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Cuifang DUAN

Etude de l'interaction entre l'éthylène et le jasmonate, hormones impliquées dans la production de caoutchouc naturel chez *Hevea brasiliensis*

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Résumé :

Les jasmonates et l'éthylène sont d'importants signaux de régulation du développement des plantes et de réponse aux stress biotiques et abiotiques. La production de jasmonates est induite à la suite d'une blessure mécanique ou des agents pathogènes. L'acide jasmonique et l'éthylène agissent en synergie sur l'activation de l'expression des gènes de défense tels que PDF1.2. Le Facteur de Réponse à l'Ethylène1 (ERF1) est un intégrateur clé de ces signaux hormonaux chez *Arabidopsis*. ERF1 appartient à la superfamille des facteurs de transcription AP2/ERF, lesquels jouent un rôle crucial dans le développement et la réponse aux stress. *Hevea brasiliensis* est la seule source commerciale de caoutchouc naturel, lequel est synthétisé dans les cellules laticifères. Le latex s'écoule du tronc des hévéas après la saignée. L'éthéphon, un générateur d'éthylène, est un stimulant exogène adopté largement dans les plantations d'hévéa pour améliorer la production de latex en prolongeant l'écoulement de latex et en stimulant le métabolisme des cellules requis pour la régénération du latex. Les jasmonates sont aussi impliqués dans la formation des laticifères. Etant donné l'implication de l'éthylène et de l'acide jasmonique dans la réponse coordonnée à la saignée et à la stimulation par l'éthéphon chez *Hevea brasiliensis*, leur interaction est supposée jouer un rôle important dans la production de latex.

L'objectif de cette thèse est de découvrir les régulateurs clés de l'interaction entre la blessure, le jasmonate et l'éthylène chez *Hevea brasiliensis.* A travers l'analyse de l'expression de 25 gènes impliqués dans les voies de transduction du jasmonate, de l'éthylène et dans le métabolisme cellulaire, nous avons montré que des voies de réponse dépendantes et indépendantes à l'éthylène et au jasmonate coexistent chez *Hevea brasiliensis*. La régulation temporelle influence aussi l'expression des gènes. L'étude s'est alors focalisée sur les facteurs de transcription de la superfamille des AP2/ERF. A partir de bases de données de séquences transcriptomiques de différents tissus obtenu par pyroséquençage, 173 membres AP2/ERF ont été identifiés chez *Hevea brasiliensis* dont 142 pleines longueurs. Cette superfamille est divisée en 3 familles majeures : AP2, ERF et RAV. Cinquante neuf membres sont exprimés dans le latex ce qui suggère qu'ils ont une fonction importante dans le métabolisme des laticifères. En plus du microARN172 connu pour cibler les transcrits AP2/ERF, six autres microARNs ont été prédits pour inhiber les transcrits de cette superfamille. L'identification de l'orthologue à AtERF1 a été aussi menée chez *Hevea brasiliensis*. L'expression de 14 gènes *HbERF* du groupe IX a été étudiée en réponse à la blessure, au méthyl jasmonate et à l'éthylène. L'accumulation relative des transcrits est remarquable pour trois gènes : *HbERF-IXc4, HbERF-IXc5* et *HbERF-IXc6*. Ces gènes candidats ont été caractérisés pour la localisation subcellulaire et la trans-activation du promoteur du gène *PDF1.2*. La fusion traductionnelle *HbERF-IXc4::GFP* a révélé que *HbERF-IXc4* code pour une protéine nucléaire comme les facteurs de transcription. Le HbERF-IXc5 induit la plus forte activation du promoteur du gene *PDF1.2* qui est un gène de défense induit fortement par AtERF1 et ORA59. Ces résultats suggèrent que HbERF-IXc5 est l'orthologue à AtERF1 chez *Hevea brasiliensis*, lequel est impliqué dans la communication des voies de signalisation de l'éthylène et du jasmonate.

L'identification des transcrits AP2/ERF chez *Hevea brasiliensis,* et la caractérisation des ERFs du groupe IX apportent les bases générales pour étudier la régulation moléculaire de la production de latex en réponse aux stress et de la différentiation des cellules laticifères. Nos résultats suggèrent que HbERF-IXc5 est un intégrateur essentiel des voies de signalisation éthylène et jasmonate chez *Hevea brasiliensis.*

Interaction between ethylene and jasmonate, plant hormones involved in the natural rubber production in *Hevea brasiliensis*

Abstract:
Jasmonates and ethylene are important signals in regulating the plant development and metabolism, and in response to biotic and abiotic stresses. Production of jasmonates is induced by mechanical wounding and pathogens. Jasmonic acid and ethylene are synergistically required to activate the expression of some defence related genes such as PDF1.2. Ethylene Response Factor 1 (ERF1) was demonstrated as a key integrator in the signal interaction in *Arabidopsis*. ERF1 belongs to AP2/ERF transcription factors superfamily, which plays a crucial role in plant development and response to biotic and abiotic stresses. *Hevea brasiliensis* is the sole source of natural rubber, which is synthesized in latex cells. Latex is expelled out after tapping the soft bark. Ethephon, an ethylene releaser, is an exogenous stimulant adopted widely in the rubber plantation for improving latex yield by prolonging latex flow and by stimulating the metabolism required for the latex regeneration. Jasmonates are also involved in the laticifer formation. Given the involvement of ethylene and jasmonic acid in the coordinated response to tapping and ethephon stimulation in *Hevea brasiliensis*, their interaction is speculated to play an important role in latex production.

The objective of this thesis is aiming to discover the key regulators in the interaction of wounding, jasmonate and ethylene in *Hevea brasiliensis*. Through the expression analysis on one group of 25 genes involved in the jasmonate and ethylene and cellular metabolism, we discovered that jasmonate and ethylene dependent and independent response coexist in *Hevea brasiliensis*. Temporal regulation can also have an influence on the gene expression. We then focused the study on the AP2/ERF transcription factor superfamily. Based on new generation of sequencing data, we identified 173 AP2/ERF members from several *Hevea brasiliensis* transcript libraries to which 142 are full-lenght. This superfamily is divided into 3 major families: AP2, ERF and RAV. Fifty nine members are expressed in latex which may indicate that they have an important function in the latex metabolism. In addition to the microRNA172, which is known to target AP2/ERF transcripts, six other microRNAs were predicted to inhibit transcripts of this superfamily. The identification of the AtERF1 orthologous gene was further conducted in *Hevea brasiliensis*. Expression analysis of 14 *HbERF* genes from the group IX was studied in response to wounding, methyl jasmonate and ethylene. A remarkable relative transcript accumulation was observed for genes *HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6*. These candidate genes were further analysed for subcellular localization and trans-activation of the promoter of the *PDF1.2* gene. The translational fusion *HbERF-IXc4::GFP* revealed that *HbERF-IXc4* encoded a nuclear targeted protein like transcription factor. The HbERF-IXc5 was shown to mediate the activation of the PDF1.2 promoter, which is a defence gene dramatically induced by AtERF1 and ORA59. For that reason, HbERF-IXc5 is suggested to be AtERF1 ortholog gene in *Hevea brasiliensis*, which is at the crosstalk of jasmonic acid and ethylene signalling pathways.

This identification of the *Hevea brasiliensis* AP2/ERF transcripts and the characterization of the ERF group IX provide general basis for studying the molecular regulation of both latex production in response to abiotic stresses and differentiation of latex cells. Our results suggested that the HbERF-IXc5 is an essential integrator of the jasmonic acid and ethylene signalling pathways in *Hevea.*

Mots clés : AP2/ERF, blessure, ERF1, communication hormonale, facteur de transcription, *Hevea brasiliensis*, méthyl jasmonate, éthylène, microARN, phytohormone

Key words: AP2/ERF, ERF1, ethylene, *Hevea brasiliensis*, methyl jasmonate, microRNA, plant hormone, transcription factor, wounding

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Production scientifique

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- **Riza Putranto, Christine Sanier, Julie Leclercq, Cuifang Duan, Maryannick Rio, Xavier Sabau, Xavier Argout, Pascal Montoro** 2011. Differential spatial gene expression in different types of *Hevea brasiliensis* roots **, doi:10.1016/j.plantsci. 2011.08.005**, http://www.sciencedirect.com/science/article/pii/S0168945211002263 - état : paru

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- **Virginie Gébelin, Xavier Argout, Worrawat Engchuan, Bertrand Pitollat, Cuifang Duan, Pascal Montoro and Julie Leclercq** 2011. Identification of novel microRNAs in *Hevea brasiliensis* and computational prediction of their targets (Soumis).

TABLES DES MATIERES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	1-Aminocyclopropane-1-Carboxylic-acid
ACO	ACC Oxidase
ACS	ACC Synthase
AFLP	Amplified Fragment Length Polymorphism
ANRPC	Association of Natural Rubber Producing Countries
ANT	AINTEGUMENTA
AOS	Allene Oxide Synthase
AP2	APETALA2
cDNA	Complementary Deoxyribonucleic Acid
βCAS	Beta-Cyanoalanine Synthase
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
CATAS	Chinese Academy of Tropical Agricultural Sciences
ChiB	Basic-Chitinase
CLFD	Corynespora Leaf Fall Disease
COI1	Coronatine Insensitive 1
Cp	Crossing point
DAF	DNA Amplification Fingerprinting
DNA	Deoxyribonucleic Acid
DRC	Dry Rubber Content
DRE	Dehydration-Responsive Element
DREB	Dehydration Responsive Element Binding Proteins
EAR	ERF-associated Amphiphilic Repression motif
EIN3	ETHYLENE INSENSITIVE3
EIL1	ETHYLENE INSENSITIVE3-LIKE 1
EMSA	Electrophoretic Mobility Shift Assays
EREBP	Ethylene-Responsive Element Binding Proteins
ERF	Ethylene Responsive Factors
ERF1	Ethylene Response Factor 1
EST	Expressed Sequence Tags
ET	Ethylene
FDP	Farnesyl Diphosphate
FDS	Farnesyl Diphosphate Synthase
GFP	Green Fluorescent Protein
GS	Glutamine Synthetase
HCN	Cyanide
HbSOD	*Hevea brasiliensis* Superoxide Dismutase
HEL	Hevein-like protein
HMGR	Hydroxy-3-methylglutaryl coenzyme A reductase
HMGS	Hydroxy-3-methylglutaryl coenzyme A synthase
IRSG	International Rubber Study Group
JA	Jasmonic acid
JAI1/JIN1	JASMONATE-INSENSITIVE1

JAR1	Jasmonate resistant 1
JAZ	Jasmonate ZIM-domain
IPP	Isopentenyl diphosphate (isopentenyl pyrophosphate)
LD	Latex Diagnosis
LOX	Lipoxygenase
LUC	LUCIFERASE
MAS	Markers-Assisted Selection
1-MCP	1-Methyl Cyclopropane
MAT	Met Adenosyl Transferase
MeJA	Methyl Jasmonate
MiR	MicroRNA
mRNA	Messenger RNA
MS medium	Murashige and Skoog medium
MVA	Mevalonate
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
OPDA	12-Oxo-phytodienoic acid
OPR3	12-Oxophytodienoic acid Reductase 3
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDF1.2	PLANT DEFENSIN1.2
PR genes	Pathogenesis Related genes
QTL	Quantitative Trait Locus
Q-PCR	Quantitative real time PCR
RAPD	Random Amplification of Polymorphic DNA
REF	Rubber Elongation Factor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription PCR
RuT	Rubber Transferase
SALB	South American Leaf Blight
SAM	S-adenosyl-L-methionine
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SRPP	Small Rubber Particle Protein
SSH	Suppression Subtractive Hybridization
SSRs	Simple Sequence Repeats
SUT	Sucrose Transporter
Thi2.1	Thionin 2.1
TPD	Tapping Panel Dryness
TSC	Total Solid Content
V-PPase	Vacuolar H(+)-Pyrophosphatase
W	Wounding

INTRODUCTION GENERALE

1. *Hevea brasiliensis* and natural rubber production

a. Hevea brasiliensis

Rubber tree (*Hevea brasiliensis* Willd. ex A. de Juss. Müll. Arg.), belonging to the genus *Hevea* of the Euphorbiaceae family, is a perennial tropical tree originated from the Amazonian forest. In the world, at least 2500 plant species are recognized for producing latex, but *Hevea brasiliensis* is currently the only commercial source of natural rubber production owing to its good yield of rubber and the excellent physical properties of the rubber products (Polhamus, 1962; Cornish, 2001). *H. brasiliensis* is intensively cultivated and exploited in modern rubber plantations with over 10 million hectares in about 40 countries in the world for providing the industry with natural rubber. Rubber tree is an important industrial crop for natural rubber production, natural rubber representing almost half (43.4% in 2010 according to Rubber Statistical Bulletin, 2011) of total world rubber production due to its unique mechanical properties, such as tearing resistance, compared with synthetic rubber (Sekhar, 1989; Venkatachalam et al., 2006; Clément-Demange et al., 2007; de Faÿ et al., 2010). There are more than 20 million of small growers' families that depend on natural rubber cultivation for their livelihood in the producing countries (http://www.irrdb.com).

b. Natural rubber and end uses

Natural rubber (cis-1, 4-polyisoprene) is obtained from the latex of laticifers of *Hevea brasiliensis* (Kush, 1994; d'Auzac et al., 1995). Latex is the cytoplasm of laticifers or latex vessels cells in the inner phloem of the tree specialized in the synthesis of natural rubber (cis-1,4-polyisoprene) (d'Auzac and Jacob, 1989). The latex flow expels out from laticifers upon bark tapping. Natural rubber production in practice is produced by both tapping and by hormonal stimulation (Compagnon, 1986). The tapping practice is making a panel on rubber tree trunk. The hormonal stimulation mainly refers to treating the rubber tree with ethylene generating chemical substances to activate the activation of latex cells metabolism to increase latex production and the productivity of tree (Coupé and Chrestin, 1989). The latex is then collected and treated to get natural rubber (Okoma et al., 2011).

Natural rubber is very important industrial material for various industries as the basic constituent of many products used in the transportation, industrial, consumer, hygienic and medical sectors because of its elasticity, resilience, and toughness etc. Among them, transportation is by far the largest single sector where the tyres manufacturing industry alone consumes more than 60% of the natural rubber supply (Clément-Demange et al., 2007). The rest of the production is used by the general goods as in the industry of construction for example, transmission and elevator belts, hoses and tubes; in the biomedical industry as surgical and medical examination gloves etc; in consumer products like golf or football balls and other recreational and sports goods, erasers and also for the manufacture of mattress, of shoes, of glues, of adhesives, etc.

c. Rubber production in the world and China

Natural rubber is mainly produced in South-east Asia (93%), in Africa (4%), and in Latin America (3%) (Figure 1). The total production in 2010 is 10.4 million tons. In 2010, the main producing countries include by descending order, Thailand (3.07 million tons), Indonesia, Malaysia and India, Vietnam, China and so on. Thailand accounts about for 30%, Indonesia has about 28%, Malaysia has about 10%, India has about 8% and China has about 6% of the world production respectively (data from Association of Natural Rubber Producing Countries, ANRPC). The world supply of natural rubber is barely keeping up with the global demand. There was a shortage of 377,000 tons of the supply in 2010 according to the Rubber Statistical Bulletin (International Rubber Study Group, 2011).

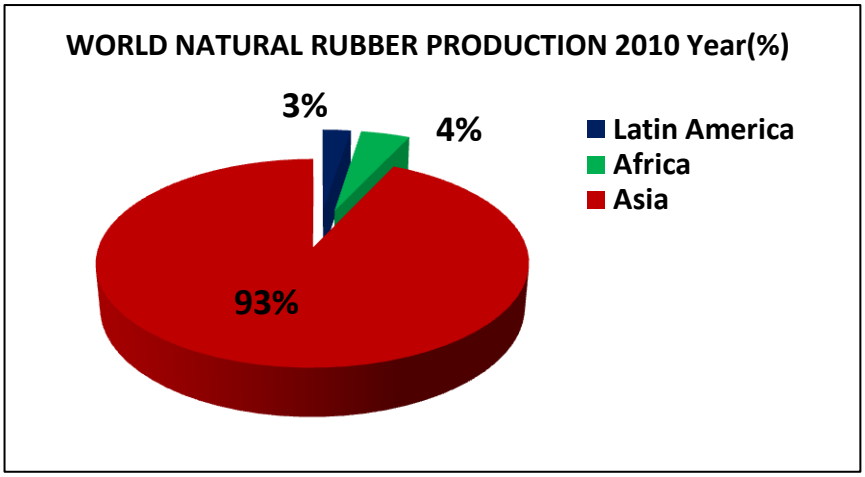

Figure 1. The percentage of total world natural rubber production in 2010 in Asia, Africa, and Latin America (%)

China has 1,002,000 hectares of rubber plantations distributed mainly in Hainan, Yunnan, and Guangdong provinces. China produced 647,000 tons of natural rubber in 2010, contributing at 6% of the world natural rubber production. In 2010, China was the largest natural rubber consumer of the world with 33% of the consumption (data from Association of Natural Rubber Producing Countries, ANRPC) (Table 1). For China, the degree of external dependence for natural rubber exceeded 80%. Therefore there is an urgent need to improve the rubber production. Since the limit of the climate element as cold, wind in China for rubber tree plantation, it is very hard to develop more suitable land for the rubber plantation. The superior clones and the tapping technique and new stimulant development are the major ways to improve the rubber production (Figure 2).

Table 1. Statistical Profile of Rubber Industry in China (data from Association of Natural Rubber Producing Countries, ANRPC)

Area and Yield	**2008**	**2009**	**2010**
Area under cultivation of rubber (Thousand hectare)	932	971	1002
Area under tapping (Thousand hectare)	520	542	566
Average annual yield per tapped area (kg/hectare)	1053	1187	1143
Natural Rubber production			
Production of NR(Thousand tonnes)	547.8	643.2	647
Gross Import of NR (Thousand tonnes)	1585	1591	1750
Consumption of NR (Thousand tonnes)	2740	3040	3300
Gross Export of NR (Thousand tonnes)	3	3	24.6

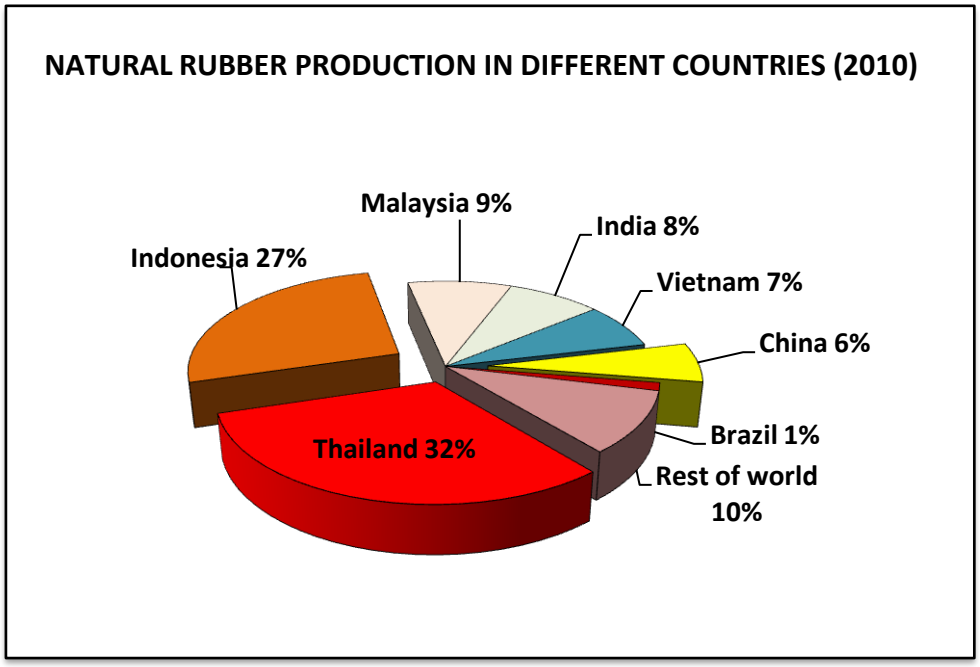

Figure 2. The percentage of natural rubber production in different countries in 2010 (%)

d. Prediction for the world rubber demand

The natural rubber shortage is predicted continuing as demand for natural rubber in China, India and Malaysia, which account for 48 percent of global usage, is expected to increase this year (data from Association of Natural Rubber Producing Countries, ANRPC). Demand in China, the largest user, may gain 9.1 percent to 3.6 million tons; India's usage may gain 5 percent to 991,000 tons and consumption in Malaysia may rise 7 percent to 490,000 tons.

It is predicted that global demand of natural rubber will rise to 16.5 million tonnes in 2020. China's consumption will reach to 4.8 million tons, accounting for 36.6% of the global consumption. The global rubber shortage mainly of natural rubber (NR) may widen to over one million tons by 2020 (International Rubber Study Group, IRSG, 2011).

Because the natural rubber is replaced by synthetic rubber, the long-term storage will lead progressively manufacturers to new industrial procedures using synthetic rubber. This scenario will provoke ecological impacts with a rubber growing disaffection. How to improve the production of the natural rubber to meet the gap in the sully and demand is now becoming a challenge for the rubber research.

2. Challenges to increase rubber production

a. Limiting factors of the rubber production

Natural rubber (cis-1, 4-polyisoprene) is from the latex of laticifers of *Hevea brasiliensis* (Kush, 1994; d'Auzac et al., 1995). The laticifers formed chains of contiguous cells that are arranged in rings parallel to the vascular cambium. When the bark is tapped, the latex of these laticifers is expelled out due to the high turgor pressure in liber tissue (Gooding, 1952; Buttery and Boatman, 1964; Adiwilaga and Kush, 1996; Hao and Wu, 2000) until coagulation processes lead to the plugging of their extremity (d'Auzac and Jacob, 1989; Yeang, 2005). The rubber particles account for up to 55% to less than 30% of the collected latex volume depending on the season, the tapping hour, the tree age, the rubber clone, and the harvesting system.

Rubber yield from *H. brasiliensis* is determined by different factors. Natural rubber (cis-1, 4-polyisoprene), is synthesized on the rubber particles in laticifer networks (Hébant and de Faÿ, 1980; Hébant, 1981) through isoprenoid biosynthesis pathway (Kekwick, 1989). Biosynthesis of natural rubber is affected by various plant hormones. The number of laticifers is one of the most important factors influencing rubber yield from *Hevea brasiliensis* (Gomez, 1982). Laticifers form a ubiquitous network of tubes in rubber tree, and are the major location of rubber biosynthesis (Gomez and Moir, 1979; Hao and Wu, 2000; Chow et al., 2007). This was validated by that the number of lactiferous cells and lactiferous rings per mm2, and lactiferous cell diameter were higher in the most productive clone RRIM 600 compared with GT1 clone for instance (Alessandro Carlos Mesquita, 2006). The latex flow rate and duration are limiting factors to rubber yield: the faster and the longer is the latex flow, the higher is the yield (d'Auzac and Jacob, 1989), but they depend on the turgor pressure in the liber tissues, and the latex dry rubber content (DRC) or total solid content (TSC), and the latex coagulation efficiency (Gooding,

1952; Buttery and Boatman, 1964; Kongsawadworakul and Chrestin, 2003; Gohet et al., 2003). Hevein, a lectin-like protein, and its receptor, and a chitinase were indicated involved in latex coagulation (Gidrol et al., 1994; H. Chrestin, 1997; Wititsuwannakul et al., 2008). Ethylene has been one practically indispensable factor concerning the rubber production being widely applied in rubber plantation as ethephon (a commercially used ethylene releaser). Ethylene was identified to delay coagulation, stimulate the latex flow and increase latex yield (d'Auzac and Ribaillier, 1969; Lynn and Redford, 1986). Bark treatment with ethephon is known to increase the latex yield by 1.5–2 folds in rubber tree (Pujade-Renaud et al., 1994; Coupé and Chrestin, 1989; Zhu and Zhang, 2009). The prolonged latex flow and acceleration of sucrose metabolism by ethylene was considered as the main reasons for the improvement of latex yield by ethylene. It was speculated recently that ethylene had little direct effect on accelerating rubber biosynthesis (Zhu and Zhang, 2009). But the mechanism of ethylene stimulation latex production is still not completely described. In addition, natural rubber production in *Hevea brasiliensis* is determined by both tapping and ethephon frequencies. However, a complex physiological disorder called Tapping Panel Dryness (TPD) also largely affected the production negatively. It was estimated that the losses due to TPD accounted to 12-14% of the annual rubber production. This syndrome is likely to be induced by environmental and latex harvesting stresses (Jacob et al., 1989; Duan et al., 2010). Currently, there is no effective prevention or treatment for this serious disease (Venkatachalam et al., 2006).

b. Tapping Panel Dryness

Over the past decades, the rubber yield has been significantly increased, due to the cultivation of high yielded clones and the utilization of ethephon (an ethylene generator). However, latex production is suffering a severe loss caused by tapping panel dryness (TPD) syndrome which caused the cessation of latex production. TPD remains the main constraint in rubber plantations worldwide. It was estimated that the losses due to TPD accounted to 12-20% of the annual rubber production, with an incidence of 20–50% of productive trees affected by TPD, in almost every rubber growing regions (de Faÿ et al., 2010; Okoma et al., 2011).

TPD syndrome and classification

The tapping panel dryness (TPD) is a serious problem occurring at the beginning of the 20th century with the increase of *Hevea brasiliensis* cultivation in Asia. It became a major problem in the 1970s, affecting the latex yield in modern industrial plantations. The TPD syndrome, first known as brown bast, was detected early by bark dryness upon tapping with partial or ultimately complete blockout of latex flow on the tapping cut. In the advanced stage, the tapping panel may even become completely dry and the other symptoms are the barks browning, bark thickening, bark cracking and bark deformations (de Faÿ and Jacob, 1989), which make the affected trees finally unsuited for latex production(Sookmark et al., 2002; de Faÿ et al., 2010).

There are two symptoms of TPD syndrome (Figure 3):

(i) Reversible overexploitation-induced TPD leading to a dry cut
(ii) Irreversible form called brown bast.

Reversible over-exploitation-induced TPD is related with the exploitation intensity. It is caused by a "physiological fatigue". The latex cell metabolism is severely disturbed when over exploiting the tree to regenerate the latex. Some authors distinguished new forms of syndrome called trunk phloem necrosis (TPN). TPN was different from TPD. Besides the obvious dryness of the cut, TPN caused a severe disturbance in the trunk phloem, from the collar towards the tapping cut (Nandris et al., 1991). TPN also occurred in immature trees which confirmed the difference between TPD and TPN, although early symptoms such as dryness of the tapping cut were similar (Nandris et al., 2004). The TPN disease was first detected in the 1980s (Nandris et al. 1984). TPN is prevalent in most modern rubber plantations worldwide.

Figure 3. The symptoms of tapping panel dryness (TPD) syndrome

The nature and mechanism of TPD

Since 1904, a great deal of effort has been invested to understand the nature and mechanism of TPD. There are two scenarios generally proposed concerning the mechanism of TPD. One is the pathological hypothesis (scenario pathogen) that TPD might be caused by pathogen; and the other is physiological scenario (scenario stress) that TPD is a multi-factor physiological disease caused by an accumulation of exogenous and endogenous stresses (Peyrard et al., 2006; de Faÿ et al., 2010).

TPD was initially thought to be caused by pathogen. However, no pathogenic agent causing TPD has been successfully isolated and no TPD occurrence of infection through TPD bark's being inoculated on healthy trees was observed. The apparent linear spread of the necrosis starting from the affected tree gradually forming a disease centre supported the initial hypothesis of a biotic causal agent for TPN. Similar epidemic extension of the disease was observed at other sites (Nandris et al., 2004). However, aetiological investigations on TPN (Nandris et al., 1991; Pellegrin et al., 2004) focusing on a range of potential pathogens (fungi, bacteria, mycoplasma, virus and viroid) failed to identify any biotic causal agent. Although all these molecular aetiological analyses could not find any evidence to characterize a given pathogen, it is still not possible to fully invalidate the existence of a biotic causal agent (Peyrard et al., 2006).

On the other hand, an alternative hypothesis that TPD is a physiological syndrome obtained the support of several lines of evidence (Chrestin et al., 1997; de Faÿ and Jacob, 1989; Jacob et al., 1989). According to this view, mechanical damage in barks caused by excessive tapping and intense ethylene stimulation would eventually lead to TPD. TPN is now considered to be a physiological disease of the trunk caused by an accumulation of different stresses.

Physiological research evidence on TPD

Physiological studies suggested that the TPD syndrome was a complex physiological disorder resulted from over tapping and over exploitation (excessive tapping as well as overstimulation with ethylene) (Jacob et al., 1989). During the process of TPD, the lutoids burst and consecutive in situ latex coagulation caused by membrane destabilization, which has been proposed to be associated with the occurrence of an uncompensated oxidative stress within the latex cells (Chrestin et al., 2004). It was found that the content of protein, nucleic acid, thiols and ascorbic acid decreased in TPD trees, whereas the activities of RNase and proteinase were increased in general (Tupy, 1988). The levels of variable peroxidise and superoxide dismutase isozyme (SOD) also decreased in TPD trees.

TPD might be directly related to cyanogenesis (de Faÿ et al., 2010). The impaired cyanide metabolism causes damage in *Hevea brasiliensis* barks. Normally, rubber trees also contain β - glucosidases and β - diglucosidases, which gradually degrade the cyanogenic glucosides, which results in the release of cyanide, but also the key enzyme of cyanide detoxification β cyanoalanine synthase (CAS) (Lieberei et al., 1989; Gruhnert et al., 1994; de Faÿ et al., 2010). Applications of linamarin and KCN to the bark were shown to cause bark dryness in clones in which β - CAS activity is low (as low as in noncyanogenic plants), and β glucosidases and β diglucosidases relatively high (de Faÿ et al., 2010).

Molecular research evidence on TPD

TPD syndrome is apparently genetically determined, as different clones of rubber tree showed different degrees of TPD in field tests (Jacob et al., 1989), and the molecular mechanism of TPD syndrome has made some progress in rubber tree.

Some genes and proteins have been identified as markers for the early diagnosis of TPD (Siswanto et al., 1997). For instance, SDS-PAGE analysis indicated that several unknown proteins increased in C-serum (the disperse phase of the latex) and B-serum (cytosol of the lutoid) from TPD tree (Darussamin A et al., 1995; Dian et al., 1995; Lacrotte et al., 1997); but the properties and function of these proteins remain unknown. Through proteomics, some researchers have identified proteins related to TPD by comparing the expression patterns between healthy and TPD trees (Darussamin A, 1995; Lacrotte et al., 1997; Sookmark et al., 2002), but their functional relations with TPD still remain unknown.

The expression of *HbMyb1* transcription factor gene was demonstrated to be closely associated with TPD with significantly decreased expression level in barks and latex of TPD tree comparing with healthy tree (Chen et al., 2003). Functional analyses indicated that *HbMyb1* negatively regulated programmed cell death (PCD) in transgenic tobacco plants (Peng et al.,

2011), 134 genes were identified associated with TPD in rubber tree by SSH method. Moreover, many TPD associated genes up-regulated in TPD trees during the onset of TPD syndrome (Venkatachalam et al., 2007). One gene *HbTOM20* was shown to be associated with TPD by mRNA differential display. *HbTOM20* might play an important role in the alteration of mitochondrial metabolism, which finally resulted in impaired latex biosynthesis (Venkatachalam et al., 2007, 2009). 237 unique genes were identified from the SSH libraries to be associated with TPD. Systematic analyses of the functional categories and expression patterns of the genes associated with TPD suggested that different signal pathways as the production and scavenging of reactive oxygen species (ROS), ubiquitin proteasome pathway, programmed cell death and rubber biosynthesis might play important roles in TPD.

The other factors related with the TPD

More specifically, disease spread was related to heterogeneous soil constraints such as soil compaction (obviously not the only factor). The development of tapping panel dryness could be influenced by climatic factors as rainfall and temperature (Okoma et al., 2011). TPN affected trees was shown to have a spatially structured progression of the disease (Peyrard et al., 2006).

The treatment of TPD

At present, there are no effective measures to prevent or treat TPD in rubber plantation. But in Indonesia, the NoBB^{TM} (No brown bast) is used in some plantations to treat the trunk for helping bark regeneration. It was considered useful to bark dryness recovery on rubber plants using NoBB (No Brown Bast) application (Siswanto, pers. com.)

It was also reported that a surprising number of rubber trees affected by TPN had recovered after an indirect treatment with sodium hypochlorite (through disinfection of the tapping knife). It was suggested that sodium hypochlorite has a curative effect in the earliest stages of the disease as a clue for controlling this disease (Peyrard et al., 2006).

c. *Hevea brasiliensis* diseases

As in other crops, rubber production is influenced by various plant physiological conditions and pathogenic diseases. It is susceptible to a number of diseases. South American Leaf Blight (SALB) caused by the fungus *Microcyclus ulei*, is the major rubber tree disease in all Central and South America as Brazil (Le Guen et al., 2003). It was reported to be controlled by spraying with Dithane. Mildew is the most serious leaf disease in Africa and the Far East, but is being effectively treated with sulfur dusts. Phytophthora foliage blight is increasing, but is being controlled by Dithane and Vancide (Control of Rubber Diseases by Chemicals, PESTICIDES IN TROPICAL AGRICULTURE, 1955, Chapter 7, pp 31–42). Corynespora Leaf Fall Disease (CLFD) has become a major threat for rubber cropping in South-East Asia and West Africa. *Corynespora cassiicola*, a non-specific host fungus, is affecting increasingly and rapidly the rubber plantations (Qi et al., 2009).

d. Abiotic stresses and climate changes

Hevea brasiliensis is frequently confronting the abiotic stresses from the tapping (wounding) and ethylene stimulant for the rubber production. Climate changes affect rubber production and productivity. The climate elements such as climate warming, wind and storm, drought etc can also cause a big threat for the rubber tree cultivation. Considering the importance of climate change and its impact on natural rubber cultivation, there are two major international conferences were targeted on this subject to review the climate change in the major natural rubber producing countries of the world and the impact on the growth and productivity of rubber in order to take strategies for rubber cultivation in stressful environments and rubber processing and manufacturing industries for energy efficiency and reducing carbon dioxide emission to mitigate climate changes. One is International Rubber Research and Development Board (IRRDB) Annual Meetings and International Rubber Conference, Oct. 18-22, 2010, Sanya: Climate Change, Low Carbon Economy and Sustainable Natural Rubber Industry; the other is the International Workshop on "Climate change and natural rubber cultivation: Research and Development Priorities" at the Rubber Research Institute of India, Kottayam, 28-30 July, 2010.

3. Genetic improvement of rubber tree

a. Conventional breeding

Hevea clones are grafted on unselected rootstock seedlings. The basic goal of rubber breeding is to provide the farmers with adapted superior clones represented by mature budded clones for latex production and also for rubber wood production. In addition to the yield improvement, new traits are considered such as: the quality of the rubber product, the resistance to the different stresses (drought, cold, wind, pathogen, etc) (Clément-Demange et al., 2007). Conventional breeding faces huge challenges and tasks due to the long juvenile cycle in the *Hevea brasiliensis* breeding.

Beyond the continued conventional breeding, biotechnology will be needed to maximize the probability of success (Ruttan, 1999; Huang et al., 2003) for improving the rubber tree breeding. The use of DNA markers in breeding or marker-assisted selection (MAS) breeding, tissue culture and genetic transformation maintains the most promising techniques to provide the superior breeding material for the future rubber tree breeding (Collard and Mackill, 2008).

b. Molecular-assisted breeding

Marker-assisted selection (MAS) using DNA markers could have enormous potential to improve the efficiency and precision of conventional plant breeding (Collard and Mackill, 2008). The large number of quantitative trait loci (QTLs) mapping studies for diverse crops species have provided an abundance of DNA marker–trait associations (Collard and Mackill, 2008). In rubber, neutral molecular genetic markers have already been useful for varied applications (clonal conformity, parentage identification, diversity analysis). With genetic mapping, QTLs have shown their capacity to assist in understanding the genetic determinism of some traits. South

American leaf blight (SALB) is a disease of the rubber tree caused by the fungus *Microcyclus ulei*. The search for QTLs was performed. Quantitative trait loci (QTLs) for resistance were mapped. Several QTL was detected responsible for the resistance (Lespinasse et al., 2000; Le Guen et al., 2003). Theoretically, molecular genetic markers, independent from the environment, could be used at a very early stage for effective screening of large populations of rubber seedlings, which would be particularly useful for a tree crop such as the rubber tree (early selection).

QTLs also have some limits since it is often related with the specific linkage pattern of one family, and cannot frequently be extended to a wider population. But genetic maps can now be developed quite fast and routinely, and the development of the methodology for their application to rubber selection still in expecting (Clément-Demange et al., 2007).

c. Biotechnologies for the improvement of conventional breeding

Conventional breeding is limited by the difficulties for controlled pollination. Indeed, rubber trees produced a low fruit set, so it is difficult to establish large progenies from one cross. Some specific crosses aborted and required embryo rescue technique from immature embryos to generate a progeny (Clément-Demange et al., 2007). This technique is under control in some countries (India, Thailand, etc.) but not well-applied in breeding programme over the world. Some inter-specific or intra-specific crosses required protoplast fusion and plant regeneration from the formed embryogenic callus. However, this technique is very difficult in *Hevea* and only a few numbers of papers were published (Cailloux and Lleras, 1979; Cazaux and d'Auzac, 1994; Sushamakumari et al., 1999; Sompong et al., 2000).

i. The multiplication of the improved plant material

Tissue cultures as microcuttings or the induction of somatic embryogenesis are both investigated in *Hevea brasiliensis*. There is no large scale commercial application of tissue culture techniques for mass propagation of clonal *Hevea brasiliensis* yet, either by microcuttings or by somatic embryogenesis. However, there is sufficient progress at research level to suggest that tissue culture of *Hevea brasiliensis* can and should be further developed (Nayanakantha and Seneviratne, 2007).

Micropropagation, which is a form of tissue culture, increases the amount of planting material to facilitate distribution and large scale planting. Propagation by in vitro culture of apex and axillary buds has been termed microcutting or microprapagation in *Hevea brasiliensis*. This technique isolated the stem pieces of young seedlings bearing a node to progressively extend to clonal material (Enjalric and Carron, 1982); the cultivation of buds includes primary culture, multiplication, rooting and acclimatization. The primary explant can be multipliplied several times to obtain the sufficient numbers. After root induction, rooting with acclimatization is carried out for 4–6 weeks in greenhouses before transferring the plantlets to the field (CIRAD). Mendanha et al. (1998) reported that shoots were obtained from axillary buds culture on Murashige and Skoog (MS) medium supplemented with growth regulators (Clément-Demange et al., 2007) ; There are several reports on *Hevea brasiliensis* micropropagation using different explants, mostly derived from seedlings (Thulaseedharan et al., 2000).

Somatic embryogenesis is a type of rapid and efficient vegetative propagation based on plant cell totipotency which offers a powerful alternative to other vegetative propagation methods, i.e. cuttings or grafting (Jean-Paul Ducos, 2007). An efficient plant regeneration pathway by somatic embryogenesis is essential for crop improvement through transgenic approaches besides using this as a micropropagation system.

Somatic embryogenesis as an *in vitro* propagation technique in *H. brasiliensis* was started to develop in the early 1970s by a Chinese team at the Rubber Research Institute (Baodao) and a Malaysian team at the Rubber Research Institute of Malaysia, simultaneously. The Institute de Recherche sur le Caoutchouc (France) began work in this field in 1979 (Carron et al., 1995). Different explants has been used somatic embryogenesis for plant regeneration in *H. brasiliensis* such as immature anther (Satchuthananthavale and Irugalbandara, 1972; Wang et al., 1980), inner integument of seed (Carron et al., 1995), immature inflorescence (Sushamakumari and Sobha, 2000), root explants. Improvement of somatic embryogenesis *H. brasiliensis* using the temporary immersion technique was reported (H. Etienne, 1997). Embryogenic capacities of integument explants excised from three different age and sources of the *Hevea brasiliensis* clone PB 260 were compared. It was suggest that the chronologic, ontogenetic and physiologic aging had effects on explant capacity for somatic embryogenesis and on the overall efficiency of the process in *H. brasiliensis* (Lardet et al., 2009).

ii. Genetic transformation

Genetic improvement of the rubber tree with conventional breeding has been very slow because a full evaluation of new genotypes takes a number of years (Jayashree et al., 2003)Many factors limited the improvement of rubber tree through conventional breeding for example, the narrow genetic base of the cultivated clones, long juvenile, highly heterozygous nature of the seed propagated plants, poor seed set, etc (Nayanakantha and Seneviratne, 2007). The tapping panel dryness (TPD), drought, and leaf fall diseases caused by *Corynospora* and *Phytophthora* etc elements causes a heavy yield loss to the rubber production in natural rubber producing countries. Currently, there is no effective prevention or treatment for these serious problems. Therefore, there is an urgent need to introduce resistant genes into high yielding clones (Nayanakantha and Seneviratne, 2007). As it is a perennial tree crop with a long breeding cycle, the integration of specific desired characters through conventional breeding is both time-consuming and labor-intensive (Jayashree et al., 2003)

Genetic engineering is a promising approach for genetic improvement. Genetic transformation with conventional breeding offers a valuable tool to introduce the specific gene into the existing *Hevea brasiliensis* genotype without loss their desirable genetic constitution and integrity in a short time period (Thulaseedharan et al., 2004). Genetic transformation, however, is still difficult in *Hevea brasiliensis*. Genetic transformation requires not only the insertion of foreign DNA into the plant genome, but also its expression in the transformed cells and an efficient protocol by which to regenerate plants via *Agrobacterium* and other methods (Jayashree et al., 2003). Thus, a reproducible plant regeneration system for each genotype of *Hevea brasiliensis* through tissue culture is essential for crop improvement programmes (Nayanakantha and Seneviratne, 2007; Venkatachalam et al., 2006). Recent advances showed some breakthrough in the genetic transformation efficiency in the study of (Leclercq et al.; Blanc et al., 2006).

The genetic transformation of *Hevea brasiliensis* using *Agrobacterium* has been reported, and transgenic plants were obtained via *Agrobacterium tumefaciens* (Arokiaraj and Wan Abdul Rahaman, 1991; Arokiaraj et al., 1996; Arokiaraj et al., 1998). Transgenic plants have also been produced using particle bombardment (Arokiaraj et al., 1994). Montoro et al. (2000) studied the response of exogenous calcium on *Agrobacterium*-mediated gene transfer in *Hevea brasiliensis* friable calli (Montoro et al., 2000). However, the transformation efficiency was generally low. It was reported that one HbSOD superoxide dismutase gene has been successfully transformed into *Hevea brasiliensis* via *Agrobacterium*-mediated genetic transformation using immature anther-derived calli as initial explants, consequently developed transgenic plants. A transformation frequency of 4% was achieved (Jayashree et al., 2003; Venkatachalam et al., 2006). Due to the consideration of the long term environmental control, the genetic transformation in rubber plantlet is now mainly used to validate the gene functional analysis.

4. Regulation of the latex production

a. Tapping and ethephon stimulation on latex production

Natural rubber is exploited by tapping, a process involving repeated wounding of the tree along a cut made on the bark of the tree. Modern tapping systems have been evolved based on the structure of the laticiferous system inside the bark and stimulation. The most common system adopted is alternatively tapping on a half spiral cut. In the case of high yielding clones like RRII 105, third daily tapping has been recommended to avoid incidence of the serious physiological disorder, tapping panel dryness. Based on the latex diagnosis (LD), the clones have been classified into three main groups as slow metabolising (PR 261, AVROS 2037), intermediate (GTI, PR 107, RRIM 600) and active metabolising clones (PB 235, PB 260 etc.). Latex diagnosis is used for determining tapping frequencies and stimulation methods (Gohet et al., 2003) also underlined the necessity to exploit each *Hevea brasiliensis* clone according to the metabolic typology of its laticiferous system (Serres et al., 1994). The intensity of tapping and stimulations must be adapted to the clone and age of the tree (Gohet et al., 2003). In 1969, d'Auzac and Ribaillier confirmed that ethylene generating chemicals could be used as yield stimulants in *Hevea brasiliensis*. It was suggested that yield response to stimulation is related to the concentration of the stimulant (Tupy, 1988). Yield response reached a maximum at 1.25% Ethephon (Tupy, 1988), and 2.5% ethephon, further increase in concentration did not improve production. However, response to concentration depends on the method of application/and age of the tree, and the tapping system adopted. Stimulation under low intensity tapping produces better yields than under high intensity tapping. With stimulation, the frequency of tapping could be reduced. Slow metabolising clones like PB 217 must be strongly stimulated to express the production potential, intermediate metabolism clones (GT1, PR 107, RRlM 600) need moderate stimulation while those like PB 235 with active metabolism require little stimulation to express their production potential (Gohet et al., 2003). It was suggested that each *Hevea brasiliensis* clone should be exploited according to the metabolic typology of its laticiferous system. There exist differences among the clones in response to stimulation and tapping (Serres E. et al., 1994).

b. Physiology of laticifers

The origin of laticifers in *Hevea brasiliensis* had been revealed by Scott in 1882 (Mahlberg, 1993). Three types of laticifers were identified as hypodermal, principal and medullar by Clavert in 1887 (Mahlberg, 1993). Two regions were distinguished as the inner soft zone near the cambium and the outer hard zone (Figure 4). Latex vessels have an inclination to the long axis of the trunk with a variation of 2'-7' (Gomez, 1982). Latex vessels are present in all *Hevea* plant tissues. Two types of laticifers are found as articulated and nonarticulated. In *Hevea brasiliensis,* the latex vessels are the articulated. The latex vessels were in a zig-zag fashion from base to the top of the trunk with a clonal variation.

Reports indicate that the structure and number of latex vessels have a correlation on productivity (Gomez, 1982; Sethuraj and Mathew, 1992; Gomez and Moir, 1979). The number of latex vessel rings is under the control of genetics (Bobiloff, 1923). The age of the tree display a linear relationship with the number of latex vessels. The latex vessel density is significantly different in various clones. It was suggested that this could be related with the productivity of the clone. Continuous wounding of the bark by tapping can be regenerated by the cambial activity. Thus, regenerated bark can be tapped again and continues to be productive (de Faÿ and Jacob, 1989). The latex flow rate after tapping was related to the diameter of the latex vessels with the clonal variation (Gomez and Moir, 1979). Latex exploitation is one of the most important factors that influence the formation of laticifer rings (Hao and Wu, 1984).

c. Molecular biology and genomics of laticifers

Laticifer-specific gene expression in *Hevea brasiliensis* was firstly reported by (Kush et al., 1990). It was shown that plant defence genes encoding chitinases, pathogenesis-related protein, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, cinnamyl alcohol dehydrogenase, and 5-enolpyruvylshikimate-3-phosphate synthase had a 10- to 50-fold higher expression in laticifers than in leaves, indicating the probable response of rubber trees to tapping and ethylene treatment (Kush et al., 1990).

Transcriptome analyses were recently reported in the researches on the mechanisms of the latex biosynthesis and TPD syndrome. In the latex transcriptome, Han etc reported about 16% of the database-matched ESTs encode rubber biosynthesis-related proteins such as rubber elongation factor (REF) and small rubber particle protein (SRPP). The defence and stress genes and protein metabolism-related genes accounts for 12.6%, suggesting that defence is one of the functions of laticifers. REF (rubber elongation factor) and 22 kDa SRPP (small rubber particle protein) are the most abundant genes in the latex. Montoro took the NGS 454 techniques to establish a large database for the transcriptomics research on the latex metabolism and stress regulation (Han et al., 2000; Ko et al., 2003; Chow et al., 2007; Montoro et al., 2010)

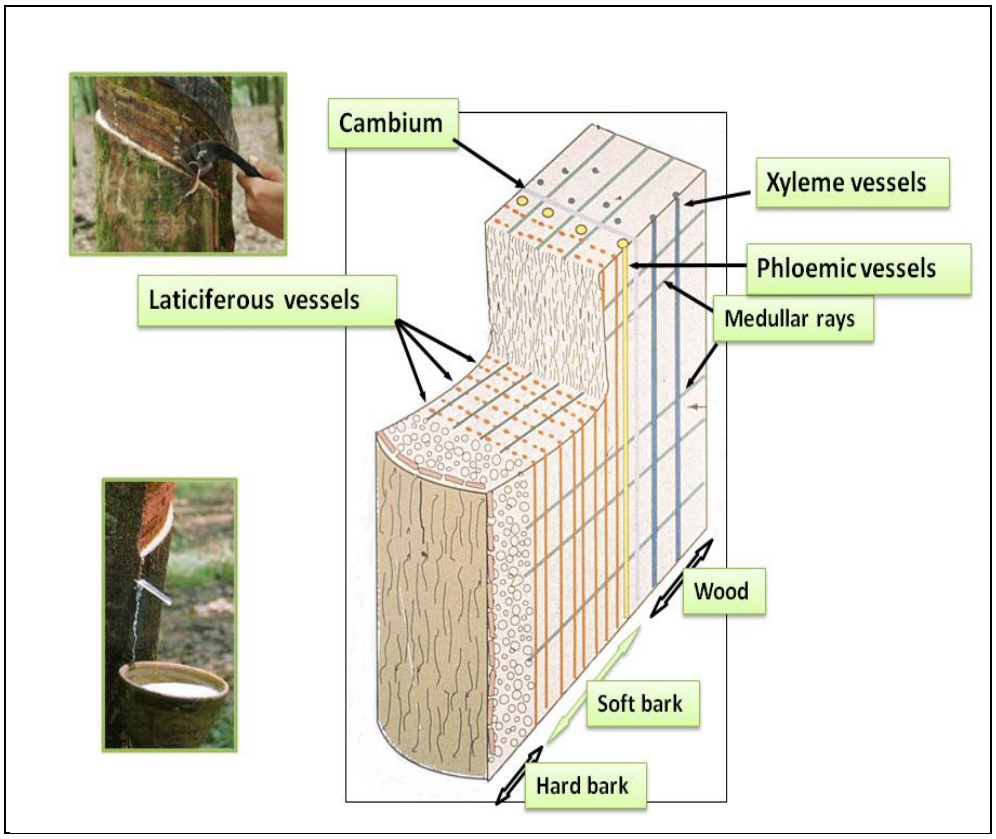

Figure 4. Anatomy of bark at the tapping cut in *Hevea brasiliensis*

5. Involved signalling pathways in latex cells

a. Relation between tapping, wounding and jasmonic acid

Tapping can be considered as an osmotic stress due to the plasmolysis of laticifers and a mechanical wounding stress. ABA and JA signalling pathways are known to be involved in the response to these stresses. Ethephon is an ethylene releaser. So ethylene signalling pathway is supposed to play a major role to coordinate the response to ethephon. Jasmonic acid is a wounding signal molecular. Wounding and exogenous jasmonates could induce the laticifer differentiation.

b. Ethephon, an ethylene releaser

Ethephon is used commercially to prolong the flow of latex from the rubber tree after tapping (Yield stimulation). The compound is applied to the bark in the region of the tapping cut and the effect on latex flow is due to the ethylene released by chemical decomposition, since gaseous ethylene itself is also a very effective stimulant. It was absorbed and ethylene formation commences immediately at the site of application, and the gas is quickly translocated throughout the plant (Audley et al., 1976).

c. Importance of ethylene and jasmonate signalling pathways in latex production and laticifer differentiation

The mechanism of ethylene stimulation of latex yield was shown in many aspects in regulating the latex metabolisms. This includes increased osmotic pressure in the laticifers, lowered the latex viscosity caused by slight decrease in DRC (dry rubber content), increased cell wall plasticity, modified the formability of plasmalemma, increased latex stability, decreased destabilising activity in latex, increased drainage areas of the bark, and an increase in the initial flow rate after stimulation, an increase in turgor pressure in the latex vessels after stimulation and prolonging the flow. Stimulation enhances the sucrose metabolism of laticifers (Tupy, 1988). Wounding induces accumulation of hevein in laticifers. Gidrol *et al.* (1994) reported that hevein, a lectin like protein involved in the coagulation of latex in related with the pH of latex and the cytosol (Gidrol et al., 1994). The cumulative effect leads to a prolonged latex flow and subsequent increase in yield. The increase in latex production occurred between 5 to 6 hours and 12 to 24 hours after application of the stimulant to bring about substantial increase in latex production(Tupy, 1988).

Exogenous jasmonic acid was indicated to have important functions on rubber productions in *Hevea brasiliensis*. Application of exogenous jasmonic acid and linolenic acid, the precursor of JA biosynthesis, can induce laticifer formation and differentiation in *Hevea brasiliensis*, but ethephon had no detectable effect on laticifer differentiation (Figure 5, Hao and Wu, 2000). Mechanical wounding can also induce laticifer differentiation and the induction may be regulated by the jasmonate biosynthesis pathway (Wu et al., 2002). Latex exploitation can influence the formation of laticifer rings (Hao and Wu, 1984). Latex flow can accelerate the laticifer differentiation induced by exploitation. Ethephon caused no obvious change in the number of laticifer ring when applied to the trees without latex exploitation (Hao and Wu, 1984). JA may play an important role in regulation of the rubber biosynthesis in *H. brasiliensis.* The key enzyme of rubber biosyntheses in *H. brasiliensis* farnesyl diphosphate (FDP) synthase is expressed in latex and in the epidermal region. The expression of this gene is not significantly affected by ethylene, but tapping increases its expression level (Adiwilaga and Kush, 1996). An appreciable increase in the expression of *Am-FaPS-1* (FDP synthase) in *Aquilaria microcarpa* was reproducibly observed upon methyl jasmonate treatment (Kenmotsu et al.). *Hbvp1* was suggested as a JA-inducible V-PPase (vacuolar H (+)-pyrophosphatase) located on the rubber particles and might play an important role in regulation of the rubber biosynthesis of *H. brasiliensis* (Zeng et al., 2009).

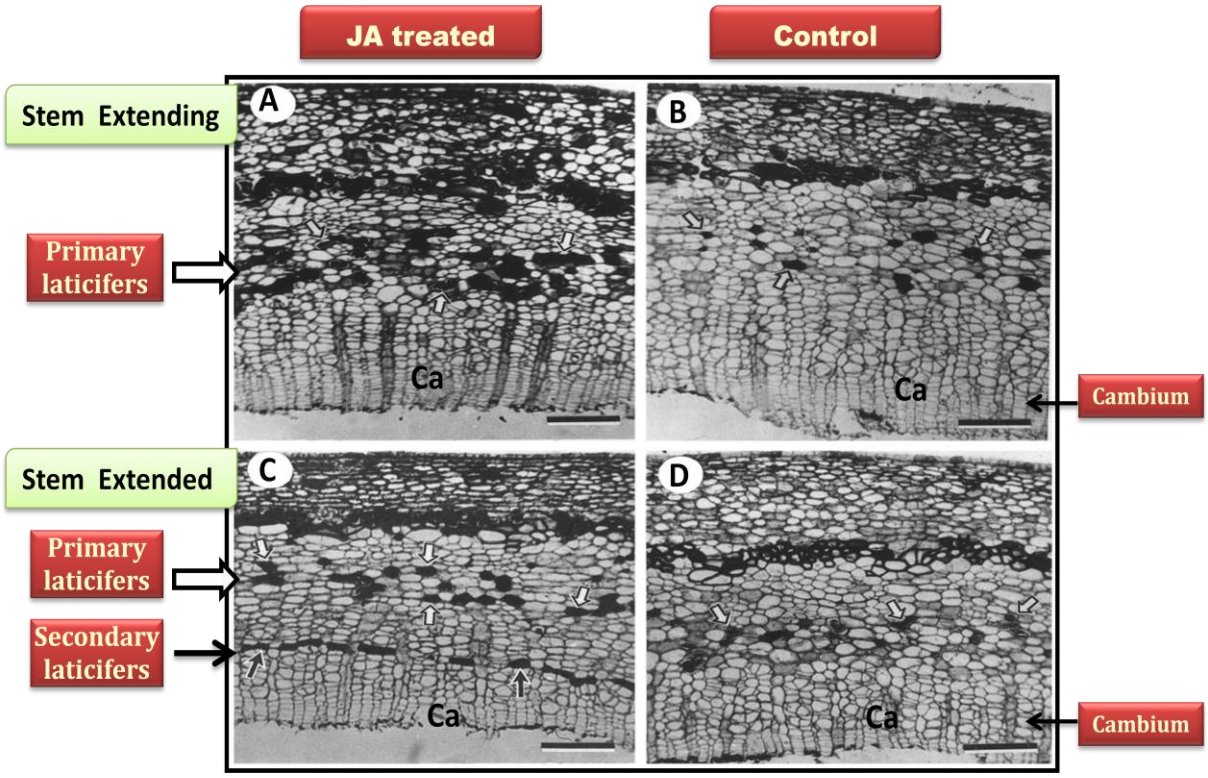

Figure 5. JA induced the laticifer differentiation. Cross-section of bark (Hao, 2000). Fig. A: JA-treated stem which was extending whilst being treated. Note primary laticifers. Fig. B: Control for Fig. A. Note there are fewer primary laticifers. Fig. C: JA-treated stem which had stopped extending when being treated. Note secondary laticifers. Fig. D: Control for Fig. C. White arrow, Primary laticifer; black arrow, secondary laticifer; Ca, cambium. Bars=200 µm

6. Ethylene and jasmonate biosynthetic and signalling pathways

a. Ethylene biosynthesis, transduction and signalling pathways in plant

Ethylene plays a key role in the regulation of plant growth, development including seed germination, root initiation, root hair development, flower development, senescence, and responses to biotic (such as pathogen attack) and abiotic (such as wounding and chilling) stresses. Ethylene signalling pathway involves the perception and the transduction of the ET signal, then the regulation on the ethylene-responsive genes (Peiser et al., 1984; Chen et al., 2005; Lin et al., 2009).

Ethylene biosynthesis starts with conversion of the amino acid methionine to S-adenosyl-L-methionine (SAM, also called Adomet) by the enzyme Met Adenosyltransferase (MAT). SAM is then converted to 1-aminocyclopropane-1-carboxylic-acid (ACC) by the enzyme ACC synthase (ACS); the activity of ACS determines the rate of ethylene production, therefore regulation of this enzyme influences the ethylene biosynthesis. The final step requires oxygen and involves the action of the enzyme ACC oxidase (ACO). Hydrogen cyanide, formed in the last step of ethylene synthesis, is detoxified by beta-cyanoalanine synthase (β-CAS) (Peiser et al., 1984; Kende, 1989; Chen et al., 2005; Etheridge et al., 2005; Lin et al., 2009). Ethylene biosynthesis can be induced by endogenous or exogenous ethylene.

A number of components of the ethylene response pathway have been characterized. After synthesis, ethylene then is perceived by a family of five membrane bound receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) in *Arabidopsis* (Hua et al., 1998; Bleecker, 1999; Hall et al., 2000; Schaller, 1997; Solano et al., 1998), and six in tomato LeETR1–LeETR6 that have similarity to bacteria two-component histidine kinase (Wilkinson et al., 1995; Lashbrook et al., 1998; Tieman and Klee, 1999; Lin et al., 2009). Ethylene receptors are negative regulators of the ethylene response (Hua and Meyerowitz, 1998). Ethylene receptor ETR1 is localized in the endoplasmic reticulum (ER) (Chen et al., 2002). The C-terminal domains of the *Arabidopsis* ethylene receptor ETR1 and ERS1 have direct protein–protein interaction with the N-terminus of the Raf-like serine/threonine protein kinase CTR1, which is co-localized with the receptors to the ER membrane (Clark et al., 1998; Gao et al, 2003). CTR1 has similarity to a mitogen-activated protein kinase kinase kinase (MAPKKK), suggesting the involvement of a MAP kinase-like signalling cascade in the regulation of ethylene signalling. CTR1 is a negative regulator of ethylene signalling (Kieber et al., 1993; Huang et al., 2003). EIN2 is the next downstream component identified in the signalling pathway, and has similarity to members of the Nramp metal-ion transporter family (Alonso et al., 1999). EIN2 plays a major role in the ethylene response and is a positive regulator of the pathway (Alonso et al., 1999). EIN2 may regulate ethylene responses partially by altering ion concentrations, such as calcium (Raz and Fluhr, 1992; Kwak and Lee, 1997). EIN2 is predicted to be membrane-localized, but the actual function of EIN2 in the pathway has not yet been determined. Recent research indicated that EBF1/EBF2 are indispensable ethylene signalling components that act genetically downstream of CTR1 and EIN2. Plant responses to ethylene are mediated by regulation of EBF1/2-dependent degradation of the ETHYLENE INSENSITIVE3 (EIN3) transcription factor (Guo H, 2003; Olmedo G., 2006; Binder BM., 2007; An FY, 2010). Functioning downstream afterwards is a small family of transcription factors that includes EIN3 and various EIN3-like (EIL) proteins (Roman et al., 1995; Chao et al., 1997). The EIN3/EIL family are involved in regulation and can stimulate the transcription of other transcription factors such as ERF1 (ethylene response factor) (Solano et al., 1998; Alonso et al., 2003), a member of the ERF family of transcription factors (also sometimes called the EREBP family for ethylene response element binding protein) (Fujimoto et al., 2000). These transcription factors have been shown to act as activators or repressors of additional downstream ethylene-responsive (Shinshi et al., 1995). This family of transcription factors plays a key role in the immediate response of plants to ethylene (Wang et al., 2002) (Figure 6).

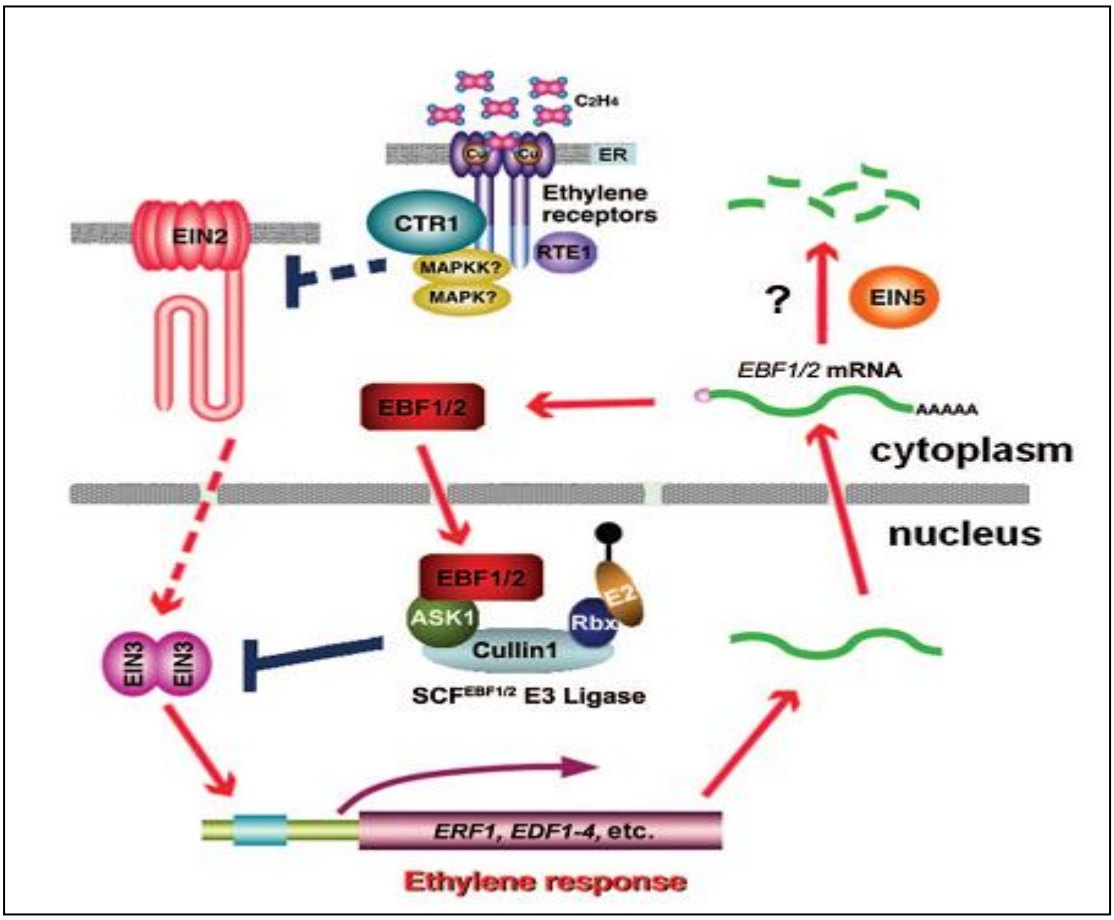

Figure 6. Ethylene signal transduction pathway. Ethylene (C2H4) is perceived by repressing the action of receptor complexes including ETR_ERS_EIN4 receptors, RTE1, and Raf-like protein ki-nase CTR1, which negatively regulates downstream signaling component EIN2. Upon ethylene treatment, EIN2 is derepressed and could thus transmit the signal into the nucleus to activate a number of transcription factors, including EIN3 and EIL1. EIN3 directly binds to the regulatory elements of target genes and induces the expression of yet other transcription factors (i.e.,ERFs and EDFs) that would ultimately regulate a series of ethylene responses. In the absence of ethylene signal, a Skp1-Cullin1-F-box complex consisting of one of two F-box proteins, EBF1 and EBF2, targets EIN3 protein for degradation via an ubiquitin proteasome pathway. Interestingly, EBF1_EBF2 gene expression is induced by ethylene in an EIN3-dependentmanner, which forms a negative feedback regulation on the EIN3 function. EIN5, a 5_33_ exoribo-nuclease, is involved in facilitating the turnover of EBF1_EBF2mRNA througha yet unknown mechanism. Therefore, EIN5 is proposed to antagonize the negative feedback regulation on EIN3 by promoting EBF1 and EBF2 mRNA decay, which consequently allows the accumulation of EIN3 protein to trigger the ethylene response. Red arrows and blue bars represent positive and negative regulations, respectively. The dotted lines represent regulatory steps in which a direct physical link between upstream and downstream components has yet to be demonstrated (Olmedo G., 2006).

b. Jasmonic acid biosynthesis and signalling pathways in plant

Jasmonates, naturally-occurring octadecanoid-based compounds are playing important roles in defence responses against pathogen and insect attack, and in plant growth and development (Sembdner and Parthier, 1993; Liechti et al., 2006).

JA biosynthesis starts with the oxygenation of α-linolenic acid (Creelman and Mullet, 1997; McConn et al., 1997; Reymond and Farmer, 1998). Oxygenation of linolenic acid is catalyzed by 13-lipoxygenase (LOX). The resultant 13-hydroperoxide is dehydrated by allene oxide synthase (AOS) to an unstable allene oxide intermediate before cyclization, guided by allene oxide cyclase (AOC), to the cyclopentenone ring-containing 12-oxo-phytodienoic acid (OPDA). OPDA can be further metabolized, by reduction of the ring double bond catalyzed by OPDA reductase 3 (OPR3), yielding a cyclopentanone intermediate. This intermediate is then subjected to three rounds of β-oxidation, which yield the best-known jasmonate family member, the 12-carbon regulator jasmonic acid (JA) (Creelman and Mullet, 1997). JA have can be conjugated by esterification to other molecules, a methyl group (Liechti and Farmer, 2002; Florian Schaller，2005；Liechti et al., 2006; Wasternack, 2007)（Figure 7）.

JA has been considered to have a role in central wound signalling (Leon et al., 2001; Schilmiller and Howe, 2005; Wasternack, 2007). One of the immediate responses to wounds is the production of JA (Strassner et al., 2002). And the genes encoding JA-biosynthetic enzymes such as lipoxygenase (LOX), allene oxide synthase (AOS) and 12-oxophytodienoic acid reductase (OPR) are also activated (Bell et al., 1995; Laudert and Welter, 1998; Schaller et al., 2000). All JA biosynthetic genes such as LOX2, AOS and OPR3 are JA-inducible, implying the existence of a feed-forward mechanism for rapid and sustainable accumulation of JA in response to stresses (Sasaki et al., 2001). Wounding activates the gene transcription by stimulating both JA-dependent and -independent wound signalling mechanisms (Devoto et al., 2005; Leon et al., 2001). However, the modes of action of these mechanisms, and their involvement in local as opposed to systemic wound responses, remain unknown (Delessert et al., 2004; Matsuda et al., 2009).

Three JA signalling main components including coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) and Jasmonate insensitive 1/MYC2 (JIN1/MYC2) are very important factors in JA signal pathway. COI1 encodes an F-box protein involved in the SCF-mediated protein degradation by the 26S proteasome and is required for most JA-mediated responses (Xie et al., 1998). JAR1 encodes a JA amino acid synthetase involved in the conjugation of isoleucine to JA (JA-Ile) which is considered to be the bioactive JA molecule perceived by plants (Staswick and Tiryaki, 2004; Thines et al., 2007). JIN1/MYC2 transcription factor regulates some JA responsive gene expression (Lorenzo et al., 2004). Jasmonate ZIM-domain (JAZ) proteins are repressors of JA signalling, JAZ1 and JAZ3 were shown to interact with JIN1/MYC2 to inhibit the expression of JA-responsive genes. COI1 or COI1-JAZ complex acts as a receptor for JAIle in *Arabidopsis* (Katsir et al., 2008). JA (more specifically JA-Ile) promotes interaction between JAZ proteins and the SCFCOI1 ubiquitin ligase, leading to the

ubiquitination and subsequent degradation of JAZ proteins by the 26S proteasome. The degradation of JAZ proteins allows transcription factors (such as MYC2) to activate the expression of JA-responsive genes (Chini et al., 2007; Thines et al., 2007). JAZ genes are induced by JA. In addition, it was highlighted that the existence of two branches in the JA signalling pathway, antagonistically regulated by AtMYC2 and ERF1, that are coincident with the alternative responses activated by JA and ET to two different sets of stresses, namely pathogen attack and wounding(Lorenzo et al., 2004). JA signalling could be a mobile signal to transmit long-distance information leading to systemic immunity in *Arabidopsis* (Truman et al., 2007; Bari and Jones, 2009); the volatile nature of MeJA led to the discovery of its role as a signal in plant cellular responses, plant–herbivore interactions and plant–plant interactions (Cheong J J and Choi YD, 2003).

Among transcription factors acting downstream of JA in stress responses are the ethylene response factor 1 (ERF1), the bHLHzip-type transcription factor ATMYC2 (Lorenzo et al., 2004), WRKY70 and the newly found family of ORAs. ORAs are the Arabidospis homologs of ORCAs initially identified in Catharanthus roseus cell suspension cultures (Memelink et al., 2001). Among them, ORA47 is a COI1-dependent positive regulator of JA biosynthesis, whereas ORA59, ERF1, ORA37, MYC2 and WRKY70 act positively or negatively on different groups of defence genes (Wasternack, 2007).

c. Crosstalk between ethylene and jasmonic acid signalling pathways

Interaction of JA and ET signal pathways enable plants to optimize their defence strategies more efficiently and economically (Baldwin, 1998). Generally, the interaction between JA and ET signalling pathways happens on three basic levels (Figure 8): (i) regulation of key hormone biosynthesis genes; (ii) JA and ET crosstalk through the common components of the amplified signal transduction pathways, such as *ERF1* (Lorenzo et al., 2003), *ORA59* (Pre et al., 2008), *GhERF-IXa1*, *GhERF-IXa2* and *GhERF-IXa5* (Champion et al., 2009) which were highly induced synergistically by JA in combination with ET; (iii) regulation of common target genes (Benavente and Alonso, 2006) *as PDF1.2, THI2.1, HEL* and *CHIB* etc (Reymond and Farmer, 1998; Rojo et al., 1998). *H. brasiliensis* could be an interesting system to study the interaction between wounding, JA and ET, since they may act together to induce changes in phloem tissues, such as controlling the differentiation of latex cells and their metabolism (Duan et al., 2010).

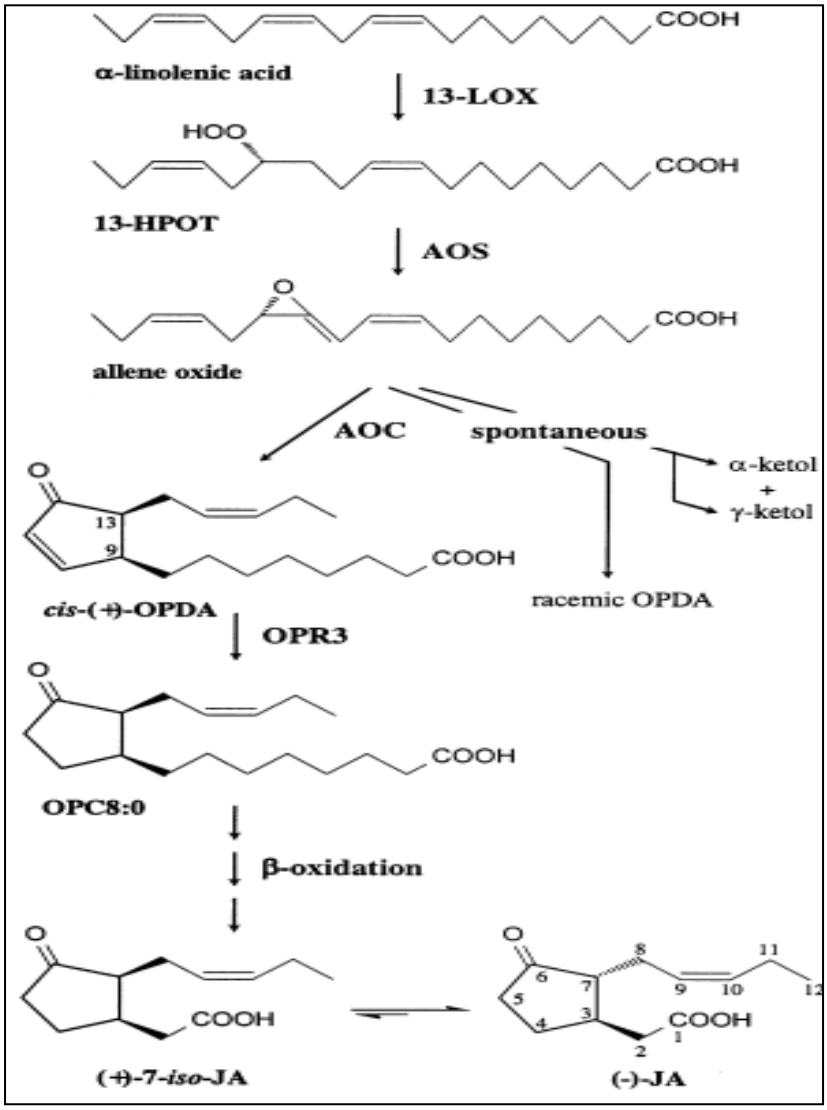

Figure 7. the pathway for JA biosynthesis. Pathway intermediates are abbreviated as 13-HPOT for (9Z11E15Z13S)-13-hydroperoxy-9,11,15- octadecatrienoic acid (that is, 13(S)-hydroperoxy linolenic acid), allene oxide for (12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid, cis-(+)-OPDA for cis-(+)-12-oxophytodienoic acid, and OPC8:0 for 3-oxo-2(2_(Z)-pentenyl)-cyclopentane-1-octanoic acid. The enzymes areindicated as 13-LOX for 13-lipoxygenase, AOS for alleneoxide synthase, AOC for allene oxide cyclase, and OPR3for 12-oxophytodienoate reductase 3 (Figure adapted from Wasternack and Hause 2002； Schaller F，2005)

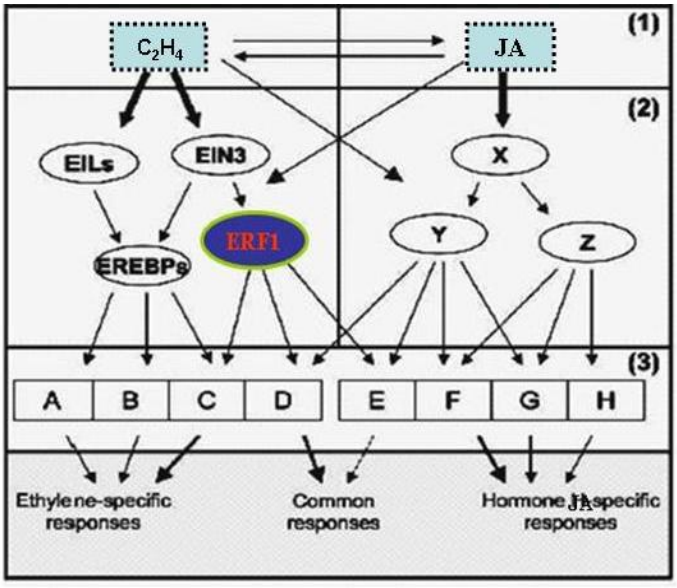

Figure 8. Simplified diagram of hormonal crosstalk in plants based on the current knowledge of the interactions between ethylene and other plant hormones in *Arabidopsis*. Three basic levels of interactions are presently known. (1) Hormones may influence the synthesis of each other through the mutual regulation of expression or activity of key biosynthetic genes, as in the case of ethylene and auxin. (2) Crosstalk may be achieved through sharing of common components of the signal transduction machineries, as exemplified by ERF1 in the ethylene–jasmonate crosstalk. (3) Signals may converge on the regulation of expression of common target genes, an interaction mode exhibited by both ethylene–auxin and ethylene– jasmonate crosstalks (Lorenzo et al., 2003).

Jasmonate (JA) and ethylene (ET) are two major plant hormones that synergistically regulate plant development and responses against biotic and abiotic stresses. Both JA and ET induce the expression of defence-related genes. In certain defence responses, JA and ET signalling pathways synergize to activate a specific set of defence genes. However, the molecular basis of JA/ET co-action and signalling interdependency is largely unknown. Concomitant activation of jasmonate and ethylene response pathways is required to activate the induction of some defence-related proteins like the antimicrobial plant defensin (PDF1.2), hevein-like protein (HEL), the basic chitinase PR-3, thionin2.1 (THI2.1) , chitinase b (CHIB), osmotin and PR1b, etc. (Xu et al., 1994; Penninckx et al., 1996; Penninckx et al., 1998; Norman-Setterblad et al., 2000; Lorenzo et al., 2003; Rojo et al., 1998; Champion et al., 2009). And these genes are commonly used to monitor JA-dependent defence responses (Reymond and Farmer, 1998). Evidence that JA and ET are co-ordinately regulated many other defence-related genes was also obtained in an *A. thaliana* microarray experiment that monitored gene expression in response to various defence-related stimuli (Schenk et al., 2000; Kunkel and Brooks, 2002). Many members of AP2/ERF transcription factors superfamily were shown to be involved in response to jasmonate and ethylene signals in different species. Little evidence suggested antagonistic interactions between the JA and ET defence pathways though ET suppresses JA induction of gene expression in nicotine biosynthesis (Shoji et al., 2000).

The key factors of the interaction between ethylene and jasmonate pathways in *Arabidopsis* have been reported. ETHYLENE RESPONSE FACTOR1 (ERF1) was shown as a key factor integrating the crosstalk of ethylene and jasmonate pathways (Lorenzo et al., 2003). The AP2/ERF domain transcription factor ORA59 was also revealed to integrate jasmonic acid and ethylene signals in plant defence (Pre et al., 2008; Memelink et al., 2001; Memelink, 2009; Zarei et al., 2011). Both ORA59 and ERF1 are able to function as activators of the PDF1.2 promoter (Pre et al., 2008). The research on the importance of AP2/ERF domain transcription factor family has been observed in different species.

7. Objectives of this PhD thesis

In *Hevea brasiliensis*, wounding, jasmonate and ethylene are important signals generated by tapping and ethephon stimulation. These signals are coordinating responses controlling latex production, either in favourable manner by stimulating the latex regeneration between two tappings and the latex flow, or in an unfavourable manner by triggering the Tapping Panel Dryness. Recently, exogenous jasmonic acid was shown to play an important role as a signalling molecule that involved in regulation of rubber biosynthesis (Zeng et al., 2009; Duan et al., 2010). Exogenous jasmonate and mechanical wounding can also significantly induce laticifer differentiation (Hao and Wu, 2000; Wu et al., 2002).

Little is known about interactions between ethylene and jasmonate in *Hevea brasiliensis*. Several questions intrigue our interest such as:

- Which role these interactions play on rubber production?
- What kind of effect do they have on defence responsive genes?
- Which genes are key factors involved in the interaction?

In this thesis, the objective is to study the mechanism of interaction between jasmonate and ethylene signal pathways in order to discover key genes regulating the expression of stress-responsive genes in *H.brasiliensis* (see figure 9).

The strategy developed in this PhD can be classified at three levels (Figure 10). First, after setting up a methyl-jasmonate treatment to *Hevea* plants, the general gene expression profile after ET and JA treatments was characterized for genes involved in the signal pathway in order to select ethylene or jasmonate-specific inducible genes to serve as control in further experiments. Synergistic induction of these genes by both ethylene and jasmonate led to set up protocol for plant hormone application, and to study the interactive effects of exogenous factors (Chapter 1). Second, this study identified the whole AP2/ERF superfamily by the transcriptomics analysis to allow us to analyse the function of the family genes (Chapter 2). Third, the research was focused on genes from the ERF family group IX, which are known to have important members integrating the ethylene and jasmonate signalling pathways (Chapter 3). The genes dramatically induced by treatments combining ethylene and jasmonate will serve as the candidate genes for the function analysis.

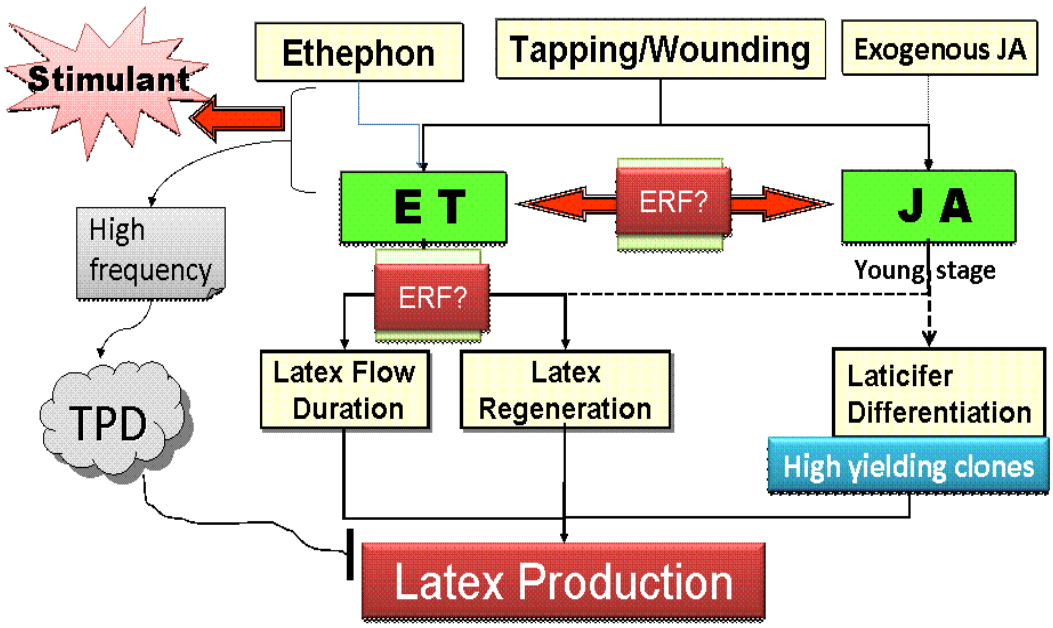

Figure 9. Objective of this thesis: study the mechanism of interaction between jasmonate and ethylene signalling pathways to discover key genes regulating the expression of stress-responsive in *H.brasiliensis*

This manuscript is organized as follows:

The chapter 1 is an article entitled "Gene expression pattern in response to wounding, methyl jasmonate and ethylene in bark of *Hevea brasiliensis*" published in Tree Physiology, 2010, 30, 10, 1349-59. The protocols for ethylene and wounding treatments were set up by Kuswanhadi (Kuswanhadi, 2006). This paper described the method set up for methyl-jasmonate treatment. This study allowed identifying some genes specifically induced by one of these studied factors of wounding, ethylene and methyl jasmonate in order to use them as control for further experiments.

The chapter 2 is a paper entitled "Transcriptomic analysis of the *Hevea brasiliensis* AP2/ERF superfamily in latex" submitted for publication to BMC Genomics on 5 October 2011. Based on a 454 transcript sequence database, AP2 domain-containing transcripts were identified and classified into families and groups, in order to identify putative key regulators belonging to the ERF family group IX.

The chapter 3 is a project of publication entitled "*Hevea brasiliensis* ERF-IXc5 functions like Ethylene Response Factor 1 (ERF1) in *Arabidopsis thaliana*". Among transcripts of the ERF family group IX, a few candidates as HbERF-IXc2, HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6 are supposed to be orthologous to the ERF1 and ORA59 in *Arabidopsis thaliana*.
Finally, a **General Discussion** was developed in order to integrate all information acquired during this thesis.

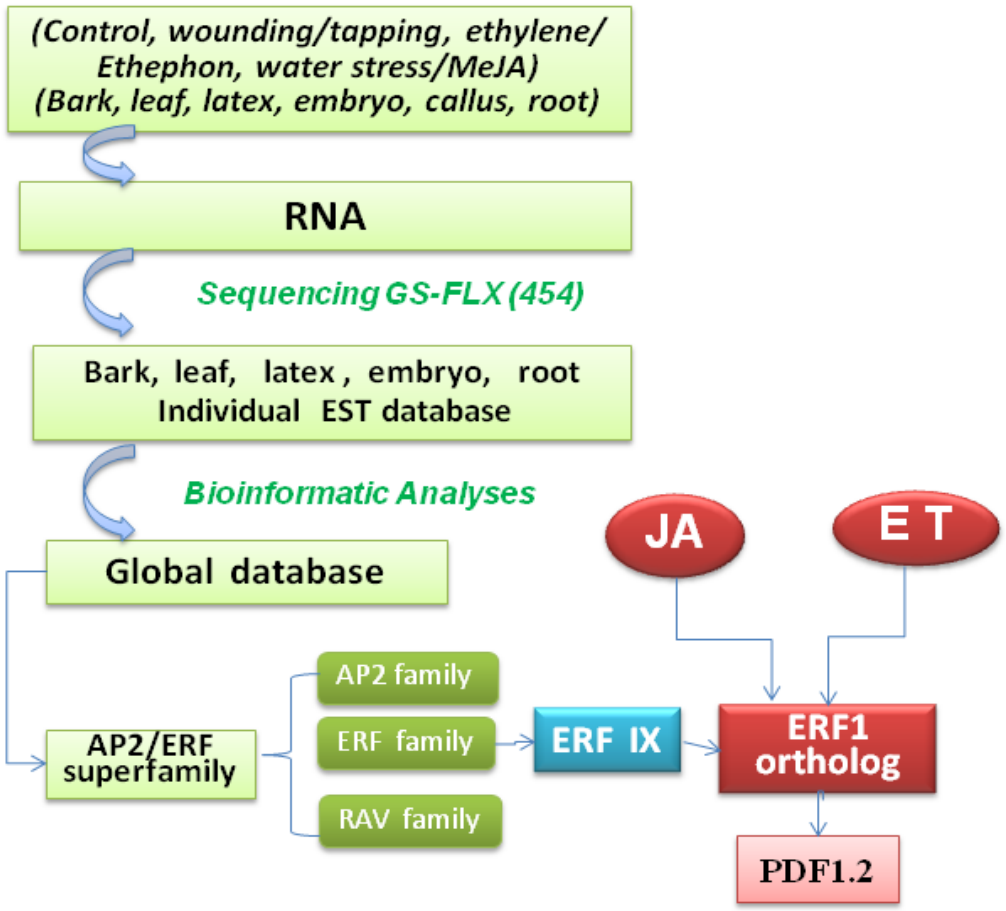

Figure 10. The strategy of this research on the interaction of ET and JA signalling pathway in *H. brasiliensis*

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CHAPITRE 1

Article intitulé "Gene expression pattern in response to wounding, methyl jasmonate and ethylene in bark of *Hevea brasiliensis*"

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Gene expression pattern in response to wounding, methyl jasmonate and ethylene in bark of *Hevea brasiliensis*

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Summary Natural rubber production in *Hevea brasiliensis* is determined by both tapping and ethephon frequencies. It is affected by a complex physiological disorder called Tapping Panel Dryness. This syndrome is likely to be induced by environmental and latex harvesting stresses. Defence responses, including rubber biosynthesis, are dramatically mediated by wounding, jasmonate and ethylene among other factors. Using real-time RT-PCR, the effects of wounding, methyl jasmonate and ethylene on the relative transcript abundance of a set of twenty-five genes involved in jasmonate and ethylene signalling and metabolic pathways were studied in bark of three-month-old epicormic shoots. Temporal regulation was observed for nine out of twenty-five genes. Wounding treatment regulated the transcript abundance of ten genes. Wounding-specific regulation was noted for the *HbMAPK, HbBTF3b, HbCAS1, HbLTPP* and *HbPLD* genes. Methyl jasmonate treatment regulated the transcript abundance of nine genes. Among them, the *HbMYB, HbCAS2, HbCIPK* and *HbChi* genes were shown to be specifically methyl-jasmonate-inducible. Ethylene response was accompanied by regulation of the transcript abundance of eight genes, and six genes *HbETR2, HbEIN2, HbEIN3, HbCaM, HbPIP1 and HbQM* were specifically regulated by ethylene treatment. Additionally, the transcript level of genes *HbGP* and *HbACR* was enhanced by all three treatments simultaneously. Overall, a large number of genes was found to be regulated 4h after application of treatments. This study nevertheless revealed JA-independent wound signalling pathways in *Hevea brasiliensis*, and provided a general characterization of signalling pathways, and will serve as a new base from which to launch advanced studies of the network of pathways operating in *H. brasiliensis.*

Keywords: wounding ethephon, jasmonic acid, latex, laticifer, rubber tree, temporal regulation

Introduction

Hevea brasiliensis (Willd. *ex* A. Juss.) Müll. Arg. is a perennial tropical species from the Amazon basin, and a member of the family Euphorbiaceae. This sole commercial source of natural rubber is widely cultivated in Southeast Asia, supplying world consumption. The economic importance of natural rubber and its ever growing consumption have prompted numerous investigations into the related biochemical and biological processes (Kush et al. 1990; Norton et al. 2007; Priyadarshan et al. 2006**;** Sando et al. 2009). Natural rubber-containing latex is harvested by tapping the bark at frequencies ranging from 2 to 5 days. Natural rubber biosynthesis takes place in latex cells and is a typical secondary metabolism with isopentenyl pyrophosphate (IPP) as the precursor of rubber molecules (Kush 1994). In the phoem, latex vessels form a reticulated network of anastomosed cells dedicated to the synthesis of natural rubber. Upon tapping, which consists in an excision of a thin layer of trunk bark about 1 mm thick, the latex vessels are opened and latex flows out (d'Auzac 1989; Kush et al. 1990; Montoro et al. 2003; Priyadarshan et al. 2006**;** Sando et al. 2009). The number of latex vessels and the metabolic activity of latex cells are therefore the most important factors influencing rubber yield in *H. brasiliensis* (Gomez 1982).

For some *H. brasiliensis* clones, ethephon (chloro-2-ethylphosphonic acid) stimulation is required to ease latex flow and activate the metabolism for latex regeneration between tappings (Audley et al. 1976; Tungngoen et al. 2009). This stimulation is associated with marked changes in the physiology and metabolism of latex cells, which induces a large but transient increase in latex production (Gidrol et al. 1988).

Mechanical damage to bark tissues caused by excessive tapping, and over-stimulation with ethephon, can cause oxidative stress inside latex cells, leading to the Tapping Panel Dryness (TPD) syndrome in *H. brasiliensis*. In that case, latex flow is stopped, with *in situ* coagulation of rubber particles, or deep degeneration of tissues related to cell death mechanisms (Chrestin 1989; Krishnakumar et al. 2006; Sookmark et al. 2006; Venkatachalam et al. 2007; Venkatachalam et al. 2009; Zeng et al. 2009). A by-product of the ethylene biosynthetic pathway is cyanide (HCN), which is a cytotoxic component inhibiting the respiration system. HCN is detoxified by beta-cyanoalanine synthase (□CAS), thereby recycling the reduced nitrogen of cyanide for amino acid synthesis (Fujita et al. 2006; Abe et al. 2008; Asumaniemi et al. 2009). In *H. brasiliensis*, it was reported that a lethal imbalance between cyanogenic and CN-detoxifying activities (CAS) in the phloem of necrotic trees, could lead to the poisoning of neighbouring cells and to the spread of tissue necrosis towards the tapping cut (Chrestin et al. 2004; Nandris et al. 2004). Ethylene, jasmonic acid (JA) and wounding are important factors involved in coordinating the responses to tapping and stimulation in *H. brasiliensis*, such as latex cell metabolism, laticifer differentiation, rubber biosynthesis, and cell senescence. Exogenous jasmonate and mechanical wounding can significantly induce laticifer differentiation (Hao and Wu 2000). Recently, jasmonate was shown to play an important role as a signalling molecule that regulates rubber biosynthesis (Zeng et al. 2009).

Genes involved in signalling pathways play an important role in stress regulation. Ethylene perception and signalling is well described in Arabidopis, from receptors to transcription factors, but the complexity of ethylene response regulation has emerged (Kendrick and Chang 2008). Ethylene receptors are negative regulators of the ethylene response pathway (Guo and Ecker 2004). In contrast, EIN2 and EIN3 are positive regulators of the ethylene signalling pathway (Angelini et al. 2008). When EIN2 is activated, it triggers a transcriptional cascade involving EIN3/EIL and ERF transcription factors. EIN3 protein accumulates in the nucleus and initiates a transcriptional cascade, resulting in the activation and repression of

hundreds of genes in *A. thaliana* (Stepanova and Alonso 2005). EIN2 has been recognized as a molecular link between distinct hormone response pathways of ethylene and jasmonic acid in *A. Thaliana* (Leon et al. 2001). However, the intrinsic nature, the mechanism and the role of signal interaction in plants has yet to be identified. Jasmonates are major signals synthesized in plants via the octadecanoid pathway (Seo et al. 2001). A critical component of the jasmonic acid receptor complex is Coronatine Insensitive 1 (COI1), an F-box protein (Alabadi et al. 2002; Katsir et al. 2008). Jasmonate ZIM-domain (JAZ) repressors are targeted by the SCF(COI1) complex for proteasome degradation in response to jasmonate (Chini et al. 2009; Chini et al. 2007). Degradation of JAZ repressors leads to the release of MYC2, a bHLH transcriptional activator that binds the G-box (CACGTG) or the T/Gbox (AACGTG) in promoters, allowing the activation of JA responses (Dombrecht et al. 2007). In plants, wounding is common damage resulting from biotic and abiotic stresses (Cheong et al. 2002). To effectively cope with wounding, plants have evolved the capacity to activate defence mechanisms that prevent further damage, which largely depend on the transcriptional activation of specific genes (Leon et al. 2001). Many signals are involved in the complex wounding signalling network, including jasmonates (Farmer and Ryan 1990) and ethylene (O'Donnell et al. 1996). The production of jasmonic acid and ethylene and their consequent signalling pathways mediate various defence responses in plants, either independently or collaboratively, to initiate an induced systematic resistance (Dong 1998).

Crosstalk between jasmonic acid and ethylene pathways may enable plants to optimize their defence strategies more efficiently and economically (Baldwin 1998). Generally, the interaction between jasmonic acid and ethylene signalling pathways occurs on three basic levels: (i) regulation of key hormone biosynthesis genes, (ii) jasmonic acid and ethylene crosstalk through the common components of the amplified signal transduction amplified, such as ERF1 (Lorenzo et al. 2003); it has been demonstrated that ORA59 is also an essential integrator of the JA and ethylene signal transduction pathways (Pre et al. 2008; Memelink 2009); several ERF genes of group IXa, such as GhERF-IXa1, GhERF-IXa2 and GhERF-IXa5, were highly induced synergistically by JA in combination with ET in cotton (Champion et al. 2009) (iii) regulation of common target gene signals that converge towards the interaction between jasmonic acid and ethylene (Benavente and Alonso 2006).

H. brasiliensis is an interesting system in which to study the interaction between wounding, jasmonic acid and ethylene, since they may act together to induce changes in phloem tissues including the control of latex cell differentiation and their metabolism. In order to define conditions for a transcriptomic analysis of these interactions, this study aimed to identify the influences of timing and genes specifically regulated by wounding, methyl jasmonate and ethylene signals, to be used as an internal control for further transcriptomic analyses. We report here on the regulation of the relative transcript abundance of 25 genes involved in the jasmonic acid and ethylene signalling pathways and in cellular responses using real-time RT-PCR.

Materials and methods

Plant material and treatments

Budded plants of clone PB 260 were grown at 28°C in a greenhouse under natural light. Three-month-old epicormic shoots from budded plants and leaves were treated at the same time. The leaves were mechanically wounded by squeezing the entire surface of the leaves with pincers whilst the bark was wounded every 0.5 cm by scarification with a razor blade. Timings after treatment were selected according to various preliminary experiments in kinetics. Tissues were

wounded at 8.00 am and collected 15 min and 4 hours after treatment. For the ethylene and methyl jasmonate gas treatment (MeJA), plants were placed in a 300-litre open-door Plexiglas box overnight before the treatment. One ppm of pure ethylene gas (0.3 mL/300 L) was injected into the sealed air-tight box. The concentration was controlled by gas chromatography (Type HP 5280 with FID detector). Tissues were collected 4 and 24 hours after treatment. For the methyl jasmonate treatment, 20 µL of liquid ≥ 95% methyl jasmonate solution (Sigma, St. Louis, US) was diluted in 500 µL of absolute ethanol, and then placed on Whatmann paper inside the box for gas release. Tissues were collected 1 h and 4 h after treatment. An inhibitor of ethylene action, 1-methyl cyclopropane (1-MCP), was used to demonstrate the specific effect of ethylene. Plants were pre-treated for 16 hours with 1 ppm 1-MCP prepared with 480 mg of a 1-MCP-releasing powder (0.14% SmartFresh^{TM}, PA, USA) dissolved in 7.2 mL of water. After ventilation, plants were then treated with 1 ppm ethylene for 4 h or 24 h. Control plants used for the ethylene, 1-MCP/ethylene, and methyl jasmonate treatments were placed in the box and exposed to air only. In order to avoid variation due to the daytime and biological development, each treatment was compared to a specific control sampled at the same time and with the same culture conditions in three biological replications. After treatment, tissues were collected and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA extraction

Leaf and bark samples were collected from three individual plants for each control or treatment. One gram of tissue was taken and ground in liquid nitrogen. Total RNA was extracted using the cesium chloride cushion method adapted from (Sambrook et al. 1989). Briefly, the powder was transferred to a tube containing 30 mL of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% PVP, and 1% ß-mercapto-ethanol. After mixing, tubes were kept on ice and then centrifuged at 10,000 rpm at 4°C for 30 minutes. The supernatant was transferred to a new tube containing 8 mL of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 25,000 rpm at 20°C for 20 hours. The supernatant and cesium cushion were discarded whilst the RNA pellet was washed with 70% ethanol. After 30 minutes of air drying, the pellet was dissolved in 200 µL of sterile water. RNAs were conserved at -80°C.

Choice of a set of representative genes

A representative set of 25 genes was selected for further gene expression analysis (Table 1). These genes were identified in *H. brasiliensis* EST and cDNA sequence databases (Chow et al. 2007; Duan et al. 2006; Duan et al. 2007; Han et al. 2000; Montoro et al. ; Zeng et al. 2003). They are respectively involved in the jasmonate and ethylene signalling pathways and defence responses: five genes (*HbETR1*, *HbETR2*, *HbEIN2*, *HbMAPK* and *HbCOI1*) related to ethylene perception and transduction of jasmonate and ethylene signals, five other genes encoding transcription factors (*HbEIN3*, *HbSAUR*, *HbWRKY*, *HbMYB* and *HbBTF3b*), twelve genes encoding functional proteins (*HbPIP1*, *HbGS*, *HbUbi*, *HbLTPP*, *HbACR*, *HbACBP*, *HbCaM*, *HbCIPK*, *HbGP*, *HbCAS1*, *HbCAS2*, *HbPLD*) and three genes related to plant defence and programmed cell death (*HbChi*, *HbDef* and *HbQM*).

Gene expression analysis by real-time RT-PCR

Primers were designed for the 25 genes selected from the EST sequences with Vector NTI software (Invitrogen, USA) in order to provide a PCR product of about 200-bp for further gene expression analyses by real-time PCR (Table 2). cDNAs were synthesized from 2 µg of total

RNA to the final 20 µL reaction mixture using a RevertAid^{TM} M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Quantitative gene expression analysis was finally carried out by real-time RT-PCR using the Light Cycler 480 (Roche, Basel, Switzerland). Real-time PCR reaction mixtures consisted of 3µL of RT product cDNA, 1.5µL of 5µM of each primer, and 7.5µL 2×SYBR green PCR master mix (LightCycler® 480 SYBR Green I Master, Roche Applied Sciences) in a 15 µL volume. PCR cycling conditions comprised one denaturation cycle at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 60°C for 15s, and 72°C for 20s). Firstly, melting curves were analysed to check the specificity of PCR amplification. The PCR products were then cloned and sequenced to ensure that the correct gene was amplified. Then, the cloned PCR products were used as calibrators in real-time RT-PCR analysis. The concentration of cDNA to be used was determined by testing various cDNA dilutions (1/5, 1/10, 1/25, 1/50, 1/100). The optimum cDNA dilution was chosen with a crossing point (Cp) between 18-22 cycles for all samples. The standard curve was generated using a fivefold dilution series of 10 points in triplicate from a mixed cDNA sample. This standard curve allowed the calculation of primer efficiencies. Expression analysis was performed in triplicate in a 384-well plate. Samples were loaded using an automation workstation (Biomek NX MC, Beckman Coulter). The homogeneity of *HbActin* gene Cp enabled use of this gene as an internal reference gene. The *HbActin* gene was amplified in parallel with the target gene. The level of transcript abundance of each gene was relatively quantified by normalization with the transcript abundance of the stable reference *HbActin* gene. Relative transcript abundance was calculated on 3 biological replicates taking into account primer efficiencies. Data from different PCR plates could be compared through normalization using calibrators All the data were calculated automatically by Light Cycler Software version 1.5.0 provided by the manufacturer.

Statistical analyses of the data

For each treatment, reactions were set up with three biological replications. For the analysis of temporal variation in control plants, relative transcript abundance was expressed as the mean of three replicates. ANOVA followed by a Student Newman-Keuls test was used in the statistical analyses after logarithmic transformation of raw data. For the treatments, changes in relative transcript abundance were expressed as the ratio between treatments and its specific control collected at the same time and grown under the same conditions (greenhouse or in box). It was calculated with the mean value of the triplicate. The statistical analysis was carried out from the logarithm of raw data using the two-tailed probability values of the *t* test. The ratio with a *p*-value ≤ 0.05 was retained as significant for down or up-regulation.

Results

HbActin is a suitable internal control for gene expression analysis

Actin transcript abundance is not prone to change in response to treatments. Primers were designed for *HbActin* and *HbUbi* genes in *H. brasiliensis* (Table 2). The stability of the transcript abundance of these 2 housekeeping genes was assessed by real-time RT-PCR in bark tissues from plants subjected to various treatments at different timing. Melting curves revealed the high specificity of the primers (Figure 1). The crossing point value for *HbActin* was more stable than *HbUbi* in response to the 19 conditions. This stability enabled *HbActin* to be used as a reference gene (Figure 2).

Temporal regulation of the expression of 25 genes

The temporal expression of a set of 25 selected genes putatively involved in the jasmonate and ethylene signalling pathways and their molecular responses was monitored during the daytime at 8:00 am, 12:00 pm and 4:00 pm (Table 3). The transcripts of the *HbMYB*, *HbGP* and *HbChi* genes were accumulated at a very low level (up to $6.79x10^{-7}$) compared to *HbWRKY, HbACBP, HbLTPP, HbUbi, HbPIP1, HbDef* and *HbQM* transcripts which accumulated at a very high level (up to $8.15\ x10^{+1}$). The accumulation of *HbSAUR* and *HbEIN2* transcripts gradually increased from 8:00 am to 4:00 pm whilst the quantity of the *HbDef* transcripts decreased. Interestingly, transcripts of 6 genes (*HbMYB, HbBTF3b, HbACBP, HbLTPP, HbPIP1*) were first significantly reduced at 12:00 pm only to increase again at 4:00 pm. This dramatic regulation of the relative transcript abundance of 9 out of 25 genes highlights the need to use a specific control sampled at the same time for each treatment in further experiments.

Regulation of the expression of 25 genes by wounding, MeJA and ethylene treatments

The collection of 25 selected genes showed differential gene expression in bark in response to wounding, methyl jasmonate and ethylene treatments (Table 4). The relative transcript abundance was quantified by referring to the stable reference gene *HbActin*. The regulation of transcript level was exhibited with the ratio of mean values of three individual relative quantifications of treated and control samples specifically collected at the same time to avoid any daytime variation. In this analysis, the variation in relative transcript abundance between the treatment and the control was considered significant when the p-value was below 0.05. An up-regulation was estimated when the ratio was higher than 1, whilst a down-regulation was noted for ratios below than 1.

Mechanical wounding triggered early (15 min) or late (4 h) responses in gene expression for 10 out of 25 genes. Ratios below 1 were significant for 6 genes only 15 min after wounding: *HbMAPK, HbBTF3b, HbCAS1, HbLTPP, HbPLD* and *HbDef*. Low ratios were maintained 4 hours after wounding for *HbMAPK, HbLTPP* and *HbDef*, but it was significant only for *HbPLD* and *HbCOI1*, which was late down-regulated. Three genes (*HbSAUR, HbACR, HbGP*) were up-regulated only 4 h after treatment presenting a ratio between 1.7 and 5.6 times. Among non-significantly wounding-induced genes (*HbETR1, HbETR2, HbEIN3, HbMYB, HbCAS2, HbChi, HbQM*), the early dramatic induction of the *HbChi* gene (212 times) by wounding remained noteworthy compared to the other treatments.

Application of exogenous MeJA gas significantly impacted on the relative transcript abundance of 9 out of the 25 genes studied 1 h or 4 h after treatment. There were 5 genes up-regulated by MeJA treatment (*HbSAUR, HbACR, HbCAS2, HbCIPK, HbGP*) and 4 genes down-regulated (*HbCOI1, HbMYB, HbChi, HbDef*). The *HbCAS2* and *HbGP* genes were induced as early as 1 h after MeJA treatment, and the maximum accumulation of *HbCAS2* transcripts was observed 4 h after treatment. The relative transcript abundance of three other genes (*HbSAUR, HbACR, HbCIPK*) was stimulated by MeJA 4 h after treatment. The *HbMYB* and *HbChi* genes were down-regulated as early as 1 h after treatment. We could therefore consider that *HbChi* was totally inhibited 4 h after treatment despite the high p-value (0.22).

The application of exogenous ethylene gas significantly influenced the relative transcript abundance of 8 genes. Four hours after ethylene application, the expression of 5 genes (*HbETR2, HbACR, HbPIP1, HbGP, HbQM*) was up-regulated from 1.8 to 4.3 times. After 24 h, the accumulation of transcripts of the *HbETR2* gene was continuously enhanced up to 8.1 times but with a higher p-value (0.08). In contrast, the expression of 3 genes (*HbEIN2, HbEIN3, HbCaM*) was totally inhibited.

Interestingly, 6 genes (*HbETR1, HbMAPK, HbWRKY, HbACBP, HbGS* and *HbUbi*) did not display any changes under the three treatments (wounding, MeJA and ET) in our data set condition and system.

Effect of a 1-MCP pre-treatment on the expression of ethylene-responsive genes

The effect of an inhibitor of ethylene action, 1-MCP, was tested on the relative transcript abundance of 5 ethylene-responsive genes induced 4 h after treatment (Table 5). Although p-values were higher than 0.05, pre-treatment with 1-MCP prevented the induction by ethylene of 4 genes in bark (*HbETR2, HbPIP1, HbGP, HbQM*). In contrast, the accumulation of the transcript of gene *HbACR* was not affected by 1-MCP pre-treatment. In this case, indirect ethylene action was assumed to affect the regulation of this gene.

Discussion

The tapping and ethephon stimulation of rubber trees trigger molecular mechanisms in phloem tissues that involve wounding, jasmonate and ethylene signalling pathways. Interaction between these components is presumed to control major parameters of latex production, such as the differentiation of latex cells, rubber biosynthesis and Tapping Panel Dryness (Chrestin 1989; Krishnakumar et al. 2006; Sookmark et al. 2006; Venkatachalam et al. 2007; Venkatachalam et al. 2009; Zheng et al. 2009). In order to decipher these mechanisms, experimental conditions have to be defined that are suitable for identifying key genes. To that end, we studied a simple plant model, developed a robust high-throughput real-time RT-PCR and identified specifically-regulated genes among a set of target genes selected for their involvement in the studied pathways. We reported here on the regulation of the relative transcript abundance of 25 genes involved in the jasmonic acid and ethylene signalling pathways and in cellular responses.

Given the complexity of the interaction between tapping, ethephon stimulation and environmental stresses in rubber trees, the identification of wounding, methyl jasmonate and ethylene-responsive genes was initiated in a simple model represented by the bark of 3-month-old epicormic stems cultivated under controlled conditions in the greenhouse. This plant material can be regularly generated from budded clones and our results showed that variability was sufficiently low to observe significant variation in relative transcript abundance between treated and control plants.

The *HbActin* gene proves to be the most stable internal control for relative quantification of transcript abundance under stress. A reference gene with stable expression in one organism may not be suitable for normalization of gene expression in another organism under a given set of conditions and needs to be validated before its use (Jain et al. 2006). Indeed, housekeeping genes may exhibit differential expression patterns and should be carefully chosen for relative quantification (Yan and Liou 2006). *HbUbi* proved to be sensitive to treatments. In addition, although some other genes (*HbETR1, HbWRKY, HbACBP, HbGS* and *HbUbi*) did not display any significant changes under the three treatments (wounding, MeJA and ET), the variation in their ratio did not allow the use of these genes as an internal control to study the effect of such treatments. In contrast, the *HbActin* gene revealed stable transcript abundance in response to treatments. It was therefore chosen as the internal control for relative quantification of gene expression.

A robust real-time RT-PCR gene expression method using a 384-well plate with an automation workstation was developed for studying a large set of target genes. Performed with the Light Cycler 480 (Roche, Basel, Switzerland), the expression analysis of 25 genes for plants treated by wounding, MeJA and ethylene was implemented at 2 timings in triplicate. Of the almost one thousand PCR reactions carried out, 9 out of 25 and 30 out of 150 conditions (genes x treatments) provided significant changes in relative transcript abundance with a p-value lower than 0.05 when only 1.25 (25 x 0.05) and 7.5 (150 x 0.05) would be expected by random chance for experiments described in tables 3 and 4 respectively. Our results showed that the variability between biological replicates is sufficiently low to observe significant variation in relative transcript abundance between treated and control plants.

The variability in relative transcript abundances during the day required the application of a specific plant control growing under the same conditions and collected at the same time. Temporal regulation of gene expression has been well documented for a long time in plants (Bowman et al. 1988; Bustos et al. 1991; Granell et al. 1992; Jain et al. 2007; Zhao et al. 1994; Zhu-Shimoni et al. 1997). To our knowledge, we showed for the first time that a large proportion of genes, 9 of the 25 studied, is regulated during the day in *Hevea brasiliensis*. Interestingly, this regulation affects genes expressed in bark tissues, which appear photosynthetic since chlorophyll cells were observed.

Four hours after treatment was the best time to observe variation in the expression of most of the genes. Seventeen genes were regulated 4 h after treatment (5 genes for wounding, 7 genes for MeJA, and 5 genes for ethylene) compared to other times (6 genes for wounding after 15 min, 4 genes for MeJA after 1 h, 3 genes for ET after 24 h). This confirmed preliminary studies conducted in kinetics for the ACC oxidase multigene family involved in ethylene biosynthesis (Kuswanhadi et al. in press).

A representative distribution of genes regulated by wounding, MeJA and ethylene was carried out (Figure 3). Fifteen of the 25 studied genes revealed significant regulation by wounding, MeJA or ethylene. Of them, 5 (*HbMAPK, HbBTF3b, HbCAS1, HbLTPP, HbPLD),* 4 (*HbCAS2, HbMYB, HbCIPK, HbChi*), and 6 (*HbETR2, HbEIN2, HbEIN3, HbCaM , HbPIP1, HbQM*) were specifically regulated by wounding, MeJA and ethylene respectively. Interestingly, only three genes (*HbCOI1, HbSAUR, HbDef*) could be induced by either wounding or methyl jasmonate. Two other genes (*HbGP, HbACR*) could be induced by any of the three treatments studied. However, the specific down-regulation of the *HbChi* gene by MeJA should be considered with caution because that gene was highly induced by wounding although the changes in relative transcript abundance were not significant. This study reveals that the response to wounding in the bark of *H. brasiliensis* is not strictly dependent on the jasmonate signalling pathway. Indeed, five genes were shown to be specifically down-regulated by wounding without any ethylene or MeJA effect. In the literature, wounding signals are known to be transmitted via at least two pathways, one being JA-independent whereas the other is JA-dependent (Konstantinov and Titarenko Ia 1997). We suggested here the existence of JA-independent wound signalling pathways in *H. brasiliensis*.

Our results are consistent with data from the literature. For instance, the *HbCOI1* gene isolated from bark tissue in this study was regulated by wounding and MeJA, but not by ethylene in rubber tree clone PB260. In clone RRIM 600, it was reported that *COI1* was present as a single copy and had high transcription in laticifers, and low in bark and leaves (Peng et al. 2009). Interestingly, its transcription was induced in latex by tapping and jasmonate but not by ethephon. This confirmed that *HbCOI1* expression is ethylene-independent in *H. brasiliensis,* and

has a differential response under the regulation of wounding and JA. It has also been reported that the expression of the *HbMYB1* transcription factor was significantly decreased in the bark of TPD trees and is likely to be associated with TPD. HbMyb1 may act as a negative regulator for programmed cell death induced genes; intense tapping and ethylene stimulation resulted in decreased expression of *HbMYB1* (Chen et al. 2003), and this was validated by Venkatachalam (Venkatachalam et al. 2007). The sequence of *HbMYB* selected in this analysis was similar to the *MYB* gene reported earlier (Chen et al. 2003; Venkatachalam et al. 2007). Our data showed that the *HbMYB* gene was MeJA-dependent, and down-regulated by MeJA stress in *H. brasiliensis*. This *HbMYB* gene is suspected of being possibly involved in JA-mediated stress signalling pathways, as demonstrated in *A. thaliana* (Jung et al. 2007, Yanhui et al. 2006). Five receptors were identified in *Arabidopsis* and are organized in two groups with histidine kinase activity (Klee 2004). Subfamily I is characterized by three amino terminal transmembrane domains and a histidine kinase activity, while subfamily II have four transmembrane domains and a serine/threonine kinase activity (except in the case of the *Arabidopsis* subfamily I receptor ERS1, which has both activities) (Klee 2004). More recently, the complexity of the ethylene signalling and regulation has emerged (Kendrick and Chang 2008). ETR1 and ETR2 can be differentially regulated by ethylene (Ziliotto et al. 2008). In *Hevea*, the induction of ETR2 gene by ethylene suggested an important role of the gene in the response to ethylene.

We reported for the first time in *Hevea brasiliensis* that several genes are regulated during the day and can take independent signalling pathways. With a view to discovering the network of genes regulated under tapping and ethephon stimulation, and functions related to latex production and the TPD syndrome, experimental conditions might be suggested based on our study. On the one hand, wounding, MeJA and ethylene treatments proved to be very efficient in triggering the regulation of a large number of genes in *Hevea*. On the other hand, a large number of genes were shown to be regulated 4 h after treatments, and consequently proposed for identifying new genes.

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Table 1. List and description of ESTs studied in the *H. brasiliensis* clone PB 260

	Hevea brasiliensis			Homology		
Function	Gene	Description	GenBank accession	GenBank accession (nucleic acid / protein)	Identity (%)	Species
Signal transduction	*HbETR1*	Ethylene receptor 1	HO004788	XM_002302696/XP_002302732	86	*Populus trichocarpa*
	HbETR2	Ethylene receptor 2	HO004786	XM_002315681/XP_002315717	78	*Populus trichocarpa*
	HbEIN2	Ethylene insensitive protein 2	EC603676	XM_002326149/XP_002326185	88	*Populus trichocarpa*
	HbMAPK	Mitogen-activated protein kinase	EC602045	DQ132852/ABA00652	80	*Gossypium hirsutum*
	HbSAUR	Auxin responsive SAUR protein	EC609830	XM_002321934/XP_002321970	88	*Populus trichocarpa*
Transcription factors	*HbEIN3*	Ethylene insensitive 3 or EIN 3	EC601444	XM_002530146/XP_002530192	86	*Ricinus communis*
	HbCOI1	Coronatine insensitive protein 1	EC606578	XM_002328835/XP_002328871	82	*Populus trichocarpa*
	HbWRKY	DNA-binding WRKY	EC603811	XM_002533823/XP_002533869	90	*Ricinus communis*
	HbMYB	Myb Transcription factor superfamily	EC608839	XM_002301314/XP_002301350	87	*Populus trichocarpa*
	HbBTF3b	BTF3b-like transcription factor	HO004787	XM_002523198/XP_002523244	98	*Ricinus communis*
Cellular metabolism	*HbACR*	Acyl CoA reductase	HbAY461413	AY461413/AAR88762	*100*	*Hevea. brasiliensis*
	HbACBP	Acyl-CoA-binding protein	HO004790	DQ452088/ABE72959	90	*Jatropha curcas*
	HbCaM	Calmodulin	HO004795	AY836672/AAX63770	81	*Populus tomentosa*
	HbCAS1	Latex beta-cyanoalanine synthase	HbAY207389	AY207389/AAP41852	100	*Hevea brasiliensis*
	HbCAS2	Phloem beta-cyanoalanine synthase	HbAY207388	AY207388/AAP41851	100	*Hevea brasiliensis*
	HbCIPK	CBL-interacting protein kinase	HO004789	DQ997698/ABJ91215	84	*Populus trichocarpa*
	HbGS	Glutamine synthetase	HbAF003197	AF003197/AAB61597	97	*Hevea brasiliensis*
	HbLTPP	Lipid transfer precursor protein	HbAY057860	AY057860/AAL25839	100	*Hevea brasiliensis*
	HbPLD	Phospholipase D	HO004793	RCU72693/AAB37305	80	*Ricinus communis*
	HbUbi	Ubiquitin	HbAY275680	AY275680/AAP31578	99	*Hevea brasiliensis*
	HbPIP1	Plasma membrane aquaporin	EC608562	GQ479823/ACV66985	100	*Hevea brasiliensis*
	HbGP	G protein beta subunit-like	HO004794	Z71750/ CAA96432	81	*Nicotiana plumbaginifolia*
Cellular & defence response	*HbChi*	Chitinase	HbAJ010397	AJ010397/CAA09110	100	*Hevea brasiliensis*
	HbDef	Defensin precursor	HO004791	EF421192/ABN46979	72	*Nelumbo nucifera*
	HbQM	QM family protein	HO004796	AY641733/AAT68777	83	*Camellia sinensis*
House keeping	*HbActin*	Actin	HO004792	AY114679/AAM47998	97	*Arabidopsis thaliana*

Table 2. List of primer sequences, primer efficiencies and expected length of amplicons after amplification by real-time PCR for selected cDNA in *H. brasiliensis* clone PB 260.

Genes	Primer sequences		Primer efficiency	PCR product (bp)
	Forward	Reverse		
HbActin	AGTGTGATGTGGATATCAGG	GGGATGCAAGGATAGATC	1.870	194
HbETR1	GGTAATGCTGTCAAGTTCAC	GGGCTGAGTGAATTTAGT	2.000	202
HbETR2	TGCCCTGACATCAAGTGCT	TCAGACCCCAAAACCGAAG	1.984	208
HbEIN2	TTGCCATATCTTGCCGAAAG	GCCCTATATTTACCCAGTTGAG	1.902	210
HbMAPK	GCCAAGTTGAGCGATAGT	ACCAACTTCACAATCCGC	1.912	194
HbSAUR	GGTGAGGAGCAACAGAGGTT	AGATGGTGATGGTGGTGGTG	1.975	200
HbEIN3	CAATGTCCTTACAGCCAAC	AGTTCACCATCGGGACAG	1.990	194
HbCOI1	ACATCAAGCAGAGACCGCAACG	TCATGCGGGACCAGGTTGAAGT	1.927	200
HbWRKY	TCAGTCAAAATTCAACCGATTC	GCAAATTCATCTGTACCTTCCC	1.885	200
HbMYB	TAGCAAGTCTTCTTCACTGC	TCCAACAAGATCACGAAT	2.000	183
HbBTF3b	TGTGTTTCCATCGGCTCGCTCT	TTCTTCTCATGCTGCCCTTCCC	1.820	207
HbACR	TGTGAAGCGACTAGTTGAAC	CCATGCCAGGAATATGTG	1.972	186
HbACBP	GTTTAACATGAGGGACAGAG	TTTGAACACAAGGAACCT	1.872	180
HbCaM	TGAGGAGGTTGATGAGATG	TGAAGATACCCTACAGCG	1.946	200
HbCAS1	CACGAGGCTGCTATCCAC	TCCCTGATTCTTTGAGCA	1.977	194
HbCAS2	CTCTTGTATGCAATCATTTGT	GCCAAACATTTCTTGAAC	1.990	181
HbCIPK	GACGGGTGAACTGATCCT	GTGACCATCTTTGCTCCT	1.988	188
HbGS	TTGCAGAAACCACAATCCTGTG	AAGGGCGAACTGGCGAAACA	1.957	195
HbLTPP	CCTTACAAGATCAGCCTG	AGGTAAGAAGAAGGAGCC	1.867	180
HbPLD	CCCAAAGGAACTTTCACT	TCAGAGAACCATAACAGG	1.939	197
HbUbi	CTGGAAAGACAATCACACTGG	TTCAGTAATCACCACCGC	1.819	214
HbPIP1	CTTGCTGCTGTGTACCAC	GCTTAATACAGCACCACTAG	1.785	200
HbGP	TGTTGAGCAGGCTTTCATGG	ACACGCCACAAAGAACACG	1.954	193
HbChi	AAGTACGGAGGTGTTATGC	GTACTCCCTCTCTCCTTATT	1.848	214
HbDef	CGTCTATTTTCAGCACTTTCCC	TTCCTAGTGCAGAAGCAGCG	1.872	212
HbQM	GCAGAGAACCGCATTGTATC	CGACACCTCACTAGCTTCATAT	1.908	214

Table 3. Temporal regulation of the expression of selected genes by real-time RT-PCR in epicormic stem bark of budded plants from clone PB260. Values are the means of the relative transcript abundance of three replicates. The data were analysed with XLSTAT software after log transformation. Statistical analysis was performed with an ANOVA followed by the Student Newman-Keuls test. Values with the same letter are not significantly different at the 0.05 probability level.

Genes	Daytime		
	8:00 am	12:00 am	4:00 pm
HbETR1	1.20E-01a	7.49E-02a	8.70E-02a
HbETR2	1.43E-01a	2.12E-01a	2.18E-01a
HbEIN2	7.66E-02c	1.05E-03b	1.73E-01a
HbMAPK	1.29E-02a	1.55E-02a	2.48E-02a
HbSAUR	5.34E-03b	1.52E-02a	1.85E-02a
HbEIN3	2.46E-01a	2.07E-01a	4.06E-01a
HbCOI1	3.34E-01a	3.26E-01a	4.38E-01a
HbWRKY	4.59E+00a	4.95E+00a	6.02E+00a
HbMYB	2.33E-06a	6.79E-07b	1.87E-06a
HbBTF3b	5.57E-01a	2.76E-01b	5.25E-01a
HbACR	5.99E-03a	1.12E-02a	1.01E-02a
HbACBP	1.15E+00a	3.44E-01c	6.91E-01b
HbCaM	6.32E-02a	8.79E-02a	6.62E-02a
HbCAS1	3.39E-03a	5.87E-03a	7.74E-03a
HbCAS2	4.10E-03b	9.15E-03a	5.16E-03ab
HbCIPK	5.32E-02a	7.75E-02a	8.00E-02a
HbGS	1.20E-02a	1.71E-02a	1.70E-02a
HbLTPP	3.16E+01a	1.77E+01b	4.30E+01a
HbPLD	2.30E-02a	4.47E-02a	6.11E-02a
HbUbi	5.59E+00a	4.82E+00a	6.92E+00a
HbPIP1	4.22E+00a	1.50E+00c	2.61E+00b
HbGP	5.40E-05a	4.15E-05a	5.30E-05a
HbChi	1.91E-04a	1.16E-04a	1.94E-04a
HbDef	6.13E+00a	2.02E+00b	1.15E+00c
HbQM	6.60E+01a	5.76E+01a	8.15E+01a

Table 4. Analysis of the expression of selected genes by real-time RT-PCR in bark of 3-month-old epicormic shoots of clone PB 260. The level of expression was calculated as the ratio between the relative transcript abundances of treated and control plants on average. It was considered as an up-regulation when the ratio >1.0, and a down-regulation when the ratio <1.0. The *p*-value corresponds to the Fisher test of the ANOVA.

Genes	Wounding				MeJA				Ethylene			
	15 min		4 h		1 h		4 h		4 h		24 h	
	ratio	*p*- value	ratio	*p*-value	ratio	*p*-value	ratio	*p*-value	ratio	*p*-value	ratio	*p*-value
HbETR1	1.97	0.81	0.57	0.10	1.23	0.99	0.14	0.14	1.74	0.07	1.77	0.65
HbETR2	2.26	0.31	1.05	0.86	1.50	0.97	1.35	0.29	4.26	0.01	8.09	0.08
HbEIN2	0.84	0.78	1.18	0.65	0.84	0.21	1.51	0.16	0.91	0.57	0.00	0.00
HbMAPK	0.30	0.05	0.61	0.10	0.68	0.08	0.68	0.08	1.28	0.20	0.37	0.35
HbSAUR	1.34	0.63	5.61	0.00	1.29	0.47	7.67	0.00	1.49	0.08	0.32	0.50
HbEIN3	1.41	0.75	2.38	0.06	1.07	0.91	0.70	0.37	1.17	0.36	0.00	0.00
HbCOI1	1.11	0.83	0.67	0.05	0.57	0.19	0.49	0.05	1.22	0.07	1.03	0.84
HbWRKY	1.44	0.32	1.16	0.85	0.95	0.81	1.36	0.22	0.94	0.65	1.03	0.89
HbMYB	0.22	0.08	5.82	0.06	0.51	0.03	1.13	0.02	1.12	0.48	1.46	0.40
HbBTF3b	0.44	0.03	1.04	0.91	0.78	0.35	1.02	0.58	1.05	0.90	1.398	0.08
HbACR	0.44	0.15	4.42	0.01	0.82	0.53	3.85	0.00	2.40	0.04	1.80	0.59
HbACBP	0.24	0.06	1.37	0.48	1.02	0.85	0.97	0.47	3.68	0.19	0.85	0.90
HbCaM	0.67	0.41	0.62	0.14	1.19	0.35	0.94	0.73	1.17	0.60	0.00	0.00
HbCAS1	0.33	0.00	1.05	0.92	0.84	0.62	1.15	0.26	2.40	0.06	1.07	0.74
HbCAS2	0.95	0.75	2.11	0.16	2.48	0.05	3.20	0.00	2.13	0.14	1.60	0.87
HbCIPK	1.13	0.60	1.62	0.39	0.83	0.12	2.22	0.03	0.82	0.64	0.92	0.58
HbGS	1.05	0.90	1.18	0.62	0.58	0.12	1.16	0.48	1.74	0.11	2.23	0.46
HbLTPP	0.50	0.04	0.81	0.64	0.97	0.95	0.62	0.18	1.62	0.69	0.42	0.07
HbPLD	0.48	0.01	0.25	0.01	0.70	0.34	0.58	0.27	0.74	0.20	0.34	0.41
HbUbi	1.37	0.76	0.79	0.32	1.08	0.67	1.45	0.18	2.41	0.25	0.42	0.06
HbPIP1	0.77	0.23	1.10	0.70	1.00	0.93	1.05	0.84	1.93	0.04	0.94	0.66
HbGP	1.00	0.89	1.73	0.01	2.01	0.04	2.15	0.11	2.39	0.01	1.11	0.47
HbChi	212.28	0.30	6.76	0.48	0.32	0.01	0.00	0.22	3.25	0.32	0.66	0.31
HbDef	0.17	0.05	0.38	0.09	0.60	0.56	0.48	0.04	2.25	0.06	1.22	0.38
HbQM	0.79	0.10	2.30	0.17	1.54	0.07	1.56	0.06	1.77	0.04	1.27	0.44

Table 5. Effect of a 16-hour 1-MCP pre-treatment on the expression of ethylene-responsive genes. The level of expression was calculated as the ratio between the relative transcript abundances of treated plants (T) and control plants (C) on average. It was considered as an up-regulation when the ratio >1.0, and a down-regulation when the ratio <1.0. The *p*-value corresponds to the Fisher test of the ANOVA

Genes	Ethylene 4 h		1-MCP / Ethylene 4 h	
	T:C ratio	*p*-value	T:C ratio	*p*-value
HbETR2	4.26	0.01	0.58	0.33
HbACR	2.40	0.04	2.19	0.27
HbPIP1	1.93	0.04	0.93	0.79
HbGP	2.39	0.01	1.12	0.53
HbQM	1.77	0.04	1.21	0.26

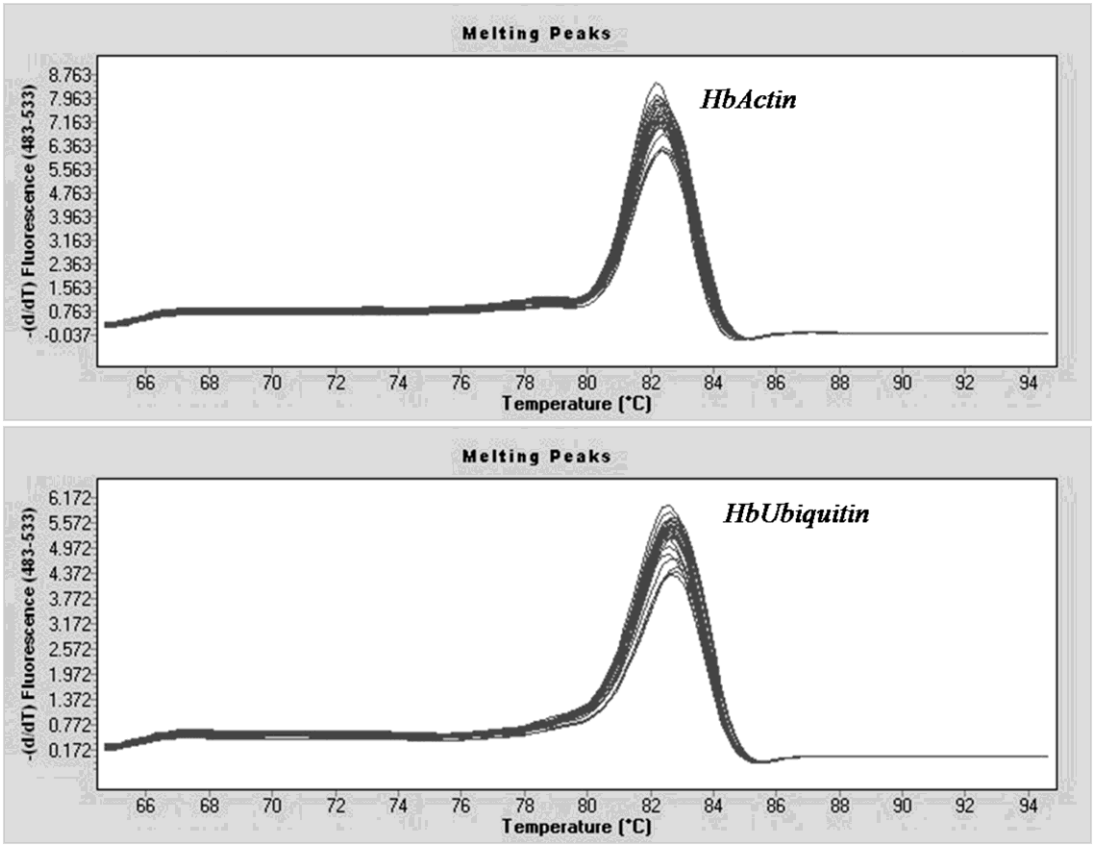

Figure 1. Specificity of real-time PCR amplification determined from single peak melting curves for *HbActin* and *HbUbi* genes. Curves were calculated for 72 reactions from 24 treatments with 3 biological replicates in the *H. brasiliensis* clone PB260.

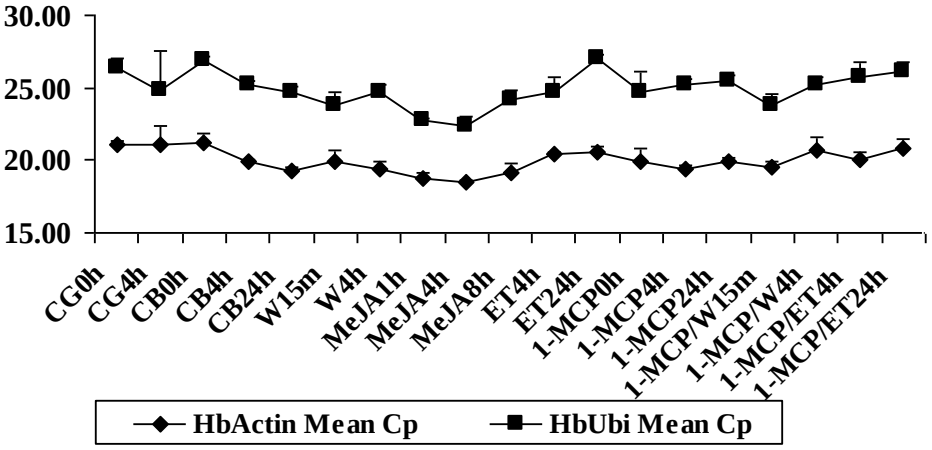

Figure 2. Evolution of the crossing point value (Cp) for *HbActin* and *HbUbi* genes under various stresses in *H.brasiliensis* clone PB260. Treatments were applied at 8:00 am. Samples were collected at 8:00 am, 12:00 am (4h after treatment), 4:00 pm (8h after treatment), or 8:00 am the day after (24h after treatment). Real-time RT-PCR was carried out from bark tissue. CG: control plants; CB: control plants placed in box; W: wounded plants; MeJA: plants treated with 0.03 µM methyl jasmonate; ET: plants treated with 1 ppm of ethylene gas; 1-MCP: plants pre-treated for 16 hours with 1-methyl cyclopropane. Error bars were calculated from 3 separate runs of real-time RT-PCR using 3 biological replicates.

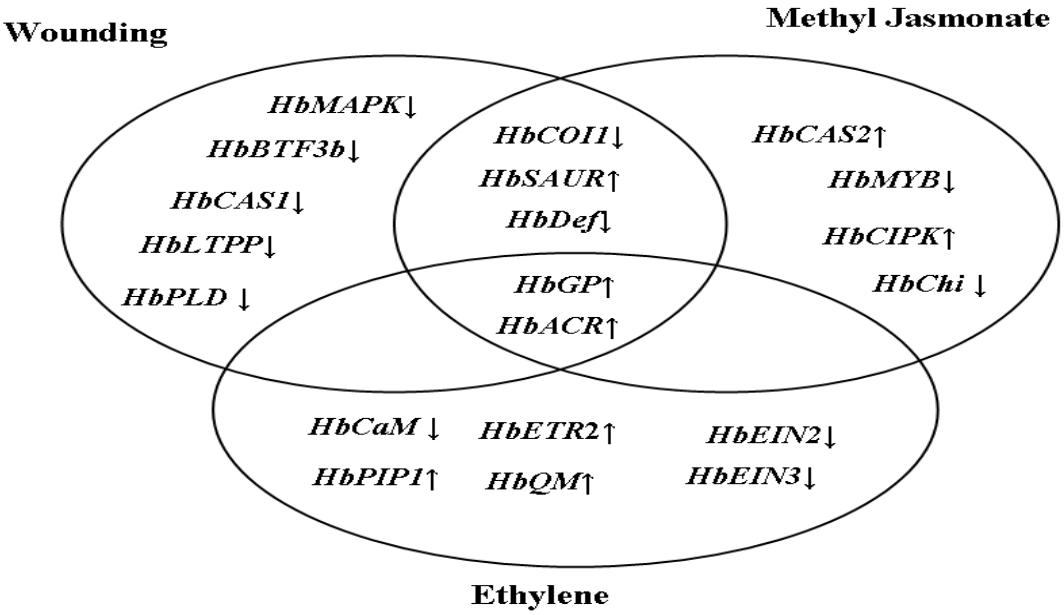

Figure 3 Representative distribution of genes regulated by one or more treatments wounding (W), methyl jasmonate (MeJA), or ethylene (ET). (↑) up-regulated and (↓) down-regulated genes.

CHAPITRE 2

Article intitulé "Transcriptomic analysis of the *Hevea brasiliensis* AP2/ERF superfamily in latex"

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Transcriptomic analysis of the *Hevea brasiliensis* AP2/ERF superfamily and the characteristics

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Abstract

Background: AP2/ERF transcription factors play a crucial role in plant development and response to biotic and abiotic stresses. This superfamily includes Ethylene-Response Factors. Rubber tree (*Hevea brasiliensis*) laticifers are the source of natural rubber. Rubber production depends on endogenous and exogenous ethylene (ethephon). To date there has been little information on the AP2/ERF superfamily in the rubber tree.
Results: NGS technique was used to generate five tissue (leaf, bark, latex, embryos and root) specific transcriptomics libraries. By searching the transcriptomics libraries, one hundred and seventy-three AP2/ERF transcripts were identified by *in silico* analysis based on the amino acid sequence of the conserved AP2 domain. The 142 transcripts with full AP2 domain were classified into three main families (20 AP2 members, 115 ERF members divided into 11 groups, and 4 RAV members) and 3 soloist members. Transcript abundance based on the read count indicated that AP2/ERF transcripts were more abundant in roots, followed by bark, embryos, latex and leaves. Sixty-six AP2/ERF transcripts were found in latex. In addition to microRNA 172, six additional microRNAs were predicted to inhibit *Hevea* AP2/ERF transcripts.
Conclusions: *Hevea brasiliensis* has a similar number of *AP2/ERF* genes to that of other dicot species. We adapted the alignment and classification methods to next-generation sequencing, which provided reliable information. We observed several features in the ERF family.Three HbSoloist members forms a group in *Hevea*. Several *AP2/ERF* genes highly expressed in latex could be related to a specific function in *Hevea brasiliensis*. The analysis of AP2/ERF transcripts in *Hevea brasiliensis* presented here provides the basis for studying the molecular regulation of latex production in response to abiotic stresses and latex cell differentiation.

Background

Natural rubber accounts for 43% of the 23.9 million tons of rubber consumed worldwide [1]. *Hevea brasiliensis* is the sole commercial source of natural rubber. The increasing demand for natural rubber calls for improved productivity in rubber plantations. The cis-1,4-polyisoprene chains are synthesized in the rubber particles of latex cells. Rubber particles account for up to 90% of the dry matter in latex cytoplasm, which is harvested by tapping the soft bark of rubber trees [2]. Latex production depends on genetic, environmental and harvesting components. Harvesting systems use ethephon, an ethylene (ET) releaser applied to the tapping panel, to stimulate latex production by improving the flow and regeneration of latex. The tapping and ethephon stimulation frequency is adjusted to *Hevea* clones according to their metabolism [3]. Given the high pressure in the phloem tissue, latex is expelled after tapping. Tapping and ethephon are likely to be sources of stress conducive to the production of secondary metabolites and consequent rubber, but over a certain stress limit they lead to tapping panel dryness (TPD) [4]. Mechanical wounding and osmotic stresses related to tapping trigger the production of endogenous ethylene and oxylipins such as jasmonic acid (JA) [5, 6]. Both mechanical wounding and methyl-jasmonate treatments induce the differentiation of secondary latex cells [7-9]. These secondary latex cells then anastomose to create laticifer vessels [10]. Ethephon

application also induces several biochemical processes in laticifers, such as sucrose loading, water uptake, nitrogen assimilation or synthesis of defence proteins [](Anaïs Dusotoit-Coucaud, 2009; CHAORONG TANG, 2010), involving a large number of ethylene-response genes [12-15], whereas its direct role in rubber biosynthesis is controversial [16].

Given the major role of ethylene and jasmonic acid in regulating latex cells, the involvement of Ethylene-Response Factors in latex cell functioning is strongly expected. Indeed, ET and JA signalling pathways involve transcription factors such as Ethylene-Responsive Element Binding Proteins (EREBP), also called the Ethylene-Response Factors (ERF) family [17]. ERFs have been shown to act as activators or repressors of additional downstream ethylene responsive genes. ERFs function as a transcription factor that integrates signals from the ethylene and jasmonic acid pathway. ERFs are a control point for crosstalk with other signals. Multiple signalling pathways converge on ERFs by transcriptional and post-transcriptional regulation [18]. Ethylene and jasmonate pathways converge in the transcriptional activation of ETHYLENE RESPONSE FACTOR1 (ERF1), which regulates *in vivo* the expression of a large number of genes responsive to both ethylene and jasmonate. ERF1 acts downstream of the intersection between ethylene and jasmonate pathways and suggest that this transcription factor is a key element in the integration of both signals for the regulation of defence response genes. [19, 20]. AP2/ERF transcription factor ORA59 acts as the integrator of the JA and ET signalling pathways and is the key regulator of JA- and ET-responsive PDF1.2 expression [21, 22].

The ERF family was first discovered in *Nicotiana tabaccum* by Ohme-Takagi and Shinshi H [17]. The ERF family is one of the most important families of transcription factors with 122 genes among the 2016 predicted transcription factors from 58 families in *Arabidopsis* [23, 24]. The ERF family belongs to the AP2/ERF superfamily like the AP2 and RAV families. The AP2/ERF superfamily encodes transcriptional regulators that serve a variety of functions in plant development and responses to biotic and abiotic stimuli [25-27]. The availability of the whole genome sequence of several plant species has made it possible to confirm a relatively well-conserved organization of the AP2/ERF superfamily with 147, 149, 202 and 180 genes in *Arabidopsis thaliana, Vitis vinifera, Populus trichocarpa* and *Oryza sativa*, mostly represented by the ERF family [23-25, 28]. Transcript sequencing is also an alternative for identifying such gene families. For instance, 156 *AP2/ERF* genes consisting of 148 ERFs, 4 AP2s and 4 RAVs were identified in *Gossypium hirsutum* from EST databases [29].

Members of the AP2/ERF superfamily contain at least one AP2 domain, which consists of about 60 to 70 amino acids. This domain is involved in DNA binding to a conserved AGCCGCC sequence called the GCC-box [17, 30] or to a dehydration response element (DRE: TACCGACAT) containing C-repeat [31, 32]. The structure of the AP2 domain was first reviewed by Riechmann and coll. [33]. Initially, the *APETALA2* gene was isolated by T-DNA insertional mutagenesis in *Arabidopsis* [34]. This gene encodes a 432-amino acid protein with two copies of a 68-amino acid direct repeat called the AP2 domain. The AP2 domain consists of three anti-parallel □-sheets and one □-helix. Two conserved elements, YRG and RAYD, have been identified. The latter is an 18-amino acid core region that is predicted to form an amphipathic □-helix [35]. In addition to the AP2 domain, this gene contains a 37-amino acid serine-rich acidic domain putatively functioning as an activation domain, and a 10-amino acid domain including a putative nuclear localization

sequence KKSR [34]. While previously thought to be plant-specific transcription factors, AP2 domain-containing genes were recently found in bacteria and viruses, which are predicted to be HNH endonucleases [36].

Several ways of classifying the AP2/ERF superfamily have been proposed in plants. Although all of them were analysed according to the number of AP2 domains, some differences exist. Sakuma et al described five subfamilies including AP2, RAV, DREB (Dehydration Responsive Element Binding Proteins), ERF (ethylene responsive factors) and others based on a homology of the DNA binding domain, and the DNA sequences that bind it, namely the DRE element or GCC-box separately [37]. The AP2, ERF/DREB and RAV subfamilies have two AP2 domains, one AP2 domain, or one AP2 and one B3 domain, respectively. Groups A1 to A6 and B1 to B6 have been assigned to the DREB and ERF families [37]. Nakano *et al* classified these proteins in only three major families: AP2, ERF and RAV [24]. The ERF family was then divided into ten groups according to the structure of the AP2 domain, groups I to IV corresponding to the DREB subfamily in Sakuma's classification. More recently, the use of a Bayesian method for phylogenetic analysis led to two additional groups in the *Arabidopsis* ERF family [38]. To date, Nakano's classification method has remained a reference for organizing the AP2/ERF superfamily in three families (AP2, ERF, RAV) and the ERF family in ten groups. In the construction of phylogenetic trees, methods for multiple sequence alignment and tree reconstruction have to be considered with caution. In the analyses by Sakuma and Nakano, a neighbour-joining distance method was chosen. Currently, although computationally intensive, maximum likelihood (PhyML) and multiple sequence alignment (MUSCLE) methods are more relevant [39-42]. This approach was chosen to improve the quality of the AP2/ERF superfamily phylogenetic tree.

AP2/ERF transcription factors play a crucial role in developmental plasticity and in the response to environmental cues and the complexity of their multi-level regulations became evident (for review, [43, 44]. At transcriptional level, AP2/ERF members are involved in gene networks, such as in cross-talk between sugar and hormones, and between light and ethylene [44, 45]. At post-transcriptional level, some AP2 genes are targeted by microRNA networks, such as miR172 targeted AP2 genes involved in flower development and patterning [46-48]. At protein level, several AP2/EREBP members have been described as being part of gene regulatory networks which integrate different pathways. This is the case of ETHYLENE RESPONSE FACTOR1 (ERF1) which acts as an integrator of ethylene and jasmonate signalling [18, 19]. In addition to their integrator function for several inputs, some members have been demonstrated to bind multiple *cis*-elements in the promoter of their target genes. This is the case of the DREB transcription factor TINY, which connects abiotic stress signalling via dehydration-responsive element (DRE)-driven genes to biotic stress signalling via Ethylene-Responsive Element–driven genes [49]. AP2/ERF members also integrate redox condition inputs by changing their protein conformation, a common mechanism to control protein function in response to a changing redox environment [50]. RAP2.4a has been described as dimerizing under normal redox conditions and as being separated as a mononer under highly reducing conditions [45]. At sub-cellular level, AP2/ERFs could also integrate input signals derived from organelles. Several members have been identified as nuclear-encoded transcription factors with potential targeting to chloroplast or mitochondria [44].

Given the involvement of wounding, jasmonate and ethylene in natural rubber production, we examined in detail the organization of the AP2/ERF superfamily in

Hevea brasiliensis. Firstly, transcripts were intensively sequenced using GS-FLX next-generation sequencing (NGS) technologies. RNAs were isolated from different tissues of plants at several stages of development growing under various conditions. Secondly, contigs harbouring at least one AP2 domain were identified in tissue-specific libraries from leaves, bark, latex, roots and embryogenetic tissues and from a global library which mixed all the reads from all tissue specific libraries. AP2 domain-containing genes were aligned with the *Arabidopsis* AP2/ERF sequences and classified according to Nakano's method based on a phylogenetic analysis of the conserved AP2 domain optimized by using a maximum likelihood method (PhyML) [39-41]. Tissue-specific transcripts were identified by *in silico* analysis. Post-transcriptional regulation was checked by predicting microRNA-targeted *AP2/ERF* genes. This study suggested that some *HbAP2/ERF* genes are specifically expressed in latex cells and could be involved in new biological processes.

Results

Identification and classification of the AP2/ERF superfamily genes in *Hevea brasiliensis*

CIRAD's *Hevea brasiliensis* transcript sequence databases (http://bassigny/cgi-bin/esttik_dev/quick_search.cgi) were searched with tblastn using the amino acid *Arabidopsis thaliana* AP2 domain sequences as the query. In all, 173 putative AP2/ERF superfamily contigs were identified in the global transcript sequence database, which incorporated the pooled information from all the tissue specific libraries. These contigs contained full-length and partial transcripts. Of them, 142 contigs had the full-length AP2 domain sequences of 58–59 amino acids. Multiple alignment analysis was performed on full-length AP2 domain sequences from *Hevea*, *Arabidopsis* and *Populus*. Group classification was firstly achieved by constructing the general phylogenetic tree of AP2 domains in *Arabidopsis* and *Hevea* with the neighbour-joining method (data not shown), and then the phylogenetic relationships between these genes were analysed by constructing another phylogenetic tree using the PhyML method only for *H. brasiliensis* (Figure 1). Using the Nakano classification method, the *Hevea brasiliensis* AP2/ERF superfamily was organized in families and groups. The alignments indicated three clusters corresponding to the AP2, ERF and RAV families, the ERF family being divided into eleven major groups including an additional VI-L group, and the three soloists being rooted with the AP2 family.

Twenty-five genes were assigned to the AP2 family based on the similarity of their amino acid sequences with the *A. thaliana* AP2 proteins and the presence of a double AP2 domain in their sequences (Table 1). This number included contigs with one or two partial domains. Ten genes containing single complete/partial AP2 domain were classified into AP2 family given their higher homology with AP2 family. The largest family was the ERF family with 141 genes harbouring a single AP2 domain, including twenty six contigs with a partial sequence of the AP2 domain. Four genes were classified in the RAV family, which had one single AP2 domain and one B3 domain. Three additional contigs with the same characteristics as the soloist in *Arabidopsis*, *Populus* and *Vitis* were distributed as HbSoloists. *HbSoloist1* shared 84% identity with *HbSoloist2* and 93% with *HbSoloist3*, *HbSoloist2* had 86% identity with *HbSoloist3* over the nucleotide length. The AP2/ERF superfamily has a similar number of genes in *Vitis* (149) and *Arabidopsis* (147) (Table 1). This number is

higher for *Gossypium* (218, data from 3 species), *Populus* (202) and *Hevea* (173), while it is lower for *Solanum* (112) and *Triticum* (117). These differences were mostly induced by a change in the number of ERF genes. With regard to the soloists, all the cited species have one sequence except *Solanum* and *Gossypium*. Interestingly, *Hevea* has three soloist sequences, which could represent a small group. Of the 173 identified AP2/ERF superfamily contigs, there were 31 contigs that had no complete AP2 domain, which were discarded for further analyses (5 AP2 and 26 ERF contigs).

Characteristics of the *Hevea* AP2/ERF superfamily

Nakano's classification method was compared with Sakuma's for the 142 *Hevea* genes with complete AP2 domain (Table 2). Families and groups were noted as subfamilies and subgroups previously by Sakuma. ERF genes were classified into two subfamilies consisting of thirty-three DREB and eighty-two ERF genes (Table 2). ERF subfamily genes were twice as large as the DREB subfamily in *Hevea*.

The AP2 family was composed of two AP2 domains, AP2 Repeat1 (R1) and AP2 Repeat2 (R2), which were 58-68 amino acids long. The AP2 family was organized in two groups including AINTEGUMENTA (ANT) and APETALA2 (AP2). Of the twenty AP2 family genes, eight *ANT* and twelve *AP2* genes were identified in *H. brasiliensis*.

One hundred and fifteen *Hevea* genes with full AP2 domain from the ERF family were organized in eleven groups according to the Nakano classification (Table 3). AP2 domains of the *Hevea* ERF family consisted of 58 amino acid residues except for group IX, which contained an additional amino acid to have 59 amino acid residues. The number of *Hevea ERF* family genes was comparable to those of *Arabidopsis* and *Vitis* (122, 122 and 135, respectively), but lower than those of *Gossypium* and *Populus* (200 and 169 genes, respectively). This difference between the species was mostly due to a variation in the number of genes for groups II, III and VII, VIII and IX to which the differences between the largest and the fewest number of genes for each group were 13, 24, 30, 22 and 25, respectively. For *Gossypium*, the largest number of ERF family genes is explained by the combination of three species of *Gossypium*, reflected in the largest number of genes for group VII and VIII with 30 and 22 additional genes, respectively, compared with the species with the fewest in the relative group. The *Hevea* ERF groups showed several characteristics. Firstly, several ERF groups and subgroups were not found in *Hevea* in comparing with *Arabidopsis* such as subgroup IIc and groups IVb, Xc and Xb-L. Secondly, the number of genes in group VII (23 genes) was the largest of the ERF groups in *Hevea*, and fourfold larger in *Hevea* than in *Arabidopsis* and *Populus*, with the smallest number of genes for group IV (3 genes) in *Hevea*. Thirdly, three soloist genes were identified in *Hevea* whereas only one has been reported for *Arabidopsis* and *Populus*, and no soloists have been identified in *Gossypium*(Table 1).

Structure and group-specific residues of the AP2 domains of the *ERF* genes

The amino acid sequences of the AP2 domain from fifty-five representative *ERF* genes with full-length transcript sequences were aligned in order to identify the structure and the group-specific residues. Tertiary structures of the AP2 domain were predicted and revealed similarity to AtERF1 for each gene that consisted of a three-stranded anti-parallel β-sheet and one α-helix (Protein Database number 2GCC) (Figure 2). Specific amino acid residues were also identified for each group (Figure 2). AP2 domains from ERF family proteins contained the WLG motif and most of

them also contained the YRG and RAYD elements. The positions of the AP2 domain were numbered according to the three-dimensional structure of AtERF1. Eight amino acids were totally conserved in each group (G148, R150, R152, G155, E160, I161, G174 and A182 amino acid in black background, Figure 2). Most AP2 domain sequences had conserved amino acid residues: V158 and E163 for groups I to IV and A158 and D163 for groups V to X, which corresponded to the V14 and E19 for DREB and A14 and D19 for the ERF subfamilies according to Sakuma's classification, respectively (Figure 2 and Supplementary Figure 1). A few members that did not show conservation at these positions 158 and 163 were categorized based on their placement in the phylogenetic tree. A conservative sequence motif of 5 amino acid residues (KREYD) only occurred in group VI-L.

The group-specific amino acid residues observed in *Hevea* were compared with those of *Arabidopsis* and *Gossypium* (Table 4). At least one specific-residue could be identified for each group, two for groups II and VIII, and three for group VII. *Hevea* group VI-L revealed one more group-specific residue (M196) in addition to the K189 found in all species. For group IX, one additional residue at position 167 was identified for all species leading to an AP2 domain 59 amino acids long as opposed to 58 for the other groups. In the AP2 family, the AP2 domains contain a conserved amino acid, T150 (92%) or A150 (8%), in *Hevea*, *Arabidopsis* and *Gossypium*. The AP2 domains of the RAV family have a conserved amino acid residue, V150 (100%), in *Hevea*, *Arabidopsis* and *Gossypium* (Table 4).

Tissue Characteristics of *AP2/ERF* genes by *in silico* analysis

An analysis of the number of AP2/ERF contigs and reads constituting these contigs, including their library origin, led us to evaluate the transcript accumulation. The number of AP2/ERF contigs was 136 in roots, 120 in bark, 111 in embryogenic tissues, 98 in leaves and 66 in latex (Supplementary Table 1). The transcript abundance for all *AP2/ERF* genes was evaluated by the number of reads from each tissue library. The AP2/ERF transcripts were more abundant in roots (1,996 reads), bark (1,480 reads), followed by latex (1,312 reads), embryos (1,312 reads) and then leaves (706 reads) (Figure 3; Supplementary Table 1). Although a smaller number of different *AP2/ERF* genes was expressed in latex (66 transcripts), their corresponding contigs were constructed from a large number of reads (20.1% of sequenced reads) reflecting higher transcript accumulation in this tissue.

Latex-expressed *AP2/ERF* genes by *in silico* analysis

Transcripts of fifty-nine *AP2/ERF* genes were present in latex plus seven putative contigs with a partial AP2 domain (Table 5; Supplementary Table 1). The transcripts expressed in latex belonged to the AP2, all ERF groups, RAV families and soloist genes. The genes most represented in the latex library were from the ERF family, of which groups VIII (12 genes), VII (10 genes), I (6 genes) and the AP2 family (5 genes). Thirty-seven transcripts were detected in all 5 tissues and could be considered as constitutively expressed genes. In addition to the latex-specific *HbERF-IIb5* gene, transcripts of fourteen other *AP2/ERF* genes were mostly accumulated in latex.

Based on contigs assembled in the global library, twelve genes accounted for more than 40% of reads from the latex library were identified: *HbERF-Ib4*, *HbERF-IIa1*, *HbERF-IIb3*, *HbERF-IIb4*, *HbERF-Va2*, *HbERF-VIIa4*, *HbERF-VIIIa1*, *HbERF-VIIIa2*, *HbERF-VIIIa4*, *HbERF-VIIIa6*, *HbAP2-4* and *HbAP2-6*

(Supplementary Table 1). Three of them (*HbERF-Ib4*, *HbERF-VIIa4* and *HbERF-VIIIa4* genes) and two other genes (*HbERF-VIIa7* and *HbERF-VIIa12*) showed contigs built with more than 50 reads also revealing their high expression status in latex. *HbERF-VIIa12* displayed the highest transcript abundance with 1,200 reads in total for all tissue libraries.

Prediction of microRNA-targeted *AP2/ERF* genes

Seven conserved microRNA families (miR156, miR159, miR172, miR393, miR395, miR396, miR408) were predicted targeting twelve *Hevea AP2/ERF* genes (Table 6). Of them, we identified four AP2 genes (*HbAP2-4*, *HbAP2-18*, *HbAP2-9*, *HbAP2-20*), seven *ERF* genes (*HbERF-IIId1*, *HbERF-VIIa9*, *HbERF-VI-L1*, *HbERF-VIIa13*, *HbERF-IXb1*, *HbERF-IXc2*, *HbERF-IXc3*) and one RAV gene (HbRAV-2). Although inhibition mostly involved a transcript cleavage, the inhibition of translation was predicted for two genes (*HbERF-IXc3*, *HbERF-IIId1*). Predicted microRNA sites were in the conserved AP2 domain for the *HbERF-IXb1* gene in CDS, outside the AP2 domain for ten genes and in the 5'UTR for gene *HbERF-VI-L1*.

Discussion

NGS data combined with an optimized method of alignment and classification led to the identification of the *Hevea brasiliensis* AP2/ERF superfamily

The AP2/ERF superfamily has been identified in several species from both genome and EST sequences. For the first time to our knowledge, this study presented the identification of most members of the AP2/ERF superfamily using the 454 sequencing technology for crop plants for which few data are available. The one hundred and seventy-three *AP2/ERF* genes identified in *Hevea brasiliensis* were clustered into three main families (25 AP2, 141 ERF, and 4 RAV members) and a group of 3 soloists using a maximum likelihood phylogenetic analysis. Then, the ERF family was subdivided into 11 major groups, which corresponded to group I to X, and group VI-like described by Nakano [24].

The number of *Hevea AP2/ERF* genes was comparable to the number observed in other species. For species whose genome has been sequenced, such as *Arabidopsis thaliana*, *Vitis vinifera*, *Populus trichocarpa* and *Oryza sativa*, 147, 149, 202 and 180 genes were counted, respectively [24, 25, 28]. From EST databases, 112 and 117 genes were found for *Solanum lycopersicum* and *Triticum aestivum*, respectively [23, 51], while 218 *AP2/ERF* genes were identified in cotton but from several species of *Gossypium* [29]. Considering the large repertoire of EST data available, the number of AP2/ERF family genes is not expected to change much [51]. However, it has been estimated that most EST sequencing projects fail to cover 20–40% of transcripts, which usually include rare or very long transcripts as well as transcripts with highly specific expression patterns [52]. By contrast, NGS technologies have the potential for providing much deeper coverage than the traditional EST libraries.

However, the first phylogenetic analyses came up against the low quality of contig sequences from NGS. The minimum overlap length was increased to 60 bp compared to the 40 bp used in *Jatropha curcas* for instance, with a minimum overlap identity of 95% [53]. Finally, the assembly strategy of *Hevea* reads delivered robust contigs from current programs since the clustering method discriminated conserved domains from the various *AP2/ERF* genes. In addition, homopolymer correction by mapping Solexa reads was not required. The error rate for 454 genome sequencing

provides a modest increase 0.04% in 454 sequencing *vs* 0.01% compared with the Sanger technique in genome sequencing [54-58].

The Neighbour-Joining tree built from the protein distance matrix with manual correction proposed by Nakano was widely adopted for the classification of the ERF family. Based on NGS contigs, the classification method proposed by Nakano provided non-consistent results due to errors and the accuracy rate of contig sequences. A partial AP2 domain of 57 amino acids was selected for the alignment of 142 sequences with a full AP2 domain using a combination of MUSCLE and Gblock softwares. The use of Gblocks reduces the need for manual editing of multiple alignments. This method facilitated the construction of a consistent phylogenetic tree with PhyML software without requiring a Bayesian Inference method. This latter method was successfully used for classification of the *A. thaliana* ERF protein family [38]. These authors included groups VI-like and Xb-like described by Nakano *et al* in their phylogenetic reconstruction, and finally placed these groups as new groups XI and XII, respectively. Group VI-L genes are close to group VI with a modification in the second element suggesting that the evolution of group VI-L is more recent than the other groups. This independent cluster on the *Hevea* phylogenetic tree led us to propose it as a new group (see below in the discussion).

Hevea AP2/ERF genes have common and several specific features compared to other species

Plant AP2 domain-containing genes were derived from bacterial or viral endonucleases [36]. The common ancestor of AP2 and ERF genes has one AP2 domain. The ERF family diverged with a duplication of the AP2 domain, probably before the *Chlorophyta* lineage from the *Streptophyta* lineage [59]. So the evolution of genes from the AP2 family by duplication occurred before the mosses [59]. The AP2 family is composed of two AP2 domains, R1 and R2 (repeat domains), which are 58-68 amino acids long. The AP2 family is divided into two groups, AINTEGUMENTA (ANT) and APETALA2 (AP2) [59, 60]. An additional ten specific amino acid residues are present in the AP2 R1 domain of the ANT homologs [59]. Of the twenty five genes from the AP2 family present in *Hevea*, 10 contained a single complete/partial AP2 domain. Based on their high homology with the *Arabidopsis* AP2 family, these 10 genes were classified into AP2 family. This is possibly because that the second AP2 motif was not identified due to incomplete sequence of the transcripts as in tomato [51]. Of 20 genes of the AP2 family with full AP2 domain, eight genes contained the characteristics of ANT genes. The RAV family includes one AP2 and one B3 domain, which are highly conserved among different species with 1 to 7 members only (http://planttfdb.cbi.pku.edu.cn/family.php?fam=RAV). Members of the RAV family were reported to be induced in ethylene response and in brassinosteroid response and to be involved in flower senescence [24, 61]. The AP2 domains of RAV1 and RAV2 are more diverged from those of the EREBP-like proteins and may be considered a third subfamily of the AP2/EREBP family [62]. *Hevea* ERF proteins have a 58-amino-acid-residue AP2 domain except for group IX, which has one additional amino acid at position 167. In some other species, the AP2 domain of ERF genes can be longer for some members, such as DR455472 in cotton. Given that all the groups are represented in monocot and dicot species, this suggests that diversification of the ERF family predates monocot-dicot divergence [27].

Several functionally important conserved motifs described in *Arabidopsis* and tomato were also found in *Hevea* AP2/ERF deduced proteins suggesting that they are likely to function as transcription factors [51]. The putative nuclear localization signal (NLS) motif near the R1 domain was found in *Hevea* AP2/ERF transcription factor sequences (data not shown). The residues G148, R150, R152, G155, E160, I161, G174 and A182 were completely conserved among all 437 ERF proteins collected from three species (*Hevea*, *Arabidopsis* and *Gossypium*). These observations are generally consistent with earlier reports on this topic [24, 33, 37]. The conserved Ala-37 (corresponding to A182 in this paper) in the ERF domain was suggested to play a major role in the stability of the ERF domain or DNA binding with the DRE element or GCC box [51, 63]. For the ERF of group II, the ERF-associated amphiphilic repression (EAR) motif was found [64]. DEAR1, a DREB protein-containing EAR motif, has been shown to mediate crosstalk between signalling pathways for biotic and abiotic stress responses [65]. The EAR motif exists in all members of ERF group VIII in tomato [51]. Licausi *et al* mentioned that the smaller number of genes (5) in group I could be a common feature for woody species (*Vitis* and *Populus*) [28]. This assumption is no longer valid since the number of *Hevea* ERF group I genes is twelve. Interestingly, Licausi *et al* proposed that the function of groups I and V overlaps since the increase in group V genes balances the smaller number of group I genes. In *Hevea*, as in *Arabidopsis* and *Gossypium*, the larger number of genes in group I was compensated for by a smaller number in group V, contrary to *Vitis* and *Populu*s, supporting the hypothesis of Licausi *et al*.

The features of the AP2 domain are helping to classify the AP2/ERF superfamily. The AP2 domain sequences in groups I to IV had conserved amino acid residues V158 and E163, and groups V to X had A158 and D163, which correspond to V14 and E19 for DREB and A14 and D19 for the ERF subfamilies, respectively, according to Sakuma's classification [37]. They are related to DNA-binding specificity. The members that did not show conservation at these positions 158 and 163 were categorized in accordance with the phylogenetic analysis. The position of the conserved residues within a group proved to be similar for *Arabidopsis*, *Gossypium* and *Hevea*. This conservation suggests that the diversity of ERF groups appeared before the divergence of monocots and dicots [29].

Several features were observed in the AP2 domain of *Hevea* genes compared to other species. Firstly, subgroup IIc and groups IVb, Xc and Xb-L. were found not to exist in *Hevea* by contrast with *Arabidopsis*, But this characteristics will be considered with the validation of *Hevea* genome. Secondly, it was shown that three soloist genes were identified in *Hevea* whereas only one has been reported for *Arabidopsis* and *Populus* [29]. Soloists have been characterized by low conservation at the ERF DNA-binding domain in all plant genomes considered [28]. In our case, we showed that this low conservation could be explained by 6 missing amino acid residues in their AP2 domain, including R152, which directly contacts the GCC box [66]. The three *HbSoloist* genes shared between 84% and 93% identity in their nucleotide sequences only, which led us to consider these as three different *HbSoloist* genes. Although the three *HbSoloist* genes have only a single AP2 domain, they formed a group and clustered together with the AP2 family, as has been reported in *Vitis vinifera* [28]. However, no functional information has been published for soloist genes. Thirdly, based on an analysis of 437 AP2 domain sequences of ERF genes from three species, ten amino acid residues were shown to be strictly group-specific for all ERF groups except for group II and group VIII. A previous study on 315 AP2

domain sequences from *Arabidopsis*, *Gossypium* and *Oryza* led to the identification of 14 group-specific residues with a certain error rate [29]. The group-specific residues reported in this study could be proposed as a group marker of the ERF family for several species. In addition, *Hevea AP2/ERF* genes harboured unique group-specific residues in their AP2 domain, such as VI-L (M196), which are not found in other species. This difference could be explained by the distance between *Gossypium* and *Arabidopsis* in the Eurosides II (Brassicales and Malvales, respectively) and *Hevea* in the Eurosides I (Malpighiales) [67]. We also identified that position 150 was conserved in *Hevea*, *Arabidopsis*, *Gossypium*, *Populus* with T150, T150 and V150 for the ERF, AP2 and RAV family, respectively. Position 150 directly contacts with DNA, These interactions determine the geometry of the GCC-box binding domain (GBD) relative to DNA and thereby comprise a framework for specific base recognition [66].

Several *AP2/ERF* genes highly expressed in latex could be related to a specific function in *Hevea brasiliensis*

AP2/ERF genes are regulated by developmental processes and environmental cues [68]. As rubber trees are subjected to frequent mechanical wounding and osmotic stress upon tapping to collect latex, and ethephon stimulation to increase latex yield, some of these transcription factors are likely to play a unique role in *Hevea* defence mechanisms and latex production. Latex cells are differentiated in phloem tissue from cambium [10]. Consequently, a proportion of latex cells can be found in bark, roots and leaves. Transcripts present in these tissues may therefore come from latex cells. In order to identify most members of the *Hevea* AP2/ERF superfamily and study the function of genes from the AP2/ERF superfamily in development, tissue-specific transcript libraries were sequenced from *Hevea* plants at various stages of development and grown under various conditions (water deficit, ethylene, wounding, etc.) in order to mimic the environmental conditions that may affect rubber tree development. Some additional genes of the AP2/ERF superfamily in *Hevea* may exist given the absence of flower and fruit libraries. Members of the AP2 family play an important role in angiosperm reproductive organ development [69-72]. Members of the RAV family were reported to be induced in ethylene response and in brassinosteroid response and to be involved in flower senescence [61]. Consequently, the genes of the AP2/ERF superfamily are suggested to play an important role in *Hevea* development.

It has been reported that numerous *AP2/ERF* genes exhibit tissue specificity [62, 73]. Genes

Several of the sixty-six AP2/ERF transcripts accumulated in latex could be related to responses to stress. Indeed, most of them belong to the ERF family including DREB (groups I to IV) and ERF subfamilies (groups VII, VIII and IX), which are recognised as very important transcription factors regulating responses to abiotic stress. The most represented were groups VII and VIII with 10 and 12 genes, respectively. Several genes from group VII have been reported to be involved in salt tolerance (*JERF3* gene) in *Lycopersicon esculentum* [76], and freezing tolerance (*CaPF1* gene) in *Capsicum annuum* [77]. For group VIII, the *AtERF4* gene has been shown to be a negative regulator of the expression of ethylene, jasmonate, and ABA-responsive genes [78, 79]. The *Zea mays DBF1* gene belongs to the group I and has been shown to activate the drought-responsive element 2 (DRE2)-dependent transcription of ABA responsive rab17 in transiently transformed maize callus [80].

ERF genes of group IX including ORA59, ERF1, AtERF1 and AtERF2 have been shown to play crucial roles in biotic stress responses and have been linked to JA and ET signalling pathways [20, 22, 29, 81].

Latex cells are differentiated in roots, leaves and bark. This might explain why latex expressed genes could alsobe identified in the other tissues. In addition, sixteen other transcripts were highly accumulated in latex compared to other tissues: two for the AP2 family and 12 for the ERF family. The ERF transcripts highly accumulated in latex were distributed as follows: one for group I, three for group II, one for group V, three for group VII and four for group VIII. In *Arabidopsis*, the ERF group II includes *RAP2.1*, *RAP2.9* and *RAP2.10* genes. RAP2.1 acts as a negative transcriptional regulator in defence responses to cold and drought stress in *Arabidopsis* [82]. The four *Hevea* members of group VIII belonged to subgroup VIIIa, which are close to the *AtERF7* gene. AtERF7 has been shown to play an important role in ABA response in plants [83]. In *Arabidopsis*, other members of group VIII encode LEP [84], and ESR1/DRN [85, 86], which are involved in the differentiation and development of organs. The ERF group VIII genes expressed in latex are therefore expected to have a function in the defence against abiotic stress and the regulation of latex production in *Hevea.*

A few members of the AP2/ERF superfamily have been previously reported in *Hevea brasiliensis*. *HbERF1*, *HbERF2*, *HbERF3* and *HbRAV1* genes were suggested to be induced by JA in bark during JA-induced laticifer differentiation [87]. According to our analysis, *HbERF1*, *HbERF2* and *HbERF3* genes corresponded to *HbERF-VIIa3*, *HbERF-VIIa17* and *HbERF-VIIa1* in our classification with 99%, 98%, 99% identity, respectively. The *HbCBF1* gene [88], and the *HbCBF2* gene [89] have been reported to be regulated by cold and drought stresses, like other members of the DREB subfamily. We classified these genes in group III. The *HbCBF1* gene corresponded to the *HbERF-IIIc1* gene with a identity of 100%, and the *HbCBF2* gene to the *HbERF-IIIb2* gene with 82% identity. Another member of the AP2/ERF superfamily is the *HbEREBP1* gene recently identified by Chen *et al* from *Hevea* laticifers [5]. This gene was down-regulated by tapping and mechanical wounding in laticifers from adult trees, and was also regulated by both exogenous ethephon or methyl jasmonate treatments. This suggests that the *HbEREBP1* gene may be a negative regulator of defence mechanisms in laticifers [5]. The *HbEREBP1* gene corresponded to the *HbERF-VIIIa12* gene with 100% identity in our analysis.

Six new microRNAs are predicted to inhibit *Hevea* AP2/ERF transcripts

The mode of action of miR172-regulated *AP2* genes has been well described in reproductive and vegetative organs as well as in the transition of developmental phases [43, 90], where multiple feedback loops involve the microRNAs miR156e targeting Squamosa Promoter Binding Protein-like (SPL) and miR172b targeting AP2 [91]. Seven gymnosperm AP2 homologs were found to contain a sequence corresponding to miR172 with an average similarity of approximately 84.4%, suggesting that mechanisms regulating gene expression using microRNAs have been conserved over the three hundred million years since the divergence of gymnosperm and flowering plant lineages [59]. The cleavage site of miR172 is conserved between plant lineages and is located between the second AP2 domain and the 3′ terminus [59]. This site is also observed in *Hevea brasiliensis*. However, miR172 regulates flowering time by down-regulating *AP2-like* target genes by a translational mechanism rather than by RNA cleavage [90], and could explain our failure in

detecting cleaved *HbAP2-18* and *HbAP2-20* transcripts (data not shown). In addition to miR172, six other microRNAs (miR156, miR159, miR393, miR395, miR396, miR408) were predicted to inhibit *Hevea* transcripts of twelve *HbAP2/ERF* genes. Four microRNAs (miR159, miR395, miR396, miR408) are predicted to inhibit tissue-specific transcripts *HbERF-IXc2* in roots, *HbERF-IXc3* in leaves, *HbRAV-2* in roots, *HbERF-IIId1* in bark, *HbERF-VIIa9* in embryos and *HbERF-VIIa13* in roots. Of the *AP2/ERF* genes expressed in latex, none of them were predicted to be regulated by microRNAs. To our knowledge, no potential cleavage sites into the AP2 domain and in other parts of the CDS have been reported yet for these new microRNAs.

Conclusions

Our study led to the identification of 173 AP2 domain-containing transcripts, of which 31 had a partial domain in the rubber tree. We have proposed an optimized alignment and classification method enabling the use of NGS data with repeatable outputs. *In silico* analysis of transcript accumulation led to the prediction that ERF genes had a major role in laticifers. A comparison with *Populus* and *Vitis* did not provide any specific features for woody species as assumed earlier, but the AP2 family appeared to be well represented for these species. Several *AP2/ERF* genes highly expressed in latex could be related to a specific function in *Hevea brasiliensis.* Further studies focusing on latex cells should provide a clearer understanding of the involvement of genes from the AP2/ERF superfamily in the regulation of latex production and latex cell differentiation.

Methods

Plant material

The clone PB 260 plant material was produced by somatic embryogenesis at the CIRAD laboratory [92, 93]. Total mRNAs were isolated from different tissues. TheEmbryo tissues sample was a mix of embryogenic callus and somatic embryos. Leaf, root and bark tissues were taken from *in vitro* plantlets and grown for up to 1 month, 1 year and 3 years after acclimatization. At each time point, *in vito* plants were treated for 4 and 24 h with 1 ppm of ethylene or by wounding, or by water deficiency up to wilting leaves (Duan, 2010; Putranto, 2011). Conventional budded plants were produced at the IRRI Sembawa Centre. Leaf, root and bark tissues were also taken from three-month-old budded plants treated by wounding or ethylene. Latex was sampled from 5-year-old trees that were either untapped, tapped or both tapped and stimulated with 2.5% ethephon before RNA isolation.

Total RNA isolation

Leaves, bark, roots, somatic embryos and callus were frozen in liquid nitrogen and stored in the freezer at -80°C pending total RNA extraction. Total RNA was extracted using the caesium chloride cushion method adapted from Sambrook [94] by Duan and coll. [95]. One gram of fresh matter was ground and transferred to a tube containing 30 ml of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone and 1% ß-mercapto-ethanol. After homogenization, tubes were kept on ice and then centrifuged at 10,000 g at 4°C for 30 minutes. The supernatant was transferred to a new tube containing 8 ml of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 89,705 g at 20°C for 20 hours. The supernatant and caesium cushion were discarded whilst the RNA pellet

was washed with 70% ethanol. After 30 minutes of air drying, the pellet was dissolved in 200 µl of sterile water. RNAs were conserved at -80°C. For latex, samples were first centrifuged for 30 min at 15,000 g. The aqueous fraction was treated with a phenol:chloroform solution twice, including centrifugation for 15 min at 10,000 g at 4°C. RNAs were precipitated overnight at 4°C after the addition of 8M LiCl to the cleaned aqueous phase. After centrifugation for 30 min at 10,000 g at 4°C, the RNA pellet was resuspended in 400 uL of DEPC water on ice and then treated with a phenol:chloroform solution twice. The RNAs were finally precipitated with 1/10 volume Na acetate 3M pH 5.2 and 3 volumes of absolute ethanol. After centrifugation, the RNA pellet was resuspended and the solution kept at -80°C.

Sequencing techniques and contig assembly

Total RNA samples from plants at different stages of development and having undergone different types of stress were pooled together for five tissues separately (leaves, bark, latex, embryos and roots), in order to have the most complete representation of the expressed genome in each tissue. Single-strand cDNA was synthesised from pooled RNA samples. Pyrosequencing was carried out using GS-FLX 454 (Roche Applied Science) by the GATC-Biotech company in Germany. A half-run of 454 sequencing generated more than 500,000 reads for each library. Reads were analysed using the ESTtik tool (Expressed Sequence Tag Treatment and investigation kit) [96] modified for the analysis of 454 data. Reads were first cleaned to avoid miss-assembly by discarding sequences that were both lower than 120 bp and of low quality. We then discarded non-coding reads by comparing the reads against the fRNAdb database using the Megablast algorithm with an e-value cutoff of 1e-20 [97]. More than 400,000 cleaned reads were obtained for each library. Reads were then assembled in contigs using the TGICL program integrated in the ESTtik pipeline. Clustering was carried out for reads with an overlap of at least 60 bp and 94% identity between reads. The second step was an assembly of reads from each cluster with greater stringency: the length of sequence overlap was then 60 bp with 95% identity between reads. The transcript sequence database consisted of contigs. An automatic annotation of each contig was attempted using the BLAST algorithm to find similar sequences using the *Arabidopsis thaliana* peptide database Tair9, the Uniprot databases Swissprot and TrEMBL, the non-redundant protein sequence database NR and the nucleotide sequence database NT from GenBank. Contigs were then annotated with Gene Ontology terms using Blast2GO on our Blast results [98]. We predicted peptide sequences for each contig using the Prot4EST pipeline [99]. The peptide sequences were then annotated comparing the sequences on the InterPro signature database using the InterProScan web service [100]. A first assembly set was generated from reads of each tissue separately to create tissue-specific transcript databases. For the leaf, bark, latex, embryogenic tissue, and root databases, the number of contigs was 29910, 45114, 29016, 44988 and 50146 respectively. The reads of all 5 tissue specific libraties were then collected together to generate one general transcript sequence database for *Hevea* clone PB260, subsequently called the global database. A combination of contigs??? for all tissues provided 94,981 unique transcripts that overestimated the number of genes in *Hevea* by considering alternative splicing and short transcripts that could combine together.

The global database

All five tissue specific EST libraries from NGS were mixed together to generate a global libraries to avoid the repetition of the information on the identification of AP2/ERF family in *Hevea brasiliensis.*

Identification of AP2 domain-containing contigs

Firstly, we downloaded the AP2 domain of the 147 *Arabidopsis thaliana AP2/ERF* genes from the *Arabidopsis* Transcription Factor Database (ArabTFDB) (http://plntfdb.bio.uni-potsdam.de/v3.0/). BLASTX (Basic Local Alignment Search; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out using the 147 AtAP2 domain amino acid sequences as protein subjects and nucleic acid sequences of contigs assembled in the HbPB260 transcript database as the query. Conversely, TBLASTN was carried out using nucleic acid sequences of contigs as the subject and the 147 AtAP2 domain amino acid sequences as the query. The two BLAST files were combined in order to keep information obtained in both BLASTX analyses. The AP2 domain was identified in each AP2 domain-containing contig after their translation using Pro4EST (http://www.nematodes.org/bioinformatics/prot4EST/index.shtml) or FrameDP (http://iant.toulouse.inra.fr/FrameDP/) and an analysis performed with the Conserved Domain Database (CDD) and Resource Group on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.). The method led to the identification of contigs with a full and partial AP2 domain.

Phylogenetic analysis of the AP2 domain from putative *AP2/ERF* genes

A multiple alignment analysis was performed on full-length AP2 domain sequences from *Hevea, Arabidopsis* and *Gossypium*. Phylogenetic trees were firstly generated with the Neighbour-Joining method for *Hevea, Arabidopsis* and *Gossypium* (data not shown) in order to classify the groups. The full AP2-domain sequences derived from 142 *H. brasiliensis* AP2-domain proteins of around 60 amino acids were then aligned using MUSCLE software [42, 101], which uses a progressive multiple alignment method. The alignment was curated by Gblocks software [102], searching for at least 10-amino-acid-long conserved blocks, and the block with 57 amino acids was extracted. This block of 57 amino acids was used to construct the phylogenetic tree using PhyML software [39], which implements a maximum likelihood tree reconstruction method, using the LG+gamma model, starting from a BioNJ tree [103]. The tree was drawn and displayed with the Dendroscope program, and rooted on the branch separating the AP2 and RAV family from the rest of the tree. Branch supports were computed using the aLRT-SHlike method, and those under 0.70 were discarded. For genes of the AP2 family having two AP2 domains, the sequence of the first AP2 domain (repeat-1 or R1) was preferentially selected for alignment. For three partial transcripts, the second AP2 domain (repeat-2 or R2) was chosen for alignment.

Comparison of the classification between various species

Genes from the AP2/ERF superfamily are listed in tables 1, 2 and 3 from publications on *Arabidopsis thaliana* [24], *Populus trichicarpa* [25], *Vitis vinifera* [28], *Solanum lycopersicum* [51], *Gossypium hirsutum/raimondii/arboretum* [29] and *Triticum aestivum* [23]. For *Hevea brasiliensis*, the classification of the AP2/ERF superfamily was based on the phylogenetic analysis presented in this paper. In addition to data

from the phylogenetic analysis, contigs corresponding to partial transcripts harbouring either a partial AP2 domain sequence or only one AP2 domain instead of two for genes of the AP2 family are included in the presentation of Table 1.

Identification of conserved motifs and specific amino acid residues

AP2 domain amino acid sequences from the *Hevea* ERF genes were aligned using CLUSTALX. Conserved residues observed in *Hevea* sequences were compared with those of other species such as *Gossypium* and *Arabidopsis* in order to identify ERF group-specific residues [29, 66].

Evaluation of transcript abundance based on the read number for each AP2/ERF contigs

An *in silico* analysis was used to compare the relative transcript abundances for the *AP2/ERF* genes in various tissues, based on transcript presence in the *Hevea* tissue-specific transcript sequence databases for bark, leaves, latex, embryos and roots. Perl script was used to parse the alignment .ace file provided by the MIRA assembler in order to count the number of reads for each transcript and to identify the number of reads for each tissue (bark, leaves latex, embryos and roots). Tissue-specific genes were identified when transcripts existed in only one tissue library.

Prediction of microRNA-targeted *AP2/ERF* genes

Deep sequencing of *Hevea brasiliensis* was performed with Solexa/Illumina technology and led to the identification of miRNA sequences conserved between plant species and putative novel miRNAs specific to *Hevea* [104] using the LeARN pipeline [105].The AP2/ERF sequences from *Hevea* were scanned with conserved and non-conserved miRNA sequences using both psRNATarget server (http://plantgrn.noble.org/psRNATarget/, [106] and Miranda, which is included in the LeARN pipeline [105] with custom parameters (gap_value=2, mm_value=1, gu_value=0.5, score_threshold=3, min_length_alignment=18 and no_mismatch_positions=10;11).

List of abbreviations used

AIL: AIntegumenta-like; ANT: AINTEGUMENTA; AP2: APETALA 2; CBF: Cold responsive element binding factor; DREB: Drought Responsive Element Binding protein; ERF: Ethylene Responsive Factor; RAP2: Related to APETALA2; RAV: Related to ABI3/VP1; RTqPCR: Real Time quantitative Polymerase Chain Reaction.

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Authors' contributions
XA and MS carried out contig assembly and generated the transcript sequence database. CFD and JFD carried out the phylogenetic analysis. CFD and AC studied the features of the AP2 domain. MR carried out RNA isolations. VG and JL identified microRNAs and their target genes. PM and CFD planned the experiments. PM, CFD, JFD and MS participated in drafting the manuscript. All the authors read and approved the final manuscript.

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Table 1. Summary of the classification of the *Hevea brasiliensis* AP2/ERF superfamily compared with several species, *Arabidopsis thaliana*, *Populus trichicarpa*, *Vitis vinifera*, *Solanum lycopersicum*, *Gossypium hirsutum/raimondii/arboretum*, *Triticum aestivum*. *AP2/ERF* gene sequences were obtained either after genome sequencing or transcriptome sequencing.

Family	Conserved domain		Number of members in each AP2/ERF gene family from different species						
			Genome			Transcriptome			
			Arabidopsis	*Populus*	*Vitis*	*Solanum*	*Gossypium*	*Triticum*	*Hevea*
AP2	Double AP2/ERF domain	**Total**	18	26	20	16	11	9	25
		Two full-length domains	14	26	20	11	11	9	9
		One full-length domain plus one partial domain	-	-	-	-	-	-	4
		Two partial domains						-	2
	One AP2/ERF domain	One full-length domain	4	-	-	5	-	-	7
		One partial domain	-	-	-	-	-	-	3
ERF	Single AP2/ERF domain	**Total**	122	169	122	93	200	104	141
		Full-length domain	122	169	122	85	200	104	115
		Partial domain	-	-	-	8	-	-	26
RAV	Single AP2/ERF domain plus one B3 domain		6	6	6	3	7	3	4
SOLOIST	Short single AP2/ERF domain		1	1	1	-	-	1	3
TOTAL NUMBER			147	202	149	112	218	117	173

Table 2. Correspondence between Nakano's and Sakuma's classification methods for the *Hevea brasiliensis* AP2/ERF superfamily genes. In this presentation, AP2/ERF genes with at least one full-length domain were kept.

Classification of the *H. brasiliensis* AP2/ERF superfamily				
Nakano's method		Sakuma's method		
Family	Group	Subfamily	Subgroup	Number of genes
AP2 family	-	AP2	-	20
ERF	I to IV	DREB	A-1 to A-6	33
	V to X	ERF	B-1 to B-6	76
	VI-L & Xb-L		B-6	6
RAV	-	RAV	-	4
SOLOIST	-	SOLOIST	-	3
Total				142

Table 3. Classification of the *Hevea brasiliensis* ERF family based on the phylogenetic analysis compared with the *Arabidopsis thaliana*, *Gossypium hirsutum*, *Populus trichocarpa* and *Vitis vinifera* species according to Nakano's method.

	Number of members in each group of the ERF family for different species				
Group	*Arabidopsis*	*Gossypium*	*Populus*	*Vitis*	*Hevea*
I	10	14	5	5	12
II	15	20	20	8	7
III	23	35	35	22	11
IV	9	2	6	5	3
V	5	3	10	11	5
VI	8	12	11	5	5
VII	5	33	6	3	23
VIII	15	33	17	11	15
IX	17	39	42	40	19
X	8	9	9	10	9
VI-L	4	0	4	2	6
Xb-L	3	0	4	0	0
Total	122	200	169	122	115

Table 4. Group-specific residues present in the AP2 domain representative of each family and each ERF group. The presented residues were the most conserved for the three compared species (*Hevea brasiliensis, Arabidopsis thaliana, Gossypium hirsutum*). (*) *Hevea*-specific residue compared to the other two species. (+) Additional amino acid residue compared to the other groups. (X) not conserved residue.

Family	Group	Group-specific residues			Conservation
		Arabidopsis	*Gossypium*	*Hevea*	(%)
AP2		T150/A150	T150/A150	T150/A150	100
ERF	I	R168	R168	R168	100
	II	S175 -Y176	S175- Y176	S175 -Y176	97-97
	III	M181	M181	M181	100
	IV	G168	G168	G168	100
	V	K168	K168	K168	100
	VI	P153	P153	P153	100
	VI-L	K189	K189	K189 - M196*	100
	VII	I149 - G168 - V169	I149 - G168 - V169	I149 - G168 - V169	100-100-100
	VIII	P153 - K168	P153 - K168	P153 - K168	98-98
	IX	+X167	+X167	+X167	100
	X	A168	A168	A168	100
RAV		V150	V150	V150	100

Table 5. Read distribution list for the 59 AP2/ERF latex-expressed transcripts.

Gene	Contig accession number in the global library	Total reads	Read number from tissue-specific libraries				
		(No)	Latex	Bark	Leaf	Root	Embryo
HbERF-Ib2	*hevea*_454_rep_c703	323	19	66	16	172	50
HbERF-Ib3	*hevea*_454_rep_c1969	163	4	28	3	115	13
HbERF-Ib4	*hevea*_454_rep_c9307	104	61	23	1	18	1
HbERF-Ib5	*hevea*_454_rep_c4396	107	12	24	7	38	26
HbERF-Ib6	*hevea*_454_rep_c5080	62	1	31	7	21	2
HbERF-Ib7	*hevea*_454_rep_c15743	41	11	9	4	8	9
HbERF-IIa1	*hevea*_454_rep_c8625	56	27	0	12	8	9
HbERF-IIb3	*hevea*_454_c54212	22	17	0	1	0	4
HbERF-IIb4	*hevea*_454_rep_c20637	29	28	0	1	0	0
HbERF-IIb5	*hevea*_454_rep_c12007	27	27	0	0	0	0
HbERF-IIIc1	*hevea*_454_rep_c22270	17	5	3	3	5	1
HbERF-IVa1	*hevea*_454_rep_c10035	24	4	3	0	14	3
HbERF-IVa2	*hevea*_454_rep_c6678	61	15	15	3	21	7
HbERF-IVa3	*hevea*_454_rep_c22480	8	1	3	0	4	0
HbERF-Va2	*hevea*_454_c35642	5	2	2	0	0	1
HbERF-Vb2	*hevea*_454_rep_c37727	9	1	1	0	2	5
HbERF-VI3	*hevea*_454_rep_c37430	6	1	0	0	5	0
HbERF-VI5	*hevea*_454_c22933	14	1	2	1	9	1
HbERF-VI-L1	*hevea*_454_rep_c17780	27	5	10	2	7	3
HbERF-VI-L2	*hevea*_454_rep_c30774	19	2	4	2	8	3
HbERF-VI-L3	*hevea*_454_rep_c6308	84	3	31	2	30	18
HbERF-VI-L4	*hevea*_454_rep_c13569	33	9	16	4	3	1
HbERF-VIIa3	*hevea*_454_rep_c18953	82	22	16	2	23	19
HbERF-VIIa4	*hevea*_454_rep_c1157	247	164	11	1	39	32
HbERF-VIIa7	*hevea*_454_rep_c945	761	232	169	35	231	94
HbERF-VIIa11	*hevea*_454_rep_c1772	184	20	23	49	60	32
HbERF-VIIa12	*hevea*_454_rep_c110	1200	225	282	42	455	196
HbERF-VIIa13	*hevea*_454_rep_c8113	95	11	29	2	26	27
HbERF-VIIa15	*hevea*_454_rep_c74800	3	1	2	0	0	0
HbERF-VIIa17	*hevea*_454_rep_c7333	57	12	17	16	0	12
HbERF-VIIa20	*hevea*_454_rep_c710	228	49	68	16	59	36
HbERF-VIIa21	*hevea*_454_rep_c2193	82	1	26	3	28	24
HbERF-VIIIa1	*hevea*_454_rep_c5746	54	23	10	4	7	10
HbERF-VIIIa2	*hevea*_454_rep_c3523	87	39	15	6	14	13
HbERF-VIIIa3	*hevea*_454_rep_c6880	57	18	14	2	19	4
HbERF-VIIIa4	*hevea*_454_rep_c2227	174	84	23	32	14	21
HbERF-VIIIa5	*hevea*_454_rep_c8903	41	13	0	6	12	10
HbERF-VIIIa6	*hevea*_454_rep_c37861	14	7	0	0	4	3
HbERF-VIIIa7	*hevea*_454_rep_c16802	18	3	2	3	4	6
HbERF-VIIIa8	*hevea*_454_rep_c10220	29	2	3	16	6	2
HbERF-VIIIa9	*hevea*_454_rep_c4136	83	19	8	33	13	10
HbERF-VIIIa10	*hevea*_454_rep_c1402	138	2	32	68	30	6
HbERF-VIIIa12	*hevea*_454_rep_c4279	54	8	12	20	12	2
HbERF-VIIIa14	*hevea*_454_rep_c34700	8	1	1	2	1	3
HbERF-IXa2	*hevea*_454_rep_c14033	23	1	9	7	3	3
HbERF-IXb2	*hevea*_454_c19752	13	1	4	7	0	1
HbERF-IXb4	*hevea*_454_rep_c12196	32	7	9	14	1	1
HbERF-IXc4	*hevea*_454_rep_c3873	57	5	25	5	22	0
HbERF-Xa4	*hevea*_454_rep_c67487	3	1	0	0	1	1
HbERF-Xa5	*hevea*_454_rep_c26270	9	1	7	0	1	0
HbERF-Xa6	*hevea*_454_rep_c36967	9	1	3	0	5	0
HbAP2-4	*hevea*_454_c60993	4	3	1	0	0	0
HbAP2-6	*hevea*_454_rep_c16078	31	24	0	0	4	3
HbAP2-10	*hevea*_454_rep_c22185	28	2	1	3	17	5
HbAP2-16	*hevea*_454_rep_c23347	9	3	1	4	1	0
HbAP2-18	*hevea*_454_rep_c16704	31	1	4	6	12	8
HbRAV-3	*hevea*_454_rep_c8782	62	19	16	10	9	8
HbSoloist2	*hevea*_454_rep_c8142	50	18	3	8	10	11
HbSoloist3	*hevea*_454_rep_c46638	10	2	0	6	1	1

Table 6. List of putative targets of conserved miRNAs and their mode of inhibition predicted both by psRNATarget server (http://plantgrn.noble.org/psRNATarget/) and by Miranda included in the LeARN pipeline. Target accessibility is represented as the maximum energy needed (UPE) to unpair the secondary structure around target site on target mRNA. The lower the energy the greater the possibility that small RNA is able to contact (and cleave) target mRNA. The lower the free energy the greater the possibility that small RNA is able to contact target mRNA.

microRNA family		Target gene		UPE	Free energy	miRNA size	miRNA aligned fragment	Target aligned fragment	Inhibition	MiR position	Position binding of CDS
Name	Accession No	Gene name	Contig accession No			(No bases)				(bp)	
miR156	acc_480780	*HbAP2-9*	*hevea*_454_rep_c24306	24.478	-24.41	23	*UGACAGAAGAGAGAGAGCACAUC*	*UACUCUCUUUUUUCUGCCAA*	Cleavage	1001-1020	Inside CDS After AP2 domain
miR159	acc_19665	*HbERF-IXc2*	*hevea*_454_c72747	13.691	-25.86	23	*UUUUGAUUGAAGGGAGCUCUAAU*	*GAGCACCCUUCAAUUAAG*	Cleavage	297-314	Inside CDS After AP2 omain
miR159	acc_19665	*HbERF-VI-L1*	*hevea*_454_rep_c17780	16.359	-27.80	23	*UUUUGAUUGAAGGGAGCUCUAAU*	*GUUCUAGCUUCCUUCAAGCAGAG*	Cleavage	50-72	outside CDS,5'UTR before AP2 domain
miR172	acc_502684	*HbAP2-18*	*hevea*_454_rep_c22185	15.621	-21.57	21	*UAGCAUCAUCAAGAUUUUUAU*	*AAGAGAAUCCUGAUGAUGCUG*	Cleavage	1473-1493	Inside CDS After AP2 domain
miR172	acc_502684	*HbAP2-20*	*hevea*_454_rep_c45080	17.625	-24.25	21	*UAGCAUCAUCAAGAUUUUUAU*	*AUGAGAAUCCUGAUGAUGCUG*	Cleavage	990-1010	Inside CDS After AP2 domain
miR393	acc_112860	*HbAP2-4*	*hevea*_454_c60993	24.258	-22.79	25	*UUCCAAAGGGAUCGCAUUGAUUAUC*	*AGCAAUGUUAUUCCUUUGGC*	Cleavage	198-217	Inside CDS Before AP2 domain
miR395	acc_262739	*HbERF-IXc3*	*hevea*_454_c37716	15.053	-24.47	25	*CUGAAGUGUUUGGGGGACCUCAUC*	*GAGAAAGUUCUCCAAUCACUUCAA*	Translation	243-266	Inside CDS After AP2 domain
miR396	acc_7978	*HbRAV-2*	*hevea*_454_rep_c13430	22.097	-22.40	24	*CCACAGCUUUCUUGAACUGCAAUC*	*GAGUUCAAGAAAGCGGUU*	Cleavage	562-579	Inside CDS After AP2 domain
miR396	acc_490677	*HbERF-IIId1*	*hevea*_454_rep_c79493	12.805	-13.21	24	*UCCCACAGCUUUCUUGAACUUAUC*	*AAUUGAAGAAAACUGUGCAG*	Translation	107-126	Inside CDS BeforeAP2 domain
miR408	acc_135004	*HbERF-IXb1*	*hevea*_454_c13287	14.501	-23.60	23	*UGCACUGCCUCUUCCCUGCCAUC*	*AAGAGAAGAGGCAGUACA*	Cleavage	118-135	Inside CDS Cut AP2 domain
miR408	acc_184014	*HbERF-VIIa9*	*hevea*_454_rep_c64305	24.62	-28.45	24	*UACACUGCCUCUUCCCUGGCUAUC*	*UGCGAGCAGGAGGAGAGGCAGU*	Cleavage	207-228	Inside CDS BeforeAP2 domain
miR408	acc_184014	*HbERF-VIIa13*	*hevea*_454_rep_c8113	24.62	-28.45	24	*UACACUGCCUCUUCCCUGGCUAUC*	*UGCGAGCAGGAGGAGAGGCAGU*	Cleavage	518-539	Inside CDS BeforeAP2 domain

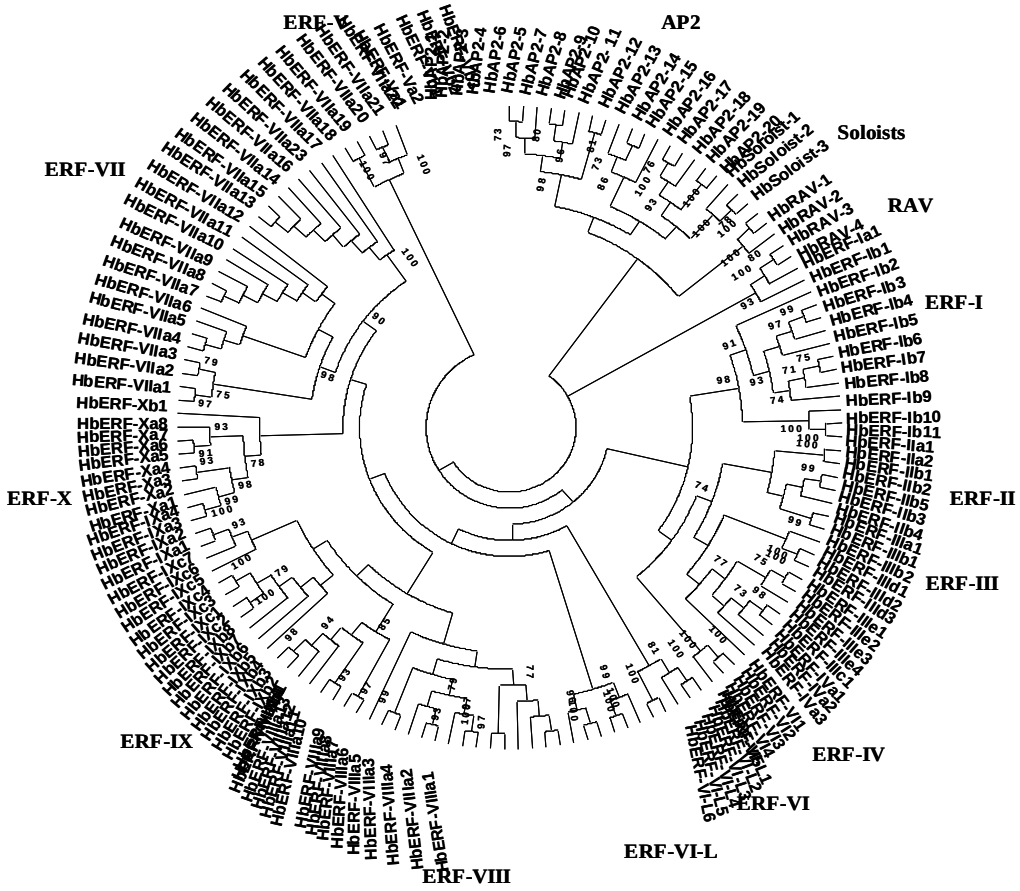


Figure 1. Phylogenetic tree of *Hevea brasiliensis* AP2/ERF proteins. The amino acid sequences of the AP2 domain were aligned using Muscle (Supplemental Fig. 1), and the phylogenetic tree was constructed using the PhyML with a LG+T model. The name of total 142 AP2/ERF proteins of all families and groups are indicated.

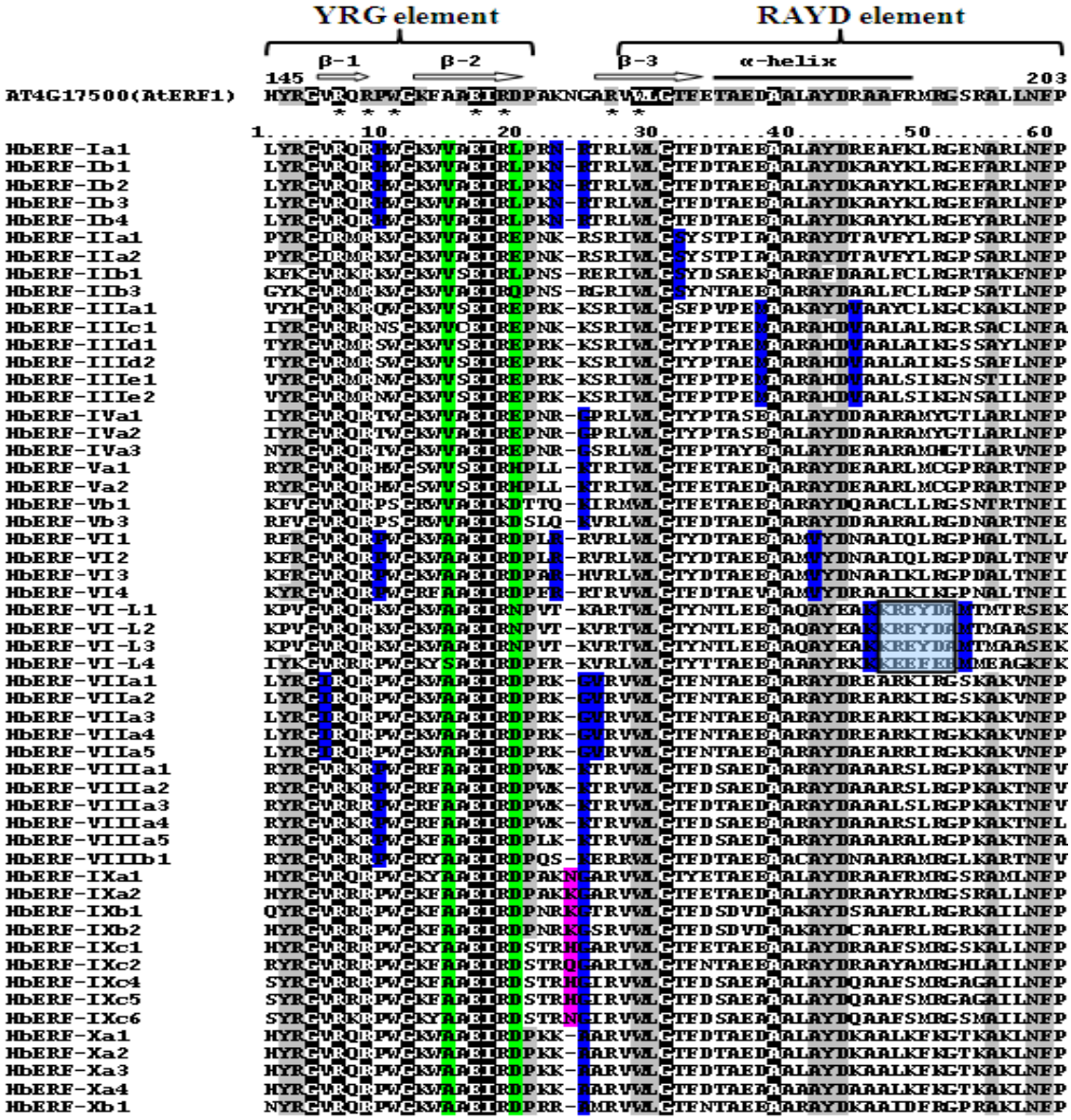


Figure 2 Alignment of the AP2/ERF domains in *H.brasiliensis* (55 representative members). Black and light gray shading indicate identical and conserved amino acid residues, respectively. Light blue shading indicates conserved amino acid residues in group VI-L. Green colour indicates the V14, E19 residue conserved (Yoh Sakuma, 2002); blue colour indicates the residue conserved in each group individually; pink colour indicates the supplementary residue in group IX. The black bar and block arrows represent predicted a-helix and b-sheet regions, respectively, within the AP2/ERF domain (Allen et al., 1998). Asterisks represent amino acid residues that directly make contact with DNA (Allen et al., 1998). The YRG, RAYD elements are indicted according to (Okamuro, 1997).

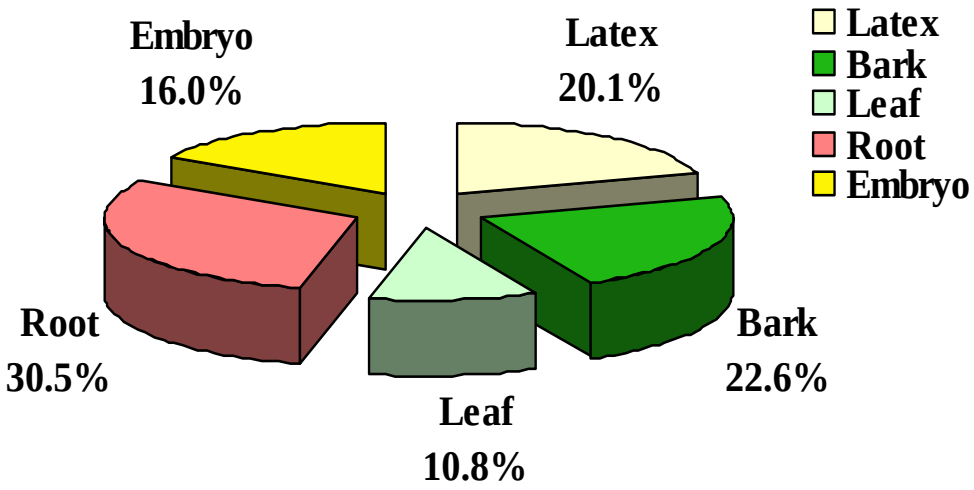
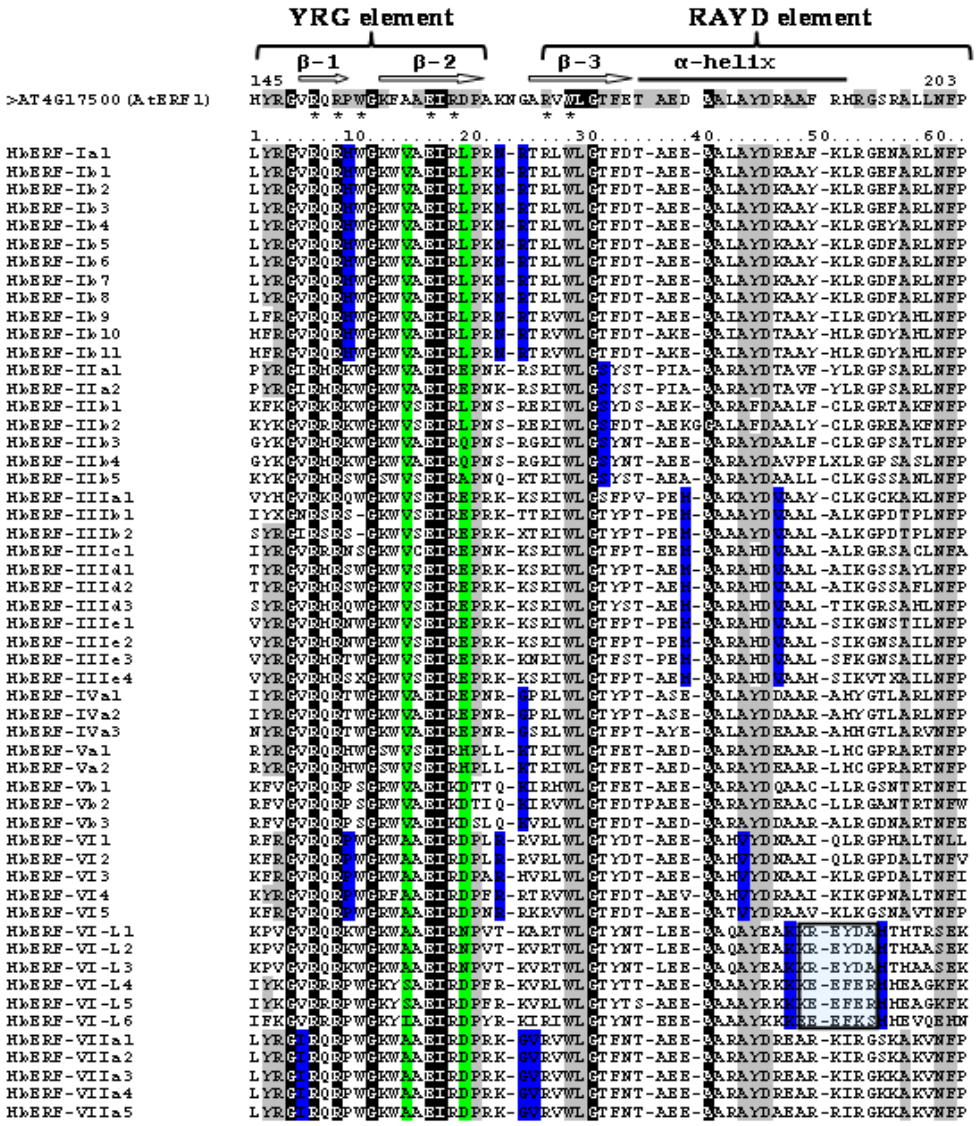
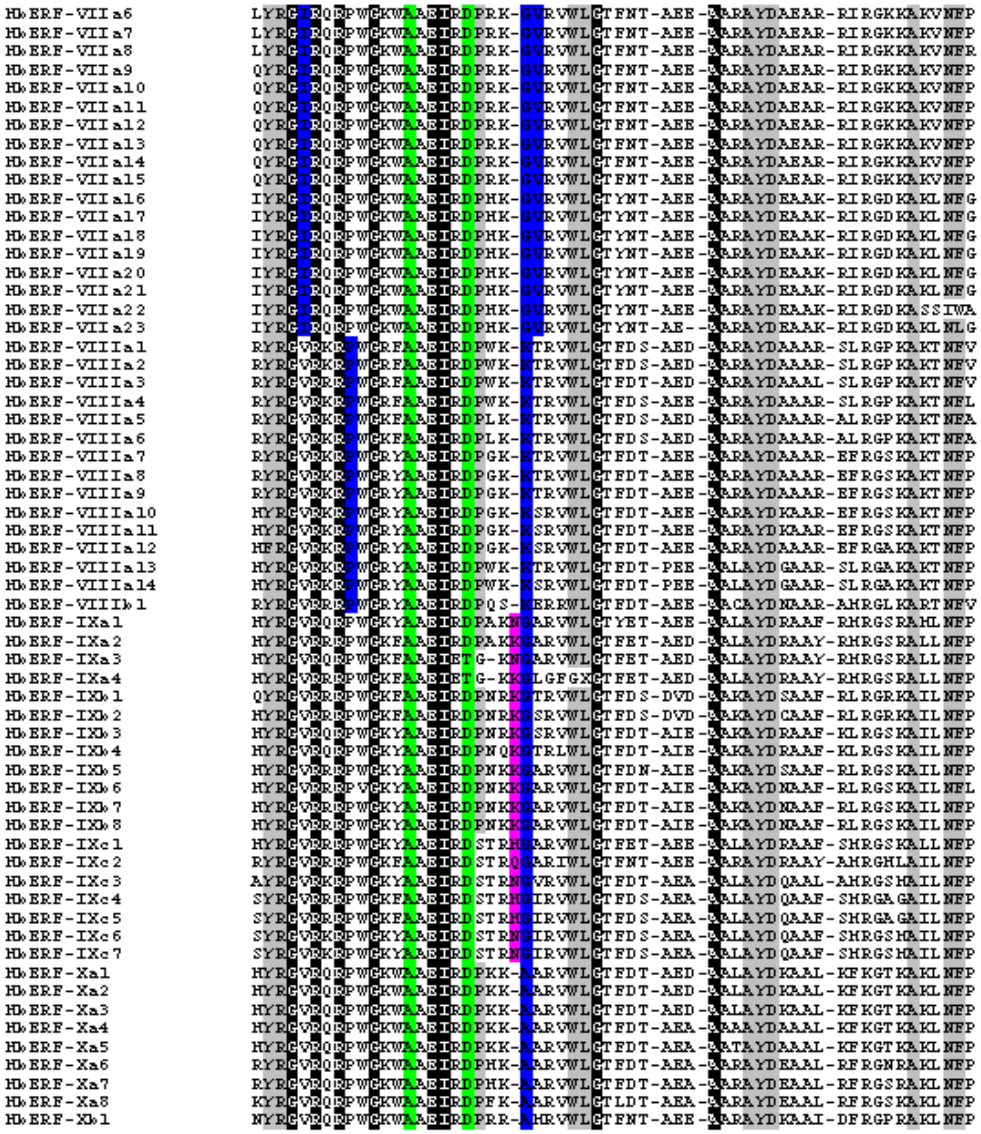


Figure 3. Distribution of AP2/ERF transcription factors transcripts in different tissues in *H. brasiliensis*. The expression profile was determined by analyzing the reads counts of AP2/ERF transcripts in five different tissues from *H. brasiliensis*



Supplementary Figure 1. Alignment of the AP2/ERF domains from 115 *H.brasiliensis* ERF family members. Black and light gray shading indicate identical and conserved amino acid residues, respectively. Light blue shading indicates conserved amino acid residues in group VI-L. Green color indicates the V14, E19 residue conserved (Yoh Sakuma,2002); blue color indicates the residue conserved in each group individually; pink color indicates the supplementary residue in group IX. The black bar and block arrows represent predicted a-helix and b-sheet regions, respectively, within the AP2/ERF domain (Allen et al., 1998). Asterisks represent amino acid residues that directly make contact with DNA (Allen et al., 1998). The YRG, RAYD elements are indicted according to (Okamuro, 1997).

Supplementary Figure 1 continued



Supplementary Figure 1 continuing. Alignment of the AP2/ERF domains from 115 *H.brasiliensis* ER family members. Black and light gray shading indicate identical and conserved amino acid residue respectively. Light blue shading indicates conserved amino acid residues in group VI-L. Green colo indicates the V14, E19 residue conserved (Yoh Sakuma,2002); blue color indicates the residue conserve in each group individually; pink color indicates the supplementary residue in group IX. The black bar ar block arrows represent predicted a-helix and b-sheet regions, respectively, within the AP2/ERF domai (Allen et al., 1998). Asterisks represent amino acid residues that directly make contact with DNA (Allen e al., 1998). The YRG, RAYD elements are indicted according to (Okamuro, 1997).

Supplementary Table 1

List of all AP2/ERF transcription factor genes identified in *Hevea brasiliensis*

Family	Group	Gene name	Global library contig name	AP2/ERF domain amino acid sequecnes in *Hevea brasiliensis*	Number of reads in the various tissue libraries					
					Total reads	Bark	Embryo	Latex	Leaf	Root
ERF	Ia	*HbERF-Ia1*	*hevea*_454_rep_c31876	LYRGVRQRHWGKWVAEIRLPRNRTRLWLGTFDTAEEAALAYDREAFKLRGENARLNFP	6	1	0	0	0	5
ERF	Ib	*HbERF-Ib1*	*hevea*_454_rep_c30352	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGEFARLNFP	7	6	0	0	0	1
ERF	Ib	*HbERF-Ib2*	*hevea*_454_rep_c703	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGEFARLNFP	323	66	50	19	16	172
ERF	Ib	*HbERF-Ib3*	*hevea*_454_rep_c1969	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGEFARLNFP	163	28	13	4	3	115
ERF	Ib	*HbERF-Ib4*	*hevea*_454_rep_c9307	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGEYARLNFP	104	23	1	61	1	18
ERF	Ib	*HbERF-Ib5*	*hevea*_454_rep_c4396	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGDFARLNFP	107	24	26	12	7	38
ERF	Ib	*HbERF-Ib6*	*hevea*_454_rep_c5080	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGDFARLNFP	62	31	2	1	7	21
ERF	Ib	*HbERF-Ib7*	*hevea*_454_rep_c15743	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGDFARLNFP	41	9	9	11	4	8
ERF	Ib	*HbERF-Ib8*	*hevea*_454_rep_c87922	LYRGVRQRHWGKWVAEIRLPKNRTRLWLGTFDTAEEAALAYDKAAYKLRGDFARLNFP	2	1	0	0	0	1
ERF	Ib	*HbERF-Ib9*	*hevea*_454_c35100	LFRGVRQRHWGKWVAEIRLPRNRTRVWLGTFDTAEEAAIAYDTAAYILRGDYAHLNFP	6	0	0	0	0	6
ERF	Ib	*HbERF-Ib10*	*hevea*_454_rep_c41155	MFRGVRQRHWGKWVAEIRLPRNRTRVWLGTFDTAKEAAIAYDTAAYMLRGDYAHLNFP	3	0	0	0	0	3
ERF	Ib	*HbERF-Ib11*	*hevea*_454_rep_c35059	MFRGVRQRHWGKWVAEIRLPRNRTRVWLGTFDTAKEAAIAYDTAAYMLRGDYAHLNFP	5	0	0	0	0	5
ERF	IIa	*HbERF-IIa1*	*hevea*_454_rep_c8625	PYRGIRMRKWGKWVAEIREPNKRSRIWLGSYSTPIAAARAYDTAVFYLRGPSARLNFP	56	0	9	27	12	8
ERF	IIa	*HbERF-IIa2*	*hevea*_454_rep_c7563	PYRGIRMRKWGKWVAEIREPNKRSRIWLGSYSTPIAAARAYDTAVFYLRGPSARLNFP	40	15	1	0	7	17
ERF	IIb	*HbERF-IIb1*	*hevea*_454_rep_c18428	KFKGVRKRKWGKWVSEIRLPNSRERIWLGSYDSAEKAARAFDAALFCLRGRTAKFNFP	25	2	2	0	21	0
ERF	IIb	*HbERF-IIb2*	*hevea*_454_rep_c19971	KYKGVRRRKWGKWVSEIRLPNSRERIWLGSFDTAEKGGALAFDAALYCLRGREAKFNFP	14	8	0	0	5	1
ERF	IIb	*HbERF-IIb3*	*hevea*_454_c54212	GYKGVRMRKWGKWVAEIRQPNSRGRIWLGSYNTAEEAARAYDAALFCLRGPSATLNFP	22	0	4	17	1	0
ERF	IIb	*HbERF-IIb4*	*hevea*_454_rep_c20637	GYKGVRMRKWGKWVAEIRQPNSRGRIWLGSYNTAEEAARAYDAVPFLXLRGPSASLNFP	29	0	0	28	1	0
ERF	IIb	*HbERF-IIb5*	*hevea*_454_rep_c12007	KYKGVRMRSWGSWVSEIRAPNQKTRIWLGSYSTAEAAARAYDAALLCLKGSSANLNFP	27	0	0	27	0	0
ERF	IIIa	*HbERF-IIIa1*	*hevea*_454_rep_c58255	VYHGVRKRQWGKWVSEIREPRKKSRIWLGSFPVPEMAAKAYDVAAYCLKGCKAKLNFP	2	0	2	0	0	0
ERF	IIIb	*HbERF-IIIb1*	*hevea*_454_rep_c38925	IYXGNRSRSGKWVSEIREPRKTTRIWLGTYPTPEMAAAAYDVAALALKGPDTPLNFP	8	2	2	0	2	2
ERF	IIIb	*HbERF-IIIb2*	*hevea*_454_rep_c70889	SYRGIRSRSGKWVSEIREPRKXTRIWLGTYPTPEMAAAAYDVAALALKGPDTPLNFP	2	1	0	0	1	0
ERF	IIIc	*HbERF-IIIc1*	*hevea*_454_rep_c22270	IYRGVRRRNSGKWVCEIREPNKKSRIWLGTFPTEEMAARAHDVAALALRGRSACLNFA	17	3	1	5	3	5
ERF	IIId	*HbERF-IIId1*	*hevea*_454_rep_c79493	TYRGVRMRSWGKWVSEIREPRKKSRIWLGTYPTAEMAARAHDVAALAIKGSSAYLNFP	2	2	0	0	0	0
ERF	IIId	*HbERF-IIId2*	*hevea*_454_rep_c21603	TYRGVRMRSWGKWVSEIREPRKKSRIWLGTYPTAEMAARAHDVAALAIKGSSAFLNFP	11	6	0	0	3	2
ERF	IIId	*HbERF-IIId3*	*hevea*_454_c39573	SYRGVRMRQWGKWVSEIREPRKKSRIWLGTYSTAEMAARAHDVAALTIKGRSAHLNFP	5	0	0	0	2	3
ERF	IIIe	*HbERF-IIIe1*	*hevea*_454_rep_c4496	VYRGVRMRNWGKWVSEIREPRKKSRIWLGTFPTPEMAARAHDVAALSIKGNSTILNFP	52	29	11	0	1	11

ERF	IIIe	*HbERF-IIIe2*	*hevea*_454_rep_c7722	VYRGVRMRNWGKWVSEIREPRKKSRIWLGTFPTPEMAARAHDVAALSIKGNSAILNFP	41	15	10	0	1	15
ERF	IIIe	*HbERF-IIIe3*	*hevea*_454_rep_c58258	VYRGVRMRTWGKWVSEIREPRKKNRIWLGTFSTPEMAARAHDVAALSFKGNSAILNFP	3	0	1	0	1	1
ERF	IIIe	*HbERF-IIIe4*	*hevea*_454_rep_c45776	VYRGVRMRSXGKWVSEIREPRKKSRIWLGTFPTAEMAARAHDVAAMSIKVTXAILNFP	5	2	1	0	0	2
ERF	IVa	*HbERF-IVa1*	*hevea*_454_rep_c10035	IYRGVRQRTWGKWVAEIREPNRGPRLWLGTYPTASEAALAYDDAARAMYGTLARLNFP	24	3	3	4	0	14
ERF	IVa	*HbERF-IVa2*	*hevea*_454_rep_c6678	IYRGVRQRTWGKWVAEIREPNRGPRLWLGTYPTASEAALAYDDAARAMYGTLARLNFP	61	15	7	15	3	21
ERF	IVa	*HbERF-IVa3*	*hevea*_454_rep_c22480	NYRGVRQRTWGKWVAEIREPNRGSRLWLGTFPTAYEAALAYDEAARAMHGTLARVNFP	8	3	0	1	0	4
ERF	Va	*HbERF-Va1*	*hevea*_454_c59307	RYRGVRQRHWGSWVSEIRHPLLKTRIWLGTFETAEDAARAYDEAARLMCGPRARTNFP	3	0	0	0	1	2
ERF	Va	*HbERF-Va2*	*hevea*_454_c35642	RYRGVRQRHWGSWVSEIRHPLLKTRIWLGTFETAEDAARAYDEAARLMCGPRARTNFP	5	2	1	2	0	0
ERF	Vb	*HbERF-Vb1*	*hevea*_454_rep_c20790	KFVGVRQRPSGRWVAEIKDTTQKIRMWLGTFETAEEAARAYDQAACLLRGSNTRTNFI	14	0	1	0	0	13
ERF	Vb	*HbERF-Vb2*	*hevea*_454_rep_c37727	RFVGVRQRPSGRWVAEIKDTIQKIRVWLGTFDTPAEEAARAYDEAACLLRGANTRTNFW	9	1	5	1	0	2
ERF	Vb	*HbERF-Vb3*	*hevea*_454_rep_c54314	RFVGVRQRPSGRWVAEIKDSLQKVRLWLGTFDTAEDAARAYDDAARALRGDNARTNFE	2	0	0	0	0	2
ERF	VI	*HbERF-VI1*	*hevea*_454_rep_c51930	RFRGVRQRPWGKWAAEIRDPLRRVRLWLGTYDTAEEAAMVYDNAAIQLRGPHALTNLL	3	1	0	0	0	2
ERF	VI	*HbERF-VI2*	*hevea*_454_rep_c63953	KFRGVRQRPWGKWAAEIRDPLRRVRLWLGTYDTAEEAAMVYDNAAIQLRGPDALTNFV	3	0	2	0	0	1
ERF	VI	*HbERF-VI3*	*hevea*_454_rep_c37430	KFRGVRQRPWGKWAAEIRDPARHVRLWLGTYDTAEEAAMVYDNAAIKLRGPDALTNFI	6	0	0	1	0	5
ERF	VI	*HbERF-VI4*	*hevea*_454_c64026	KYRGVRQRPWGRFAAEIRDPFRRTRVWLGTFDTAEVAAMVYDRAAIKIKGPNALTNFI	3	0	1	0	0	2
ERF	VI	*HbERF-VI5*	*hevea*_454_c22933	KFRGVRQRPWGRWAAEIRDPNRRKRVWLGTFDTAEEAATVYDRAAVKLKGSNAVTNFP	14	2	1	1	1	9
ERF	VI-L	*HbERF-VI-L1*	*hevea*_454_rep_c17780	KPVGVRQRKWGKWAAEIRNPVTKARTWLGTYNTLEEAAQAYEAKKREYDAMTMTRSEK	27	10	3	5	2	7
ERF	VI-L	*HbERF-VI-L2*	*hevea*_454_rep_c30774	KPVGVRQRKWGKWAAEIRNPVTKVRTWLGTYNTLEEAAQAYEAKKREYDAMTMAASEK	19	4	3	2	2	8
ERF	VI-L	*HbERF-VI-L3*	*hevea*_454_rep_c6308	KPVGVRQRKWGKWAAEIRNPVTKVRTWLGTYNTLEEAAQAYEAKKREYDAMTMAASEK	84	31	18	3	2	30
ERF	VI-L	*HbERF-VI-L4*	*hevea*_454_rep_c13569	IYKGVRRRPWGKYSAEIRDPFRKVRLWLGTYTTAEEAAAAYRKKKEEFERMMEAGKFK	33	16	1	9	4	3
ERF	VI-L	*HbERF-VI-L5*	*hevea*_454_rep_c32440	IYKGVRRRPWGKYSAEIRDPFRKVRLWLGTYTSAEEAAAAYRKKKEEFERMMEAGKFK	11	8	0	0	0	3
ERF	VI-L	*HbERF-VI-L6*	*hevea*_454_c36326	IFKGVRRRPWGKYIAEIRDPYRKIRIWLGTYNTEEEAAAAYKKKEEEFKSMMEVQEHN	9	4	0	0	4	1
ERF	VIIa	*HbERF-VIIa1*	*hevea*_454_rep_c16874	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDREARKIRGSKAKVNFP	25	0	23	0	0	2
ERF	VIIa	*HbERF-VIIa2*	*hevea*_454_rep_c40731	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDREARKIRGSKAKVNFP	4	1	3	0	0	0
ERF	VIIa	*HbERF-VIIa3*	*hevea*_454_rep_c18953	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDREARKIRGKKAKVNFP	82	16	19	22	2	23
ERF	VIIa	*HbERF-VIIa4*	*hevea*_454_rep_c1157	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDREARKIRGKKAKVNFP	247	11	32	164	1	39
ERF	VIIa	*HbERF-VIIa5*	*hevea*_454_rep_c91560	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	2	0	1	0	0	1
ERF	VIIa	*HbERF-VIIa6*	*hevea*_454_rep_c91075	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	5	0	3	0	0	2
ERF	VIIa	*HbERF-VIIa7*	*hevea*_454_rep_c945	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	761	169	94	232	35	231
ERF	VIIa	*HbERF-VIIa8*	*hevea*_454_rep_c46995	LYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFR	4	2	1	0	0	1
ERF	VIIa	*HbERF-VIIa9*	*hevea*_454_rep_c64305	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	18	16	1	0	1	0

ERF	VIIa	*HbERF-VIIa10*	*hevea*_454_rep_c32212	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	2	0	0	0	0	2
ERF	VIIa	*HbERF-VIIa11*	*hevea*_454_rep_c1772	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	184	23	32	20	49	60
ERF	VIIa	*HbERF-VIIa12*	*hevea*_454_rep_c110	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	1200	282	196	225	42	455
ERF	VIIa	*HbERF-VIIa13*	*hevea*_454_rep_c8113	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	95	29	27	11	2	26
ERF	VIIa	*HbERF-VIIa14*	*hevea*_454_rep_c33173	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	6	1	0	0	0	5
ERF	VIIa	*HbERF-VIIa15*	*hevea*_454_rep_c74800	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAEEAARAYDAEARRIRGKKAKVNFP	3	2	0	1	0	0
ERF	VIIa	*HbERF-VIIa16*	*hevea*_454_rep_c5852	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	44	16	12	0	3	13
ERF	VIIa	*HbERF-VIIa17*	*hevea*_454_rep_c7333	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	57	17	12	12	16	0
ERF	VIIa	*HbERF-VIIa18*	*hevea*_454_rep_c46573	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	4	2	0	0	0	2
ERF	VIIa	*HbERF-VIIa19*	*hevea*_454_rep_c84232	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	2	1	0	0	0	1
ERF	VIIa	*HbERF-VIIa20*	*hevea*_454_rep_c710	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	228	68	36	49	16	59
ERF	VIIa	*HbERF-VIIa21*	*hevea*_454_rep_c2193	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	82	26	24	1	3	28
ERF	VIIa	*HbERF-VIIa22*	*hevea*_454_rep_c58062	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKASSIWA	2	0	0	0	1	1
ERF	VIIa	*HbERF-VIIa23*	*hevea*_454_rep_c88669	IYRGIRQRPWGKWAAEIRDPHKGVRVWLGTYNTAEAARAYDEAAKRIRGDKAKLNLG	4	1	0	0	0	3
ERF	VIIIa	*HbERF-VIIIa1*	*hevea*_454_rep_c5746	RYRGVRKRPWGRFAAEIRDPWKKTRVWLGTFDSAEDAARAYDAAARSLRGPKAKTNFV	54	10	10	23	4	7
ERF	VIIIa	*HbERF-VIIIa2*	*hevea*_454_rep_c3523	RYRGVRKRPWGRFAAEIRDPWKKTRVWLGTFDSAEDAARAYDAAARSLRGPKAKTNFV	87	15	13	39	6	14
ERF	VIIIa	*HbERF-VIIIa3*	*hevea*_454_rep_c6880	RYRGVRRRPWGRFAAEIRDPWKKTRVWLGTFDTAEDAARAYDAAALSLRGPKAKTNFV	57	14	4	18	2	19
ERF	VIIIa	*HbERF-VIIIa4*	*hevea*_454_rep_c2227	RYRGVRKRPWGRFAAEIRDPWKKTRVWLGTFDSAEEAARAYDAAARSLRGPKAKTNFL	174	23	21	84	32	14
ERF	VIIIa	*HbERF-VIIIa5*	*hevea*_454_rep_c8903	RYRGVRKRPWGKFAAEIRDPLKKTRVWLGTFDSAEDAARAYDAAARALRGPKAKTNFA	41	0	10	13	6	12
ERF	VIIIa	*HbERF-VIIIa6*	*hevea*_454_rep_c37861	RYRGVRKRPWGKFAAEIRDPLKKTRVWLGTFDSAEDAARAYDAAARALRGPKAKTNFA	14	0	3	7	0	4
ERF	VIIIa	*HbERF-VIIIa7*	*hevea*_454_rep_c16802	RYRGVRKRPWGRYAAEIRDPGKKTRVWLGTFDTAEEAARAYDAAAREFRGSKAKTNFP	18	2	6	3	3	4
ERF	VIIIa	*HbERF-VIIIa8*	*hevea*_454_rep_c10220	RYRGVRKRPWGRYAAEIRDPGKKTRVWLGTFDTAEEAARAYDAAAREFRGSKAKTNFP	29	3	2	2	16	6
ERF	VIIIa	*HbERF-VIIIa9*	*hevea*_454_rep_c4136	RYRGVRKRPWGRYAAEIRDPGKKTRVWLGTFDTAEEAARAYDAAAREFRGSKAKTNFP	83	8	10	19	33	13
ERF	VIIIa	*HbERF-VIIIa10*	*hevea*_454_rep_c1402	HYRGVRKRPWGRYAAEIRDPGKKSRVWLGTFDTAEEAARAYDKAAREFRGSKAKTNFP	138	32	6	2	68	30
ERF	VIIIa	*HbERF-VIIIa11*	*hevea*_454_rep_c64121	HYRGVRKRPWGRYAAEIRDPGKKSRVWLGTFDTAEEAARAYDKAAREFRGSKAKTNFP	2	2	0	0	0	0
ERF	VIIIa	*HbERF-VIIIa12*	*hevea*_454_rep_c4279	HFRGVRKRPWGRYAAEIRDPGKKSRVWLGTFDTAEEAARAYDAAAREFRGAKAKTNFP	54	12	2	8	20	12
ERF	VIIIa	*HbERF-VIIIa13*	*hevea*_454_rep_c20048	HYRGVRKRPWGRYAAEIRDPWKKTRVWLGTFDTPEEAALAYDGAARSLRGAKAKTNFP	16	4	3	0	8	1
ERF	VIIIa	*HbERF-VIIIa14*	*hevea*_454_rep_c34700	HYRGVRKRPWGRYAAEIRDPWKKSRVWLGTFDTPEEAALAYDGAARSLRGAKAKTNFP	8	1	3	1	2	1
ERF	VIIIb	*HbERF-VIIIb1*	*hevea*_454_rep_c11109	RYRGVRRRPWGRYAAEIRDPQSKERRWLGTFDTAEEAACAYDNAARAMRGLKARTNFV	57	0	57	0	0	0
ERF	IXa	*HbERF-IXa1*	*hevea*_454_c20829	HYRGVRQRPWGKYAAEIRDPAKNGARVWLGTYETAEEAALAYDRAAFRMRGSRAMLNFP	12	1	1	0	0	10
ERF	IXa	*HbERF-IXa2*	*hevea*_454_rep_c14033	HYRGVRRRPWGKFAAEIRDPAKKGARVWLGTFETAEDAALAYDRAAYRMRGSRALLNFP	23	9	3	1	7	3

ERF	IXa	*HbERF-IXa3*	*hevea*_454_c31433	HYRGVRQRPWGKFAAEIETGKNGARVWLGTFETAEDAALAYDRAAYRMRGSRALLNFP	12	1	0	0	8	3
ERF	IXa	*HbERF-IXa4*	*hevea*_454_rep_c63160	HYRGVRRRPWGKFAAEIETGKKGLGFGXGTFETAEDAALAYDRAAYRMRGSRALLNFP	2	1	0	0	1	0
ERF	IXb	*HbERF-IXb1*	*hevea*_454_c13287	QYRGVRRRPWGKFAAEIRDPNRKGTRVWLGTFDSDVDAAKAYDSAAFRLRGRKAILNFP	20	3	3	0	14	0
ERF	IXb	*HbERF-IXb2*	*hevea*_454_c19752	HYRGVRRRPWGKFAAEIRDPNRKGSRVWLGTFDSDVDAAKAYDCAAFRLRGRKAILNFP	13	4	1	1	7	0
ERF	IXb	*HbERF-IXb3*	*hevea*_454_rep_c9973	HYRGVRQRPWGKYAAEIRDPNRKGSRVWLGTFDTAIEAAKAYDRAAFKLRGSKAILNFP	32	12	3	0	2	15
ERF	IXb	*HbERF-IXb4*	*hevea*_454_rep_c12196	HYRGVRQRPWGKYAAEIRDPNQKGTRLWLGTFDTAIEAAKAYDRAAFKLRGSKAILNFP	32	9	1	7	14	1
ERF	IXb	*HbERF-IXb5*	*hevea*_454_rep_c25450	HYRGVRRRPWGKYAAEIRDPNKKGARVWLGTFDNAIEAAKAYDSAAFRLRGSKAILNFP	17	1	3	0	7	6
ERF	IXb	*HbERF-IXb6*	*hevea*_454_rep_c86034	HYRGVRRRPVGKYAAEIRDPNKKGARVWLGTFDTAIEAAKAYDNAAFRLRGSKAILNFL	2	2	0	0	0	0
ERF	IXb	*HbERF-IXb7*	*hevea*_454_rep_c9858	HYRGVRRRPWGKYAAEIRDPNKKGARVWLGTFDTAIEAAKAYDNAAFRLRGSKAILNFP	38	15	5	0	3	15
ERF	IXb	*HbERF-IXb8*	*hevea*_454_rep_c16062	HYRGVRRRPWGKYAAEIRDPNKKGARVWLGTFDTAIEAAKAYDNAAFRLRGSKAILNFP	23	10	1	0	7	5
ERF	IXc	*HbERF-IXc1*	*hevea*_454_rep_c12109	HYRGVRRRPWGKYAAEIRDSTRHGARVWLGTFETAEEAALAYDRAAFSMRGSKALLNFP	23	3	4	0	7	9
ERF	IXc	*HbERF-IXc2*	*hevea*_454_c72747	RYRGVRRRPWGKFAAEIRDSTRQGARIWLGTFNTAEEAARAYDRAAYAMRGHLAILNFP	2	0	0	0	0	2
ERF	IXc	*HbERF-IXc3*	*hevea*_454_c37716	AYRGVRKRPWGKYAAEIRDSTRNGVRVWLGTFDTAEAAALAYDQAALAMRGSMAILNFP	4	0	0	0	4	0
ERF	IXc	*HbERF-IXc4*	*hevea*_454_rep_c3873	SYRGVRRRPWGKFAAEIRDSTRHGIRVWLGTFDSAEAAALAYDQAAFSMRGAGAILNFP	57	25	0	5	5	22
ERF	IXc	*HbERF-IXc5*	*hevea*_454_rep_c36947	SYRGVRRRPWGKFAAEIRDSTRHGIRVWLGTFDSAEAAALAYDQAAFSMRGAGAILNFP	21	12	4	0	2	3
ERF	IXc	*HbERF-IXc6*	*hevea*_454_rep_c18341	SYRGVRKRPWGKYAAEIRDSTRNGIRVWLGTFDSAEAAALAYDQAAFSMRGSMAILNFP	22	1	0	0	8	13
ERF	IXc	*HbERF-IXc7*	*hevea*_454_rep_c27017	SYRGVRKRPWGKYAAEIRDSTRNGIRVWLGTFDSAEAAALAYDQAAFSMRGSMAILNFP	13	3	0	0	5	5
ERF	Xa	*HbERF-Xa1*	*hevea*_454_c58761	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFDTAEDAALAYDKAALKFKGTKAKLNFP	12	1	7	0	0	4
ERF	Xa	*HbERF-Xa2*	*hevea*_454_rep_c8880	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFDTAEDAALAYDKAALKFKGTKAKLNFP	33	1	16	0	2	14
ERF	Xa	*HbERF-Xa3*	*hevea*_454_rep_c30115	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFDTAEDAALAYDKAALKFKGTKAKLNFP	11	0	7	0	0	4
ERF	Xa	*HbERF-Xa4*	*hevea*_454_rep_c67487	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFDTAEAAAAAYDAAALKFKGTKAKLNFP	3	0	1	1	0	1
ERF	Xa	*HbERF-Xa5*	*hevea*_454_rep_c26270	HYRGVRQRPWGKWAAEIRDPKKAARVWLGTFDTAEAAATAYDAAALKFKGTKAKLNFP	9	7	0	1	0	1
ERF	Xa	*HbERF-Xa6*	*hevea*_454_rep_c36967	RYRGVRQRPWGKWAAEIRDPHKAARVWLGTFDTAEAAARAYDEAALRFRGNRAKLNFP	9	3	0	1	0	5
ERF	Xa	*HbERF-Xa7*	*hevea*_454_rep_c24581	RYRGVRQRPWGKWAAEIRDPHKAARVWLGTFDTAEAAARAYDEAALRFRGSRAKLNFP	7	3	0	0	2	2
ERF	Xa	*HbERF-Xa8*	*hevea*_454_c51284	KYRGVRQRPWGKWAAEIRDPFKAARVWLGTLDTAEAAARAYDEAALRFRGSKAKLNFP	3	1	0	0	2	0
ERF	Xb	*HbERF-Xb1*	*hevea*_454_rep_c21830	NYRGVRQRPWGKWAAEIRDPRRAMRVWLGTFNTAEEAARAYDKAAIDFRGPRAKLNFP	13	5	0	0	6	2
AP2		*HbAP2-1*	*hevea*_454_c41136	SIYRGVTRHRWTGRYEAHLWDNSCRIEGQTRKGRQVYLGGYDKEEKAARAYDLAALKYWGTTTTNFP	7	0	5	0	0	2
AP2		*HbAP2-2*	*hevea*_454_rep_c74324	SIYRGVTRHRWTGRYEAHLWDNSCRREGQSRKGRQVYLGGYDKEEKAARAYDLAALKYWGPTTTTNFPISNYQKELEEMKHMTRQEFVASLRRKSSGFSRGASVYRGVTRHHGTSGRWQARIGRVAGNKXLIVGNF	2	0	1	0	0	1
AP2		*HbAP2-3*	*hevea*_454_c24965	SIYRGVTRHRWTGRYEAHLWDNSCRREGQARKGRQVYLGGYDREXKAARAYDLAALKYWGHTATTNFPVANYTKELEEMKYVSKQEFIASLRRKSSGFSRGASIYRGVTRHHQQGRWQARIGRVAGNKDLYLGTFATEEEAAEAYDIAAIKFRGMNAVTNFE	22	0	19	0	0	3
AP2		*HbAP2-4*	*hevea*_454_c60993	SIYRGVTRHRWTGRYEAHLWDKNCWNESQNKKGRQVYLGAYDDEEAAAHAYDLAALKYWGQDTILNFP	4	1	0	3	0	0

AP2		*HbAP2-5*	*hevea*_454_rep_c25399	SIYRGVTRHRWTGRYEAHLWDKSTWNQNQNKKGKQVYLGAYDDEEAAARAYDLAALKYWGPGTLINFPVTDYTRDLEEMQNVSREEYLASLRRKSSGFSRGISKYRGLSSRWDSSFGRMPGSEYFSSINYGDDPAAESDYVGSLCFERKIDLTSYIK	11	8	1	0	0	2
AP2		*HbAP2-6*	*hevea*_454_rep_c16078	SIYRGVTRHRWTGRFEAHLWDKSSWNNIQNKKGRQVYLGAYDNEEAAAHTYDLAALKYWGPDTTLNFPIETYSKELEEMQKMSKEEYLASLRRRSSGFSRGVSKYRGVARHHHNGRWEARIGRVFGNKYLYLGTYNTQEEAAAAYDMAAIEYRGANAVTNFD	31	0	3	24	0	4
AP2		*HbAP2-7*	*hevea*_454_c17771	HSIEVLPGIDGRADMKPISGIIVASRRAXTRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHINFPLENYQEELEEMKNMSRQEYVAHLRRKSSGFSRGASMYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGVNAVTNFD	21	5	0	0	16	0
AP2		*HbAP2-8*	*hevea*_454_rep_c12362	HNTEVLQGTDGTGRYEAHLWDNSCKKEGQSRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHINFPLENYQKELEEMKNMTRQEYVAHLRRKSSGFSRGASIYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGVNAVTNFD	33	19	4	0	2	8
AP2		*HbAP2-9*	*hevea*_454_rep_c24306	SQNRGVTFYRRTERWESHSWDEGKLVHLGEFDTAHAAAARAYDRTSIKFKGVEADINFS	21	7	3	0	2	9
AP2		*HbAP2-10*	*hevea*_454_rep_c22185	SQYRGVTFYRRTGRWESHIWDCGKQVYLGGFDTAVAAAKAYDRAAIKFRGVDADINFXNLSDYDEDMKQMRNLGKEEFIHTLRRKITGYVRGSSKYRGVNLHKCGRWEARMGQFHGKKAYDIEAIKCNGRDAVTNFE	28	1	5	2	3	17
AP2		*HbAP2-11*	*hevea*_454_c58670	SQYRGVTFYRRTGRWESHIWDCGNKCXLGGFDTAHAAARAYDRAAIKFRGVDADINFNVSDYEEDIKQMSNFSKEEFVHILRRQSTGFSRGSSKFRGVTLHKC	3	1	1	0	0	1
AP2		*HbAP2-12*	*hevea*_454_c40524	SQYRGVTYYRRTGRWESHIWDCGKQVYLGGFDTAHAAARAYDKAAIKCNGKEAVTNFD	8	0	5	0	0	3
AP2		*HbAP2-13*	*hevea*_454_c19894	SVYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGLNAVTNFD	11	6	1	0	0	4
AP2		*HbAP2-14*	*hevea*_454_rep_c59394	SIYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFXGLNAVTNFD	6	0	4	0	0	2
AP2		*HbAP2-15*	*hevea*_454_c50010	DMEEKAARAYDLAALKYWGPSTHINFPLENYQEELEEMKNMSRQEYVAHLXKESSGFSRGASMYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGVNAVTNFD	13	2	3	0	8	0
AP2		*HbAP2-16*	*hevea*_454_rep_c23347	SKYRGVARHHHNGRWEARIGRVFGNKYLYLGTYATQEEAATAYDMAAIEYRGLNAVTNFD	9	1	0	3	4	1
AP2		*HbAP2-17*	*hevea*_454_c60010	VTWSYDEEESAARAYDLAALKYWGTSTFTNFPISDYEKEIEIMQTVTKEEYLASLRRKSSGFSRGVSKYRGVARHHHNGRWEARIGRVFGNKYLYLGTYSTQEEAARAYDIAAIEYRGINAVTNF	2	0	0	0	0	2
AP2		*HbAP2-18*	*hevea*_454_rep_c16704	SQYRGVTYYRRTGRWESHIWDCGKQVYLGGFDTAHAAARAYDRAAIKFRGVEADINFNIEDYEDDLKQMSNLTKEEFVHVLRRQSTGFPRGSSKYRGVTLHKCGRWEARMGQFLGKKYVYLGLFDTEIEAARAYDKAAIKCNGKEAVTNFD	31	4	8	1	6	12
AP2		*HbAP2-19*	*hevea*_454_rep_c24432	DCGKQVYLGGFDTAHAAARAYDRAAIKFRGVDADINFNVSDYEEDIKQMSNFSKEEFVHILRRQSTGFSRGSSKFRGVTLHKCGRWEARMGQFLGKKYMYLGLFDSEIEAARAYDKAAIKCNGREAVTNFE	13	8	3	0	1	1
AP2		*HbAP2-20*	*hevea*_454_rep_c45080	SQYRGVTFYRRTGRWESHIWDCGKQVYLGGFDTAHAAARAYDRAAINLXGVDADINFNLTDYEEDLKQMKNLTKEEFVHILRRQSTGFSRGSSKYRGVTLHKCGRWEARMGQFLGKKYIYLGLFDSEVEAARAYDKAAIKFNGREAVTNFE	5	1	2	0	1	1
RAV		*HbRAV-1*	*hevea*_454_rep_c15925	SKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAAKAYDIAAQRFRGRDAITNFKPQGAETEEDDIETAFLNSHSKAEIVDMLRKHTYNDELEQSKRNYRIDGQGKQNRNPGANNVAVYGSDRVLKAREQLFEKAVTPSDVGKLNRLVIPKQHAEKHFPLQSGSNSTKGVLLNFEDITGKVWRFRYSYWNSSQSYVLTKGWSRFVKEKNLKAGDIVSFQRSTG	34	17	3	0	1	13
RAV		*HbRAV-2*	*hevea*_454_rep_c13430	SKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAAKAYDIAAQRFRGRDAITNFKPQGAETEEDDIETAFLNSHSKAEIVDMLRKHTYNDELEQSKRNYRIDGQGKQNRNPGANNVAVYGSDRVLKAREQLFEKAVTPSDVGKLNRLVIPKQHAEKHFPLQSGSNSTKGVLLNFEDITGKVWRFRYSYWNSSQSYVLTKGWSRFVKEKNLKAGDIVSFQRSTG	39	15	5	0	4	15
RAV		*HbRAV-3*	*hevea*_454_rep_c8782	SKYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEARAYDIAAQRFRGRDAITNFKPQGAEIEDDDIETAFLNSHSKAEVVDMLRKHTYNDELEQSKRNYRIDGQGKQHRNPGANNVALSGSGRVLKEREQLFEKAVTPSDVGKLNRLVIPKQHAEKYFPLQSGSNSTKGVLLNFEDITGKVWRFRYSYWNSSQSYVLTKGWSRFVKEKNLKAGDIVSFQRSTG	62	16	8	19	10	9
RAV		*HbRAV-4*	*hevea*_454_rep_c24256	SKFRGVVAHQSGHWGCQIYANHQRVWLGTFKYEQEAAMAYDSAALKLRSGDSRSKFXPFTNITVEEENFQSSYSTEAVLSMIKDGTYRSKFADFLRTRAQNFEADLSLKLMKTQSSRRLTCKQLFQKELTPSDVGKLNRLVIPKKFATKFFSPLSEGVQENAADVRQLSFYDKAMKLWKFRYCYWRSSQSYVFTRGWSGFVKEKQLKANDIICF	13	0	3	0	0	10
SOLOIST		*HbSoloist1*	*hevea*_454_rep_c14983	LMRGVYFKNMKWQAAIKVDKKQIHLGTVGSQEEAAHLYDRAAFMCGREPNFE	37	12	1	0	13	11
SOLOIST		*HbSoloist2*	*hevea*_454_rep_c8142	LMRGVYFKNMKWQAAIKVDKKQIHLGTVGSQEEAAHLYDRAAFMCGREPNFE	50	3	11	18	8	10
SOLOIST		*HbSoloist3*	*hevea*_454_rep_c46638	LMRGVYFKNMKWQAAIXVDKKQIHLGTVGSQEEAAHLYDRAAFMCGREPDFE	10	0	1	2	6	1
–		*partial*	*hevea*_454_rep_c52465	IYRGVRQRTWGKWVAEIREPNRGPRLWLGTYPTASEATASNHSEVCADEDTKEHIVKNWG	2	0	0	0	0	2
–		*partial*	*hevea*_454_rep_c88128	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTAETAFQGKLVSEFEXXLNCTEPDYFN	2	1	0	0	0	1

–		*partial*	*hevea*_454_rep_c64029	QYRGIRQRPWGKWAAEIRDPRKGVRVWLGTFNTARKXKVNFPDEAPRASPKRTVKAKXPQKPLSKENLS	13	9	0	2	1	1
–		*partial*	*hevea*_454_rep_c94406	IYRGIRQRPWGXXWAAEIRDPHKGVRVWLGTYNTAEEALLRCLLLPSKPAAYQHRKHTWVLASDIKKNWG	2	0	0	0	0	2
–		*partial*	*hevea*_454_rep_c51770	AKLQLKQRPPRNXDAEIRDPHKGVRVWLGTYNTAEEAARAYDEAAKRIRGDKAKLNFG	4	2	0	0	0	2
–		*partial*	*hevea*_454_rep_c43836	SDTAVSVKAXWGSWVSEIRHPLLKTRIWLGTFETAEDAARAYDEAARLMCGPKARTNFP	5	0	0	0	4	1
–		*partial*	*hevea*_454_c45860	FGPILLPRFCQPIVTATIGSDTCNVCKINGCLGCNFFPPNNQEEAARAYDKAAIDFEGRXAKLNFPFPDNVSNTTNLE	4	0	0	0	2	2
–		*partial*	*hevea*_454_rep_c39126	GKSPDTEESGKDHGADLLPRSEIPGKTRVWLGTFDSAEEAARAYDAAARSLRGPKAKTNFLISDSHLSPFIYENPP	4	2	0	1	1	0
–		*partial*	*hevea*_454_rep_c56042	EPNGSAPQNITNGKEXPDTEESEKDHGADLPLRSEIPGKTRVWLGTFDSAEDAARAYDAAARSLRGPKAKTNFV	5	0	3	2	0	0
–		*partial*	*hevea*_454_rep_c45447	QYRGIRQRPWGKWAAEIRDPRXEDANPLKKMKPDSGNVVPVEENNGKSLSEELLAFDNQV	5	3	1	1	0	0
–		*partial*	*hevea*_454_c42618	ARAYDKAAIKCNGKEAVTNFD	4	1	3	0	0	0
–		*partial*	*hevea*_454_c71098	KYLYLGTYATQEEAATAYDMAAIEYRGLNAVTNFD	2	0	0	0	0	2
–		*partial*	*hevea*_454_rep_c27567	MRSWGKWVSEIREPRKKSRIWLGTYPTAEMAARAHDVAALAIKGSSAYLNFP	5	3	1	0	0	1
–		*partial*	*hevea*_454_rep_c29766	LYRGVRQRHWGKWVAEIRLPKNRTRQLIS	6	2	0	0	0	4
–		*partial*	*hevea*_454_rep_c34934	MRQWGKWVSEIREPRKKSRIWLGTYSTAEMAARAHDVAALTIKGRSAHLNFP	4	3	0	0	1	0
–		*partial*	*hevea*_454_rep_c35089	WGKFAAEIRDPNRKGTRVWLGTFDSDV	8	0	4	0	0	4
–		*partial*	*hevea*_454_rep_c64570	PARRVRLWLXTYNTAEEAAMVMTMLQSXARGPDALTNFI	2	0	1	0	0	1
–		*partial*	*hevea*_454_rep_c65247	RDSTRQGARIWLGTFNTAEEAARAYDRAAYAMRGHLAILNFP	2	0	0	0	2	0
–		*partial*	*hevea*_454_rep_c67322	RYRGVRQRSWGKWVAEIREPRKRTRKWLGNFCYCGGRSSSLX	2	0	2	0	0	0
–		*partial*	*hevea*_454_rep_c78875	VYRGVRMRNWGKWVSKIREPRKKSRIWLGTFPTPEMAARAHDVAALSI	2	0	2	0	0	0
–		*partial*	*hevea*_454_rep_c85825	HYRGVRQRPWGKYAAEIRDPNRKGSRVWLGTFDAAIEAAKAYDRAAFKL	3	1	0	0	0	2
–		*partial*	*hevea*_454_rep_c89100	LYRGVRQRHWGKWVAEIRLPKTGQDSGLALLTQLK	2	2	0	0	0	0
–		*partial*	*hevea*_454_c51892	IAAIKFRGMNAVTNFE	4	0	4	0	0	0
–		*partial*	*hevea*_454_c54709	AYAYDRAAYKLRGEYARLNFP	3	0	1	0	2	0
–		*partial*	*hevea*_454_rep_c42539	LHSCDTQFITLNSHPQYRSIQHVXPFDFLELRAYDKAAIKCNGREAVTNFE	7	2	2	2	1	0
–		*partial*	*hevea*_454_rep_c59277	KXERAYDAEARRIRGKKAKVNFP	3	1	0	1	0	1
–		*partial*	*hevea*_454_c74808	SQYRGVTFYRRTGRWESHIWSISS	2	1	1	0	0	0
–		*partial*	*hevea*_454_c47582	IFRGVTKRPWGKYAAEIRDSTRNGIRVGXGTLTVRRQLLSLRPSSISMRGSMAILNFP	4	0	0	0	2	2
–		*partial*	*hevea*_454_rep_c35500	RGVTRHHQHGRWQAREFGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGVNAVTNFX	8	4	0	0	3	1
–		*partial*	*hevea*_454_rep_c41834	GQTRKGRQVYLGGYDKEVKAARAYDLAALKYWGPTTHINFPLSTYEKELEEMKHLTRQEFVANLRRKSSGFSRGASAYRGVTRHHQHGRWQARIGRVAGNRTCTSEHLAHKKKLLRPMILQLLI	4	0	4	0	0	0
–		*partial*	*hevea*_454_rep_c55387	KGRQVYLGGYDKEEKAARAYDLAALKYWGTSTTTNFPISNYEKELEEMKHMTRQEFVASIRRKSSGFSRGASMYRGVTRHHQHGRWQARIGRVAGNKDLYLGTFSTEEEAAEAYDIAAISSEG	3	0	1	2	0	0
				Total number of reads	6541	1480	1047	1312	706	1996
				Proportion of reads in each tissue		22,63%	16,01%	20,06%	10,79%	30,52%

CHAPITRE 3

Projet d'article intitulé
"Several *Hevea brasiliensis* ERF-IX from the subgroup c function like the *Arabidopsis thaliana* Ethylene Response Factor 1 (ERF1) and ORA59"

Several *Hevea brasiliensis* ERF-IX from the subgroup c function like the *Arabidopsis thaliana* Ethylene Response Factor 1 (ERF1) and ORA59

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Abstract
Ethylene-response factors (ERFs) are transcription factors involved in plant development and defence. ERF1 and ORA59 play a crucial role in the activation of plant defence responses. These genes belong to the group IX of ERFs, which are involved in the response to biotic stresses through jasmonic acid (JA) and ethylene (ET) signalling pathways. These two plant hormones play an important role in *Hevea brasiliensis* on the latex production and latex cell differentiation, respectively. The regulation of 14 *HbERF* genes from group IX were characterized in response to wounding, methyl jasmonate (MeJA) and ET treatments in order to identify key regulators in latex cells. Transcripts of several members of group IX were accumulated after wounding, MeJA and ET treatments. Furthermore, transcripts of three *ERF* genes from subgroup IXc (*HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6*) were dramatically accumulated by combining wounding, JA and ET treatments. *Hevea* ERF family group IX genes were aligned with the group IX members of the seven other species. Conserved amino acid residues and phylogeny reconstruction using the AP2 conserved domain of ERF family group IX genes of different species suggest that the group IX genes were well classified into 3 subgroups for different species. *HbERF-IXc3*, *HbERF-IXc6* and *HbERF-IXc7* cDNA sequences are closed to the *Arabidopsis ORA59* gene, and *HbERF-IXc4* and *HbERF-IXc5* are closed to the *ERF1*. Another phylogenetic analysis carried out with full length ERF protein sequences revealed that HbERF-IXc4 and HbERF-IXc5 are putative ortholog to ERF1, and HbERF-IXc2 is a possible ortholog to ORA59. Given ORA59 and ERF1 are positive regulators of the *PLANT DEFENSIN1.2* (*PDF1.2*) gene expression, a transactivation experiment of the PDF1.2::GUS was carried out in *Arabidopsis* leaf protoplast. HbERF-IXc6 and above all HbERF-IXc5 showed a high transient GUS activity compared to HbERF-IXc4. A translational fusion HbERF-IXc4-GFP was tested and revealed that HbERF-IXc4 has a nuclear localization as transcription factor. Our results suggest that HbERF-IXc3, HbERF-IXc4, HbERF-IXc5, HbERF-IXc6 or HbERF-IXc7 could be essential integrator of the JA and ET signalling pathways in *Hevea*. Further characterization of these candidate genes will provide new insight into the nature of the molecular components involved in the crosstalk between these two hormones.
Keywords: AP2/ERF, ERF1, ethylene, methyl jasmonate, ORA59, wounding, rubber, PDF1.2.
Abbreviation: At: *Arabidopsis thaliana*; ET: ethylene; Hb: *Hevea brasiliensis*; JA: jasmonic acid; P: *Populus*; Nt: *Nicotiana tabacum*; Sl: *Solanum lycopersicum*; Vv: *Vitis vinifera*; G: *Gossypium hirsutum, G. Raimondii, G. Hirsutum* and *G. Arboretum*; MeJA: methyl-jasmonate; SA: salicylic acid; W: wounding.

1. Introduction

ETHYLENE RESPONSE FACTOR (ERF) genes belong to the superfamily of AP2/ERF. They encode transcription factors involved in plant development and response to biotic and abiotic stresses (Hu et al., 2008). Biological processes regulated by ERF proteins include embryo development (Boutilier et al., 2002), leaf petiole development (van der Graaff et al., 2000) and leaf epidermal cell identity (Moose and Sisco, 1996), flower development (Elliott et al., 1996) and fruit ripening (Wang et al., 2004). ERF proteins also participate in plant responses to biotic stimuli. For example, ERF proteins modulate the expression of pathogenesis related (PR) genes (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998; Fujimoto et al., 2000; Gu et al., 2000; Onate-Sanchez et al., 2007). Recent investigations showed that several ERF-like proteins, such as ERN1, ERN2 and ERN3 and EFD from *Medicago truncatula*, regulate legume root nodule development to establish a symbiosis with nitrogen-fixing bacteria (Andriankaja et al., 2007; Middleton et al., 2007). Genes of the ERF family are predominant transcription factors responsive to both JA and incompatible necrotrophic pathogen *Alternaria brassicicola* (McGrath et al., 2005).

ERF1 was suggested to be a key component for the defence responses through the integration of ethylene (ET) and jasmonic acid (JA) signalling pathways (Lorenzo et al., 2003). The crosstalk between these two plant hormones determines the activation of an important set of genes involved in the defence against pathogens and herbivores. For instance, ERF1 has been shown to confer resistance to several fungi (Berrocal-Lobo and Molina, 2004). In addition, overexpression of the TERF1 and JERF1 isolated in tomato improved the tolerance of transgenic rice and tobacco to osmotic stress (Zhang et al., 2004; Wu et al., 2007; Zhang et al., 2007; Quan et al., 2010). The activation of genes encoding the PLANT DEFENSIN1.2 (PDF1.2) is commonly observed in the jasmonate-dependent defence responses (Brown et al., 2003). Constitutive overexpression of the *ERF1* gene activates the expression of several defence-related genes, including the *PDF1.2*, the *thionin2.1* (*Thi2.1*) and the *basic-chitinase* (*ChiB*) in *Arabidopsis thaliana* (Manners et al., 1998; Solano et al., 1998; Brown et al., 2003). More recently, ORA59 was found to be another integrator of JA and ET signals in plant defence (Pre et al., 2008). ORA59 is strictly required to activate a specific set of defence genes including PDF1.2 by synergizing the JA and ET signalling pathways after infection with necrotrophic fungi (Zarei et al., 2011). ORA59 caused increased resistance against *B. Cinerae* (Pre et al., 2008). The jasmonates are indispensable metabolites in mediating the activation of direct plant-defence responses (Chehab et al., 2008).

The ERF family is one of the largest families of transcription factors with a total of 122 and 139 members in the *Arabidopsis* and rice genomes, respectively (Gutterson and Reuber, 2004). This family is organized in ten groups (Nakano et al., 2006). In *Arabidopsis*, ERF1 gene (At3g23240) and ORA59 (At1g06160) belong to the subgroup IXc of ERF family. These transcription factors are characterized by a single conserved DNA-binding AP2 domain of about 60 amino acids. The complex between the *Arabidopsis thaliana* ERF1 and its target DNA fragment was determined by nuclear magnetic resonance (NMR) based on the analysis of the three-dimensional structure of the DNA-binding AP2 domain (Allen et al., 1998). The domain consists of a three-stranded, anti-parallel □-sheet and a □-helix packed approximately parallel to the □-sheet. The AP2 domain of ERF proteins specifically interacts with a conserved AGCCGCC sequence called the GCC-box (Menke et al., 1999). Although genes of the AP2/ERF superfamily encode transcription factors in plant, AP2 domain-containing genes were recently found in bacteria and viruses, which are predicted to be HNH endonucleases (Magnani et al., 2004).

Ethylene and jasmonate signalling pathways converge in the transcriptional activation of ERF1. Transcriptome analysis supports that ERF1 regulates *in vivo* the expression of a large number of genes responsive to both ethylene and jasmonate. It was suggested that ERF1 acts downstream of the intersection between ethylene and jasmonate pathways and is a key element in the integration of both signals for the regulation of defence response (Lorenzo et al., 2003). JASMONATE-INSENSITIVE1 (JAI1/JIN1) encodes AtMYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor, whose expression is rapidly up-regulated by JA, in a CORONATINE INSENSITIVE1 (COI1)-dependent manner (Lorenzo et al., 2004). It was highlighted that the existence of two branches in the JA signalling pathway, antagonistically regulated by AtMYC2 and ERF1, that are coincident with the alternative responses activated by JA and ET to two different sets of stresses, namely pathogen attack and wounding. ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE 1 (EIL1) integrate ethylene and jasmonate signals in the regulation of the expression of genes involved in root development and defence against necrotrophic pathogens. JA enhances the transcriptional activity of EIN3/EIL1 by removal of JA-ZIM domain (JAZ) proteins (Zhu et al., 2011). JAZ proteins are repressors of MYC2 and targets of SCF(COI1), which is the likely jasmonate receptor (Chini et al., 2009). Analysis of transgenic plants in which *ORA59* gene expression was silenced by RNAi, whereas the *ERF1* gene was normally expressed, showed that ORA59 is strictly required for the expression of the *PDF1.2* gene in response to JA, JA/ET and the infection with necrotrophic fungi (Pre et al., 2008). Studies of the promoter of the *PDF1.2* gene identified a GCC-box at positions -256 to -261 which is involved in the JA response through ORA59 and ERF1 trans-activates the *PDF1.2* promoter via binding to two GCC boxes (Manners et al., 1998; Brown et al., 2003). Interestingly, mutation of a single GCC box at positions -256 to -261 reported to be important for JA-responsive expression completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ethylene-releasing agent ethephon (Zarei et al., 2011). JA and ET signalling pathways converge to a single type of GCC box sequence (Zarei et al., 2011).

Ethylene and jasmonate-regulated ERFs are assumed to play a key role in the production of natural rubber. *Hevea brasiliensis* is the sole commercial source of natural rubber. Rubber biosynthetic pathway occurs in specialized latex cells. Latex is harvested by tapping on the *Hevea* soft bark. Latex production depends on the flow and regeneration of latex between two tappings. For *Hevea* clones with a low metabolism, ethephon, an ethylene releaser is applied to the tapping panel to stimulate latex production. Ethephon application also induces several biochemical processes in laticifers, such as sucrose loading, water uptake, nitrogen assimilation or synthesis of defence proteins (d'Auzac, 1992), involving a large number of ethylene-response genes (Pujade-Renaud et al., 1994; Tungngoen et al., 2009; Duan et al., 2010), whereas its direct role in rubber biosynthesis is controversial (Zhu and Zhang, 2009). Tapping and ethephon are likely to be sources of stress conducive to the production of secondary metabolites and consequent rubber, but over a certain stress limit they lead to tapping panel dryness (TPD) (Venkatachalam et al., 2009). Mechanical wounding related to tapping trigger the production of endogenous ethylene and oxylipins such as jasmonic acid (JA) (Kuswanhadi et al., 2010; Chen et al., 2011). Both mechanical wounding and methyl-jasmonate treatments induce the differentiation of primary latex cells into secondary latex cells (Hao and Wu, 1982; Hao and Wu, 2000; Wu et al., 2002). These secondary latex cells then anastomose to create laticifer vessels (de Faÿ and Jacob, 1989). The AP2/ERF superfamily has been identified in *Hevea brasiliensis* but a little is known with regards to their functions (Duan et al., Submitted). *HbERF1*, *HbERF2*, *HbERF3* and *HbRAV1* genes were suggested to be induced by JA in bark during JA-induced laticifer differentiation (Wu, 2010).

Another member, the HbEREBP1, was proposed to be a negative regulator of defence mechanisms in laticifers (Chen et al., 2011). The loss of latex also represents an osmotic stress involving ABA-dependant response. Some ERF of group I to IV are called Dehydration Responsive Element Binding proteins. Their transcriptional regulation has been shown both ABA dependent or independent.

The aim of this study was to identify the orthologous gene to the *Arabidopsis* ERF1 (At3g23240) and ORA59 (At1g06160) within the *Hevea* ERFs from group IX. First, a phylogenetic analysis led to predict *HbERF* genes from the subgroup IXc as putative orthologous gene to the *Arabidopsis* ORA59 and ERF1 genes. Second, the relative transcript abundance of 14 ERF genes in response to ethylene, wounding and methyl jasmonate treated applied alone or in combination on bark of 1-year-old plants allowed identifying *HbERF* genes dramatically induced by a treatment combining ET and MeJA. The transactivation of the AtPDF1.2::GUS was tested for three ethylene and jasmonate responsive genes. Then, the subcellular localization of these putative transcription factors was checked using translational fusion between HbERF-IXc candidates and the GFP.

2. Materials and methods

Plant material and treatments

Hevea brasiliensis clone PB 260 buds were grafted on seedling rootstocks. These budded plants were grown at 28°C in a greenhouse under natural light. Three-month-old epicormic shoots from budded plants and leaves were treated at the same time. Leaves and bark of these plants were subjected or not to various factors alone or in combination (treatments): mechanical wounding (W), methyl jasmonate (MeJA), ethylene (ET), WxMeJA, WxET, MeJAxET, WxMeJAxET. Leaves were mechanically wounded by squeezing the entire surface of the leaves with pincers whilst the bark was wounded every 0.5 cm by scarification with a razor blade. For the ethylene and methyl jasmonate gas (MeJA) treatments, plants were placed in a 300-litre open-door Plexiglas box overnight before the treatment. One ppm of pure ethylene (ET) gas (0.3 mL/300 L) was injected into the sealed air-tight box. The concentration was controlled by gas chromatography (Type HP 5280 with FID detector). For the methyl jasmonate treatment, 20 µL of liquid ≥ 95% methyl jasmonate solution (Sigma, St. Louis, US) was diluted in 500 µL of absolute ethanol, and then placed on Whatmann paper inside the box for gas release. Each treatment was compared to a specific control sampled at the same time and with the same culture conditions in three biological replications. Plants were treated at 8.00 am and tissues were collected 4 hours after treatment based on various preliminary experiments in kinetics (Duan et al., 2010). After treatment, bark tissues were collected and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA isolation

Total RNAs from bark tissues were isolated using the caesium chloride cushion method adapted from the Sambrook's protocol by Duan et al. (Sambrook et al., 1989; Duan et al., 2010). One gram of fresh matter was ground and transferred to a tube containing 30 ml of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone and 1% ß-mercapto-ethanol. After homogenization, tubes were kept on ice and then centrifuged at 10,000 g at 4°C for 30 minutes. The supernatant was transferred to a new tube containing 8 ml of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 89,705 g at 20°C for 20 hours. The supernatant and caesium cushion were discarded whilst the RNA pellet was washed with 70% ethanol. After 30 minutes of air drying, the pellet was dissolved in 200 µl of sterile water. RNAs were conserved at -80°C.

Phylogenetic analysis of the AP2 domain of the ERF group IX genes for seven various species

Firstly, we downloaded the AP2 domain of the 147 *Arabidopsis thaliana* AP2/ERF genes from the *Arabidopsis* Transcription Factor Database (ArabTFDB) (http://plntfdb.bio.uni-potsdam.de/v3.0/). BLASTX (Basic Local Alignment Search; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out using the 147 AtAP2 domain amino acid sequences as protein subjects and nucleic acid sequences of contigs assembled in the HbPB260 transcript database as query (Duan et al., Submitted). Genes of the ERF group IX were listed for seven different species from publications on *Arabidopsis thaliana* (Nakano et al., 2006), *Populus trichicarpa* (Zhuang et al., 2008), *Vitis vinifera* (Licausi et al., 2010), *Solanum lycopersicum* (Sharma et al., 2010), *Gossypium hirsutum/raimondii/arboretum* (Champion et al., 2009), *Triticum aestivum* (Zhuang et al., 2010).

Two hundred twenty two full AP2-domain sequences of about 60 amino acids from seven species were aligned using MUSCLE software (Edgar, 2004, 2004), which use a progressive multiple alignment method. The alignment was curated by the Gblocks software searching at least 10-amino acid long conserved blocks (Talavera and Castresana, 2007). A block of 57-amino acid long was abstracted and used in the construction of a phylogenetic tree using the PhyML software (Guindon et al., 2010), which implements a maximum likelihood tree reconstruction method, using LG+gamma model, starting from a BioNJ tree (Gascuel, 1997). The tree was drawn and displayed with the Dendroscope program, and rooting on the branch of the AP2 and RAV family. The sequence of the first AP2 domain (R1) of gene AT4G36920.1 APETALA2.1 (AP2R1) was used to root tree.

Identification of conserved motifs and specific amino acid residues

Amino acid sequences of the AP2 domain from 222 ERF genes of group IX were aligned using CLUSTALX for *Hevea brasiliensis* (19 genes), *Arabidopsis thaliana* (17 genes), *Populus* (31genes), *Gossypium* (39 genes), *Nicotiana tabacum* (57 genes), *Vitis vinifera* (40 genes), *Solanum lycopersicum* (18 genes). Conserved residues were identified after comparing the entire group IX sequences of the seven species.

Analysis of transcript abundances by real-time RT-PCR

Gene expression analysis through real-time RT-PCR was carried out for fourteen *HbERF-IX* genes in *Hevea* bark tissues using gene specific primers (Table 1). Several rules were applied in order to reduce the risk of errors in relative gene expression data. *HbActin* was selected as the best reference gene according to its stability in response to various stresses and temporal regulation in our experimental conditions (Duan et al.). Integrity of total RNA and full-length cDNA synthesis were checked by electrophoresis and PCR amplification of full-length Actin cDNA using primers at the cDNA ends. Primers were designed at the 3' side of each sequence in order to reduce the risk of error due to short cDNA synthesis using Primer 3 module of Geneious (Biomatters Ltd., New Zealand). cDNAs were synthesized from 2 µg of total RNA to the final 20 µl reaction mixture using a RevertAidTM M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Full-length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA for using primers at the cDNA ends. Quantitative gene expression analysis was finally carried out by real-time RT-PCR using a Light Cycler 480 (Roche, Switzerland). Real-time PCR reaction mixtures consisted of 2 µl of RT product cDNA, 0.6 µl of 5 µM of each primer, and 3 µl 2×SYBR green PCR master mix (LightCycler® 480 SYBR Green I Master, Roche Applied Sciences) in a 6-µl volume. PCR cycling conditions comprised one denaturation

cycle at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 60°C for 15s, and 72°C for 20s). Firstly, melting curves were analysed to check the specificity of PCR amplification. The PCR products were then cloned and sequenced to ensure that the correct gene was amplified. The cloned PCR products were then used as calibrators in real-time RT-PCR analysis. The concentration of cDNA to be used was determined by testing various cDNA dilutions (1/5, 1/10, 1/25, 1/50, 1/100). The optimum cDNA dilution was chosen with a crossing point (Cp) between 18-22 cycles for all samples. The standard curve was generated using a five-fold dilution series of 10 points in triplicate from a mixed cDNA sample. This standard curve allowed the calculation of primer efficiencies. Expression analysis was performed in three biological replications in a 384-well plate. Samples were loaded using an automation workstation (Biomek NX, Beckman Coulter). The homogeneity of the *HbActin* gene Cp confirmed that it could be used as an internal reference gene. The *HbActin* gene was amplified in parallel with the target gene. The transcript abundance level for each gene was relatively quantified by normalization with the transcript abundance of the reference *HbActin* gene. Relative transcript abundance took into account primer efficiencies. Data from different PCR plates were normalized with *HbActin* expression and calibrators allowing the comparison between several cDNAs and also different 384-well plates. All the normalized ratio corresponding to the transcript accumulation were calculated automatically by Light Cycler Software version 1.5.0 provided by the manufacturer using the following calculation: Normalized Ratio = Efficiency (Cp target-Cp Actin) sample / Efficiency (Cp target-Cp Actin) calibrator.

Statistical analysis for the comparison of relative transcript abundances and for the analysis of the interaction between W x MeJA x ET

The experimental design includes three factors (wounding, methyl jasmonate, ethylene) alone and in combination leading to eight treatments (Control (C), W, MeJA, ET, WxMeJA, WxET, MeJAxET, WxMeJAxET). The experimental unit is one plant. Three biological replications were generated for each treatment (three plants). Each relative transcript abundance value is the mean of two technical replications (two PCR reactions from the same cDNA samples). Statistical analysis was performed after logarithmic transformation of raw data.

The comparison of relative transcript abundances between treated and control plants was carried out using ANOVA followed by a Fisher test. The level of expression was calculated as the ratio between the mean values of relative transcript abundances of treated and control plants. It was considered as an up-regulation when the ratio >1.0, and a down-regulation when the ratio <1.0. The ratio with a p-value ≤ 0.05 was adopted as significant for down or up-regulation.

The level of interaction between W, MeJA and ET was assessed for each tested genes in a variance table. This table included F values for each interaction and the corresponding P-values were noted as follows: <0.001 (***); <0.01 (**); <0.05 (*); <0.1 (°).

The construction of the expression vector using gateway system

Gateway system was used to transfer candidate genes in transformation vector (Curtis and Grossniklaus, 2003). Candidate genes were directional cloned in the vector pENTR™/TEV/D-TOPO® (InvitrogenTM) using a forward primer containing 4 additional base pair sequences (CACC) at the 5' end. The TOPO® Cloning reaction was set up with a 2:1 molar ratio of PCR product:TOPO® vector. One Shot® Chemically Competent E. coli bacteria were transformed with the reaction according the manufacturer conditions. After an overnight culture in SOB medium containing 50 µg/ml kanamycin, positive bacterial colonies were selected and their plasmid DNA was isolated using Miniprep Kit (Promega).

Amplification of a partial sequence of candidate genes was carried out by PCR to confirm the presence and correct orientation of the insert using plant specific primers in combination with the M13 Forward or M13 Reverse primers. In order to avoid the multiplication of pENTR-D-TOPO::HbERF after LR recombination, the TOPO cloning reaction is restricted by MluI.

The pMDC32 and the pMDC83 vectors are respectively used for the trans-activation of the promoter of the *PDF2.1* gene and the subcellular localization by GFP. The LR recombination reaction between the digested pENTR-D-TOPO::HbERF and two destination vectors pMDC32 and pMDC83 (including a GFP translational fusion) was then carried out using the Gateway® LR Clonase™ II enzyme by overnight incubation at room temperature. After proteinase K treatment, the LR recombination reaction was introduced into the TOP10 competent cells. Resistant bacteria colonies to kanamycin were checked by PCR amplification, restriction mapping and sequencing to verify the directional cloning of the ORF sequences.

3. Results

Phylogenetic analysis of ERF genes from group IX in seven species

Two hundred seventeen sequences of ERF genes from group IX were collected for seven different species: *Arabidopsis thaliana* (Nakano et al., 2006), *Gossypium* (Champion et al., 2009), *Hevea brasiliensis* (Duan et al., 2010), *Nicotiana tabacum* (Rushton et al., 2008), *Populus trichocarpa* (Zhuang et al., 2008), *Solanum lycopersicum* (Sharma et al., 2010) and *Vitis vinifera* (Licausi et al., 2010). The number of genes ranged from 17 for *Arabidopsis thaliana* to 57 in *Nicotiana tabaccum*. In *Hevea*, this number is among the lowest with 19 identified genes (Table 2).

A first phylogenetic analysis of the AP2 domain amino acid sequences of the 217 group IX ERF genes was carried out in order to assign ERF genes in specific subgroups (Figure 1). The tree showed three clades corresponding to the three subgroups a, b, c previously described by Nakano (Nakano et al., 2006). The subgroup IXa has the largest number of members for several species like tobacco. Several genes from the subgroup IXa were reported to play important functions: *AtERF1* in *Arabidopsis*, *Nt210* in *Nicotiana*, *Pti4* in *Solanum* and *ORCA3* in *Catharanthus*. The subgroup IXb includes also important members such as *Nt165* gene in tobacco. In the ERF subgroup IXc, the most known members are ERF1 and ORA59 in *Arabidopsis* (Table 4).

A second phylogenetic analysis of the AP2 domain amino acid sequences from ERF group IX genes of several species was performed separately for each subgroup a, b and c. These analyses showed that ERF1 and ORA59 belong to the subgroup c. HbERF-IXc4 and HbERF-IXc5 are homologous to ERF1, and HbERF-IXc3, HbERF-IXc6 and HbERF-IXc7 to ORA59 (Figure 2).

A third phylogenetic analysis was carried out with full length ERF protein sequences of the subgroup IXc from *Arabidopsis thaliana* and *Hevea brasiliensis* (Figure 3). This phylogenetic analysis revealed that HbERF-IXc4 and HbERF-IXc5 are putative ortholog to ERF1, and HbERF-IXc2 is a possible ortholog to ORA59.

Identification of ERF1-specific residues in the AP2 domain of *HbERF* genes from group IX compared to *ERF1*

According to Allen (Allen et al., 1998), four specific residues are localized at position 164, 165, 167 and 168. AP2 domains of group IX have one supplementary residue +167 compared with other groups of the ERF family, and showed one conserved specific residue at the position G168 for all seven species (Table 3).This could be used as a recognition marker for

the group classification. The three subgroups have two conserved residues at positions 164 and 165. At the position 164, subgroups IXa, IXb and IXc showed P164 (59/64), P164 (76/81) and S164 (62/78,12P), respectively. S164 is a specific residue present at a high frequency (62/78) that could be considered as a marker of the subgroup IXc. At the position 165, subgroups IXa, IXb and IXc showed A165 (32/64), N165 (58/81,15T) and T165 (50/78), respectively. So, the high frequency for each subgroup-specific amino acid residues at position 165 could also used for the prediction of the assignment of ERF sequences in subgroup. ERF1 and ORA59 have specific and conserved residues discussed here such as S164, T165, +167N (for ERF1) or +167K (for ORA59) and G168.

Analysis of the relative transcript abundance of *ERF* genes from group IX

In *Hevea brasiliensis*, genes of the ERF family group IX are assumed to be involved in the responses to wounding, ethylene and jasmonate signalling pathways. Genes of the ERF group IX were demonstrated to respond to the JA in several different species such as *Arabidopsis* (Zarei et al., 2011), *Gossypium* (Champion, 2009), *Nicotiana* (Rushton, 2008), *Solanum* (Sharma, 2010) and *Vitis* (Licausi, 2010) (Table 4). The most important members identified in *Arabidopsis* are the ERF1 and ORA59. There are several other members responding to JA in the group IX such as ORCA3, AtERF1, Nt210, etc. These genes from the group IX were shown to be involved in different functions like disease resistance, secondary metabolism, ripening, or salt and oxidative stresses (Table 4).

Relative transcript abundance was analysed in bark tissue for genes of all subgroups IX in response to mechanical wounding, methyl jasmonate and ethylene in order to check the specificity of gene induction in response to ethylene and jasmonate (Table 5). These factors were applied alone or in combination in order to study the interaction between ethylene and methyl jasmonate. Specific primers have been designed for 14 ERF genes of group IX. For each gene, the ratio of relative transcript abundance was calculated between treated and control plants in order to estimate the level of up- or down-regulation. Application of at least one treatment significantly changed this ratio for 8 genes out of the 14 tested.

Transcripts of six ERF genes were significantly accumulated in response to these treatments compared with the control. HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6 genes were induced by all treatments. The relative transcript abundance of HbERF-IXc4 increased up to 39, 54 and 306 fold for W, MeJa and ET treatments, respectively. When ethylene was combined with MeJA, the relative transcript abundance was dramatically increased to 1754 and 3143 fold. HbERF-IXc5 and HbERF-IXc6 showed similar but lower gene induction. For these genes, the highest induction, 502 and 589 times respectively, is obtained for the combination W x MeJA x ET. HbERF-IXb1 and HbERF-IXb2 genes were induced 9 and 5 fold by ET, respectively, and 30 and 12 fold by the combination W x MeJA x ET. Transcripts of the HbERF-IXa2 gene were also accumulated 125 fold compared with the control for the same combination.

By contrast, three genes (*HbERF-IXb2, HbERF-IXb4* and *HbERF-IXc2*) were significantly down-regulated by these treatments. The relative transcript abundance of the *HbERF-IXc2* gene was remarkably reduced by any of these treatments, when it was mainly after wounding for the *HbERF-IXb4* gene. For the gene *HbERF-IXb2*, which was induced by ET, the combinations W x MeJA and W x ET reduced by 2-3 times its relative transcript abundance.

Interactions between wounding, ethylene and methyl-jasmonate were studied through the variance of the expression of 13 *HbERF-IX* genes in response to the eight different combinations of factors (Control (C), W, MeJA, ET, WxMeJA, WxET, MeJAxET, WxMeJAxET). The complete analysis of variance table of the variable HbERF-IXa1 was

reported as example in Table 6. The summary of variance tables for each genes showed that the expression of 8, 5 and 7 genes was significantly changed by wounding, MeJA and ET treatments, respectively (Table 7). The relative transcript abundance of *HbERF-IXa2*, *HbERF-IXb1* and *HbERF-IXc4* genes were significantly modified for the three factors applied alone. Two genes are responsive to the W x ET treatment (*HbERF-IXb4* and *HbERF-IXc4*). Three genes are significantly regulated by the MeJA x ET treatment (*HbERF-IXa1*, *HbERF-IXb1* and *HbERF-IXb2*). Finally, the combination of all factors (W x MeJA x ET) led to predict that four genes (*HbERF-IXb1*, *HbERF-IXb2*, *HbERF-IXc4* and *HbERF-IXc6*) are strongly involved in the interaction of JA/ET signalling pathways.

Transactivation of the promoter of the *AtPDF1.2* gene by several HbERF-IXs

Cassettes consisting of a fusion of the dual CaMV 35S promoter with full length cDNA of *HbERF-IXb7, HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6* genes were cloned into the pMDC32 vector. These plasmids were co-transformed with the plasmid carrying the PDF1.2 promoter fused to the *gusA* gene by electroporation into *Arabidopsis* leaf protoplasts. Trans-activation of the promoter of the *Arabidopsis PDF1.2* gene fused with the *gusA* gene was evaluated by analyzing the GUS and Luciferase activities. The relative GUS/LUC activity increased gradually from 98 to 1205 for *HbERF-IXc4* and *HbERF-IXc5* but remained much lower than in the positive control using the AtORA59 to which the relative GUS/LUC activity reached 3886 (Figure 4).

Nuclear localization of the HbERF-IXc4

Subcellular localization of the HbERF-IXc4::GFP was carried out in *Arabidopsis* leaf protoplasts. Protoplasts were transformed with plasmids carrying the HbERF-IXc4::GFP translational fusion driven by the dual CaMV 35S promoter. Nuclear GFP activity could be observed in leaf protoplasts (Figure 5). This revealed that the HbERF-IXc4 protein is targeted in the nucleus and may play a role as transcription factor.

4. Discussion

Structural and functional features of ERF genes from group IX

Ethylene and jasmonic acid are plant hormones involved in the coordinated response to tapping and ethephon stimulation in *Hevea brasiliensis*. Application of ethephon stimulates the latex production. However, overexploitation of rubber trees causes an oxidative stress that leads to stopping the flow of latex. Ethylene Response Factors are the last transcription factors of the ethylene transduction pathway. ERFs are known to be at the crosstalk of different signalling pathways such as the abscisic acid or the JA. They constitute good candidates to explain molecular bases of both harvesting stresses and latex production. Although often related to the expression of genes involved in response to pathogen infection (Gu et al., 2000; Berrocal-Lobo and Molina, 2004), *ERF* genes of group IX have been shown differentially induced by ethylene and jasmonate and salicylic acid (Gu et al., 2000; Onate-Sanchez et al., 2007; Champion et al., 2009). This study showed that several HbERFs are strongly governed by the ET and JA interaction.

The members of the *Hevea brasiliensis* ERF family were divided into 12 groups (Duan et al., Submitted). Groups VII and IX have the largest number of genes with 23 and 19 members for *Hevea brasiliensis*, respectively. *Arabidopsis*, *Lycopersicum* and *Hevea* have the smallest number of ERF genes for the group IX (17, 18 and 19 respectively) compared to other species such as *Populus*, *Vitis* or *Nicotiana*, which counted 31, 41 and 57 members. This group have some specific features such as one additional amino acid at position 167 compared

to the 58-amino-acid-residue AP2 domain found in other groups. Group IX is also subdivided in three subgroups (a, b and c).

The classification based on the phylogenetic analysis of the amino acid sequences of the AP2 domain also corresponds to some functional features. Genes of group I to IV encoded dehydration-responsive element-binding (DREB) proteins. Two members of the *Hevea* DREB subfamily HbCBF1 and HbCBF2 were isolated (Cheng, 2005). They genes showed a high identity (99% and 82%) to HbERFIIIc1 and HbERF-IIIb2 according to the Duan's classification (Duan et al., Submitted). Genes of groups V to X are more related to the response to jasmonate and ethylene. Interestingly, ERF1, ERF2 and ERF3 genes are induced by JA in bark during JA-induced laticifer differentiation in *Hevea brasiliensis* (Wu, 2010). These genes correspond to three genes of group VII according to the Duan et al classification (Duan et al., Submitted): *HbERF-VIIa3, HbERF-VIIa17* and *HbERF-VIIa1*.

The genes of group IX have been implicated in regulating defence responses mediated by methyl jasmonate (MeJA) in a number of plant species (van der Fits et al., 2000; McGrath et al., 2005; Rushton et al., 2008). In *Catharanthus roseus* and *Nicotiana tabaccum*, the ERF genes from group IX such as ORCA3 has been shown to be a master regulator of primary and secondary metabolism during jasmonate responses (van der Fits et al., 2000; De Boer et al., 2011). The rubber biosynthesis going that route, genes of this ERF group IX are assumed to play important role in the regulation of latex cell metabolism.

Ethylene and jasmonate-responsive ERF genes from group IX

The phylogenetic analysis carried out with full length ERF protein sequences revealed that HbERF-IXc4 and HbERF-IXc5 are putative orthologs to ERF1, and HbERF-IXc2 is a possible ortholog to ORA59. The analysis with the AP2 domain only led to a different conclusion for ORA59 since *HbERF-IXc3, HbERF-IXc6* and *HbERF-IXc7* were the closest homologs. Given ORA59 and ERF1 are known to dramatically induce the expression of a *PDF1.2* gene (Pre et al., 2008; Champion et al., 2009), the dramatic accumulation of transcripts after the ethylene plus jasmonate treatment for genes *HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6* led to consider these three candidate genes as the putative orthologs. According to this experiment, *HbERF-IXc2* gene expression was not induced at all, and data for *HbERF-IXc3* are missing.

Given all combinations between wounding, methyl-jasmonate and ethylene were tested, this experimental design (2^3) allowed studying the interaction between these three factors. Values of relative transcript abundance have different orders of magnitude and required a Log transformation. Log values led to more correct residues. The main effects observed on Log values were additives and corresponded to a multiplicative effect on raw data. In the ANOVA, the expression of 8 genes was significantly changed whereas 11 genes showed significant regulations in the variance table. In the variance table, the major effects of factors (W, MeJA and ET) were significant. Nevertheless, although the relative transcript abundance of *HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6* genes was increased, no significant interaction between ET and MeJA was observed in the variance table. Any significant interaction in the ANOVA from Log values can reflect either a more multiplicative effect or by contrast to a less multiplicative effect. The significant effect for *HbERF-IXb1* and *HbERF-IXb2* could be reflecting an effect by attenuation. By contrast, the absence of effect for *HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6* genes could be explained by the fact that a simple additive model with significant effect of W, MeJA and ET using Log values correspond to a multiplicative model using raw data. The MeJA and ET interaction was not observed for *HbERF-IXc4* because the increase was already very high for the MeJA and ET factors when used alone. So the increase in transcript abundance for the combined treatment

MeJA plus ET compared to the factors alone was not so important than the increase between factors alone (W, MeJA or ET alone) and the control. However, *HbERF-IXc4* could not significantly transactivate the promoter PDF1.2 compared to the two other candidates. Pending more complete results for the *HbERF-IXc3* and *HbERF-IXc7* genes, which are not missing in this analysis, these results suggest that *HbERF-IXc5* and *HbERF-IXc6* could likely to be orthologs to ERF1 and ORA59, respectively.

5. Conclusions

The striking induction of some ERF genes by different combinations of factors (wounding, ET, and MeJA) was one of the first features of the *Hevea* orthologs to ERF1 and ORA59. In addition, phylogenetic analyses of ERF genes from the group IX based on seven different species revealed that HbERF-IXc4/HbERF-IXc5 and HbERF-IXc3/HbERF-IXc6/HbERF-IXc7 were found in small clades that contain ERF1 and ORA59, respectively. This partial characterization of 14 ERF genes for the group IX should be extended to the total 22 identified ERF genes. Especially the gene expression analysis in response to wounding, methyl-jasmonate and ethylene should be carried out for the *HbERF-IXc3* and *HbERF-IXc7* candidates identified in the phylogenetic analysis. The method of interaction analysis carried out on 13 genes looks appropriate for an analysis of this full set of genes. Then additional PDF1.2 transactivation experiments should be conducted on both *HbERF-IXc3* and *HbERF-IXc7* transcripts and the *Arabidopsis* ERF1 should be added as internal control in addition to the ORA59. Finally, the encouraging nuclear localization seen for the *HbERF-IXc4* should be done for all candidates.

Table 1. List of primer sequences for 14 HbERF- IX group genes cloned and expected length of amplicons after amplification by real-time PCR in *H. brasiliensis* clone PB 260.

Gene	Contig accession number in the global library	Primer sequences		Primer efficiency	PCR product (bp)
		Forward	Reverse		
HbERF-IXa1	*hevea*_454_c20829	*GAGGAGAAAAAGGTCGGTGGCT*	*AACACTCATAGGCTCGTTCGGG*	2.00	184
HbERF-IXa2	*hevea*_454_rep_c14033	*GTGGTTCACCGAAGAGGAG*	*TGCAGATGTGTAGAGCACTC*	1.94	198
HbERF-IXb1	*hevea*_454_c13287	*GGAAACTGAAGAGAGCGGAAG*	*ACAAGCCAGGGAACTCAAGG*	2.00	207
HbERF-IXb2	*hevea*_454_c19752	*GATTTTGGTGACGGGTCAT*	*GTCTACGCTCTACAGCAGATG*	2.00	184
HbERF-IXb3	*hevea*_454_rep_c9973	*ATTCTTTGAAGCGGTCTTGG*	*AGTCAGACACAGACTGAGAG*	2.00	189
HbERF-IXb4	*hevea*_454_rep_c12196	*CGTCCTGTTGAATTGCTCTT*	*TGTCAAAAACCTACAAGCCA*	2.00	185
HbERF-IXb6	*hevea*_454_rep_c86034	*CCTCTTGAAGTTGGGGATTCCG*	*GCTGCCTTGCCTTCCCTTTCC*	2.00	198
HbERF-IXb7	*hevea*_454_rep_c9858	*CCGATTCCAATAATACCCAGCA*	*ATTGAGCTGCCTTGCCTTCCCT*	1.98	184
HbERF-IXb8	*hevea*_454_rep_c16062	*AGAGCAGAAGGGGAGGGATAAC*	*CGACTACTGGTTGTTGCGCCAT*	1.97	185
HbERF-IXc1	*hevea*_454_rep_c12109	*AATTGGAGTCGGAGAGCAGT*	*AACTCACTCTTGCTGTAATGGC*	2.00	196
HbERF-IXc2	*hevea*_454_c72747	*TTAGCAATCCTCAACTTCCCG*	*CTTCTCTGCCTTGTTCAGTCCC*	2.00	179
HbERF-IXc4	*hevea*_454_rep_c3873	*TCAAGTGTAGTCAAGAAGAGGG*	*CACCAAGGTCTAGCATTCTCAG*	2.00	180
HbERF-IXc5	*hevea*_454_rep_c36947	*CAGTTGAAAGAGTGAAGGAATC*	*TCCAAGTAATCAGCACCCAAG*	2.00	186
HbERF-IXc6	*hevea*_454_rep_c18341	*GGAGCTGAGTATTTAGAAGAGC*	*CGGAGTGGATAACAAGATGTG*	2.00	187
HbActin	(Duan et al., Submitted)	AGTGTGATGTGGATATCAGG	GGGATGCAAGGATAGATC	1.94	194

Table 2. ERF transcription factor group IX genes members collected in different species. * Some publications mentioned 42 genes for this group but according to our analysis some errors of gene assignment have been noted and 30 genes only can be assigned to group IX.

Species	Group IX gene number
Arabidopsis	17
Populus	30*
Solanum	18
Nicotiana	53
Hevea	19
Gossypium	39
Vitis	41
Total	217

Table 3. Conserved amino acid residues in group IX for different species

ERF subgroup	Position of conserved amino acid residues according to Allen (1998) among different species						
	164		165		167	168	
	Residue	Percentage	Residue	Percentage	Supplementary Residue	Residue	Percentage
IXa	P	59/64	A	32/64	+X	G	62/64
IXb	P	76/81	N	58/81	+X	G	81/81
IXc	S	62/78	T	50/78	+X	G	77/78

Table 4. Summary of the characterization of ERF transcription factors from the group IX in various species. Ethylene (ET), jasmonic acid (JA), semi-quantitative reverse transcription-PCR (RT-PCR), real-time RT-PCR (Q-PCR)

Species	Generic name	Gene name	Group	JA response	ET response	Method	References	Function
Arabidopsis	At2g44840	*AtERF13*	IXa	Up-regulated		Q-PCR/microarray	McGrath *et al.* , 2005 ; Pauwels *et al.* , 2008	
Arabidopsis	At4g17500	*AtERF1*	IXa	Up-regulated	Up-regulated	Q-PCR/microarray	McGrath *et al.* , 2005 ; Pauwels *et al.*, 2008 ; Fujimoto *et al*., 2000	
Arabidopsis	At5g47220	*AtERF2*	IXa	Up-regulated	Up-regulated	Q-PCR/microarray	McGrath *et al.* , 2005; Pauwels *et al.* , 2008 ; Fujimoto et al, 2000	
Arabidopsis	At1g04370	*AtERF14*	IXc	Up-regulated		Q-PCR	McGrath *et al*.,2005	Disease resistance
Arabidopsis	At1g06160	*ORA59*	IXc	Up-regulated	Up-regulated	Q-PCR	McGrath *et al* ., 2005; Pré *et al* . , 2008	Disease resistance
Arabidopsis	At3g23230	*TDR1*	IXc	Up-regulated		Q-PCR	McGrath *et al* . , 2005	
Arabidopsis	At3g23240	*ERF1*	IXc	Up-regulated	Up-regulated	Q-PCR	McGrath *et al.* , 2005 ; Solano et al. , 1998	Disease resistance
Gossypium		*GhERF-IXa5*	IXa	Up-regulated		Q-PCR	Champion *et al*., 2009	Disease resistance
Gossypium		*GhERF-IXa1*	IXa	Up-regulated		Q-PCR	Champion *et al*,, 2009	Disease resistance
Gossypium		*GhERF-IXa2*	IXa	Up-regulated		Q-PCR	Champion *et al*., 2009	Disease resistance
Nicotiana		*NtERF210*	IXa	Up-regulated		RT-PCR	Rushton *et al*., 2008	Secondary metabolism
Nicotiana		*NtERF179*	IXa	Up-regulated		RT-PCR	Rushton *et al.* , 2008	Secondary metabolism
Nicotiana		*ORC1*	IXa	Up-regulated		RT-PCR	Rushton *et al.* , 2008	Secondary metabolism
Nicotiana		*Jap1*	IXb	Up-regulated		RT-PCR	Rushton *et al.* , 2008	Secondary metabolism
Nicotiana		*NtERF165*	IXb	Up-regulated		RT-PCR	Rushton *et al.* , 2008	Secondary metabolism
Nicotiana		*ACRE1*	IXc	Up-regulated		RT-PCR	Rushton *et al.* , 2008	Secondary metabolism
Solanum		*SlERF68*	IXc	Up-regulated		Q-PCR & GeneChip arrays	Sharma *et al.* , 2010	Salt & oxidation stress
Solanum		*Pti4*	IXa	Up-regulated	Up-regulated	Northern-blot	Gu *et al.* , 2002	Disease resistance
Catharanthus		*ORCA3*	IXa	Up-regulated	Up-regulated	Northern-blot	Van Der Fits *et al.*, 2001	Secondary metabolism
Vitis		*VvERF94*	IX	Up-regulated		Q-PCR	Licausi *et al.* , 2010	Ripening
Vitis		*VvERF85*	IX	Up-regulated		Q-PCR	Licausi *et al*,2010	Ripening
Vitis		*VvERF78*	IX	Up-regulated		Q-PCR	Licausi *et al*,2010	Ripening

Table 5. Analysis of the relative transcript accumulation of 14 ERF family group IX genes by real-time RT-PCR in bark of control (C) and treated plants by seven different treatments: (W) wounding; (MeJA) methyljasmonate; (ET) ethylene individually or a combination of treatments (T): a combination of MeJA and ET, or combination of W and ET, or combination of W, MeJA and ET in 3-month-old epicormic shoots of clone PB 260. The levels of expression were calculated as the ratio between the treatment and the control on average. The ANOVA test and the Student Neuman-Keuls test were used in the statistical analyses ($p<0.05$). It was considered as an up-regulation when the ratio >1.0, and a down-regulation when the ratio <1.0.

Gene	Treatments													
	W		MeJA		ET		W x MeJA		W x ET		MeJA x ET		W x MeJA x ET	
	ratio T/C	*p-value*	ratio T/C	*p-value*	ratio T/C	*p-value*	ratio T/C	*p-value*	ratio T/C	*p-value*	ratio T/C	*p-value*	ratio T/C	*p-value*
HbERF-IXa1	0.45	0.49	0.05	0.53	0.08	0.70	0.68	0.35	0.46	0.53	0.31	0.60	33.29	0.15
HbERF-IXa2	2.60	0.13	1.63	0.20	0.65	0.37	2.35	0.14	9.88	0.06	7.68	0.06	124.96	0.05
HbERF-IXb1	0.40	0.14	2.53	0.37	8.84	0.01	0.41	0.14	0.32	0.11	4.09	0.03	30.35	0.01
HbERF-IXb2	0.73	0.27	0.62	0.24	5.26	0.01	0.36	0.02	0.42	0.03	1.95	0.14	12.40	0.05
HbERF-IXb3	0.06	0.16	0.69	0.80	0.21	0.57	0.28	0.63	0.08	0.22	1.18	0.78	0.14	0.37
HbERF-IXb4	0.03	0.04	0.27	0.44	0.16	0.21	0.23	0.33	0.14	0.19	0.28	0.40	0.76	0.68
HbERF-IXb6	0.39	0.62	0.90	0.64	0.60	0.93	1.52	0.36	1.37	0.46	0.87	0.71	1.25	0.53
HbERF-IXb7	0.01	0.55	0.01	0.64	0.01	0.56	0.02	0.89	0.02	0.88	0.02	0.82	0.01	0.75
HbERF-IXb8	0.41	0.72	0.32	0.58	0.43	0.81	1.78	0.30	1.70	0.32	1.50	0.44	28.69	0.22
HbERF-IXc1	0.35	0.49	2.85	0.64	0.15	0.89	1.08	0.25	3.03	0.16	0.93	0.33	32.76	0.25
HbERF-IXc2	0.00	0.001	0.04	0.04	0.00	0.19	0.00	0.003	0.00	0.01	0.00	0.005	0.00	0.002
HbERF-IXc4	38.81	0.01	53.90	0.00	306.20	0.003	38.82	0.01	396.07	0.001	1754.05	0.000	3143.14	0.003
HbERF-IXc5	5.38	0.03	2.95	0.04	38.53	0.002	4.36	0.02	224.85	0.001	120.67	0.004	502.54	0.02
HbERF-IXc6	17.09	0.01	29.52	0.01	57.90	0.003	4.28	0.08	124.79	0.002	257.63	0.002	588.72	0.01

Table 6. Analysis of variance table of the variable HbERF-IXa1. Df: degree of freedom.

Interaction	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wounding	1	5.3027	5.3027	14.0901	0.001734	**
Methyl-jasmonate	1	0.8137	0.8137	2.1621	0.160841	
Ethylene	1	0.9313	0.9313	2.4747	0.135256	
Wounding x Methyl-jasmonate	1	0.4400	0.4400	1.1692	0.295590	
Wounding x Ethylene	1	0.0508	0.0508	0.1351	0.718011	
Ethylene x Methyl-jasmonate	1	1.9592	1.9592	5.2059	0.036539	*
Wounding x Ethylene x Methyl-jasmonate	1	0.0094	0.0094	0.0249	0.876504	
Residuals	16	6.0215	0.3763			

Table 7. Summary of analysis of variance tables of each tested genes from the group IX of the ERF family. Data correspond to F values. P-values are indicated as follows: 0.001 (***); 0.01 (**); 0.05 (*); 0.1 (°).

Gene	W	MeJA	ET	W x MeJA	W x ET	MeJA x ET	W x MeJA x ET
HbERF.IXa1	14.09**	2.16	2.47	1.17	0.14	5.2*	0.02
HbERF.IXa2	13.49**	8.76**	11.63**	3.38°	0.09	0.41	0.42
HbERF.IXb1	14.44**	20.68***	44.89***	20.37***	0.63	8.70**	33.91***
HbERF.IXb2	3.68°	0.42	33.15***	15.78**	0.16	11.44**	18.94***
HbERF.IXb3	12.15**	5.83*	0.24	0.15	0.16	0.01	0.90
HbERF.IXb4	3.36°	2.33	0.08	3.58°	5.76*	0.00	2.09
HbERF.IXb6	0.45	2.96	0.61	0.27	0.61	1.78	1.22
HbERF.IXb7	0.01	0.05	0.01	0.16	0.42	0.05	2.32
HbERF.IXb8	4.58*	2.86	4.63*	1.24	0.72	0.83	0.80
HbERF.IXc1	4.50*	1.72	1.44	0.29	0.20	0.00	0.22
HbERF.IXc4	9.63**	33.63***	138.81***	17.20***	6.29*	0.48	8.18*
HbERF.IXc5	7.33*	0.85	77.89***	3.82°	0.12	0.63	0.01
HbERF.IXc6	2.54	10.72**	92.20***	18.84***	0.08	0.02	11.01**

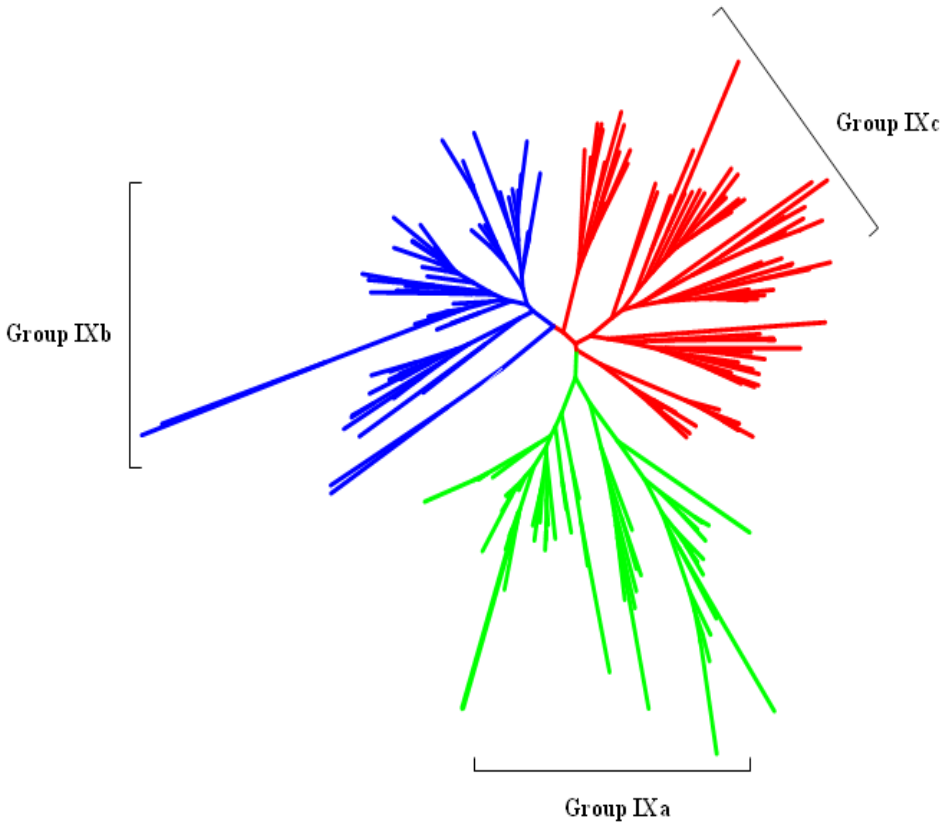

Figure 1. Phylogenetic tree of 222 deduced amino acid sequences of ERF from the group IX for seven species. The phylogenetic tree was constructed using the BioNJ method with the amino acid sequences of the AP2/ERF domain. The name of total 222 AP2/ERF proteins are indicated. Gh, Ga,Gr: *G. hirsutum, G. arboreum, G. raimondii* . P.: *Populus*; At: *A. thaliana* ; Hb: *H. brasiliensis*; Nt: *N. tabacum* , Sl: *S. lycopersicum*; Vv:*V. vinifera*. Color red: subgroup IXc; Color blue: subgroup IXb; Color green: subgroup IXa; the tree is rooted on the branch of APETALA2 (AP2R1)(not shown).

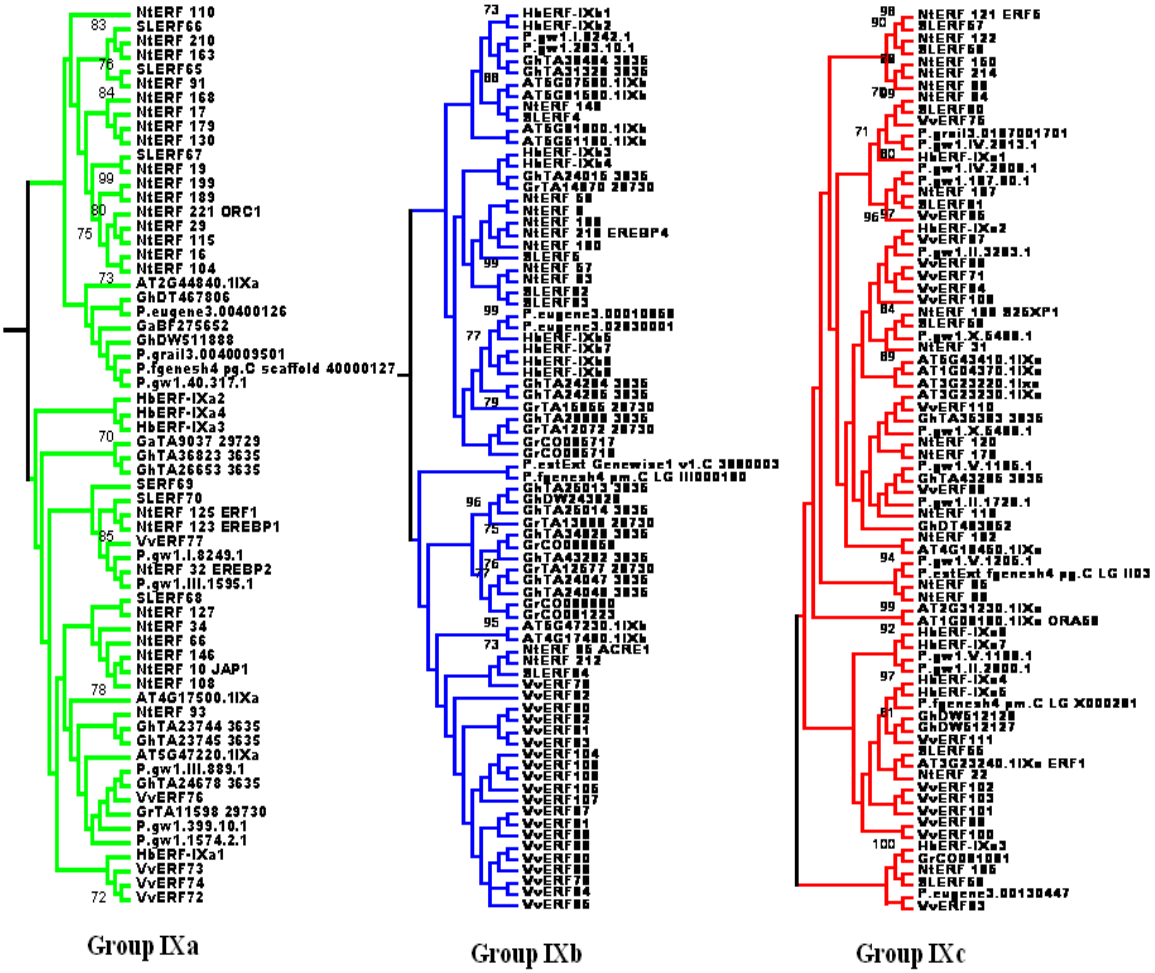

Figure 2. Phylogenetic tree of 222 deduced amino acid sequences of ERF from each subgroup Ixa, Ixb and IXc for seven species. The phylogenetic tree was constructed using the BioNJ method with the amino acid sequences of the AP2/ERF domain. The name of the seven species are indicated as: Gh: *G. hirsutum,* , Ga: *G. arboreum,* Gr: *G. Raimondii,* P.: *Populus*; At: *A. thaliana* ; Hb: *H. brasiliensis*; Nt: *N. tabacum* , Sl: *S. lycopersicum*; Vv:V. vinifera. Color red: group IXc; Color blue: group IXb; Color green: group IXa

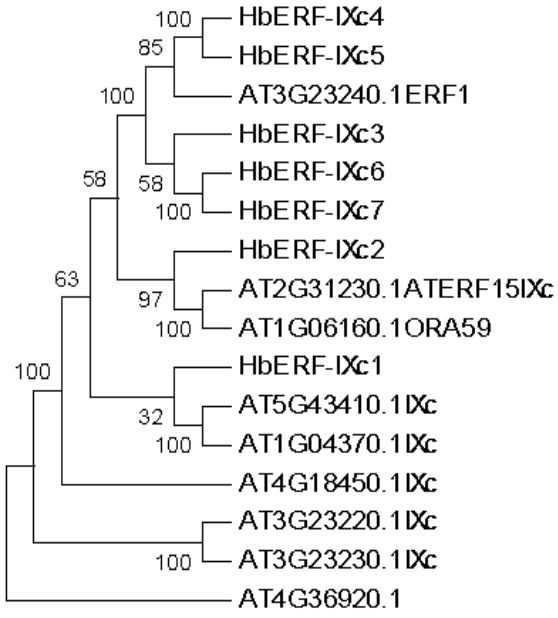

Figure 3. Phylogenetic tree of ERF from the subgroup c for *Hevea brasiliensis* and *Arabidopsis thaliana*. The phylogenetic tree was constructed using the BioNJ method with the deduced amino acid sequences of full lenght ERF transcripts. At: *A. thaliana* ; Hb: *H. brasiliensis*.

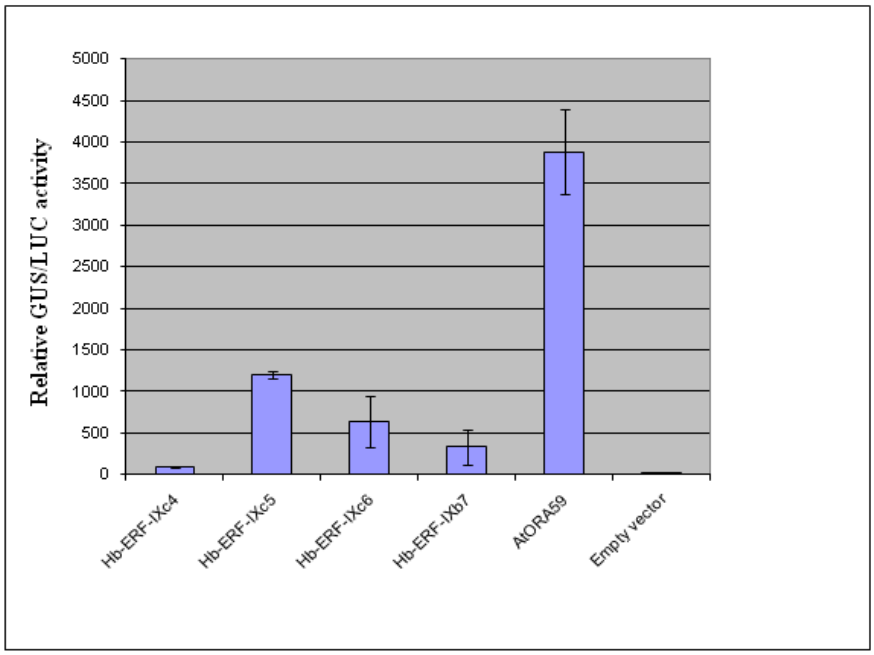

Figure 4. Transactivation of the promoter of the *Arabidopsis PDF1.2* gene by HbERF-IXb7, HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6. Protoplasts were co-transformed with plasmids carrying the PDF1.2 promoter fused to the *gusA* gene and overexpression vectors harbouring respectively the *AtORA59, HbERF-IXb7, HbERF-IXc4, HbERF-IXc5* or *HbERF-IXc6* genes driven by the dual CaMV 35S promoter. Relative GUS/LUC activity was observed in *Arabidopsis* leaf protoplasts after electroporation.

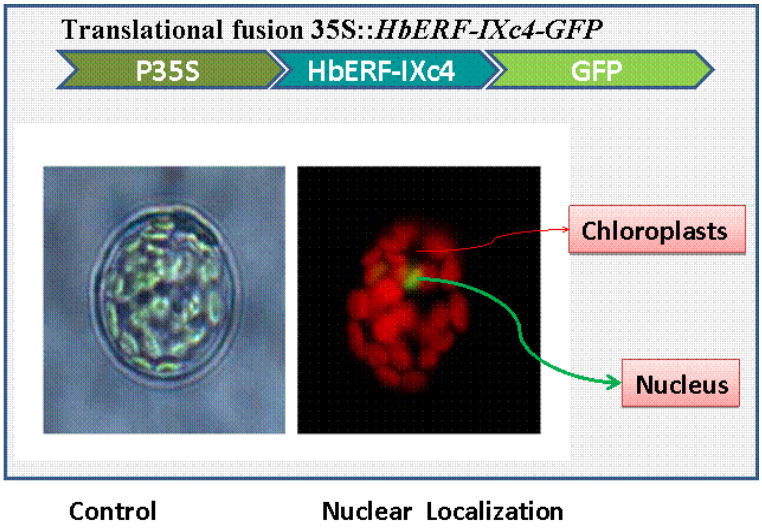

Figure 5 Subcellular localization of the HbERF-IXc4::GFP in *Arabidopsis* leaf protoplasts. Protoplasts were transformed with plasmids carrying the *HbERF-IXc4::GFP* translational fusion driven by the dual CaMV 35S promoter.

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DISCUSSION GENERALE

Rubber yield from *H. brasiliensis* is depending on many different factors such as the number of laticifer rings, the metabolic activity of laticifers, the tapping/ethephon stimulation systems, etc. Ethylene is an important factor in promoting the latex production as it could delay coagulation, prolonged the latex flow and increase latex yield. ET has been widely applied in rubber plantation as a stimulant in form of ethephon. Both tapping and ethephon frequencies influence the natural rubber production. However, the mechanism of ethylene stimulation in latex production is still not completely clarified. Exogenous JA was indicated to have important functions on rubber productions in *H. brasiliensis* since application of exogenous jasmonic acid and linolenic acid can induce laticifer formation and differentiation in *H. brasiliensis* (Hao and Wu, 2000). Mechanical wounding can also induce laticifer differentiation (Wu et al., 2002). Exogenous JA was also involved in the regulation of the rubber biosynthesis of *H. brasiliensis* (Zeng et al., 2009). Given a mechanical wounding triggers the JA&ET production, JA and ET are supposed to play an important role in the regulation of latex cell metabolism in response to harvesting stress in *H. brasiliensis*. These plant hormones are shown to synergistically regulate plant development and response to biotic or abiotic stresses. Concomitant activation of JA and ET response pathways is required to activate the induction of some defence-related proteins like *PDF1.2, THI2.1* and *CHIB,* etc. These genes are commonly used to monitor JA-dependent defence responses (Reymond and Farmer, 1998). However, the molecular basis of JA/ET co-action and signalling interdependency is largely unknown. Many members of AP2/ERF transcription factor superfamily were shown to be involved in response to jasmonate and ethylene signals in different species. AP2/ERF domain transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1) was shown as a key factor integrating the crosstalk of ethylene and jasmonate pathways (Lorenzo et al., 2003). Another AP2/ERF domain transcription factor, the ORA59, was also revealed to integrate jasmonic acid and ethylene signals in plant defence (Pre et al., 2008; Memelink, 2009; Zarei et al., 2011). Both ORA59 and ERF1 are able to function as activators of the PDF1.2 promoter (Pre et al., 2008). The research on the importance of AP2/ERF domain transcription factor family has been observed in different species.

In *H. brasiliensis*, wounding, JA and ET are important signals in coordinating responses to tapping and stimulation, such as latex cell metabolism, laticifer differentiation, and rubber biosynthesis as well as cell senescence. But the mechanism of interaction of JA and ET signalling pathways in *H. brasiliensis* has not been reported.

In this thesis, we have firstly investigated one group of 25 genes involved in the JA and ET signalling pathways and in cellular responses, and discovered that some genes have dependent and independent expression in response to wounding, MeJA and ET. Some candidate genes specifically regulated by wounding, methyl jasmonate (MeJA) and ET signals could be used as an internal control in further transcriptomic analyses. Influences of time (temporal regulation) were also observed on gene expression. Then we further identified the AP2/ERF transcription factor superfamily comprising 173 members. The putative function of AP2/ERF transcription factors in the latex metabolism and the tissue characteristics were analysed in *H. brasiliensis*. In order to discover which member of AP2/ERF family is responsible of the interaction of JA and ET, we identified the *HbERF-IXc5* and *HbERF-IXc6* as a putative orthologs in *H. brasiliensis* of ERF1 and ORA59 respectively. We studied the regulation of *HbERF-IXc5* gene on the expression of defence gene PDF1.2 to prove it is a key factor of the transduction of MeJA and ET signals.

This general discussion integrates the research advances on latex physiology and molecular bases of the rubber production. We discussed ethylene signalling and response; wounding and JA signalling; interaction ET x JA in *H. brasiliensis* and prospects of this study.

1. Ethylene signalling and response in *H. brasiliensis*

In *H. brasiliensis*, ethylene biosynthesis can be induced by endogenous or exogenous ethylene. Three *HbACO*1, *HbACO*2, *HbACO*3 genes and one *HbACS1* genes have been identified (Kuswanhadi, 2006; Kuswanhadi et al., 2010). Three other partial *HbACS* cDNAs were isolated in *H. brasiliensis* later (personal communication, Pascal Montoro). Ethylene biosynthesis is known to be determined by the activity of ACC synthase (ACS) and ACC-oxidase (ACO) (Yang and Hoffman, 1984). ET is perceived by a family of five membrane bound receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) in *Arabidopsis* (Hua J et al.,1998), and six in tomato LeETR1–LeETR6 (Wilkinson et al., 1995; Lashbrook et al., 1998; Tieman and Klee, 1999). Ethylene receptors are negative regulators of the ethylene response pathway (Hua et al., 1998). EIN2 plays a major role in the ethylene response and is a positive regulator of the pathway (Alonso et al., 1999). The EIN3 family are involved in regulation, and can stimulate the transcription of other transcription factors such as ERF1 (Solano et al., 1998; Alonso et al., 2003). They act as activators or repressors of additional downstream ethylene-responsive genes (Shinshi et al., 1995). In our research in *H. brasiliensis*, *HbETR2, HbEIN2, HbEIN3* were specifically regulated by ET treatment (cf. Chapter 1). The *HbETR2* transcripts were accumulated by 1.8 to 8.1 times after 24hours treatment, whereas the abundance of *HbEIN2* and *HbEIN3* transcripts was dramatically reduced. Early induction of the *HbETR2* gene by ET is suppressed by the ET inhibitor 1-MCP (Duan et al., 2010). These data are in accordance with results published for apple and peach, indicating that ETR2 gene expression is affected by 1-MCP (Dal Cin et al., 2007; Ziliotto et al., 2008).

Ethylene-response factor (ERF) transcription factors have been shown to play a crucial role in the JA and ET signalling pathways by activating plant defence responses in *Arabidopsis.* AP2/ERF superfamily genes have been identified in many species such as *A. thaliana*, *Populus*, *N. tabacum*, *S. lycopersicum*, *V. vinifera*, G. *hirsutum*, etc. In *H. brasiliensis*, the AP2/ERF superfamily was first characterized thoroughly in this study. This superfamily consists of one hundred and seventy-three members in *H. brasiliensis* by *in silico* analysis based on the conserved AP2/ERF domain (cf. Chapter 2). ERF family genes were revealed to be involved in the pathogen and abiotic stress response (Licausi et al.; Nakano et al., 2006; Champion et al., 2009). In *H. brasiliensis*, 115 members with full AP2/ERF domain are belonging to ERF family further classified into 11 groups. The functions of a few members of the AP2/ERF superfamily genes in *H. brasiliensis* were reported to be regulated by ET and JA. For example, *HbERF1*, *HbERF2*, *HbERF3* (belonging to HbERF-VIIa) and *HbRAV1* genes were suggested to be induced by JA in bark during JA-induced laticifer differentiation (Chen et al.). *HbCBF1* (belonging to HbERF-IIIc) and *HbCBF2* (belonging to HbERF-IIIb) were regulated by cold and drought stresses (Cheng, 2005; Cai, 2008)}. *HbEREBP1* gene (belong to HbERF-VIIIa) down-regulated by tapping or exogenous ethephon or methyl jasmonate at early stage was suggested to be a negative regulator of defence mechanisms in laticifers (Chen et al.). Sixty-six AP2/ERF transcripts in latex may have important role in contributing the rubber production. The functions of ERF family genes

in rubber production and the ET and JA signalling transductions in *H. brasiliensis* remain further to be clarified.

The mechanism of regulation of latex production by ethylene has not been completely elucidated in *H. brasiliensis*. Physiological and biochemical research evidences showed that ethylene increase the membrane permeability, leading to prolonged latex flow and activate general regenerative metabolism (Coupé and Chrestin, 1989; Pujade-Renaud et al., 1994). Ethylene increased the activity of invertase to accelerate glycolysis to improve the supply of carbon source (such as Acetyl coenzyme A) for rubber biosynthesis. Latex adenylate pool, polysomes and rRNA contents as the indications of metabolic activation were obviously accumulated in laticifers (Coupé, 1989 ; Amalou et al., 1992). Furthermore, the activities of glutamine synthetase (GS) and chitinase have been shown to be modulated by ethylene in *H. brasiliensis* (Coupé and Chrestin, 1989).

Ethylene has been shown to regulate the expression of genes involved in different signalling pathways in *H. brasiliensis*. Some laticifer specific genes were reported for the first time to be induced by ethylene in *H. brasiliensis* by Kush et al (Goyvaert et al., 1991). Hevein, a lectin-like protein involved in the coagulation of latex was regulated by ethylene. MnSOD functions as a superoxide scavenger (Broekaert et al., 1990; Gidrol et al., 1994; Sivasubramaniam et al., 1995). High levels of MnSOD induced by ethephon might aid in preventing lutoid disruption caused by superoxide radicals to speed up the latex flow (Miao and Gaynor, 1993). The *HbCuZnSOD* gene displays a differential expression in the three genotypes PB260, PB217, RRIM600, and in response to ethylene and wounding in young budded plants (Leclercq et al., 2010; Jiahong Zhu, 2009).

But ethylene was thought to have little direct effect on accelerating natural rubber biosynthesis (Zhu and Zhang, 2009). Natural rubber is biosynthesized by the isoprenoid biosynthesis pathway using as precursor the isopentenyl pyrophosphate (IPP) metabolized in the mevalonate (MVA) pathway. It was shown that the plastidic 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (MEP) pathway could also generate the IPP in latex cells (Kekwick, 1989; Chow et al., 2007; Sando et al., 2008). Three key enzymes in rubber biosynthesis were shown closely related to the yield and quality of rubber as the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), Farnesyl-diphosphate (FDP) synthase and Rubber transferase (RuT) (Miao and Gaynor, 1993; Adiwilaga and Kush, 1996). The enzymes HMGS (3-hydroxy-3-methylglutaryl coenzyme A synthase) and HMGR are involved in early steps of rubber biosynthesis. Ethephon influenced the expression and activity of *HMGS* gene (Suwanmanee et al., 2004; Sirinupong et al., 2005). Three HMGR genes as *hmg1*, *hmg2* and *hmg3* have been identified in *H.brasiliensis* (Chye et al., 1991; Chye et al., 1992). The *hmg1* gene may involve in rubber biosynthesis, and was induced by ethylene. The *Hmg2* could be linked to the defence reactions against wounding and pathogens and *hmg3* was possibly involved in isoprenoid biosynthesis of (Chye et al., 1992; Wittsuwannakul et al., 1998). However, ethylene could not influence the activity of HMGR (Wititsuwannakul et al., 1986). FDP synthase could catalyze the synthesis of the last common substrate in rubber biosynthesis. The expression of FDP synthase was not significantly affected by ethylene (Adiwilaga and Kush, 1996). RuT is one of the key enzymes in rubber biosynthesis to initiate the rubber molecule and IPP to form the polymer. But ethephon did not affect the gene expression and the activity of RuT. Small GTPases HbArf2 and HbRab4 were down-regulated significantly by Ethrel (a commercial form of ethephon) (Qin et al.,2011).

Ethephon treatment results in the prolonged flow of latex. Latex flow is regulated by some ethylene-induced genes (Coupé and Chrestin, 1989; Zhu and Zhang, 2009). Sucrose is the unique precursor of rubber synthesis. Sucrose content and metabolic intensity were considered as a limiting factor for rubber biosynthesis (Tupý J., 1973; Tupy, 1988). Sucrose transporters *HbSUT1A* and *HbSUT2A* could be distinctly induced by ethylene specifically in laticifers. They are suggested to increase the sucrose import into laticifers, and required for the stimulation of latex yield by ethylene (Coupé and Chrestin, 1989; Dusotoit-Coucaud et al., 2009)). It was also found that ethylene stimulation of latex yield depends on the expression of a sucrose transporter (*HbSUT1B*) in *H. brasiliensis* (Dusotoit-Coucaud et al., 2010). Water circulation mediated by aquaporins between the laticifers and their surrounding tissues has been linked with the latex stimulation by ethylene. Aquaporins HbPIP2;1 and HbTIP1;1 were suggested to play an important role in water fluxes across the laticifer plasmalemma to control the latex water content, and influence the processes of latex flow (de Faÿ and Jacob, 1989; Tungngoen et al., 2009). Aquaporin HbPIP2;1 was shown to have a higher efficiency of than HbTIP1;1 in increasing plasmalemma water conductance. *HbPIP2;1* gene was expressed in all liber tissues including the laticifers. Ethylene induces the expression of *HbPIP2;1* gene in both liber tissues and laticifers, whereas decreases the expression of *HbTIP1;1* in liber tissues but increases in laticifers. Glutamine synthetase (GS) is a key enzyme of nitrogen metabolism, and the GS-glutamate synthase cycle might be the major pathway for the amino acid and protein synthesis required for latex regeneration (Pujade-Renaud et al., 1994). Ethylene could up regulate GS expression in *H. brasiliensis* latex cells, suggesting that GS was involved in stimulation of rubber production by ethylene (Pujade-Renaud et al., 1994). Ethylene induces an over-expression of chitinase in latex. The higher over-expression of one chitinase can explain the partial deglycosylation of the hevein receptor at the surface of rubber particles and resulted in the delay of coagulation of rubber particles. The level of hevein and chitinase expression in latex is a clonal characteristic, linked to the characteristics of the latex flow (Chrestin et al., 1997).

2. Wounding and JA signalling in *H.brasiliensis*

One of the immediate responses to wounds **is** the production of endogenous JA (Laudert and Welter, 1998), which starts with the oxygenation of a-linolenic acid (LA) (Creelman and Mullet, 1997). The genes encoding JA-biosynthetic enzymes such as lipoxygenase (LOX2), allene oxide synthase (AOS) and 12-oxophytodienoic acid reductase (OPR3) will be activated (Laudert and Welter, 1998; Schaller et al., 2000). All JA biosynthetic genes such as LOX2, AOS and OPR3 are JA-inducible, implying the existence of a feed forward mechanism for rapid and sustainable accumulation of JA in response to stresses (Sasaki et al., 2001; Wasternack, 2007). In *H. brasiliensis*, AOS was identified as the key enzyme of JA synthesis (Norton et al., 2007; Duan et al., 2005). Wounding activates the gene transcription by stimulating both JA-dependent and –independent wound signalling mechanisms (Leon et al., 2001; Devoto et al., 2005). However, the modes of action of these mechanisms, and their involvement in local as opposed to systemic wound responses, remain unknown (Fonseca et al., 2009; Matsuda et al., 2009). Application of exogenous JA or its methyl ester can induce a variety of wound-responsive genes, including *Pin2* and *Vsp* (Titarenko et al., 1997). Meanwhile, some research also indicated that methyl jasmonate might act as the long distance signal as it could diffuse to distal parts of the plant via the vapor phase or by intercellular migration, possibly through the phloem to activate the same gene response in the neighbor cells thereby propagating MeJA response in the whole plant or the neighbor plant(Cheong JJ and Choi YD, 2003).

In *H.brasiliensis*, several key factors of the jasmonic acid signalling pathway have been characterized, which will aid to understand the molecular mechanism of laticifer differentiation induced by mechanical wounding and JA (Hao and Wu, 2000; Wu et al., 2002). The response of these genes to wounding or JA was either JA-dependent or JA-independent. Three JA signalling main components including coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) and Jasmonate insensitive 1/MYC2 (JIN1/MYC2) are very important factors in JA signalling pathway.

This study showed that *HbCOI1* gene in bark was regulated by wounding and MeJA, but not by ET (Duan et al., 2010). The *COI1* gene encodes an F-box protein involved in the SCF-mediated protein degradation by the 26S proteasome, which is required for most JA-mediated responses (Xie et al., 1998). The *JAR1* gene encodes an enzyme for the conjugation of isoleucine and JA in JA-Ile, which is considered to be the bioactive JA molecule perceived by plants (Staswick et al., 2002; Thines et al., 2007). For the clone RRIM 600, it was reported that *HbCOI1* was present as a single copy and had high transcription in laticifers and low transcription in bark and leaves (Peng et al., 2009). The transcription of *HbCOI1* gene was induced in latex by tapping and jasmonate but not by ethephon. This confirmed that *HbCOI1* expression is ET independent in *H. brasiliensis* and displays a differential response under the regulation of wounding and JA.

H. brasiliensis HbJAZ1 gene was strongly up-regulated by tapping and mechanical wounding, but not by Ethrel. JIN1/MYC2 transcription factor regulates some JA responsive gene expression (Lorenzo et al., 2004). Jasmonate ZIM-domain (JAZ) proteins are repressors of JA signalling, JAZ1 and JAZ3 were shown to interact with JIN1/MYC2 to inhibit the expression of JA-responsive genes. COI1 or COI1-JAZ complex acts as a receptor for JA-Ile in *Arabidopsis* (Katsir et al., 2008). JA (more specifically JA-Ile) promotes interaction between JAZ proteins and the SCF^{COI1} ubiquitin ligase, leading to the ubiquitination and subsequent degradation of JAZ proteins by the 26S proteasome. The degradation of JAZ proteins allows transcription factors (such as MYC2) activating the expression of JA-responsive genes (Chini et al., 2007; Thines et al., 2007). JAZ genes are induced by JA. Characterization of *HbJAZ1* will help to understand JA signalling pathway in *H. brasiliensis* (Zhao et al.,2011).

In addition, *HbMYC1* and *HbMYC2* genes were identified in *H. brasiliensis*. It was highlighted in *Arabidopsis* that the existence of two branches in the JA signalling pathway, antagonistically regulated by AtMYC2 and ERF1, that are coincident with the alternative responses activated by JA and ET to two different sets of stresses, namely pathogen attack and wounding. The antagonistic action of MYC2 and ERF1 may cause the independence between wound signalling and pathogen-defence signalling (Lorenzo et al., 2004; Lorenzo and Solano, 2005). JA signalling could be a mobile signal to transmit long-distance information leading to systemic immunity in *Arabidopsis* (Truman et al., 2007). *HblMYC1* was up regulated by tapping and Ethrel. *HblMYC2* was down-regulated by successive tapping significantly. Upon response to methyl jasmonate, *HblMYC2* and *HblMYC1* expression displayed a mutual movement and flow relationship. *HblMYC1* and *HblMYC2* present most abundantly in latex, but nearly undetected in bark tissues and roots (Zhao et al.,2011).

In *H. brasiliensis*, genes responsive to wounding and JA displayed at least two different models of JA-dependent and JA-independent. The response to wounding is not

strictly dependent on the jasmonate signalling pathway, which is consistent with reports in the literature. Wounding signals are transmitted via at least two different pathways including jasmonic acid independent gene activation at the wound site and JA dependent systemically in non-damaged tissues (Titarenko et al., 1997; Leon et al., 2001). Indeed, five genes (*HbMAPK*, *HbBTF3b*, *HbCAS1*, *HbLTPP* and *HbPLD*) were shown to be specifically down-regulated by wounding without any ET or MeJA effect in bark of the young plantlet of clone PB260 (Duan et al., 2010). Four genes (*HbMYB*, *HbCAS2*, *HbCIPK* and *HbChi*) were shown to be methyl jasmonate specifically inducible. The fact of three genes *HbCOI1*, *HbSAUR* and *HbDef* induced by either wounding or MeJA suggested that the responses could be JA-mediated co-regulation (cf. Chapter 1).

In *H. brasiliensis*, the key enzyme of rubber biosynthesis, farnesyl diphosphate (FDP) synthase, is expressed in latex producing cells and in the epidermal cells suggesting a dual role of this gene in the biosyntheses of rubber and other isoprenoids. The expression of this gene is not significantly affected by ethylene, but tapping increases its expression level (Adiwilaga and Kush, 1996).An appreciable increase in the expression of *Am-FaPS-1* (FDP synthase) in *Aquilaria microcarpa* was reproducibly observed upon methyl jasmonate treatment (Kenmotsu et al., 2011).

Transcripts of the *HbWRKY1* gene were shown to be significantly accumulated in latex and flowers, lower in bark, leaves and roots (Putranto et al., 2011 on line). The transcription of *HbWRKY1* in latex was strongly induced by tapping, ethylene and jasmonic acid. HbWRKY1 mainly functions in response to mechanical wounding, some wounding-related hormones, as an ethephon-induced WRKY transcriptional factors in *H. brasiliensis* (Zhu and Zhang, 2009).

JA may play an important role in flower development and cell senescence in *H. brasiliensis.* Members of the MADS box gene family play important roles in flower development (Dornelas and Rodriguez, 2005). Three MADS-box genes *HbMADS1*, *HbMADS2* and *HbMADS3* were highly expressed in laticifer cells. *HbMADS1* and *HbMADS3* genes were induced by jasmonic acid. Ethephon had no effect (Li et al.). Rubber tree FLORICAULA/LEAFY (FLO/LFY) orthologous to *HbLFY* is expressed in lateral meristems that give rise to inflorescences and in all flower meristems (Dornelas and Rodriguez, 2005). Transcription factor *HbMyb1* was reported to be likely associated with TPD. The expression of *HbMyb1*was significantly decreased in the bark of TPD. Intense tapping and ET stimulation resulted in decreased expression of *HbMyb1*. HbMyb1 may act as a negative regulator to suppress the programmed cell death-induced genes (Chen et al., 2003; Venkatachalam et al., 2007). Our research on one member of *HbMYB* gene showed that *HbMYB* was MeJA dependent and down-regulated by MeJA stress in *H. brasiliensis*, and is possibly involved in JA-mediated stress signalling pathways, as MYB21 and MYB24, are JA-inducible in *A. thaliana* (Mandaokar and Browse, 2009). This may show that they are two different members of the MYB transcription factors superfamily in *H.brasiliensis* (Duan et al., 2010).

3. Response to wounding and JA in *Hevea brasiliensis*

Natural rubber is practically exploited by tapping. This process represents the repeated wounding applied to the bark of the tree. Among a number of stresses that plants encounter in nature, mechanical damage can be particularly detrimental. Plants undergoing the mechanical

wounding activate defence mechanisms. Responses to mechanical damage are either local or systemic or both and hence involve the generation, translocation, perception, and transduction of wound signals to activate the expression of wound inducible genes (Leon et al., 2001; Lorenzo and Solano, 2005; Wasternack, 2007). Wounding and jasmonic acid are important factors involved in coordinating responses to tapping and stimulation in *H. brasiliensis*, such as latex cell metabolism, laticifer differentiation, rubber biosynthesis and cell senescence. Exogenous jasmonate and mechanical wounding can significantly induce laticifer differentiation (Hao and Wu, 2000; Wu et al., 2002). Recently, jasmonate was shown to play an important role as a signalling molecule that regulates rubber biosynthesis (Zeng et al., 2009; Duan et al., 2010). *Hbvp1* was suggested as JA-inducible and play an important role in rubber biosynthesis in *H. brasiliensis* (Zeng et al., 2009).

In *Hevea*, wounding and MeJA may regulate the latex metabolism differentially. It has also been reported that a lethal imbalance between cyanogenic and CN detoxifying activities (CAS) in the phloem of necrotic trees can lead to the poisoning of neighbouring cells and the spread of tissue necrosis towards the tapping cut (Chrestin et al., 2004; Nandris, 2004). HCN is the by-product of the ethylene biosynthetic pathway. HCN is detoxified by the beta-cyanoalanine synthase (βCAS) (Fujita et al., 2006; Abe et al., 2008). Our research indicated that *HbCAS1* (latex βCAS) was shown to be specifically down-regulated by wounding without any ET or MeJA effect. By contrast, the *HbCAS2* (phloem βCAS) was induced by MeJA treatment specifically in *H. brasiliensis* (Duan et al., 2010). JA might induce CAS2 to detoxify HCN and could decrease the rate of TPD. This implied that wounding and MeJA have different impact on the latex metabolism.

Genes encoding small GTPases (*HbArf1*, *HbRab2*, *HbRab3* and *HbRab4*) were upregulated by latex exploitation and exogenous methyl jasmonate, especially *HbRab1* and *HbArf2*. Wounding might up-regulate the expression of *HbRab1* and *HbArf2* through a jasmonic acid-mediated signalling pathway. *HbRab1* was speculated to be involved in latex regeneration (Qin et al., 2011), The transcription of *Hb14-3-3c* gene in latex was induced by jasmonate and ethephon. It is proposed that the Hb14-3-3c protein may participate in regulation of rubber biosynthesis (Yang et al., 2011).

Biotic and abiotic stresses can induce higher expression of chitinases in plants (Collinge et al., 1993). Chitinolytic enzymes in plants are numerous and highly diverse (Michal Shoresh, 2010). Our research on one *HbChi* gene showed it could be down regulated by MeJA (Duan et al., 2010). But *BjCHI1* is induced by wounding and methyl jasmonate (MeJA) treatment and unaffected by ethylene (Chye et al., 1991).

4. Interaction ET x JA in *H.brasiliensis*

Wounding and ethylene both have an impact on the rubber production, but the molecular mechanism of wounding and ethylene interaction is still not clear yet. Tapping (wounding) is one important exploiting factor which determines the rubber production(Gidrol *et al.*,1994; Gidrol et al., 1994). Ethylene is a major stimulating factor for natural rubber production with the effect of a prolonged latex flow and subsequent increase in yield. Many genes were activated by both wounding and ethylene signals simultaneously. For example, ethylene biosynthesis key enzyme ACC oxidase (ACO) genes could be regulated by both

wounding and ethylene but with different expression profiles. The ACC oxidase (ACO) gene *HbACO1* was expressed at a higher level than the other two genes, and was down-regulated by ethylene and wounding in leaf and bark. *HbACO2* and *HbACO3* were transiently induced in response to ethylene and wounding. 1-MCP could abolish the ethylene induction of *HbACO2* and *HbACO3* expression with a positive feedback regulation. All *HbACO* multigene family genes were expressed at all stages of development from in vitro callus to the exploited plant (Kuswanhadi, 2006). *HbACO1* suggested being responsible for basal levels of ethylene production while *HbACO2* and *HbACO3* are up-regulated in response to external factors (Kuswanhadi et al., 2007; Kuswanhadi et al., 2010).

The identification of key factor genes involved in the crosstalk of MeJA and ET signals in *H. brasiliensis* lead us to understand the mechanism of JA and ET crosstalk on the regulation of latex metabolism. ERF1 and ORA59 genes were known as a cross point genes in the jasmonate and ET signalling pathways (Lorenzo et al., 2003). In this study, we found that *HbERF-IXc2* gene is an ortholog of ORA59, *HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6* genes are the putative ortholog genes to ERF1. Further study found that *HbERF-IXc5* functions like the ERF1. *HbERF-IXc5* was induced by both ET and MeJA in *H.brasiliensis.* And the response of *HbERF-IXc5* to the combined treatment of wounding, MeJA and ET was significantly mulplified. This key gene *HbERF-IXc5* in MeJA and ET signal in *H. brasiliensis* will serve as an integrative centre of JA and ET interaction on the regulation of latex metabolism. It will help us to establish the model of wounding, JA and ET signalling pathway in *H.brasiliensis.*

In addition, the relative transcript abundances of the *HbGP* and *HbACR* genes were enhanced by all three treatments of wounding, MeJA and ET respectively. This may also give a clue that JA and ET crosstalk can regulate the rubber biosynthesis.

CONCLUSIONS & PERSPECTIVES

1. Perspectives

i. Functional analysis of ORA59 and ERF1 ortholog genes in *H. brasiliensis*

Phylogenic analysis on the ERF group IX members among the different species using the AP2 domain and the full length deduced proteins, respectively, showed that *HbERF-IXc4, HbERF-IXc5* and *HbERF-IXc6* are putative orthologs of *ERF1* gene.*HbERF-IXc2* is the putative ortholog of *ORA59* gene. The expression profile of *HbERF-IXc4, HbERF-IXc5 & HbERF-IXc6* genes are all responsive to the regulation of wounding, JA, ET and their combination treatment. Given the importance of ERF1 and ORA59 in the ET and JA interaction, these genes will be studied together for the function analysis.

Isolation of the Full-lenght HbERF-IXc2 cDNA

In our research, it was suggested that the *HbERF-IXc2* gene is the ortholog of ORA59 based on the phylogenetic analysis among the different species. Given the important role of ORA59 in the signal interaction of JA and ET, our next step will continue to work on the *HbERF-IXc2* gene, to isolate the whole length of this gene, and analyse its characterisation and study its function in the signalling pathway, and investigate its possible regulation on the latex production.

Firstly, it will be necessary to clone the whole length cDNA of *HbERF-IXc2*. According to the contig length in the cDNA library, we will design primers to further get the full length. The response to JA and ET signal regulation will be examined by Q-PCR in different combined treatment. A housekeeping gene *Hbactin* will be used to normalize amounts of cDNAs among the samples. These analyses will be conducted with three biological replications. Gene expression of *HbERF-IXc2* in response to the interaction of JA and ET signals will be compared with the other HbERF-IX group members. If it showed strong regulation by JA and ET signals, we will continue the verify characteristic of this transcription factor.

Downstream Activation of Ethylene Responses by HbERF-IXc2, HbERF-IXc4, HbERF-IXc5, HbERF-IXc6

To verify the characteristic of these genes as a transcription factor, the trans-activation experiments will be required to evaluate their capability of binding the *PDF1.2* gene promoter to modulate the gene expression. Several constructs of the expression vector will be needed. By using gateway system, ORF of these genes will be inserted into the vector pMDC32 to get the expression vector 35S:: HbERF-IX for transformation. *Arabidopsis* leaf protoplasts will be co-transformed with a reporter plasmid carrying PDF1.2 promoter fused to GUS, over expression plasmids carrying *HbERF-IXc2, HbERF-IXc4, HbERF-IXc5, HbERF-IXc6,* or ORA59 genes driven by the dual CaMV 35S promoter, and a reference plasmid carrying the Renilla LUCIFERASE (LUC) gene under the control of the CaMV 35S promoter. As controls, PDF1.2 promoter-GUS will be co-transformed with the corresponding empty effector vectors. Protoplasts will be transformed with the three constructs in a ratio of 1:1:3 (GUS:LUC:effector plasmid). Protoplasts will be harvested at 18 h after transformation and frozen in liquid nitrogen. GUS reporter gene expression will be related to LUC expression to correct for transformation and protein extraction efficiency. Average GUS-LUC ratios from

triplicate experiments were expressed relative to the respective vector controls (Pre et al., 2008).

Subcellular Localization of HbERF-IXc2, HbERF-IXc4, HbERF-IXc5, HbERF-IXc6

The termination codon of the ORF of HbERF-IX candidates will be removed by PCR and then subcloned in-frame to the 5′-terminus of the coding region of green fluorescent protein (GFP), under the control of the cauliflower mosaic virus 35S promoter in the pMDC83 vector (Mark D. Curtishttp://www.plantphysiol.org/content/133/2/462.full - COR1#COR1, 2003). The resulting 35S::HbERF-IX-GFP fusion construct and the control vector 35S::GFP, will be transformed into *Arabidopsis* protoplast or white onion epidermal cells for transient expression. The green fluorescence of GFP can be visualized by a Confocal Laser Scanning Microscope.

HbERF-IXc2 or HbERF-IXc5 is a GCC-box-binding Protein

To verify that the AP2/ERF domain of the proteins of these candidate genes binds to the GCC box, the coding region of each AP2/ERF domain-containing gene will be amplified and subcloned as GST gene in the pGEX-4T-1 vector (Amersham, England), resulting in the expressing vector of the recombinant protein pGST-HbERF-IX. The pGST-HbERF-IX proteins will be expressed in *Escherichia coli* BL21 cells. The purified GST-HbERF-IX proteins have to be prepared. Electrophoretic mobility shift assays (EMSA) could be conducted according to the protocol (Woo et al., 2002). Probe containing a GCC box probe, a mutant GCC (mGCC) box probe or a DRE box probe synthesized and purified will be mixed with the purified protein in the binding buffer (Zhang et al., 2007).

RNA Interference to Silencing the Gene Expression

Conserved microRNA miR159 was predicted to cut the HbERF-IXc2 sequence, and its position localised inside the coding sequence region and after AP2 domain. Silencing of the HbERF-IXc2 gene can be performed by expressing double-stranded RNA. For the RNAi-HbERF-IXc2 constructs several methods exists. First method, the open reading frame of HbERF-IXc2 could be cloned as an inverted repeat into the pHANNIBAL vector (GenBank accession no. AJ311872) to get the RNAi- HbERF-IXc2 constructs. For the RNAi control line GUS 5, the GUS open reading frame will be cloned into pHANNIBAL. The pHANNIBAL expression cassettes will be cloned into the binary vector pART27 (Pre et al., 2008). Second method, a partial HbERF-IXc2 cDNA fragment could be amplified using specific primers, and recombined into the pHANNIBAL vector as plasmid RNAi-HbERF-IXc2. All plasmids will be then transformed into *Agrobacterium tumefaciens* strain GV3101.The double-stranded RNA interference (dsRNA) construct can be produced via a PCR using the amplification products from a unique N-terminal region (300 bp) spanning a portion of the 5′-untranslated region and adjacent coding region of the *HbERF-IXc2* gene. The sense strand will be then amplified using a primer combination that generated a selective restriction site on the opposed ends of the product, whereas the anti-sense strand was amplified using a primer combination that added selective restriction sites on the opposite ends of the product. Third method, primer design is extremely important for PCR. The primer sequences could use the methods as an option: forward primer, 5'-loop region-anti-sense-TTTTTT-SV40 annealing region-3'; reverse primer, 5'-homologous region (complementary to loop) -anti-sense-hU6 annealing region-3'(Jun Xu et al., 2008).

Overexpression of HbERF-IXc2 by Genetic Transformation

According to the transactivation result and the RNAi result, it will be useful to confirm the function of the candidate gene in *H. brasiliensis* by gene overexpression to check the function in *H. brasiliensis*, and observe its phenotype. Or transfer this gene to RNAi lines to analyze the recovery effect of *HbERF-IXc2 etc.*

Roles in the Latex Metabolism and on the Laticifer Differentiation
By in situ hybridization, the localization of the expression of HbERF-IXc candidates will be checked. They are expected to be expressed in the laticifer. By latex diagnosis, check the activities of some key enzymes to see the difference.

ii. Characterization of *AP2/ERF* genes involved in *H. brasiliensis* development

Genes from the AP2 (APETALA2) family play a variety of roles throughout the plant life cycle: from being key regulators of several developmental processes, like floral organ identity determination or control of leaf epidermal cell identity, to forming part of the mechanisms used by plants to respond to various types of biotic and environmental stress. For example, *Ap2*, *Aintegumenta* (*Ant*), *Glossy15* (*Gl15*), and *Indeterminate spikelet 1* (*Ids1*) transcription factors contain all two AP2 domains and play a role in plant development. *Ant* is required for ovule and female gametophyte development (Elliott et al. 1996 ✦; Klucher et al. 1996), *Gl15* is required for the maintenance of juvenile traits of epidermal cells in maize leaves from node 2 to node 6 (Moose and Sisco 1996), and *Ids1* controls maize spikelet meristem fate (Chuck et al. 1998). Members of the AP2 family of transcription factors, such as BABY BOOM (BBM), play important roles in cell proliferation and embryogenesis in *Arabidopsis thaliana* (atbbm) and *Brassica napus* (bnbbm) and *GmBBM1* control somatic embryogenesis and embryo development in soya (El Ouakfaoui et al.). RAV subfamily transcription factor contains an AP2- and B3-DNA binding domain, which belongs to the AP2/ERF family. It encodes transcriptional regulators with a variety of functions involved in the developmental and physiological processes in plants. The involvement of members of the RAV family in ethylene response and in brassinosteroid response was reported.

In *H. brasiliensis*, 20 AP2 family members and 4 RAV family members are identified in our research. In order to study the functions of AP2 and RAV family in the somatic embryogenesis development in *H. brasiliensis*, it will be interesting to check the expression profile of AP2 and RAV family genes in response to abiotic stress like JA, ET, and analyze their tissue specificity. Gene expression in different metabolism clones allowed selecting the candidate genes involved in the secondary metabolism, and development, and identify the ortholog gene of the important members such as AtBBM from AP2 and RAV family in *H.brasiliensis*.

iii. Characterization of AP2/ERF genes in response to abiotic stresses

Latex production depends on genetic, environmental and harvesting components. We know that harvesting systems use ethephon, an ethylene (ET) releaser applied on the tapping panel, to stimulate the latex production by improving the flow and regeneration of latex and also tapping that will induce laticiferous cell differentiation by JA-dependent pathway. AP2/ERF superfamily as one of the most important transcription factor in plants could be involved in latex production by regulating the downstream genes by ET or JA-dependent pathways. In addition, environmental stresses in form of abiotic stresses such as cold, dehydration, salt could generate an osmotic stress that might pass through ABA-dependent

pathway. The imbalance of osmotic stress in the cell prevents latex production which could lead to the tapping panel dryness (TPD). It will be interesting to study the response of AP2/ERF superfamily against abiotic stresses in order to understand the mechanism of regulation of these genes in transcriptional level, and their expression in latex cell to identify specific genes for genetic transformation.

To have the immediate response of stress, first step is to perform a screening experiment for 142 AP2/ERF genes in response to several abiotic stresses (dehydration, cold, mechanical wounding, ET) using kinetic expression analysis approach by real-time PCR. Second screening would be done to obtain several genes candidates that would be studied for their expression in laticiferous cell. Further, the expression level of candidate genes in three clones with contrasting metabolism (PB260, PB235, PB217, and RRIM 600) will be studied. Several candidate genes will be expected to be found for genetic transformation to understand their function. Other perspective, these genes could also serve as markers for molecular breeding.

MiRNAs direct cleavage and translational repression of partially complementary mRNA target transcripts. Computational predictions are based on high degrees of complementarity. The plant small RNA target (psRNAtarget) analysis server (Dai and Zhao et al., 2011) analyses reverse complementary matching between small RNA and target transcript using a proven scoring schema and target accessibility evaluation by calculating unpaired energy (UPE) required to open secondary structure around small RNA's target site on mRNA. Several miRNA have been predicted to inhibit *H. brasiliensis* AP2/ERF transcripts (HbIX-C3/miR395, HbERF/miR159, and HbERF-IXb1/miR408). To confirm these couples, an experimental validation is necessary.

Molecular techniques are commonly used to validate miRNA targets (Chen et al,2010.; Mallory and Bouche, 2008). The cleavage site can be identified in vivo using a modified 5′ RLM RACE technique (Llave et al., 2011). MiRNA processing activity generates 3'ends cleaved products that are relatively stable and contain ligation competent 5'monophosphate ends rather than conventional 5'cap. A RNA oligonucleotide adapter is ligated directly to the 5'terminus of the cleavage product using T4 RNA ligase and without any further enzymatic pretreatment required for the classical 5'RLM RACE methods. The ligate RNA is then reverse transcribed into first strand cDNA and PCR amplified using a reverse gene specific primer (GSP), designed downstream the predicted miRNA::target binding site, in combination with a forward primer homologous to the RNA adapter sequence. The first PCR product is then used for the nest amplification performed with nested primers. The PCR products were gel purified, cloned and sequenced. MiRNA targets generally are cleaved between the positions that pair to nucleotides 10 and 11 of the miRNAs. This features can be used to predict the method of inhibition in psRNA target analysis.

If the cleavage site is validated, further analyses are needed to validate the inhibition at transcriptional or translational level. The co-expression of the couple miRNA/target gene needs to be performed by real-time RT-PCR. It is necessary for that purpose to analyze the expression of the precursor of miRNA by real-time RT-PCR or the accumulation or diminution of mature miRNA by RNA gel blot if the precursor sequence is not available. Two transcript products can be amplified to analyze the expression of target gene, the full length using primers on both sides of the miRNA::target binding site and the cleaved targets genes using primers after the miRNA::target binding site.

Other methods are used to validate the target in other plants (Mallory and Bouche, 2008): transient or stable overexpression of MIR genes that lead to reduced accumulation of their full length mRNAs and increased accumulation of cleavage fragments, indicative of miRNA-directed cleavage or a MIR gene mutations, that decrease accumulation of miRNAs, lead to increased target mRNA accumulation. The accumulation or diminution of target mRNAs transcripts (full length or cleaved) can be visualized by RNA gel blot, QRT-PCR, *in situ* hybridization and transcriptome analyses.

A genetic transformation of rubber tree with HbERF-IXc3 miR-resistant form can be planned. Genetic transformation experiment is performed by inoculation of highly embryogenic callus line of clone PB260 as described by Blanc *et al.* 2006 (Blanc et al., 2006).

Other methods are necessary to discover plant miRNA targets that are less complementary and which are potentially regulated in a non-cleavage mechanism (Lanet et al., 2009). These methods could include a biochemical approach to isolate mRNAs associated with AtAGO1 (Karginov et al., 2007).

2. General conclusion

Rubber yield from *H. brasiliensis* is determined by different factors (Figure 4). Ethylene has been one practically indispensable factor in the rubber production, which has been widely applied in rubber plantation as ethephon (an ethylene releaser). Ethylene was identified to delay coagulation, stimulate the latex flow and acceleration of sucrose metabolism to increase latex yield. Bark treatment with ethephon is known to increase the latex yield by 1.5–2 folds in rubber tree. But the mechanism of ethylene stimulation latex production is still not completely described. Natural rubber (cis-1, 4-polyisoprene), is synthesized into rubber particles of laticifer networks through isoprenoid biosynthesis pathway. The number of laticifers is one more of the most important factors influencing rubber yield from *H. brasiliensis.* Biosynthesis of natural rubber is affected by various plant hormones. Exogenous jasmonate and mechanical wounding were shown to significantly induce laticifer differentiation, and jasmonate plays an important role as a signalling molecule that regulates rubber biosynthesis. Jasmonate (JA) and ethylene (ET) are often synergistically regulating plant development and against biotic or abiotic stresses. Wounding, JA and ET are important signals in coordinating responses to tapping and stimulation, such as latex cell metabolism, laticifer differentiation, and rubber biosynthesis as well as cell senescence. But the mechanism of the interaction of JA and ET in *H. brasiliensis* has never been reported.

Our research attempt firstly to investigate the genes involved in the JA and ET signalling pathways and in cellular responses to discover the general response profile upon the regulation of wounding, MeJA and ET and to identify the influences of timing and of genes specifically regulated by wounding, methyl jasmonate (MeJA) and ET signals for use as an internal control in further transcriptomic analyses. Then we further studied the AP2/ERF transcription factor superfamily, which is shown to be important in response to abiotic stresses. The putative function of AP2/ERF genes was studied in the latex metabolism and tissue characteristics in *H. brasiliensis*. ERF1 ortholog gene then further is identified in *H.*

brasiliensis as it will serve as the integrative key factor in the transduction of MeJA and ET signalling pathway.

According to our analysis, it was discovered that in *H. brasiliensis* there are several expression patterns existing in response to the wounding, methyl jasmonate and ethylene signals regulation. Firstly, each signal has the specific responsive genes. It was indicated as wounding-specific regulation genes, methyl jasmonate specifically inducible genes and ethylene specifically regulated genes. Secondly, the co-regulations by two individual signals were demonstrated. Three genes could be induced by either wounding or MeJA. Thirdly, the effect of co-regulations by three signals respectively was displayed. As the transcript level of the *HbGP* and *HbACR* genes was enhanced by all three treatments simultaneously. Temporal regulation was observed for some genes. Four hours after the treatments was shown to be a time point to activate the response of a large number of genes to the stress. This study provided a general characterization of signalling pathways, and will serve as a new base to launch advanced studies of the network of pathways in *H. brasiliensis.*

AP2/ERF transcription factor superfamily was identified in order to study the key regulatory factors in the interaction of jasmonates and ethylene in *H. brasiliensis*. The number of AP2/ERF superfamily members in *H. brasiliensis* is relatively comparable with the other species. Based on the conserved AP2/ERF domain, one hundred and seventy-three AP2/ERF members are identified by NGS techniques. Highly expressed AP2/ERF latex genes could have important function in *H. brasiliensis*. ERF family of the AP2/ERF superfamily was considered related with the defence response against biotic and abiotic stress. The tissue specificity of AP2/ERF transcripts was analysed. MicroRNA regulation on the AP/ERFfamily was detected. Six microRNAs including microRNA 172 were predicted to inhibit *H. brasiliensis* AP2/ERF transcripts.

We predict that ERF group IX genes are involved in jasmonate (JA), ethylene (ET) responses in *H. brasiliensis*. *Hb*ERF-IXc5 could function as the Ethylene Response Factor 1 (ERF1) in *Arabidopsis*. The key factor gene ERF1 and ORA59 genes in *Arabidopsis* are belonging to group IX of the ERF family. Group IX genes of ERF family have been shown to involve in pathogen stress responses and have been linked to JA and ET signalling pathways in several species but little is known about the functions of groups or individual ERFs in this process in *H. brasiliensis*. Fourteen genes of group IX of the ERF family were analysed in the regulation of wounding MeJA and ET signals. The expression of several members of group IX was induced by wounding MeJA and/or ET signals. Furthermore, the expression of several ERF genes of subgroup IXc was induced synergistically by wounding, JA in combination with ET including HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6, suggesting that the encoded ERF proteins may play key roles in the integration of both signals. The alignment of *H. brasiliensis* ERF genes of group IX with members of the same group from seven other species suggests that HbERF-IXc4 and HbERF-IXc5 are putative orthologs of the ERF1. HbERF-IXc2 is supposed to be ortholog of the *AtORA59* gene. By the analysis of ERF1 regulation on PLANT DEFENSIN1.2 (PDF1.2) gene expression using transient over expression approaches in *Arabidopsis*, we found that HbERF-IXc5 was able to activate PDF1.2 gene expression in contrary to the related proteins in response to the JA and ethylene signalling pathway. Our results suggest that HbERF-IXc5 might be one of the essential integrator of the JA and ethylene signalling transduction pathways in *H. brasiliensis*.

The results obtained in this thesis demonstrated a general mode of wounding, JA and ET acting on the genes involved in the signal pathway from three different levels including signal biosynthesis level, signal transduction level and cellular response level. They showed that wounding, JA and ET have individual effects on the gene expression and also they could combine together to regulate the genes response to the abiotic stresses.

The identification of the AP2/ERF transcription factor superfamily in *H. brasiliensis* showed many factors work on the transcriptional level to regulate the gene expression. The identification of the HbERF-IXc5 as putative ortholog to ERF1 gives us a first view of the mechanism of interaction of JA and ET in *H. brasiliensis*. It showed the evidence first time in *H. brasiliensis* that there is an interaction between JA and ET, which is mediated by one AP2/ERF transcription factor HbERF-IXc5.

Important transcription factors of the AP2/ERF superfamily could be characterized during the process of latex harvesting under various conditions of tapping and ethephon stimulation and in relation with the appearance of TPD. The allelic variation of major AP2/ERF sequences could be then used to determine molecular markers useful for further marker-assisted selection for identifying high-yielding and TPD-tolerant clones. Besides, further functional analysis using genetically modified plants for these genes will provide a general characterization of the ethylene and jasmonate signalling pathways. This information will serve as a new base to launch advanced studies of the network of pathways in *H. brasiliensis.* The analysis of AP2/ERF superfamily in *H. brasiliensis* provides the basis for studying the regulation of latex metabolism and the response to biotic and abiotic stresses. Identification of key factor HbERF-IXc5 in the interaction of the JA and ethylene signalling pathways in *H. brasiliensis* will help to understand the mechanism of JA and ET crosstalk on the regulation of the latex metabolism.

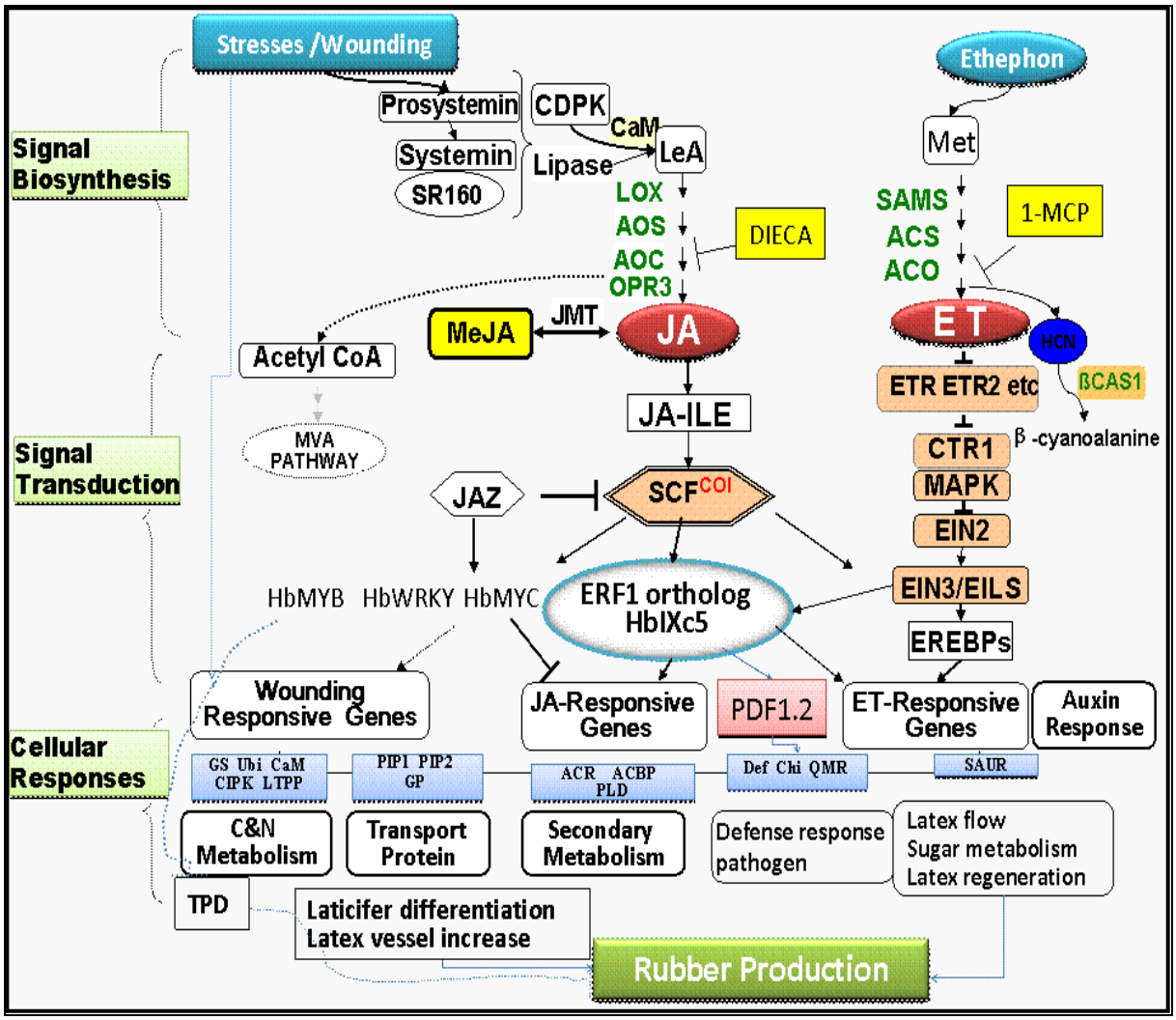

Figure 11. Integrative scheme of molecular factors (ethylene and jasmonate signalling pathways) involved in the response to latex harvesting stresses (tapping, ethephon stimulation) for the rubber production. Tapping involves a mechanical wounding. Jasmonates could act as a wounding signal regulating the defense responses to wounding.

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