Songklanakarin J. Sci. Technol.
32 (6), 555-559, Nov. - Dec. 2010

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

CDNA library from the latex of *Hevea brasiliensis*

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Received 19 June 2008; Accepted 31 August 2009

Abstract

Latex from *Hevea brasiliensis* contains 30-50% (w/w) of natural rubber (cis-1,4-polyisoprene), the important raw material for many rubber industries. We have constructed a cDNA library from the latex of *H. brasiliensis* to investigate the expressed genes and molecular events in the latex. We analyzed 412 expressed sequence tags (ESTs). More than 90% of the EST clones showed homology to previously described sequences in public databases. Functional classification of the ESTs showed that the largest category were proteins of unknown function (30.1%), 11.4% of ESTs encoded for rubber synthesis-related proteins (RS) and 8.5% for defense or stress related proteins (DS). Those with no significant homology to known sequences (NSH) accounted for 8.7%, primary metabolism (PM) and gene expression and RNA metabolism were 7.8% and 6.6%, respectively. Other categories included, protein synthesis-related proteins (6.6%), chromatin and DNA metabolism (CDM 3.9%), energy metabolism (EM 3.4%), cellular transport (CT 3.2%), cell structure (CS 3.2%), signal transduction (ST 2.2%), secondary metabolism (SM 1.7%), protein fate (PF 2.2%), and reproductive proteins (RP 0.7%).

Keywords: cDNA library, expressed sequence tag, *Hevea brasiliensis*, latex, rubber tree

1. Introduction

More than 12,000 species of the plant kingdom produce latex (de Fay and Jacob, 1989; Yeang *et al*., 2002). The living cytoplasm of specialized lactiferous cells, contains 30-50% (w/w) of natural rubber (cis-1, 4-polyisoprene) (Metcalfe, 1967; Kekwick, 1989). *Hevea brasiliensis* (Willd. *ex* A, Juss.) Mull. Arg is the only species that produces commercially viable quantities of high quality rubber (de Fay and Jacob, 1989; Ko *et al*., 2003). Therefore, the expression of genes and other molecular events in the rubber tree are of interest to scientists to provide information that might help them to improve the productivity of the plant.

After ultracentrifugation of the latex from *Hevea brasiliensis*, at 53,000 g, three main fractions can be detected, these are: the rubber phase (RP), the C-serum and the bottom fraction (B-serum). There are proteins associated with each fraction, those from the B-serum originating mainly from the lutoids. The C-serum is the aqueous medium in which all the latex organelles are suspended and contains a large variety of proteins associated with cellular metabolism and various enzymes specific to latex, for example the enzymes associated with the rubber biosynthetic pathway (Yeang *et al*., 2002). Rubber itself is synthesized on the surface of particles suspended in the C-serum. A physiological function for the latex and stored rubber molecules has not been confirmed. Plant genomics could offer a unique opportunity to change this deficiency. The identified genes expressed about 16% in the latex of the rubber tree encode for rubber biosynthesis-related proteins while 12.6% are related to plant defense genes (Han *et al*., 2000). Among the redundantly expressed genes, REF (rubber elongation factor) was expressed most frequently, followed by the gene for SRPP (small rubber particle protein). The differential expression of several rubber biosynthesis-related genes in the latex of *H. brasiliensis* was investigated (Kush *et al*., 1990). Transcripts encoding enzymes involved in rubber biosynthesis and plant defenses

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in latex were enriched 20- to 100-fold and 10 to 50-fold as compared to leaf, respectively. Although, transcripts of a cDNA library in latex have been reported, a cDNA library of latex from different sources of latex may provide different outcomes of transcripts that will lead to further insides on the molecular mechanisms of the rubber tree.

In order to obtain a comprehensive profile of genes expressed in the rubber tree, we constructed a cDNA library from the latex of *H. brasiliensis* and analysed the expressed sequence tag (EST) of these *H. brasiliensis* cDNA libraries. Identification of several novel genes expressed in the rubber tree latex could provide information that would provide us with a better understanding of the molecular events involved in rubber biosynthesis and/or plant defensive mechanisms against pathogens.

2. Materials and Methods

2.1 Plant material

Latex samples were obtained from mature rubber plants *(H. brasiliensis* RRIM 600), grown in Phatthalung Province, Thailand.

2.2 Total RNA and poly(A)$^+$ RNA isolation

Ten ml of latex was collected in a 50 ml Folcon tube and total RNA was extracted with Trizol reagent (Gibco/Life Technologies, Grand Island, NY, USA). Poly(A)$^+$RNA was isolated with the messenger RNA isolation Kit (Stratagene, La Jolla, CA). Briefly, from 1 to 5 mg of total RNA was hybridized with the oligo(dT) cellulose resin in a 50 ml conical tube by gently shaking for 15 min at room temperature. Then, the supernatant was removed by centrifugation at 700 g for 3 min. The pellet was washed with high-salt buffer and re–suspended in low-salt buffer. 400 µl of the elution buffer at 68°C was added to elute the mRNA and the mRNA was stored at -70°C until used.

2.3 Construction of the cDNA library

A cDNA library was constructed with the ZAP Express™ cDNA synthesis kit (Stratagene, La Jolla, CA, USA). The mRNA (5.0 µg) was reverse transcribed and double-stranded cDNA was synthesized following the manufacturer's instructions. Synthesized cDNA was ligated to the *EcoR*I adapters and then digested with *Xho*I. The digested cDNA was size fractionated by a drip column containing the Sepharose CL-2B gel and directionally cloned into the ZAP Express vector. The cDNA library was amplified and the lambda phage was excised *in vivo* from the vector with ExAssit Helper Phage. Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA, USA) was used to package the ligated products. Mass excision was performed and the cDNA inserts from the amplified phage library were rescued as pBK-CMV phagemid in XLOLR *Escherichia coli* according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Recombinant cells were plated onto LB agar containing 50 µg/ml kanamycin. Plasmids from selected colonies were sequenced and further characterized.

2.4 sequence and data analysis

Plasmid DNA harboring inserted genes from the bacterial cultures were purified with a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and the purified plasmid DNA was sequenced (Macrogen, Seoul, South Korea) by the Automated DNA sequencer, ABI Prism 377, Applied Biosystems. A National Centre for Biotechnology Information (NCBI) non-redundant nucleotide BLASTX search was carried out to find any similarities to the edited sequences (Altschul *et al*., 1990). Scores and E values from the BLASTX results were considered when determining significant similarities. The sequence homology of the EST clones was compared by the ClustalX software.

3. Results

3.1 Construction of cDNA library

We constructed a cDNA library from the latex of *H. brasiliensis* using the ZAP Express™ cDNA synthesis kit (Stratagene, La Jolla, CA, USA). After packaging and titreing, the primary library contained the phage at $1x10^7$ pfu/ml. The phage were excised *in vivo* to form plasmids and transformed to XLOLR *E. coli* cells. To evaluate and estimate the quality of the library, we sequenced the 3' end of 413 randomly selected cDNA clones and analyzed the results against the non-redundant database (NCBI, http://www.ncbi.nlm.nih.gov/) with the BLASTX algorithm (Altschul *et al*., 1990). The clones with homologies $\geq 10^{-5}$ to the NCBI public database were classified.

3.2 Functional classification of the EST sequences

More than 90% of the EST clones showed homology to previously described sequences in public databases. The database-matched ESTs were classified based on their putative functions (Table 1). Functional classification of the ESTs showed that the largest group were proteins of unknown function (UF) (30.1%). 11.4% of ESTs encoded rubber synthesis-related proteins (RS) (Table 2) which were genes encoding the rubber elongation factor (REF), Hev b3, hydroxymethlglutaryl coenzyme A synthase, small rubber particle protein (SRPP) and cis-prenyl transferase. Sequences with no significant homology to any known gene (NSH) accounted for 8.7%. The defensive or stress related proteins (DS 8.5%), for example, an ASR-like protein 1, Pro-hevein and a disease-resistant-related protein were abundant. In addition, primary metabolism (PM), gene expression and RNA metabolism (GERM) and protein synthesis-related proteins were 7.8%, 6.6%, 6.6%, respectively. Small proportions i n

Table 1. Functional calssification of all sequenced ESTs in the cDNA library from the latex of *H. brasiliensis*.

Function category	Number in category	Number in category (%)
Rubber synthesis (RS)	47	11.4
Defense or stress (DS)	35	8.5
Primary metabolism (PM)	32	7.8
gene expression and RNA metabolism (GERM)	27	6.6
Protein synthesis (PS)	27	6.6
Chromatin and DNA metabolism (CDM)	16	3.9
Energy metabolism (EM)	14	3.4
Cellular transport (CT)	13	3.2
Cell structure (CS)	13	3.2
Protein fate (PF)	9	2.2
Signal transduction	9	2.2
Secondary metabolism (SM)	7	1.7
Reproductive proteins (RM)	3	0.7
Unknown function (UF)	124	30.1
No significant homology (NSH)	36	8.7
Total ESTs	412	100

Table 2. Rubber synthesis transcripts in the cDNA library from the latex of *H. brasiliensis*.

Putative protein	Accession number	Hit acc no.	No. of ESTs
Rubber elongation factor protein (REF) (Allergen Hev b 1)	100%homology to P15252	P15252	39
Hev b 3	FE969191	CAA11305	5
Hydroxymethylglutaryl coenzyme A synthase	FE969192	AAL18930	1
Cis-prenyltransferase 7	FE969193	AAM92886	1
Small rubber particle protein (SRPP) (22 kDa rubber particle protein) (22 kDa RPP) Latex allergen Hev b 3	FE969191	O82803	1

other categories included chromatin and DNA metabolism (CDM 3.9%) energy metabolism (EM 3.4%), cell structure (CS 3.2%), secondary metabolism (SM 1.7%), cellular transport (CT 3.2%), protein fate (PF 2.2%), and reproductive proteins (RP 0.7%). The most abundant transcripts are reported in Table 3.

4. Discussion

Natural rubber (cis-1,4-polyisoprene) of *H. brasiliensis* is synthesized on the surface of particles suspended in latex. We postulated that genes uniquely or preferentially expressed in the latex might be important for rubber biosynthesis. Therefore we constructed a cDNA library from latex to investigate the genes expressed in the latex of *H. brasiliensis* using expressed sequence tags (ESTs) for analysis. Functional classification of the ESTs obtained from the cDNA library revealed that of those with recognized homology, the rubber biosynthesis-related genes exhibited the highest frequency of expression followed by defense-related genes. More than 90% of the rubber biosynthesis-related genes were for REF (rubber elongation factors). This protein is closely associated with rubber particles and may be directly involved in rubber biosynthesis (Dennis and Light, 1989; Yeang *et al*., 1996). Reverse northern blot analysis had shown that the products of REF and SRPP genes were accumulated more than 10-fold in the latex (Ko *et al*., 2003). SRPP have also been shown highly expressed in latex than in leaves and that it enhances

Table 3. Most abundant transcripts in the cDNA library from the latex of *H. brasiliensis*.

Putative protein	Hit acc no.	No. of ESTs	% of total ESTs
Rubber elongation factor protein (REF) (Allergen Hev b 1)	P15252	39	9.44
RNA-binding protein-like	AAM47313	11	2.66
Transcription factor	AAS97942	6	1.45
Hev b 3	CAA11305	5	1.21
Zinc finger protein	AAN15629	4	0.97
Protease inhibitor protein 1	AAP46156	4	0.97
ATSWI3B; DNA binding	NP_180919	4	0.97
Catalytic/ hydrolase	NP_566932	4	0.97
ATP binding / kinase/ protein kinase/ protein serine/ threonine kinase/ protein-tyrosine kinase	NP_001031758	4	0.97
S-adenosylmethionine decarboxylase	AAM44307	3	0.73
ASR-like protein 1	AAP46155	3	0.73
Disease-resistant-related protein	AAL78367	3	0.73
Receptor activity modifying protein 1	CAC85626	3	0.73
REF-like stress related protein 2	AAP46160	3	0.73
Prohevein	CAA05978	2	0.48
Cinnamyl alcohol dehydrogenase	AAK28509	2	0.48
Myb transcription factor	ABK20308	2	0.48
Skp1	AAD34458	2	0.48
Cytosolic acetoacetyl-coenzyme A thiolase	AAU95618	2	0.48
Chorismate synthase 1	CAA79859	2	0.48
Chloroplast ATP synthase (delta subunit)	CAA45153	2	0.48
Cytochrome b5 isoform Cb5-C	AAT84460	2	0.48
Electron transporter	NP_175507	2	0.48
40S ribosomal protein S23	P46297	2	0.48
40S ribosomal protein S16	AAF34799	2	0.48
putative 24 kDa seed maturation protein-like protein	ABC01908	2	0.48

rubber synthesis (Han *et al*., 2000). In contrast, SRPP was not as highly expressed as previously described while Hev b3 was present in high amounts (Table 2).

The second most frequently expressed genes in the latex were the defense-related genes, for example, an ASR-like protein 1 and a disease-resistance-related protein. A role for latex in defense has been proposed (Farrell *et al.,* 1991). Isoprenoids including terpenes and steroids seem to be particularly resistant to biological degradation. The transcript levels of plant defense- or stress-related genes were observed 10 to 50-fold higher in latex than in leaves (Kush *et al*., 1990). Possibly the most popular justification for the presence of laticifers has been a presumed protective role. The protection of *H. brasiliensis* from fungi and insect pests by latex has been supported by the characterization of a chitin binding protein in latex called hevein and detection of chitinase activity, both of which are found in the lutoid organelle (Kekwish, 2001). A chaperone and heat shock proteins (HSP) were also found. These latter proteins are a family induced by different stresses and involved in the defence against reactive oxygen species or chemicals that generate oxidative stress (Hansen *et al*., 2007). There were other genes, involved in detoxifying, found in the library: glutathione-dependent formaldehyde dehydrogenase, glutathione transferase and a superoxide dismutase including a Thioredoxin H protein that is believed to interact with a reactive oxygen species-detoxifying enzyme. Many genes are involved in gene expression and RNA metabolism such as a zinc finger protein and transcription factors and a Myb transcription factor is crucial to control the proliferation of a number of cell types. The comparison of the expression of the Myb transcription factor gene in healthy trees and rubber trees with tapping panel dryness (TPD) syndrome was reported (Venkatachalam *et al*., 2007). The results showed that the Myb transcription factor gene was decreased in the TPD tree. It is possible that TPD trees lack active cell division or proliferation and this is revealed by the decreased expression of Myb genes.

There could be others as 30.1% of the genes encode for proteins of unknown function and some of these may provide information about function and molecular events in the rubber latex. Therefore, functional analysis of these unknown proteins should be investigated.

Acknowledgment

This work was supported by a research grant from Center for Genomics and Bioinformatics Research, Prince of Songkla University. We thank B. Hodgson, Faculty of Science, Prince of Songkla University, for reading the manuscript and for valuable comments.

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