Full Length Research Paper

Molecular characterization of *HbEREBP2,* a jasmonate-responsive transcription factor from *Hevea brasiliensis* Muell. Arg.

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Transcription factors of AP2/ERF superfamily are generally involved in defense responses of plants to biotic and abiotic stresses. Although, defense proteins are present in abundance in laticifers of rubber tree, little is known about their transcriptional regulation. In this study, a full length cDNA, referred to as *HbEREBP2* was characterized by means of bioinformatic analysis and quantitative real-time RT-PCR. The *HbEREBP2* was 786-bp in length and contained a 480-bp open reading frame (ORF) encoding a protein of 159 amino acid residues. Bioinformatic analysis showed that the deduced amino acid sequence of HbEREBP2 had a specific domain of AP2 superfamily and shared relative high identity with members of CBF/DREB subfamily from different plant species. Quantitative real-time RT-PCR revealed that methyl jasmonate was more effective than ethylene and rapidly than mechanical wounding on up-regulating *HbEREBP2* expression. The results suggest that HbEREBP2 may be involved in the regulation of jasmonate-mediated defense responses in laticifers of rubber tree.

Key words: *Hevea brasiliensis*, Laticifer, defense proteins, AP2/ERF transcription factor, Methyl jasmonates, Ethephon, mechanical wounding.

INTRODUCTION

AP2/ERF transcription factors are characterized by a 60 to 70 amino acid DNA-binding domain specifically binding to GCC box or GCC-like box in the ethylene-responsive genes (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). This domain is first identified in APETALA2 (AP2) from the Arabidopsis (*Arabidopsis thaliana*) and in the ethylene-responsive element binding proteins (EREBPs)

from tobacco (*Nicotiana tabacum*). The EREBPs are currently renamed ERFs (Nakano et al., 2006). The AP2/ERF superfamily contains three families, ERF, AP2 and RAV (Nakano et al., 2006). The ERF family possesses only one AP2 domain and is further divided into two major subfamilies, the ERF subfamily and the CBF/DREB subfamily (Nakano et al., 2006). The ERF binding *cis*-element is referred as the GCC box, which is found in the promoters of several PR (Pathogenesis-related) genes and confers ethylene responsiveness (Ohme-Takagi and Shinshi, 1995). The DREB binding element is called C-repeat (CRT)/ dehydration-responsive element (DRE) (similar to GCC box), which is involved in the regulation of the expression of dehydration- and low-temperature-responsive genes (Singh et al., 2002). Based on the characteristic structure of DREB transcription factors, the CBF/DREB subfamily is further divided into six groups from A-1 to A-6 (Sakuma et al., 2002; Nakano et al., 2006). Members of AP2/ERF superfamily play important roles in regulating

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Table 1. Primers used for full length cDNA cloning and real-time PCR.

Name	Sequence (5' to 3')	Name	Sequence (5' to 3')
3HbEREBP1	GGGTAGCGGAGATTCGTCAA	YEREBPF	TGGATACAAGGGAGTGAGGATG
3HbEREBP2	TCTCAATTTCCCAATGAATCCG	YEREBPR	GGGAAATTGAGAGTCGCAGAAG
HbEREBPF	TCTTCTAGCTCTTTATTGCATTACC	Y18Sf	GCTCGAAGACGATCAGATACC
HbEREBPR	TATACTGACTTTCTAGGGTCCAAAA	Y18Sr	TTCAGCCTTGCGACCATAC

appropriate defense responses of plants against pathogens and herbivores (Kimura et al., 2011; Leon-Reyes et al., 2010).

Laticifers in rubber tree are the sole site for natural rubber biosynthesis and storage. They are also considered as a kind of defense structure for the presence of abundance of defense proteins, hevein, chitinase and β-1,3-glucanase (van Parijis et al., 1991; Gidrol et al., 1994; Subroto et al., 1996). In the practice of natural rubber production, latex is exploited from laticifers by tapping which results in a repeatedly mechanical wounding and latex flow of rubber tree. Meanwhile, ethephon, an ethylene releaser, is found to be effective on increasing rubber yield and thus commonly applied in rubber plantation since 1970s (Coupe and Chrestin, 1989). It is well known that either tapping or ethephon application increases the level of defense proteins in laticifers (Kush et al., 1990). These defense-related proteins are suggested to act as a biochemical barrier at the end of severed laticifers (Hao et al., 2004). To elucidate the transcriptional regulation of the defense proteins, this study focused on the molecular characterization of a member of AP2/ERF transcription factors from laticifers of rubber tree.

MATERIALS AND METHODS

Plant material and treatments

7-year-old virgin trees of rubber tree clone CATAS 7-33-97 were planted at the Experimental Farm of the Chinese Academy of Tropical Agricultural Sciences on Hainan Island of P.R. China. The trees were treated with mechanical wounding (cut the trunk barks in a half spiral pattern without latex flow) or tapping (cut the trunk barks in a half spiral pattern with latex flow) while epicormic shoots were treated with 0.5% ethephon or 0.1% methyl jasmonate. Latex samples from every three of either wounded virgin trees or tapped virgin trees for each interval were collected by tapping at 2 h, 6 h, 1 d, 3 d, 5 d, and 7 d after treatments. As control, latex samples were collected from every three of virgin trees without any treatments at the corresponding intervals. The latex samples from three of epicormic shoots for each interval were collected by cutting at 2 h, 6 h, 1 d, 3 d, and 5 d after treatments. As control, latex samples were collected from three of epicormic shoots respectively treated with water (the solvent of ethephon) and 6% ethanol (the solvent of methyl jasmonate) at the corresponding intervals.

Isolation of total RNA

Total RNA was extracted from latex according to the method (Tang

et al. 2007; Xiao et al. 2009). The quality and concentration of the extracted RNA were checked by agarose gel electrophoresis and measured by a spectrophotometer (GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer, USA).

Full length cDNA cloning

A 704-bp fragment (GenBank Accession No.: EC 607860) with conserved domain of EREBP was isolated from latex EST database of rubber tree clone RRIM600 (http://genome.ukm.my/nrestdb/). In order to obtain the full length cDNA from rubber tree clone CATAS 7-33-97, primers were designed for RACE and PCR (Table 1). The 3'-ready cDNA was synthesized by reversely transcribing 1 μg total RNA with oligo dT-3 site adaptor primer (5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TT-3') supplied by 3'-RACE kit (Takara, Dalian, China). The specific primers for 3'-RACE were designed as primer 3HbEREBP1 and the nested primer 3HbEREBP2 based on the blasted EST sequence, respectively. The first round of 3'-RACE was performed using the universal primer (5'-CCA GTG AGC AGA GTG ACG-3') and the specific primer 3HbEREBP1 in a total volume of 50 μL under the conditions of 94℃ for 5 min, 55℃ for 5 min, 72℃ for 40 min followed by 31 cycles of amplification (94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min). The PCR product was ten-fold diluted as a template for the second round of 3'-RACE using the universal primer (5'-GAG GAC TCG AGC TCA AGC-3') and the specific primer 3HbEREBP2 under the same conditions as the first round 3'RACE. The products were purified and cloned into pGEM-T easy vector, followed by sequencing. After alignment and assembly of the sequences of the blasted EST sequence and the 3'-RACE products, the full-length cDNA sequence were deduced and subsequently obtained by PCR. Its open reading frame (ORF) was amplified by RT-PCR using primers HbEREBPF and HbEREBPR with the PCR kit (Takara, Dalian, China) under the conditions of 94℃ for 5 min, followed by 32 cycles of amplification (94℃ for 1 min, 55℃ for 1 min, 72℃ for 2 min). The products were purified and cloned into pGEM-T easy vector (Promega, Fitchburg, Wisconsin.USA), and sequenced.

Multiple alignments and bioinformatics analyses

The full-length cDNA sequence was compared to the non-redundant peptide database at the National Centre for Biotechnology Information (NCBI) (http: //www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) version 2.2.17 and DNAMAN version6.0. The *cis*-acting elements in the promoters of defense proteins were analyzed online using Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Real-time RT-PCR

Prior to cDNA synthesis, RNA samples were treated with Ambion

DNA-free Dnase treatment and Removal reagents to remove the contaminating genome DNA. First strand cDNA was synthesized from 2 µg of RNA with M-MuLV reverse transcriptase and random hexamer primers (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA was diluted 1:20 with nuclease-free water. Aliquots of the same cDNA sample were used for real-time PCR with primers YEREBPF/YEREBPR designed for *HbEREBP2*, and Y18Sf/Y18Sr for *18S rRNA* (as a house-keeping gene) (Table1). Reactions were performed in a 25 µL volume containing 500 nM of each primer and 1×SYBER Green PCR master mix (Takara, Dalian, China). The primers for *18S rRNA* was used at the concentration of 50 nM.

Real-time RT-PCR was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the LightCycler 2.0 system (Roche Diagnostics, Germany). The reactions were carried out as follows: 30 s at 95℃ for denaturation, 5 s at 94℃, 20 s at 60℃, and 20 s at 72℃ for amplification. The relative abundance of transcripts was calculated according to the LightCycler Relative Quantification Software 4.05 instructions. The specificity of each primer pairs was verified by determining the melting curves at the end of each run and by sequencing the amplified fragments from agarose gel electrophoresis. The statistical significance of the values was determined by the *t*-test. The *P* values<0.05 and 0.01 were respectively considered to be significant and very significant in all cases.

RESULTS

Isolation and characterization of *HbEREBP2*

A 704-bp fragment (GenBank Accession No.: EC607860) from rubber tree clone RRIM 600 was found in latex EST library which showed similarity to some extent with the EREBP genes from *Arabidopsis* as revealed by a BlastX search. The start codon ATG was found in this EST. In this study, a homologous fragment was amplified and cloned from rubber tree clone CATAS7-33-97. It was identical with the EST. 3'-RACE generated a 328-bp fragment with the primers 3HbEREBP1 and 3HbEREBP2 (Table 1). By alignment and assembly of these two fragments, a full-length cDNA was deduced. The cDNA, referred to as *HbEREBP2* (GenBank accession number: HQ171931), was 786-bp in length and contained a 480-bp ORF encoding a putative protein of 159 amino acids, flanked by a 192-bp 5'-UTR (untranslated region) and a 144-bp 3'-UTR. The predicted molecular mass of the putative protein, HbEREBP2, is 17.7 kDa with a pI of 9.71. The deduced amino acid sequence of HbEREBP2 had specific domain of AP2 superfamily, sharing 62.11, 60.87, 37.74, 37.69, 34.18 and 28.51% identity with PtAP2, RcTINY, BpDREB, *GmDREB3*, AtERF018 and MdAP2, respectively (Figure 1). Phylogenetic tree analysis clustered HbEREBP2 into DREB A5 IIb sub-group although, it had no LWSY motif (Figures 1 and 2).

Effect of tapping and mechanical wounding on the expression of *HbEREBP2*

In the practice of natural rubber production, people regularly cut the trunk bark of rubber tree, normally with a two-day interval, for the purpose of latex exploitation. This action is called tapping which resulted in repeatedly mechanical wounding and latex flow of rubber tree. The level of *HbEREBP2* expression was flat and low in virgin trees without any treatments (Figure 3). There was no significant difference in the level of *HbEREBP2* expression within 1 d upon treatment with either tapping or mechanical wounding while the expression of *HbEREBP2* was significantly up-regulated at 3 d and remained relatively high level within 7 d after the treatments (Figure 3). The expression pattern of *HbEREBP2* was similar between the treatment with tapping and the treatment with mechanical wounding (Figure 3), indicating that latex flow had little effect on up-regulating *HbEREBP2* expression.

Effect of methyl jasmonate and ethephon on the expression of *HbEREBP2*

HbEREBP2 expression was rapidly and strongly up-regulated by methyl jasmonate, which was in contrast to the effect of tapping and mechanical wounding. The expression of *HbEREBP2* was up-regulated more than two times at 2 h and four times at 6 h and thereafter returned to the level of control after treatment with methyl jasmonate (Figure 4). The range of up-regulated expression of *HbEREBP2* was much lower upon ethephon than that upon methyl jasmonate (Figure 4).

DISCUSSION

62 CBF/DREB members have been identified in *Arabidopsis* and are divided into six groups, namely A1-A6 (Nakano et al., 2006). The members of group A5 were further divided into subgroup IIa, IIb, and IIc. Of which IIa is characterized by one AP2/ERF domain and a ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif D(L/M)NxxP, IIb is characterized by one AP2/ERF domain and a LWSY motif while IIc contains only one AP2/ERF domain (Nakano et al., 2006). HbEREBP2 possessed an AP2 superfamily domain and shared relative high identity with the CBF/DREB members from other plant species. It is quite conservative among different rubber tree clones. Phylo-genetic tree analysis, however, clustered HbEREBP2 into subgroup IIb other than IIc although, it had no LWSY motif. This discrepancy may be mainly ascribed to the difference in criteria between phylogenetic tree analysis and classification of the subgroups. Members of the three subgroups of group A5 are generally involved in the regulation of responses of plants to biotic and abiotic stresses and several members of subgroup IIb mediated the salicylic acid (SA)-, jasmonate- and ethylene-dependent responses (Table 2). There are several defense proteins in abundance in lutoids, a kind of

CLUSTAL multiple sequence alignment

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AtERF018   ---------------MVKQAMKEEEKKRNTAMQSK-YKGVRKRKWGKWVSEIRLPHSRER
BpDREB     --------------MVKQTSAENTFADQRSDGSCR-YKGVRKRKWGKWVSEIRLPNSRDR
GmDREB3    -----------------MAKPSSEKPEEHSDSKYYKGVRKRKWGKWVSEIRLPNSRQR
HbEREBP2   ------------------MVRQNGARGERR--DGGYKGVRMRKWGKWVAEIRQPNSRGR
MdAP2      MSNHFSKIFDTSSSSSSSSMLNQTSGEPSERNDVK-YKGVRKRKWGKWVSEIRLPNCRER
PtAP2      ------------------MVRERRERGDD---SGRYKGVRMRKWGKWVAEIRQPNSRDR
RcTINY     ------------------MVRSPNS--------SRYKGVRMRKWGKWVAEIRQPNSRGR
                                              ***** ******* *** *  * *
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AP2/ERF domain

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AtERF018   IWLGSYDTPEKAARAFDAAQFCLRGGDANFNFPNNPPSIS-VEKSLT-PPEIQEAAARFA
BpDREB     IWLGSYDTAEKAARAFDAALFCLRGRTAKFNFPENPPEIT-GGRSLS-PAEIQTAAARFA
GmDREB3    IWLGSYDTPEKAARAFDAAMFCLRGRNAKFNFPDNPPDIA-GGTSMT-PSQIQIAAAQFA
HbEREBP2   IWLGSYNTAEEAARAYDAALFCLRGPSATLNFPMNPPDIH-LTTDLS-PLQIREAALRHS
MdAP2      IWLGSYGTPEKAARAFDAALFCLRGRTAKFNFPDNPPDIP-GGRSLS-PAEIQVEAARFA
PtAP2      IWLGSYNTAEEAARAYDAAVLCLRGPSATFHFPTNIPEIPAMTDQVLSPMQIREVASRHA
RcTINY     IWLGSYNTAEEAARAYDAALFCLRGPSVTLNFPMNPPDINPAQTDLS-PLQIREVAFRHA
           ****** * * ****.***  ****    .** * * *    . * .*. * . 
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AP2/ERF domain

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AtERF018   NTFQDIVKGEEESGLVPGSEIR-----PESPSTSASVATSTVDY------DFSFLDLLPM
BpDREB     NSENPNSHPGRTVPEMQMESP----SPSLSQSVSEGSVTVHIDS--EVTTDGSFMDMFAN
GmDREB3    NAGPHEGHSGRPE-HPPMESP--------SPSVSEGTIQTDSDVP---TLNGSVTDLFTP
HbEREBP2   RRG-VSEITASQDLSSRVPAS------GLFQESSELRGEVYLPS------TEHRVESLDR
MdAP2      NSEIPRSHQSMSAPQAECQSMSELQAESQSPSLSEGSGTAFMDTDLQVTPNESLSDLFGS
PtAP2      RRG--STVEPAERIVG----P-------GLCEVSSGRSGEVYLGG------GENMEECLEG
RcTINY     RKGDESTTAAAEDLG------------------------YLPG------ESG---SLDG
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AtERF018   NFGFDSFSDDFSGFSGGDRFTE---------ILPIEDYGGE-----SLLD-ESLILWDF
BpDREB     -IGSGNYTADYGLFPGFDDLAGDFFMP----PLPTVDYGEENYNVDGVIP-QESFLWNF
GmDREB3    -VGSSGYASDYGIFPGFDDFSGDFYVP----EMPNVNYGEE--NGEGFIV-DESFLWNF
HbEREBP2   -ISGGAYYQASGVWTV------------------------------------------
MdAP2      -FGSGNYATEYGLFPGFDEMNGQFFSPASAAPTPAVDYVEENLDGQFFNPAQESFLWNF
PtAP2      -IFSGAYYQTPGVWTV------------------------------------------
RcTINY     -ISSGGFYQTPGAWTI------------------------------------------
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LWSF motif

Figure 1. Amino acid alignment of HbEREBP2 protein and other AP2/ERF proteins. The identical amino acids were marked with asterisk and the well-conserved residues were marked with dot. The conserved domain and motif was underlined. The aligned *HbEREBP2* protein was from *Hevea brasiliensis* (GenBank Accession No.: HQ171931) and AP2/ERF proteins were from *Arabidopsis thaliana*, AtERF018 (GenBank Accession No.: Q9S7L5.1); *Populus trichocarpa*, PtAP2 (GenBank Accession No.: ABQ62999.1); *Ricinus communis*, RcTINY (GenBank Accession No.: EEF46236.1); *Malus domestica*, MdAP2 (GenBank Accession No.: ADE41099.1), *Broussonetia papyrifera*, BpDREB (GenBank Accession No.: ABU84809.1); and *Glycine max*, GmDREB3 (GenBank Accession No.: ABB36646.1), respectively.

organelle in laticifer cells. The accumulation of these proteins released from the broken lutoids at the end of

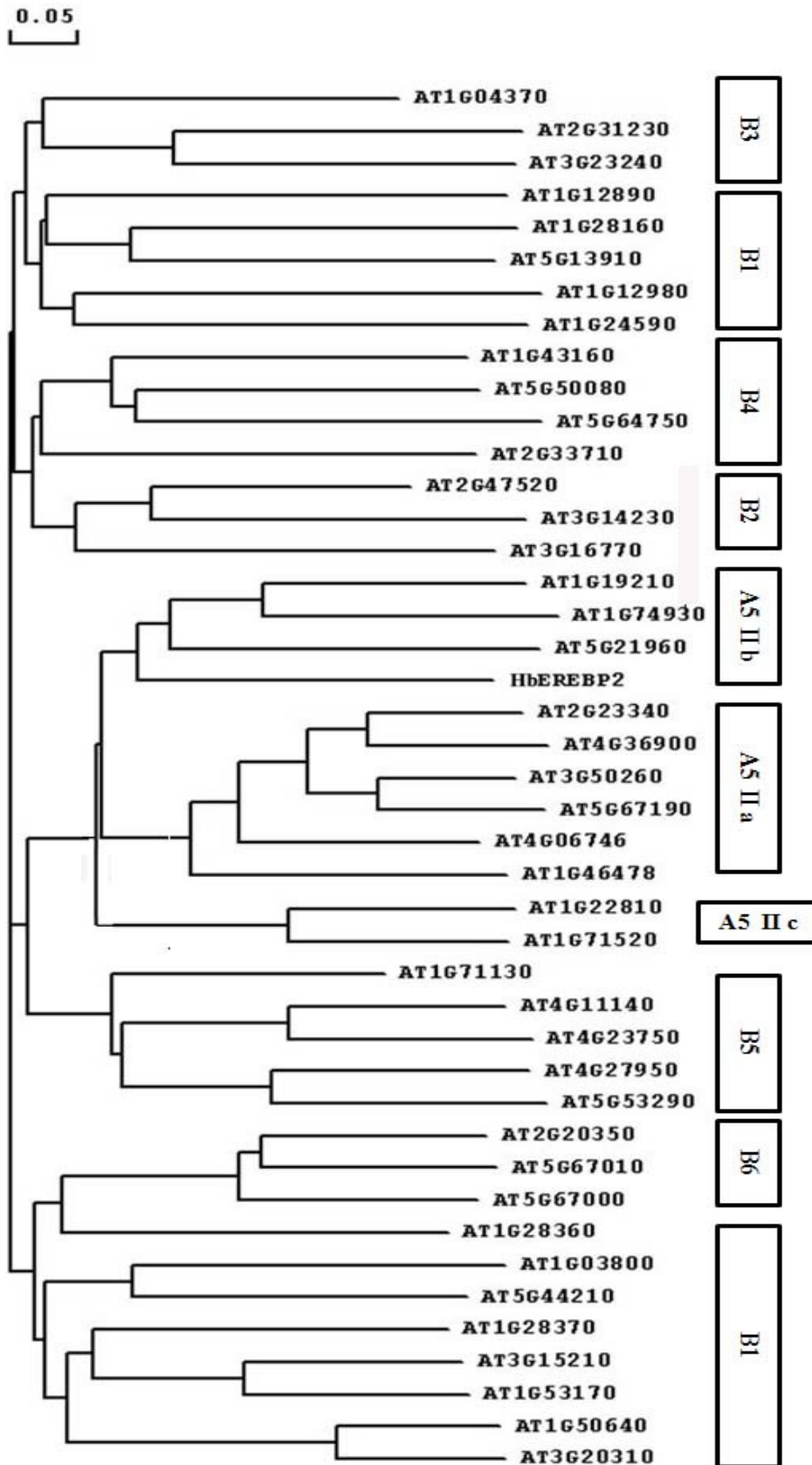the severed laticifers after tapping is believed to act as a biochemical barrier against pathogen and herbivore

Figure 2. Phylogenetic tree of *HbEREBP2* from rubber tree and members of ERF family from *Arabidopsis*.
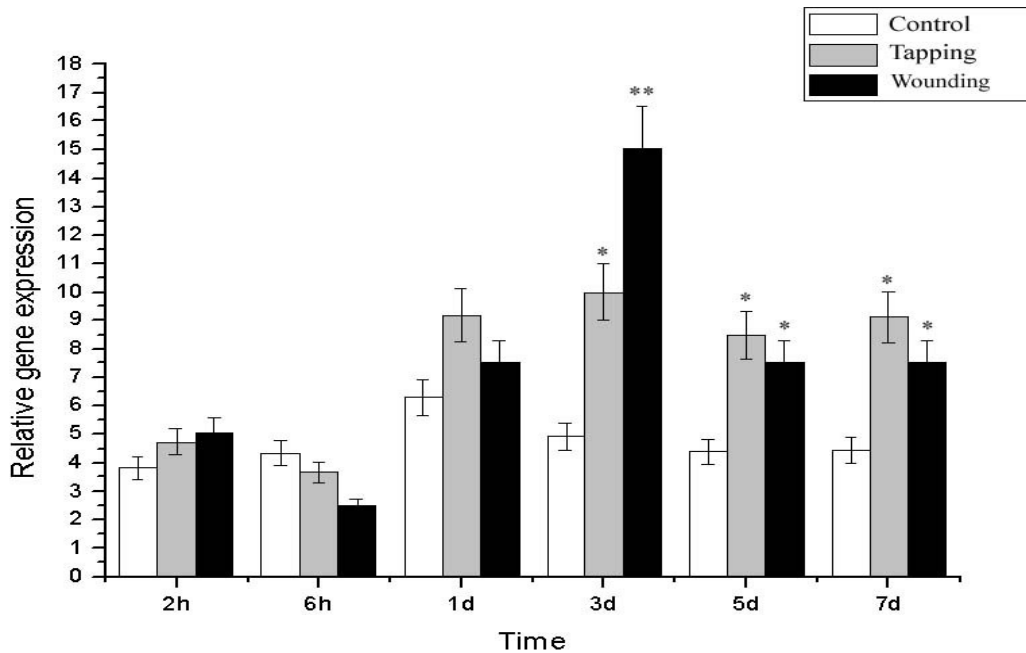
Figure 3. Transcription pattern of *HbEREBP2* in laticifer cells of rubber tree clone CATAS7-33-97 treated with tapping and wounding. * Significant difference contrast to control; ** very significant difference contrast to control.
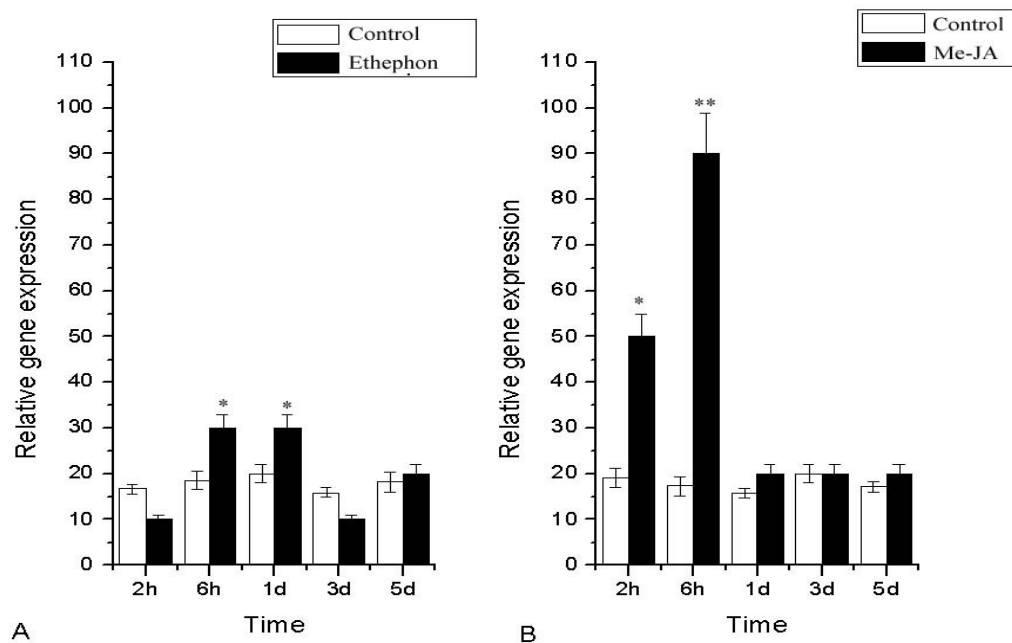

Figure 4. Transcription pattern of *HbEREBP2* in laticifer cells of epicormic shoots in rubber tree clone CATAS7-33-97 treated with ethephon and methyl-jasmonate. * Significant difference contrast to control; ** very significant difference contrast to control.

attacks (Hao et al., 2004). The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are of the most important players in regulating the induced defense responses that are differentially effective against specific types of attackers (Leon-Reyes et al., 2009). In general, SA is active against biotrophic pathogens while

Table 2. Members of subgroup A5 with identified functions in arabidopsis.

Type	TAIR number	Gene name	Function	Reference
A5 IIa	At1g46768	RAP2.1	Involved in response to drought and cold stress via an ABA-independent pathway.	(Dong and Liu, 2010)
A5 IIa	At4g06746	RAP2.9	Involved in response to both pathogen infection and cold treatment.	(Tsutsui et al., 2009)
A5 IIa	At2g23340	DEAR3	Involved in response to both pathogen infection and cold treatment.	(Tsutsui et al., 2009)
A5 IIa	At4g36900	RAP2.10/DEAR4	Enhanced tolerance to photoinhibitory light and exogenous H_2O_2, increased expression of antioxidative genes.	(Welling and Palva, 2008; Rossel et al., 2007)
A5 IIa	At5g67190	DEAR2	Involved in defence responses to bacterial pathogens between *Arabidopsis thaliana* and *Pseudomonas syringae*; salt, osmotic, and cold stress.	(Truman et al. 2006; Kreps et al., 2002)
A5 IIa	At3g50260	DEAR1	Involved in response to salt, osmotic, and cold stress; cooperatively regulated by ethylene and jasmonate; functionally redundant roles in aspects of development in addition to flowering time.	(Kreps et al., 2002; Veley and Michaels, 2008)
A5 IIb	At1g21910	DREB26	Involved in plant developmental processes as well as biotic and/or abiotic stress signaling.	(Krishnaswamy et al., 2011)
A5 IIb	At1g77640	T5M16.23	Involved in salt stress pathways.	(Lee et al., 2007; Ma et al., 2006)
A5 IIb	At1g44830	T12C22.10	Involved in MAP kinase 4 regulated salicylic acid- and jasmonic acid/ethylene-dependent responses.	(Brodersen et al., 2006)
A5 IIb	At4g31060	F6I18.30	Involved in jasmonate-mediated activation.	(Brown et al., 2003)
A5 IIb	At5g21960		Involved in the epigenetic regulation of pollen tube growth and fertilization.	(Cartagena et al., 2008)
A5 IIb	At1g19210	T29M8.8	Involved in light regulation of development.	(Lee et al., 2007)
A5 IIb	At1g74930	ORA47	Involved in impacting pathogen response and cell cycle during geminivirus Infection; jasmonate-mediated transcriptional reprogramming of metabolism.	(Ascencio-Ibanez et al., 2008; Pauwels et al., 2008)
A5 IIc	At1g22810	T22J18.2	Involved in salt stress pathways; 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-Induced gene expression in arabidopsis.	(Ma et al., 2006; Taki et al., 2005)
A5 IIc	At1g71520	F26A9.11	Involved in atrazine toxicity and sucrose-induced tolerance.	(Ramel et al., 2007)

JA is effective against necrotrophs and insect herbivores (Grant and Lamb, 2006). SA signaling act antagonistically on JA-dependent defenses in a nonexpressed pathogen related 1 (NPR1)-dependent manner (Spoel et al., 2003) while ethylene is involved in the regulation of the crosstalk between these two signalings (El Oirdi et al., 2011). The expression of genes encoding for the defense proteins, such as hevein, chitinase and β-1,3-glucanase is up-regulated by mechanical wounding and the application of methyl jasmonate (Yang et al., 2008). Meanwhile, the application of ethephon increases the level of defense-related proteins in laticifers (Kush et al., 1990). These data suggest that jasmonate signaling and ethylene signaling act synergistically in positively regulating the defense proteins in laticifer cells of rubber tree. In this study, however, ethephon has less effect than methyl jasmonate on up-regulating the expression of *HbEREBP2*, suggesting that HbEREBP2 may be involved in the transcriptional regulation of jasmonate-mediated defense responses which are effective against necrotrophs and insect herbivores at the end of the severed laticifers. It is interesting that both the GCC box and the CRT/DRE which are respectively characterized as the ERF and DREB binding *cis*-elements in ethylene-responsive genes (Ohme-Takagi and Shinshi, 1995) and dehydration- and low-temperature-responsive genes (Singh et al., 2002) are not found in the available promoter sequences of genes encoding for hevein and β-1,3-glucanase by online analysis with plant care. Whether the HbEREBP2 is involved in transcriptionally regulating on the defense proteins remains to be elucidated in rubber tree.

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