

Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree)

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Abstract

Latex of *Hevea brasiliensis* (Willd. ex A. Juss.) Mull. Arg. (Brazilian rubber tree) contains 30–50% (w/w) of natural rubber (*cis*-1,4-polyisoprene), which is an important raw material for many industrial uses. In order to gain insights into the molecular events occurring in latex, we analyzed more than 20,000 cDNA-AFLP-based TDFs (transcription-derived fragments) and 1176 ESTs. The results revealed several novel features of the latex transcriptome. First, the repertoire of the genes expressed in latex is unique. Only seven gene families accounted for more than 51% of the latex transcriptome. Among them, two of the most abundant ESTs were the genes encoding rubber particle proteins REF (rubber elongation factor) and SRPP (small rubber particle protein), comprising 29% of the total ESTs. Unexpectedly, several genes involved in the rubber biosynthesis were expressed at low levels in the latex. In fact, genes encoding *cis*-prenyltransferase (CPT), a potential candidate for rubber polymerase, were not present in the EST pool because of their low expression level. However, we were able to clone four full-length cDNAs by screening the same latex cDNA library used in the EST analysis and confirmed their enzyme activity *in vitro*. The second most abundant transcripts were defense- or stress-related genes, suggesting that defense is one of the functions of laticifers. Finally, the presence of the non-mevalonate DXP/MEP pathway for IPP synthesis in latex was noted by up-regulation of the 1-deoxy-D-xylulose 5-phosphate synthase gene.

Abbreviations: AFLP, amplified restriction fragment length polymorphism; CPT, *cis*-prenyltransferase; DMAPP, dimethylallyl diphosphate; DXP/MEP, deoxyxylulose/2-C-methyl- D-erythritol 4-phosphate; EST, expressed sequence tag; IPP, isopentenyl diphosphate; SRPP, small rubber particle protein; TDF, transcription-derived fragment

Introduction

Natural rubber (*cis*-1,4-polyisoprene), a secondary metabolite for which the producing plant has no obvious use, is an important raw material in commercial, defense, and transportation industries. Despite the availability of petroleum-base synthetics, natural rubber is highly valued because no synthetic substitute has comparable elasticity, resilience, and resistance to high temperature (Davis, 1997). Although rubber is produced by about 2000 plant species (Backhaus, 1985), *Hevea brasiliensis* (Willd. ex A. Juss.) Mull.

Arg. (the Brazilian rubber tree) is the only species that produces commercially viable quantities of high-quality rubber (30–50% by volume of fresh latex). Diminishing acreage of rubber plantations and a life-threatening allergy to *Hevea* rubber, coupled with increasing worldwide demand, have renewed interest in the study of rubber biosynthesis and the development of an alternative rubber source.

In *H. brasiliensis*, rubber is synthesized on the surface of particles suspended in latex, which constitutes the cytoplasm of laticifers. Laticifers are specialized vessels located inside the phloem of many angio-

sperms. The physiological roles of latex and rubber molecule remain a scientific mystery. For instance, rubber appears to be a metabolic dead-end because there are no enzymes capable of breaking down the rubber present in plants (Bonner and Galston, 1947). Why then would the plant invest so much energy into rubber biosynthesis? And how? Recent advances in plant genomics offer a unique opportunity to attack these age-old questions. Analysis of transcriptome in latex might provide information on metabolic activities in the specialized tissue. Kush *et al.* (1990) have shown the differential expression of several rubber biosynthesis-related genes in the latex of *H. brasiliensis*. They demonstrated that laticifer was 20- to 100-fold enriched in transcripts encoding enzymes involved in rubber biosynthesis and plant defense. The rubber elongation factor (REF), a protein related to the rubber biosynthesis (Dennis and Light, 1989), is highly expressed in laticifers (Goyvaerts *et al.*, 1991). Other rubber synthesis-related genes, such as HMG-CoA reductase (Chye *et al.*, 1991) and small rubber particle protein (SRPP) (Oh *et al.*, 1999), are also highly expressed in the latex. REF and SRPP genes are showing similar expression patterns in latex although not by wounding per se, ethylene or ABA (Sookmark *et al.*, 2002). About 200 distinct polypeptides are present in the latex (Posch *et al.*, 1997). Previously, we reported a small-scale expressed sequence tag (EST) analysis of the latex of *Hevea* rubber tree, and showed that the genes coding for rubber particle-associated proteins (REF and SRPP) were the most abundantly expressed among the 125 ESTs studied, along with defense-related genes and some of which were latex-specific (Han *et al.*, 2000). However, these studies cover only a small fraction of the transcriptome and, therefore, may not represent an accurate account of latex biology.

In order to gain better understanding of the molecular events taking place in the latex of *Hevea* rubber tree, we carried out analyses on a large number of redundancy-reduced ESTs and more than 20 000 transcription-derived fragments (TDFs) generated by cDNA-AFLP (amplified restriction fragment length polymorphism) technique (Bachem *et al.*, 1996). Unlike differential display methods that use small random primers, high annealing temperatures and specific primers can be used and, therefore, cDNA-AFLP is more stringent, reliable and highly reproducible (Bachem *et al.*, 1996; Durrant *et al.*, 2000; He *et al.*, 2003; Yang *et al.*, 2003). The EST analysis revealed that the latex transcriptome is surprisingly unique

compared to other transcriptomes reported in the literature. In addition, the analyses identified several novel genes that are abundantly expressed in latex. We also discovered that 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) was highly up-regulated in latex compared to leaf tissue, suggesting that latex might use both the DXP/MEP pathway and the well-known mevalonate pathway for synthesis of isopentenyl diphosphate (IPP).

Materials and methods

Plant material

Latex and leaf tissues were obtained from mature rubber plants (*H. brasiliensis* clone RRIM 600) growing at the Rubber Research Institute of Malaysia (RRIM), Selangor, Malaysia. Latex collection was performed as described in Oh *et al.* (1999). Briefly, 20 ml of the latex was collected in a 50 ml Falcon tube containing 20 ml of 2× RNA extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 0.01 M EDTA, 10% SDS, pH 9.5) and stored at −70 °C until use.

Construction of full-length cDNA library

Total RNA was extracted with Trizol reagent (Gibco/Life Technologies, Grand Island, NY). Poly(A)⁺ RNA was isolated from 150 µg of total RNA with the PolyA purist MagBeads kit (Ambion). We used the SMART strategy (Switching Mechanism At 5' end of the RNA Transcript; Clontech, Palo Alto, CA) to construct a high-content full-length cDNA library from the latex according to the manufacturer's instructions. After packaging and titrating, the primary library contained 1.4×10^6 (pfu/ml) phage and more than 90% were recombinant clones. The phage library was converted through mass excision to a phagemid library in the vector pTriPLEX (Clontech, Palo Alto, CA), according to the manufacturer's protocols.

EST generation and data analysis

The sequencing was carried out at the Genomics Technology Support Facility (GTSF) at Michigan State University. Plasmid DNA from bacterial cultures was purified at GTSF using Qiagen 3000 robots (Qiagen, Chatsworth, CA). After the fluorescent labeled sequencing products were generated by PCR amplification, the products were separated and sequenced

by capillary electrophoresis on an ABI prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The sequence quality trimming program *phred* (<http://bozeman.mbt.washington.edu/>; Ewing *et al.*, 1998) was used to trim vector sequences and low-quality sequences with Q20. *Phred* performs basecalling and assigns a quality score to each base. By definition, a phred quality score (where $Q \geq 20$) indicates that there is a less than 1% chance that the base calls are incorrect. Consequently, 'Q20' bases are considered 'high-quality' calls, and these regions are identified. Edited sequences were also inspected visually. Since high levels of sequence error can hinder subsequent assembly of ESTs into contiguous sequences (Liang *et al.*, 2000), sequences containing greater than 5% ambiguous base calls (Ns) were eliminated from the analysis. The nucleotide sequences were loaded into the National Center for Biotechnology Information (NIH, USA) form for BLAST searching. The searches were performed with the default settings of the BLASTX algorithm (Altschul *et al.*, 1990; Gish and States, 1993). Both scores and *E* values from the BLASTX results were considered when determining significant similarities. Any similarity with a score greater than 50 and an *E*-value less than $1.0E-04$ was considered a hit.

cDNA-AFLP analysis

The template for cDNA-AFLP was prepared according to Bachem *et al.* (1996) with slight modifications. For first-strand synthesis, oligo dT25N was used in combination with the SuperscriptII System (Invitrogen). Second-strand synthesis was done with DNA polymerase I (Promega) and RNaseH (Invitrogen). Double-stranded cDNA (500 ng) was digested with *ApoI* and *MseI* and ligated to respective adaptors. Sequences of primers and adapters used for amplified fragment length polymorphism (AFLP) reactions are as follows (N can be any nucleotide): *ApoI* adapter top strand, 5'-CTCGTAGACT-GCGTACC-3'; *ApoI* adapter bottom strand, 5'-AATTGGTACGCAGTCTAC-3'; *MseI* adapter top strand, 5'-GACGATGAGTCCT-GAG-3'; *MseI* adapter bottom strand, 5'-TACTCAGGACTCAT-3'; *ApoI* pre-amplification primer, 5'-CTCGTAGACT-GCGTACCAATT-3'; *ApoI* selective amplification primer, 5'-GACTGCGTACCAATTNN-3'; *MseI* pre-amplification primer, 5'-GACGATGAGTCCTGAG-TAA-3'; and *MseI* selective amplification primer, 5'-GATGAGTCCTGAGTAANN-3'. The ligated pro-

ducts were subjected to pre-selective amplification by using pre-amplification primers (15 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min) and selective amplification by using selective amplification primers. One of the primers was end-labeled with [γ - ^{33} P]dATP. The PCR cycles used for selective amplification consisted of 10 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min and 24 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. The annealing temperature between the first and tenth cycles was linked by a touch-down phase of decreasing 0.7 °C in each cycle. Selective amplification products were separated on a 5% polyacrylamide gel and run at 90 W until the xylene-cyanol dye reached the 1/3 position from the bottom. Gels were dried onto 3MM Whatman paper (Whatman, Maidstone, UK) and positionally marked before exposing to Kodak Biomax film (Sigma) for 1 day.

Isolation and sequencing of TDFs

Films were aligned with markings on the gels. The bands of interest were marked, cut out with a razor blade, and incubated overnight in 100 μ l TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) at 37 °C. The gel slices were then incubated at 95 °C for 10 min. The eluted cDNA after ethanol precipitation, eluted cDNA was re-amplified with the same primers under the same conditions as for cDNA-AFLP analysis. The fragments were sequenced as described above.

Reverse northern blot analysis

Selected sequenced TDFs were amplified by PCR with *ApoI* pre-amplification primer and *MseI* pre-amplification primer. PCR products were diluted to a final concentration of 5 ng/ μ l, denatured with NaOH (0.2 M final concentration) for 10 min, and spotted onto Hybond-N⁺ membranes (Stratagene, La Jolla, CA) with a 96-well dot-blot apparatus (BioRad, Hercules, CA). Controls were spotted at specific positions on the filters, consisting in distilled water and 8 leaf abundant TDFs. Total RNA from latex and leaf tissues were used for complex probes by reverse transcription with the SMART cDNA synthesis kit (Clontech, Palo Alto, CA) and labeled with [α - ^{32} P]-dCTP with a Prime-it II Random Primer Labeling kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Array filters were hybridized with Ultrahyb (Ambion) according to the manufacturer's instructions. Half of the membranes were hybridized with the latex probes and the other half with leaf probes.

After high stringency washing ($0.1 \times$ SSC, 0.1% SDS, 65 °C), filters were exposed to Kodak Biomax film (Sigma) for 1–3 days. The raw images were digitized with LabScan (Amersham-Pharmacia Biotech) software. Quantification of the signals was performed with ImageQuant5.2 software (Molecular Dynamics). The local background value for each membrane was calculated on the basis of no DNA spotted area. Net signal was determined by subtraction of this mean background from the intensity of each spot.

Northern blot analysis

Total RNA (15 μ g) from the latex and leaf tissues was denatured and separated by a 1% agarose formaldehyde gel. RNA was transferred onto Hybond-N⁺ membrane (Stratagene, La Jolla, CA) by capillary techniques. Probes were prepared by PCR and labeled with [α -³²P]-dCTP with the Prime-it II Random Primer Labeling kit (Stratagene). Hybridization was carried out as described above and then exposed to Kodak Biomax film (Sigma). Ethidium bromide-stained ribosomal RNA was used as a loading control.

cDNA library screening and heterologous expression of HbCPT protein in Escherichia coli

The full-length *HbCPT* gene (accession number AY124466) was used to screen 4×10^6 colonies of the latex cDNA library. Colony hybridization was performed at 60 °C in a $2 \times$ SSC hybridization solution (Sambrook *et al.*, 1989). The cDNA clones hybridized to the probe were sequenced for confirmation purposes. The *HbCPT* genes were cloned into the pGEX-4T-1 (Amersham Pharmacia Biotech) to construct pGEX-*HbCPT*1~4. The *E. coli* BL21 (DE3) transformed with pGEX-*HbCPT*1~4 were grown to mid-stationary phase in TB medium (1.2% bacto-treptone, 2.4% yeast extract, 0.4% glycerol, and 10% potassium phosphate solution) containing 500 μ g/ml carbenicillin at 30 °C with vigorous aeration. The cultures were induced by adding IPTG to a concentration of 1 mM and then incubating for another 2 h. All subsequent steps were carried out at 4 °C. The cells were harvested, washed with 0.1 M potassium phosphate pH 7.4 by centrifugation ($5000 \times g$, 10 min), and then disrupted by repeated freeze-thawing (3 times). The lysate was subjected to SDS-polyacrylamide gel electrophoresis according to the standard method of Laemmli (1970).

Assay of HbCPT activity

Enzyme activity was measured in a 50 μ l reaction mixture containing 100 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM dithiothreitol, 20 μ M FPP, and 80 μ M [¹⁴C]IPP (55 mCi/mmol, Amersham Pharmacia Biotech). The reaction was initiated by the addition of crude *E. coli* lysates (10 μ g of total protein). The mixture was incubated for 1 h at 30 °C and then treated with 1-butanol saturated with water to extract the products of the prenyltransferase reaction. The butanol phase was collected and mixed with Ready Solv HP scintillation mixture (Beckman), and radioactivity was determined by a liquid scintillation counter (Beckman).

Results

The latex transcriptome is unique

As a first step toward the analysis of latex transcriptome, we constructed a full-length cDNA library from the latex of *H. brasiliensis*. To evaluate and estimate the quality of the library, 5'-end sequences of 87 randomly selected cDNA clones were obtained and analyzed against the non-redundant database (NCBI, <http://www.ncbi.nlm.nih.gov/>) with the BLASTX algorithm (Altschul *et al.*, 1990; Gish and States, 1993). Of these clones, 89% were full-length and 68% matched to known sequences. Two of the most abundant genes encode REF (rubber elongation factor) and SRPP (small rubber particle protein), representing about 29% of the matched ESTs (15.6% and 13.1%, respectively). In an attempt to increase the depth of EST sequencing, we eliminated these redundant clones by virtual subtraction. Nylon membrane filter arrays carrying 1344 cDNA clones were prepared and hybridized with labeled probes made from REF and SRPP genes. We found that in a high-stringency hybridization/washing condition, 390 clones were strongly hybridized with the probes, suggesting that 29% of the latex transcripts are from REF and SRPP. This confirms the initial estimate by the small EST sequencing described above. The remaining 954 non-hybridized clones were selected for 5'-end sequencing, 835 of which produced high-quality sequences with an average length of 549 bp. Considering the 29% redundancy of REF and SRPP transcripts, the final 835 ESTs obtained after virtual subtraction amounted to a total of 1176 ESTs analyzed in this study (i.e., 71% of 1176 EST = 835 ESTs).

Table 1. Highly expressed genes in the latex of *Hevea brasiliensis*.

Contig	GenBank accession number	Gene description	Number of ESTs	% of total ESTs	Putative function ^a	NR <i>E</i> -value ^b	<i>Arabidopsis</i> homologue ^c	At <i>E</i> -value ^d
ct41	X56535	rubber elongation factor (REF)	187	15.9	RS	1.12E-71	At3g05500	8.42E-25
ct27	AF051317	small rubber particle protein (SRPP)	154	13.1	RS	5.70E-97	At3g05500	7.78E-48
ct17	AY221983	<i>Hevea</i> C3HC4-type RING zinc-finger protein (HbRGZF)	80	6.8	UF	4.83E-35	At2g04240	8.82E-34
ct13	AY221984	<i>Hevea</i> ASR like protein1 (HbASRLP1)	57	4.8	DS	2.79E-21	no	–
ct11	AY221985	<i>Hevea</i> protease inhibitor1 (HbPI1)	42	3.6	DS	4.1E-17	At2g38870	4.01E-15
ct30	AY058080	<i>Hevea</i> unknown protein1 (HbUNK1)	29	2.5	UF	1.66E-25	At5g39570	1.66E-25
ct25	AY221987	<i>Hevea</i> ASR like protein2 (HbASRLP2)	25	2.1	DS	7.45E-07	no	–
ct29	AJ003196	prohevein	18	1.5	DS	1.83E-111	At3g04720	8.38E-71
ct14	U42640	latex allergen Hev b 5	16	1.4	UF	7.19E-25	no	–
ct04	AY221988	<i>Hevea</i> REF-like stress-related protein 1 (HbRLP1)	15	1.3	DS	7.11E-31	At3g05500	1.44E-26
ct37	Y08996	acyl-CoA-binding protein (ACBP)	15	1.3	PM	2.21E-32	At1g31812	1.00E-25
ct05	AY221989	<i>Hevea</i> REF-like stress-related protein 2 (HbRLP2)	13	1.1	DS	1.74E-22	At3g05500	4.58E-19
ct24	AY221990	<i>Hevea</i> unknown protein2 (HbUNK2)	13	1.1	NSH	–	no	–
ct26	AY221991	<i>Hevea</i> unknown protein3 (HbUNK3)	13	1.1	NSH	–	no	–
ct36	AF0323593	thioredoxin H	11	0.9	CT	4.65E-47	At3g51030	4.67E-47
ct23	CB376619	<i>Hevea</i> unknown protein4 (HbUNK4)	8	0.7	NSH	–	no	–
ct18	AC006284	unknown protein	7	0.6	UF	3.28E-16	At2g03440	4.72E-15
ct47	AF104221	low-temperature/salt-responsive protein LTI6B	7	0.6	DS	8.12E-16	At3g05880	8.16E-16
ct03	AL391150	farnesylated protein ATP6-like protein	6	0.5	PF	4.86E-54	At1g71050	8.12E-53
ct49	X07160	18.5 kDa class I heat shock protein	6	0.5	DS	1.21E-52	At5g59720	2.25E-49
ct28	CB376430	<i>Hevea</i> unknown protein 5 (HbUNK5)	5	0.4	NSH	–	no	–
ct54	CB376810	<i>Hevea</i> unknown protein 6 (HbUNK6)	5	0.4	NSH	–	no	–
ct61	AP002544	unknown protein	5	0.4	UF	3.09E-52	At5g06240	5.36E-51
ct19	AJ243325	profilin 6 (Hev b 8.0204)	4	0.3	CS	3.5E-72	At4g2934	0.93E-60
ct31	X59751	calmodulin	4	0.3	ST	5.67E-81	At3g43810	1.04E-79
ct35	AF0183904	actin-depolymerizing factor 2 (ADF 2)	4	0.3	CS	5.61E-68	At3g46010	1.21E-65
ct42	CB376646	<i>Hevea</i> unknown protein 7 (HbUNK7)	4	0.3	NSH	–	no	–
ct43	U15019	arginase	4	0.3	PM	2.31E-79	At4g08900	4.16E-78
ct50	CB376648	<i>Hevea</i> unknown protein 8 (HbUNK8)	4	0.3	NSH	–	no	–
ct53	U88711	copper homeostasis factor	4	0.3	IH	6.25E-27	At1g66240	1.03E-25
ct57	AAN13206	putative polygalacturonase	4	0.3	PM	3.57E-54	At1g80160	6.28E-53

Table 1. (Continued.)

Contig	GenBank accession number	Gene description	Number of ESTs	% of total ESTs	Putative function ^a	NR <i>E</i> value ^b	<i>Arabidopsis</i> homologues ^c	At <i>E</i> value ^d
ct60	AL133315	transcription factor Hap5a	4	0.3	GERM	4.47E-31	At3g48590	7.82E-30
ct71	AF091455	translationally controlled tumor protein	4	0.3	UF	6.71E-82	At3g05540	5.31E-69
ct02	CB376370	<i>Hevea</i> unknown protein 9 (HbUNK9)	3	0.3	NSH	–	no	–
ct08	AB016886	NADH dehydrogenase 10.5K chain-like protein	3	0.3	EM	1.66E-37	At5g47890	2.89E-36
ct33	AY050918	lectin-like protein	3	0.3	PF	8.79E-07	At2g02250	7.65E-06
ct34	AL021712	nifU-like protein	3	0.3	PM	3.46E-64	At4g22220	6.19E-63
ct38	Z25537	low-temperature-induced protein 1t101.1 (Blt101)	3	0.3	DS	4.55E-22	At3g05880	2.91E-19
ct45	X73419	ubiquitin-conjugating enzyme E02	3	0.3	PF	6.86E-81	At5g41700	1.6E-80
ct46	AF0191302	disease resistance protein RTM1	3	0.3	DS	1.69E-21	At2g43740	3.06E-20
ct62	CB376916	<i>Hevea</i> unknown protein 10 (HbUNK10)	3	0.3	NSH	–	No	–

^aCS, cell structure; DS, defense or stress; EM, energy metabolism; GERM, gene expression and RNA metabolism; CT, cellular transport; PM, primary metabolism; PF, protein fate; RS, putative rubber synthesis; ST, signal transduction; UF, unknown function; NSH, no significant Homology.

^b*E* value from BlastX against NR (all non-redundant GenBank CDS translations + RefSeq Proteins + PDB + SwissProt + PIR + PRF).

^c*Arabidopsis thaliana* gene index (AGI) number.

^d*E* value from BlastX against *Arabidopsis thaliana* database (<http://arabidopsis.org/Blast/>).

This number was used as a base for calculating the frequency of EST clusters or functional categories. The EST sequences were subjected to contig analysis with the StackPACK v2.2 (Electric Genetics) program and clustered into 72 contigs. A very low number of singletons (394; 33.5% of total ESTs) were present in this specialized tissue, indicating a high redundancy of latex transcripts. A total of 41 highly redundant genes, having at least three ESTs in the latex, represented more than 68% of the latex transcriptome analyzed (Table 1). Of these, 12 (29.3% of 41 genes) had no *Arabidopsis thaliana* homologues and 9 genes (named *HbUNK2–HbUNK10*) showed no significant homology (*e*-value >1.0E-04) to known sequences in the current NCBI database, indicating that they may be novel genes specific to the rubber tree. Functional classification of the ESTs showed that the largest category was ‘proteins of unknown function’ or ‘no significant homology to known sequences’ (36.9%), followed by ‘putative rubber biosynthesis’ (29.1%) and ‘defense or stress related’ (17.7%) (Figure 1A). Other categories with small proportions include ‘protein fate’ (4.5%), ‘primary metabolism’ (3.2%), ‘signal transduction’ (2.0%), and ‘secondary metabolism’ (0.3%). Detailed information about the latex-expressed genes is presented in our project web site (<http://genomics.msu.edu/~han>).

Latex abundant or specific genes

To our surprise, the members of only seven gene families made up more than 50% of the latex transcriptome (Figure 1B). Northern blot analysis confirmed that five out of seven genes were abundantly or specifically expressed in the latex compared to leaf tissues (Figure 2). We found four genes that are homologous to REF (>70% identity in amino acid sequence), thus named HbRRLPs (*H. brasiliensis* REF-like protein 1–4), and also abundantly expressed (2.8%; Figure 1B) in the latex. While their functions are unknown, BLASTX analysis showed that HbRRLPs had high amino acid sequence homology (>65%) with stress-related proteins (Figure 3). After REF and SRPP, the next most abundant genes were homologous to tomato *ASR* (abscisic acid, stress and ripening) gene family (Rossi *et al.*, 1996), thus named as HbASRLP (*H. brasiliensis* ASR-like protein 1 and 2; 4.8% and 2.1%, respectively) (Table 1, Figure 1B). Transcripts of these genes were highly accumulated in the latex, but not in the leaves (Figure 2).

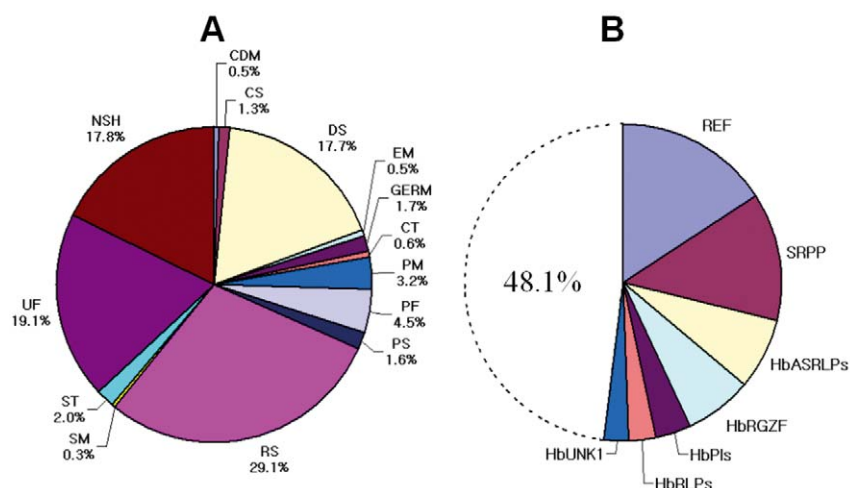


Figure 1. Functional classification and characteristics of latex ESTs. A. Functional classification of a total of 1176 latex ESTs by the following categories: CDM (chromatin and DNA metabolism), CS (cell structure), DS (defense or stress), EM (energy metabolism), GERM (gene expression and RNA metabolism), CT (cellular transport), PM (primary metabolism), PF (protein fate), PS (protein synthesis), RS (putative rubber synthesis), SM (secondary metabolism), ST (signal transduction), UF (unknown function) and NSH (no significant homology). B. Seven gene families make up about 51.9% of the ESTs analyzed. REF (rubber elongation factor), SRPP (small rubber particle protein), HbASRLPs (*Hevea* ASR-like proteins), HbRGZF (*Hevea* C3H4-type RING zinc-finger protein), HbPIs (*Hevea* proteinase inhibitors), HbRLPs (*Hevea* REF-like proteins), and HbUNK1 (*Hevea* unknown protein 1).

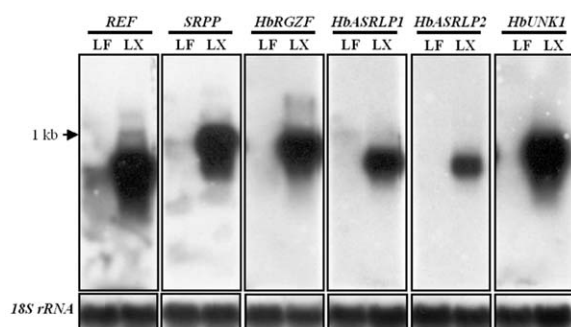


Figure 2. Expression of genes identified by EST analysis of the latex tissue. RNA gel blot analysis was performed as described in Materials and methods. Membranes were exposed to Kodak X-ray film for 1 days. LF, leaf tissue; L, latex. 18S rRNA serves as a RNA loading control.

Another highly abundant transcript is a C3H4-type RING zinc-finger protein (named HbRGZF; 6.8%), ranking third most abundant in the latex as a single gene (Table 1). While this gene is not characterized yet, a conserved domain database (CD) search of NCBI predicted it to have a RING-finger-containing ubiquitin ligase (COG5540) related to posttranslational modification, protein turnover, and chaperones. The RING finger is a specialized type of Zn finger of 40–60 residues that binds two atoms of zinc, and is probably involved in mediating protein-protein interactions (Borden and Freemont, 1996; Saurin *et al.*,

1996). It is notable that acyl-CoA binding protein (ACBP), a small cytosolic protein known to have a role in sequestering cellular acyl-CoA esters (Færgeman and Knudsen, 1997), was highly expressed in the latex (15 ESTs; 1.3% of total ESTs). The basal isoform is highly conserved and is found in all eukaryotic species examined, ranging from yeast and plants, to reptiles, birds and mammals (Kragelund *et al.*, 1999). In addition, ESTs involved in the membrane trafficking were highly expressed. Three ADP-ribosylation factors (M95166), two membrane-trafficking factor (AL161499) and several GTP-binding proteins such as RAB1 (U38471), RAB2A (Z97343) and Ras-like GTP-binding protein (U73810) were found in the latex EST database.

Differential gene expression in latex

We carried out cDNA-AFLP analysis to identify the genes that are differentially expressed in latex compared to leaf tissues. In this analysis, cDNAs synthesized from latex and leaf tissue mRNAs were digested by two restriction enzymes (*ApoI* and *MseI*). Oligonucleotide adapters were then ligated to the resulting restriction fragments to generate template DNA for PCR. Sequence analysis of 37 randomly selected *H. brasiliensis* full-length cDNAs from the NCBI database showed that 84% of the surveyed cDNAs

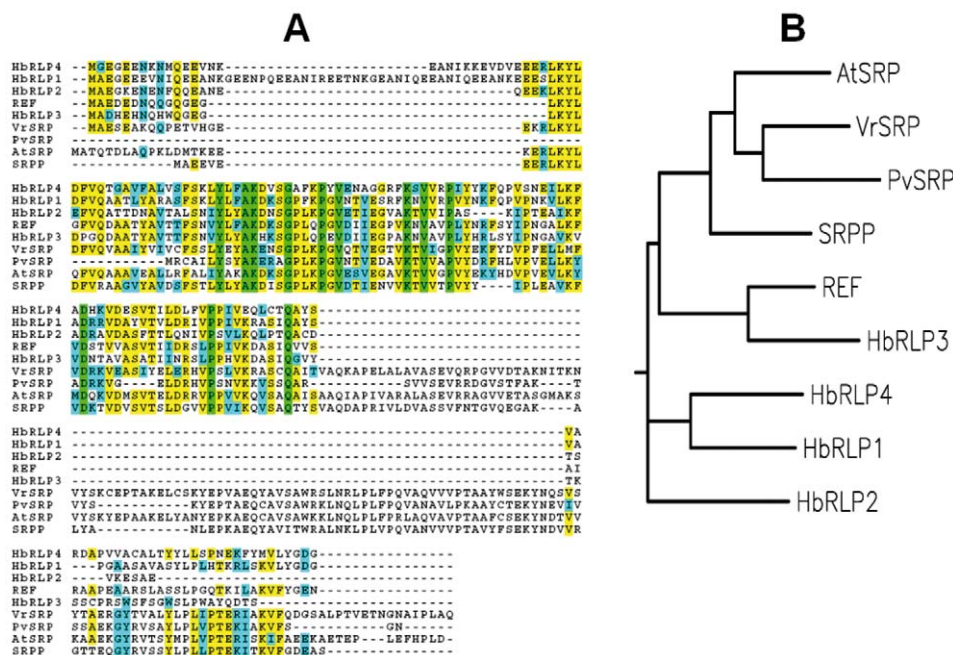


Figure 3. HbRLPs have sequence similarity with REF, SRPP and various stress-related proteins. A. Alignment of amino acid sequences. PvSRP (*Phaseolus vulgaris* stress-related protein; Q41112), VrSRP (*Vitis riparia* stress-related protein; Q9SW70), AtSRP (*Arabidopsis thaliana*; stress related protein – related; NP_187201). B. Phylogenetic tree of the genes indicated in A.

contained *ApoI*-*MseI* restriction sites that could generate restriction fragments (50–400 bp) suitable for cDNA-AFLP analysis. This suggests that roughly over 80% of the genes expressed in the latex could be studied by using this enzyme combination. We have obtained over 20 000 TDFs (transcript-derived fragments), from which 354 differentially expressed TDFs (346 abundant in latex and 8 in leaf) were isolated and sequenced. Expression pattern was confirmed by reverse northern blot analysis (Table 2). A total of 55 genes including 13 unknown function and 24 no hit were accumulated more than 10-fold in the latex. The latex abundant genes such as REF, SRPP, HbRGZF, acyl-CoA-binding protein (ACBP), HbPI1, HbUNK6 and prohevein, found from EST analysis, were up-regulated in the latex compared to leaf tissue (Table 2). Detailed expressions and a list of TDFs can be found at our web site (<http://genomics.msu.edu/~han>).

Rubber biosynthesis

Natural rubber (*cis*-1,4-polyisoprene) is made by head-to-tail condensation exclusively in *cis* configuration of isoprene units derived from IPP. Although the details of the isoprenoid pathway have been described, the final steps that lead to rubber synthesis

have not been identified. The polymerization of the isoprene polymer is thought to be catalyzed by the enzyme 'rubber polymerase,' a *cis*-prenyltransferase (CPT). Contrary to our expectation, we could not find *cis*-prenyltransferase genes among the ESTs and TDFs analyzed in the current study. Sequence analysis of a previously deposited *Hevea cis*-prenyltransferase gene in GenBank (*HbCPT*; accession number AY124466) revealed that this gene does not have the restriction sites for the enzymes (*ApoI* and *MseI*) used in the cDNA-AFLP analysis. In order to clone full-length *HbCPT* genes, we screened the same latex cDNA library used in the EST analysis with the AY124466 gene as a probe and isolated a total of 27 cDNA clones. Based on sequence information, we grouped them into four different *HbCPTs* (named *HbCPT1*–4; 7 clones to *HbCPT1*, 17 clones to *HbCPT2*, 2 clones to *HbCPT3*, and 1 clone to *HbCPT4*) (Figure 4). All of them lack the restriction sites for the enzymes used in the cDNA-AFLP analysis. Phylogenetic analysis of *HbCPTs* with other known CPTs shows that *HbCPTs* belonged to the same clade with eukaryotic CPTs (e.g., yeast and *Arabidopsis thaliana*) (Figure 4A and B). The enzyme activities of the *HbCPT1* and *HbCPT3*, which have the least similarity among the four *HbCPTs*, were analyzed by measuring the incorporation of ^{14}C -IPP into

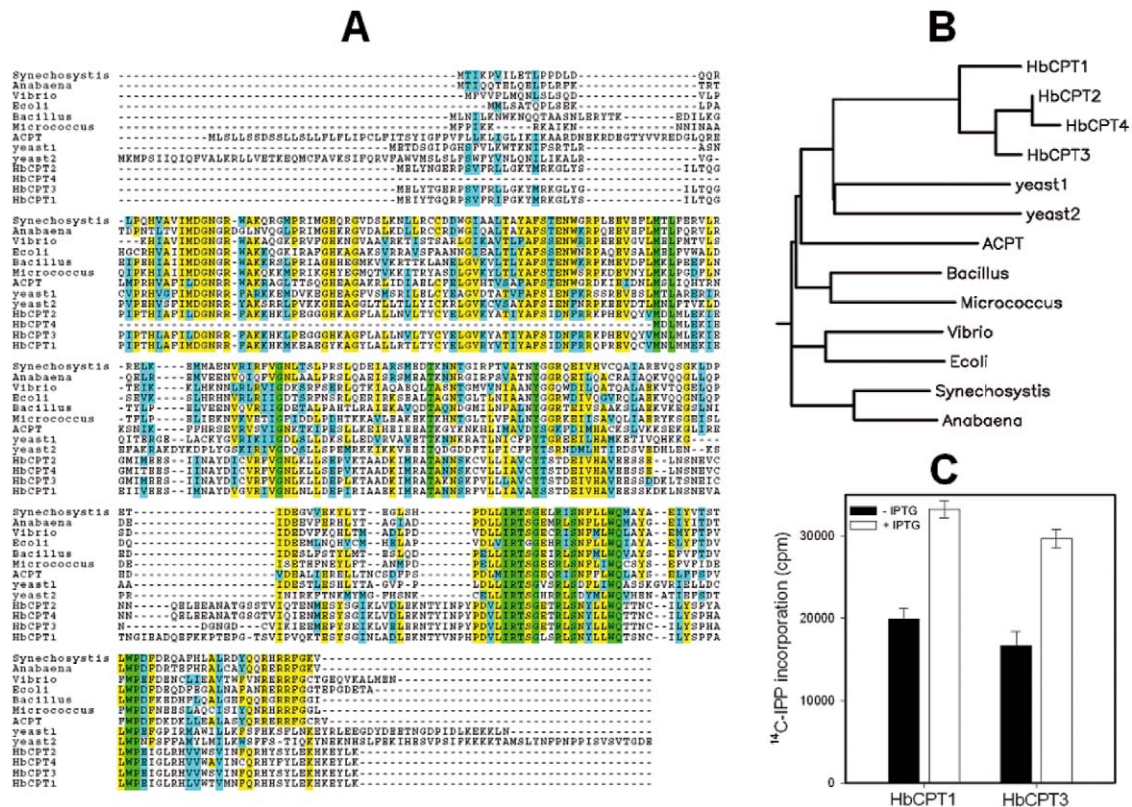


Figure 4. Amino acid sequence comparison of HbCPTs with known CPTs and expression analysis. **A.** Amino acid sequence alignment with known CPTs. ACPT (*Arabidopsis thaliana*, AF162441), Anabaena (*Anabaena variabilis*, Q9ZEJ7), Bacillus (*Bacillus subtilis*, O31751), Ecoli (*Escherichia coli*, NP_752159), Micrococcus (*Micrococcus luteus*, O82827), Synechocystis (*Synechocystis* sp. PCC 6803, NP_442758), Vibrio (*Vibrio cholerae*, NP_231887), Yeast1 (NP_009556), Yeast2 (NP_013819). **B.** Phylogenetic tree of genes aligned in A. **C.** Measurement of ^{14}C -IPP incorporation by HbCPT. HbCPT enzyme assay was performed as 27 described in Materials and methods with crude lysates of *E. coli* with or without IPTG induction. Each value is the mean of three separate experiments. Error bars mean standard error.

the initiation molecule (farnesyl diphosphate, FPP) in an *in vitro* rubber assay system as described by Oh *et al.* (1999). Both HbCPT1 and HbCPT3 incorporated the labeled IPP into FPP and had similar enzymatic activity (Figure 4C).

The fact that the current EST depth does not pick up any *HbCPT* suggests their low level of expression. To test whether that was the case, we performed northern blot analysis with an *HbCPT1* and other isoprenoid pathway genes as probes. Hydroxy-3-methylglutaryl CoA reductase 1 (HMGR1) was highly accumulated in the latex (Table 2, Figure 5A). However, the *HbCPT1* gene transcript was detected exclusively in the latex but at a low level (Figure 5A). Interestingly, the expression of another key enzyme in isoprenoid pathway, isopentenyl diphosphate isomerase (IPPI), was very low in both of latex and leaf tissues (Figure 5A). Plants have two different pathways for IPP synthesis: (1) the mevalonate pathway

that starts from condensation of acetyl-CoA, and (2) the 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) pathway (Rohmer *et al.*, 1993), in which pyruvate and D-glyceraldehyde-3-phosphate is a precursor of IPP (Figure 5B). We found that a gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) was expressed in the latex, suggesting the presence of DXP/MEP pathway in the latex of *H. brasiliensis*.

Discussion

Analysis of expressed genes in a given tissue can provide information about the biochemical and molecular events occurring in the tissue at the time of investigation. Here, we employed EST and cDNA-AFLP analysis to study the genes expressed in the latex of *Hevea* rubber tree. Although the EST num-

Table 2. Selected TDFs identified from cDNA-AFLP analysis.

TDF ID	Gene Description	Gene Bank Accession	E-value	RN ^a (lx:lf)	FC ^b (latex/leaf)
<i>Latex abundant TDFs</i>					
HbAFLP01F07	Rubber elongation factor (REF)	X56535	1.12E-71		37.9
HbAFLP04D04	C3HC4-type RING zinc finger protein (HbRGZF)	AY221983	6.16E-09		28.2
HbAFLP01D04	Small rubber particle protein (SRPP)	AF051317	5.70E-97		25.1
HbAFLP02E02	ACYL-COA-BINDING PROTEIN (ACBP)	Y08996	4.09E-32		25.0
HbAFLP02A04	<i>Hevea</i> protease inhibitor1 (HbPI1)	AY221985	3.09E-12		20.3
HbAFLP04F06	Putative aminotransferase	AC010871	2.94E-40		14.9
HbAFLP02H01	Putative pyrrolidone carboxyl peptidase	AC082644	1.87E-14		11.7
HbAFLP02G03	Prohevein	AJ003196	7.29E-12		10.3
HbAFLP04E06	Dormancy related protein	AC069144	1.25E-11		8.9
HbAFLP04G06	Putative splicing factor	AY093182	4.26E-31		8.4
HbAFLP02D02	RNA-binding protein-like	AP003375	2.01E-05		8.1
HbAFLP01A08	Unknown protein (HbUNK6)	CB376810	-		7.9
HbAFLP02H02	Similar to polyubiquitin	U16852	7.6E-10		7.6
HbAFLP03D08	Putative aminotransferase	AP002899	1.71E-08		7.0
HbAFLP04A07	Spermidine synthase 2	AF043109	2.77E-06		6.7
HbAFLP03B01	Putative serine/threonine protein kinase	AC079828	5.87E-21		6.7
HbAFLP03F09	Putative RNA-binding protein	AY063782	2.41E-19		6.7
HbAFLP02B03	IDS-4-like protein	AY055748	1.29E-32		6.6
HbAFLP03G12	Putative nuclear transport factor	AY042889	5.52E-30		6.4
HbAFLP01H08	(1-4)-beta-mannan endohydrolase-like protein	AL162351	4.67E-29		6.3
HbAFLP04C01	Hydroxy-3-methylglutaryl coenzyme A reductase 1	X54659	2.74E-40		6.2
HbAFLP04F01	Putative protein phosphatase-2C	AC011620	1.91E-24		5.6
HbAFLP01D08	1-deoxy-D-xylulose-5-phosphate synthase (DXPS)	AF0443590	1.8		5.2
<i>Leaf abundant TDFs</i>					
HbAFLP02D09	Putative protein	AY054681	1.15E-11		-2.40
HbAFLP02B09	Caffeoyl-CoA O-methyltransferase	Z54233	5.36E-25		-4.40
HbAFLP02A09	Oxygen evolving enhancer protein 2	AB043961	6.17E-11		-4.60
HbAFLP02C09	Glutathione S-transferase	AF288192	6.15E-26		-5.00
HbAFLP02H08	Unknown protein	AF0360323	9.51E-19		-7.80
HbAFLP02G08	Plastocyanin	B61320	1.81E-07		-11.00
HbAFLP02E09	Plastocyanin b	P00298	3.74E-21		-35.00

a, RN; Reverse northern blot analysis (lx:lf = latex : leaf).

b, FC; fold changes of gene expression (latex versus leaf) derived from reverse northern analysis.

ber is limited, we have tried virtual subtraction with abundantly expressed genes as probes to enhance the depth of EST database. In addition, the coverage of transcription profiling was further increased by the cDNA-AFLP analysis, which surveyed more than 20 000 genes expressed in the latex and found 354 differentially expressed genes. The analyses of latex transcriptome offer several novel insights about latex physiology.

Characteristics of the latex transcriptome

An interesting finding in this study is that the latex transcriptome is very simple in its complexity. Transcripts from only two of the most abundant genes (REF and SRPP) comprised 29% of the transcriptome. REF, a 14 kDa protein tightly associated with the large rubber particles, was suggested to be a rub-

ber elongation factor (Dennis and Light, 1989), but the direct role of this protein in rubber biosynthesis has not been proven. More recently, SRPP gene was isolated from *H. brasiliensis*. SRPP encodes a major rubber particle protein of 24 kDa, is tightly associated with small rubber particles, and was found to play a positive role in IPP incorporation into the rubber polymer (Oh *et al.*, 1999). Furthermore, only seven gene families including REF and SRPP accounted for over 50% of the latex expressed genes. To our best knowledge, this kind of characteristic and low complexity of transcriptome in any given plant tissue has not been reported before. However, this is not entirely unexpected since latex is a highly specialized tissue that produces a large amount of a single product (i.e., rubber). Of these abundantly expressed genes, 12 genes have no counterpart in *Arabidopsis thaliana*, implying

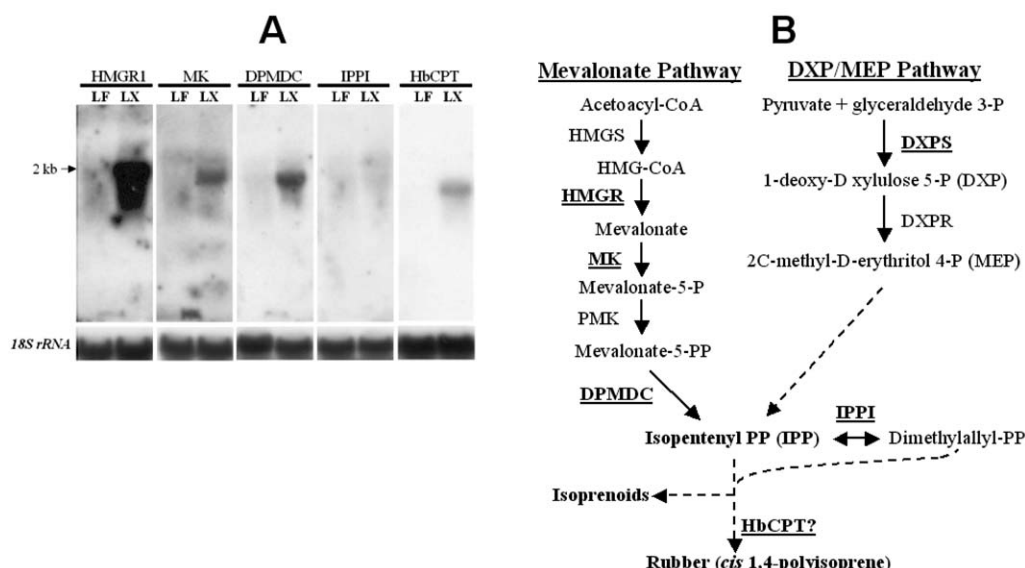


Figure 5. Schematic pathway of rubber biosynthesis in the latex of *H. brasiliensis*. **A.** Expression of genes involved in rubber biosynthesis. RNA gel blot analysis was performed as described in Materials and methods. Membranes were exposed to Kodak X-ray film for 3 days. LF, leaf tissue, LX, latex. 18S rRNA serves as a RNA loading control. **B.** Mevalonate and DXP/MEP pathway in latex. Underlined genes are identified in this study. HMGS (hydroxy 3-methylglutaryl CoA synthase), HMGR1 (hydroxy 3-methylglutaryl CoA reductase 1), MK (mevalonate kinase), PMK (phosphomevalonate kinase), DPMDC (diphosphomevalonate decarboxylase), IPPI (isopentenyl diphosphate isomerase), DXPS (1-deoxy-D-xylulose 5-P-synthase), DXPR (DXP reductoisomerase), HbCPT1 (*Hevea cis*-prenyltransferase1).

the unique nature of latex since *Arabidopsis thaliana* is regarded as having all of the genes a plant needs. In addition, latex has many ESTs involved in membrane trafficking, including ADP-ribosylation factor (M95166), membrane trafficking factor (AL161499) and several GTP-binding proteins. ADP-ribosylation factor is a family of small GTP-binding proteins involved in the formation of coated transport vesicles, which are used to carry important cellular components (Balch *et al.*, 1992; Spang *et al.*, 2001; Lee *et al.*, 2002). This may partially explain why the laticifer uses an active vesicle transport system as an internal secretory system.

The role of laticifer in the rubber tree

The physiological roles of laticifer in the rubber tree have yet to be clearly understood clearly. Latex has been implicated to play a role in defense (Dussourd and Eisner, 1987; Farrell *et al.*, 1991). Kush *et al.* (1990) also observed that the transcript levels of plant defense- or stress-related genes were 10- to 50-fold higher in latex than leaves. Hydrolytic enzymes such as β -1,3-glucanase (Chye and Cheung, 1995), cellulase, and polygalacturonase (Kush *et al.*, 1990) were also highly expressed in laticifers. In addition, the

in vitro antifungal activity of latex has been demonstrated for several species including *Lactuca sativa*, *Asclepias curassavica*, *Carica papaya*, and *Candida albicans* (Moulin-Traffort *et al.*, 1990; Giordani *et al.*, 1991, 1996, 1999). Hevein, a serine protease, has been described as an antifungal protein (Lynn *et al.*, 1984; van Parijs *et al.*, 1991; Koo *et al.*, 1998). In the current study, hevein comprised a high proportion (18 ESTs; 1.5% of total EST) in the EST pool and was accumulated more than 10- fold in the latex when compared to the leaf tissues (Tables 1 and 2).

In fact, defense- or stress-related genes are abundantly expressed in the latex. For example, transcripts of *HbASRLP1* and *HbASRLP2* were the most abundant (82 ESTs; 6.9%) next to those of REF and SRPP, and had high levels of accumulation exclusively in the latex. Putative proteins coded by these genes have an ABA_WDS domain (pfam02496.6), which is the characteristic domain of plant proteins induced by water deficit stress (WDS) or abscisic acid (ABA) stress and ripening, suggesting their role in defense. In addition, a latex up-regulated gene family encodes HbRLPs (REF-like proteins), which are homologous to stress-related proteins. It is also interesting that SRPP is structurally closer to stress-related proteins than REF in the phylogeny tree analysis. Furthermore,

another latex-abundant gene, HbPI1 (*H. brasiliensis* proteinase inhibitor 1), has a conserved domain from the potato inhibitor 1 family (pfam00280.6) and is homologous (>70% in similarity) to well known proteinases including protease inhibitor (accession number P82381), serine proteinase inhibitor (accession number S12897), and pathogenesis-related protein (accession number CAA87073). Other plant defense genes highly expressed in latex include LTI6B (low-temperature- and salt-responsive protein; AF104221), Blt101 (low-temperature-induced protein; Z25537), and RTM1 (disease resistance protein; AF0191302). These results suggest that the laticifer of rubber trees also have a functional role in defense.

Rubber biosynthesis in the laticifer of Hevea rubber tree

IPP and dimethylallyl diphosphate (DMAPP) are the fundamental building blocks of rubber polymer in rubber producing plants. Therefore, sufficient and efficient supply of IPP is one of the most important factors in rubber synthesis (Cornish, 2001). IPP can be synthesized from the mevalonate pathway through condensation of three molecules of acetyl-CoA. Acyl-CoA-binding protein (ACBP) is a small cytosolic protein of 10 kDa that binds medium and long chain acyl-CoA esters with high affinity (Knudsen *et al.*, 1989). The ability of ACBP to control the pool of acyl-CoAs was demonstrated by over-expression of a bovine ACBP gene in yeast (Mandrup *et al.*, 1993). The ACBP gene is one of the most abundantly expressed genes in latex comprising 1.3% of the total ESTs and its up-regulated (>25-fold) in latex compared to leaf tissues. The exact functional role of ACBP in the physiology of *H. brasiliensis* latex is not known. Both HMGS and HMGR have been shown to be involved in early steps of rubber biosynthesis (Hepper and Audley, 1969) and are regulatory enzymes in isoprenoid biosynthesis (Bach, 1995). Kush *et al.* (1990) showed that these two genes were highly expressed in laticifers compared to leaves. We also found that HMGR1 was highly expressed in the latex. However, IPP isomerase was expressed at a very low level (almost not detectable by northern blot analysis) in both latex and leaf tissues.

Until recently, it was generally assumed that IPP derives solely from the mevalonate pathway. However, independent studies demonstrated the existence of a novel, mevalonate-independent pathway for IPP synthesis known as the 1-deoxy-

D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) pathway (Rohmer *et al.*, 1993; for review, Eisenreich *et al.*, 1998). The discovery of DXPS gene from *Hevea* latex in this report suggests that the DXP/MEP pathway may also work in latex. However, latex has no detectable expression levels of genes involved in photosynthesis according to the current analysis and previous reports (Kush *et al.*, 1990), through which pyruvate and glyceraldehyde 3-phosphate, the substrates for DXP/MEP pathway, are produced. Therefore, it is plausible that those substrates may come from glycolysis of transported sugars. Evidence of multiple sugar uptakes across the plasma membrane of laticifer protoplasts was reported from *H. brasiliensis* (Bouteau *et al.*, 1999), suggesting that this peculiar sink has a wide capacity for the uptake of sugars. The high level of DXPS gene expression in latex implies that this non-mevalonate pathway might also contribute to the rubber biosynthesis together with the well-known mevalonate pathway.

In an effort to identify the genes responsible for rubber biosynthesis, we employed two extensive transcriptome analysis approaches: EST and cDNA-AFLP analysis. Surprisingly, we were not able to find well-known genes such as mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, IPP isomerase, and *cis*-prenyltransferase (*HbCPT*) in the isoprenoid pathway. When examined by northern analysis, the overall expressions of these genes were very low compared to *HMGR1*. These results suggest that the genes directly involved in rubber biosynthesis including rubber polymerase may be regulated at low levels in the laticifer. Furthermore, recent western and immunogold label analyses showed that the rubber particles of *F. carica*, *H. brasiliensis*, and *F. benghalensis* did not contain the HbCPT (Singh *et al.*, 2003). These observations suggest that HbCPT itself may not be a part of the rubber polymerase complex. We also report nine novel genes (HbUNK2–HbUNK10) that are highly expressed in latex but have no homology with current NCBI databases. Their functional role with regard to rubber biosynthesis remains to be investigated.

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