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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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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

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, isopentyl 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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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, C_{15}) and *E,E,E*-geranylgeranyl diphosphate (GGPP, C_{20}). 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 RNAgents 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 *Escherichia coli* SOLR strain. The resultant SOLR cells were plated onto Luria and Bertani (LB) media containing 50 μg/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 K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM ammonium sulfate, 4.4% (v/v) glycerol [16] and 50 μg/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′-ATTAACCCTC 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.

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