J. Rubb. Res., **18(3)**, 148–158

Identification and Characterisation of Endo-1,4-β-glucanase Family Members in Hevea brasiliensis

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In order to study wood formation in Hevea brasiliensis*, endo-1,4-β-glucanase (EGase) was selected for molecular genetic analysis. EGase sequences were isolated from bark, leaf and latex transcriptomes (clone RRIM 928). Altogether, 22 sequences were obtained from the bark transcriptome while 14 and 8 sequences were obtained from leaf and latex transcriptomes, respectively. Based on multiple sequence alignment, only twelve sequences encoded full protein open reading frames while the others contained partial proteins. Translation of twelve EGase sequences (designated HbEg1-12) produced peptide sequences ranging from 494-622 amino acids. Based on protein motif conservation and phylogenetic analysis, these EGases were separated into three classes, A, B and C. Expression of EGase was found to be generally low in latex, bark and leaves.*

Keywords: Wood formation; rubber wood; endo-1,4-β-glucanase; transcriptome

Wood formation in plants is typically governed by two mechanisms, merismatic cell growth and expansive cell growth. Although both processes occur through different cellular machinery, the same rate limiting factor lies on the degree of flexibility of the plant cell walls to allow such expansion to occur[1]. In plant cell walls, cellulose microfibrils are tightly crosslinked by xyloglucans chains which contribute to the rigidity of the cell walls. To enable cellular expansion and division during plant development, cellulose microfibrils need to be loosened in order for cells to elongate[2]. This process which has been previously identified as the rate limiting step for successful cell elongation may be achieved through several proposed enzymatic mechanisms which involves endo-1,4-β-glucanases[3,4,5,6], endo-xyloglucan tranferases[7,8] and expansins[1,9,10].

Endo-1,4-β-glucanases (EC 3.2.1.4), or better known as cellulases, are enzymes which metabolise xyloglucan and cellulose in higher plants. These apoplastic enzymes participate in various physiological aspects of

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plant growth, for example, loosening of the primary cell wall, vascular differentiation, leaf abscission, fruit ripening and symbiosis[11,12,13]. Further molecular characterisation established that this EGase is essential for the formation of vascular tissue particularly the xylem[14]. In fact, knockdown studies in *Arabidobsis thaliana* plants with an anti-sense cDNA of EGase resulted in plants with stunted growth or slow in development[15].

A deeper understanding of the genetic and biochemical processes of rubberwood formation is important for developing strategies for enhancing wood production. Given the well documented functions of EGase, we selected this class of wood forming genes for molecular characterisation. In this paper, we have isolated three classes of EGase from leaf, latex and bark transcriptomes (tree clone RRIM 928). In addition, we show results of preliminary expression analysis of such genes in different *Hevea* tissues.

MATERIALS AND METHODS

Bioinformatics Analysis

Six EGase sequences were retrieved from Genebank *Populous* and *Arabidopsis* databases: AtGH9A1 (NM_124350.2), AtGH9B1 (X98544.1), AtGH9C1 (NM_103786.2), PtGHA1 (HQ331249.1), PtGHB1 (D32166.1) and PtGHC1 (HQ331271.1) and searched against Trinity-assembled transcripts from RNA-Seq libraries previously generated from three *Hevea* tissues[16]. Transcript search was done using the BLASTn programme[17] and putative *Hevea* EGase matches were selected based on a minimum e-value of $1.0e^{-5}$. Transcripts were selected and protein translation was performed using Expasy-Translate Tool[18]. Identification of untranslated regions (UTRs) and open reading frames (ORFs) was carried out using InterProScan search programme[19]. Motif identification among EGase sequences was performed using the MEME programme[20]. Phylogenetic analysis was conducted using Multiple Sequence Alignment by CLUSTALW[21].

Plant Material

Latex was collected by tapping the *Hevea* trees (clone RRIM 928) grown in the fields of the Sungai Buloh Experiment Station, Malaysian Rubber Board for RNA isolation. Latex was collected directly into an equal volume of 2X RNA extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 10 mM EDTA, 10% SDS, pH 9.5) in a 50 ml Falcon tube and immersed in liquid nitrogen (N_2). Total RNA from latex was extracted according to the method of Kush *et al*.[22] *Hevea* tree bark was collected from the same trees where latex was collected and sealed in a zip lock bag before immersing into liquid N_2. Bark RNA extraction was performed using a modified protocol by Mackenzie *et al*.[23] Young fully green *Hevea* leaves were collected and sealed in a zip lock bag before immersing in liquid N_2. Leaves were ground in liquid N_2 in a mortar into fine powder before extraction in 25 mL of 1X RNA extraction buffer[22]. Total RNA from leaves was extracted according to the method of Kush *et al*.[22] Total RNA was electrophoresed using 1% agarose gel to check its integrity[24] and quantified using Nanodrop ND1000 (Thermo Scientific).

Primer Design

Primers were designed using the Primer3.0 software[25]. Degenerate primer sequences for gene expression studies of each class of EGase and of the *Hevea* 18S rRNA (AB268099.1) are listed in *Table 1*. For amplification of full ORFs of HbEg1-12, specific primers targeting

the 5' start codon and 3' stop codon are listed in *Table 1*. All primers were synthesised by AITBiotech Pte Ltd. (Singapore).

Semi-Quantitative Expression Analysis and HbEg ORF Amplification

Reverse transcription was performed using the ImPromII™ Reverse Transcription System (Promega) with 1 µg of total RNA from bark, latex and leaf according to manufacturer's protocol. Semi-quantitative PCR analysis was performed using 1 µl of reverse transcription product and PCR reaction products sampled at 5 time points (cycles 15 20, 25, 30 and 35). The PCR reaction mix consisted of 1X Green GoTaq flexi buffer, 1.9 mM $MgCl_2$, 0.4 mM dNTP mix, 1 µM forward primer, 1 µM reverse primer, 1.25 U GoTaq DNA polymerase to a final reaction volume of 25 µl. PCR products were analysed on a 1.5% agarose gel. Amplification of ORF regions of HbEg1-12 ORFs was performed using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) according to the manufacturer's protocol. For these reactions, the DNA template for each EGase was the reverse transcription reaction of latex, leaf or bark depending on the tissue origin of each EGase (*Table 2*).

RESULTS AND DISCUSSION

Identification of EGase Isoforms

To isolate EGases from *Hevea brasiliensis*, three previously generated bark, latex and leaf RNA-Seq transcriptomes[16] were used. EGase nucleotide sequences of *A. thaliana* and *Populus trichocarpa* were used as query sequences to search the *Hevea* transcripts assembled by the Trinity programme. Altogether, 44 sequences were retrieved from the transcriptome databases with transcript length ranging from 236-5126 bp (*Table 2*). Within these, 22 EGase sequences were obtained from the bark transcriptome while 14 and 8 sequences were derived from the leaf transcriptome and latex transcriptome respectively (*Table 2*).

Next, the 44 EGase sequences were translated for analysis of protein coding regions. The expected size of EGases is approximately 490 amino acids for Class B EGases and 620 amino acids for Classes A and C EGases[26]. By comparing *Hevea* EGase peptide sequences through multiple sequences alignment showed that, only 35 displayed conserved EGase domains while the remaining nine transcripts did not show any similarity to EGase (*Table 2*). However, out of the 35, only 12 sequences encoded complete ORFs inclusive of 5' and 3' UTRs while others contain partial ORFs. The 12 EGases were then designated as HbEg1 to HbEg12 (*Table 2*).

EGase Classification and Phylogenetic Analysis

Three different classes of EGases (A, B and C) have been characterised previously in *A. thaliana*[26], *Solanium lycopersicum*[27], maize[28] and rice[29]. Subsequently, the 12 EGase protein isoforms found in this study were aligned in order to identify motif conservation and distribution as shown in *Figure 1*. The alignment revealed that HbEg5, HbEg6, HbEg7, HbEg9 and HbEg10 share a high level of sequence similarity and exhibited highest homology with Class A EGases (*Figure 1*). Interestingly, HbEg5, HbEg6, HbEg9 and HbEg10 have less than seven amino acid difference between them (detailed sequence region not shown). HbEg7 slightly differs in its motif distribution in that it lacks an extra six-hairpin glycosidase motif (*Figure 1*, Motif 5). Five isoforms (*ie*. HbEg2, HbEg3, HbEg8, HbEg11 and HbEg12) were identified as Class

TABLE 1. PRIMER SEQUENCES FOR ORF AMPLIFICATION AND FOR SEMI-QUANTITATIVE PCR

Gene	Primer Sequence (FW:Forward; RV: Reverse)	Amplicon Length (bp)	Annealing Temperature (ºC)	GenBank Accession No. of cDNA
HbEgA	FW:TGGACGAGACWCAACAGAGC RV:CTCCAGCTCARCATSGTCA	424	53	-
HbEgB	FW:TGGGCTACYGATTATCTGYTM RV:CCAGCRTGCTTRTTRTCCC	497	53	-
HbEgC	FW:GACCATTACTGCTGGCAAAGR RV:CCAGCATAYTTRACATCCCARC	460	53	-
18S rRNA	FW:AAAGACGAACAACTGCGAAAG RV:GCTCCACCAACTAAGAACGG	377	53	-
HbEg1	FW:ATGAAGCCCTCTCCTTTCTTCT RV:TCAACTAAAATTAGGATTTGCTGCG	1482	45	KM388877
HbEg2	FW:ATGGAGAAGCATTTTAAGCTCATTTC RV:TCAGGCTAAAGTGTAGCTTGAAAC	1866	48	KM388878
HbEg3	FW:ATGGAGAAGCATTTTAGGCTCATTTC RV:TCAGGCTAAAGTGTAGCTTGAAAC	1866	48	KM388879
HbEg4	FW:ATGGCGCCAAAATCCCACTCATTGT RV:TCAAGAGTGGGCTGAAAAGTAAGCG	1491	52	KM388880
HbEg5	FW:ATGAGTATGTACGGAAGGGATCCCT RV:TTAGGGTTTCCAGGGTGCTGGA	1860	52	KM388881
HbEg6	FW:ATGTACGGAAGGGATCCATGGG RV:TTAGGGTTTCCAGGGTGCTGGA	1854	52	KM388882
HbEg7	FW:ATGCATTCAGGGAATAACTGGGGAG RV:TCAAGGCTTCCAAGGTGGAGGAG	1863	52	KM388883
HbEg8	FW:ATGGAGAAGGAGAAGAAGAACCAAC RV:CTAATAAGAAGCAATCAAAGGCTGATT	1563	48	KM388884
HbEg9	FW:ATGAGTATGTACGGAAGGGATCCCT RV:TTAGGGTTTCCAGGGTGCTGGA	1860	52	KM388885
HbEg10	FW:ATGAGTATGTACGGAAGGGATCCCT RV:TTAGGGTTTCCAGGGTGCTGGA	1860	52	KM388886
HbEg11	FW:ATGGAGAAGCATTTTAGGCTCATTTC RV:TCAGGCTAAAGTGTAGCTTGAAAC	1866	48	KM388887
HbEg12	FW:ATGGAGAAGCATTTTAGGCTCATTTC RV:TCAGGCTAAAGTGTAGCTTGAAAC	1866	47	KM388888

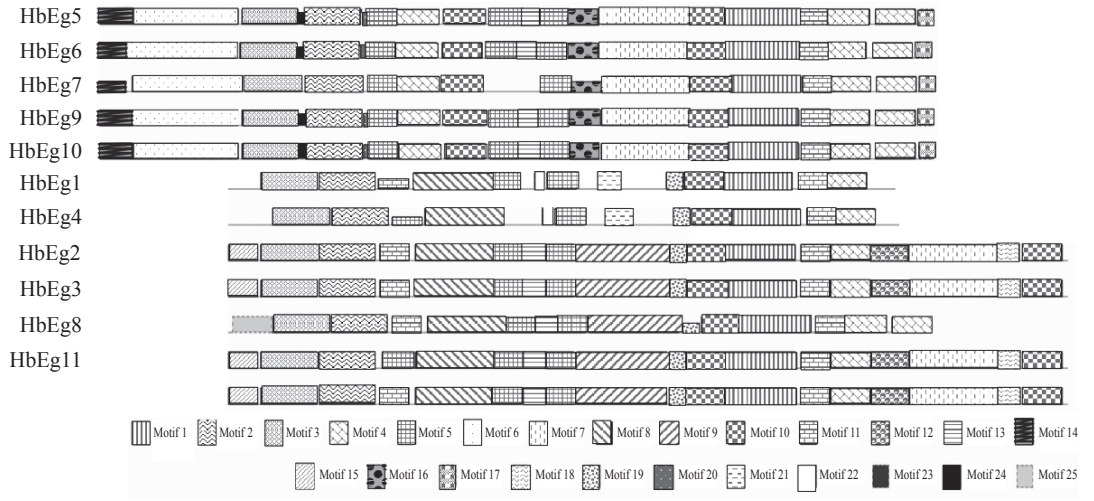
C EGases based on a consensus signal peptide at the N terminal region (*Figure 1*, Motif 15) and a conserved catalytic domain (*Figure 1*, Motif 9). However, HbEg8 does not possess a carbohydrate binding module (CBM; *Figure 1*, Motif 7) similar to the other Class C EGases but contained a stop codon and a repeat of Motif 4 at C terminal region. Additionally, HbEg8 also has a dissimilar signal peptide region at the N terminal. Nonetheless, motif distributions of its catalytically essential six-hairpin glycosidase motifs well aligned with the other Class C EGases. Both HbEg1 and HbEg4 were identified as Class B EGases

TABLE 2. FORTY-FOUR SEQUENCES IDENTIFIED WHICH HAS SIGNIFICANT SIMILARITY IN ITS AMINO ACID SEQUENCES TO THE EGASES IN THE PUBLIC DOMAIN (GENEBANK)

No.	Transcript	Transcript length (bp)	Nature of transcripts	Total	Source Transcriptome	Query sequence	E-value	Protein coding (a.a)	New Designation	New Classification
1	comp73840_c0_seq3	3130	5'UTR + ORF + 3'UTR	12	bark	PaGH9B1	3.00E-07	494	HbEg1	B
2	comp84883_c0_seq1	2544			bark	PtGH9C1	0.0	622	HbEg2	C
3	comp84883_c0_seq2	2675			bark	PtGH9C1	0.0	622	HbEg3	C
4	comp77557_c0_seq2	2169			bark	AtGH9B1	5.00E-49	497	HbEg4	B
5	comp82487_c0_seq2	2496			bark	PtGH9A1	0.0	620	HbEg5	A
6	comp82487_c0_seq1	2566			bark	PtGH9A1	0.0	618	HbEg6	A
7	comp82737_c0_seq1	4085			bark	AtGH9A1	1.00E-06	621	HbEg7	A
8	comp81336_c0_seq1	1884			bark	AtGH9C1	2.00E-12	521	HbEg8	C
9	comp37348_c0_seq2	4081			leaf	PtGH9A1	0.0	620	HbEg9	A
10	comp37348_c0_seq1	5126			leaf	PtGH9A1	0.0	620	HbEg10	A
11	comp36383_c0_seq2	2147			leaf	PtGH9C1	0.0	622	HbEg11	C
12	comp23396_c0_seq1	2481			latex	PtGH9C1	0.0	622	HbEg12	C
13	comp72620_c1_seq1	577	5'UTR + partial ORF	3	bark	PaGH9B1	6.00E-95	157	-	-
14	comp71925_c0_seq1	1909			bark	PtGH9A1	6.00E-12	588	-	-
15	comp23081_c0_seq1	1970			latex	PtGH9A1	0.0	604	-	-
16	comp72620_c0_seq1	1228	Partial ORF + 3'UTR	4	bark	PaGH9B1	0.0	344	-	-
17	comp53468_c0_seq3	236			bark	PtGH9A1	2.00E-18	63	-	-
18	comp24145_c0_seq2	1207			leaf	AtGH9B1	7.00E-06	281	-	-
19	comp37348_c0_seq3	831			leaf	PtGH9A1	1.00E-91	129	-	-
20	comp1526975_c0_seq1	255	Partial ORF	16	leaf	AtGH9B1	4.00E-32	85	-	-
21	comp24145_c0_seq1	841			leaf	AtGH9B1	7.00E-06	279	-	-
22	comp511910_c0_seq1	344			leaf	PtGH9C1	6.00E-07	114	-	-
23	comp13054_c0_seq1	409			leaf	PtGH9C1	6.00E-07	136	-	-
24	comp933589_c0_seq1	254			leaf	AtGH9C1	4.00E-05	84	-	-
25	comp598650_c0_seq1	615			latex	PtGH9A1	1.00E-12	204	-	-
26	comp2949_c0_seq1	465			latex	PtGH9A1	1.00E-12	154	-	-
27	comp73840_c0_seq2	2696			bark	AtGH9B1	2.00E-05	375	-	-
28	comp83103_c0_seq36	2577			bark	AtGH9A1	2.00E-09	118	-	-
29	comp83103_c0_seq24	898			bark	AtGH9A1	2.00E-09	118	-	-
30	comp83103_c0_seq10	2467			bark	AtGH9A1	2.00E-09	114	-	-
31	comp81336_c0_seq2	2010			bark	AtGH9C1	2.00E-12	444	-	-
32	comp34390_c0_seq2	637			leaf	PtGH9A1	1.00E-11	118	-	-
33	comp34390_c0_seq1	683			leaf	PtGH9A1	1.00E-11	114	-	-
34	comp23053_c0_seq4	2407			latex	PtGH9A1	6.00E-12	125	-	-
35	comp23053_c0_seq1	2475			latex	AtGH9A1	4.00E-10	114	-	-
36	comp46052_c0_seq1	431	No conserved domain (no significant similarity to EGases)	9	leaf	AtGH9A1	6.00E-07	-	-	-
37	comp36383_c0_seq1	680			leaf	PtGH9C1	4.00E-17	-	-	-
38	comp475471_c0_seq1	239			latex	AtGH9A1	2.00E-05	-	-	-
39	comp23081_c0_seq2	429			latex	AtGH9A1	4.00E-16	-	-	-
40	comp53468_c0_seq2	648			bark	PtGH9A1	2.00E-18	-	-	-
41	comp53468_c0_seq1	401			bark	PtGH9A1	2.00E-18	-	-	-
42	comp361399_c0_seq1	504			bark	PtGH9A1	9.00E-08	-	-	-
43	comp73105_c0_seq1	260			bark	PtGH9A1	1.00E-06	-	-	-
44	comp73105_c1_seq3	269			bark	PtGH9A1	2.00E-05	-	-	-

since it lacks the transmembrane region (*Figure 1*, Motif 6) such as that found in Class A EGases and the CBM (*Figure 1*, Motif 7) of Class C EGases. In conclusion, the 12 protein isoforms consisted of five Class A, two Class B and five Class C EGases.

Previously, Class A EGases has been known as membrane bound protein where it possess a characteristic transmembrane domain at its N-terminal region[29] while Class B is known as extracellular proteins located in the apoplastic regions due to its lack of a transmembrane



Motif 1-5, 8-10 -Six hairpin glycosidase; Motif 6- Transmembrane region; Motif 7- Carbohydrate binding module (CBM); Motif 15 – Signal peptide; Motif 11-14, 16-25 – No match was found using InterProScan search program.

Figure 1. Motif conservation and distribution of HbEg1-12 protein isoforms.

region. Class C enzymes have an additional carbohydrate binding domain (CBD) present at their C-terminal which allows effective binding to its substrates for effective catalysis[27]. Without the CBM, glycoside hydrolases attack on their substrate was deemed ineffective as the chemical and physical structure of the plant cell wall restricts enzyme access to cellulose-xyloglucan linked microfibrils. To maximise such hydrolytic activity, plants and microbial EGases have evolved with one or more CBM which allow the enzymes to bring the appended target sites into intimate contacts with the catalytic domain which in other words, CBMs promote the association of the enzyme with the substrate[30].

To further validate HbEg1-12 classification by motif characterisation, phylogenetic analysis of 12 *Hevea* EGases with known endo-1,4-β-glucanases from various plant species was performed. As shown in *Figure 2*, the 12 EGase proteins found in this study can be grouped into three separate subclasses *i.e*. Class A, B and C (*Figure 2*). This phylogenetic classification is consistent with that from the motif conservation and distribution analysis in *Figure 1*.

PCR Amplification of 12 HbEgs

As explained previously, EGase transcripts HbEg1-12 had been generated from *de novo* assembly of RNA-Seq reads. We therefore decided to verify transcript presence by ORF-specific RT-PCR amplification of HbEg1-12 using RNA templates corresponding to the tissue of origin of each EGase (*Table 2*). Agarose gel analysis showed that all reactions, with the exception of that for HbEg1, produced single amplification products although HbEg8 product was barely detectable (*Figure 3*). The molecular weights of these products

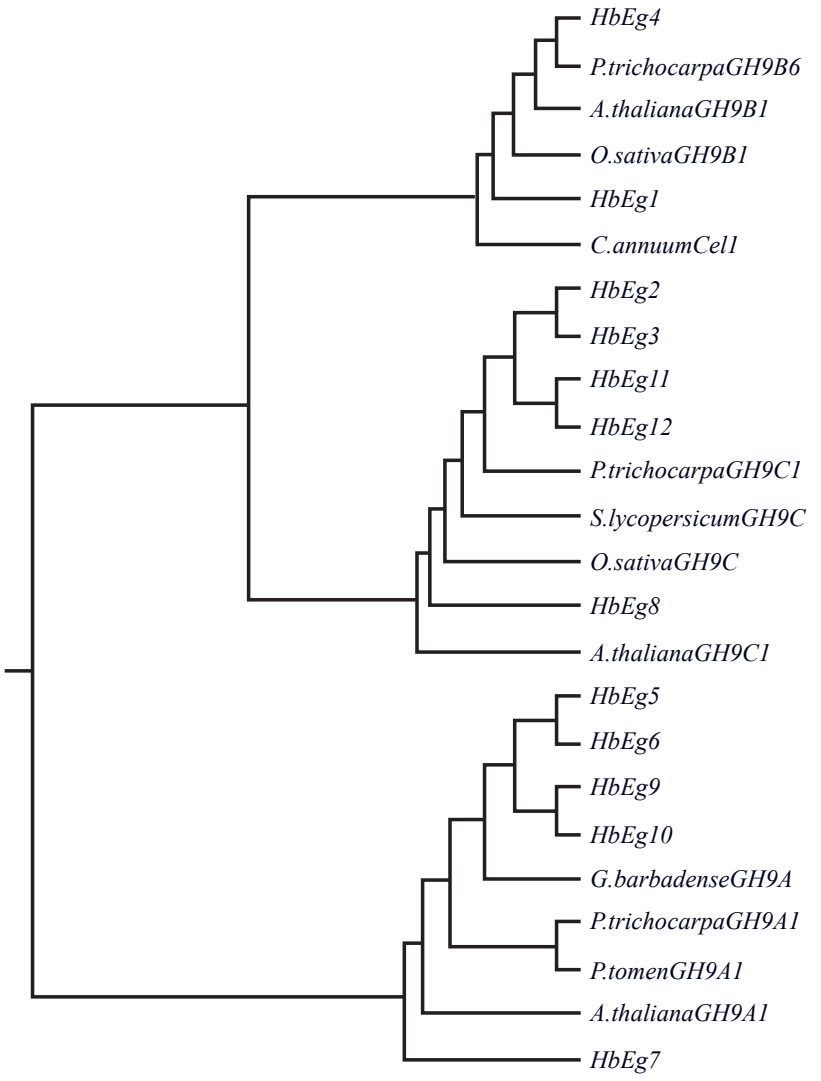


Figure 2. Phylogenetic analysis of 12 Hevea *EGase family members with endo-1,4-β-glucanases from several other plant species.*

corresponded with their respective ORF sizes between 494 to 622 amino acids (*Table 2*). In conclusion, this analysis lent support to the fact that up to ten of the EGase transcripts isolated in this study (*i.e.* with the exception of HbEg1 and HbEg8) may be found *in vivo*.

EGase Expression Analysis in *Hevea* tissues

Class A, B and C EGases were found among 12 transcripts as described previously. In *Table 2*, the tissue origins of these 12 EGases show that all three classes of EGases are found in

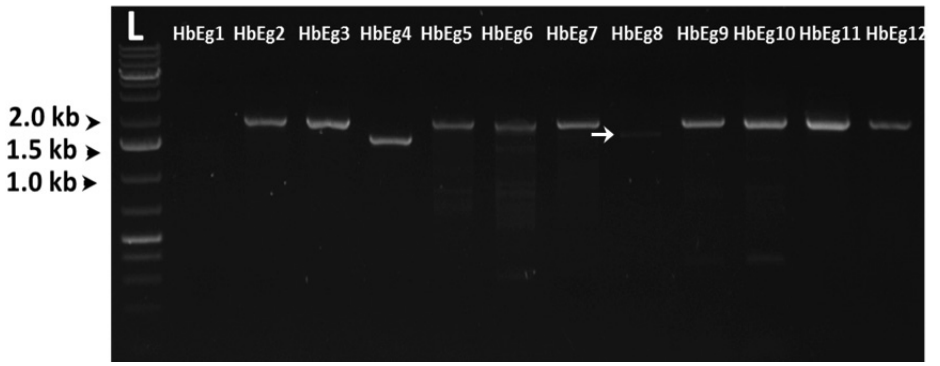


Figure 3. PCR amplification and verification of HbEg1-12 using ORF-specific primers and reaction templates derived from tissues corresponding to their source transcriptomes. A single PCR product was obtained in all cases except for HgEg1 (arrow indicates faint band of HgEg8 product). Lane L: 1 kb DNA ladder; Lane 1-12: HbEg 1-12.

bark, Classes A and C only in leaf and Class C only in latex. Since eight EGases originated from the bark transcriptome, this implied that EGase expression is most abundant in bark compared to the other two tissues. Subsequently, to study the expression pattern of *Hevea* EGase, degenerate primers were designed for semi-quantitative PCR detection of each class of EGase in three tissues (*Figure 4*). Overall, EGase expression in bark, latex and leaves was low, with most transcripts detected after more than 25 cycles of PCR amplification. Among the three classes, Class A EGase expression appeared to be the highest in all tissues. The relative abundance of each EGase Class in latex, leaf and bark is shown in *Figure 4*.

Although most of the EGases transcripts were identified from the bark transcriptome in this study, surprisingly, semi-quantitative analysis of all three tissues indicated a lower level of EGases expression in bark tissue compared to leaf; which is particularly noticeable with Class B EGases expression. In this case, it is postulated that the transcripts of this apoplastic enzyme may not be detected due to the nature of bark tissue consisting primarily of rigid non-dividing secondary cell wall. On the other hand, failure to detect Class B expression in latex is largely expected as well, as the latter is basically made up of the cytoplasmic content of laticifers. These laticifers are long parallel running hollow tubes which were once individual cellular unit and has since been extensively differentiated[31]. All three EGase classes were found to be expressed in leaf tissue since young leaves are actively dividing tissue which essentially requires EGases for tissue expansion.

CONCLUSION

Endo-1,4-β-glucanases belongs to the glycosyl hydrolases family and consist of three subclasses based on the functional domains presence on its peptide sequence. In this study, all three classes of *Hevea* EGase, A-C, were successfully isolated. Of the 44 transcripts isolated from bark, leaf and latex transcriptomes, only 12 transcripts were found to code for full length ORF. Initially, five were categorised as Class

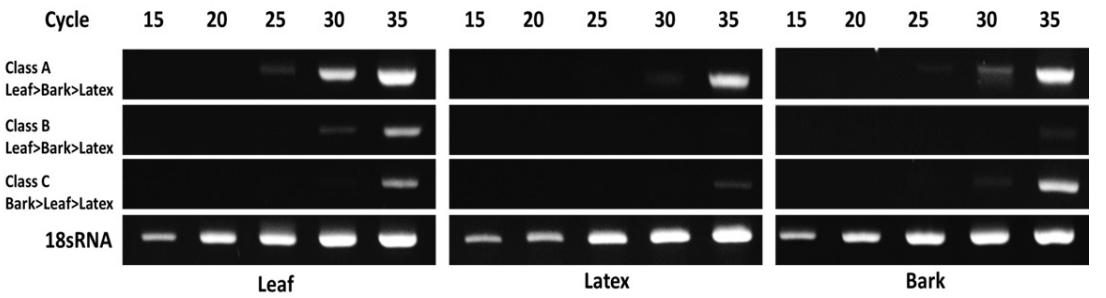


Figure 4. Gene expression analysis of Class A, B and C EGases by semi-quantitative PCR and agarose gel electrophoresis.

A and C EGase while two as B EGase. Subsequently, only ten of these transcripts were validated by RT-PCR, whereby two EGases, HbEg1 and HbEg8 were removed since it could not be efficiently amplified. Only the bark tissue contained all three EGase Classes while leaf and latex contained Class A and C EGases, respectively. As compared to the number of EGase members detected in *A. thaliana* (25), maize (29) and barley (24), the number of EGases detected was considered small which may require further identification work. Although 8 out of 12 full length EGases transcripts were identified from the bark transcriptome, semi-quantitative analysis indicated that only Class C EGase showed highest abundance in bark, in comparison with leaf and latex. For Classes A and B EGase, expression in bark was second highest across the three tissues. These observations suggest that regardless of the number of isoforms found in any of these tissues, specific isoforms may undergo tighter transcriptional control. Information on the *Hevea* EGase gene family and expression pattern from this study is a first step towards understanding the role and regulation of this gene in wood formation in the rubber tree. Future work should be performed on identifying a more comprehensive wood forming genes network in *Hevea* to allow further understanding of rubberwood formation.

Date of receipt: November 2014
Date of acceptance: April 2015

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