of dry rubber owing to TPD is 15-20% of the annual rubber production. Despite the number of studies attempted so far, the original cause for the onset of TPD still remains poorly understood. As different clones of rubber tree showed varying degrees of susceptibility to TPD in field tests, the gene associated with TPD syndrome is yet to be identified.[5]

With the advent of recombinant DNA technology, different types of molecular markers became available to breeders, geneticists, and germplasm specialists. Molecular markers serve as useful aids in understanding the genetics of the rubber tree, and already they have been applied to investigate the genetic variations between clones and tagging genes in the recent past.[4] A range of molecular marker systems is available to detect DNA polymorphism. Random amplified polymorphic DNA (RAPD) "fingerprinting" yields patterns of DNA fragments that reflect differences in template sequence,[6] which is straightforward and reproducible.[7] In addition to their value in mapping, map-based cloning, MAS, and gene "tagging" studies, RAPDs have been deployed in various plant species including *Hevea* for detection of genetic variations.[4,7-10] Presence or absence of DNA bands in the gel may be used as RAPD markers to study inter- and intra-specific genetic variation[11] for the identification of specific genes[4,12,13] and to study the pattern of gene expression.[14] Although few protein markers associated with TPD have been reported,[15-17] no DNA marker is available for identification and/or early detection of TPD syndrome in this important commercial tree crop at present.

It has been reported that TPD is genetically controlled in *Hevea*,[5] and there is still the possibility of developing DNA markers for this syndrome. The main focus of our present study is to detect genetic variations within 37 cultivated rubber clones using RAPD fingerprint-

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ing. This paper reports identification, cloning, and sequence characterization of a polymorphic band, and its expression pattern is correlated between healthy and TPD-affected rubber trees to consider it as a potential molecular marker for the detection of the TPD syndrome.

Materials and Methods

Plant material

In total, 37 clones originating from six countries were used in this investigation. These clones had genetic variation in several desirable traits including high yield potential, tolerant and/or susceptible to leaf diseases, TPD, wind damage, drought, and cold (Table 1). Plants selected for this experiment were cultivated in the nursery as well as experimental field at the Rubber Research Institute of India (RRII). Fully expanded and disease-free leaves on the main stem were utilized for genomic DNA extraction.

Genomic DNA extraction

Total genomic DNA was isolated from young leaves according to the procedures described by Venkatachalam *et al.*[9] Briefly, fresh leaf tissue (1 g) was ground to a fine powder under liquid nitrogen in a mortar-and-pestle and homogenized in DNA isolation buffer [2% cetyltriethylammonium bromide (CTAB); 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), 1% polyvinyl polypyrolidone (PVPP), 1% 2-mercaptoethanol]. The homogenate was kept in a water bath at 65°C for 30 min and the tubes were agitated frequently. The extracts were centrifuged for 15 min (8000 rpm) and the supernatant was transferred to fresh centrifuge tubes and re-extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and spun at 10,000 rpm for 10 min. The top aqueous phase was transferred carefully to new tubes and incubated at 37°C for 1 h after the addition of 10 μL of RNase A (10 mg/mL). The samples were extracted with an equal volume of chloroform (100%) and spun at 10,000 rpm for 5 min and re-extracted until a clear aqueous phase was obtained. The DNA was precipitated with 0.6 volume of ice-cold isopropanol (100%). After 15 min of centrifugation at 10,000 rpm, the DNA pellet was washed with 70% (v/v) ethanol, air dried, and dissolved in about 300 μL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). DNA was quantified by comparison with ethidium bromide-stained standard concentrations in 0.8% (w/v) agarose gels and stored at -20°C until used for polymerase chain reaction (PCR) amplification.

Table 1. Morphological characteristics of 37 cultivated rubber tree (*Hevea brasiliensis*) clones used in this study.

No.	Clones	Major characteristics	Parentage	Origin
1.	RRII105	High yield, ALF tolerance, TPD severe	Tjir1 x Gl1	India
2.	RRIM600	High yield, ALF susceptible, TPD susceptible	Tjir1 x PB86	Malaysia
3.	PB217	Average yield, TPD mild, PM severe, ALF low	PB5/51 x PB6/9	Malaysia
4.	PB235	High yield, TPD mild, PM severe, ALF medium	PB5/51 x PB S/78	Malaysia
5.	PB255	High yield, ALF susceptible, PM tolerance, TPD medium	PB5/51 x PB32/36	Malaysia
6.	PB260	High yield, ALF medium, PM severe, TPD medium	PB5/51 x PB49	Malaysia
7.	PB280	High yield, ALF medium, PM severe, TPD mild	PBIG seedlings	Malaysia
8.	PB310	PM susceptible, TPD medium	RRIM600 x PB5/51	Malaysia
9.	PB311	High yield, PM medium, TPD mild	RRIM600 x PB235	Malaysia
10.	PB312	Average yield, TPD mild	RRIM600 x PB235	Malaysia
11.	PB314	ALF tolerance, TPD mild	RRIM600 x PB235	Malaysia
12.	SCATC93-114	Cold tolerance	TR31-45xHaiken3-11	China
13.	Haiken1	Wind tolerance, TPD mild	Primary clone	China
14.	KRS25	ALF average, TPD medium	Primary clone	Thailand
15.	KRS128	ALF average	RRIM501 x PB5/63	Thailand
16.	KRS163	ALF tolerance	RRIM501 x PB5/65	Thailand
17.	RRII201	High yield, TPD tolerance, ALF susceptible	Tjir1 x PB25	India
18.	RRII202	High yield, TPD susceptible	PB86 x Mil3/2	India
19.	RRII203	High yield, disease tolerance, timber clone	PB86 x Mil3/2	India
20.	RRII204	High yield, TPD mild, disease susceptible	PB86 x Mil3/2	India
21.	RRII205	Average yield, TPD tolerance	PB86 x BD10	India
22.	RRII206	High yield, TPD tolerance	Mil3/2 x AVROS255	India
23.	RRII207	Average yield, disease tolerance, TPD susceptible	Mil3/2 x AVROS255	India
24.	RRII208	Moderate yield, cold tolerance, wind damage low, TPD medium	Mil3/2 x AVROS255	India
25.	RRII209	High yield, wind damage high, disease susceptible, TPD mild	Mil3/2 x BD10	India
26.	PR107	Average yield, ALF susceptible, PM, wind tolerance	Primary clone	Indonesia
27.	PB5/63	Wind damage, Gloeosporium susceptible	PB56 x PB24	Malaysia
28.	Mil3/2	Low yield, disease tolerance	Primary clone	Sri Lanka
29.	BD10	TPD medium, PM tolerance	Primary clone	Indonesia
30.	AVROS255	Low yield, ALF and PM susceptible	Primary clone	Indonesia
31.	Tjir1	ALF and PM susceptible, TPD medium	Primary clone	Indonesia
32.	PB6/9	High yield, TPD medium	PB24 x PB28	Malaysia
33.	Gl1	ALF tolerance, TPD susceptible, drought tolerance	Primary clone	Malaysia
34.	PB86	ALF susceptible, PM tolerance, TPD mild	Primary clone	Malaysia
35.	RRIM501	High yield, TPD tolerant, Pink and wind damage susceptible	PilA44 x LunN	Malaysia
36.	PB5/51	ALF tolerance, PM susceptible, TPD mild	PB86 x PB24	Malaysia
37.	PB25	TPD & wind damage susceptible	Primary clone	Malaysia

ALF, abnormal leaf fall; PM, powdery mildew; TPD, tapping panel dryness.

Random amplified polymorphic DNA fingerprinting and gel electrophoresis

RAPD reactions were carried out with different oligonucleotide primers (Operon Technologies Inc., USA). PCR reaction mixtures (20 μL) consisted of 10 mM Tris-HC1, 50 mM KCl, 1.5 mM each dNTP (dATP, dCTP, dGTP, dTTP), 250 nM primer, 1.5 mM MgCl, 10 ng DNA template, and 0.5 units (U) *Taq* DNA polymerase, overlaid with 50 μL of mineral oil to prevent evaporation. RAPD reactions were performed in a thermal cycler (Perkin Elmer 480) with the following PCR conditions: 94°C for 4 min followed by 39 cycles of 94°C for 1 min, 37°C for 1.30 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Reaction products were separated by electrophoresis in 1.5% (w/v) agarose gels using a Tris-Borate-EDTA (TBE) buffer system.[18] Permanent records were obtained by photographing ethidium bromide-stained gels under UV light. Band sizes were determined by comparison with the DNA ladder (λ-DNA double digest with *Hind*III/*Eco*RI, Bangalore Geni., Bangalore) in each gel. Reactions were kept at 4°C until analyzed

and four repeat reactions were carried out to ensure the fidelity and reproducibility of results. For each clone, the presence and/or absence of fragments was recorded as 1 or 0, respectively. The selection of polymorphic bands for inclusion in the data set was based on band reliability, clarity, signal strength, and resolution. Faint RAPD bands were not considered in this analysis.

Random amplified polymorphic DNA data analysis

All bands were scored as either present or absent. Polymorphisms at most of the loci were confirmed by repeating tests on all the relevant genotypes. A conservative approach to scoring of the amplified fragments was adopted and only consensus bands were included for the final analysis. Individual amplified bands were indicated by the primer used, its size in bp. Data were scored for computer analysis on the basis of the presence or absence of the amplified product of a given length. We retained only the major fragments that were unambiguously present. If a product was present in a genotype it was designated "1"; if absent it was designated "0". Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products were used to generate Jaccord's similarity coefficients. The matrix of similarities was used to construct a dendrogram according to the UPGMA (unweighted pair-group method with arithmetical average) using a computer program.

Cloning of a polymorphic band and nucleotide sequence analysis

One of the polymorphic DNA fragments amplified from TPD- tolerant clone PB235 was excised from low melting agarose gel and the DNA was purified using the standard protocol.[18] The DNA fragments were ligated into the pGEM-T easy vector (Promega Inc., USA) and cloned into a competent *Escherichia coli* (DH5α) strain. The selection of transformed clones was performed by PCR analysis using white colonies directly as templates. Positive colonies were grown overnight in 10 mL of Luria Bertani (LB) medium containing 50 μg/mL ampicillin. To confirm the presence of the cloned DNA fragment, purification of plasmid DNA was carried out from five independent transformed clones using a mini preparation. The DNA insert size was checked by double digestion with Not I enzyme followed by separation in a 1.5% (w/v) agarose gel. The complete sequence of cloned fragment was obtained using an Automated DNA sequencer (Indian Institute of Science, Bangalore, India). The nucleotide sequences were analyzed for homologies in the GenBank data base using the BLAST programme at NCBI (*http://www.ncbi.nlm.hih.gov*).[19] Alignment of amino acid sequences and phylogenetic tree analysis were performed using the ClustalW program.[20]

Genomic Southern hybridization analysis

Total genomic DNA extracted from the PB235 clone was digested with different restriction enzymes (*Hind*III, *EcoR*I, *EcoR*V, *BamH*I, and *Xba*I) and digested DNA fragments were electrophoresed on 1.0% (w/v) agarose gel in TBE buffer (0.045 M Tris-borate and 0.001 M EDTA) at 50 V for 6 h. After depurination in 0.25 M HCl for 10 min, denaturation of the DNA in the gels was carried out in a buffer containing 1.5 M NaCl and 0.5 M NaOH for 30 min, followed by neutralization in 1.5 M NaCl and 1.0 M Tris-HCl (pH 7.4) buffer for 30 min. The DNA was then transferred onto a nylon membrane (Hybond N^+, Amersham-Pharmacia, UK) in 10×SSC (1×SSC is 0.15 M NaCl, 0.015 M trisodium citrate) for 18 h.[18] After DNA transfer, the nylon membranes were rinsed in 2×SSC buffer, UV-crosslinked, and stored at 4°C until use. The TK gene fragment was amplified from the plasmid that contained the RAPD fragment by PCR, and the purified DNA fragment (25 ng) was used for labeling. The radioactive probe was synthesized with [α-^{32}P]-dCTP (BARC, Trombay, Mumbai, India, 4000 Ci/mmol) using the random primer labeling kit (Amersham-Pharmacia, UK) according to the manufacturer's protocol, followed by purification with Sephadex G-50 spin columns. The nylon membranes with DNA were placed in hybridization bottles and prehybridized for 2 h (hybridization buffer is 6×SSC, 5×Denhardt's, 0.5% SDS, 0.2 mg/mL salmon sperm DNA) at 65°C. Then the denatured [α-^{32}P]-dCTP TK gene probe was added into the prehybridization buffer and hybridization was continuously performed at 65°C for 20 h in a rotary hybridization oven (Amersham-Pharmacia, UK). After completion of hybridization, membranes were washed at low stringency at room temperature twice in 2×SSC + 0.1% SDS for 5 min and 1×SSC + 0.1% SDS for 15 min and high stringency at 65°C, once in 0.5×SSC + 0.1% SDS for 15 min and 0.1×SSC + 0.1% SDS for 15 min, followed by radioactive signal generation. Then the labeled blots were exposed to X-ray film (X-Omat, Kodak) with intensifying screens at -80°C for 1-3 days.

Total RNA isolation and Northern blot analysis

For Northern blot analysis, high latex yielding rubber clones PB235 (TPD tolerant) and RRII 105 (TPD susceptible) were selected and trees regularly tapped [tapping is a controlled shaving of a thin bark section (about 2 mm) with a special kind of tapping knife under d/2 (trees are being wounded alternate days for latex collection) tapping system for latex collection for the past 13 years]. Some of these trees developed TPD syndrome, but tapping them was continued along with the healthy trees to maintain uniform conditions before sample collection. Bark tissues and latex (laticifer cell cytoplasm) samples were collected from five healthy and TPD- affected trees and pooled before total RNA extraction.[21] The mRNA quantity and quality were determined spectrophotometrically at 260 and 280 nM.

Aliquots (15 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 (0.2M 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. RNA was then transferred onto a Hybond N+ nylon membrane (Amersham) by capillary blotting overnight using 10×SSC. RNA was crosslinked in a UV-crosslinker. The membrane was prehybridized for 2 h in 10 mL of a prehybridization buffer (50% formamide, 6×SSPE, 5×Denhardt's, 0.5% SDS, 0.2 mg/mL salmon sperm DNA) in a hybridization incubator (Amersham) with constant rotation at 42°C. The hybridization probe was prepared as described previously. Then the denatured [α-^{32}P]-dCTP TK gene probe was added and the membrane was hybridized overnight in the same buffer at 42°C. The membrane was washed twice (10 min each) in 2×SSC + 0.1% SDS at room temperature. After this, high stringency washing was carried out for 10 min in 0.1×SSC + 0.1% SDS at 42°C and then exposed to an X-ray film for 1-3 d with an intensifying screen for signal detection.

Results

Enhanced latex (rubber) production has been achieved substantially by releasing new high yielding rubber clones during the last two decades (Figure 1A). However, TPD is considered as a serious malady affecting rubber biosynthesis in high-yielding rubber clones worldwide (Figure 1B, C, D). The major goal of this experiment was to develop a nondestructive, early DNA diagnostic system to implement marker-assisted selection (MAS) in breeding programs. Therefore, we made an attempt to develop DNA markers using RAPD fingerprinting. DNAs extracted from 37 *Hevea* clones were used for RAPD reactions. A total of 140 arbitrary sequence decamers were tested as primers for RAPD fingerprinting. Of these, 16 primers gave reproducible polymorphic profiles, while one primer produced a monomorphic banding pattern within the 37 clones analyzed. PCR products amplified by these primers varied in size from 300 bp to 3500 bp.

Reproducibility of the RAPD fingerprinting pattern was tested by repeating each preparation at least thrice without alteration in the method. Different primers produced a RAPD profile of variable intensity from each clone examined. Although several polymorphic bands were detected among 37 clones, most of them were found to be faint and not considered as potential markers. Among the primers examined, OPA-17 primer amplified a bright and distinct uncommon band of approximately 2.0 kb with some clones, along with several common bands. The RAPD profile observed with OPA-17 primer (5′-GACCGCTTGT-3′) is depicted in Figure 2. This polymorphic band was amplified from 21 clones and it was absent or poorly amplified (not scorable) in the remaining 16 clones used for RAPD analysis.

RAPD profiles were used to construct dendrograms and clones originating from different countries were clearly distinguished (Figure 3). The rubber clones are classified into seven major groups based on DNA markers. The phenogram showed that RRII105 (India) and RRIM600 (Malaysia) clustered together and these were susceptible to the TPD syndrome. Further, it is interesting to note that most of the TPD-susceptible clones formed together in the same clusters. Although the rubber clones originated from different countries (India, Malaysia, and Indonesia), TPD-tolerant clones have been put in the same cluster with three major groups according to their origin. The two clusters formed by the Indian clones were distinct, with one group (RRII201, and RRII204, RRII205, RRII206, RRII208, and RRII209) clustering with Malaysian clones. The clones PB217, PB260, PB312, and PB314 were grouped together. In our present study, it is interesting to note that most of the primary clones, such as TJIR1, PB86, and BD10, are closely clustered. In most cases, clones with a common pedigree, such as PB311, PB312, PB314, PB217, PB255, PB260, RRII204, RRII209, were observed to cluster together.

To characterize this polymorphic band further, it was cloned successfully into the pGEM-T vector and the presence of the cloned RAPD fragment in the recombinant plasmid was confirmed by both PCR and restriction digestion analysis. The cloned DNA fragment was sequenced subsequently (the nucleotide sequence is illustrated in Figure 4) and also deposited in the NCBI GenBank database (Accession no. AY130829). The first ten nucleotides from either side of the sequences obtained showed exact homology with the corresponding RAPD primer used for PCR amplification. The cloned RAPD marker consists of 2017 bp. BLAST analysis of the sequenced product identified it as the nuclear encoded novel putative plant thymidine kinase (TK) gene (378 bp; 126 aa) in which one intron (202 bp) was also detected. Attempts to isolate the

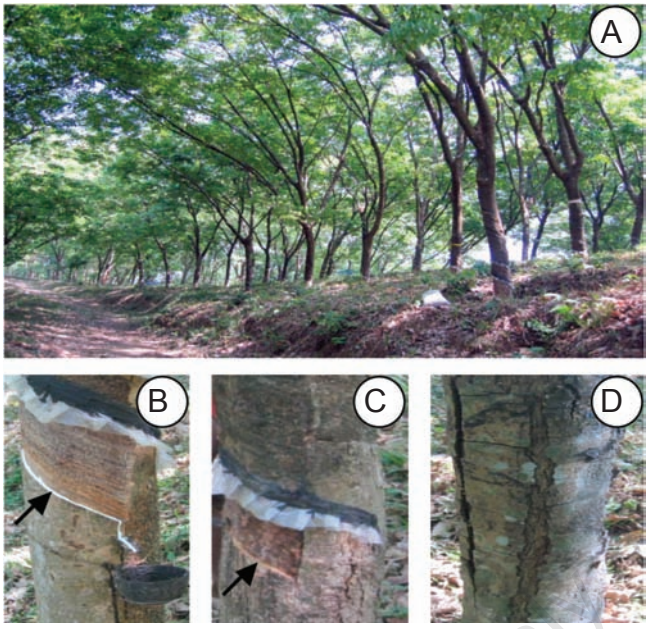

Figure 1. (A) *Hevea* rubber trees are growing at an experimental field of the Rubber Research Institute of India. (B) A healthy rubber tree with normal latex flow into the cup (arrow). (C) A TPD-affected tree with no latex flow at the tapped site (arrow). (D) Advanced stages of TPD in the affected tree, showing cracked bark tissue that is flaking.

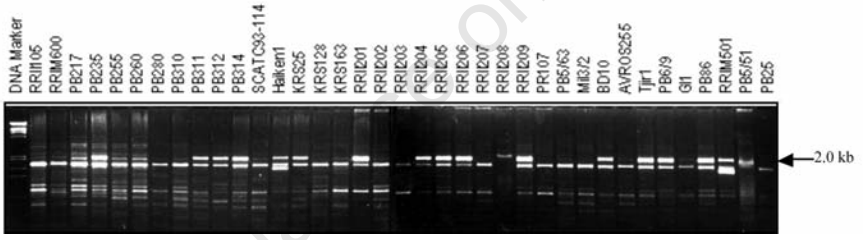

Figure 2. Genomic DNA from 37 *Hevea* clones was amplified using OPA-17 primer. The arrow indicates positions of the polymorphic band at 2.0 kb.

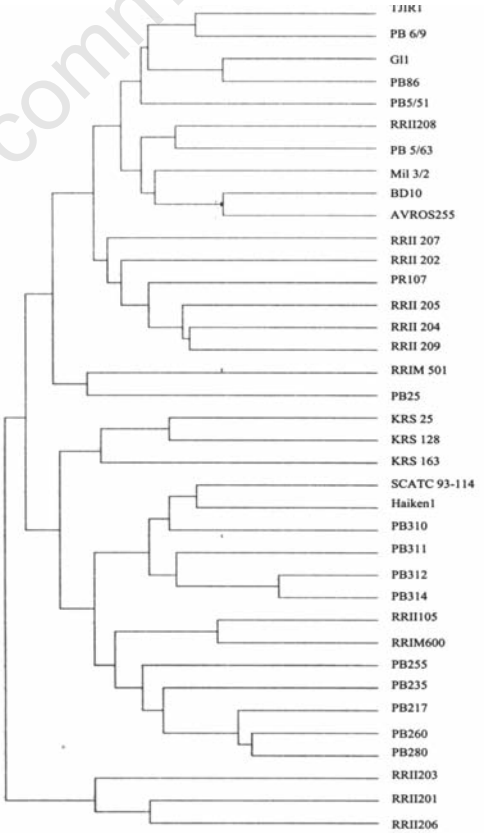

Figure 3. The dendrogram generated by UPGMA analysis. The bar on the top represents the dissimilarity index based on Jaccord's coefficients.

full-length cDNA by RT-PCR were unsuccessful. The multiple amino acid alignment is presented in Figure 5A. According to pair-wise BLAST analysis, the truncated *HbTK* gene shares 67% homology with the *Medicago* TK gene (ABE88233), 65% amino acid sequence identity with the *Oryza TK* gene (AF066050, 212 aa), 63% similarity with the *Lycopersicon TK1* gene (AF514775, 234 aa), and 64% homology with the *Arabidopsis TK* gene (AF370344, 238aa). All the TKs used for the analysis have a TK-conserved domain in the C-terminal half that is involved in nucleotide transport and metabolism. SignalP prediction suggests the presence of a signal peptide near the N terminus of the HbTK protein (MCILF FLVSV ILTHD EYMVQ LDVIG IDEAQ) and it is predicted to be located in the cytosol. The phylogenetic relationship of TK proteins from various taxa is shown in Figure 5B. The TK of *Hevea* formed a cluster with the TK of *Medicago* and branched with *Oryza* and *Lycopersicon* homologues.

To investigate the copy number and distribution of the TK gene in the *Hevea* genome, Southern hybridizations were performed on genomic DNA using the *HbTK* gene as hybridization probe (Figure 6A). On the blot, a major signal was detected in the DNA digests with *Eco*RI, *Eco*RV, *Hind*III, and XbaI, together with few weak signals (Figure 6B). This result suggests that *HbTK* is encoded by a small gene family in *Hevea*. Further, we compared the expression pattern of the *HbTK* gene in mature bark tissues and latex cells between healthy and TPD-affected trees using Northern blot analysis (Figure 6C, D). As expected, mRNA transcript accumulation was down-regulated in TPD-affected trees compared to healthy ones. The presence of low levels of *HbTK* transcripts detected in TPD-affected trees supports the notion of a biological function of TK even in the absence of active meristematic cell division. Of the two tissues examined, increased expression was noticed in bark tissues compared to that of latex cells. Northern blot data showed that the level of *HbTK* mRNA transcripts accumulated seven-fold higher in the healthy tree than in the TPD-affected one. These results together suggest that up-regulated expression of the *HbTK* gene in the healthy tree is implicated in active cell division and cellular metabolism.

Discussion

In our present study, we evaluated the potential of the RAPD method to detect variation at the DNA level among *Hevea* clones. The RAPD assay performs well in terms of number of bands, product yield, and clarity of the profiles. Alterations in RAPD "fingerprints" produced by a random decamer primer set under optimized conditions were seen in one of two ways: there was a gain or loss of a band. In some patterns, changes of a single amplification product appeared; in others, more complicated patterns of gains and losses of bands were observed. RAPD patterns generated by various primers were different among the clones evaluated. It is interesting to note that in this investigation, a distinct polymorphic band was detected with OPA-17 primer, and its nucleotide sequence showed a significant homology to the putative plant *TK* gene. It has been recognized that RAPD fingerprinting is an effective tool for detection of DNA variation in the nuclear genome because there are a large number of unique nucleotide sequences available for primer binding in the

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   1 GACCGCTTGT agtcatggag gtttgattga tgaagcttgg gattatttct catcaatgaa
  61 aagagattat ggaatccagc ccagagggca acactatgca gcaatggtcg atcttttagg
 121 tcgtgctggc aggttacaag aggcttatga gtttgtactg gaggcacctt gccaggaaca
 181 ctcagttgta tggggtgctt tgcttggggc ttgtaggatt catggagaca tggacctggt
 241 aaattgtgca gctaagaagt actttgaatt ggatccagaa aacgctggaa aatatgtggt
 301 cttgtctaat acatatgccg catttgggtt ctgggatgat gttgcaaagg ttaggagtat
 361 gatgagagat tcaggaatat tgaaggaacc tgcttatagc aggattggaa gttcaaggag
 421 aagtccactg cttcctatgg gtgataaatc tcatagaaaa tctgaggaga tatgtaggac
 481 aattatcaaa atgatgcgca ttttaagaga tgctggctat gttcctgata taagtgacca
 541 ttaattcatg aagaagactg atatgcttta catttagatc caaaatctca cctctcttat
 601 tggctgagct tcccattgca tttctgggct caggttgtta acttgaggta aagctgtttg
 661 tgtactaact aaccacataa agtactggat ctgggctttt ttttttttta aaaaaataca
 721 ttaagtaatt acctttttat atggttgtat ataattaata attcaggttg agtttggtat
 781 gttgctatta tacctttttg gcttggcaga actctaggtt aggatttctg tttttacctt
 841 gcaattttcc taggcactat tgcagtatat aacagcgaga ctaattttct gcatatatat
 901 cttataggat ttttatttct agttttagca atttcagtga gagatggtga tgatttaaag
 961 cattgttcta tgtagaatct gaaatatatt aaatcttcag caaaattatt attttaaaat
1021 tcatttttcc tttgtgtaca tttgttatgc agaaatgtag cagttataaa atcaaacagg
1081 gatacaagat atggtttgga ttcaatagtg acacatgatg gggtgaaact gccatgtatg
1141 gcactgccta atttgtcatc atttagacaa aaatttggcg ctgatgcata tgagcaggta
1201 tctgaattgc aaaacatgat gaactgtatt tttaagaatg atatctaatg tagcctcaaa
1261 aaaaagaaag gaatgatatt aatgcatata ttcaatcaag actgaatgtg aactatggaa
1321 agagtcatct agcagttaat attacctgta catttatttg tgttttgagt aaatcatttc
1381 tgtgaaagtc aatgtgacta atttttgaaa gaatgctacc agaaaattct taatgctgaa
1441 tatatgtgta tattgttctt tctagtttcc tgagtttaca gtgtatacta ggtgatatta
1501 actcatgatg agtacatggt gacagctgga tgtgattggt attgacgaag cacaattttt
1561 tgaagaccta tatgatttct gccgcgaagc tgctgatcat gatggaaaaa ctttaatagt
1621 tgctggtcta gatggtaact atttgaggta tatgctcgtt gtcaaaccta ctgatgttat
1681 atatatatag ttttatgctc attacatatt tcttctcctc tggaattctt gcttactagt
1741 taatccatcg gttaattgct taaaatcaca ttacattact atcagagctc cttttgatgg
1801 tggattaaat attgttgaca ggaggagttt tggttgtgta cttgatataa ttcctcttgc
1861 tgattcggta actaagttaa ctgcacggtg tgaactttgt ggaaaacgtg ctttctttac
1921 cttgagaaag acagaggaga caaggataga acttattggt ggggctgatg tctacatgcc
1981 tgtatgtcga caccactatg tcagtggACA AGCGGTC
```

Figure 4. Nucleotide sequence of RAPD polymorphic DNA obtained using primer. The primer sequence (upper case letters) is underlined (Accession No. AY130829).

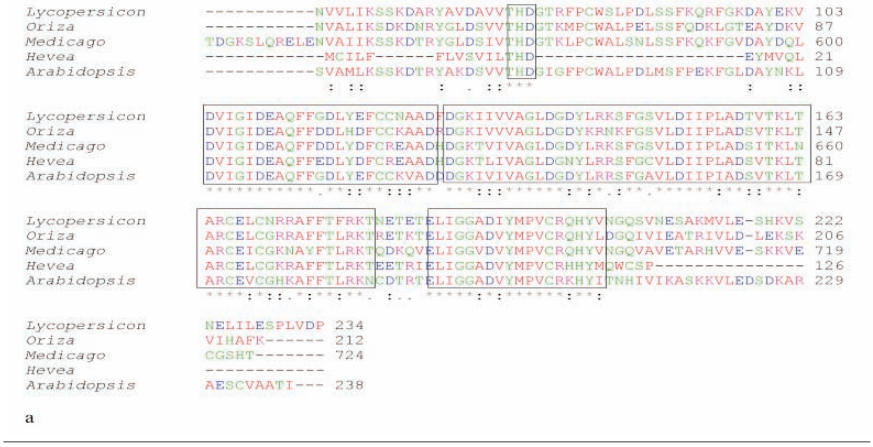

Figure 5. (A) Multiple alignments of the amino acid sequence of *HbTK* from *Medicago, Lycopersicon*, *Oryza*, and *Arabidopsis*. The alignments were generated using the ClustalW program.[19] (B) Phylogenetic analysis deduced amino acid sequences of TK genes. The phylogenetic analysis was performed using the TreeView program.

nuclear genome relative to the organelle genome.[4,7,8,22] The dendrogram clearly showed that the TPD-tolerant clones clustered together compared to that of the susceptible one. This result suggests that the identified RAPD marker is specific to TPD-tolerant clones.

The identified polymorphic band nucleotide sequence confirms that the cloned DNA fragment was coding for a putative plant TK protein. So far, few TK gene sequences have been reported from the following plant species, viz. *Oryza*, *Medicago*, *Lycopersicon*, and *Arabidopsis*, but no detailed information is available. A bioinformatics search with TK sequences indicated that the RAPD fragment had significant homology between 1550 bp and 2017 bp. A sequence similarity tree was generated to establish correlation and detection of possible genetic relationships by grouping of *Hevea TK* with other plant *TK* genes. The *Hevea* TK was clustered with *Medicago* and branched next to *Lycopersicon* and *Oryza* and its position within the tree suggests that *Hevea TK* gene is more similar to other plant species. As the probe hybridized to the *Hevea* genomic DNA, we putatively concluded that *Hevea* has a TK homolog in its genome. In addition, multiple hybridization signals suggest that *TK* represents as one of the members of a small gene family. In order to obtain insight about the TK expression, we studied possible differences in *TK* gene expression between mature bark tissue and latex cells.

In the *Hevea* tree, mature bark tissues were tapped (wounded) regularly to harvest latex. Therefore, it is quite possible that the occurrence of DNA damage within the latex-producing cells followed by tapping (wounding) may be one of the causes for the onset of TPD, which leads to cell death (bark dryness) at the tapped site of the rubber tree. It is well characterized that intensive tapping stress results in significant reduction of latex biosynthesis largely mediated via the generation of reactive oxygen species (ROS) and lutoid burst (latex cell damage) within the laticifer cells of TPD-affected trees.[23] The Northern blot result indicates that down-regulated expression of the *TK* gene correlates with the lack of meristematic activity in TPD-affected tree bark tissues.[24] This supports the notion that up-regulated TK expression may be involved in the DNA damage repair mechanism in the healthy tree. An earlier study indicated that TK expression has been found in various tissue types, including mature tissues, in response to DNA damage repair.[25] Although there are reports on putative TK activity from plants,[26] purification of the enzyme has not been reported from plants, and in addition it is poorly understood. In plant cells, ROS is the primary cause for DNA damage, which makes a continuous threat to the integrity and viability of the cell even in the absence of external stress.[27] Therefore, one can hypothesize that increased ROS production owing to tapping in TPD-affected trees may cause DNA damage, which could probably inhibit active cell division at the tapped site of the *Hevea* tree. These results further support the notion that decreased latex biosynthesis in TPD-affected trees may be because of lack of active nucleotide metabolism.

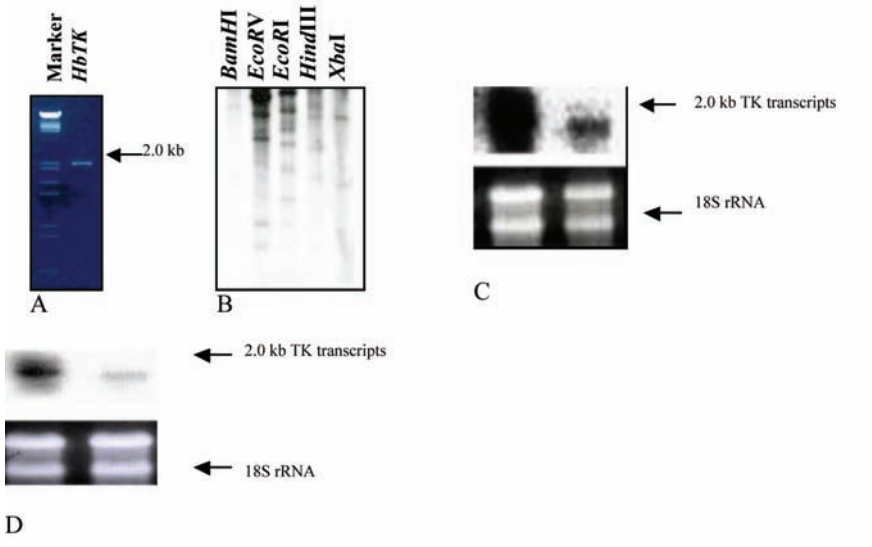

Figure 6. (A) PCR-amplified TK gene fragment used for ^{32}P labeling. (B) Southern blot analysis of genomic DNA (10 µg) digested with *Bam*HI, *EcoR*I, *EcoR*V, *Hind*III, and *Xba*I enzymes. Northern blot analysis of *HbTK* transcripts in mature bark tissues (C) and latex cell (D). Each lane contains 15 µg of total RNA. Ribosomal RNA was used to normalize RNA loading.

In conclusion, the RAPD fingerprinting has been successfully used to identify the *HbTK* gene. Further, decreased expression of the *HbTK* gene is associated closely with TPD syndrome development in the rubber tree. This is the first report of the cloning and characterization of the *TK* gene from *Hevea*. It would be interesting to study the possible involvement of the identified TK gene in various rubber clones affected by TPD, to consider as potential molecular marker for TPD diagnosis.

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