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Review

The *Hevea brasiliensis* AP2/ERF superfamily: from ethylene signalling to latex harvesting and physiological disease response

Superfamili AP2/ERF pada Hevea brasiliensis*: dari sinyalisasi etilen hingga penyadapan lateks dan respons terhadap penyakit fisiologis*

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Abstrak

*Etilen merupakan hormon yang dikenal karena perannya dalam proses penyadapan lateks di tanaman karet (*Hevea brasiliensis*). Hormon tersebut memfasilitasi aliran lateks melalui aktivasi metabolisme endogen dalam sel lateks saling terhubung yang disebut latisifer. Etilen memiliki peran ganda yaitu menguntungkan karena memacu produksi lateks dan tidak menguntungkan pada tingkat tertentu sehingga menyebabkan munculnya penyakit fisiologis yang disebut sebagai kering alur sadap (KAS). Beberapa penelitian mendalam telah dilakukan untuk mengungkap aktor molekuler dalam biosintesis dan sinyalisasi etilen pada* Hevea brasiliensis*. Salah satu superfamili yang penting dan terlibat sebagai faktor transkripsi terakhir yang diketahui pada sinyalisasi etilen adalah APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF). Saat ini, 114 sekuen unik yang menyandi superfamili AP2/ERF pada* Hevea *telah diidentifikasi dan dikarakterisasi. Karakterisasi spesifik pada saat kondisi stres penyadapan dan kejadian KAS telah berhasil mengidentifikasi 36 marka ekspresi gen (GEMs). Delapan belas GEMs diprediksi memiliki ortologi dengan 19 gen* AP2/ERF *pada* Arabidopsis*. Meskipun karakterisasi ini difokuskan pada regulasi tingkat transkripsi, regulasi pasca-transkripsi dan pasca-translasi potensial dari* HbAP2/ERFs *juga diprediksi. Dikarenakan akumulasi transkrip yang tinggi pada latisifer dan dalam respon terhadap multi-stres abiotik, tiga grup HbERF (HbERF-VII, HbERF-VIII dan HbERF-IX) diduga memiliki peran penting dalam toleransi* Hevea *selama produksi lateks. Untuk beberapa gen kunci, analisis fungsional lanjut perlu dilakukan untuk sepenuhnya memahami regulasi dari* HbAP2/ERFs*. Akhirnya, penanda molekuler dalam kaitannya untuk pemuliaan tanaman karet kemungkinan dapat dikembangkan dari superfamili ini.*

[Kata kunci : etilen, pohon karet, faktor transkripsi, analisis ekspresi, kering alur sadap].

Abstract

Ethylene is a hormone known for its involvement in the process of latex harvesting in *Hevea brasiliensis*. It facilitates latex flow by activation of endogenous metabolism in the anastomosed latex cells called laticifers. In regard to its ambivalent role, ethylene is both favourable to the latex production and unfavourable to a certain level, to the apparition of a physiological disease termed as tapping panel dryness (TPD). Comprehensive researches have been carried out to reveal the molecular actors in ethylene biosynthesis and signalling pathways in *Hevea brasiliensis*. One of the most important superfamily implicated as the last transcription factor known in plant ethylene signalling is the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF). Currently, 114 unique sequences related to the *Hevea AP2/ERF* superfamily have been identified and characterized. Specific characterizations under the condition of harvesting stress and the occurrence of TPD have identified 36 gene expression markers (GEMs). Eighteen of these GEMs were predicted as ortholog with 19 *Arabidopsis AP2/ERF* genes. The characterization was mainly focused on transcriptional regulation, whilst potential post-transcriptional and post-translational regulations of *HbAP2/ERF* genes were formerly predicted. Three HbERF groups (HbERF-VII, HbERF-VIII and HbERF-IX) were hypothesized to have an important role in *Hevea* tolerance during latex production as they highly accumulated in laticifers and in response to multiple abiotic stresses. Further functional analysis of several key genes is suggested in order to fully understand the regulation of *HbAP2/ERFs*. Finally, the molecular markers for future *Hevea* breeding could be possibly developed from this superfamily.

[Keywords: ethylene, rubber tree, transcription factor, expression analysis, tapping panel dryness].

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Introduction

Ethylene is a hormone known for its involvement in the process of latex harvesting in *Hevea brasiliensis*. It facilitates latex flow by activation of endogenous metabolism in the anastomosed latex cells called laticifers (Pujade-Renaud *et al.,* 1994). Latex regeneration disrupts the metabolism homeostasis in laticifers and triggers the production of ROS in particular the O_2^- form by lutoidic NADPH oxidase. In extreme cases of stress, this leads to an oxidative burst provoking coagulation of the rubber particles (d'Auzac *et al.,* 1993). This condition will cause the onset of a physiological disease known as tapping panel dryness (TPD). Thus, the ethylene is subjected to an ambivalent role, both favourable for the latex production and unfavourable in certain level leading to the apparition of TPD. For several years, comprehensive researches have been carried out to reveal the molecular actors in ethylene biosynthesis and signalling pathways in *Hevea brasiliensis*.

One of the most important superfamily implicated as the last actor known in plant ethylene signalling is the APETALA2/ ETHYLENE RESPONSE FACTOR (AP2/ERF). Members of the AP2/ERF superfamily encode transcription factors involved in the regulation of biological processes, including floral and ovum development, responses to phytohormones, and adaptation to biotic and abiotic stress (Mizoi *et al.,* 2012; Licausi *et al.,* 2013a, Li *et al.,* 2012; Xu *et al.,* 2011). These transcription factors were characterized by a conserved DNA-binding AP2 domain of about 60 amino acids (Ohme-Takagi & Shinshi, 1995). The AP2 domain was able to interact specifically with GCC-box (Ohme-Takagi & Shinshi, 1995) or dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting elements in the promoter region of target genes (Ito *et al.,* 2006; Sakuma *et al.,* 2006). The AP2/ERF superfamily was classified according to the number of AP2 domain. The AP2 family consists of proteins with a double domain of AP2 in repeated tandem (Okamuro *et al.,* 1997). The ERF family encodes transcription factors containing a single AP2 domain. Finally, the Related to AB1/VP1 (RAV) family encodes proteins with a single AP2 and an additional domain of B3-type (Kagaya *et al.,* 1999). There are different classifications of the superfamily. Sakuma *et al.* (2002) have described five subfamilies consisting of the APETALA2 (AP2), Related to AB1/VP1 (RAV), Dehydration Responsive Element Binding Proteins (DREB) and Ethylene-Responsive Element Binding Proteins (EREBP) also called the Ethylene Response Factor (ERF). By contrast, Nakano *et al.* (2006) have categorized these proteins in three families consisting of the AP2, ERF and RAV.

The current *state-of-the-art* of ethylene research in *Hevea brasiliensis* especially in relation to the characterization of AP2/ERF superfamily involved in diverse physiological mechanisms of *Hevea brasiliensis* is reviewed in this paper. The main ideas discussed are consisted of (1) characterization of the ethylene biosynthesis and signalling pathways in rubber tree, (2) identification of AP2/ERF gene expression markers related with potential function in rubber tree, (3) potential post-transcriptional and post-translational regulations of *Hevea AP2/ERF* genes, (4) potential important role of *Hevea AP2/ERF* genes in reversible tapping panel dryness generated by oxidative stress, and (5) future perspective for functional analysis of *AP2/ERF* candidate genes.

Characterization of the ethylene biosynthesis and signalling pathways in rubber tree

The ethylene biosynthesis may be induced by the presence of endogenous or exogenous ethylene in *Hevea brasiliensis*. The aminocyclopropane-1-carboxylic acid (ACC) synthase activity or ACS and the ACC oxydase or ACO are the last two enzymes of the ethylene biosynthetic pathway (Yang & Hoffman, 1984). The genes involved in the ethylene biosynthesis pathway have been identified and partially characterized (Kuswanhadi, 2006). Three *HbACO* genes (*HbACO1*, *HbACO2*, and *HbACO3*) and three *HbACS* genes (*HbACS1, HbACS2,* and *HbACS3*) have been identified (Kuswanhadi *et al.* 2007; Kuswanhadi *et al.,* 2010). The transcript of *HbACO1* was the most abundant among three *HbACOs* in bark tissue of 3-month-old budded plants. However, it was reduced upon stimulation by ethylene in leaves and bark (Kuswanhadi, 2006). The *HbACO2* and *HbACO3* genes were transiently induced in response to ethylene. The application of an ethylene inhibitor, i.e. 1-MCP, abolished the induction of ethylene in both of the mentioned genes demonstrating the positive feedback regulation. All *HbACOs* genes were expressed at all developmental stages. All these works have suggested that *HbACO1* gene was responsible for the ethylene biosynthesis regulating at the basal level while the *HbACO2* and *HbACO3* genes were regulated in response to external factors (Kuswanhadi *et al.,* 2010, Kuswanhadi *et al.,* 2007).

The molecular actors involved in ethylene perception and signalling were also studied in *Hevea brasiliensis* (Duan *et al.,* 2010). Two genes of ethylene perception, *Hevea brasiliensis* Ethylene Receptor 2 (*HbETR2*) and *Hevea brasiliensis* Ethylene-insensitive 2 (*HbEIN2*), and a gene of ethylene signalling (*HbEIN3*) were differentially regulated by ethylene treatment. The transcript of *HbETR2* gene was rapidly

accumulated upon stimulation of ethylene while the transcripts of *HbEIN2* and *HbEIN3* genes were significantly reduced. This early induction *HbETR2* was suppressed by an ethylene inhibitor, 1-MCP. In this work, Duan *et al.* (2010) have shown the effect of ethylene and wounding treatments on the downstream-response genes of ethylene signalling pathway. Thus, it opened the perspective of crosstalk signalling between phytohormones in *Hevea brasiliensis*. In search of transcription factors involved in this mechanism, the *Hevea* AP2/ERF superfamily has been the focus subject of study. Based on known previous knowledge in model plants such as *Arabidopsis thaliana* and *Oryza sativa*, these transcription factors were involved in the hormonal response of various biotic and abiotic aspects.

Duan *et al.* (2013) have classified 142 members of *Hevea* AP2/ERF superfamily based on the AP2 domain sequence from full length transcripts of RNA sequencing from rubber clone PB 260. The authors have classified HbERF family into 10 functional groups. The transcriptomic database was generated from somatic embryo tissue, leaves, bark, latex, and roots. The work of Duan and colleagues has further focused on the study of transcription factors involved in the ethylene and jasmonate signalling. Among those Ethylene Response Factors, two *Arabidopsis* genes (*ERF1* and *ORA59*) were found at the intersection of both hormonal signalling (Huang *et al.,* 2015, Pre *et al.*, 2008). In *Hevea*, the *HbERF-IXc4* and *HbERF-IXc5* genes were the orthologue to *ERF1* while the *HbERF-IXc6* gene was the orthologue of *ORA59* (Duan, 2011). A further study revealed that *HbERF-IXc4* and *HbERF-IXc5* genes behaved like the *Arabidopsis ERF1*. Both of genes were induced by ethylene and methyl jasmonate (MeJA). When all the treatments such as MeJA and ethylene were combined, the transcript abundance has multiplied. A preliminary functional study using trans-activity and subcellular localization experiments was performed for *HbERF IXc4* and *HbERF IXc5* genes. The HbERF-IXc4 and HbERF-IXc5 proteins were able to bind to the promoter of *AtPDF1.2* gene and were localized in the nucleus (Duan, 2011).

The *Hevea brasiliensis* AP2/ERF super-family was further characterized by Piyatrakul and colleagues in 2012. Firstly, the work has focused in the role of AP2/ERF transcription factors in the developmental aspect of rubber tree. The transcript accumulation of AP2/ERF genes was analyzed during the process of somatic embryogenesis from callus lines with different regeneration capacity in various vegetative and reproductive tissues (Piyatrakul *et al.,* 2012). Secondly, Piyatrakul *et al.* (2014) focused the experiment on the search for ERF family having an important role in the laticifers related to the latex production. The transcriptomic database was improved by supplementing RNA samples from reproductive tissues (immature and mature male flowers, immature and mature female flowers, and zygotic embryos). The conducted work has confirmed 114 contigs encoding *Hevea* AP2/ERF genes. These transcription factors were further confirmed by *in silico* analysis of genomic sequences from rubber clone CATAS-7-33-97. The transcriptomic and genomic database showed a difference in the number of AP2/ERF genes. Potential isoforms of the genes were hypothesized since the clones used for sequence comparison were unlike. In addition, the absence of certain tissues in the transcriptomic database (such as cambium and xylem) could explain the absence of several *Hevea* AP2/ERF genes. However, the work has identified the *Hevea* ERF Group VII as gene expression markers in latex suggesting a potential role in the hypoxia regulation in laticifers. Functional analysis by trans-activity and subcellular localization has confirmed that members of *HbERF-VII* was an activator-type transcription factor (Piyatrakul *et al.,* 2014).

In parallel with the work of Piyatrakul, the characterization of *Hevea* AP2/ERF superfamily was similarly addressed by studying their stress response as well as during the occurrence of tapping panel dryness (Table 1) (Putranto *et al.,* 2015b, Putranto *et al.,* 2015a). A polyclonal test was initiated in 2003 at the Sembawa Research Station, Palembang, under collaboration of the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) and the Indonesian Rubber Research Institute (IRRI). The first tapping of mature trees was carried out in 2010. Since, latex diagnostic have been regularly measured in order to monitor the physiological state of rubber tree under different harvesting system. Latex and bark samples under the effect of harvesting system, including tapping and ethephon stimulation, and tapping panel dryness were then collected. The *HbERFs* as gene expression markers (GEMs) was identified using high-debit qPCR analysis from the juvenile plant samples which were generated from the somatic embryogenesis prepared in CIRAD (Putranto *et al.,* 2015a).

Identification of AP2/ERF gene expression markers related with potential function in Rubber tree

The identification of gene expression markers was focused on the strongly expressed genes during latex harvesting and during the occurrence of tapping panel dryness. In regard to known function and well-characterized AP2/ERF superfamily, the *Arabidopsis* database was selected as comparison to *Hevea brasiliensis*.

Duan *et al.* (2013) was also used this database to construct the classification of *Hevea AP2/ERF* transcription factors. The bibliographic and phylogenetic analyses were able to reveal the potential functions of these GEMs (Putranto *et al.,* 2015a, Piyatrakul *et al.,* 2014). However, these GEMs may or may not have all important function in rubber tree. Putranto *et al.* (2015b) and Putranto *et al.* (2015a) have identified 36 *HbAP2/ERF* genes including 5 genes of AP2 family and 31 genes of ERF family as GEMs (Table 1). Eighteen GEMs were predicted as ortholog with 19 *Arabidopsis AP2/ERF* genes. Nevertheless, several GEMs without known-ortholog could have a specific and important function in *Hevea*. For now, the *HbERF-IXb2* gene is linked to the occurrence of tapping panel dryness. It was the sole gene induced by the disease in laticifers.

In general results, high relative abundance of transcripts were detected in several groups of *HbAP2/ERFs* (Putranto *et al.,* 2014; 2015a; 2015b). Although some members of these groups have not proven to be GEMs, high level of expression leads us to suggest their involvement in some regulation at the molecular level. The transcripts of the HbERF-I, HbERF-VII, HbERF-VIII and HbERF-IX were highly accumulated in various responses such as development process, latex harvesting and tapping panel dryness. Among members of ERF group I, WOUND INDUCED DEDIFFERENTIATION 1 (WIND1) and RELATED TO AP2.4 (RAP2.4) proteins have known functions in *A. thaliana*. The *WIND1* gene was associated with the control of wounding-induced cellular dedifferentiation (Iwase *et al.,* 2011a; 2011b). This gene was rapidly induced in the wounded site promoting cell dedifferentiation leading to cell proliferation until a mass of pluripotent cells called callus were formed (Iwase *et al.,* 2013). The *RAP2.4* gene was highly expressed in stems and roots under abiotic stress such as cold, dehydration and osmotic stress (Lin *et al.,* 2008). It has been shown that the abundance of RAP2.4 protein was regulated by a BR-C, ttk and bab/Pox virus Zinc finger (BTP/POZ-MATH) or briefly known as BPM protein family (Weber & Hellmann, 2009). In addition, it is suggested that this gene plays a role in the regulation of water homeostasis (Rae *et al.,* 2011). The *HbERF-Ib7* gene is a GEM in response to latex harvesting stress but it has no known ortholog in *Arabidopsis*.

The ERF-VII was characterized by a protein motif MCGGAI(I/L) at the N-terminus (van Veen *et al.,* 2014; Piyatrakul *et al.,* 2014; Nakano *et al.,* 2006). This N-end rule regulation was associated with the targeted protein degradation by the proteasome (Licausi *et al.,* 2011). The elimination of the methionine at N-terminal by methionine aminopeptidase (MAP) left the cysteine residue to be exposed and destabilized. Under the normal condition of the oxygen rate called normoxia, this cysteine was oxidized by oxygen or nitric oxide (Gibbs *et al.,* 2014). This oxidized cysteine induced the addition of argynil residue at the N-terminal end which promotes polyubiquitination leading to the degradation of ERF-VII (Licausi *et al.,* 2013b). The ERF-VII in *Arabidopsis* has been reported as a group of hypoxia- or anoxia-responsive genes such as *HYPOXIA RESPONSIVE 1* and *2* (*HRE1* and *HRE2*), *RELATED TO AP2.2 (RAP2.2)* and *RELATED TO AP2.12 (RAP2.12)* (Seok *et al.,* 2014; Licausi *et al.,* 2010b). The ethylene-induced over-expression of *RAP2.2* gene improves the plant survival under hypoxia (Hinz *et al.,* 2010). By contrast, the overexpression of *HRE1* gene increased tolerance to anoxia (Licausi *et al.,* 2010b). Its role as repressor of ethylene during hypoxia has been demonstrated (Yang *et al.,* 2011). The constitutive regulation of *RAP2.12* and *RAP2.3* genes involving a membrane protein named Acyl-CoA-binding protein (ACBP) has been shown (Bailey-Serres *et al.,* 2012; Licausi *et al.,* 2011). In rubber tree, a hypoxic condition has been suggested in the laticifers since the fermentative metabolism of pyruvate is the main degradation pathway of sugar in the latex cytosol (Tupý, 1989). The intermediate product, pyruvate can be used by both aerobic and anaerobic pathways to generate acetyl-CoA (Jacob *et al.,* 1989a). The transcripts of *HbERF-VIIs* were accumulated in laticifers, suggesting a potential role of regulation to hypoxia in *Hevea brasiliensis*. Based on a phylogenetic analysis, the *HbERF-VIIa12* and *HbERF-VIIa20* genes were orthologous with *Arabidopsis* RAP2.12 and RAP2.3, respectively (Piyatrakul *et al.,* 2014).

The ERF-IX group probably contains the transcription factors whose involvement in the response to pathogens has been most extensively studied (Licausi *et al.,* 2013a; Zarei *et al.,* 2011; Moffat *et al.,* 2012). Members of this group were characterized by a protein motif of activator-type EDLL in the C-terminal (Tiwari *et al.,* 2012). The involvement of ERF-IX (*ERF1*, *ORA59*, *ERF5*, and *ERF6* genes) in the ethylene and jasmonate signalling was clearly demonstrated in *Arabidopsis* (Cheng *et al.,* 2013a; Moffat *et al.,* 2012; Zarei *et al.,* 2011). The *ERF1* and *ORA59* genes belong to the subgroup of ERF-IXc while *ERF5* and *ERF6* genes are within the subgroup of ERF-IXb. Recently, it was shown that ERF5 also called AAL MODULATOR OF CELL DEATH (MACD1) was upregulated in response to programmed cell death via the ethylene signalling during the attack of pathogens (Mase *et al.,* 2013). In *Hevea*, the *HbERF-IXa3, HbERF-IXb3*, *HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6* genes were orthologous to *AtERF1*, *ERF5/MACD1*, *ERF1* and *ORA59* genes in *Arabidopsis*, respectively (Piyatrakul *et al.,* 2014).

Table 1. The AP2/ERF gene expression markers (GEMs) during latex harvesting and under the occurrence of tapping panel dryness (TPD) in *Hevea brasiliensis*. The upregulated GEMs were shown in red meanwhile the downregulated GEMs were in blue colour. LH = latex harvesting; T = tapping; Et- = no ethephon treatment; Et+ = ethephon treatment. The GEMs were extracted from Putranto *et al.* (2015a, 2015b).

Tabel 1. Marka ekspresi gen (GEMs) dari AP2/ERF saat penyadapan lateks dan kejadian kering alur sadap (KAS) pada Hevea brasiliensis*. Regulasi positif dari GEMs ditunjukkan dengan warna merah, sedangkan regulasi negatif ditunjukkan dengan warna biru. LH = penyadapan lateks; T = penyadapan; Et- = tanpa perlakuan ethephon; Et+ = perlakuan ethephon. Marka-marka tersebut disarikan dari Putranto et al. (2015a, 2015b).*

Gene / *Gen*	GEMs				Bibliographical and phylogenetic analyses / *Analisis bibliografi dan filogenetik*			
	LH		TPD		Ortholog / *Ortolog*	*Arabidopsis* accession / Aksesi	Known function / *Fungsi yang diketahui*	Reference / *Referensi*
	T	Et+	Et-	Et+				
HbAP2-3								
HbAP2-6					*WRI1*	At3g54320	Seed development	(Focks & Benning, 1998; Cernac *et al.*, 2006)
HbAP2-7								
HbAP2-10								
HbAP2-13								
HbERF-Ib7								
HbERF-IIb2					*ORA47*	At1g74930	JA biosynthesis	(Pauwels *et al.*, 2008)
HbERF-IIIb1								
HbERF-IVa3								
HbERF-Va2					*ESE3*	At5g25190	Salinity stress response	(Zhang *et al.*, 2011)
HbERF-VI1								
HbERF-VI3								
HbERF-VI5								
HbERF-VI-L3					*CRF10*	At1g68550.1 At1g68550.2	Leaf development	(Rashotte & Goertzen, 2010)
HbERF-VI-L4								
HbERF-VIIa12					*RAP2.12*	At1g53910	Hypoxia response	(Bailey-Serres *et al.*, 2012)
HbERF-VIIa20					*EBP/RAP2.3*	At3g16770	Defence against pathogen	(Büttner & Singh, 1997)
HbERF-VIIIa4					*ERF3*	At1g50640	Programmed cell death response	(Ohta *et al.*, 2001; Ogata *et al.*, 2013)
HbERF-VIIIa8								
HbERF-VIIIa9								

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Tabel 1. Marka ekspresi gen (GEMs) dari AP2/ERF saat penyadapan lateks dan kejadian kering alur sadap (KAS) pada Hevea brasiliensis*. Regulasi positif dari GEMs ditunjukkan dengan warna merah, sedangkan regulasi negatif ditunjukkan dengan warna biru. LH = penyadapan lateks; T = penyadapan; Et- = tanpa perlakuan ethephon; Et+ = perlakuan ethephon. Marka-marka tersebut disarikan dari Putranto et al. (2015a, 2015b) (lanjutan).*

Gene / *Gen*	GEMs				Bibliographical and phylogenetic analyses / *Analisis bibliografi dan filogenetik*			
	LH		TPD		Ortholog / *Ortolog*	*Arabidopsis* accession / Aksesi	Known function / *Fungsi yang diketahui*	Reference / *Referensi*
	T	Et+	Et-	Et+				
HbERF-VIIIa10					*ERF11*	At1g28370	Repressor of ethylene biosynthesis	(Li *et al.*, 2011)
HbERF-VIIIa12								
HbERF-VIIIa13					*ERF12*	At1g28360	Repressor of ABA and ET	(Yang *et al.*, 2005)
HbERF-VIIIa14								
HbERF-VIIIb1					*DRN-LIKE*	At1g24590	Regeneration of apical meristem	(Kirch 2003; Chandler *et al.*, 2007)
HbERF-IXa3					*AtERF1*	At4g17500	Division of vascular cells	(Etchells *et al.*, 2012)
HbERF-IXb1								
HbERF-IXb2						At5g07580		(Nakano *et al.*, 2006)
HbERF-IXb3					*ERF5/MACD1*	At5g47230	Programmed cell death response	(Moffat *et al.*, 2012; Mase *et al.*, 2013)
HbERF-IXc1								
HbERF-IXc4					*ERF1*	At3g23240	Defense against pathogen	(Lorenzo *et al.*, 2003; Cheng *et al.*, 2013a)
HbERF-IXc5								
HbERF-IXc6					*ORA59*	At1g06160	Defense against pathogen	(Pre *et al.*, 2008; Zarei *et al.*, 2011)
HbERF-Xa2					*RAP2.6-LIKE*	At5g13330	Multiple abiotic stress response	(Krishnaswamy *et al.*, 2011)
HbERF-Xa8					*ABR1*	At5g64750	Repressor of ABA response	(Pandey *et al.*, 2005)
HbERF-Xb1					*RRTF1*	At4g34410	Redox homeostasis	(Khandelwal *et al.*, 2008)

Taken together, the result of differential gene expression analysis by Duan *et al.* (2013) and Putranto *et al.* (2015a) has brought into conclusion that *HbERF-IXc4* and *HbERF-IXc5* could have a major role in the regulation of latex production as they were highly expressed in bark during latex harvesting.

The ERF group VIII contains an ERF-associated amphiphilic repression (EAR)-motif known as the transcriptional repressors (Ohta *et al.,* 2001). These ERFs of repressor-type were demonstrated to be able to induce a programmed cell death (Ogata *et al.,* 2011; 2013). The multifunctional co-repressors, TOPLESS (TPL) and TPL-related (TPR) interact directly with the EAR motif of ERF group VIII. The interaction of TPL/TPR and other transcription factors was co-opted several times to modulate the expression of genes in various biological processes, including hormonal signalling and stress responses (Causier *et al.,* 2012). The *Arabidopsis ERF3*, *ERF4*, *ERF7*, *ERF10*, *ERF11*, *ERF12* and *ESR1/DRN* genes belong to the ERF-VIII. However, these genes showed differential expression and distinct roles in plant growth and development as well as in response to environmental stress. For instance, the *ERF4* and *ERF7* genes play an important role in the response to ABA (Yang *et al.,* 2005; Song *et al.,* 2005). The overexpression of ERF7 increased the sensitivity to ABA in guard cells (Song *et al.,* 2005). The suppression of ERF-VIII also requires interactions with other transcription factors. The interaction of *ERF11* gene with the bZIP-type transcription factor, HY5 has modulated the ethylene biosynthesis *via* ABA signalling (Li *et al.,* 2011). The *ESR1/DRN* genes were involved in the development of petiole, the structuration of embryo and the development of cotyledons (Chandler & Werr, 2011; Cole *et al.* 2013). The high abundance of HbERF-VIII transcripts in bark and latex under harvesting stress suggest a post-transcriptional regulation of other *HbERFs* by their role as repressor (Putranto *et al.,* 2015a).

Two GEMs such as *HbERF-IIIB1* and *HbERF-IVa3* did not have orthologs in *Arabidopsis*. However, these genes could have an important role in osmotic stress response especially linked to the production of latex. In the classification of Sakuma (2002), the *HbERF-III* genes were included in the CBF/DREB1 subfamily. The *CBF/DREB1* genes have been extensively characterized in plants linked to the cold stress (Kurepin *et al.,* 2013; Rehman & Mahmood, 2015). In *Hevea*, a small number of Cold Binding Factor/Dehydration Responsive Element Binding 1 (CBF/DREB1) genes were identified (Duan *et al.,* 2013). This could be explained by a minor adaptation of *Hevea* tree to the cold zone. Additionally, *HbERF-IV* genes correspond to the DREB2 subfamily involved in the response to dehydration. The expression of *HbERF-IVa3* gene was induced by tapping, ethephon stimulation and tapping panel dryness (Putranto *et al.* 2015b; Putranto *et al.,* 2015a). The expression of *HbERF-IVa3* gene was consistent with the hypothesis since laticifers are subject to a recurrent osmotic stress during the exploitation of rubber tree. In the future, these ERF groups, which have a strong expression but no known functions, deserve a deep characterization to determine their involvement in cell processes, including latex production.

Potential post-transcriptional and post-translational regulations of hevea AP2/ERF genes

The characterization of AP2/ERF superfamily in *Hevea brasiliensis* was focused on transcriptional regulation. Yet, previous research works in different plant species has demonstrated that the AP2/ERF genes can be regulated at the post-transcriptional level, including the regulation by alternative splicing, control of protein stability, acetylation and nitrosylation (Licausi *et al.,* 2013a). A strong accumulation of transcripts was observed for *HbERF-I, HbERF-VII, HbERF-VIII* and *HbERF-IX* members under harvesting stress and tapping panel dryness in bark and latex tissues (Putranto *et al.,* 2015a; 2015b). The constitutive expression of several *HbERFs* led us to consider a post-transcriptional regulation controlling the activity of these proteins. Yet, it has been shown in grapevine, tomato and *Arabidopsis* as ERF groups (ERF-II, ERF-III, ERF-VII and ERF-IX) were strongly expressed in different tissues (Licausi *et al.,* 2010a; Pirrello *et al.,* 2012; Bailey-Serres *et al.,* 2012). A high abundance transcript in non-stressed condition was potentially a default defence system being present before the stress condition (Cheng *et al.,* 2013b).

Post-transcriptional regulation of Dehydration Responsive Element Binding Proteins 2 (DREB2) genes by alternative splicing has been reported in *Arabidopsis*, tomato and wheat (Rehman & Mahmood, 2015; Pirrello *et al.,* 2012; Lata & Prasad, 2011; Lucas *et al.,* 2011; Mizoi *et al.* 2012). In the case of alternative splicing, two types of transcripts may exist. The first is an inactive portion having a transcribed intron sequence which generates a stop codon before the DNA binding domain. The second is an active transcript encoding the full-length protein. This type of regulation was also observed in the ERF-VII in tomato and *Arabidopsis* (Pirrello *et al.,* 2006 , Licausi *et al.,* 2010b). Furthermore, post-transcriptional regulation of *AP2/ERF* genes by microRNAs was also demonstrated. The *JcDREB* gene in *Jatropha*

curcas has been targeted by miR156 (Galli *et al.,* 2014) while a repression of expression of *OitaAP2* gene in *Orchis italica* was controlled by miR172 (Salemme *et al.,* 2013). In some cases, the action of miRs can be specific. In *Arabidopsis*, the miR319b targeted transcript isoform of RAP2.12 containing an intron in order to inactivate the gene (Sobkowiak *et al.,* 2012). In *Hevea*, several microRNAs including the well-known miR172 were predicted to target AP2/ERF transcripts (Duan *et al.*, 2013). Although the inhibition mode was being achieved mainly by cleavage of target RNA messenger, translation inhibitions has been predicted for two genes (*HbERF-VI5* and *HbERF-IXc5*).

The AP2/ERF genes are likewise regulated at the post-translational level. For now, the DREB2 belonging to ERF-IVa contains a rich region of Ser/Thr is a potential site of phosphorylation. A deletion of a Ser/Thr motif led to a constitutively active form of DREB2A in *Arabidopsis* (Sakuma *et al.,* 2006). This finding was validated by overexpression of DREB2A in *Arabidopsis* and *Oryza* that did not result to an induction of downstream genes in normal conditions. Post-translational modification was necessary to activate this phosphorylation site (Dubouzet *et al.,* 2003; Liu *et al.,* 1998). Phosphorylation regulation *via* histidine kinases has also been suggested for the ERF-VI (Raines *et al.,* 2016). Another ERF belonging to the subgroup of ERF-IXb, *AtERF104* required an interaction with the mitogen-activated protein kinase 6 (MPK6) for its stability (Bethke *et al.,* 2009). The stability of ERF proteins occasionally involves the 26S proteasome. Once more, the ERF-VII gives an example of ubiquitin ligase involvement such as PROTEOLYSIS6 (PRT6) in the degradation of ERF-VII proteins *via* the N-end rule pathway (Gibbs *et al.,* 2011; Licausi *et al.,* 2011). There are not yet any reports about the post-translational regulation of *AP2/ERF* genes in *Hevea brasiliensis*. However, the low expression of *HbERFs* during the onset of TPD has brought a hypothesis in which a post-translational regulation was taken place (Putranto *et al.,* 2015b).

Potential important role of hevea AP2/ERF in reversible tapping panel dryness generated by oxidative stress

The occurrence of tapping panel dryness depends on various factors, such as plant material (genotype sensitivity and rootstock-scion interaction), environment aspects (harvesting system, biotic and abiotic stresses, and soil compaction) (Putranto *et al.,* 2015b). These factors result in a complex signalling involving molecular responses at transcriptional and post-transcriptional levels. Previous studies in *Hevea* have identified gene expression markers for TPD such as *HbMyb1*, *HbTOM20* and *HbTCTP* genes (Venkatachalam *et al.,* 2009; Peng *et al.,* 2011). These genes were negative regulators of genes that positively regulate the programmed cell death. The inhibition of these genes in TPD-affected trees thus causes the activation of programmed cell death. *HbTOM20* gene encodes a translocate protein of the outer mitochondrial membrane regulating the transport of nuclear proteins within mitochondria (Venkatachalam *et al.,* 2009). It is likely that inhibition of this protein results to the mitochondrial dysfunction leading to impaired respiration and a low level of ATP. *HbTCTP* gene encodes cytosolic calcium binding protein that exact function remains to be elucidated in rubber tree. The transcript of this gene was also 60% lower during the occurrence of TPD in comparison to normal condition (Venkatachalam *et al.,* 2007). In their study, Gébelin *et al.* (2013) predicted that the microRNA, *HbmiR159b* could target the Myb-like transcription factor (*HbMyb1*) and glutathione peroxidase (*HbGPX*) in *Hevea brasiliensis*. These post-transcriptional regulations may result in a change in the activity of target genes. Glutathione peroxidase is one of the key enzymes in the regulation of thiols in plants which function is to reduce the hydro-lipid peroxides in a form of alcohol as well as to hydrogen peroxide into H_2O (Noctor *et al.,* 2012).

The characterization of *Hevea AP2/ERFs* has led to the possible involvement of these transcription factors during the occurrence of TPD. In transcriptional level, Putranto *et al.* (2015b) has observed the induction of two *HbERF* genes (*HbERF-IIb2* and *HbERF-IXb2*) and the inhibition of *HbERF-VIIIa14* gene. The participation of jasmonate biosynthetic pathway could be suggested since the *HbERF-IIb2* gene (ortholog of *Arabidopsis ORA47*) was induced. The *ORA47* gene was demonstrated to induce the expression of *LOX3,* one of key genes to jasmonate (JA) biosynthesis (Khurshid 2012). Recently, Chen *et al.* (2016) has demonstrated the dual function of ORA47 protein in both JA and ABA biosynthesis leading to a clear involvement of ORA47 under osmotic stress. By contrast, the inhibition of *HbERF-VIIIa14,* a member of ERF-VIII during the occurrence of TPD suggests a control of the cell death regulation.

These responses at the molecular level generate a biochemical change in response to TPD. It is known that the thiol concentration and the enzymatic activities of catalase (CAS) and superoxide dismutase (SOD) were reduced during the occurrence of TPD (d'Auzac *et al.,* 1993). Some of these results have been confirmed in *Hevea* (Putranto *et al.,* 2015b). In addition, the reduction of the CAS and SOD activities was followed by

the increase of NAD(P)H oxidase activity (Chrestin, 1989). These parameters highlighted the appearance of oxidative burst that could also become a response signal of TPD. From this oxidative stress, Putranto *et al.* (2015a) hypothesized two events by known mechanisms which can be drawn. First, the peroxidation of lutoidic membranes releases coagulation factors leading to reactive oxygen species-type of TPD (ROS-TPD) (Jacob *et al.,* 1989b). Secondly, oxidative stress may lead to lipid peroxidation in the plasma membrane, release of linamarase and linamarin resulting to cyanide production (Kadow *et al.,* 2012). In a worse condition, a bark necrosis can occur resulting finally brown bast-type of TPD (BB-TPD).

Future perspective for functional analysis of AP2/ERF candidate genes

HbAP2/ERF genes, which were responsive to multiple harvesting stress and TPD, have been identified and characterized in transcriptional level. Further characterization of several *HbAP2/ERF* candidate genes using transgenesis was suggested in order to fully understand their functions, especially in latex production of *Hevea brasiliensis*. Targeted candidate genes should be selected from HbERF-VII, HbERF-VIII, and HbERF-IX previously described as the most abundant and potentially important groups involved in the tolerance to harvesting stress and tapping panel dryness.

The metabolism activities of laticifers consume oxygen and generate reactive oxygen species. Interestingly, the GEMs of HbERF-VII are potential activators of downstream genes in response to hypoxia such as *HbADH* and *HbSUS1* (Piyatrakul *et al.,* 2014). Among the members of this group, *HbERF-VIIa12* gene, a potential ortholog of *RAP2.12*, could have an important role in the regulation of homeostasis of oxygen in the laticifers. In *Arabidopsis*, the RAP2.12 protein was known to be able to interact with alcohol dehydrogenase (ADH) and regulate their activity throughout the fermentation route. Further characterization of the gene, including a study of co-expression with response to hypoxia genes such as *HbADH*, a study of regulation by microRNAs and an analysis of transgenic line overexpressing or dowregulating *HbERF-VII* should clarify its involvement during a hypoxic condition in laticifers.

The rubber biosynthetic pathway belongs to the secondary metabolism (d'Auzac *et al.,* 1993). For this reason, the HbERF-IX group could play an important role controlling latex metabolism, e.g. during sucrose loading into laticifers. Among members of this group, two genes (*HbERF-IXc4* and *HbERF-IXc5*) were dramatically induced by the combination of ethylene and methyl jasmonate treatments in *Hevea* (Putranto *et al.,* 2015a). These genes are potential ortholog to *ERF1* famously known to be highly regulated at transcriptional level during multiple abiotic stresses. A further characterization of these genes requires the identification of their regulons (downstream target genes) by Chromatin Immunoprecipitation Sequencing (ChIP-seq) method and the functional analysis in transgenic lines. Transgenic lines overexpressing *HbERF-IXc4* and *HbERF-IXc5* genes have been regenerated (Lestari *et al.,* 2014). These lines showed a promising phenotype of highly tolerant to abiotic stress which will confirm the major role of both genes in latex production.

Another full characterization should be carried out on the ERF-VIII having an important role in the induction of programmed cell death biotic and abiotic stresses. In relation to TPD, members of this group should be considered as important candidate genes. An ortholog of *Arabidopsis ERF12*, *HbERF-VIIIa13* gene has been demonstrated to be repressor-type transcription factor (Putranto *et al.,* 2015a). In addition, *HbERF-VIIIa14* gene was a gene expression marker specifically induced under the occurrence of TPD. The protein-protein interaction analysis with known repressor network such as TOPLESS protein could be an important aspect to be elucidated.

Conclusion and Remarks

The ethylene biosynthesis and signalling pathway is one of the most studied aspects in *Hevea brasiliensis*. These studies have been driven by the practice of latex harvesting involving an ethylene releaser. Understanding the hormonal regulation of latex production in cellular and molecular levels is an important aspect in *Hevea brasiliensis*. However, the crosstalk of other phytohormones such as jasmonate and ABA with ethylene cannot be taken aside. Clear evidence has shown that their regulation could be also key factors in latex production. As a transcription factor, the *Hevea brasiliensis* AP2/ERF superfamily has shown its potency to be the master regulator of phytohormones related directly to latex production, regulator of tolerance to abiotic and biotic stress, and regulator of tolerance to physiological disease. In the near future, molecular markers could be developed from HbAP2/ERFs in order to select *Hevea* clones tolerant to abiotic and biotic factors.

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