



Identification of laticifer-specific genes and their promoter regions from a natural rubber producing plant *Hevea brasiliensis*[☆]

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ABSTRACT

Latex, the milky cytoplasm of highly differentiated cells called laticifers, from *Hevea brasiliensis* is a key source of commercial natural rubber production. One way to enhance natural rubber production would be to express genes involved in natural rubber biosynthesis by a laticifer-specific overexpression system. As a first step to identify promoters which could regulate the laticifer-specific expression, we identified random clones from a cDNA library of *H. brasiliensis* latex, resulting in 4325 expressed sequence tags (ESTs) assembled into 1308 unigenes (692 contigs and 617 singletons). Quantitative analyses of the transcription levels of high redundancy clones in the ESTs revealed genes highly and predominantly expressed in laticifers, such as *Rubber Elongation Factor* (REF), *Small Rubber Particle Protein* and putative protease inhibitor proteins. *HRT1* and *HRT2*, *cis*-prenyltransferases involved in rubber biosynthesis, was also expressed predominantly in laticifers, although these transcript levels were 80-fold lower than that of REF. The 5'-upstream regions of these laticifer-specific genes were cloned and analyzed *in silico*, revealing seven common motifs consisting of eight bases. Furthermore, transcription factors specifically expressed in laticifers were also identified. The common motifs in the laticifer-specific genes and the laticifer-specific transcription factors are potentially involved in the regulation of gene expression in laticifers.

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Abbreviations: ABA, abscisic acid; cPT, *cis*-prenyltransferase; EST, expressed sequence tag; FPP, *E,E*-farnesyl diphosphate; GGPP, *E,E,E*-geranylgeranyl diphosphate; GO, gene ontology; PI, protease inhibitor-like protein; REF, Rubber Elongation Factor; RP, rubber particle; JA, jasmonic acid; IPP, isopentenyl diphosphate.

[☆] The nucleotide sequence reported in this paper is available through international databases (DDBJ/GenBank/EMBL) under accession numbers; AB861873: REF 5' upstream sequence; AB861874: SRPP 5' upstream sequence; AB861875: PI 5' upstream sequence; AB861876: HRT1 5' upstream sequence; HX869926–HX874249: *Hevea* latex ESTs.

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1. Introduction

Natural rubber consists mainly of *cis*-1,4-polyisoprene and is the most important raw material produced by plants, because of its unique physical properties, including resilience, elasticity, abrasion and impact resistance, efficient heat dispersion and malleability at cold temperatures [1]. Although more than 2500 higher plants produce natural rubber, only the Para rubber tree (*Hevea brasiliensis*) has been established as a key commercial source of natural rubber because of its high yield and excellent physical properties. In *H. brasiliensis*, natural rubber is obtained from latex, the milky cytoplasm of laticifers, which are highly specialized cells in the vascular tissues [2].

Although the precise structure and biosynthetic pathway of natural rubber has not been fully elucidated, many genes have

been suggested to participate in the natural rubber biosynthesis. The basic backbone structure of natural rubber, comprising of *cis*-1,4-polyisoprene with two or three *trans*-isoprene units at the ω -terminus [3], suggests that its biosynthesis involves sequential condensation of the C₅ isoprene unit, isopentenyl diphosphate (IPP), with *cis*-configuration onto an all-*trans* short-chain prenyl diphosphate, such as *E,E*-farnesyl diphosphate (FPP, C₁₅) and *E,E,E*-geranylgeranyl diphosphate (GGPP, C₂₀). Therefore, the key enzyme responsible for the biosynthesis of natural rubber is considered to be a member of the *cis*-prenyltransferase (cPT) enzyme family, which catalyze the sequential *cis*-1,4-condensation of IPP and exist ubiquitously in all organisms for the formation of *Z,E*-mixed isoprenoids, such as polyprenols, and dolichols. Two cPT homologs, *HRT1* and *HRT2*, cloned from *H. brasiliensis* latex, are proposed to be key enzymes responsible for the formation of natural rubber. Recombinant *HRT2* protein expressed in *Escherichia coli* is significantly activated by the addition of a centrifuged fraction of latex, resulting in the formation of polyisoprenes corresponding to natural rubber [4]. The involvement of cPT in natural rubber biosynthesis is also indicated by the observation that RNA interference-mediated depletion of cPTs in the laticifers of transgenic *Taraxacum brevicorniculatum* plants resulted in significant reduction in rubber biosynthesis [5].

To meet the ever-increasing demand for natural rubber, especially for the manufacture of tires, metabolic engineering of *H. brasiliensis* to improve its natural rubber production is required. One method of enhancing the productivity of natural rubber would be the simple overexpression of genes involved in natural rubber biosynthesis, such as *HRT2*. However, constitutive overexpression of genes for a specific isoprenoid biosynthetic pathways in whole plant tissues is predicted to affect plant growth and development because of the metabolic disorder of other physiologically important isoprenoids, such as quinones, sterols, and phytohormones [6]. To avoid this problem, the development of a laticifer-specific high-level gene expression system is required. However, no *cis*- and *trans*-acting regulatory elements that function in the laticifer-specific expression of genes in *H. brasiliensis* have been elucidated.

Expressed sequence tags (ESTs), which are single-pass sequence reads from reverse-transcribed mRNAs, provide a substantial representation of the transcriptome. ESTs have played significant roles in accelerating gene discovery; several reports conducted EST analysis on latex of *H. brasiliensis* to provide a comprehensive view of the transcriptional regulation of natural rubber biosynthesis [7–9]. These reports revealed unique transcriptional profiles in latex, showing high-redundancy of ESTs for two major genes, *Rubber Elongation Factor* (*REF*) [10] and *Small Rubber Particle Protein* (*SRPP*) [11], corresponding to about 30% of total ESTs. The high redundancy of these genes among the EST is considered to correlate with the high expression levels of the corresponding genes in latex, which have also been reported at the transcriptional [9,11,12] and the protein levels [10,13,14]. In addition, the expression levels of *REF* and *SRPP* in laticifers (latex) are much higher than those in leaves [7,8,11,12]. However, neither the precise expression levels nor the transcriptional regulation mechanisms of these laticifer-abundant genes have been elucidated.

To develop a laticifer-specific overexpression system, the regulation mechanism for gene expressions of laticifer-specific highly expressed genes must be determined. Here, we report the construction and analyses of ESTs comprising 5760 sequences obtained by sequencing a cDNA library from latex of *H. brasiliensis* to discover genes specifically expressed in laticifers. In addition, we successfully cloned the promoter regions of those genes, which are expected to be useful for laticifer-specific high level gene expression.

2. Materials and methods

2.1. Plant materials and total RNA extraction

Latex and various tissue samples were obtained from ten-year-old rubber plants (*H. brasiliensis* clone RRIM 600) grown at the Rubber Research Center of Songkla, Thailand. Latex collection by tapping of the bark of the tree trunks was performed basically as described by Kush et al. [15]. Young leaves with size from 15 to 20 cm, green stems which were 5 cm below the shoot apex and less than 1.5 cm in diameter, and lateral roots less than 0.5 mm in diameter were collected, immediately frozen in liquid nitrogen, and stored at -80°C until the total RNA extraction. Total RNA from latex for the cDNA library construction was extracted using RNA-gents Total RNA Isolation System (Promega, Fitchburg, WI, USA), and Poly(A)⁺ RNA was isolated from the total RNA with Oligodex-dT30 mRNA Purification kit (TaKaRa, Ohtsu, Japan). Total RNAs from other tissues were extracted using the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). Contaminating DNA in the total RNA sample was eliminated by treatment with DNase I (RNase-free; Takara Bio, Ohtsu, Japan) at 37°C for 30 min.

2.2. cDNA library construction and DNA sequencing

A latex cDNA library was constructed using a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The cDNA library was converted into a phagemid form by mass *in vivo* excision and transfected into *E. coli* SOLR strain. The resultant SOLR cells were plated onto Luria and Bertani (LB) media containing 50 $\mu\text{g}/\text{ml}$ ampicillin and incubated for 16 h at 37°C . 5760 colonies were picked by sterile toothpicks and inoculated into 384-well plastic plates containing 60 μl LB medium supplemented with 36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM sodium citrate, 0.4 mM MgSO₄, 6.8 mM ammonium sulfate, 4.4% (v/v) glycerol [16] and 50 $\mu\text{g}/\text{ml}$ ampicillin, and incubated for 16 h at 37°C . The insert of each plasmid was amplified by direct PCR from the 384-well plates using primers T3 (5'-ATTAACCTC ACTAA AGGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3'). The PCR program was as follows: 94°C for 2 min; 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 3 min; and 72°C for 10 min. The PCR products were purified by ethanol precipitation and sequenced using primer T3 and a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an automated DNA sequencer (ABI PRISM 3730xl DNA sequencer, Applied Biosystems).

2.3. Bioinformatics

The sequence trace files were submitted to the PHRED program [17] using the parameter – trim.alt 0.01 (quality value = 20). Sequences longer than 100 bases were selected and submitted to the CROSS-MATCH program using the parameters, – minmatch: 12 and – minscore: 20. Sequence assembly was performed using the PHRAP program with the default parameters, which resulted in clustered sequences (contigs) and unclustered sequences (singletons). The unigene set comprising the contigs and singletons was subjected to blastn and blastx searches. The blastn searches were performed against the GenBank nt nucleotide database at NCBI using default parameters, and the blastx searches were conducted against the NCBI nr protein database and the TAIR9 peptide database (The Arabidopsis Information Resource version 9; <http://www.arabidopsis.org>). For gene ontology (GO) analysis, GO slim term annotation of the best-matched Arabidopsis proteins, according to the blastx search results, was assigned to each unigene.

2.4. Quantitative reverse transcription PCR

Quantitative reverse transcription (RT)-PCR analyses were carried out using a One-step SYBR RT-PCR kit (Takara Bio), according to the manufacturer's instructions, with a LightCycler 1.2 apparatus (Roche Diagnostics, Tokyo, Japan), using gene-specific primers and thermal cycling conditions (Supplemental Table S1).

2.5. Isolation of 5'-upstream flanking regions

Genomic DNA from *H. brasiliensis* leaves was prepared by a standard genomic DNA isolation method for plant tissue using cetyltrimethylammonium bromide [18]. For *REF*, *SRPP* and *HRT1*, the genomic DNA segments of the 5' upstream flanking regions of each gene were isolated by an inverse polymerase chain reaction (IPCR) method [19]. About 1 µg of genomic DNA was separately digested with *Xba* I, *Spe* I or *Sac* I restriction enzymes (Takara Bio). After phenol/chloroform (1:1, v/v) extractions and ethanol precipitations, DNA fragments were self-circularized using a Ligation High kit (TOYOBO, Osaka, Japan). The primer sets used for the primary and secondary IPCRs were designed according to the coding sequences for *REF*, *SRPP* and *HRT1* (GenBank accession numbers: X56535, AF051317 and AB061234) (Supplemental Table S2). The primary IPCR with KOD FX (TOYOBO), using the each DNA fragment as a template, was performed with the following thermal program: 94 °C for 2 min; and 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 3 min. The secondary IPCR with nested primer sets, using the PCR product from the first IPCR as a template, was performed with the following thermal program: 94 °C for 2 min; and 30 cycles of 98 °C for 10 s, 57 °C for 30 s, and 68 °C for 3 min. For *PI*, a 5' upstream flanking region was amplified by a PCR-based genome walking method using DNA Walking SpeedUp Premix Kit (Seegene, Seoul, Korea), according to the manufacturer's instructions, with primers TSP1, TSP2 and TSP3 (Table S2), designed according to the *PI* coding sequences (GenBank accession number: AY221985). Amplified products were analyzed by agarose gel electrophoresis and purified from the agarose gel using UltraClean15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA). After the addition of 3' A-overhangs using a Mighty TA-cloning Reagent Set for PrimeSTAR (Takara Bio), purified products were subcloned into pGEM-T Easy Vector (Promega, Fitchburg, WI, USA) and sequenced using a CEQ 2000XL DNA Analysis System (BECKMAN COULTER, Brea, CA, USA).

2.6. Consensus motif prediction and database-assisted motif annotation

POCO (<http://ekhidna.biocenter.helsinki.fi/poxo/poco/>) [20] was used to predict eight base motifs that were common among the 5' upstream regions of *REF*, *SRPP*, *PI* and *HRT1*. The predicted motifs were used in homology searches of the Plant Cis-acting Regulatory DNA Elements (PLACE) database (<http://www.dna.affrc.go.jp/PLACE/>) [21] for similarity to previously described cis-regulatory elements.

3. Results and discussion

3.1. Transcriptome analyses of *H. brasiliensis* latex

In total, 5760 independent colonies of the latex cDNA library were isolated and subjected to 5'-end single-pass sequencing. After eliminating low quality sequences (quality value <20 or sequenced length ≤100 base), we obtained 4325 high quality sequences (average size: 613 base), which represented the EST dataset (Table 1). Assembly of the ESTs using PHRAP algorithm resulted in 1309

Table 1

Summary of EST analysis of *H. brasiliensis* latex.

Feature	Value
Total number of clones sequenced	5760
Number of high-quality sequences	4325
Average length of high-quality ESTs [bp]	613 ± 179
Number of contigs (A)	386
Number of ESTs in contigs	3402 (79%)
Number of singletons (B)	923 (21%)
Number of UniESTs (A + B)	1309

unigenes, comprising 692 contigs and 617 singletons (Table 1, Supplemental Table S3 and S2), which were analyzed with the blastn and blastx algorithm against the non-redundant (nr) database of NCBI. Although 1045 (80%) unigenes were assigned as identical or homologous to identified genes, exhibiting *E*-values <10^{−5}, many of the matched genes were annotated as putative proteins with unknown function. These result reflected, at least in part, the increased number of genes of unknown function deposited in the nr database from the *Euphorbiaceae* family, to which *H. brasiliensis* belongs, such as *Ricinus communis* [22], and *Jatropha curcas* [23]. To predict the biological processes in which the ESTs are involved, unigenes were also matched using the blastx algorithm against the TAIR-9 Arabidopsis database. Gene Ontology (GO) analysis was then performed, assigning GO slim terms to Arabidopsis genes that were the best matches for each EST. Among 12,929 GO slim terms belonging to the Biological Process, the top three GO slim terms assigned to the ESTs were “unknown biological processes” (51.0%), “other cellular processes” (21.2%), and “response to stress” (5.6%) (Fig. 1). The high frequency of gene of “unknown biological processes” may reflect the higher expression levels of *Euphorbiaceae* family-specific genes and/or laticifer-specific genes involved in specialized physiological roles during laticifer development. Comparative genomic analyses among *A. thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Vitis vinifera*, *Lotus japonicus*, *Glycine max* and *Euphorbiaceae* families, *R. communis*, *J. curcas* and *Manihot esculenta*, revealed that 1529 genes in *J. curcas* (4% of the putative protein-encoding genes) are specific to the *Euphorbiaceae* family [23], suggesting that such *Euphorbiaceae*-specific genes, which cannot be annotated accurately based on the Arabidopsis datasets, are expressed in laticifers of *H. brasiliensis*. Latex, estimated to occur in more than 2000 plant species in 40 families [24], is suggested to have defensive roles against herbivorous insects; however, the precise roles and functions of various latex proteins and chemicals in the plant defense systems have not been well documented [25]. Therefore, the latex ESTs categorized in “unknown biological

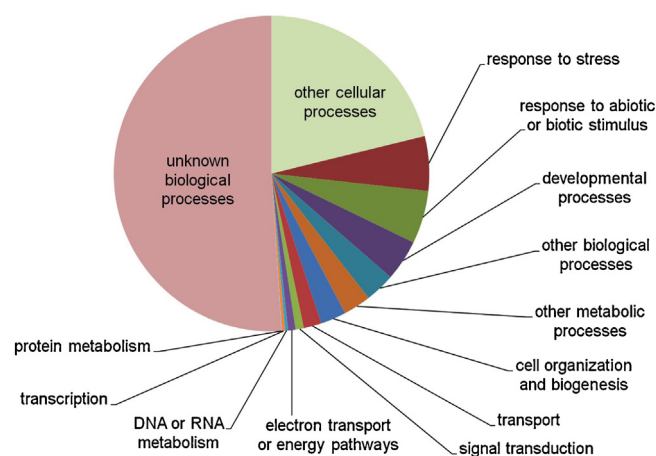


Fig. 1. Distribution of gene ontology slim term assignments.

Table 2
List of unigenes with high redundancy. Unigenes are listed according to the EST redundancy as the number of ESTs included in each contig. In this table, the top 20 unigenes are listed. The sequences were searched against the nr (NCBI-nr) and Arabidopsis TAIR-9 (TAIR9-pep) databases using the blastx program, and the annotations and corresponding E-values for top hit genes are listed.

Contig ID	Number of ESTs	NCBI-nr		E-value	TAIR9-pep	
		Accession number	Definition		AGI number	E-value
692	635	CAA39880	Rubber Elongation Factor (REF)	1.2E–70	AT3G05500	2.0E–24
691	378	CAA39880	Rubber Elongation Factor (REF)	1.1E–70	AT3G05500	1.0E–24
690	148	AAC82355	Small Rubber Particle Protein (SRPP)	3.9E–97	AT3G05500	2.0E–48
689	127	CAA39880	Rubber Elongation Factor (REF)	1.1E–70	AT3G05500	1.0E–24
688	60	CAA39880	Rubber Elongation Factor (REF)	1.8E–70	AT3G05500	2.0E–24
687	52	ABK94280	Unknown Protein 1 [<i>Populus trichocarpa</i>] (UP1)	1.2E–31	AT1G31812	1.0E–25
686	46	CAA39880	Rubber Elongation Factor (REF)	1.0E–68	AT3G05500	2.0E–24
685	40	CAA39880	Rubber Elongation Factor (REF)	2.7E–70	AT3G05500	3.0E–24
684	35	CAA39880	Rubber Elongation Factor (REF)	1.8E–70	AT3G05500	2.0E–24
683	34	AAP46156	Trypsin Inhibitor-like Protease Inhibitor Protein (PI)	8.9E–25	AT2G38870	8.0E–16
682	33	CAN79393 ^a	Unknown Protein 2 [<i>Vitis vinifera</i>] (UP2)	3.7	AT3G46310	2.0
681	33	CAA39880	Rubber Elongation Factor (REF)	9.5E–69	AT3G05500	2.0E–24
680	31	CAA39880	Rubber Elongation Factor (REF)	1.0E–70	AT3G05500	1.0E–24
679	31	CAA39880	Rubber Elongation Factor (REF)	1.1E–68	AT3G05500	3.0E–24
678	29	AAP46156	Trypsin Inhibitor-like Protease Inhibitor Protein (PI)	1.2E–23	AT2G38870	1.0E–14
677	28	AAP46156	Trypsin Inhibitor-like Protease Inhibitor Protein (PI)	9.8E–33	AT2G38870	7.0E–18
676	28	AAP46155	Absciscic Acid-, Stress-, Ripening-induced (ASR)-like Protein	4.0E–08	AT4G15070	9.5
675	26	CAA39880	Rubber Elongation Factor (REF)	1.3E–70	AT3G05500	2.0E–24
674	25	AAP46156	Trypsin Inhibitor-like Protease Inhibitor Protein (PI)	5.8E–33	AT2G38870	5.0E–18
673	24	AAP46156	Trypsin Inhibitor-like Protease Inhibitor Protein (PI)	5.5E–33	AT2G38870	6.0E–18

^a EMBL Accession No.

processes” might correlate with the, as yet undiscovered, plant defense systems.

Based on the EST redundancy of each unigene, the top 20 high redundancy unigenes, having 25–635 ESTs, corresponded to ~40% of the total number of ESTs, indicating that a limited number of genes are expressed at exceptionally high levels in laticifers (Table 2), which correlates with previous reports of latex ESTs of *H. brasiliensis* [7–9]. Among the highly expressed genes, three gene families were notable, i.e., REF, SRPP, and genes for protease inhibitor-like protein (PI). Some isoforms of REF and SRPP have been identified from latex [9]. In the top 20 high redundancy unigenes, 11 unigenes showing extremely high homology to REF, corresponding to 33% of the total ESTs, and one unigene of SRPP, corresponding to 3.4% of total ESTs, were included. REF is a well-studied latex abundant protein that is suggested to be related to the natural rubber biosynthesis in laticifers. REF is localized on the surface of rubber particles (RPs) [10,26,27], which are mainly composed of rubber molecules surrounded by a lipid monolayer and membrane proteins [28], and on which rubber biosynthesis takes place. Comparative analyses of different *H. brasiliensis* clones with contrasting latex yields demonstrated a positive correlation between the transcript levels of REF and latex yield [12]. In addition, inhibition of the rubber biosynthesis activity of latex *in vitro* by the addition of REF-specific IgG suggested the involvement of REF in natural rubber biosynthesis [10]. SPPP, which has a domain with high similarity to REF [11] and is localized on the RPs [26,27], is also a latex-abundant protein that is proposed to play a role in natural rubber biosynthesis, having a positive effect on the rubber biosynthesis activity of *Hevea* latex *in vitro* [11]. As reported in other latex-producing plant species, such as *Ficus carica* [29] and *Carica papaya* [30], putative serine protease inhibitors (trypsin inhibitors) were identified among the *Hevea* latex ESTs, showing relatively high redundancy (five unigenes in the top 20 high frequency unigenes, Table 2). Protease inhibitor proteins in laticiferous cells are thought to play defensive roles against insect herbivores, causing inhibition of their digestive processes [25]. The defensive roles of protease inhibitor proteins in latex of *H. brasiliensis* was suggested by a study that showed that the inhibitory effect of C-serum from routinely wounded trees on trypsin was higher than that from single wounded trees, indicating that the trypsin inhibitory activity in latex was induced by wounding [31]. In the top 20 high redundancy

unigenes, two unigenes coding an unknown protein were included. Unknown protein 1 (UP1) had high similarities to an unknown function protein found in *P. trichocarpa* and other plant species, but unknown protein 2 (UP2) was a novel gene because there was no gene showing significant similarity to it in public databases.

By contrast, among the ESTs in this study, a limited number of ESTs were identified as representing genes for enzymes in the isoprenoid biosynthetic pathway, which is considered to be involved in natural rubber biosynthesis (Table 3), suggesting relatively low expression levels of these genes, despite of the exceptionally high accumulation levels of polyisoprenoids, i.e., natural rubber, in laticifers.

3.2. Identification of laticifer-specific highly expressed genes

Genes represented by unigenes that showed high EST redundancy were expected to be highly expressed in laticiferous cells. To identify laticifer-specific genes with high expression levels among the ESTs, the transcript levels of the unigenes with high redundancy, represented by REF [EST Clone No.: Hebr-OJ23], SRPP [EST Clone No.: Hebr-HL13], PI [EST Clone No.: Hebr-EL15], Unknown Protein 1 (UP1) [EST Clone No.: Hebr-ID22] and Unknown Protein 2 (UP2) [EST Clone No.: Hebr-BB02], in various plant tissues, such as leaves, green stems, roots and latex (laticifers), were analyzed using quantitative RT-PCR. All five genes analyzed were expressed predominantly in latex: the expression levels of each gene in latex were much higher than those in other tissues analyzed (Fig. 2). Since all tissues of *H. brasiliensis* analyzed in this study contained latex, which was impossible to be eliminated thoroughly, the transcripts in each tissue are considered to include those originated from laticifers in each tissue. The higher accumulation levels of latex in stems, compared with those in leaves and roots, might reflect relatively higher transcript levels of each gene in stems. Consequently, these five genes were identified as laticifer-specific highly expressed genes. To compare the expression levels of enzymes corresponding to the natural rubber biosynthesis with these laticifer-specific highly expressed genes, the transcript levels of two CPTs, HRT1 and HRT2 [4,32], were also analyzed (Fig. 2). Surprisingly, transcripts of HRT1 were detected only in latex among the tissues analyzed, while transcripts of HRT2 were detected in all tissues analyzed, the highest level in latex. Extraordinary

Table 3

Putative isoprenoid metabolism-related unigenes among latex ESTs. Unigenes with high homology to the genes for enzymes involved in isoprenoid metabolism are listed. Annotations, EST-redundancies, and corresponding *E*-values for top hit genes searched against the nr database with the BlastX are indicated.

Representative EST ID (contig ID)	Number of ESTs	Genes involved in isoprenoid metabolism	Accession number	<i>E</i> -value
IJ20 (Contig578)	5	Hydroxymethylglutaryl-CoA synthase	AAL18930	0
AM12	1	Acetyl-CoA C-acetyltransferase	BAF98276	2.0E–121
BG04	1	Farnesyl diphosphate synthase	AAM98379	1.0E–140
CG06	1	Geranyl diphosphate synthase	BAF98299	6.0E–114
ND09	1	Phosphomevalonate kinase	AAL18926	2.0E–87

laticifer-specific expression of *HRT1*, despite inclusion of latex in all tissues analyzed, indicated that *HRT1* could be considered as a trunk laticifer-specific gene, at least among the tissues analyzed, and that the regulation mechanism of gene expression of *HRT1* is different from that of *HRT2*, despite the 92% nucleotide sequence identity of their coding regions. This suggested different physiological roles of these cPTs in laticifers of *H. brasiliensis*, concordant with the fact that *HRT1* shows a lower functional complementation ability of a temperature-sensitive phenotype of *rer2-2*, the yeast mutant of cPT, than *HRT2* although *in vitro* enzymatic function of recombinant proteins of *HRT1* and *HRT2* expressed in yeast are almost identical [32]. In terms of the transcriptional levels in latex, these cPTs showed 80-fold lower expression than *REF*, consistent with their low representations among the ESTs.

3.3. Cloning of the promoter regions of laticifer-specific expressed genes

To obtain laticifer-specific promoters, the 5' upstream regions of the coding sequences of *REF*, *SRPP*, *PI* and *HRT1* were cloned using thermal asymmetric interlaced (TAIL) PCR or inverse PCR. As a result, 3199, 2129, 924, and 1087 bp of sequences for *REF*, *SRPP*, *PI*, and *HRT1*, respectively, were identified as putative promoter regions (Supplemental Figs. S1–S4). To identify *cis*-regulatory elements involved in laticifer-specific promoter activity, common motifs among the four promoter regions were explored using the POCO algorithm (<http://ekhidna.biocenter.helsinki.fi/poxo/poco/>) [20]. As a result, seven common motifs consisting of eight bases were identified (Fig. 3, Supplemental Figs. S1–S4). Similarity search against a database of Plant *Cis*-acting Regulatory DNA Elements (PLACE) [21] revealed these motifs were similar to *cis*-regulatory elements involved in several biological phenomena in other plant species (Fig. 3).

Motif 4, “CTGCGTT”, commonly found in the 5' upstream region of *REF*, *SRPP* and *PI*, resembles VSF1PVGRP18 (PLACE ID: S000249), a *cis*-acting regulatory element identified as a binding sequence of VFS-1, a bZIP transcription factor participate in plant vascular development by regulating vascular-specific gene expression [33]. Latex is the cytoplasm of highly specialized cells, laticifers, which are extremely long cells ramified at maturity throughout various

tissues of the plant, such as the cortex, pith, foliar mesophyll, and especially the vascular tissues, phloem, and xylem rays [2,34]. Differentiation of articulated laticifers in *H. brasiliensis* is coordinated with the development of other phloem tissues [2]; therefore, motif 4 is potentially involved in a laticifer-specific gene expression.

Common motifs 1, 2 and 3 were found in the promoter regions of four laticifer-specific genes. Motif 1, “TAAATTAT”, bears a close resemblance to LECPLEACS2 (PLACE ID: S000465), which is a *cis*-acting regulatory element of an elicitor-induced expression of a gene encoding 1-aminocyclopropane-1-carboxylic acid synthase *Acs2*, a key enzyme in ethylene biosynthesis in *Solanum lycopersicum* [35]. Motif 3, “AAAAATGA”, was similar to the pathogen-responsive element GT1GMSAM4 (PLACE ID: S000453), identified as a target sequence of a GT-1-like transcription factor involved in the pathogen- and salinity-induced expression of *SCaM-4* [36]. Some latex genes are potentially involved in pathogen responses, such as the response to herbivores [25]. Indeed, the antifungal activity of *Hevein*, one of the laticifer-specific genes [37], against several fungi has been reported [38]. Therefore, these motifs commonly found in all four laticifer-specific genes may participate in the control of anti-pathogenic gene expressions in laticifers, concomitant with the upregulation of natural rubber biosynthesis involving in *HRT1*, *REF* and *SRPP*.

Motif 2, “ATTATTA”, has significant sequence similarity to ABA-responsive element ATHB6COREAT (PLACE ID: S000399) [39], identified as a target sequence of a homeodomain protein in *A. thaliana*, ATHB6, a regulator of the abscisic acid (ABA)-responsive gene expression. Many studies have shown the involvement of certain phytohormones in laticifer development or latex yields in *H. brasiliensis*, making them important factors in aspects of the natural rubber production. In *H. brasiliensis*, ethylene stimulates latex production. Bark treatment with ethephon (an ethylene releaser) increases the latex yield by 1.5–2-fold [40,41]. In addition, treatment of *H. brasiliensis* bark with ABA and auxin also affected latex yield positively, resulting from the sugar transporter-mediated turgor pressure change in laticifers [42]. In addition, in plant immunosystems, ABA acts agonistically on jasmonic acid (JA)-mediated signaling in response to herbivores, whereas it suppresses salicylic acid (SA)- and ethylene-mediated immuno-signaling pathways [43]. Motif 2 may contribute to the ABA-mediated upregulation of natural rubber biosynthesis as one of the plant immunosystems specialized in laticifers. Similar to ABA, other common motifs, which have similarity with *cis*-acting regulatory elements identified to be involved in phytohormone responses, were found. Motif 6, “TGTTTGCT”, has significant sequence similarity with a *cis*-acting element D3GMAUX28 (PLACE ID: S000330), identified in the promoter region of an auxin responsive gene, *GmAux28* [44]. Motif 5, “TCAATTAA”, has high sequence similarity with JASE1ATOPR1 (PLACE ID: S000388), a *cis*-acting regulatory element identified as a positive regulator of a senescence associate gene in response to JA and senescence in *A. thaliana* [45]. In addition to the role as a signaling molecule in the plant defense system against necrotrophic pathogens and herbivorous insects, JA also plays an important role as an inducer of laticiferous cell differentiation in *H. brasiliensis* [46], implying a principal

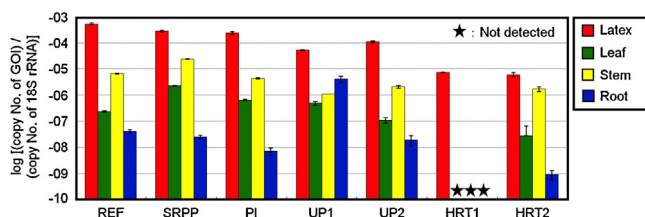


Fig. 2. Tissue specific transcript levels of unigenes with high EST redundancy and cPTs, *HRT1* and *HRT2*. The transcript levels of genes of interest (GOI) were determined as the copy number in an amount of total RNA extracted from latex, leaves, stems, and roots and then normalized by the level of the 18S rRNA in corresponding tissues. Abbreviations of the gene names correspond to those in Table 2. The results are presented as the means of three-independent determinations $\pm$ SDs.

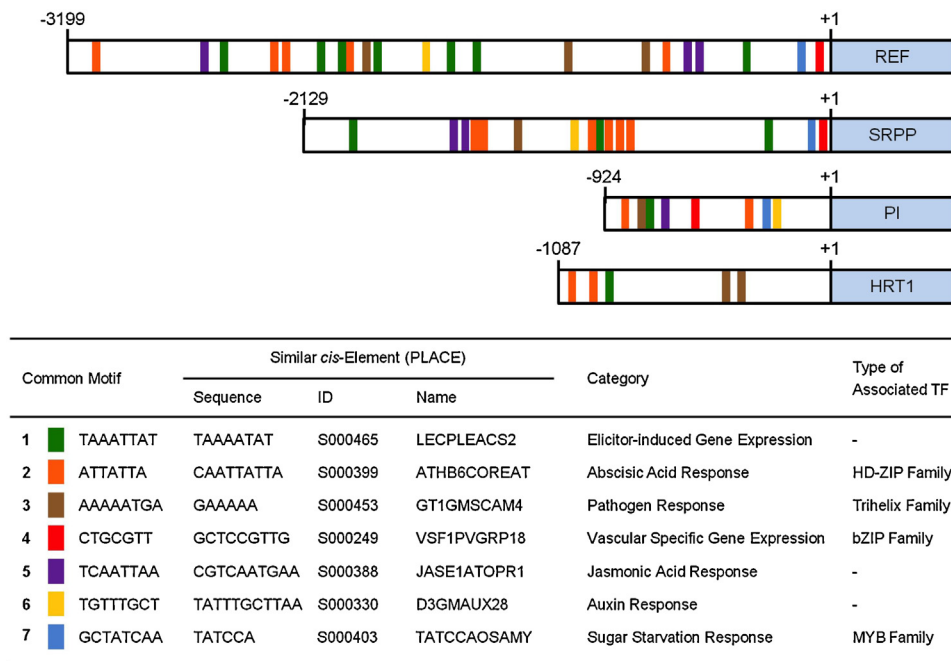


Fig. 3. Potential *cis*-acting regulatory elements commonly found in the 5'-upstream regions of the laticifer-specific genes. The POCO algorithm was used to search for motifs commonly found in a set of sequences corresponding to the promoter region plus the 5'-UTR of each gene. Similar *cis*-elements and associated transcription factors were searched in the PLACE database using the PLACE Homology Search Program.

role of latex as plant defensive agent and an involvement of phytohormone-mediated expression of laticifer-specific genes in laticifer differentiation.

Meanwhile, one putative *cis*-regulatory element involved in metabolic regulation was discovered. Motif 7, "GCTATCAA", has sequence similarity with a *cis*-acting regulatory element TATCCAOSAMY (PLACE ID: S000403, TATCCA element) and functions in sugar starvation-induced gene expression of α -amylases in rice, which is regulated by MYB transcription factors with one DNA-binding repeat [47,48]. It has been suggested [49] that the TATCCA element could be a common *cis*-acting regulatory element functioning during sugar starvation, because some variants of the *cis*-acting elements are found not only in the 5'-upstream regions of α -amylases in monocots, but also in those of other sugar starvation-induced genes, such as the cucumber malate synthase and isocitrate lyase genes, and the maize sucrose synthase gene. Although a correlation between the laticifer-specific gene expression and sugar starvation-induced responses has not been uncovered, motif 7 "GCTATCAA" might participate in metabolic regulation in laticifers, involving MYB transcription factor-mediated gene expression.

3.4. Identification of laticifer-specific transcription factors

In the EST analysis, genes that have similarities to transcription factors were identified (Table 4, Supplemental Table S3 and

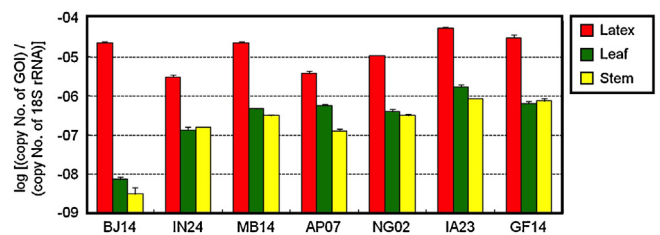


Fig. 4. Tissue specific transcript levels of putative transcription factors. Detailed annotations of putative transcription factors, indicated as representative EST IDs, were summarized in Table 4. The transcript levels of genes of interest (GOI) were determined as the copy number in an amount of total RNA extracted from latex, leaves, and stems, and then normalized by the level of the 18S rRNA in corresponding tissues. The results are presented as the means of three-independent determinations $\pm$ SDs.

S2). To identify potential transcription factors that could regulate the expressions of laticifer-specific genes in *H. brasiliensis*, transcript levels of the putative transcription factors with high EST redundancy were analyzed using total RNAs from various tissues as templates, by means of quantitative RT-PCR (Fig. 4). Notably, the redundancies of the putative transcription factors were lower than those of the laticifer-specific highly expressed genes. All of the putative transcription factors analyzed were revealed to be expressed in latex predominantly. Among them, a unigene represented by

Table 4
Putative transcription factors analyzed in this study. Putative transcription factors were annotated by searching against the Arabidopsis TAIR-9 databases with the blastx program. The family types of the TFs were determined using *A. thaliana* transcription factor database (AtTFDB) provided by the Arabidopsis Gene Regulatory Information Server (AGRIS). In this table, only putative transcription factor genes subjected to quantitative RT-PCR analysis are listed.

Representative EST ID (contig ID)	Number of ESTs	Transcription Factor Family	Arabidopsis protein name	AGI number	E-value
BJ14 (Contig554)	6	MYB Family	AtMYB17	AT3G61250	8.0E–12
IN24 (Contig430)	2	Zinc Finger, LSD1-type	L0L2	AT4G21610	1.0E–30
MB14 (Contig402)	2	bHLH Family	AtbHLH6	AT1G32640	2.0E–29
AP07 (Contig325)	2	bZIP Family	AtbZIP60	AT1G42990	7.0E–39
NG02	1	Alfin-like Family	PHD Finger Family Protein	AT5G05610	3.0E–34
IA23	1	NAC Family	NAC Domain-containing Protein	AT1G73230	5.0E–63
GF14	1	NAC Family	NAC Domain-containing Protein	AT1G73230	9.0E–56

EST clone Hebr-BJ14 showed extreme latex specific expression, showing ~1000 times higher expression levels in latex than in leaves and stems. Hebr-BJ14 had high similarities to AtMYB17 (At3g61250, renamed as *LATE MERISTEM IDENTITY2* (*LMI2*)), an R2R3 class MYB family transcription factor in *A. thaliana* identified as a direct target of the plant-specific transcription factor LEAFY (*LFY*) [50]. The R2R3 class MYB family participate in many biological processes in plants, including cell fate specification, primary and secondary metabolism, and biotic and abiotic stress responses [51,52]. Taken together with the highest redundancy in this EST (Table 4), the putative MYB transcription factor Hebr-BJ14 may play a role in laticifer-specific gene expression. Other putative transcription factors analyzed in this study are predicted to participate in latex-specific gene expression.

4. Conclusions and perspectives

In this study, we identified laticifer-specific highly expressed genes in *H. brasiliensis* by EST analysis and quantitative analyses of their transcript levels. In addition, we revealed several motifs commonly found in promoter regions of the laticifer-specific highly expressed genes, representing possible *cis*-acting regulatory elements that participate in the regulation of gene expression in laticifers, although these motifs were explored *in silico* from a limited number of the 5'-upstream regions of the laticifer-specific highly expressed genes. In order to explore such common motifs have more statistically significant co-occurrence provability, we are currently conducting large scale transcriptome analyses to identify other laticifer-specific highly expressed genes. The recent increasing availability of genomic information for *H. brasiliensis* [53] can be helpful in validating the accuracy of predicted motifs and finding other possible *cis*-regulatory elements. These promoter regions and potential *cis*-acting regulatory elements can be applied to develop laticifer-specific, high-level expression of a target gene, which could be used to improve the production of natural rubber. Material production in laticifers could be considered as a versatile plant bioprocess, where laticifers act as a sink tissue that enables the host plants to avoid the negative effects of heterologous production of valuable natural compounds and functional peptides, and to establish an easy and sustainable harvest system of the products by tapping. Moreover, these motifs could contribute to the discovery of the regulatory mechanisms of natural rubber metabolism. Further investigations of the motifs and transcription factor proteins participating in the regulation of gene expressions, in which the putative transcription factors identified in this study might be potential candidates, could accelerate our understanding of the gene regulatory network in *Hevea* laticifer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2014.05.003>.

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