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Biologie, Interactions, Diversité, Adaptative des Plantes

Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Bogor Agricultural University
Kampus IPB Dramaga, Bogor
Indonesia

Plant Biology

Graduate School of
Bogor Agricultural University

THESIS

For obtaining the double degree of
DOCTOR

Presented by: **RETNO LESTARI**

Functional analysis in *Hevea brasiliensis* of *HbERF-IXc4* and *HbERF-IXc5* genes, two potential orthologs of the *ERF1* gene from *Arabidopsis*

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ACKNOWLEDGEMENTS

Firstly, I would like to thank God Almighty, syukur Alhamdulillahirabbil'alamiin, grateful to **Allah Subhanahu wa Ta'ala** for His blessings, strength, and loving for completion this research and manuscript.

This research was supported by the **Directorate General of Higher Education (DGHE) Ministry of National Education of Republik Indonesia, French Ministry of Foreign Affairs**, **AGREENIUM**, **L'Institut Français du Caoutchouc (IFC)**, **the Michelin**, **Socfinco**, and **SIPH** companies. Thank you for financial supporting for my Doctoral study.

My sincere thanks to Centre de Coopération International en Recherche Agronomique pour le Développement (CIRAD)-Montpellier, France which allowed me to conduct my research, especially for Director of UMR AGAP research unit, **Dr. Patrice This.**

I would like to express my sincere gratitude to my supervisor at France, **Dr. Pascal Montoro** for the continuous support of my Doctoral, for his guidance, patience, motivation, support, and immense knowledge. His guidance helped me in all the time of research and writing of this manuscript. He inspired me a good managerial of a research, persistence, thoroughness, scientific spirits and personal scientific quality in the work.

I am deeply grateful for my supervisor at Indonesia, **Prof. Dr. Ir. Suharsono, DEA** for his support, great efforts to explain things clearly, simply and always give me advice, insightful comments and encouragement to finish my research.

I would like to thank **Dr. Ir. Utut Widyastuti, MS** and **Dr. Ir. Sudradjat, MS** as supervisor committee from Bogor Agricultural University for the suggestions, advices, "work smartly and without stress", help, and support during a long period of Doctoral study.

I would like to thank **Dr. Julie Leclercq** for her meaningful scientific experiment design and assistance during my research.

I would like to thank **Maryannick Rio**, **Florence Martin**, and **Florence Dessailly** for their assistance, friendliness, and friendship during my research. It is nice to know and work with you.

I would like to thank **Prof. Chantal Teulieres**, **Prof. Dr. Sri Nanan B. Widiyanto** as reviewers for their comments that greatly improved the manuscript. **Dr. Christiane Marque** as examiners for their suggestion to meet a successful dissertation.

I would like to thank **Anne Clement-Vidal**, **Dennis Fabre**, **Sandrine Roques**, and **Natthakom Azim** for collecting and analysing some data.

I would like to thank **Dr Frédérique Aberlenc** as representative Doctoral School and **Dr. Dominique This** (Montpellier SupAgro) for discussion and support.

I would like to thank **Florence Chazot** for the administrative management since my arrival at CIRAD.

I would like to thank **Dr. Julien Pirello** for his basic molecular questions and explanation.

I would like to thank **Eve Lorenzini**, **Semi Melliti**, **Christian**, and **Remi** as solid greenhouse team. There was always joy when working with you.

My sincere thanks also go to **Christine Sanier**, **Marc Lartaud**, and **Jean-Luc Verdeil** who provided and guided me an opportunity to work at Laboratory of Histology and Plant Cell Imaging Platform (PHIV) and gave access to the laboratory and research facilities.

My sincere thanks to **Prof. Dr. Ir. Nahrowi, M.Sc.**, **Prof. Dr. Ir. Marimin, M.Sc., Prof. Dr. Ir. Irwan Katili, DEA**, and **Dr. Ir. Naresworo, MS** as responsible person for mediation Double-Degree Program (DDIP) between Bogor Agricultural University and France.

Special thank you for **Dr. Didier Pillot** for encourage, financial support and standing by me to give some solution during DDIP Program.

I would like to thank Rector of Bogor Agricultural University **(Prof. Dr. Ir. Herry Suhardiyanto, M.Sc.)**, Dean of Graduate School **(Dr. Ir. Dahrul Syah, M.Sc. Agr.)** and staff, Dean of Faculty of Mathematics and Natural Sciences **(Dr. Ir. Sri Nurdiati, M.Sc.), Dr. Kiagus Dahlan,** and staff as for the support during my study at Bogor Agricultural University.

I would like to thank **Dr. Ir. Miftahudin, M.Si.** as Head of Plant Biology Study Program, **Dr. Ir. Aris Tjahjoleksono, DEA**, and **Staff**.

I would like to thank **Biology Department of FMIPA UI** and **Staff** for recommendation and support during Doctoral study.

I would like to thank to lab mates **Piyanuch**, **Yi**, and especially for **Dr. Riza-Arief Putranto** for enlightening the research, sharing expertise, and valuable guidance to me.

I would like to thank **Christian-Jacques Etienne**, **Marie-Françoise**, **Donaldo**, **Béatrice**, and all the other colleagues at CIRAD who I cannot mention one by one for help, encouragement, and friendship.

My grateful to **BOT 2010** and **DDIP 2010** for always supporting me, and **Indonesian Student Association in Montpellier (PPIM),** who together share happiness and sadness as a family during my study in Montpellier.

I would like to thank **Pandu Holding Company** for the support since the beginning of my PhD program.

I would like to thank **Supri, Ulima, Amelia**, **Putri**, **Yunita**, and **Dhani** for their help and support during writing my manuscript.

I would like to thank **all my teachers** since my childhood until now that has educated me sincerely, and also to **all my big family** and **my best friends** for sharing happiness and support.

Most importantly, my grateful especially for my beloved husband **Fajar** Reza Budiman, my beloved sons (Muhammad **Syafi** Nurhakim, **Nabil** Ariq Ahmad Nurzahid, and Muhammad Ihsan Nur**hanif)**, my parents (**Sarmiati** and Alm. **Paimin** Siswoutomo), and my parents in law (Sri **Sudarsini** and Mas **Kahar** Kusmen). Words cannot express how grateful I am to my families for all of the sacrifices that you have made on my behalf, everlasting support, understanding, and sincerity. Your prayer for me was what sustained me thus far.

Hopefully this manuscript can give a quite contribution for rubber research.

Sharing knowledge is the key to immortality.....
Education is like a wheel
The longer the distance it travels
The more knowledge we gain.....

RÉSUMÉ

Le caoutchouc naturel (CN), a *cis*-1,4-polyisoprene, est produit principalement par *Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg. Le CN est un matériau très important pour l'industrie du transport et médicale. La demande en CN augmente d'année en année. Le CN est obtenu à partir du latex. Le latex s'écoule des laticifères après saignée de l'écorce des hévéas. L'éthéphon, un libérateur d'éthylène, peut être appliqué sur certains clones d'hévéa pour stimuler la production de latex. La saignée et la stimulation à l'éthéphon sont des stress de récolte conduisant à la production de métabolites secondaires et par conséquence au caoutchouc. La biosynthèse et la signalisation de l'éthylène (ET) et de l'acide jasmonique (JA) jouent un rôle crucial dans la réponse aux stress de récolte.

Deux gènes codant des facteurs de réponse à l'éthylène (*ethylene response factor*, ERF), *HbERF-IXc4* et *HbERF-IXc5,* ont été prédits être orthologue à ERF1 d'*Arabidopsis*. ERF1 est considéré comme un facteur clé de la réponse de défense à travers l'intégration des voies de signalisation de l'éthylène et du jasmonate. Les transcrits de *HbERF-IXc4* et *HbERF-IXc5* s'accumulent drastiquement t en réponse à des traitements combinant la blessure, le méthyl jasmonate, et l'éthylène. Ces facteurs sont ainsi supposés être des régulateurs clés au croisement des voies de signalisation de l'éthylène et du jasmonate dans les laticifères. HbERF-IXc4 et HbERF-IXc5 ont plusieurs caractéristiques des facteurs de transcription révélés respectivement lors des expériences de trans-activation et de localisation subcellulaire: ils peuvent activer des éléments GCC agissant en *cis* des promoteurs des gènes cibles et ils sont présents au niveau du noyau.

Dans cette étude, l'analyse fonctionnelle des gènes *HbERF-IXc4* et *HbERF-IXc5* a été effectuée par sur-expression de ces gènes sous le contrôle de deux promoteurs, *35S CaMV* et *HEV2.1* dans des lignées transgéniques d'*Hevea* obtenues par transformation génétique *via Agrobacterium tumefaciens*. Cette sur-expression a conduit à augmenter les effets des gènes natifs *HbERF-IXc4* et *HbERF-IXc5*. Vingt-neuf lignées à activité GFP ont été sélectionnées sur un milieu contenant de la paromomycine. Au total, douze lignées des plantes ont été régénérées parmi lesquelles dix ont produit un nombre suffisant de plantes soit 1622 plantes transgéniques acclimatées en serre pour réaliser les observations de phénotypage. Ces dix lignées transgéniques ont été confirmée par hybridation moléculaire de type Southern. L'observation morphologique des plants jusqu'à un an montre que les deux gènes (*HbERF-IXc4* and *HbERF-IXc5*) favorisent une meilleure croissance, en termes de hauteur des plants, du diamètre des tiges, et du poids frais et sec des parties aériennes et racinaires, avec une plus forte vigueur et tolérance aux stress abiotiques. Les plants sur-exprimant *HbERF-IXc5* ont aussi une meilleure performance que ceux sur-exprimant *HbERF-IXc4*. Ces résultats montrent aussi un système racinaire plus vigoureux et bien équilibré par rapport à la plante entière. Les analyses de RT-PCR en temps réel révèlent que l'abondance de transcrits des gènes *HbERF-IXc4* et *HbERF-IXc5* était plus importante chez les lignées transgéniques que la lignée sauvage L'analyse fine des lignées *HbERF-IXc5* montre aussi des modifications anatomiques (activité cambiale, nombre de cellules laticifères, amidon, et largeur du xylème).

Ce travail est la première analyse fonctionnelle de facteurs de transcription chez *Hevea*. Des différences ont été observées entre les lignées *HbERF-IXc4* et *HbERF-IXc5.* Comme ERF1, HbERF-IXc4 et HbERF-IXc5 doivent diriger la réponse à certains stress. HbERF-IXc5 serait un régulateur de la différentiation des laticifers. Cette étude pourrait être complétée par des analyses dans des lignées éteintes pour ces gènes, une comparaison des transcriptomes et métabolome de lignées sauvages et transgéniques, et l'identification des gènes cibles contrôlés par HbERF-IXc4 et HbERF-IXc5. Ces résultats pourraient être appliqués à travers le développement de marqueurs génétiques pour la tolérance aux stress de récolte du latex et le développement de nouveaux stimulants pour des applications agronomiques.

Mots clés: cambium, ERF1, ethylène, *Hevea*, *HbERF-IXc4*, *HbERF-IXc5*, cellule à latex, laticifère, stress, facteur de transcription

ABSTRACT

Natural rubber (NR) (*cis*-1,4-polyisoprene) is the main production from *Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg. NR is a very important industrial material for transportation, consumer, and medical. The demand for NR is increasing from year to year. NR is obtained from latex. The latex flows out from laticifers after tapping the bark. Ethephon, an ethylene releaser, can be applied on clones to stimulate the latex production. Tapping and ethephon stimulation are sources of harvesting stresses conducing to the production of secondary metabolites and consequent rubber. Ethylene (ET) and jasmonic acid (JA) biosynthesis and signalling pathways play a crucial role in the response to latex harvesting stress.

Two *Hevea ethylene response factor* genes, *HbERF-IXc4* and *HbERF-IXc5,* were predicted to be orthologs to *ERF1* from *Arabidopsis*. ERF1 was suggested to be a component of defence responses through the involvement of ethylene and jasmonic acid signalling pathways. Transcripts of *HbERF-IXc4* and *HbERF-IXc5* were dramatically accumulated by combining wounding, methyl jasmonate, and ethylene treatment. These factors were assumed to be a regulator at the crosstalk of ethylene and jasmonate signalling pathways in latex cells. HbERF-IXc4 and HbERF-IXc5 have several features of transcription factor revealed by transactivation experiment and subcellular localization, respectively: they can activate the GCC *cis*-acting element of promoters of target genes and are localized in nucleus.

In this study, functional analysis of *HbERF-IXc4* and *HbERF-IXc5* genes have been carried out by overexpression of these genes under the control of *35S CaMV* and *HEV2.1 as a* promoter in transgenic *Hevea* lines obtained by *Agrobacterium tumefaciens*-mediated genetic transformation. This overexpression of genes led to emphasize the effect of native *HbERF-IXc4* and *HbERF-IXc5* genes. Twenty-nine GFP-positive lines were established on paromomycin selection medium. Twelve lines regenerated plants but only ten led to produce a sufficient number of plants for further phenotyping with totally 1,622 transgenic plants in greenhouse. These ten lines were confirmed as transgenic by Southern blot hybridization. Observation of morphology until one year showed both genes (*HbERF-IXc4* and *HbERF-IXc5*) promoted a better growth in terms of plant height, stem diameter, and weight of aerial and root system with higher vigour and better tolerance to some abiotic stresses. Plants overexpressing *HbERF-IXc5* have also a better performance than *HbERF-IXc4*. Data also showed a vigorous root system well balanced with regard to the whole plant. Real-time RT-PCR analyses revealed that expression of *HbERF-IXc4* and *HbERF-IXc5* genes was higher in transgenic lines compared to wild-type. Analysis in details of *HbERF-IXc5* lines also showed some changes in anatomy (cambium activity, number of latex cells, starch, and width of xylem).

This work is the first successful functional analysis of transcription factors in *Hevea*. Some differences have been observed between *HbERF-IXc4* and *HbERF-IXc5.* As ERF1, HbERF-IXc4 and HbERF-IXc5 proteins should drive the response to some stresses. HbERF-IXc5 protein might be a regulator of laticifer differentiation. This study could be completed with analysis of silenced transgenic lines, comparison of transcriptome, metabolome of wild-type and transgenic lines, and identification of target genes controlled by HbERF-IXc4 and HbERF-IXc5. These results could be applied through development of molecular genetic markers for tolerance to harvesting stress and development of new stimulants for agronomical application.

Keywords: cambium, ERF1, ethylene, *Hevea*, *HbERF-IXc4*, *HbERF-IXc5*, latex cell, laticifer, stress, transcription factor

RINGKASAN

Karet alam (*cis*-1,4-polyisoprene) dihasilkan oleh *Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg. Setiap tahun, kebutuhan karet alam semakin meningkat sebagai bahan baku dalam bidang industri transportasi, barang konsumsi, dan alat kesehatan. Lateks, bahan baku pembuatan karet alam, dihasilkan oleh sel latisifer, diperoleh dengan cara melukai bagian kulit tanaman karet (penyadapan). Stimulasi ethephon pada *Hevea brasiliensis* dapat meningkatkan aliran lateks. Penyadapan dan stimulasi ethephon dapat menjadi cekaman bagi tanaman karet. Hormon tumbuhan seperti etilen dan asam jasmonat memiliki peran penting dalam proses pertahanan tanaman untuk mengatasi cekaman pada proses penyadapan.

Dua gen *Hevea brasiliensis*, *HbERF-IXc4* dan *HbERF-IXc5*, ortolog dengan *ERF1* pada *Arabidopsis thaliana* diduga berperan penting dalam respons pertahanan tanaman. Mekanisme keterlibatan gen tersebut melibatkan sinyal dari dua hormon, yaitu etilen dan asam jasmonat. Akumulasi transkripsi gen *HbERF-IXc4* dan *HbERF-IXc5* meningkat tajam pada tanaman dengan kombinasi pemberian cekaman berupa perlukaan, perlakuan asam jasmonat, dan etilen. Faktor tersebut diasumsikan sebagai pengendali mekanisme transduksi sinyal pada sel-sel lateks. HbERF-IXc4 dan HbERF-IXc5 sebagai faktor transkripsi mampu mengaktivasi promoter elemen GCC *cis*-acting melalui percobaan transaktivasi dan lokalisasi seluler.

Analisis fungsional gen *HbERF-IXc4* dan *HbERF-IXc5* telah dilakukan pada penelitian ini. Gen tersebut dioverekspresikan pada tanaman *Hevea brasiliensis* transgenik, di bawah kendali promoter *35S CaMV* dan *HEV2.1* melalui transformasi genetik diperantarai *Agrobacterium tumefaciens.* Overekspresi bertujuan menonjolkan peran kedua gen tersebut. Dua puluh lima kalus positif penanda GFP dalam medium seleksi mengandung paromomisin telah diperoleh. Dua belas transforman berhasil diregenerasikan menjadi tanaman transgenik, namun hanya sepuluh yang mampu menghasilkan tanaman dengan fenotipe dan pertumbuhan yang baik. Total terdapat 1.622 tanaman transgenik yang ditumbuhkembangkan di dalam rumah kaca. Pengamatan morfologi selama satu tahun menunjukkan *HbERF-IXc4* dan *HbERF-IXc5* mampu menstimulasi pertumbuhan dicirikan dengan tinggi tanaman, diameter batang, berat akar, dan toleran terhadap cekaman abiotik. Tanaman transgenik *HbERF-IXc5* menunjukkan pertumbuhan dan fenotipe lebih baik daripada tanaman transgenik *HbERF-IXc4*. Tanaman transgenik menunjukkan sistem perakaran dan keseimbangan pertumbuhan yang baik. Overekspresi gen *HbERF-IXc4* dan *HbERF-IXc5* pada tanaman transgenik dianalisis dengan metode real-time RT-PCR dan dibandingkan dengan *wild-type*. Analisis histologi menunjukkan tanaman transgenik *HbERF-IXc5* mengalami beberapa perubahan anatomi pada peningkatan aktivitas kambium, jumlah sel-sel lateks, pati, dan lebar xilem.

Penelitian ini merupakan penelitian pertama yang berhasil menganalisis secara spesifik fungsi faktor transkripsi pada tanaman *Hevea.* Beberapa parameter menunjukkan perbedaan signifikan antara fungsi kedua gen. Sebagai faktor transkripsi yang ortolog dengan ERF1, HbERF-IXc4, dan HbERF-IXc5 mampu meregulasi respons tanaman terhadap cekaman. HbERF-IXc5 diasumsikan berperan pada proses diferensiasi latisifer. Analisis *silencing* pada tanaman transgenik, perbandingan transkriptome, mekanisme metabolomik pada tanaman transgenik dan *wild-type*, serta identifikasi gen-gen target dari HbERF-IXc4 dan HbERF-IXc5 dapat memperkuat analisis fungsi gen-gen tersebut sehingga menunjang penelitian ini. Hasil tersebut dapat diaplikasikan untuk pengembangan marker genetika molekular pada mekanisme respons terhadap cekaman pada proses penyadapan dan juga sebagai stimulan baru dalam pengembangan agronomi.

Kata kunci: kambium, ERF1, etilen, *Hevea*, *HbERF-IXc4*, *HbERF-IXc5*, sel lateks, latisifer, cekaman, faktor transkripsi

SCIENTIFIC PUBLICATIONS

Lestari, R., R.A. Putranto, P. Piyatrakul, C. Duan, M. Rio, F. Martin, J. Pirrello, F. Dessailly, J. Leclercq, Kuswanhadi, Suharsono, & P. Montoro. 2014. Identification of signalling factors involved in the regulation of laticifer metabolism by tapping and ethephon stimulation in *Hevea brasiliensis*. In *IRRDB International Rubber Conference,* Manila, Philippines.

Lestari, R., M. Rio, F. Martin, J. Pirrello, F. Dessailly, J. Leclercq, Suharsono, & P. Montoro. 2015. Production of *Hevea brasiliensis* transgenic lines overexpressing transcription factors involved in ethylene signalling pathway. In *Asia Pacific Rubber Conference,* Phuket, Thailand.

Lestari, R., M. Rio, F. Martin, J. Leclercq, F. Dessailly, Suharsono, & P. Montoro (2016) Establishment of *Hevea brasiliensis* lines overexpressing genes involved in ethylene signalling pathway. *Menara Perkebunan,* 84 (1), 43-48.

Lestari, R., M. Rio, F. Martin, J. Leclercq, N. Woraathasin, S. Roques, F. Dessailly, A. Clément-Vidal, C. Sanier, D. Fabre, S. Melliti, Suharsono, & P. Montoro (In preparation) Overexpression of *Hevea brasiliensis* ethylene response factor *HbERF-IXc5* enhances growth, tolerance to abiotic stress and affects laticifer differentiation. *Plant Biotechnology Journal*

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ABBREVIATIONS

13-HPOT	13S-Hydroxyperoxypeoctadecatrienoic Acid
13-LOX	13-Lipoxygenase
1-MCP	1-Methylcyclopropene
3,4D	3,4-Dichlorophenoxy-Acetic Acid
ABA	Abscisic Acid
ACBP	Acyl CoA Binding Protein
ACC	1-Aminocyclopropane-1-Carboxylic Acid
ACO	ACC Oxidase
ACS	ACC Synthase
Acxl	*Acyl-coA-oxidase1*
ANOVA	Analysis of Variance
ANPRC	Association of Natural Rubber Producing Countries
AOC	Allene Oxide Cyclise
AOS	Allene Oxide Synthase
AP2	APETALA2
APH(3')II	Aminoglycoside 3'-Phosphotransferase
ATP	Adenosine Triphosphate
ATSW	Actual Transpirable Soil Water
B	Bark
BAP	Benzylaminopurine
b-CHI	*Basic Chitinase*
C	Cork
c	Cuticle
C_2H_4	Ethylene
Ca	Cambium
CaERFLP1	Ethylene-responsive Factor like Protein 1
CaMV	Cauliflower Mosaic Virus
CCP	Pathogenic isolates of *Corynespora cassiicola*
cDNA	Complementary DNA
Chi	Chitinase
CIAA	Chloroform-Isoamyl Alcohol
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développment
CLFD	Corynespora Leaf Fall disease
COI1	Coronatine Insensitive1
COP9	Constitutive Photomorphogenesis 9
CSN	COP9 Signalosome
CTR1	Constitutive Triple Response1
dad1	Delayed Anther Dehiscence1
DF	Degree of Freedom
DEV	Development Medium
DM	Decontamination Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
DRE/CRT	Dehydration Responsive Element/C-repeat
DREB	Dehydration Responsive Element Binding Protein
DREB2	Dehydration Responsive Element Binding Protein 2

DREB2A	Dehydration Responsive Element Binding Protein 2A
DRF1	Diaphanous-Related Formin
EBP	Ethylene Binding Domain
EDTA	Ethylenediaminetetraacetic Acid
EFRs	Ethylene Responsive Factors
EILs	EIN3-like protein
EIN2	Ethylene Insensitive 2
EIN3	Ethylene Insensitive 3
EIN4	Ethylene Insensitive 4
EMEA	Europe, Middle East, America
ER	Endoplasmic Reticulum
ERBPS	Ethylene Responsive Element Binding Protein
ERF	Ethylene Response Factor
ERF1	Ethylene Response Factor 1
ERF-VII	Ethylene Response Factor-VII
ERS1	Ethylene Response Sensor 1
ERS2	Ethylene Response Sensor 2
ET	Ethylene
ETR1	Ethylene Resistant 1
ETR2	Ethylene Resistant 2
EXP	Embryogenesis Expression Medium
Fm	Maximum Fluorescence
FTSW	Fraction of Transpirable Soil Water
Fv	Variable Fluorescence
Fv/Fm	Maximum quantum yield of PSII
GA3P	Glyceraldehyde-3-Phosphate
GFP	Green Fluorescence Protein
Glu	Glucanase
GS	Glutamine Synthetase
GUS	β-Glucoronidase
HCL	Hydrochloric Acid
HCN	Hydrogen Cyanide
HEV	Hevein
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
HMGR	3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase
HMGS	3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase
HXT	Hexose Transporter
INM	The Institute for Neurosciences of Montpellier
IPP	Isopentenyl Pyrophosphatase
IRSG	International Rubber Study Group
JA	Jasmonic Acid/ Jasmonate
Jai1	Jasmonic Acid Insensitive1
JA-Ile	Jasmonoyl Isoleucine
JAR1	JA-Amino Acid Synthetase
JAs	Jasmonate
JAZ	Jasmonate-ZIM-Domain
jin1	JA insensitive mutant1
jin4	JA insensitive mutant4
JMT	Jasmonate Carboxyl Methyltransferase
L	Lamina

la	Laticifers
LC	Latex Cells
LC	Laticifer Cell
LE	Lower Epidermis
LR	Laticifer Ring
LW	Leaf Weight
MeJA	Methyl Jasmonate
MEP	2-C-Methyl-D-Erythritol 4-Phosphate
MET	Methionine
MJE	MeJA Esterase
MM	Maintenance Culture Medium
MN	Main Nerve
mRNA	Messenger RNA
MPAKKK	MAP Kinase Kinase Kinase
M0	Month-0
M2	Month-2
M6	Month-6
M12	Month-12
MVA	Mevalonic Acid
MW	Molecular Weight
NADPH	Nicotinamide Adenine Dinucleotide Phosphate reduced form
NBB	Naphtol Blue Black
nd	Not determined
NPTII	Neomycin Phosphotransferase II
NR	Natural Rubber
NRAMP	Natural Resistance-Associated Macrophage Protein
OD	Optical Density
OPC	Oxophytoenoic Acid
OPDA	Oxophytodienoic Acid
OPR	OPDA Reductase
ORA	Octadecanoid-Responsive Arabidopsis
P	Pith
P	Number of plantlets g^{-1} of callus
P/WS	Conversion percentage
PC	Palisade Cell
PC	Phenolic Compound
PCR	Polymerase Chain Reaction
PDF1.2	Plant Defensin1.2
pH	Potential Hydrogen
PHIV	Histology and Plant Cell Imaging Platform
Pi	Inorganic Phosphorus
pINDEX1 or PI	Performance Index 1
PIP	Plasma Membrane Intrinsic Protein
PLA_1	Phospholipase A_1
PLC	Primary Latex Cell
PM	Pre-culture media
PO	Polyphenol
PP	Palisade Parenchyma
PR	Pathogenesis Related
PSI	Photosystem I

PSII	Photosystem II
PVP	Polyvinylpyrrolidone
qPCR	Quantitative PCR
r	Parenchymatous rays
R	Total root weight
R	Replicate
R1	Taproot
RAP2.12	Related to AP2.12
RAP2.3	Related to AP2.3
RAV	Related-to-AB13/VP1
Rboh	Respiratory burst oxidase homologue
REF	Rubber Elongating Factor
RTE1	Reversion to Ethylene Sensitivity 1
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RW	Root Weight
s	Sclerids
S	Stomata
SA	Salicylic Acid
S-AdoMet	*S*-Adenosyl-Methionine
SAM	S-Adenosyl-Methionine
SCF	SKp-Cullin-F-box
SE	Somatic Embryogenesis
SL	Secondary Laticifer
SP	Spongy Parenchyma
SPAD	A Single-Photon Avalanche Diode
ST	Sieve Tubes
st	Starch
SUS	Sucrose Synthase
SUT	Sucrose Transporter
SW	Stem Weight
t	Tannin Cell
T	Total embryos g^{-1} of callus
T	Treatment
TAE	Tris-acetate-EDTA
T-DNA	Transfer-DNA
TFs	Transcription Factor
TPD	Tapping Panel Dryness
TSC	Total Solid Content
TTSW	Total Transpirable Soil Water
UE	Upper Epidermis
UP	Upper Epidermis
W	Wounding
WS	Number of well-shaped embryos g^{-1} of callus
WHC	Water Holding Capacity
WT	Wild type
x	Xylem
α-LeA	α -Lineloic Acid

SECTION I

INTRODUCTION

1. Natural rubber production

Natural rubber (NR) (*cis*-1,4-polyisoprene) is a milky secretion of many species of plants. Natural rubber mostly produces by a tree belonging to *Euphorbiaceae* family, such as *Hevea* spp., *Castilla* spp., and *Ficus elastica* (Jones and Allen 1992). Only limited number from many sources of rubber producing tree can be exploited and have economic importance for NR industry. The only commercial major source of NR and the best rubber producer is *Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg. *Hevea brasiliensis* is tropical tree species which is originated from the Amazonian forest. Rubber tree has 2n=36 chromosomes and behaves as a diploid (Clément-Demange et al. 2007). This plant is widely cultivated in Southeast Asia for basis rubber product (Kush 1994, Vaysse et al. 2012) (Figure 1).

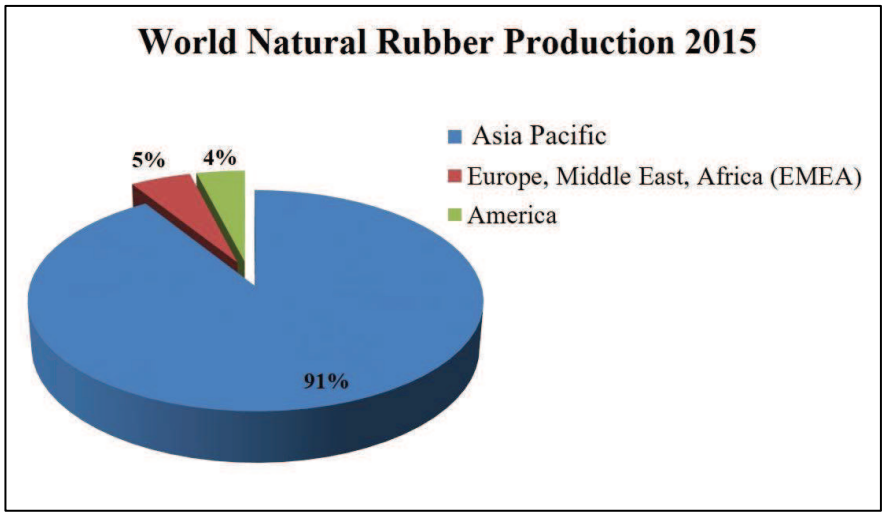


Figure 1. World source of natural rubber production in 2015. This figure is extracted from (IRSG 2015).

Natural rubber is very important industrial material for transportation, consumer, and medical sectors (Figure 2). The advantages of natural rubber are the elasticity, resilience, and toughness. The needs for NR are increasing from year to year. Nowadays, about 93% of NR world production produces in Asia, Africa (4%), and Latin America (3%). The largest NR producing countries are Thailand (38%), Indonesia (30%), and Vietnam (9%) (IRSG 2015) (Figure 3 and Table 1).

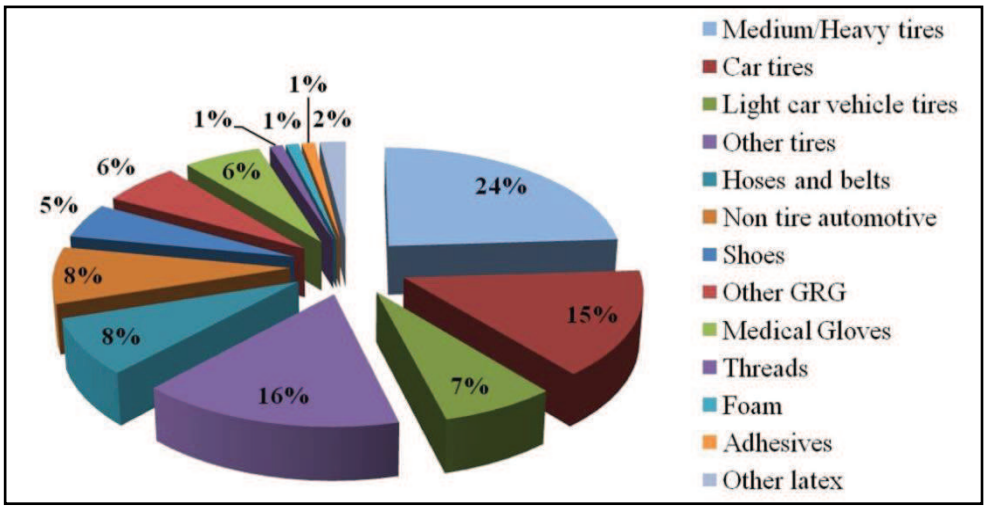


Figure 2. Fields of natural rubber application. This figure is extracted from (Vaysse et al. 2012).

Table 1. Statistical of natural rubber production from 2010-2015. Statistical of natural rubber production from 2010–2015. Data from Association of Natural Rubber Producing Countries (ANRPC 2015).

Production of natural rubber in ANRPC member countries ('000 tons)					
Country	2010	2011	2012	2013p	2014p
Thailand	3252.0	3569.0	3778.0	4170.0	3997.0
Indonesia	2735.0	2990.0	3012.0	3237.0	3153.2
Vietnam	751.7	789.3	877.1	949.1	953.7
China	687.0	727.0	802.0	865.0	857.0
India	850.9	892.7	919.0	796.0	705.5
Malaysia	939.2	996.2	922.8	826.4	655.0
Sri Lanka	153.0	158.2	152.0	130.4	99.3
Philippines	98.8	106.4	110.7	111.2	115.0
Cambodia	42.2	51.3	64.5	85.2	96.6
TOTAL	9509.8	10280.1	10638.1	11170.3	10632.3

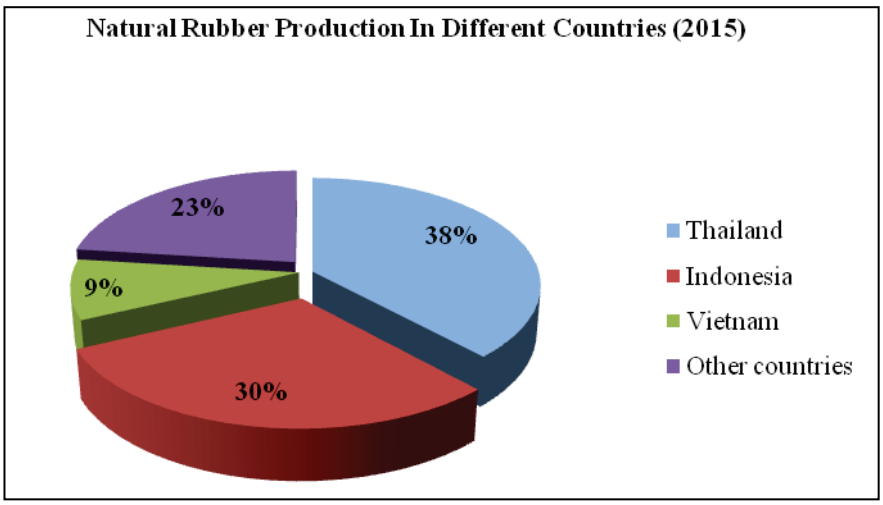


Figure 3. The tree world largest countries producing natural rubber in 2015.This figure is extracted from (IRSG 2015).

Natural rubber is one of top 20 commodities with the highest net production value in the world. It is predicted will grow fast by time to time. It proved that rubber industry will getting bigger and it needs proper production to meet the huge demand in over the world. Consumption of natural rubber increased every year as well as the production. The increase was really significant from 2005 to 2008 as much as 8 M ton to 12 M ton (IRSG 2015) due to its particular chemical structure and the consequent physical properties (Allen 1972) (Figure 4 and 5).

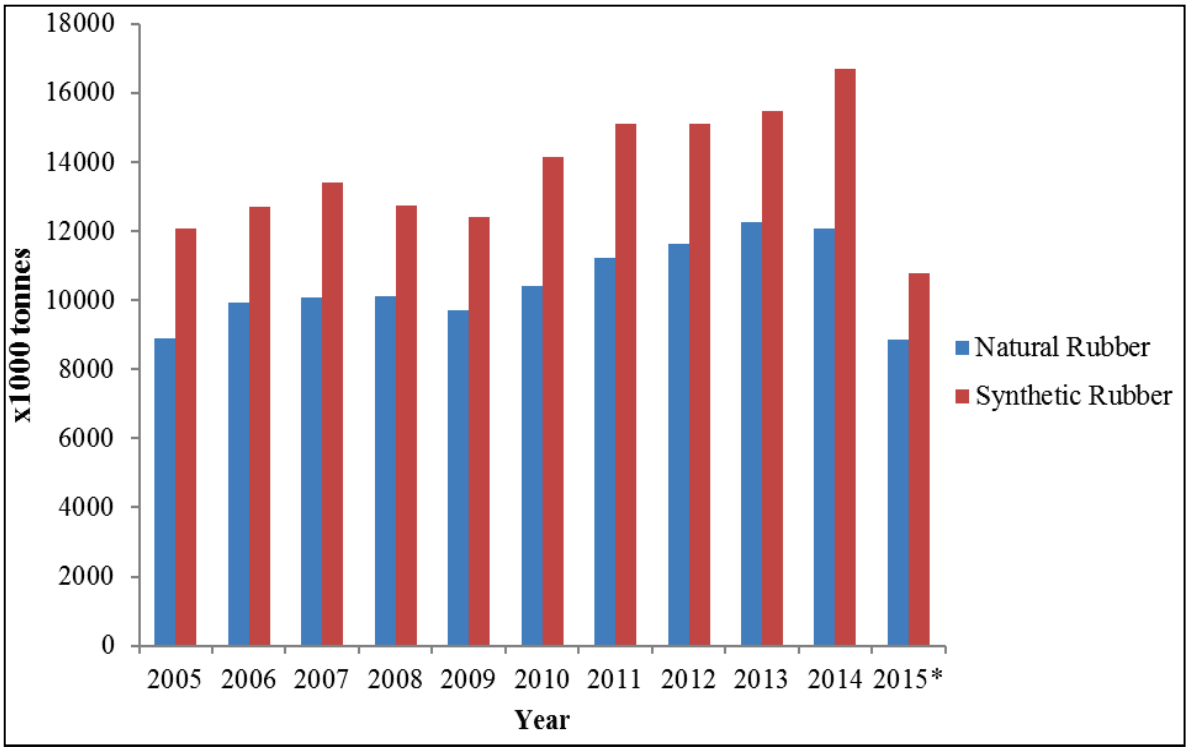


Figure 4. World natural and synthetic rubber production from 2005 until 2015.*counted from January to September (IRSG 2015).

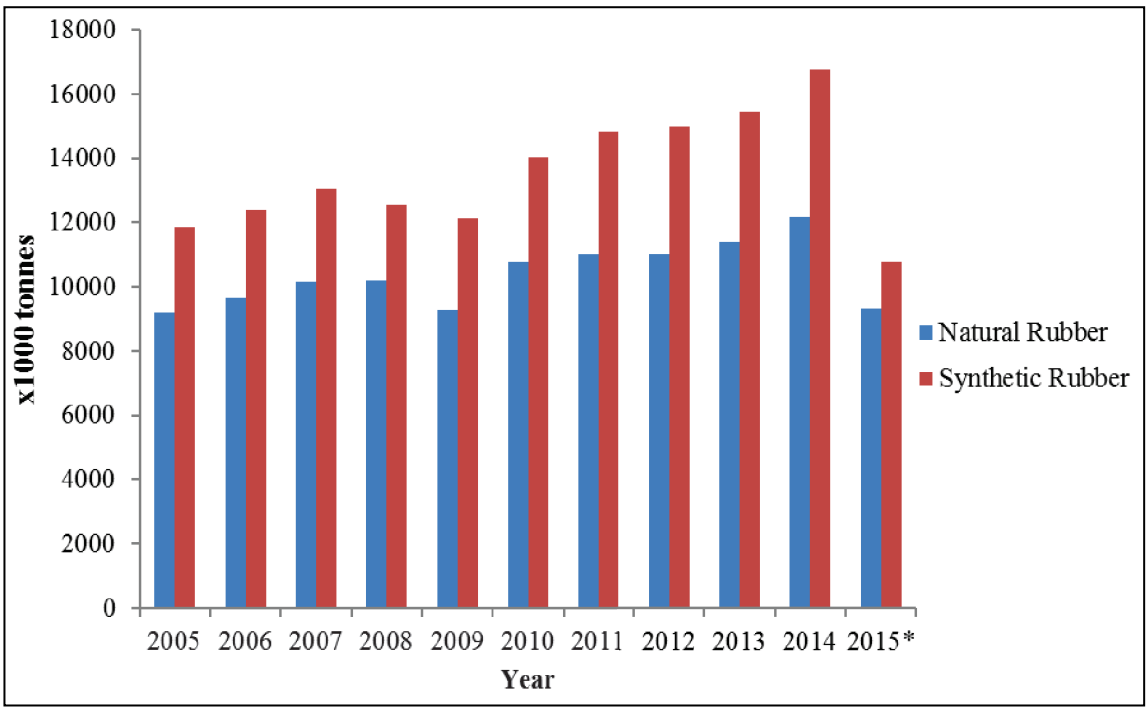


Figure 5. World natural rubber consumption from 2005 to 2015.*counted from January to September (IRSG 2015).

2. Histology of laticifers

Latex is produced and then stored in the tube structure known as laticifers, which are specialized cells that contain a slurry or suspension of many small particles in sap of unspecified composition (Figure 6 and 7) (d'Auzac and Jacob 1989, Fahn 1979, Mahlberg 1993, Premakumari and Panikkar 1992). Based on the growth of laticifers, there are two type forms; the primary and the secondary laticifers in *H. brasiliensis.* The primary laticifers develop during the primary growth while the secondary laticifers differentiate during the secondary growth (Tian et al. 2003).

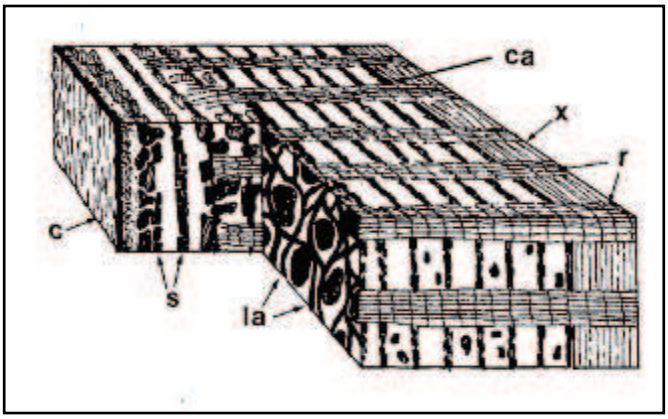


Figure 6. Histological organization of *Hevea brasiliensis* in longitudinal section: laticifers are organized in concentric rings (la) which alternate with parenchymatous rays (r) and sieve tubes (c: cork, ca: cambium, s: sclerids, x: xylem). This figure is extracted from (Nicole et al. 1986).

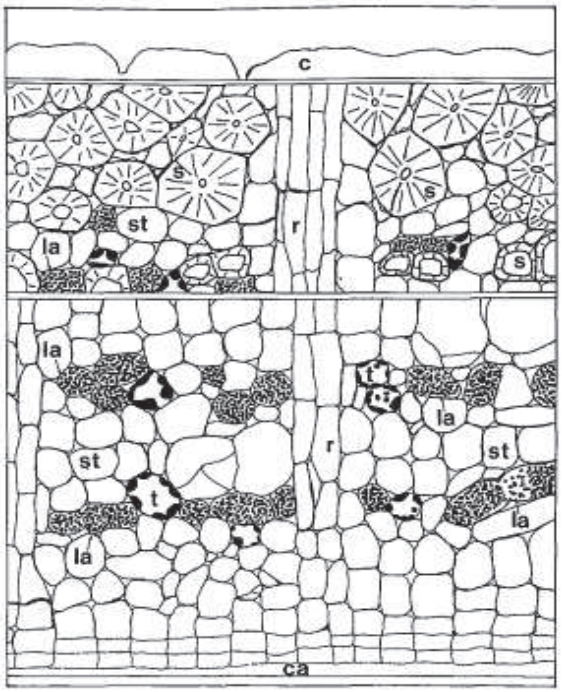
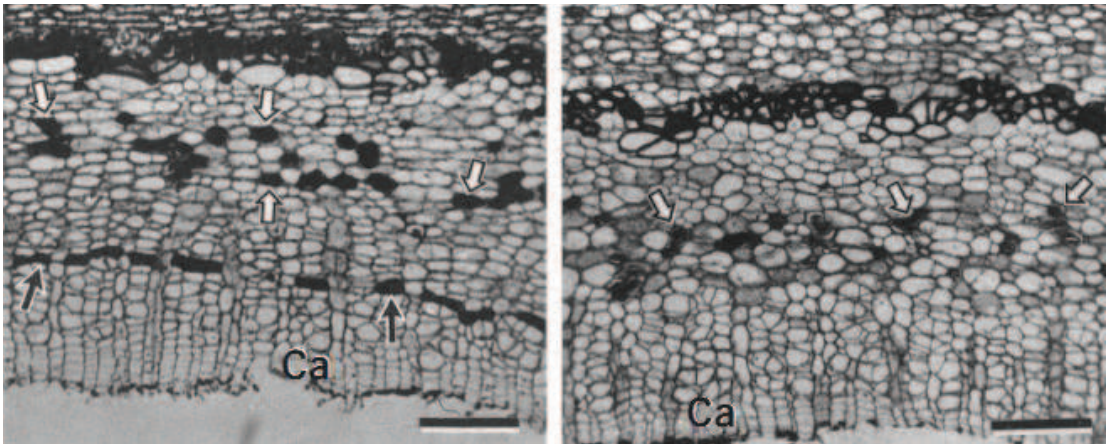


Figure 7. Histological organized of *Hevea brasiliensis* in cross-section. The conducting phloem lies near the cambium (ca) and contains the functional sieve tubes (st) and laticifers (la), which is tapped for latex production. Tannin cells (t) are associated with laticifers mantles. The differentiation of sclerids (s) in the older phloem modifies its organization, thus preventing latex production (c: cork, r: parenchymous rays). This figure is extracted from (Nicole et al. 1986).

The primary laticifer differentiates during new shoot development. These systems are differentiated from the procambium in the vicinity of the phloem (Tian et al. 2003). The distributions of primary laticifers are usually present in young organs in the primary state of growth such as in young leaves, flowers, cotyledons, and pith. After cambium has formed, it produces a special laticiferous system in the secondary phloem (de Faÿ and Jacob 1989, Sando et al. 2009).

In contrast to the primary laticifer, the secondary laticifers are differentiated by the activity of vascular cambium as in the case of vessel elements and hence the term 'latex or laticiferous vessels' in appropriate. Laticiferous vessels were formed in a rhythmic process. The contiguous walls of two adjacent laticifers become perforated in several places and producing anastomoses which create a continuous network and distributed in the bark (Premakumari and Panikkar 1992, Tian et al. 2003). Articulated and anastomosing laticiferous vessels from successive vertical network called rings or mantles (Southorn 1969). The number of rings forming each year was varying between 1.74 and 3.14 according to the soil type (d'Auzac and Jacob 1989).

In *H. brasiliensis*, secondary laticifers are the principal type of laticifers exploited commercially for its latex. Anastomoses between laticifers are believed to enable latex outflow from extensive laticifers in bark during tapping (Archer 1980, Sando et al. 2009). The differentiation of the secondary laticifer determines the number of secondary laticifer in soft bark and is genetically controlled and is also influenced by environmental condition (Hao and Wu 2000, Zhang et al. 2015b) (Figure 8).



(a) (b)

Figure 8. Cross-section of bark showing laticifer differentiation. (a) JA-treated stem which had stopped extending when being treated. (b) Control. White arrow: primary laticifers, black arrow: secondary laticifers, Ca: cambium. This figure was extracted from (Hao and Wu 2000).

Quantity of laticiferous tissue is determined by some factors such as the number of latex vessel rows, density of latex vessels within a ring, distance between vessel rows, and distribution pattern of latex vessel rings, size of laticifers, and girth of the tree. The number of latex vessel rows is the most important factor related to rubber yield, combining with plugging index and girth could account for 75% of the yield variation young trees. The accountability was reduced to 40% at the mature phase which indicated a predominant role of genotype environment interaction (Narayanan and Gomez 1973).

There are different proteins in the lutoid of the primary laticifer and secondary laticifer. Proteinous microfibrils were abundant in the lutoids of primary laticifers, but absent in the lutoid of secondary laticifers (d'Auzac and Jacob 1989). The microfibrillar protein was suggested to act as typical vegetative storage protein and not related to the development of laticifer because such microfibrils were unable to be detected in the lutoid particle in the latex of *H. brasiliensis* (Audley 1964, d'Auzac and Jacob 1989, Hao and Wu 2000, Tian et al. 2003).

3. Latex physiology and metabolism

Natural rubber is obtained from the latex in laticifers which are articulated and anastomosed latex cells (d'Auzac, Prévôt and Jacob 1995, Kush 1994). The biochemical and cytological aspect of latex are important for studying natural rubber (d'Auzac et al. 1995, de Faÿ and Jacob 1989, Kush 1994). Latex is a cytoplasmic component of laticifers (de Faÿ and Jacob 1989). It is a colloidal suspension that contains 30% to 50% dry matter, of which 90% is rubber (Chrestin et al. 1997). Latex contains organelles, rubber, and non-rubber particles (Archer et al. 1963, Nicole et al. 1986). The most abundant of a typical non-rubber bodies are lutoids, which forming 15% to 30% of fresh latex (d'Auzac et al. 1995). Latex production is dependent to: a) volume and type of laticiferous tissues in which latex is stored, b) capacity of storage vessels, c) capacity of the tree to resynthesize latex and other organic constituent within the drained area, and d) physiological and biochemical processes controlling latex flow (Pakianathan, Tata and Chon 1994).

Latex from *Hevea* is a macromolecule formed by chain of 5-carbon isoprenic unit. The amount of this unit is about 10,000 in *Hevea* (Audley and Archer 1988). The isoprenic bonds are mainly of the *cis* form (*cis*-polyisoprene). The synthesis of *cis*-polyisoprene can be

divided schematically into two distinct phases. First phase is conversion of sugar into acetate and the second phase is isoprenic synthesis itself (Jacob and Prevot 1992).

Natural rubber is synthesized through a mevalonate pathway (Sando et al. 2009). The cytosolic mevalonate pathway in *H. brasiliensis* is accepted pathway which provides isopentenyl disphosphate (IPP) for *cis*-polyisoprene (rubber) biosynthesis. Another putative source of IPP would be the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Chow et al. 2012) (Figure 9).

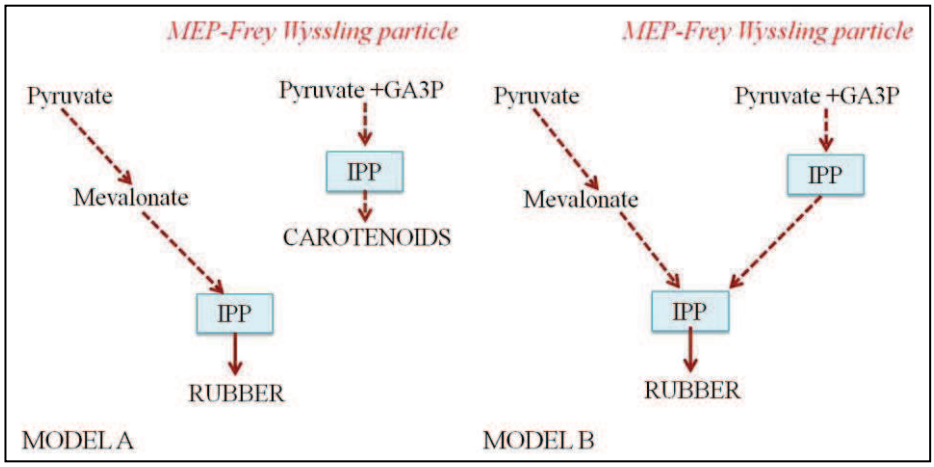


Figure 9. Biosynthesis of natural rubber in *Hevea brasiliensis*. This figure is extracted from (Chow et al. 2012).

Isoprenic anabolism is the major synthetic process in latex. Conversion of HMG-CoA into mevalonic acid (MVA) is very important in the regulation of isoprenic anabolism. It corresponds to a reduction which requires the specific presence of two molecules of NADPH by a HMG-CoA reductase enzyme (Jacob and Prevot 1992). The 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS) and the 3-hydroxy-3methylglutaryl-coenzyme A reductase (HMGR) have involved in early steps to supply of substrates for rubber biosynthesis. Three HMGR genes *hmg1*, *hmg2*, *hmg3* have been discovered in the rubber tree (Chye et al. 1991, Chye, Tan and Chua 1992). Ethephon influenced the expression of the HMG-CoA synthase gene and the activity of the enzyme (Pluang, Sirinupong and Suvachitanont 2004, Sirinupong et al. 2005).

Although the isoprene metabolism dominates among the overall metabolic pathways in laticiferous tissue, the other pathways add to the complexity of the laticifers metabolism. The synthesis of quebrachitol is having a role in the osmoticum in laticiferous tissue, the synthesis of proteins is crucial for regeneration of latex between two tapping, and synthesis of lipids which play a major role in the membrane structure of cell organelles (Jacob and Prevot 1992, Low 1978).

In the biosynthetic of rubber, energy is supplied from glycolytic process (d'Auzac 1965), ATPase transmembrane transfers (d'Auzac and Jacob 1989), and the other cell synthesis process. The metabolic pathways such as oxidative phosphorylation producing ATP may also participate in the production of biochemical energy in the laticifers (Tupy and Primot 1976).

Ethylene also has influence on the metabolism of laticiferous cells. Stimulation of latex production by the application of an ethylene on the bark is very widely used in rubber plantation. This stimulation increases the quantity of latex, causes a longer latex flow, and also activated *in situ* generation of the cell contents. It must indeed be stressed that although latex regeneration appears to be dependent on the cell metabolism, it has also been shown

that the flow itself is partly related to the availability of biochemical energy in the laticifers (Jacob, Serres and Prévôt 1988).

4. Latex harvesting

The latex is obtained by tapping and ethephon stimulation (Figure 10). Tapping and ethephon are likely to be sources of stress conducing to the production of secondary metabolites and consequent rubber. Tapping is a process involving repeated wounding of the tree along a cut made on the bark of the tree. The most common system adopted is a tapping on a half spiral cut. Rubber trees are tapped when their trunks attain 50 cm in girth (Clément-Demange et al. 2007). Upon bark wounding or deliberate tapping, the latex expelled from the laticifers (de Faÿ and Jacob 1989). Tapping causes loss of cell constituents from the laticifers. The latex flow will stop as a result of complex phenomena which lead to the coagulation of rubber particles and plunging of the wound.

(a) (b) (c)

Figure 10. The methods of latex harvesting(a) by tapping (b) by ethephon stimulation (c) latex collection.

Regeneration of the latex lost becomes necessary and involves intense metabolic activity (Southorn 1969). The availability of sugar metabolism (Tupý 1989) and nitrogen compounds (Pujade-Renaud et al. 1994) in the laticifer cell allow the reconstitution of the exported latex before the next tapping. The full regeneration of the latex after tapping was estimated to be around 72 hours (Serres et al. 1994).

Ethephon (chloro-2-ethylphosphonic acid) is an ethylene-releaser compound. Ethephon stimulation can be necessary for some *Hevea* clones to increase latex yield or to develop low tapping frequency systems (Pujade-Renaud et al. 1994). For certain rubber clones with a low latex metabolism, application of ethephon to the bark stimulates latex flow and latex regeneration between two tapping (d'Auzac et al. 1997). This process is highly stressful for the trees, and combined with environmental stress can lead to a stop in latex flow. This loss of production is related to a physiological syndrome called Tapping Panel Dryness (TPD) (de Faÿ and Jacob 1989, Venkatachalam, Thulaseedharan and Raghothama 2009).

TPD is one of the most serious threats to natural rubber production that cause 12-20% annual rubber production losses (Okoma et al. 2011). TPD is a physiological disorder resulting from abiotic stress with two forms, first, a reversible tapping cut dryness, and second, an irreversible bark necrosis (brown bast disease) (Jacob, Prevot and Lacrotte 1994). TPD is supposed for long to be related to endogenous ethylene production and oxidative stress (Chrestin et al. 2004). Environment and harvesting stresses induced an overproduction of reactive oxygen species (ROS) that cannot be overcome by ROS-scavenging system (Chrestin 1989).

Diagnosis of latex consists in measuring four main parameters: sucrose, inorganic phosphorus (Pi), thiol content, and the total solid content (TSC) of latex (Jacob et al. 1986, Jacob et al. 1989). Sucrose is the source of carbon for the biosynthesis of natural rubber. The inorganic phosphorus reflects the turn-over of ATP and metabolic activity. Thiol content is parameter to check the detoxification capacity of laticifers. TSC is for estimating the dry rubber content (Jacob et al. 1995, Jacob et al. 1989).

5. Abiotic and biotic stresses

Plant lives in the environment surrounded by biotic and abiotic factors. Any sudden change in the environment from optimal condition for growth may be disturbing the homeostasis of plant causing a stress. Understanding abiotic and biotic stress responses in plant is essential to increase knowledge of physiological and molecular mechanisms in plant defence. One of the improvements in plant stress tolerance has been attempted by genetic modification (Hirayama and Shinozaki 2010).

Many genes respond to cold, salinity, ethephon, water deficit, salt, and biotic stress at the transcriptional level and the products of these genes function in the stress response and tolerance. Various transcription factors are involved in the regulation of stress-inducible genes (Zhang et al. 2009, Shinozaki, Yamaguchi-Shinozaki and Seki 2003). Plants have developed mechanisms to adapt against environmental stress or biotic stress. Defence response genes are transcriptionally activated by different form of abiotic and biotic stress. Transcription factors play an essential role in the regulation of plant adaptation to environmental stress and take part in the crosstalk between abiotic and biotic stress signalling network (Fraire-Velázquez, Rodríguez-Guerra and Sánchez-Calderón 2011). Several members of Ethylene Response Factor (ERF) activator-type transcription factors are involved in various biological functions including plant development and response to biotic and abiotic stresses (Cheng et al. 2013, Pirrello et al. 2014).

6. Jasmonate biosynthesis and signalling

The mechanical wounding, by tapping soft bark for harvested latex, can induce endogenous hormone production such as jasmonic acid (JA) (Hao and Wu 2000). JA is an effector of a large number of plant defence genes, and is assumed to play a major role in latex production. JA is also involved in the differentiation of primary and secondary laticifers.

6.1. Jasmonate biosynthesis and signalling in plant

Plants as a sessile organism need to adapt and respond to environmental challenges. One of the plant defence strategy is the production of chemical compounds both volatile and non-volatile. The phytohormones are playing important regulatory roles in plant growth, development, and response to stresses (Avanci et al. 2010, Wasternack and Hause 2013). The phytohormones jasmonic acid and methyl jasmonate, along with their intermediate compounds are collectively called jasmonates (JAs) (Avanci et al. 2010).

JAs are present in almost all higher plants and are distributed in plant tissues and cell (Avanci et al. 2010). Some studies revealed levels of JA are higher in the hypocotyls hook, young plumules, flowers, and pericarp tissues of developing reproductive structures in soybean (Creelman and Mullet 1997, Lopez et al. 1987). JA involved in crucial processes related to plant development and survival, including direct and indirect defence responses, secondary metabolism, reproductive process, and senescence (Avanci et al. 2010, Balbi and

Devoto 2007, Creelman and Mullet 1997, Pirrello et al. 2014, Turner, Ellis and Devoto 2002).

Jasmonates are fatty acid derived cyclopentanones and belong to the family of oxygenated fatty acid derivatives called as oxylipins, which are produced by the oxidative metabolism of polysaturated fatty acids. Jasmonates are synthesized in plants via the octadecanoid pathway (Dar et al. 2015, Wasternack and Hause 2013). The biosynthesis of JA initiates in plastid, involving the release of α-linolenic acid (α-LeA) which is a precursor of JA. Afterwards, α-linoleic acid is oxidized by the action of a chloroplastic 13-lipoxygenase (13-LOX) generating the 13-hydroperoxy derivate of linoleic acid (13-HPOT). The subsequent enzyme along the pathway is the allene oxide synthase (AOS) and allene oxide cyclise (AOC) (Farmer and Ryan 1992, Vick and Zimmerman 1984).

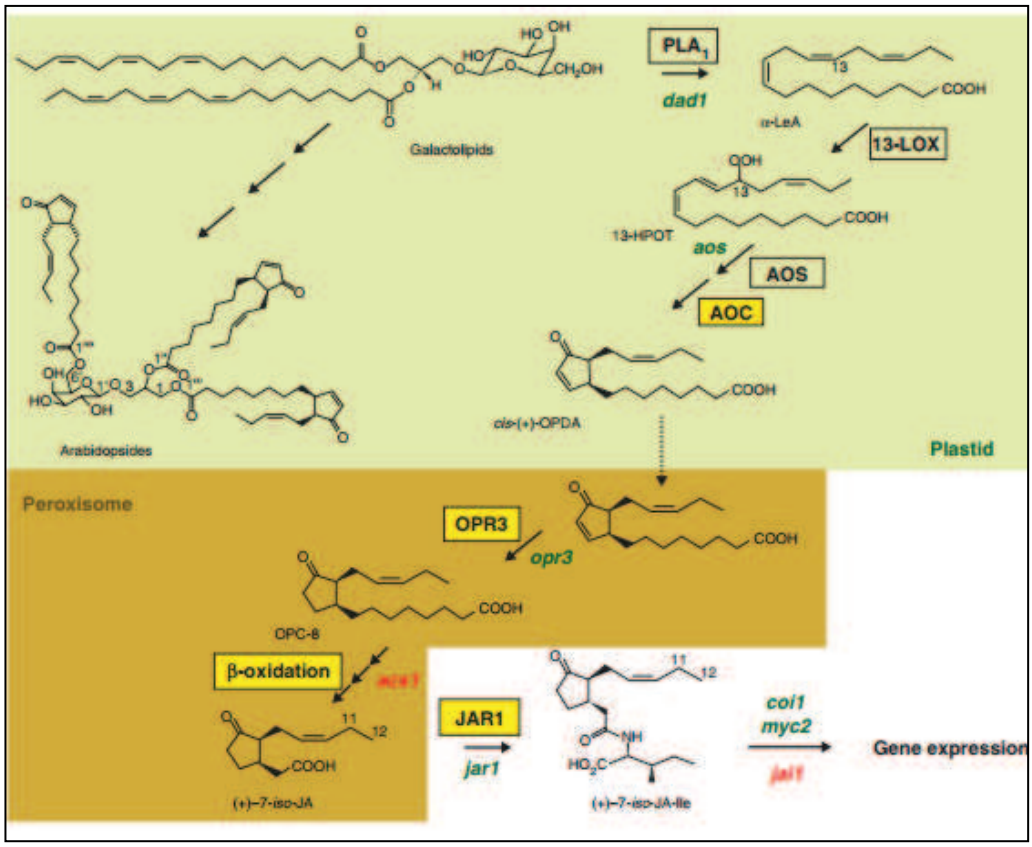


Figure 11. Synthesis of jasmonic acid (JA)/JA-Ile from α-linoleic acid generated from galactolipids. Enzymes which have been crystallized are given in yellow boxes. Steps impaired in mutant of *Arabidopsis* (green) or tomato (red) are indicated, *acxl*: *acyl-coA-oxidase1, AOC*: *Allene oxide cyclise,* AOC*: Allene oxide synthase*, *coi1*: *coronatine insensitive1*, *dad1*: *delayed anther dehiscence1*: 13-HPOT: *13S*-hydroxyperoxypeoctadecatrienoic acid, *jai1*: *jasmonic acid insensitive1*, JAR1: JA-amino acid synthetase, α-LeA: α-lineloic acid, 13-LOX: 13-lipoxygenase, *myc2*: *bHLHzip* transcription factor MYC2, OPR3: OPDA reductase3, OPC-8: 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoid acid, *cis*-(+)-12-oxophytodienoic acid, PLA_1:phospholipase A_1. This figure is extracted from (Wasternack and Hause 2013).

The next steps of JA biosynthesis take place in the peroxisome, where oxophytodienoic acid (OPDA) is reduced to 12 oxophytoenoic acid (OPC-8) by the enzyme OPDA reductase (OPR3), and subjected to three cycles of β-oxidation, to yield (+)-7-iso-JA. The β-oxidation cycles take place by a set of four enzymatic reactions such as oxidation, hydration, oxidation and thiolysis, and the cytosol. JA is further metabolized to various inactive and bioactive derivates. Jasmonate carboxyl methyltransferase (JMT) converts to methyl jasmonate (MeJA). The reverse reaction is catalysed by MeJA esterase (MJE).

Conjugation of JA by jasmonate resistance 1 (JAR1) will produce jasmonoyl isoleucine (JA-Ile) (Laudert and Weiler 1998, Schaller et al. 2000.) (Figure 11).

The JA signalling pathway plays a pivotal role in regulating response of plants to biotic and abiotic stress. The jasmonate signalling pathways involved JA signal, SCF-type E_3 ubiquitin ligase SCF^{COI1}, jasmonate-ZIM-domain (JAZ) repressor protein, and transcription factor (TFs) (Wasternack 2007). Under stress condition, JA response is controlled by a group of nuclear JAZ repressor proteins. JAZ repressors interact with the F-box protein COI1 (Coronatine Insensitive 1). F-box protein COI1 is an integral part of the SCF (SKp-Cullin-F-box) complex involved in the co-reception of biologically active JA-Ile (Chini et al. 2007, Thines et al. 2007).

The JA signalling pathway can be accessed by two massive signalling. The first one is involved SCF^{COI1} complex which is an E_3 ubiquitin ligase. In this complex, the F-box protein COI1 associates with SCF (Ssp-Cullin-F-box) and AtRbx1 to form active SCF^{COI1}. The second is the COP9 signalosome (CSN) which interacts *in vivo* with SCF^{COI1}. Together, the SCF^{COI1} and CSN complex form the core of signal pathways and control all JA responses (Devoto et al. 2002, Memelink 2009, Xu et al. 2002.).

JA interacts with receptors in the cell that activate a signalling pathway resulting changes in transcription, translation, and other responses (Ballaré 2011, Creelman and Mullet 1997). JA receptors and other components of the signal transduction pathway are more likely to be discovered through analysis of mutants that are insensitive or altered in their response to JA. There are four classes of JA insensitive mutants have been identified *coi1*, *jar1*, *jin1*, and *jin4*. The *coi1* mutant also shows MeJA-insensitive root growth phenotype (Benedetti, Xie and Turner 1995, Berger et al. 1995).

The act of JA to regulate plant growth, development, metabolism, and defence are not alone, but in complex network with other hormones like salicylic acid (SA), abscisic acid (ABA), and ethylene (ET) (Dar et al. 2015). Several studies showed the phytohormones (including JA) often act together to achieve certain physiological functions. Crosstalk between jasmonate and ethylene signalling pathway present a fascinating case of synergism and antagonism responses. JA and ET signalling pathways synergize to activate a set of defence genes (Lorenzo et al. 2003). In addition, jasmonate antagonizes ethylene effect in the regulation of apical hook development and wounding responses (Turner et al. 2002, Wasternack and Hause 2013, Zhu and Lee 2015). Response to herbivorous insects and necrotrophic pathogens induce crosstalk between JA, ethylene, and abscisic acid (ABA) (Figure 12). Attacks by herbivorous insects induce JA and ABA-dependent signalling pathways and infections by necrotrophic pathogens induce JA- and ET-dependent signalling pathways (Pieterse et al. 2012, Wasternack and Hause 2013).

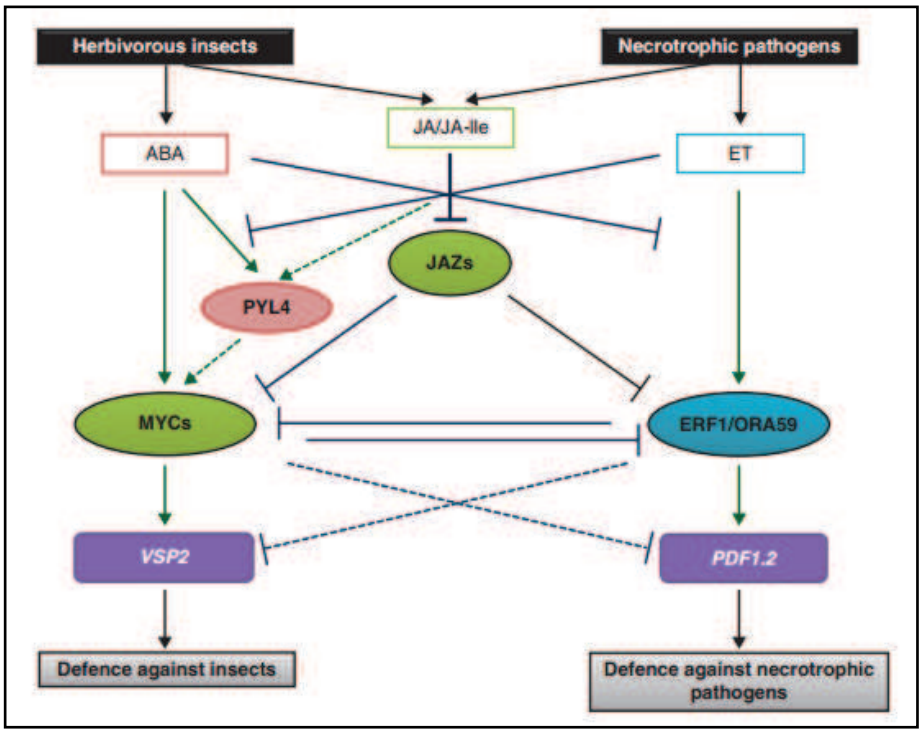


Figure 12. Crosstalk between jasmonate (JA), ethylene (ET), and abscisic acid (ABA) triggered in response to pathogens.This figure is extracted from (Pieterse et al. 2012, Wasternack and Hause 2013).

6.2. Jasmonate biosynthesis and signalling in *Hevea*

Harvesting stress like tapping as a physical wounding can produce systemin, which induce JA production. JA acts as a signal molecule in rubber biosynthesis (Hao and Wu 2000, Sun et al. 2011). Little is known on JA biosynthesis in rubber tree but a few genes encoding one of the main enzyme (allene oxide synthetase) were isolated (Duan et al. 2005). Because JA is an activator of laticifer differentiation, more studies have been reported on jasmonate signalling pathway especially in response to harvesting stress. Twenty-four contigs involved the jasmonate signalling pathway was identified in a reference transcriptome (Pirrello et al. 2014).

Studies on the expression profile of one or two members of the multigene families encoding COI, JAZ, and MYC suggest the importance of JA in latex production. *HbCOI1* is highly expressed in laticifers, the transcripts of *HbJAZ1* accumulate in response to tapping and wounding, *HbMYC1* and *HbMYC2* are abundant in latex. *HbMYC1* is induced by tapping and wounding (Tian et al. 2010, Xu et al. 2009, Zhao et al. 2011). Efficiency of wounding, MeJA, and ET treatment were controlled using *HbERF-IXc4* and *HbERF-IXc5* (orthologs to *ERF1*) (Putranto et al. 2015a).

7. Ethylene biosynthesis and signalling

Latex is harvested by tapping and latex production can be stimulated by application of ethephon on the tapping trunk panel. Both processes can alter several biochemical changes in laticifers, and involve synthesis of defence proteins. Tapping can induce biosynthesis of endogenous ethylene for certain *Hevea* clones and ethephon stimulation release exogenous ethylene. Ethylene is known as an essential compound to control latex production (Duan et al. 2010, Piyatrakul et al. 2014, Putranto et al. 2015a). This hormone acted on membrane permeability, leading to prolonged latex flow and on general regenerative metabolism (Zhu

and Zhang 2009). Research on the mechanism induced by ethylene might explain the metabolic modifications, which are responsible for latex production. Based on this, there is important to study ethylene biosynthesis and signalling in rubber.

7.1. Ethylene biosynthesis and signalling in plant

Ethylene is a gaseous plant hormone that has simple hydrocarbon chemical structure (C_2H_4) (Benavente and Alonso 2006). Ethylene is involved in many developmental and physiological processes in higher plants (Chang and Bleecker 2004). This hormone is implicated in some aspects of plant development including senescence, cell death, ripening and chlorosis (Abeles, Morgan and Saltveit 1992). Ethylene can also influence changes at the morphological, cellular, and molecular levels (d' Auzac et al. 1993).

Ethylene biosynthesis is regulated during different stages of plant growth and development (Fluhr and Mattoo 1996, Yang and Hoffman 1984). Ethylene synthesis is induced in response to developmental processes and to biotic and abiotic stresses in plants (Wang, Li and Ecker 2002). Major breakthroughs in the ethylene biosynthesis pathway were the establishment of *S*-adenosyl-methionine (*S*-AdoMet) and 1-aminocyclopropane-1-carboxylic acid ACC as the precursors of the ethylene (Wang et al. 2002, Yang and Hoffman 1984).

There are three pathways involved in ethylene production: (1) the activated methyl cycle, (2) the S-methylmethionine cycle, and (3) the Yang cycle or methionine (Adam and Yang 1979). The key reactions focused with ethylene production from methionine *via* S-adenosyl-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), catalysed by ACC synthase (ACS), and ACS to ethylene, catalysed by ACC oxidase (ACO) (Figure 13). ACC synthase (ACS) is encoded by a multi-gene family that is regulated at the transcriptional and post-transcriptional regulation (Fluhr and Mattoo 1996). ACC oxidase (ACO) catalyses the subsequent oxidation of ACC to ethylene with the release of HCN and CO_2 (Yang and Hoffman 1984).

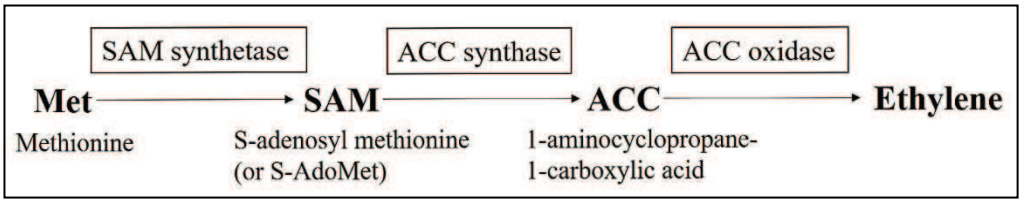


Figure 13. The biosynthesis pathway in ethylene. The synthesis of ethylene from methionine and catalysed by ACC synthase (ACS) and ACC oxidase (ACO). This figure is extracted from (Grierson 2012).

After ethylene biosynthesis, this hormone is perceived through transduction machinery to trigger specific biological responses. Ethylene signalling pathway involves the perception and the transduction of the ET signal, then the regulation on the ethylene-responsive genes (Chen et al. 2005, Lin, Zhong and Grierson 2009, Peiser et al. 1984). The key components in ethylene signalling pathway have been identified in *Arabidopsis* (Wang et al. 2002) (Figure 14).

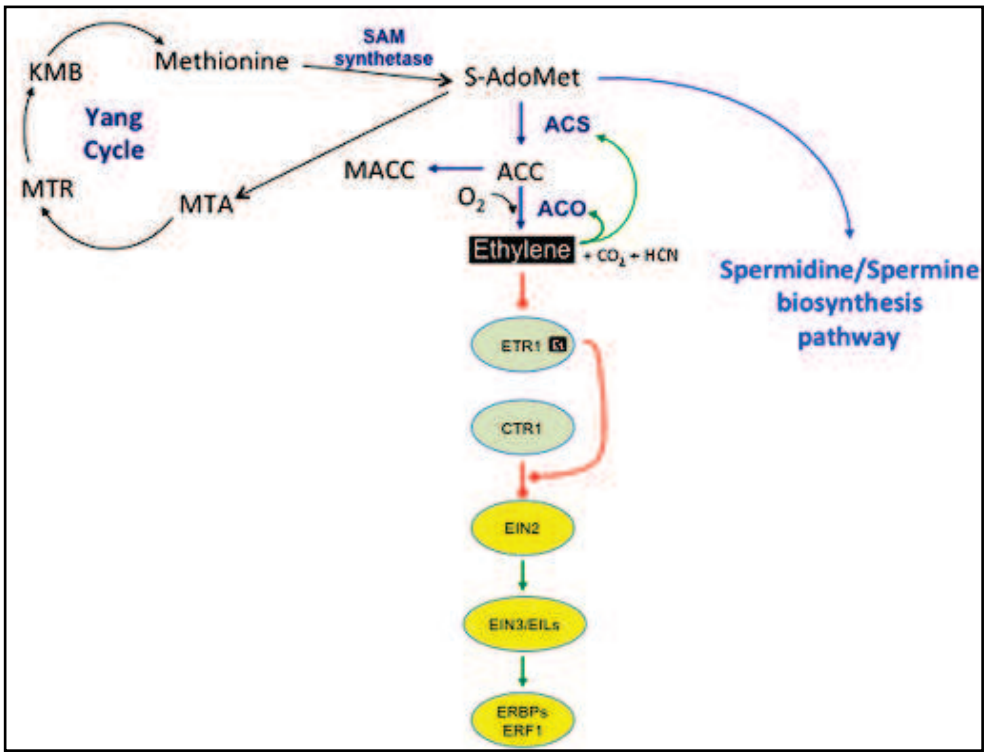


Figure 14. Ethylene biosynthesis and signalling pathways. Ethylene can stimulate by improving ACC synthesis catalysed by ACC synthase (ACS) and conversion to ethylene by ACC oxidase (ACO). Ethylene binds to receptors, ETR1 which leads to the deactivation of the receptor. Release of CTR1 inhibition allows EIN2 to act as a positive regulator of ethylene signalling pathway. EIN2 acts upstream of nuclear transcription factor, such as EIN3, EILs, ERBPS, and ERFs. This figure is extracted from (Corbineau et al. 2014).

There are five membrane-localized ethylene receptors in *Arabidopsis*, ethylene resistant 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1), ERS2, and ethylene insensitive 4 (EIN4) predominantly localized at the membrane of the endoplasmic reticulum (ER) (Figure 15). ETR1 and ERS1 contain three transmembrane domains in the N-terminus and a histidine kinase domain in the C-terminus while ETR2, EIN4, and ERS2 have four transmembrane regions and a serine-threonine kinase domain in the C-terminus (Stepanova and Alonso 2009, Wang et al. 2002). The receptors physically associate with CTR1 (Binder et al. 2012).

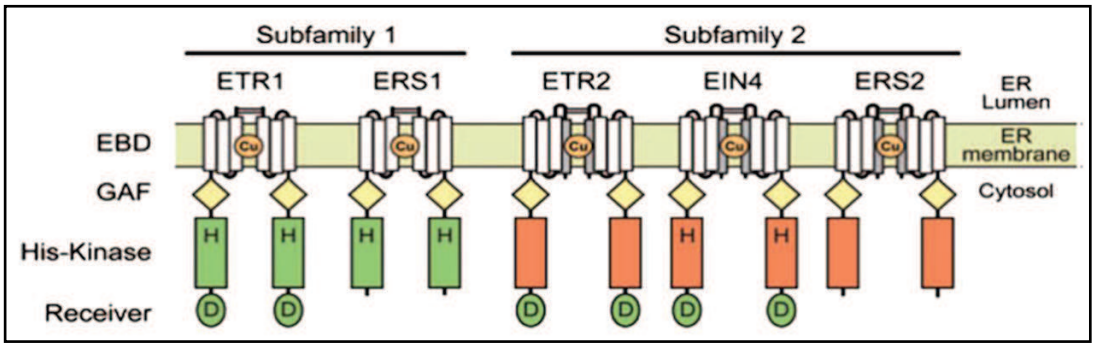


Figure 15. Five membrane-localized ethylene receptors in *Arabidopsis*. The ethylene receptor family of *Arabidopsis* is divided into subfamilies 1 and 2 based on phylogenetic analysis and structural features. This figure is extracted from (Shakeel et al. 2013).

In the absence of ethylene, the receptors positively regulate Constitutive Triple Response1 (CTR1) which acts as a negative regulator of the pathway. The role of CTR1 is to inhibit downstream components of the pathway and prevent ethylene responses (Kieber et al. 1993) (Figure 16). CTR1 phosphorylates the C-terminal plant-specific domain of EIN2 preventing EIN2 from signalling (Alonso et al. 1999, Ju et al. 2012).

In the presence of ethylene to its receptors results in inactivation of CTR1 protein kinase, which in turn activates the kinase cascade controlling EIN2 and its transcription factors in the nucleus, such as EIN3, EIN3-like protein (EILs), ethylene response element binding proteins or ethylene responsive factors (EFRs) activate the transcription of ethylene response genes, like *ERF1* (Chang et al. 2013, Guo and Ecker 2004, Solano et al. 1998).

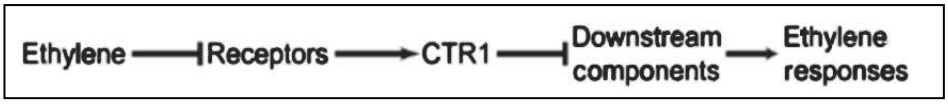


Figure 16. Negative regulator model of ethylene signal transduction. The absence of ethylene activates CTR1 that inhibit downstream components. This figure is extracted from (Binder et al. 2012).

EIN2 protein level is regulated by degradation of proteasome (Ju et al. 2012). EIN2 and EIN3 are positive regulators of the ethylene signalling pathway. The *N*-terminus of EIN2 has sequences homology with natural resistance-associated macrophage proteins (NRAMP) ion transporters (Grierson 2012). EIN2 signalling involves cleavage of the EIN2 C terminus from the ER membrane-bound N terminus, followed by translocation of the C terminus into the nucleus (Ju et al. 2012, Qiao et al. 2012). The process activates the transcription factor EIN3 and then activates expression of the transcription factor *ERF1* gene and other genes (Solano et al. 1998) (Figure 17).

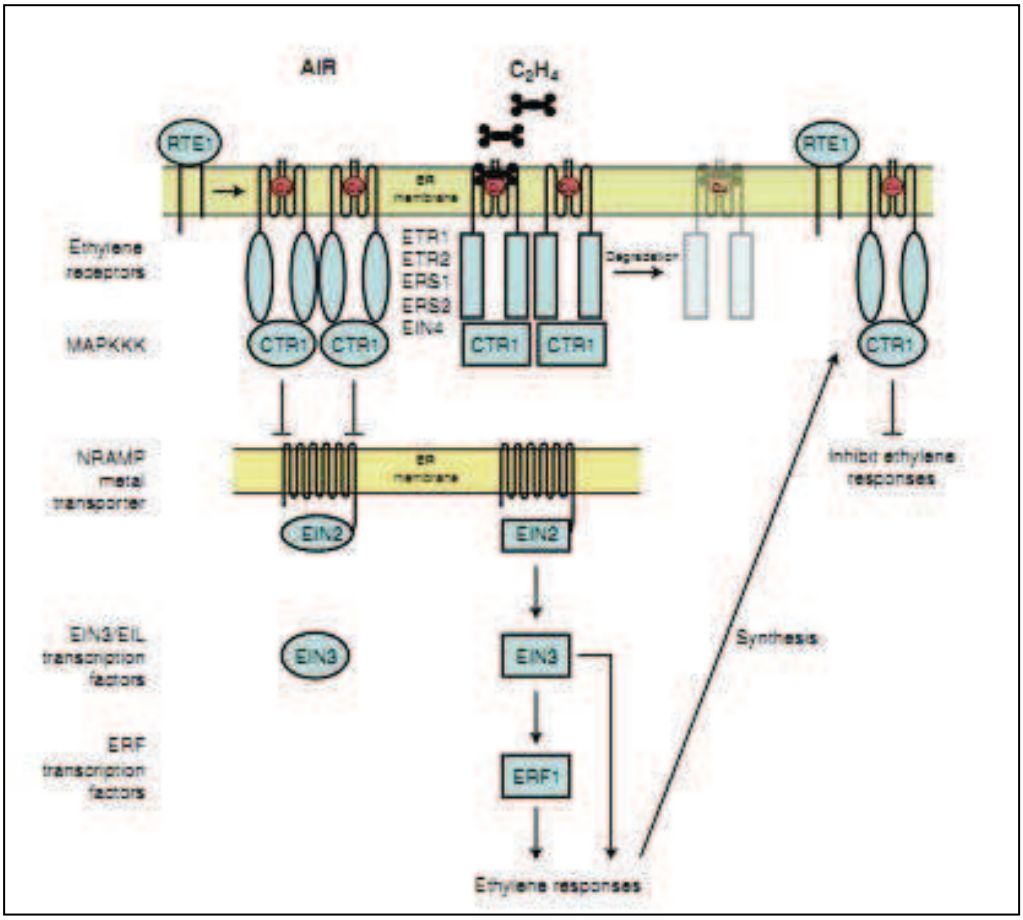


Figure 17. Ethylene signalling pathway of *Arabidopsis*. In air, ethylene receptors maintain CTR1 in active state that repress ethylene response. The presence of ethylene inactivates CTR1. Therefore, EIN2 is activated and transcriptional cascade involving the EIN3/EIL and ethylene response factor (ERF) transcription factors is initiated. This figure is extracted from (Chen et al. 2005).

According to the Nakano's classification, AP2/ERF superfamily consists of three separated families, namely ERF, AP2 and RAV families (Figure 18). Most proteins with single AP2 domain and whose genomic sequence contains a small amount of introns are assigned to the ERF family (Nakano et al. 2006). The AP2 family consists of members characterized by tandem repetition of two AP2 domains and a small number of proteins with a single AP2 domain that shows higher similarity to the one contained in double AP2 proteins than to the AP2 domain of the ERF proteins. The AP2 family was further subdivided into AP2 and ANT groups according to the amino acid sequence of the double AP2 domain and the nuclear localization sequence (Shigyo and Ito 2004). A third class of proteins possesses an ERF domain association with B3 DNA-binding domain. They constitute the RAV family (Swaminathan, Peterson and Jack 2008).

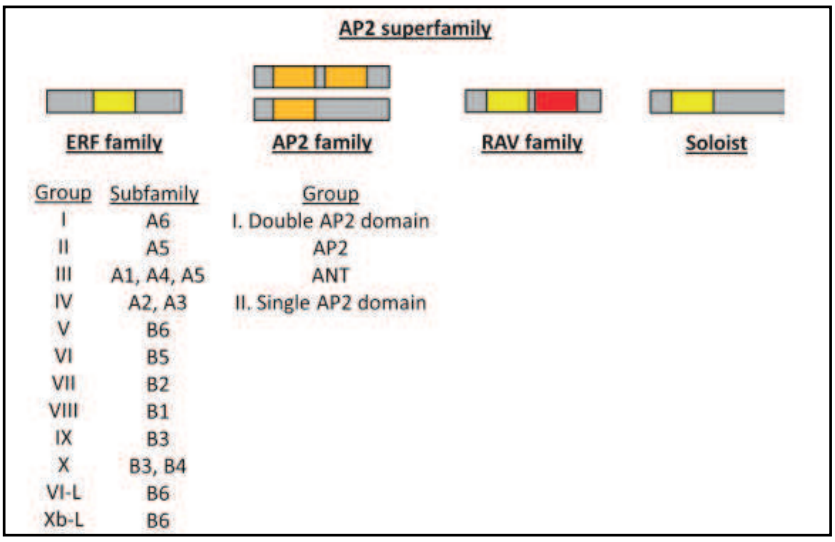


Figure 18. Structure of the APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily. The AP2 superfamily is composed of single-AP2 domain proteins (ERF family), single or double ERF domain proteins (AP2 family), proteins containing one AP2 domain plus a B3 DNA binding domain (RAV family). This figure is extracted from (Licausi et al. 2013).

Two classifications of the ERF family coexist. Nakano classified the ERF family into10 functional groups (Nakano et al. 2006), while Sakuma divided this family into 2 subfamilies, DREB and ERF, which are divided in 12 subgroups (A1 to A6, and B1 to B6) (Sakuma et al. 2002). The AP2/ERF DNA binding domain has been distinguished between domains depending on the identity of residues at specific positions (Sakuma et al. 2002). The differences in amino acid sequence reflect in the DNA affinity and specify of the two subfamilies. Many DREB proteins have been shown to bind to an A/GCCGAC element, which is often associated with ABA, drought and cold responsive genes (Stockinger, Gilmour and Thomashow 1997).

Conversely, members of the ERF subfamily specifically bind *in vitro* an AGCCGCC element, named the GCC-box often found in the genomic regions upstream of genes that respond to ethylene, pathogens, and wounding (Ohme-Takagi and Shinshi 1995). ERFs are involved in biotic and abiotic stress responses via direct interaction with GC-rich *cis*-elements such as GCC box and DRE (Dehydration Responsive Element) in the promoter of their target genes (Aharoni et al. 2004, Hao, Ohme-Takagi and Sarai 1998, Liu et al. 1998).

Several ERFs protein is also regulating the biosynthesis of ethylene. The *ERF* genes are induced by biotic and abiotic stresses such as pathogen infection, salt stress, osmotic stress, wounding, drought, hypoxia, temperature stress and the stress-related hormones ethylene, jasmonic acid and ABA (Cheng et al. 2013, Steffens 2014). A number of *ERF* genes confer tolerance to various biotic stresses when expressed ectopically in various plants and in many cases. For example, several ERFs activate the transcription of basic type defence-related genes, pathogenesis-related (PR) genes, osmotin, chitinase and β-1,3-glucanase (Lorenzo et al. 2003, Zhang et al. 2009).

Activation of ERF protein in post-transcriptional control is a key feature of several physiological processes in plants (Figure 19). Alternative splicing has been reported to play a major role in the fast accumulation of DREB2-like sequences in grass species. Barley (DRF1), wheat (WDREB2) and maize (DREB2A) orthologs show accumulation of an mRNA isoform characterized by a STOP-codon before the DNA binding domain under non-stress conditions, thus producing a non-functional protein. When stress stimuli occur, alternatively splicing takes place excluding the exon that contains the premature stop codon, rapidly

generating a functional isoform. Alternative splicing has also been reported for members of the ERF-VII group in *Arabidopsis* and tomato (Licausi et al. 2013, Pirrello et al. 2006).

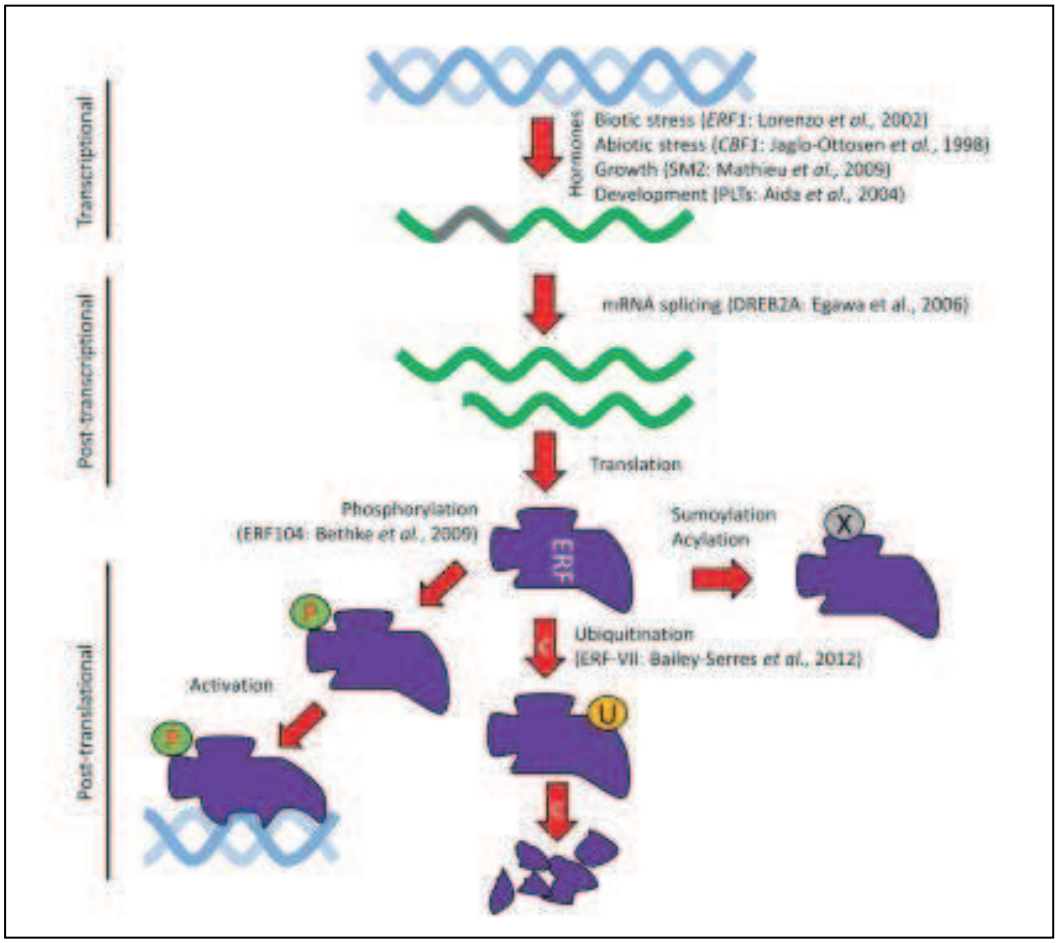


Figure 19. Regulatory mechanism affecting Ethylene Responsive Factor (ERF) protein activity. The transcription of *ERF* genes can be regulated in response to biotic and abiotic stresses or in frame of growth and development programs. Hormones are often mediators of this regulatory step. ERF mRNAs can be alternatively spliced to produce functional or non-functional proteins. Once translated, ERF transcription factors can be controlled by protein modifications that enhance or hamper their activity, restrict their localization or promote their stability or degradation. This figure is extracted from (Licausi et al. 2013).

7.2. Ethylene biosynthesis and signalling in *Hevea*

Latex is harvested by tapping and latex production can be stimulated by application of ethephon on the tapping trunk panel. Both processes can alter several biochemical changes in laticifers, and involve synthesis of defence proteins. Tapping can induce biosynthesis of endogenous ethylene for certain *Hevea* clones and ethephon stimulation release exogenous ethylene. Ethylene is known as an essential compound to control latex production (Duan et al. 2010, Piyatrakul et al. 2014, Putranto et al. 2015a). Research on the mechanism induced by ethylene might explain the metabolic modifications, which are responsible for latex production. Based on this, it is important to study ethylene biosynthesis and signalling in rubber.

Ethylene biosynthesis and signalling pathways play crucial role in response to latex harvesting stress but also during the occurrence of tapping panel dryness (TPD). Ethylene in the form of chloroethylphosphonic acid applied to *Hevea* bark in order to stimulate latex production and flow of latex biosynthesis. Both exogenous and endogenous ethylene are

essential hormones controlling latex production (d'Auzac and Jacob 1989). Endogenous ethylene induced by ethylene self-catalysis play an important role in boosting rubber yield through stimulation especially during the first several tapping after stimulation (Fan, Yang and Yang 1986). An amount of endogenous ethylene generated 9th day after treatment was still 50% higher of the exogenous ethylene. Ethephon induces some biochemical pathways in latex cells, such as sucrose loading, water uptake, nitrogen assimilation or synthesis of defence proteins, involving a large number of ethylene-response genes (Chye et al. 1992, Duan et al. 2010, Dusotoit-Coucaud et al. 2010, Gidrol et al. 1988, Jacob et al. 1989, Pujade-Renaud et al. 1994, Tang et al. 2010, Tungngoen et al. 2009, Zhu and Zhang 2009) (Figure 20).

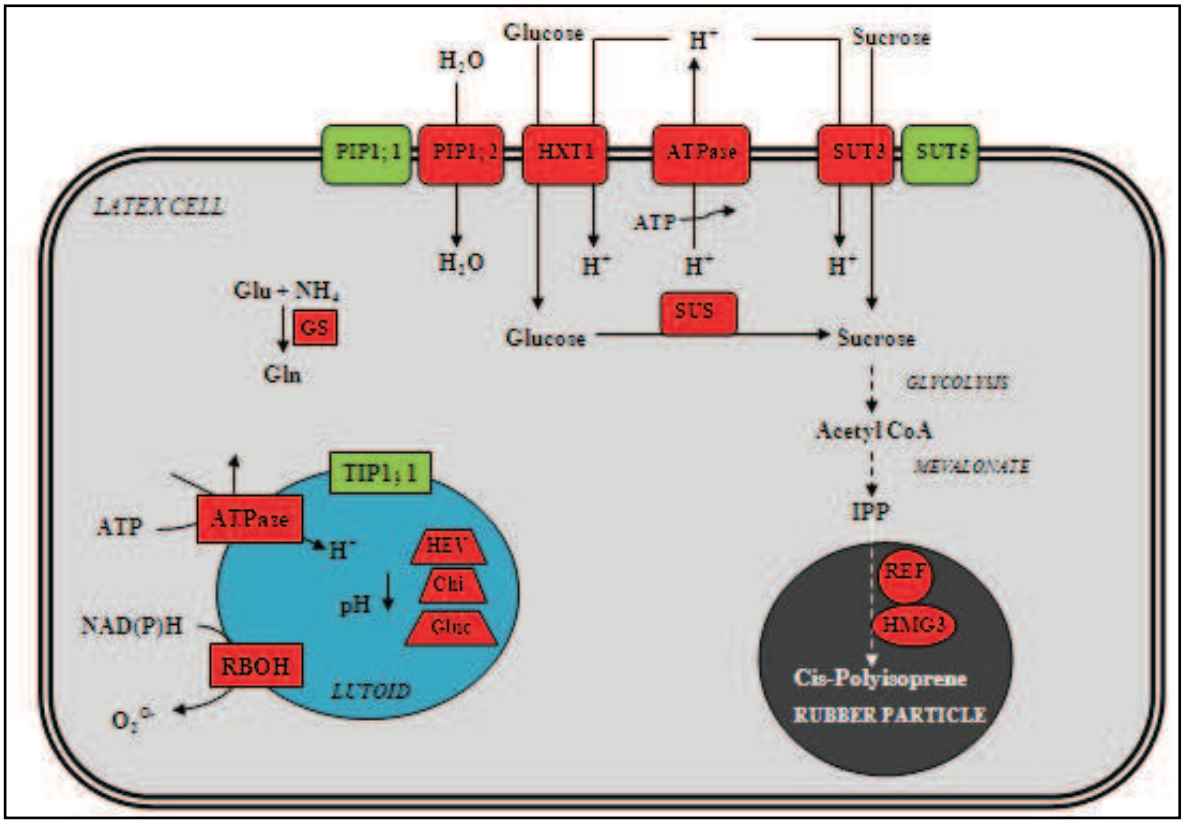


Figure 20. General scheme of ethylene-induced biochemical pathways in latex cells. Factors in red and green are activated and inhibited by ethephon or ethylene. Chi (Chitinase), Glu (Glucanase), GS (Glutamine synthetase), HEV (Hevein), HMG (3-hydroxy-3-methylglutaryl-coenzyme A reductase), HXT (Hexose transporter), PIP (Plasma membrane intrinsic protein), RBOH (NADPH oxidase), REF (Rubber elongating factor), SUS (Sucrose synthase), SUT (Sucrose transporter). This figure is extracted from (Piyatrakul et al. 2014).

Ethylene synthesis in plant tissues is catalysed by two main enzymes called 1-aminocyclopropane-1-carboylic (ACC) synthase and ACC oxidase. ACC enzyme is the precursor of ethylene. The expression of these enzymes is regulated tightly by biotic and abiotic factors, plant development, wounding and hormonal treatment. The kinetics of the ACC oxidase multigene family involved in ethylene biosynthesis. Three genes have been identified (Kuswanhadi et al. 2007, Kuswanhadi et al. 2010) (Figure 21). All *HbACO* multigene family genes were expressed at all stages of development (Kuswanhadi et al. 2010).

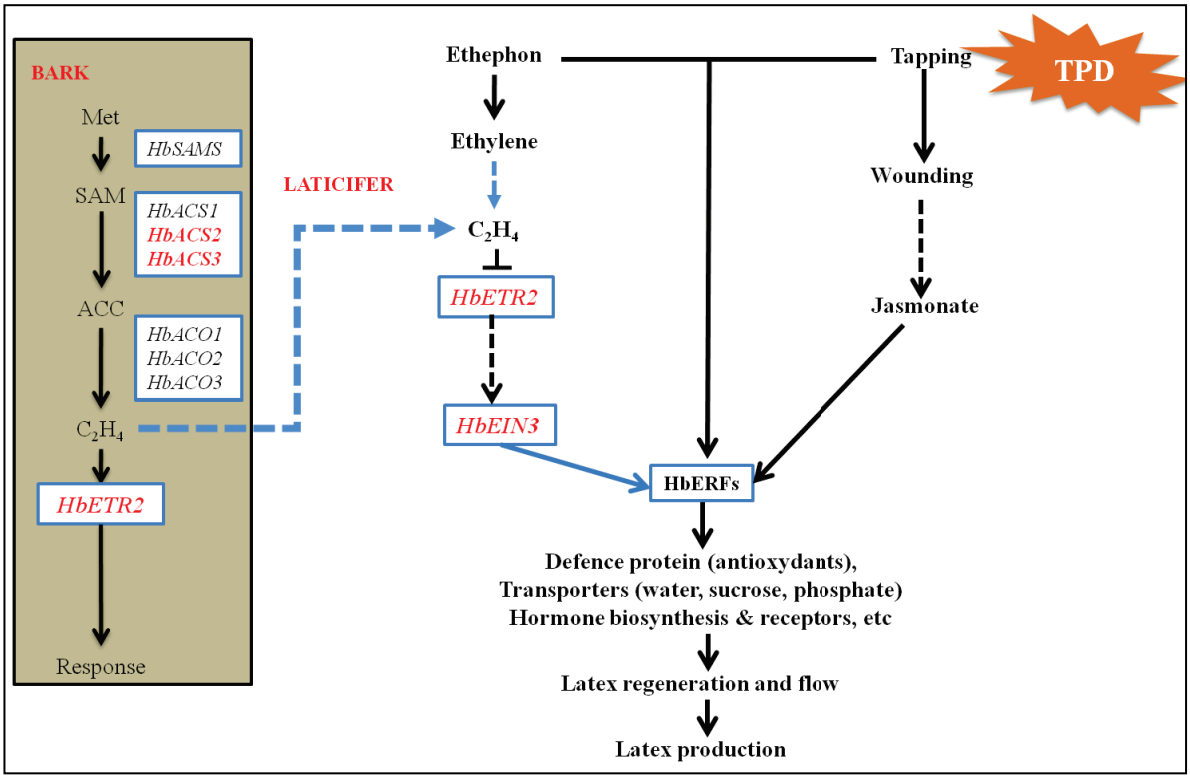


Figure 21. Molecular model on the involvement of ethylene in laticifer. This figure is a summary of conclusions from several papers (Duan et al. 2010, Kuswanhadi et al. 2007, Piyatrakul et al. 2014, Putranto et al. 2015a).

Research on mechanism of ethylene in stimulating latex production led to characterization of ethylene biosynthesis and signalling genes and more recently to the identification of the different members of the APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily (Duan et al. 2010, Piyatrakul et al. 2014, Putranto et al. 2015a). AP2/ERF is a large superfamily of plant-specific transcription factor that involves in activation process of expression of abiotic stress-responsive genes. Transcription factor plays central roles in gene expression by regulating expression of downstream gene as *trans*-acting elements via specific binding to *cis*-acting elements in the promoters of target genes (Mizoi, Shinozaki and Yamaguchi-Shinozaki 2012).

Some of *Hevea AP2/ERFs* are transcription factors and play a unique role in defence mechanisms and latex production. Natural rubber production in *Hevea* is influenced by tapping and stimulation of ethephon. The use of ethephon induces the production of reactive oxygen species (ROS) that are responsible for the coagulation of rubber particles in latex cells. A high tapping frequency and ethephon stimulation induced a physiological disorder, TPD (Duan et al. 2010, Putranto et al. 2015a).

In *Hevea*, ethylene perception and signalling pathways were also identified (Duan *et. al.* 2010). There were two genes of perception (*HbETR2* and *HbEIN2*) and for a signalling gene (*HbEIN3*) were differentially regulated by ethylene treatment. The transcripts of *HbETR2* accumulated early after ethylene stimulation while *HbEIN2* and *HbEIN3* were significantly reduced. The early induction *HbETR2* was suppressed by an inhibitor of ethylene, 1-MCP. This work has shown the impact of wounding, MeJA, and ET and for evaluating crosstalks between hormone signalling pathways and understanding defences responses in plants by finding the transcription factor involved (Duan et al. 2010).

Several genes in *H. brasiliensis* were regulated independently by the different of signalling pathways. Defence responses in *Hevea* were dramatically mediated by wounding, methyl jasmonate (JA), and ethylene (ET). Gene expression patterns in response to

wounding, MeJA, and ET in the bark of *Hevea* showed that these treatments are very efficient to trigger the regulation of a large number of genes (Duan et al. 2010). Five genes *HbETR1, HbETR2, HbEIN2, HbMAPK* and *HbCOI1* were related to ET perception and transduction of jasmonate and ET signals. Five other genes encoding transcription factors were *HbEIN3, HbSAUR, HbWRKY, HbMYB* and *HbBTF3b*. Twelve genes encoding functional proteins were *HbPIP1, HbGS, HbUbi, HbLTPP, HbACR, HbACBP, HbCaM, HbCIPK, HbGP, HbCAS1, HbCAS2* and *HbPLD*. Three genes were related to plant defence and programmed cell death: *HbChit, HbDef*, and *HbQM* (Duan et al. 2010).

Duan et al. (2013) ranked 142 super family members of AP2/ERF based on the AP2 full length from RNA sequence of clone PB260 (Duan et al. 2013). The transcript database was generated from somatic embryo, tissue, leaves, bark, latex, and roots. The study has focused on transcription factors involving ET and JA signalling. *ERF1* and *ORA59* genes are found in the crosstalk between ET and JA signalling pathway (Lorenzo et al. 2003, Pré et al. 2008). In rubber, the *HbERF-IXc4* and *HbERF-IXc5* genes are orthologs to *ERF1* from *Arabidopsis* while *HbERF-IXc6* gene is an orthologs of *ORA59* (Duan 2011). The *HbERF-IXc4* and *HbERF-IXc5* genes were induced by both ethylene and MeJA. When treatment combined the injury with the MeJA and ethylene, transcript abundance was highly increased. HbERF-IXc4 and HbERF-IXc5 proteins could bind to the promoter portion of *AtPDF1.2* gene and were localized in the nucleus (Duan 2011).

The characterization of the super family AP2/ERF in the aspect of development in rubber was studied by Piyatrakul et al. (2012). The accumulation of transcripts of genes AP2/ERF was analysed during the process of somatic embryogenesis from callus lines with different regeneration potentials in various vegetative and reproductive tissues (Piyatrakul et al. 2012). The transcript database was supplemented with reproductive tissues (immature and mature male flower and immature and mature female, zygotic embryos) (Piyatrakul et al. 2014).

There were 114 *AP2/ERF* genes and 1 soloist in *H. brasiliensis* by a comparison with genomic scaffolds of clone rubber CATAS-7-33-97. Transcript result showed that ERFs from group I and VIII were very abundant in all tissues while those of group VII were highly accumulated in latex cells. This work has identified markers of gene expression latex belongs to ERF Group VII suggesting a potential role in the regulation of hypoxia in laticifers. Functional analysis by trans-activity and subcellular localization confirmed that members of HbERF-VII is an activator-like transcription factor (Piyatrakul et al. 2014).

HbERF-VIIa04, *HbERF-VIIa07*, and *HbERF-VIIa12* are highly regulated in latex and are orthologs to *AtEBP/RAP2.3* and *AtERF74/RAP2.12*. *HbERF-VIIa17* gene orthologs to *AtEBP/RAP2.3/ERF72* might play a role in the response to the accumulation of reactive oxygen species generated during latex regeneration. Three *HbERF* genes induced upon laticifer differentiation correspond to three members of group VII (*HbERF-VIIa3*, *HbERF-VIIa17* and *HbERF-VIIa1*) (Duan et al. 2013, Piyatrakul et al. 2014).

Analyses of the relative transcript abundance were carried out for 35 *HbERF* genes in latex, in bark from mature trees, and in leaves from juvenile plants under multiple abiotic stresses for understanding the regulation of *ERF* genes during latex harvesting. ERF groups III and IV were regulated under osmotic stress during acclimatization against cold and dehydration (Mizoi et al. 2012). *HbERF-IVa3* transcripts were highly accumulated in response to tapping in latex. Two other genes, *HbERF-VIIIa9* and *HbERF-IIIa10*, also had the same pattern of induction by dehydration and cold (Duan et al. 2013, Piyatrakul et al. 2014, Putranto et al. 2015a, Mizoi et al. 2012).

Twenty-one *HbERF* genes in group IX were regulated by harvesting stress in laticifers. Subcellular localization and transactivation experiments confirmed that several members of HbERF-IX are activator-type transcription factors. Transcripts of three *HbERF-*

IX genes from *HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6* were significantly accumulated by combining of wounding, methyl jasmonate and ethylene treatments (Putranto et al. 2015a). A modification of cambial activity with a smaller number of laticifer probably resulted from TPD-affected trees. Latex harvesting is expected to induce plant responses to abiotic stress on various hormonal signalling pathways by some candidate genes (Putranto et al. 2015a). In *Hevea*, proposed model interaction of *ERF* genes function in the regulation between stresses responsive gene expression can be seen at Figure 22.

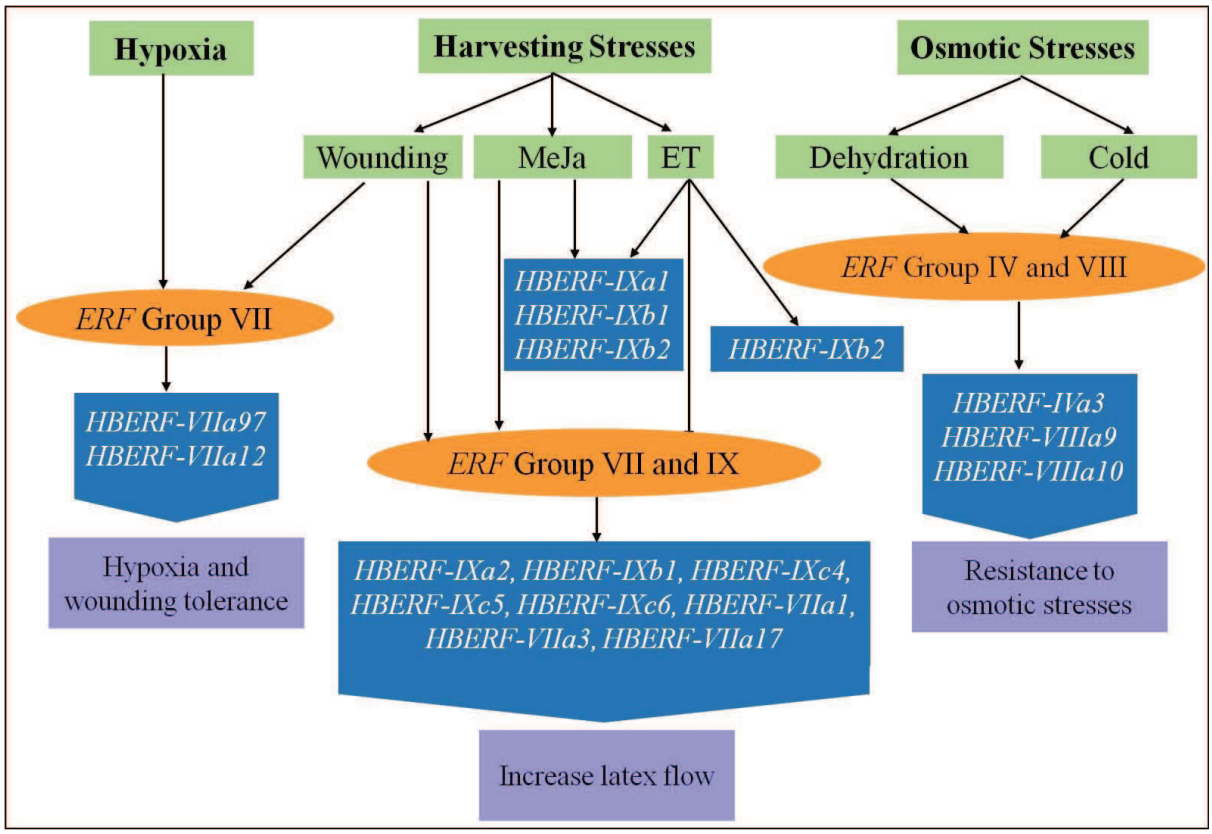


Figure 22. Proposed model interaction of *ERF* genes function in *Hevea* in the regulation between biotic and abiotic stresses responsive gene expression. This figure is a summary of conclusions from (Duan et al. 2013, Piyatrakul et al. 2014, Putranto et al. 2015a).

7.3. *Ethylene Response Factor1 (ERF1)* and its orthologs genes in Hevea

Ethylene Response Factor1 (ERF1) is one of ERFs, which constitute one of the largest transcription factor gene families in plants (Nakano et al. 2006). ERF transcription factors bind to GCC-box in the promoters of many ethylene-inducible and defence-related genes. ERF1 activates different set of stress genes in response to different stress. During adaptation salinity, ERF1 activates salt-tolerance genes by binding to the DRE-box in the promoter of these genes. By contrast, in pathogen-challenged plants, ERF1 activates defence genes by binding to the GCC-box promoter element (Achard et al. 2006).

ERF1 is an upstream key element in the integration of both signals and promotes stress tolerance by multiple mechanisms (Cheng et al. 2013, Lorenzo et al. 2003). ERF1 induced plant resistance responses to necrotrophic fungi such as *Botrytis cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo, Molina and Solano 2002). ERF1 activates the transcription of downstream effector genes such as *basic chitinase* (*b-CHI*) and *plant defensin1.2* (*PDF1.2*) (Solano et al. 1998). In *Capsicum annuum* L., the ethylene-responsive

factor like protein (CaERFLP1) can bind to GCC and DRE/CRT sequences and show resistance to pathogens and high salinity (Lee et al. 2004). In wheat, *TaERF1* increased multiple stress tolerance against cold, drought, salts, bacterial, and fungal pathogens (Xu et al. 2007). In *Arabidopsis*, *ERF1* is highly induced by high salinity and drought stress, hypersensitivity to JA and the different long of roots. The overexpressing lines with *35S*::*ERF1* are more tolerant to drought, salt stress, and increase heat stress tolerance than wild types (McGrath, Dombrecht and Manners 2005, Cheng et al. 2013). In sunflower, *ERF1* expression is fivefold higher in non-dormant embryo compare to the dormant one (Oracz et al. 2008).

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, Benavente and Alonso 2006). The crosstalk between ET and JA signalling pathways acts together synergistically during plant defence against herbivores, necrotrophic fungi infections, plant pathogens, and in responses of wounding (Lorenzo et al. 2003, Zhu and Lee 2015) (Figure 23). ET and JA signalling pathways converge in the transcriptional activation of *ERF1*. The expression of *ERF1* can be activated rapidly by ET or JA and can be activated synergistically by both hormones (Lorenzo et al. 2003).

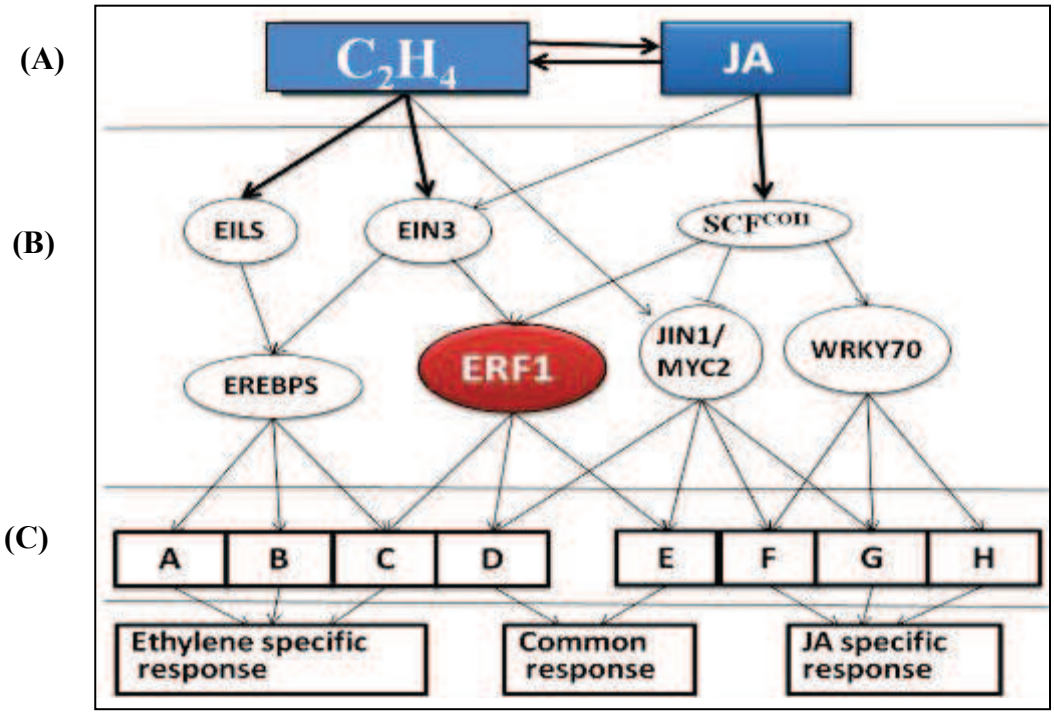


Figure 23. *ERF1* is at the crosstalk of ethylene and jasmonate signalling pathways. The three basic levels of interactions are: (A) JA influence the synthesis of each other through the mutual regulation of expression or activity of key biosynthesis genes, (B) Crosstalk may be achieved through sharing of common components of signal transduction machineries by ERF1 in the ethylene-jasmonate crosstalk, (C) Signals may converge on the regulation of expression of common target genes. This figure is extracted from (Lorenzo et al. 2003, Benavente and Alonso 2006).

Beside interaction between JA and ET, expression of *ERF1* is also controlled by interaction with ABA (Figure 24). In addition to the well-known ABA accumulation induced by abiotic stress, including drought and high salinity in plants, and JA accumulation can also be induced by drought stress in soybean leaf (Morgan and Drew 1997, Yoo, Cho and Sheen 2009). *ERF1*-overexpressing lines in *Arabidopsis* were more tolerant to drought and salt stress. It has specific characteristic such as smaller stomatal aperture and less transpirational

water loss. *ERF1*-overexpressing lines also enhanced heat tolerance and up regulation of heat tolerance genes compared with the wild type. Under salt or dehydration stress, *ERF1* expression was induced during stress JA/ET signalling can override the negative effect of ABA on *ERF1* expression. ABA production was highly induced in response to drought and salt stress. The expression of *ERF1* probably resulted from the different tissue-specific location of ABA and ET biosynthesis (Cheng et al. 2013).

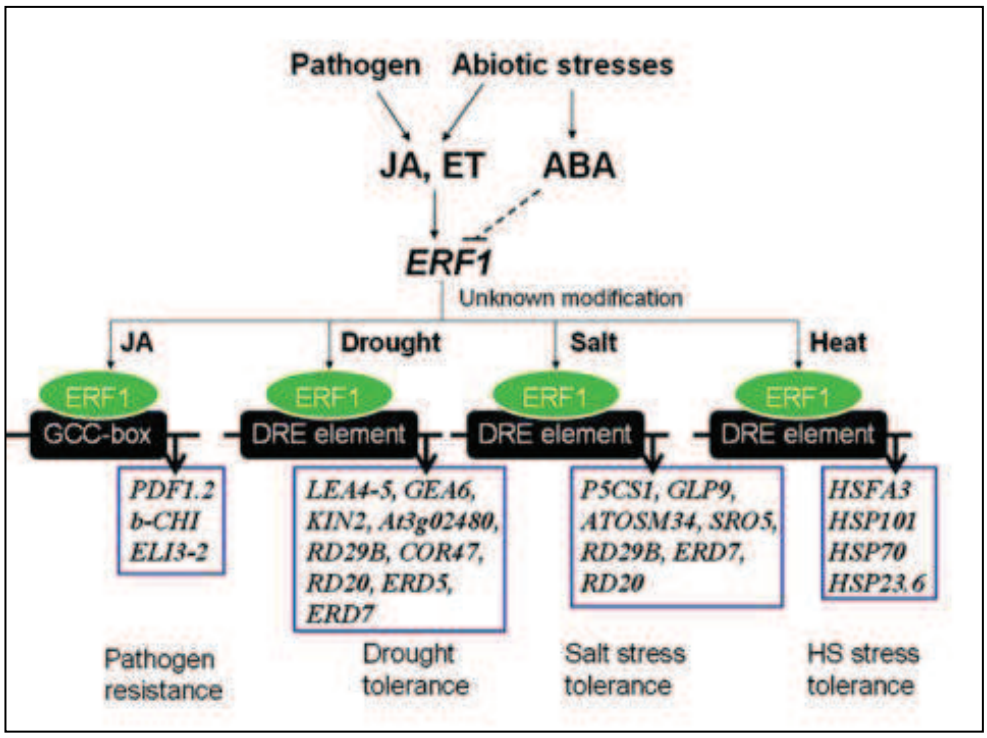


Figure 24. Proposed model of ERF1 function in the regulation of biotic stress and abiotic stress responsive gene expression in *Arabidopsis*. ERF1 positively regulates both biotic and abiotic stress responses. *ERF1* induction required both ET and JA signalling under abiotic stress and was negatively regulated by ABA. Under different stress conditions, such as pathogen infection, dehydration, high salinity, and heat shock, ERF1 activates specific sets of stress response genes by targeting to specific *cis*-elements (GCC boxes during biotic stress and DRE elements during abiotic stress). This figure is extracted from (Cheng et al. 2013).

In family-wide screening, (Atallah 2005) previously characterized 14 genes encoding AP2/ERF domain proteins that were rapidly induced by JA treatment in young *Arabidopsis* seedlings. JA induced *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes. The protein encoded by the *ORA59* gene showed high sequence similarity to *ERF1*, 40% amino acid identify over their entire length (Pré et al. 2008). *ORA59* and *ERF1* were shown to activate the *PDF1.2* promoters in transient assays in protoplast, suggesting that they bind directly to the promoter (Pré et al. 2008, Zarei et al. 2011).

In *Hevea*, *HbERF-IXc4* and *HbERF-IXc5* are two orthologs to ERF1 according to phylogenetic analyses (Figure 25) (Duan et al. 2010, Putranto et al. 2015a). These genes belong to the HbERF group IX suggesting a potential primary response in the ethylene transduction pathway. The *cis*-acting regulatory elements in HbERF-IX promoters suggested an activation of these genes by ethylene, jasmonate, auxin, cytokinin, gibberellin, abscisic acid and oxidative stress.

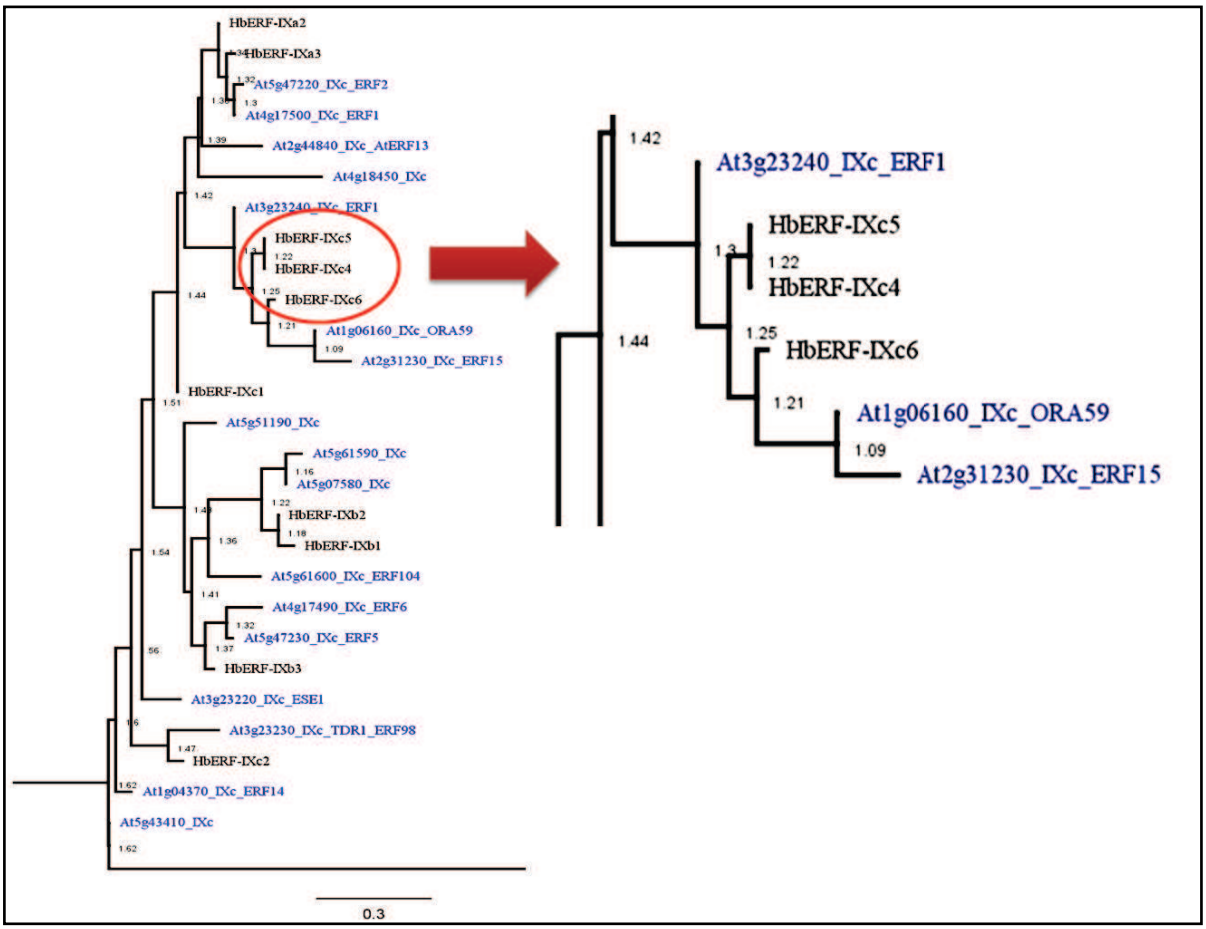


Figure 25. Phylogenetic analysis of *HbERF-IXc4* and *HbERF-IXc5*. *HbERF-IXc4* and *HbERF-IXc5* are orthologs to *ERF1* from *Arabidopsis*. This figure is extracted from (Putranto et al. 2015a).

Wounding, MeJA, and ET treatments were shown to trigger the regulation of a large number of genes in *Hevea* (Duan et al. 2010, Putranto et al. 2015a). The ERF family is divided into ten groups based on comparison with genomic scaffolds led to an estimation of 87 *ERF* genes in *H. brasiliensis* (Piyatrakul et al. 2014). *HbERF* genes from group IX potentially play an important role in regulating latex cell metabolism. Transcripts of *HbERF-IXc4* and *HbERF-IXc5* were significantly accumulated in response to different treatments: wounding, methyl jasmonate, and ethylene which show their involvement in the crosstalk between ethylene and jasmonate signalling (Putranto et al. 2015a) (Figure 26).

The experiments of transactivation and subcellular localization confirmed that HbERF-IX is activator-type transcription factor. These results suggest that several members of HbERF-IX could be regulators of complex hormonal signalling pathways during latex production in rubber (Putranto et al. 2015a). Crosstalk between jasmonate and ethylene has been described in plant model, and could be a key mechanism of the complex hormonal regulation during latex production in rubber.

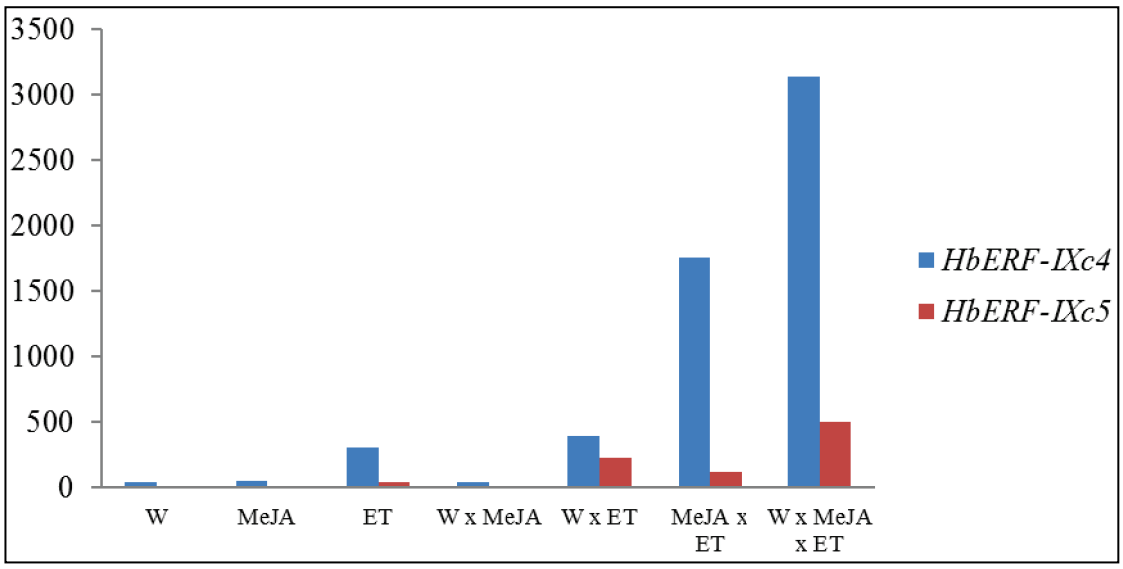


Figure 26. Analysis of the relative transcript accumulation of *HbERF-IXc4* and *HbERF-IXc5* genes by real-time RT-PCR in the bark with different treatment: (W) wounding; (MeJA) methyl jasmonate; (ET) ethylene; either individually or in a combination of treatments. This figure is extracted (Putranto et al. 2015a).

8. Towards the functional analysis of *Hevea* orthologs to *ERF1*

These genes *HbERF-IXc4* and *HbERF-IXc5* could be a regulator at the crosstalk of ethylene and jasmonate signalling pathways (Duan et al. 2010, Putranto et al. 2015a). The functional analysis of two *Hevea* orthologs to *ERF1* is possible because a robust procedure of *Agrobacterium tumefaciens*-mediated genetic transformation is available in *Hevea.* Overexpression of the candidate genes *HbERF-IXc4* and *HbERF-IXc5* under the control of *35S CaMV* and *HEV2.1* promoter could be considered.

8.1. Efficient *Agrobacterium tumefaciens*-mediated genetic transformation in *Hevea*

The current genetic transformation systems in *H. brasiliensis* are based on *A. tumefaciens* inoculation of embryogenic callus. The efficient somatic embryogenesis and *A. tumefaciens* mediated genetic transformation procedures were used for functional analysis a candidate gene *HbCuZnSOD* in *Hevea* (Blanc et al. 2006, Leclercq et al. 2010, Leclercq et al. 2012). Promoter of the *HEV2.1* gene was reported to drive expression in latex and leaves (Montoro et al. 2008).

The new era of biotechnology offers techniques that overcome the biological barriers that are common for rubber tree species such as *H. brasiliensis.* These techniques include *in vitro* cultivation of cells and tissues, genotyping selection, genetic engineering, and molecular markers. Plant regeneration from *in vitro* cultured cells can be accomplished through somatic embryogenesis (SE). Somatic embryogenesis for *H. brasiliensis* was an *in vitro* technique used for mass clonal propagation of *H. brasiliensis* (Montoro et al. 2000a, Lardet et al. 2009). Somatic embryogenesis is a process by which embryo forms and develops from bipolar structures from somatic cells that parallel the development path of zygotic embryos. Long-term somatic embryogenesis was developed in *H. brasiliensis*. Embryogenic lines were established as friable callus, which can be cryopreserved for long-term conservation (Lardet et al. 2009).

Agrobacterium tumefaciens-mediated genetic transformation was first attempt in *H. brasiliensis* in 1991 (Arokiaraj and Wan 1991). *Agrobacterium tumefaciens* has a unique mechanism to transfer genes into plant genomes. Some studies show that *A. tumefaciens* is an

effective system for mediating stable transformation of rubber tree calli with a low copy number of transgenes. Various *Agrobacterium* strains were screened for gene transfer efficiency on tissue precultured on $CaCl_2$ free medium or on calcium containing medium. The result showed that inoculation with EHA105pC2301 led to efficient transformation. These results confirmed that transgenic callus lines are useful tool for studying genes of interest on a cellular level and for regenerating transgenic rubber tree (Montoro et al. 2003).

H. brasiliensis transgenic plants have been produced by the incorporation of β-glucuronidase (GUS) and neomycin phosphotransferase gene (*NPTII)* by particle gun method (Arokiaraj et al. 1994). The neomycin phosphotransferase II (*NPTII*) gene is one of the selective marker genes in plant transformation for regenerating transgenic plants (Zhang and Blumwald 2001). The *NPTII* gene is derived from *E. coli* strain K12 (Beck et al. 1982), encodes for an aminoglycoside 3'- phosphotransferase enzyme (APH(3')II or *NPTII*) (Zhang and Blumwald 2001). *NPTII* gene catalyses the ATP-dependent phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of certain aminoglycosides including neomycin, kanamycin, geneticin, and paromomycin (Miki and McHugh 2004).

Plant cells transformed with *NPTII* gene can detoxify the antibiotic in the selection medium and remain alive, but non-transformed cells will die because they are highly sensitive to antibiotics and lack the phosphotransferase to detoxify these antibiotics (Zhang and Blumwald 2001). The overexpression gene with strong promoter like the cauliflower mosaic virus *35S* promoter can increase the level of *NPTII* enzyme activity and tolerance to antibiotic without creating instability in the expression of the *NPTII* gene (Kay et al. 1987, Lardet et al. 2009).

Selection of transgenic calli *H. brasiliensis* was based on GUS activity. Fluorimetric GUS activity is used to assess variation of transgene expression in *Hevea* transgenic *in vitro* plants and also budded sub lines (Lardet et al. 2011). As the GUS activity is destructive, selection was performed using antibiotic such as paromomycin. Paromomycin is aminoglycoside antibiotic that inhibits the growth of plant cells by binding to the 30S ribosomal subunit and inhibiting initiation of plastid translation (Moazed and Noller 1987, Wilmink and Dons 1993). Paromomycin can select transgenic plants. This antibiotic has been successfully used as a selective agent in transformation of *H. brasiliensis*. Paromomycin is more efficient than kanamycin for the selection of transformed cells and can inhibit the growth of non-transformed cells more quickly (Montoro et al. 2003, Pérez-Barranco et al. 2009).

Agrobacterium tumefaciens genetic transformation procedure has been developed from friable callus line for clone PB260 and effective system for stable transformation of rubber tree calli with a low copy number of transgenes (Montoro et al. 2003). (Blanc et al. 2006) showed that an efficient procedure to generate transgenic calli from *H. brasiliensis* clone PB260 requires a highly embryogenic callus line. Genetic transformation procedure using gene fluorescent protein (GFP) in *H. brasiliensis* clone PB260 was conducted. GFP visual selection is the development of stable or transient genetic transformation in *Hevea.* Transgenic calli lines exhibit better growth if used GFP selection rather than GUS selection. Furthermore, GFP selection marker has important rules to avoid antibiotic selection. Sometimes, antibiotic selection could damage tissue cells (Leclercq et al. 2010) (Figure 27).

Ticarcillin is one of antibiotic which used in genetic transformation to select transgenic tissues, to inhibit systemic bacteria in tissue culture (Buckley and Reed 1994) and or to suppress *A. tumefaciens* from the cultures (Zimmerman 1995, Cheng, Schnurr and Kapaun 1998). This antibiotic is belonging to the β-lactam group of penicillin group G. The suppression of *A. tumefaciens* from cultures is important, because microbial contaminants in cultured plants can reduce multiplication, induce plant death (Cassells 1991) and for

preventing the possibility of gene release when transgenic plants are transferred to the soil (Barrell et al. 2002).

Embryogenic callus lines of *H. brasiliensis* can be stored or frozen in liquid nitrogen that is called cryopreservation. Cryopreservation involves the storage of plant tissues (usually seed or shoot tips) in liquid nitrogen at -196 ^{o}C or in the vapour phase of liquid nitrogen at -135 ^{o}C. Cryopreservation is usually applied to recalcitrant species at ultra-low temperatures. The main advantage of cryopreservation is that once material has been successfully cooled to liquid nitrogen temperature, it can be conserved in long term, because at these ultra-low temperatures no metabolic processes occur. There are further advantages of cryopreservation such as low cost of storage, minimal space requirements, and reduced labour maintenance compares to living collections (Harding 2004, Kaczmarczyk et al. 2012).

Transgene copy number can greatly influence the expression level and genetic stability of the target gene in transgenic plants. Estimation of transgene copy number and determination of their expression levels are important to choose the best lines. Multiple copies are useful for overexpression experiment, and single or low copy transformation events are preferred for most applications because they are stable over several generations of subsequent breeding. Southern blot analysis is usually used to determine transgene copy number (Honda et al. 2002, Watson et al. 2004). The presence of *gusA* and neomycin phosphotransferase (*NPTII*) genes in rubber tree calli with a low copy number can be identified by Southern hybridization (Montoro et al. 2003).

The technique of Southern blot hybridization can identify the size of the particular fragments containing the gene of interest. Restriction DNA fragments that have been separated by gel electrophoresis were soaked in alkali to denature the double-stranded DNA fragments. Restriction fragments were transferred from an electrophoresis gel to a nitrocellulose membrane. The DNA bound to the membrane was then incubated with radio labelled DNA probe containing a sequence complementary of interest gene (Brown 2001, Watson et al. 2004).

Gene expression analysis was performed in various tissues of *H. brasiliensis* using real-time RT PCR and sequencing in order to identify *ERF* genes function (Piyatrakul et al. 2014). Analysis of the relative transcript abundance of *ERF* genes in *Hevea* in response to harvesting stress in bark and latex were carried out by real-time RT PCR. Result showed *ERF* genes tested in response to harvesting stress in bark and latex (Putranto et al. 2015a).

Promoter is the main determinant for the initiation of transcription and modulation of levels and timing of gene expression. A regulatory region of DNA located upstream (toward the 5' region) of a gene, providing a control point for regulated gene transcription (Roa-Rodriguez 2003). Strong promoters are frequently used to ensure abundant transcription of the selectable marker genes (Peremarti et al. 2010). One of commonly used as strong promoters in selectable marker genes is the *35S* RNA promoter sequence from the Cauliflower Mosaic Virus (*CaMV*) (Franck et al. 1980). The *35S* promoter effectively puts its downstream gene outside virtually any regulatory control by the host genome and expresses the gene at approximately two to three orders of magnitude higher, thus allowing a strong positive selection. A number of plant transformation vectors, including pPZP family vectors, the pCAMBIA series and pINDEX1, use the *35S* promoter in their selectable marker genes (Hajdukiewicz, Svab and Maliga 1994).

In *Hevea*, the hevein promoter, which is a strong latex specific promoter, was sought with the aim to improve the production of recombinant proteins in the latex cytosol. Sunderasan and Pujade-Renaud's groups cloned several *Hevein* genes from *H. brasiliensis* with the objective to isolate useful promoter to drive transgene expression in genetically engineered rubber tree (Sunderasan et al. 2012, Pujade Renaud et al. 2005). The longest promoter sequence (*PHEV2.1*) conferred a high level of expression to transgene in various

tissues of this heterologous host. Analysis by *in situ* hybridization of mRNAs showed that the expression of *HEV* genes and of the *HEV2.1* gene were in the latex cells, in roots, and stems of PB 260 plantlets (Montoro et al. 2008).

8.2. PhD research ins and outs

8.2.1. Specific background

Ethylene is a major hormone to stimulate natural rubber production. The ethylene signalling pathway through Ethylene Response Factor (ERF) transcription factors activates a large number of ethylene-responsive genes, which are involved in the response to abiotic and biotic stresses.

Two *Hevea ERF*, *HbERF-IXc4* and *HbERF-IXc5*, were predicted to be orthologs to the *ERF1* gene from *Arabidopsis* (Duan et al. 2010, Putranto et al. 2015a). Transcripts of *HbERF-IXc4* and *HbERF-IXc5* were significantly accumulated by combining wounding, methyl jasmonate, and ethylene treatment (Duan et al. 2010, Piyatrakul et al. 2014, Putranto et al. 2015a). These genes could be a regulator at the crosstalk of ethylene and jasmonate signalling pathway in latex cells. These genes were suggested to play an important role in regulating latex cell metabolism in response to tapping and ethephon stimulation. Response to tapping and ethephon stimulation in latex harvesting involved a complex hormonal regulation.

8.2.2. Problematic

Previous studies revealed the high expression of two putative genes (*HbERF-IXc4* and *HbERF-IXc5*) in latex. These two genes could be involved in the regulation of latex production but functional analysis in mature trees is restricted. To date, only a functional analysis using genetic modification can be carried out in confined conditions on juvenile material.

8.2.3. Objectives

This study aimed for analysing the specific function of *HbERF-IXc4* and *HbERF-IXc5* genes by overexpression under control of *35S CaMV* and *HEV2.1* promoter in transgenic *Hevea* lines obtained by *Agrobacterium tumefaciens*-mediated genetic transformation. The study strategy was as follows:

First step is establishment of transgenic callus lines generated by overexpressing *HbERF-IXc4* and *HbERF-IXc5* under the control of *35S CaMV* and *HEV2.1* promoter (Figure 27). Analysis of effect of two promoters was conducted as well due to the difference strength of two promoters. Transgenic plants have been regenerated by efficient somatic embryogenesis of transgenic lines obtained from *Agrobacterium tumefaciens*-mediated genetic transformation. The previous research on functional analysis of a candidate gene, *HbCuZnSOD*, was carried out in *Hevea* (Leclercq et al. 2012), Transgenic callus lines were cryopreserved in cryovials in liquid nitrogen for storage in a cryo-biological storage system.

Second step, plant regeneration and acclimatization in the greenhouse, morphological analysis of plants for one year, and then application of environmental stresses (drought, salinity, ethephon, cold, biotic) for monitoring ecophysiological parameters. Morphological analysis of effect of genes was necessary to understand the effect in plants growth and development.

Third step, the last research activities have dealt with genomic DNA extraction from leaves for determination of T-DNA copy number through Southern-blot hybridization, histo-cytological analysis, and RNA extraction from leaves and bark tissues for gene expression analysis by real-time RT-PCR. Southern-blot hybridization and real-time RT-PCR analysis were conducted to verify the insertion and overexpression of two candidate genes into *Hevea* transgenic plants genome. Histo-cytological analysis was observed in leaves, green stem, lignified stem, and taproot (R1) of *HbERF-IXc5* overexpressing transgenic plants. Histo-cytological analysis was conducted to see if there were differences tissue between wild-type and transgenic plants. This analysis could elucidate the role of putative gene in molecular metabolism of latex cell development and laticifer differentiation. The diagram and steps of general strategy of research can be seen at Figure 27 and Figure 28.

8.2.4. Novelty

Novelties of this research were:

1. The first successful establishment of somatic embryogenic callus lines and transgenic plants of *Hevea brasiliensis* overexpressing transcription factors (*HbERF-IXc4* and *HbERF-IXc5* genes).
2. The involvement of *HbERF-IXc5* in the accumulation of starch reserve, which are important for latex production.
3. The involvement of *HbERF-IXc5* gene in the regulation of laticifer differentiation.

8.2.5. Research Benefits

These results led to a better understanding of ethylene signalling pathway in *Hevea brasiliensis*. The important role of *HbERF-IXc4* and *HbERF-IXc5* genes encourages studying their sequence polymorphism in relation to genetic analysis in order to assess their involvement in agronomical traits that could finally lead to development of molecular genetic markers for tolerance to harvesting stress. Besides, this comprehensive analysis of roles of hormones in latex might highlight some factors to be used as new stimulant for agronomical application.

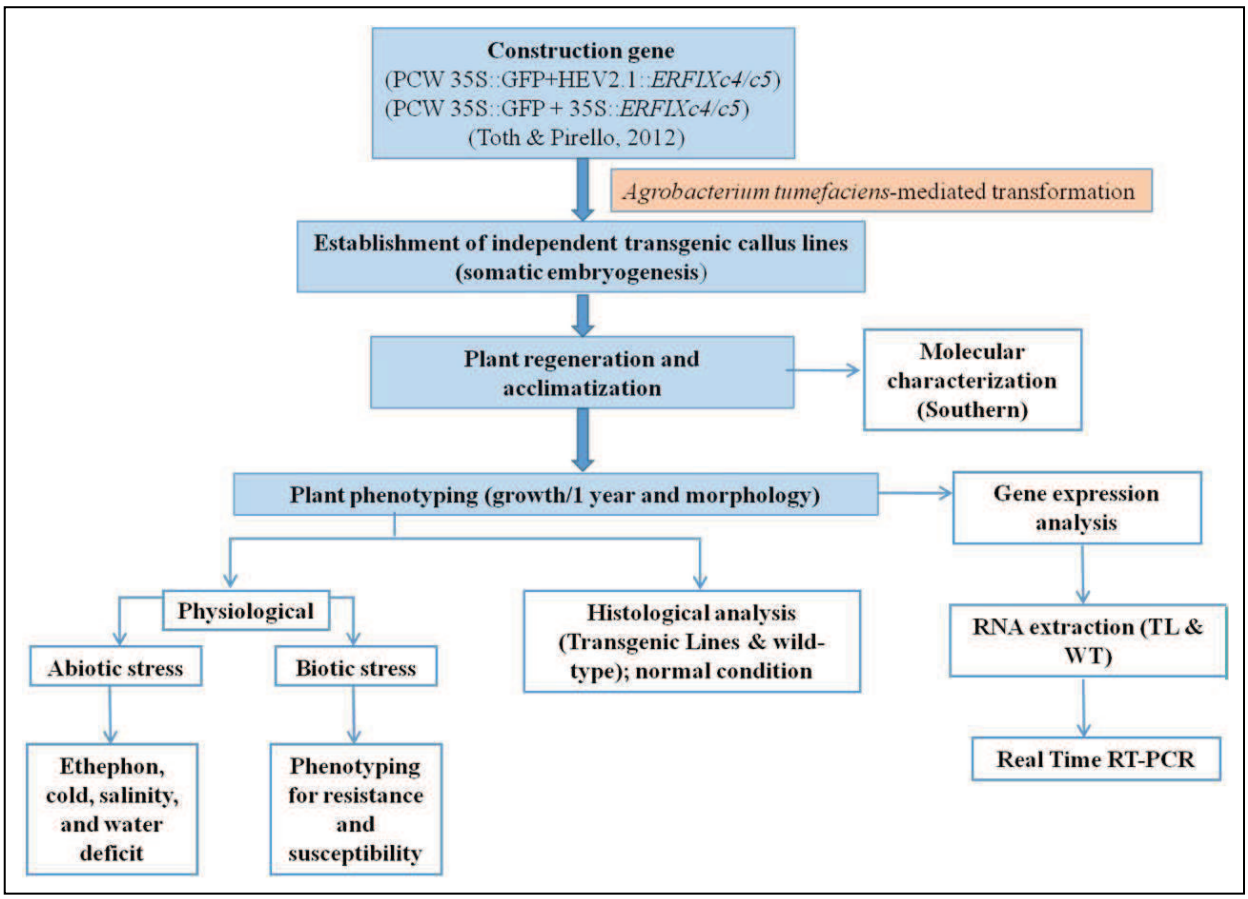


Figure 27. Diagram of general strategy for functional analysis *HbERF-IXc4* and *HbERF-IXc5* genes.

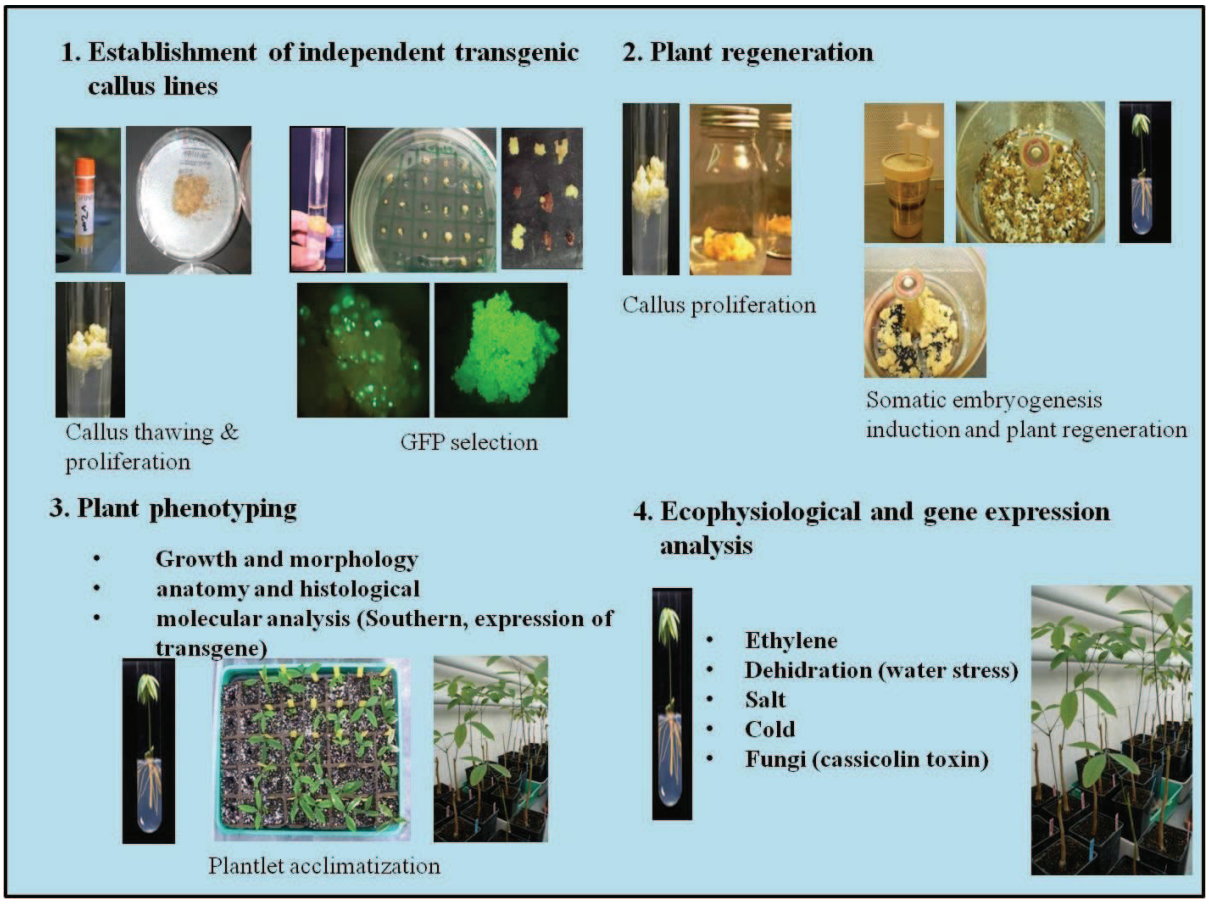


Figure 28. Steps for research from first year until third year.

SECTION II

MATERIALS AND METHODS

1. Establishment of transgenic callus lines

1.1. Plant material

Embryogenic friable callus line CI07060 was established from integument-calli of clone PB260 (Lardet et al. 2009). This callus line was sub-cultured every two weeks on a maintenance culture medium (MM) containing macro-elements (20 mM NH_4NO_3, 20 mM KNO_3, 3 mM $MgSO_4.7H_2O$, 2 mM $NaH_2PO_4.H_2O$, 9 mM $CaCl_2$), micro-elements (150.08 µM H_3BO_3, 100µM $MoSO_4.H_20$, 5 µM KI, 1.01 µM $CoCl_2.6H_20$), vitamins (300 µM inositol, 20 µM nicotinic acid, 3 µM pyridoxine-HCl, 2 µM thiamine-HCl, 0.2 µM biotine, 1 µM D-calcium pantothenate, 1 µM ascorbic acid, 0.1 µM choline chloride, 60 µM L-cysteine-HCl, 5 µM glycine, 1 µM riboflavin), 1.35 µM benzylaminopurine (BAP), 1.35 µM 3,4-dichlorophenoxy-acetic acid (3,4-D), 100 µM $FeSO_4$, 100 µM Na_2EDTA, 30 µM $AgNO_3$, 234 mM sucrose, 0.5 µM abscisic acid (ABA) and 2.3 g L^{-1} Phytagel (Lardet et al. 2007, Carron, Lardet and Montoro 2005). The pH of all media was adjusted to 5.8 prior to autoclaving. Callus cultures were grown in the dark condition at 27 ^{o}C. Before *Agrobacterium* inoculation, the callus line was pre-cultivated for 15 days in glass tubes on two different pre-culture media (PM), namely a $CaCl_2$-free MM medium supplemented either with 4.5 µM BAP and 3,4-D, or supplemented with 1,35 µM BAP and 3,4-D (Montoro et al. 2003).

1.2. Binary vectors and *Agrobacterium* strain

Two binary vectors called pCamway binary destination vectors allow cloning of a candidate gene under the control of the *35S CaMV* promoter or latex-specific *HEV2.1* promoter (Montoro et al. 2008, Leclercq et al. 2015). These vectors had a pCamway 2300 backbone with the *NPTII* gene conferring resistance to neomycin and a GFP reporter gene under the control of the *35S CaMV* promoter. Candidate genes *HbERF-IXc4* and *HbERF-IXc5* were cloned both under the control of *35S CaMV* promoter and *HEV2.1* promoter (Figure 29).

The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. For inoculation, bacteria were grown in liquid Lysogeny Broth medium (Duchefa, Haarlem, The Netherlands) supplemented with 50 mg L^{-1} kanamycin and 100 µM acetosyringone at 28 ^{o}C until OD_{600nm} = 0.6. After centrifugation at 1,000 *g* for 10 min, the pellet was dissolved to OD_{600nm} =0.06 in liquid MM from which Fe-EDTA, $CaCl_2$ and growth regulators were eliminated and 100 µM acetosyringone were added (Blanc et al. 2006).

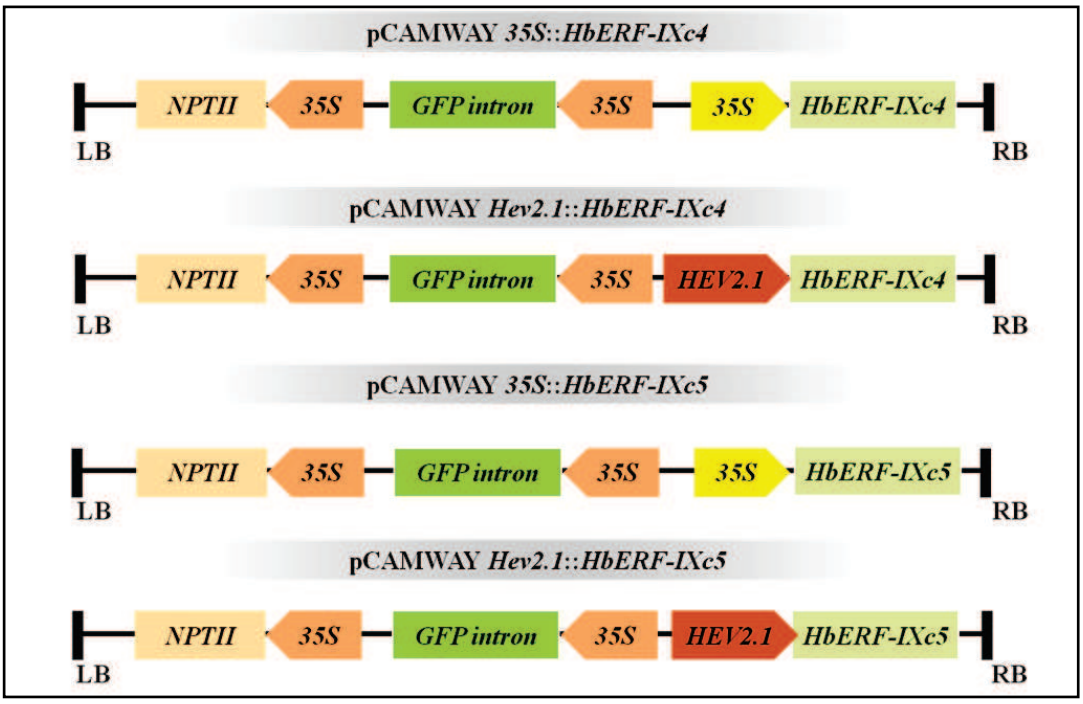


Figure 29. Structure of T-DNA constructs using pCamway 2300 *NPTII* and *GFP* genes are fused with the *35S CaMV* promoter. Candidate genes *HbERF-IXc4* and *HbERF-IXc5* are under the control of the *35S CaMV* promoter or the latex-specific *HEV2.1* promoter.

1.3. Inoculation, coculture, and selection of transgenic callus lines

Inoculation was performed as described by Blanc and collaborators (Blanc et al. 2006). Briefly, forty glass tubes containing precultured embryonic calli from clone PB260 were used (Montoro et al. 2000b). Calli were immersed directly in the tube for 1 s in the *Agrobacterium* suspension prepared as described above. Two coculture durations (4 and 5 days) were tested at 20 ^{o}C (Blanc et al. 2006). Six hundred small aggregates per treatment were then placed in 20 Petri dishes containing a decontamination medium (DM), a MM containing 500 mg L^{-1} ticarcillin (Sigma, Saint-Louis, USA), to prevent *Agrobacterium* growth.

GFP visualization was performed on callus at the end of each subculture under a fluorescence stereomicroscope and macroscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany) using the GFP2 filter (480 nm excitation filter/510 nm barrier filter). To isolate transgenic callus lines, GFP-positive aggregates were successively sub-cultured every 3 weeks on DM and then several times on DM with increasing concentrations of paromomycin from 50 to 150 mg L^{-1} (Rattana et al. 2001). Finally, transgenic callus lines were established from sub-aggregates showing full GFP activity (Leclercq et al. 2010). These calli were then subjected to molecular characterization, plant regeneration and/or cryopreservation according to the protocol described previously (Lardet et al. 2007).

2. Cryopreservation of transgenic callus lines

The cryopreservation procedure used was as described by (Engelmann et al. 1997) and (Lardet et al. 2007). It consists of two steps, first a cryoprotective treatment and then a freezing step.

2.1. Cryopreservation procedure

Embryogenic callus was sampled after 12 days of culture on MM. Liquid cryoprotective medium, which is an MM modified with 1 M sucrose, was added to the callus at a rate of 1 mL g^{-1} of callus. Dimethylsulphoxide (DMSO) was added gradually over the first 30 min period to reach a final concentration of 10%. The composition of DMSO solution was 70% DMSO + 30% H_20). The callus suspension was gently shaken for 1 min and then callus suspension was pipetted and dispensed into cryovials (1 mL per cryovial) (Lardet et al. 2007).

2.2. Freezing

Each cryovial containing 120-160 mg callus (fresh weight) was placed in Nalgene Cryo 1C in the polystyrene box was placed in a -80 °C deep freezer and the temperature was monitored by a thermocouple, which was placed in one of the cryovials. At -40 °C, the cryovials were rapidly immersed in liquid nitrogen for storage in a cryobiological storage system LocatorJR Plus (Thermolyne, Ohio, USA). The polystyrene box allowed a significant decrease in the "Cryo 1C" cooling rate, with average cooling rates of 0.20 °C ± 0.06 min^{-1} (Lardet et al. 2007).

3. Plant regeneration

Production of somatic embryos and their conversion into plantlets were carried out as described in (Lardet et al. 2007). Somatic embryogenesis was initiated for 4 weeks by sub-culturing 1 g of callus showing full GFP activity in 250 mL flasks containing 50 mL of a semi-solid embryogenesis expression medium (EXP), which was a modified MM medium supplemented with 58.5 mM sucrose, 175.5 mM maltose, 0.44 µM BAP and 0.44 µM 3,4-D. Pro-embryo development was then carried out in a temporary immersion system (RITA®, CIRAD, Montpellier, France) for two subcultures of 4 weeks each with 1 min of immersion per day in the liquid development medium (DEV), which was a MM containing 234 mM sucrose and 3 mM $CaCl_2$, without any growth regulator. Each RITA was considered as an experimental replication. Conversion of mature embryos was carried out according to (Lardet et al. 1999). Well-shaped mature embryos were collected and transferred to glass tubes on a semi-solid germination medium (DEV3), which consisted of the MM medium supplemented with 1.5 mM $CaCl_2$ solidified with 7 g L^{-1} Agar (Sigma, St. Louis, USA). Embryos were incubated under a light intensity of 60 µmol $m^{-2}s^{-1}$ and a 12 h day/dark photoperiod up to the full conversion of embryos into plants. Plantlets were then acclimatized in the greenhouse at 28 ^{o}C with 60% relative humidity.

To compare the regeneration ability of wild-type and transgenic callus lines, wild-type callus line CI07060 was cultured over the duration of the transformation experiment and regenerated. Once enough calluses were produced, plant regeneration was initiated. For both non-transformed and transgenic callus lines, the regeneration replication number, the number of total embryos g^{-1} of callus (T), the number of well-shaped embryos g^{-1} of callus (WS), the number of plantlets g^{-1} of callus (P) and the conversion percentage (P/WS) were recorded.

4. Genomic DNA extraction from leaves and Southern-blot hybridization

DNA from leaves from wild-type and transgenic lines were isolated as described in (Leclercq et al. 2010). One gram of tissue was ground in liquid nitrogen and then mixed with 6 mL of MATAB buffer (100 mM Tris-HCl pH 7.5, 2% MTAB, 0.4% w/v sodium sulphite,

1% PEG 6000, 1.4 M NaCl, 20 mM EDTA). Extracts were maintained at 74 ^{o}C for 20 min, and proteins removed using an equal volume of 24:1 chloroform-isoamyl alcohol (CIAA) followed by centrifugation at 6,220 *g* for 10 min. Supernatants were transferred to clean tubes and DNA precipated with 5 mL of isopropanol followed by centrifugation at 13,000 *g* for 15 min. DNA pellets were re-suspended in 300 L µM of TE buffer.

Ten micrograms of genomic DNA were fragmented with *Eco*RI restriction enzyme and fractionated by electrophoresis in a 0.8% agarose gel in TAE 1x buffer. After transfer onto a Hybond N^{+} nylon membrane (Amersham^{TM} Megaprime DNA Labelling System, Buckinghamshire, UK), hybridization was performed as described in (Sambrook, Fritsch and Maniatis 1989), using random primed ^{32}P radio-labelled probes corresponding *NPTII* genes amplified with the following primers:

NPTII-F: 5'-CCGGCTACCTGCCCATTCGA-3'

NPTII-R: 5'-GCGATAGAAGGCGATGCG-3'

The numbers of bands reflected the number of T-DNA insertions.

5. RNA extraction from leaf and bark

Twelve-month-old plants per line were used for gene expression analysis. Leaf and bark samples were collected from wild-type (CI07060) and transgenic lines (TS18A09, TS18A13, TS18A37, TS 19A46, TS19A59, TS19A90, TS20A69, TS20A75, and TS20A82). The RNA extraction procedure used has been describing in (Duan et al. 2010). Briefly, 1 g of leaves from fresh matter was ground in liquid nitrogen and 30 mL of extraction buffer (4 M guanidium isothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone (PVP), and 1% β-mercapto-ethanol) was added to the powder. After homogenization, tube was kept on ice and then centrifugated at 13,000 g at 4 ^{o}C for 30 minutes, the supernatant was loaded on 8 mL of 5.7 M CsCl. Ultracentrifugation was carried out at 89,705 *g*, at 20 ^{o}C for 20 hours in a swinging bucket. After discarding the supernatant and the cesium cushion, the RNA pellet was washed with 70% ethanol, air dried and dissolved in 200 µM of sterile water. Total RNAs were quantified with Nanoquant (Tecan, Männedorf, Switzerland) and conserved at -80 ^{o}C.

6. Complementary DNA (cDNA) synthesis

Before cDNA synthesis, a DNAse treatment was performed using TurboDNAse (Ambion, Life Technologies, Texas, USA). The absence of contaminating genomic DNA was checked on all RNA samples by performing a PCR reaction with *HbActin* primers following the manufacturer's instructions. Four micrograms of DNA-free RNAs was used for cDNA in a 40 µL reaction mixture using a RevertAid™ M-MuLV reverse transcriptase following the manufacturer's instructions (MBI, Fermentas, Canada). Full length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA using primers at the cDNA ends.

7. Gene expression analysis by real-time RT-PCR analysis

Primers were designed for the *HbRhb2* reference gene (internal control): *HbRhb2*-F: 5'- GAGGTGGATTGGCTAACTGAGAA -3' and *HbRhb2*-R: 5'-GTTGAACATCAAGTCCCCGAGC -3' and target gene: *HbERF-IXc5*, *HbERF-IXc4*, *HbPDF1*, *HbPDF2, HbChit1*, *HbChit2*, *HbSUT3*, *Defensin*, *ETR2*, and *HbERF-Xb1* (Table 2).

Real-time RT-PCR analysis was carried out using a Light Cycler 480 (Roche, Basel, Switzerland) as described in (Duan et al. 2010). Real-time PCR reaction mixtures consisted of 2 µL RT product cDNA, 0.6 µL of 5 µM of each primer, and 3 µL 2xSYBR green PCR master mix (Light CyclerR 480 SYBR green 1 Master, Roche Applied Sciences) in a 6 µL volume. PCR cycling condition comprised one denaturation cycle at 95 ^{o}C for 5 minutes, followed by 45 amplification cycles (95 ^{o}C for 20 s, 60 ^{o}C for 15 s, and 72 ^{o}C for 20 s). Expression analysis was performed in a 384-well plate. Samples were loaded using an automation workstation (Biomek NX, Beckman Coulter). The *HbRhb2* gene and the target genes were amplified in parallel allowing calculation of the relative gene expression ratio taking into account primers efficiencies (*Hb* target gene and *HbRhb2* primers pair: E= 1.95 (Putranto et al. 2012). All the expression data were automatically calculated by Light Cycler Software version 1.5.0 provided by the manufacturer. For the expression study by real-time PCR, the expression ratio was normalized using the Log10(X) function. For each gene, an ANOVA analysis followed by Tukey test were used in the statistical analyses ($p<0.05$).

Table 2. List of primer sequences for *Hevea brasiliensis* genes involved in ethylene signalling, ethylene biosynthesis, defence, and sucrose transporter.

Name of primer	Sequence 5' > 3'
HbERF-IXc4-F(3)623	GAAGCAAGAGAGAAAGGGATG
HbERF-IXc4-R(3)802	TTCCTACCACTGAAAGGAGGAG
HbERF-IXc5-F381	CAGTTGAAAGAGTGAAGGAATC
HbERF-IXc5-R567	TCCAAGTAATCAGCACCCAAG
HbERF-Xb1 Xb1-F(3)623	CCTATGATAAGGCGGCGATA
HbERF-Xb1 F(3)623812	TCACTTTCCTTCCCCTTTCC
HbDefensinP-F5	CGTCTATTTTCAGCACTTTCCC
HbDefensinP-R216	TTCCTAGTGCAGAAGCAGCG
HbPDF-F1-2943	TGTCCCTGCACATCTTGAAC
HbPDF-R1-3133	GCAGCAAACATCCATTCTCTC
HbPDF-F2-2519	CAATCGTTCACCGGGATT
HbPDF-R2-2704	CACACTTCTCATTTGACGGTTC
HbChit-F1-743	GCCATCAAGCCACAATGTTA
HbChit-R1-935	GGTGACACCCAAAATGTCG
HbChit-F2-103	AAGCTGGGAATGCTCTGTGT
HbChit-R2-284	AAGAGCTGGGGTGATTATGC
HbCHIT- F862	AAGTACGGAGGTGTTATGC
HbCHIT-R1076	GTACTCCCTCTCTCCTTATT
HbSUT3-F	CACCACAACCACCATCAC
HbSUT3-R	GTGGAAGAGGTTCAGAAGAG
HbETR2-F201	TGCCCTGACATCAAGTGCT
HbETR2-R403	TCAGACCCCAAAACCGAAG
HbRH2b-F259	GAGGTGGATTGGCTAACTGAGAA
HbRH2b-R432	GTTGAACATCAAGTCCCCGAGC

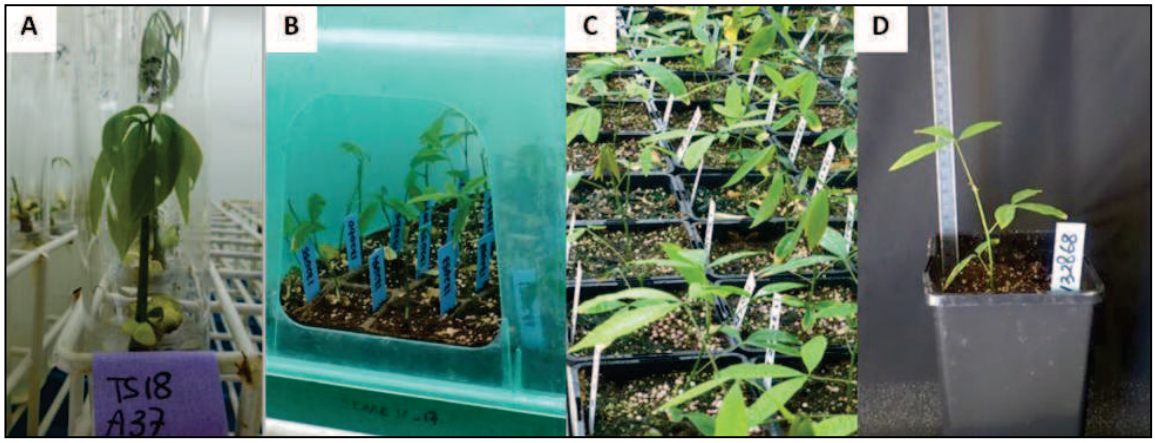


Figure 30. Acclimatization of *in vitro* plants in greenhouse. (A) Plantlet; (B) Transfer plantlet to substrate; (C) Transferred plants into pot; (D) Plants (2 months).

8. Acclimatization and plant morphology measurements

Plantlets were transferred into 120 mL paper pots (Jiffy pot, Ohio, USA) and were covered by special plastic box. After 2 months of first acclimatization, plants are transferred into 2-L plastic pots (Figure 30). Several parameters were measured on plants: before acclimatization (month 0): height of root, diameter of root, height of stem, diameter of stem, number of leaves, number of leaflets, and number of lateral roots from *in vitro* plantlets; at 2 and 6 months after acclimatization: height of plant, diameter of stem at the collar, number of leaves, and number of leaflets; and then for 12 months after acclimatization: diameter of stem, height of stem, number of leaves, number of leaflets, weight of leaves, weight of stem, weight of total root, and weight of the main root.

9. Histo-cytological analysis

9.1. Plant material

Leaf, green stem, lignified stem, and taproot (R1) were collected from one year plants of wild-type (WT) line CI07060 and transgenic lines TS19A46, TS19A90, TS20A69, and TS20A75 for *Hevea* clone PB260 (Figure 31). All parts of samples were cut in small parts for longitudinal and transversal sections. Leaves were cut in square (1 x 1) cm^2 including main nerve and lamina; green stem were cut in cylindrical slice (0.5 x 0.5 x 0.5) cm^3; lignified stem were cut in cylindrical slice (0.5 x 0.5 x 1) cm^3 and if possible cut in a half part; after washing with water root were cut in cylindrical slice (0.5 x 0.5 x 1) cm^3 and cut in a half part when it was possible like for lignified stem. After cutting, all samples were directly kept in the fixative solution in a small sample bottle (40 mL) one by one. Each bottle was filled up to the half part of the bottle (approx. volume) with fixative solution. The composition of fixative solution is detailed in Table 3. Samples in fixative solution were kept in vacuum minimum five hours and after they remained in cold room at 8-10 ^{o}C for three days. After this time, the fixative solution was changed gradually by ethanol 50% then ethanol 70%. During each bath the samples were kept under vacuum for two hours. For long storage, ethanol 70% must be changed by new ethanol 70% and samples must be kept in cold room at 8-10 °C. All methods adapted from PHIV platform (CIRAD, Montpellier, France). All the process for preparing histology samples can be seen in Figure 32.

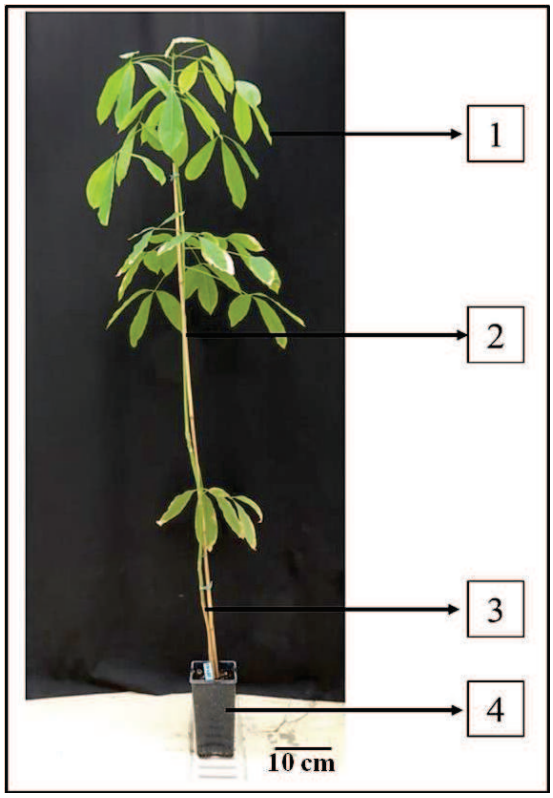


Figure 31. Part of collected samples from leaf (1), green stem (2), lignified stem (3), and taproot (4).

Table 3. Composition of stock solutions.

Final solution	Composition	Quantity of stock solution
Solution A	Anhydrous NaH_2PO_4 (MW 120) Distilled H_20	2.4 g Up to 100 mL
Solution B	Anhydrous NaH_2PO_4 (MW 142) Distilled H_20	2.84 g Up to 100 mL
Buffer phosphate pH 7.2; 0.2 M	Solution A Solution B	28 mL 72 mL
Fixative solution	Buffer phosphate pH 7.2; 0.2 M Paraformaldehyde 20% Glutaraldehyde 50% Caffein Distilled H_20	50 mL 10 mL 2 mL 1 g Up to 38 mL

9.2. Softening procedure

For big and hard samples, a pre-treatment was necessary. First, samples were put in histology cassettes. The name of samples was written on the cassette by pencil. Cassettes were rinsed by H_2O for a while and after were put in softener solution (DEAM solution, the composition can be seen on (Table 4) in specific Erlenmeyer with stirrer. After, the samples were put in HISTOS 5 instrument (Mileston Srl, Sorisole, Italy) with special program, softening, for one hour, at 80 ^{o}C (PHIV platform, CIRAD, Montpellier, France).

Table 4. Composition of DEAM solution.

Solution	Quantity
H_2O	89 mL
Glycerol	10%
Tween 20	1%

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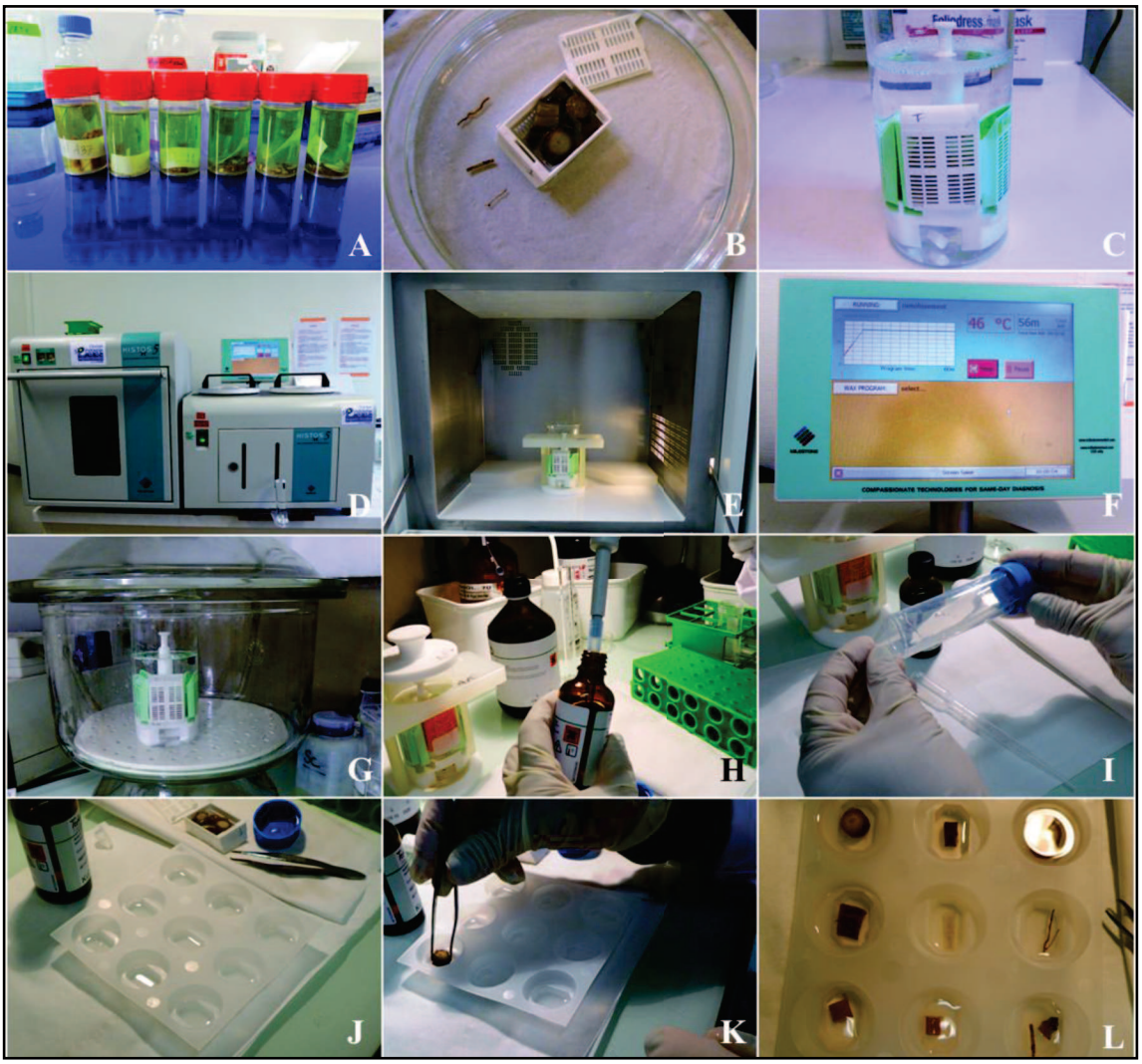

Figure 32. Preparation of samples before and after using HISTOS 5. (A) Samples kept in fixative solution, (B) samples in histology cassette, (C) samples in specific Erlenmeyer for HISTOS 5 with stirrer, (D) the rapid microwave histoprocessor HISTOS 5, (E) samples with Erlenmeyer put in HISTOS 5, (F) the monitor of HISTOS 5 for controlling the process, (G) samples in vacuum, (H, I) embedding process with resin solution, (J) preparation a plastic mould with resin solution, (K) the samples in the well, (K) the samples covered by resin solution with the desirable orientation.

9.3. Tissue processing (dehydration and impregnation)

All steps of dehydration, dehydration/impregnation, and impregnation were carried out in the rapid microwave histoprocessor HISTOS 5. Each step can be seen on the Table 5. This procedure was modified for rubber.

Table 5. Stages of dehydration, dehydration/impregnation, and impregnation of samples by HISTOS 5.

Step	Treatment	Duration of step (min)	Number of cycle	
			Standard for PHIV	Modified for rubber
1. Dehydration	Ethanol 70%	8	1	1
	Ethanol 95%	8	1	1
	Ethanol 100%	8	1	1
2. Dehydration/Impregnation	Ethanol 100%	8	1	1
	Ethanol/Butanol (1:1)	8	1	1
	Butanol 100%	13	1	1
3. Vacuum		30	0	1
4. Impregnation	Butanol/Resin	18	1	2
	Resin	90	1	2

Estimation of total times is around 5 hours.

9.4. Embedding process

After all samples were processed by HISTOS 5 the next step was embedding with resin solution (*Technovit*® 7100, Heraeus Kulzer GmbH, Wehrheim, Germany). Resin solution was made by adding 1 g hardener I (1 bag) to 100 ml Technovit 7100. For embedding, 1 mL Harderner II was added to 15 mL prepared resin in a Falcon tube and after the solution was gently mixed for homogenous. A plastic HistoMold (Leica, Nussloch, Germany) was prepared before. The sample name was noted on the plastic mould by pencil. First, a little volume of resin solution was poured in the wells, and then each sample was gently and slowly put in the well with the desirable orientation. Resin solution was added gradually until all samples covered by resin before hardening because of the polymerization process. Samples embedded in resin were put in the oven (37 ^{o}C) for polymerization achievement and long storage. All work was conducted under the fume hood (PHIV platform, CIRAD, Montpellier, France).

9.5. Sample specimen preparation

Resin-embedded specimen was ready to cut by automated rotary microtome (Leica RM2255 (Leica, Nussloch, Germany). Before resin-embedded specimen was excluded from the mould, the surface of preparation was scratched by pinsetter or scalpel. The rough surface was covered by very strong glue, affixed on the support, and ready for cutting. The cross-sections obtained from microtome were thin slices (3.5 μm). Each thin slice was put on the surface of water in a big dish. Slices of specimen were put on glass slides immersed in water then set on a hot plate (40 ^{o}C) for 30 min. Slides were dried and mounting solution (glycerol: water = 50:50) was used to fix coverslips. The edge of coverslips was covered by polish solution or Pertex (xylene 40-60% and ethylbenzene 10-20%) (Gothenburg, Sweden) for automatic microscopic scanning. The glass slides must be soaked first in alcohol 100% before used them for removing fat traces.

9.6. Sample staining

There were two staining used, first was Oil Red O-Toluidine Blue staining method adapted from (Lillie and Ashburn 1943) by PHIV platform (CIRAD, Montpellier, France). The composition of staining was Oil Red O 0.5% in alcohol 70% and Toluidine-Blue 0.1% in Walpole buffer 0.1 M pH 4.2. The solution of Oil Red O was filtered before use by the

membrane filter. Slides of samples were stained by Oil Red O for 10 minutes and rinsed by H_2O. Furthermore, slides of samples were stained by Toluidine Blue for two minutes and then rinsed by H_2O.

The other staining was periodic acid-Schiff and Naphtol Blue Black (NBB, (Fisher 1968) adapted by PHIV platform (CIRAD, Montpellier, France). The different components of staining were periodic acid solution 1% in water distilled (prepared immediately upon before staining), Schiff solution, and NBB (NBB 1 g, acid acetic 7 mL and H_2O was added until 100 mL) (Sigma-Aldrich, St. Louis, USA). Slides with sections were hydrolysed freshly with periodic acid at room temperature for 5 minutes. Slides were washed by tap water and distilled water then dried on tissue paper. Slides were stained by Schiff's reagent in darkness in fume hood at room temperature for 10 minutes and washed by running water until the water was colourless and continued with distilled water quickly. Microscopic control was done after washing with water. Before used it, NBB solution was filtered. Slides were stained in NBB solution at 50 ^{o}C for 5 minutes. After few rinses coverslips were sticked to slides using Isomount 2000 (Labonord, Templemars, France) as mounting medium.

9.7. Slide observation, qualitative, and quantitative parameters

The slides were analysed by digital slide scanner NanoZoomer 2.0-HT (Hamamatsu, Japan) at The Institute for Neurosciences of Montpellier (INM). The image bar scale was defined using image analysis software (ImageJ, Bethesda, Maryland, USA). Parameters of qualitative and quantitative for leaf, green stem, lignified stem, and root can be seen in Table 6.

Table 6. Qualitative and quantitative parameters for slide observation.

Organ	Part	Parameter	
		Qualitative	Quantitative
Leaf	main nerve	starch (St), polyphenol (PO)	laticifer cell (LC)
	lamina	starch (St), polyphenol (PO)	laticifer cell (LC), palisade cell (PC), stomata (S), the width of cuticle (C), upper epidermis (UE), palisade parenchyma (PP), spongy parenchyma (SP), and lower epidermis (LE)
Green stem	bark, cambium, xylem, pith	starch (St), polyphenol (PO)	the width of bark (B), cambium (Ca), xylem (X), and pith (P); primary laticifer cells (PLC), and laticifer ring (LR)
Lignified stem	bark, cambium, xylem, pith	starch (St), polyphenol (PO)	the width of bark (B), cambium (Ca), xylem (X), and pith (P); primary laticifer cells (PLC), and laticifer ring (LR)
Taproot (R1)	bark, cambium, xylem, pith	starch (St), polyphenol (PO)	the width of bark (B), cambium (Ca), xylem (X), and pith (P); primary laticifer cells (PLC), and laticifer ring (LR)

9.8. Statistical analysis

Histological observations were done on plants from wild-type (CI07060), TS19A46, TS19A90, TS 20A69, and TS20A75 transgenic lines. The quantitative data was manually measured and calculated. Statistical analysis was performed using an ANOVA followed by a Tukey test ($p<0.05$).

10. Evaluation of the effect of environmental stresses

12-month-old wild-type (CI07060) and transgenic plants of lines TS18A09, TS18A13, TS18A37, TS19A90, TS20A69, and TS20A75 were used for testing effects of environmental stresses (Table 7).

Table 7. Number of wild type and transgenic plants for each environmental stress experiment.

Line	Number of plants used for each experiment				
	Drought	Ethephon	Cold	Salinity	Biotic
CI07060	10	18	21	64	2
TS18A09	10	18	6	7	0
TS18A13	7	12	6	7	4
TS18A37	0	12	6	7	2
TS19A46	0	0	0	0	2
TS19A90	10	12	6	7	2
TS20A47	0	0	0	0	2
TS20A69	10	12	6	7	4
TS20A75	10	12	6	7	2

10.1. Drought treatment

The experiment was performed in a greenhouse cell, under controlled conditions with a mean temperature of 28.4 ^{o}C and 43.6% relative humidity. The daylight period in the the cell was 12 h, and the photosynthetic active radiation flux was an average of 600 µmol m^{-2} s^{-1} above the canopy. 12-month-old plants were cut and placed in pots with the same weight of soil (EGO 140 substrate, Tref group, Netherlands). Plants from the transgenic control line (CI07060) and transgenic lines were subjected to a controlled water deficit.

Drought stress was imposed by holding water from the pots. At the onset of soil dry-down, the surfaces of the pots were sealed with cellophane to prevent soil evaporation. In this way, it was possible to calculate both the dynamics of soil water depletion and plant transpiration from gravimetric observations. Soil water status was monitored using the fraction of transpirable soil water (FTSW) (Luquet et al. 2008). In order to estimate the FTSW value of each pot, full watering of all the pots the day before the start of measurements was followed by one night of drainage. On the next morning, the initial pot water capacity was determined by weighing all the pots. FTSW was estimated as the ratio of actual transpirable soil water (ATSW) to total transpirable soil water (TTSW), ATSW being the mass difference between daily and final pot weight. TTSW was calculated as the difference between initial pot capacity and the final pot weight after soil desiccation. The experiment ended when the transpiration rate of each stress pot was less than 10% of that of the fully watered pots (Sinclair and Ludlow 1986). Its value matched 1 when the plants were well watered. Drought stress continued up to FTSW= 0.1.

10.2. Ethephon treatment

Ethephon (2-chloro-ethylphosphoric acid) solutions were prepared at various concentrations by adding gelatine in water, and then the solution was heated and left to cool. After, the ethephon was put in gelatine solution (Table 8).

Table 8. Ethephon solution with various concentrations.

Final concentration of ethephon (%)	Volume of ethephon from stock solution (mL)	Quantity for 0.1% gelatine (mg)	H_2O (mL)
0	0	50	50
1	1.04	50	50
2.5	2.6	50	50
5	5.2	50	50

Leaf and stem parts of plants were covered with various solutions of ethephon using a soft brush. Plants were kept for one week in greenhouse under standard growth conditions. Number of dropped leaves and leaflet senescence were observed until six days after treatment. The control plants received non-ethephon treatment.

10.3. Cold treatment

Twenty-one 8-month-old plants from wild type (CI07060) and six transgenic lines (TS18A09, TS18A13, TS18A37, TS19A90, TS20A69, and TS20A75) were used for cold treatment. All plants in 2 L pots were put inside a climatic room at 10 °C for 96 hours with a photoperiod of 8h/16h. On day 4th, the temperature was set up to 20 °C. One day after, all plants were transferred to greenhouse and temperature was set at 28 °C. Morphological and eco-physiological measurements were conducted every 2 days for 10 days after treatment. Morphological parameters consist of number of leaves, leaflets, senescent leaves, burned leaves, abscission leaves, wilting leaves, and wilting-burned leaves. Eco-physiological parameters were Fv/Fm, Performance Index (P. Index), and SPAD.

10.4. Salinity treatment

Sixty-four 10-month-old plants from wild type (CI07060) and seven transgenic lines (TS18A09, TS18A13, TS18A37, TS19A46, TS19A90, TS20A69, and TS20A75) were used for salinity treatment. Morphology parameters (number of leaves, leaflets, leaves senescence, burned leaves, abscission leaves, wilting leaves, wilting-burned leaves, and colour of leaves) and eco physiology parameters (Fv/Fm, P. Index, and SPAD) were measured until 28 days. Pots of plant were watered by 1 L of sodium chloride [500 mM] on the third day. Morphology and eco physiology were observed every two days until four weeks in greenhouse. Each pot was washed by water during a week to prevent the increase in osmotic potential from salt.

10.5. Biotic treatment

Hevea brasiliensis leaves from clone PB260, wild type (CI07060) and 7 transgenic lines (TS18A13, TSA18A37, TS19A46, TS19A90, TS20A47, TS20A69, TS20A75) were used on the biotic stress experiment. The morphogenetic leaves stage C were detached and placed on water-soaked filter paper in large Petri, plates, abaxial side up. The lower epidermis was gently scarified (over 1 mm2) using a scalpel blade and one drop of the toxin cassiicoline Cas1 and of the filtrate CCP (15 µl) was placed on the scarified spot. The plates were maintained for 1 day to 7 days at 25 °C (dark) until symptoms were clearly visible. The observation was conducted until 8 days.

10.6. Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were performed with a Handy-PEAR chlorophyll fluorometer (Handy-Plant Efficiency Analyser, Hansatech Instruments, King's Lynn, Norfolk, UK) (Figure 33) on stressed plants at the beginning of water stress and at the several FTSW values during the dehydration treatment, always in the morning, at the same time as stomata conductance measurements. The transients were induced by 1-s illumination with an array of six light-emitting diodes providing a maximum light intensity of 3,000 µmol (photons) m^{-2} s^{-1} and uniform irradiation over a 4-mm diameter leaf area. Fast fluorescence kinetics (F0 to FM) was recorded from 10 µs to 1 s. The fluorescence intensity at 50 µs was considered as F0 (Strasserf and Srivastava 1995). Reading data were taken on the abaxial side of mature leaves, dark adapted with a lightweight plastic leaf clip for 30 min before measurement. The performance index (PI_{abs}) plant vitality indicator (Strauss et al. 2006) which comprises light energy absorption, excitation energy into electron flow, was also measured to quantify photosystem II integrity (PSII) (Strasserf and Srivastava 1995). Each measurement was performed on apparently healthy leaves.

Stomata conductance was measured at the same time with an SC-1 Decagon Devices leaf porometer (Pullman, USA). All stomata conductance measurements were carried out and compared under the same environmental conditions.

Figure 33. Instruments of chlorophyll fluorescence measurement.

10.7. Database and statistical analysis

The Biotekva database (Microsoft Access) (Leclercq et al. 2010) was designed to gather, store, and manage data arising from all the experimental steps, from callus to plant production. The data were normalized prior to statistical analysis using XLSTAT (Addinsoft, Paris, France). The number of total embryos. g^{-1} callus, the conversion percentage and the number of plantlets. g^{-1} callus, the number of well-shaped embryos. g^{-1} callus, and parameters of morphology were analysed by ANOVA with a Tukey test in the statistical analyses ($p<0.05$).

SECTION III

RESULTS

1. Establishment and cryopreservation of transgenic callus lines and plant regeneration

The wild-type embryonic callus line CI07060 was transformed using two binary pCamway 2300 vector harbouring the *NPTII*, *GFP*, and *HbERF-IXc4* or *HbERF-IXc5* candidate genes. These latter were under the control of *35S CaMV* and *HEV2.1* promoter, respectively. Our experiments were then conducted with four constructs described in Figure 29 (Materials and Methods). Each construct was tested in experiment called TS17, TS18, TS19, and TS20. Two cocultures of 4 or 5 days were tested at 20 ^{o}C (Blanc et al. 2006).

1.1. Number of GFP-positive aggregates during the selection transgenic lines

Hevea callus was sub-cultured as small aggregates on paromomycin selection medium. Six hundred small aggregates per treatment were then placed in Petri dishes containing 500 mg L^{-1} ticarcillin to prevent *Agrobacterium* growth. To isolate transgenic callus lines, GFP-positive aggregates were successively sub-cultured every 3 weeks on DM and then several times on DM with increasing concentration of paromomycin from 50 to 150 mg L^{-1} (Rattana et al. 2001). GFP activity was monitored at the end of each subculture to discard tissues without GFP activity. Transgenic callus lines were established from sub-aggregates showing full GFP activity (Figure 34). These callus lines were then subjected to cryopreservation and plant regeneration.

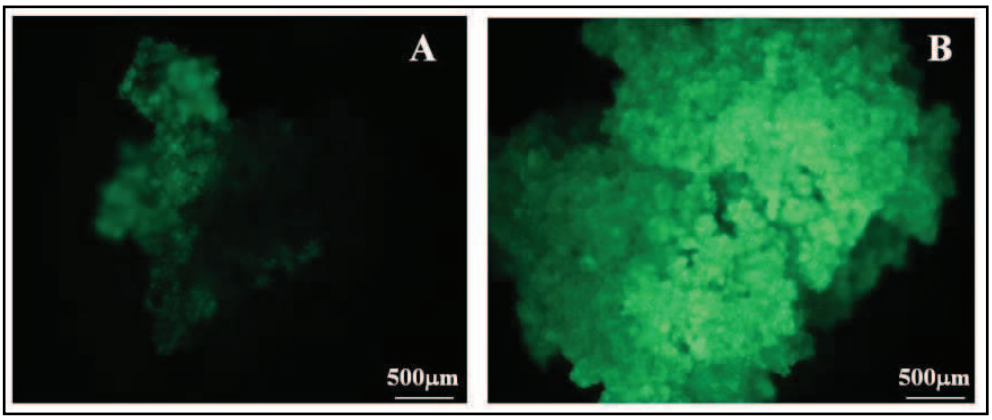

Figure 34. GFP fluorescence in callus. (A) Partially fluorescent callus and (B) fully fluorescent callus.

During the sub-culturing step, the green fluorescence was clearly visible, thus allowed the subculture of only GFP-positive-calli. The GFP-positive aggregates were fractionated into small sub-aggregates of 2 mm and their numbers were recorded. When the sub-aggregate was fully fluorescent, it was declared as a fully putative transgenic callus line. After 6 subcultures, some callus showed fully homogenous GFP fluorescence. Finally, twenty-nine GFP-positive lines were established on paromomycin selection medium (Figure 35) and then cryopreserved. The twenty-nine GFP-positive were: 5 lines for *35S*::*HbERF-IXc4* (Table 9), 6 lines for *HEV2.1*::*HbERF-IXc4* (Table 10), 7 lines for *35S*::*HbERF-IXc5* (Table 11), and 11 lines for *HEV2.1*::*HbERF-IXc5* (Table 12).

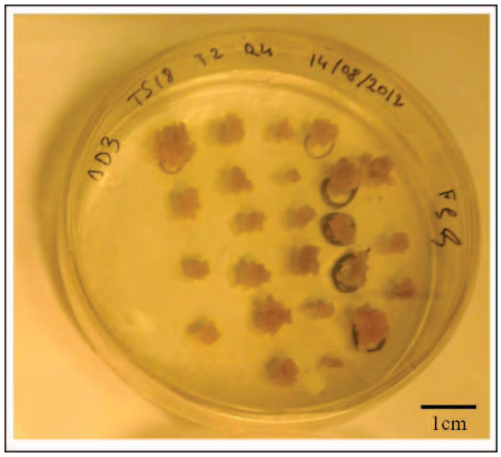

Figure 35. Selection of paromomycin-resistant calli with GFP positive aggregates.

Table 9. Number of GFP positive aggregates during the selection of transgenic lines harbouring the *35S::HbERF-IXc4* construct (TS17).

TS17		Total initial aggregate	No GFP aggregates										
Treatment (T)	Replicate (R)		DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10	DM11
4 days	1	60	10	0	0	0	0	0	0	0	0	0	0
	2	60	14	0	0	0	0	0	0	0	0	0	0
	3	60	14	2	2	2	2	0	0	0	0	0	0
	4	60	3	1	1	1	1	0	0	0	0	0	0
	5	60	19	1	1	1	1	0	0	0	0	0	0
Subtotal T1		**300**	**60**	**4**	**4**	**4**	**4**	**0**	**0**	**0**	**0**	**0**	**0**
5 days	1	60	19	1	1	1	1	0	0	0	0	0	0
	2	60	24	8	7	7	7	1	1	1	1	1	1
	3	60	8	3	2	2	2	0	0	0	0	0	0
	4	60	18	13	11	11	11	3	3	3	3	3	3
	5	60	22	8	8	8	8	2	1	1	1	1	1
Subtotal T2		**300**	**91**	**33**	**29**	**29**	**29**	**6**	**5**	**5**	**5**	**5**	**5**
Total		**600**	**151**	**37**	**33**	**33**	**33**	**6**	**5**	**5**	**5**	**5**	**5**

DM: decontamination medium

Table 10. Number of GFP positive aggregates during the selection of transgenic lines harbouring the *HEV2.1::HbERF-IXc4* construct (TS18).

TS18		Total initial aggregate	No GFP aggregates									
Treatment (T)	Replicate (R)		DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10
4 days	1	60	12	0	0	0	0	0	0	0	0	0
	2	60	25	6	6	6	6	0	0	0	0	0
	3	60	9	8	8	8	7	1	1	1	1	1
	4	60	27	16	16	15	12	0	0	0	0	0
	5	60	8	5	5	3	1	0	0	0	0	0
Subtotal T1		**300**	**81**	**35**	**35**	**32**	**26**	**1**	**1**	**1**	**1**	**1**
5 days	1	60	19	10	7	7	5	0	0	0	0	0
	2	60	24	0	0	0	0	0	0	0	0	0
	3	60	8	9	4	4	4	4	2	2	2	2
	4	60	18	14	12	12	12	10	4	4	4	4
	5	60	22	10	0	0	0	0	0	0	0	0
Subtotal T2		**300**	**43**	**23**	**23**	**21**	**14**	**6**	**6**	**6**	**6**	**6**
Total		**600**	**124**	**58**	**58**	**53**	**40**	**7**	**7**	**7**	**7**	**7**

DM: decontamination medium

Table 11. Number of GFP positive aggregates during the selection of transgenic lines harbouring the *35S::HbERF-IXc5* construct (TS19).

TS19		Total initial aggregate	No GFP aggregates									
Treatment (T)	Replicate (R)		DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10
4 days	1	60	13	5	5	3	2	0	0	0	0	0
	2	60	14	4	3	2	0	0	0	0	0	0
	3	60	9	1	1	1	1	0	0	0	0	0
	4	60	12	2	2	1	1	0	0	0	0	0
	5	60	12	2	2	2	2	0	0	0	0	0
Subtotal T1		**300**	**60**	**14**	**13**	**9**	**22**	**0**	**0**	**0**	**0**	**0**
5 days	1	60	37	11	11	11	10	4	2	2	2	2
	2	60	16	7	7	7	6	0	0	0	0	0
	3	60	19	2	1	1	1	1	1	1	1	1
	4	60	16	7	7	7	4	3	3	3	3	3
	5	60	20	4	4	4	3	1	1	1	0	0
Subtotal T2		**300**	**108**	**31**	**30**	**30**	**24**	**9**	**7**	**7**	**6**	**6**
Total		**600**	**168**	**45**	**43**	**39**	**46**	**9**	**7**	**7**	**6**	**6**

DM: decontamination medium

Table 12. Number of GFP positive aggregates during the selection of transgenic lines harbouring the *HEV2.1::HbERF-IXc5* construct (TS20).

TS20		Total initial aggregate	No GFP aggregates									
Treatment (T)	Replicate (R)		DM1	DM2	DM3	DM4	DM5	DM6	DM7	DM8	DM9	DM10
4 days	1	60	6	0	0	0	0	0	0	0	0	0
	2	60	13	0	0	0	0	0	0	0	0	0
	3	60	8	0	0	0	0	0	0	0	0	0
	4	60	8	1	1	1	1	1	1	1	1	0
	5	60	11	2	2	2	2	1	1	1	1	1
Subtotal T1		**300**	**46**	**3**	**3**	**3**	**3**	**2**	**2**	**2**	**2**	**1**
5 days	1	60	16	7	6	6	6	4	4	4	4	4
	2	60	3	0	0	0	0	0	0	0	0	0
	3	60	20	7	6	5	4	2	2	2	2	1
	4	60	13	11	10	9	8	4	4	4	4	4
	5	60	10	3	3	3	3	1	1	1	1	1
Subtotal T2		**300**	**62**	**28**	**25**	**23**	**21**	**11**	**11**	**11**	**11**	**10**
Total		**600**	**108**	**31**	**28**	**26**	**24**	**13**	**13**	**13**	**13**	**11**

DM: decontamination medium

Callus proliferation induced by subsequent subcultures of calli on DM medium resulted in the establishment of embryonic callus lines. TS18, TS19, and TS20 had very high proliferation rate on DM7 and showed a lower level of proliferation after DM7 sub-cultured (Table 13). At the beginning, the proliferation rate of TS17 showed slower than TS18, TS19, and TS20. TS17 needed more subcultures on DM medium. The best proliferation rate of TS17 was obtained when calli were sub-cultured on DM10 compare the others.

Table 13. Number of GFP positive aggregates during the selection of transgenic lines assessed by the number of GFP aggregates multiplied. Each experiment has been conducted with 10 replications of 60 aggregates.

Construct	DM1	DM 2	DM 3	DM 4	DM 5	DM 6	DM 7	DM 8	DM 9	DM 10	DM 11
35S::HbERF-IXc4	600	690	828	486	311	402	349	312	930	990	780
HEV2.1::HbERF-IXc4	600	464	1502	774	544	1367	3060	2280	900	750	-
35S::HbERF-IXc5	600	818	1167	907	285	911	2513	1530	990	960	-
HEV2.1::HbERF-IXc5	600	351	639	533	329	1694	4552	2670	1800	810	-

1.2. Morphogenetic potential of callus lines with different somatic embryogenesis capacity from wild-type (CI07060) and transgenic lines

Somatic embryogenesis was initiated for 4 weeks by sub-culturing 1 g of callus showing full GFP activity on semi-solid embryogenesis expression medium (EXP). All GFP-positive lines from DM medium continued to transfer on EXP medium. Pro-embryo development was then carried out in a temporary immersion system (RITA®, CIRAD, Montpellier) for 4 weeks with 1 min of immersion per day in the liquid development medium (DEV). Each RITA was considered as an experimental replication. Conversion of mature embryos was carried out according to (Lardet et al. 1999). Well-shaped mature embryos were collected and transferred to glass tubes on a semi-solid germination medium (DEV3). Embryos were incubated under a light intensity of 60 µmol $m^{-2}s^{-1}$ and a 12 h day/dark photoperiod up to the full conversion of embryos into plantlets.

The morphogenetic capacities of friable callus lines were tested up to somatic embryos conversion into plantlets. Most of callus lines regenerating embryos and plantlets turned brown after embryogenesis induction in EXP and DEV media. Callus turned brown at the advantage of embryo formation. Brown calli produced a large number of somatic embryos compared to yellow calli. The well-shaped embryos had an embryonic body and two well-developed cotyledons. The abnormal types of embryos were more numerous, with a single cotyledon or with malformed cotyledons, or a double embryonic body were found. Plantlets derived from normal embryos developed a taproot and a lateral root system, and a stem with leaves within a month in DEV3 medium (Figure 36).

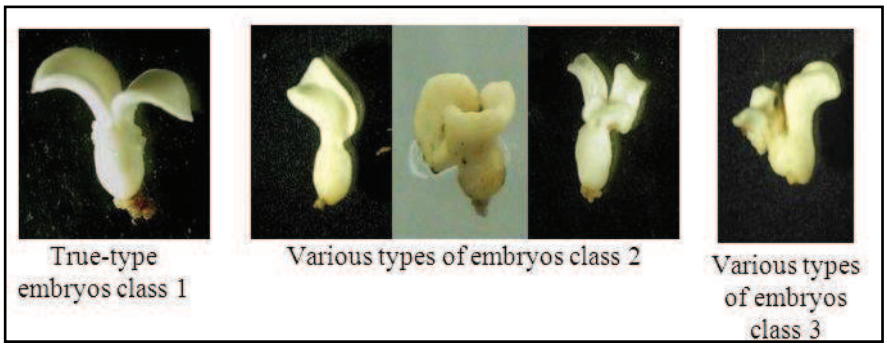

Figure 36. Various classes of somatic embryos.

Some replication of wild-type (CI07060) calli produced brown callus as well as some of transgenic lines harbouring *HEV2.1::HbERF-IXc4, 35S::HbERF-IXc5*, *HEV2.1::HbERF-IXc5* (TS18, TS19, and TS20) which were associated with the high capability of producing somatic embryos. On the contrary, some transgenic callus lines harbouring *35S::HbERF-IXc4* (TS17A24, TS17A35, TS17A53, TS17A61, and TS17A79) had yellow calli on DM, EXP, and in DEV media (Figure 37). Some calli became necrotic. These data suggest that *35S::HbERF-IXc4* had a lower morphogenetic capacity compared the others.

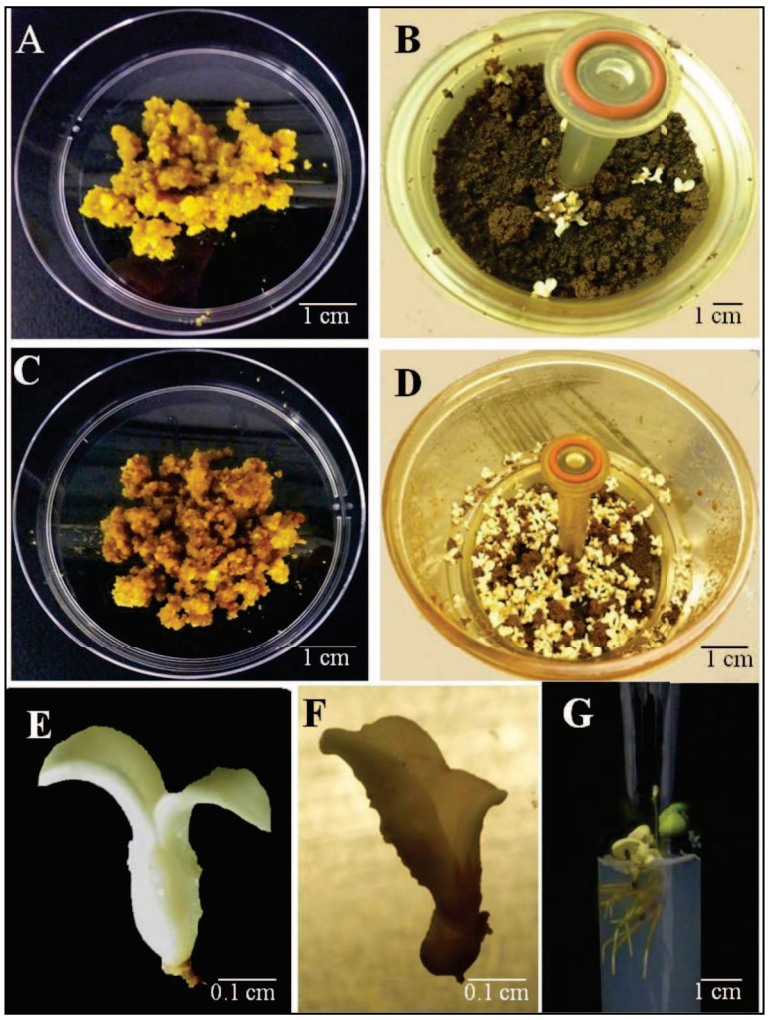

Figure 37. The differences of embryonic capacity from callus obtained from somatic embryogenesis of *Hevea brasiliensis*. (A) Yellow-callus (TS17A53) on EXP medium (B) Yellow-callus did not produce any somatic embryos on DEV2 medium. (C) Brownish-callus (TS20A75) on EXP medium (D) Brownish-callus can produce a large number of total somatic embryos on DEV2 medium. (E) Normal cotyledoned embryo. (F) Abnormal somatic embryo. (G) Plantlet from a normal somatic embryo.

1.3. Effect of construct and lines on the production of total embryos, well-shaped embryos, and plantlets

The number of somatic embryos and plantlets were quantified for at least 5 independent replications and continued analysed with XLSTAT with an ANOVA. The embryogenic line produced somatic embryos per g of callus. The total number of embryos produced by wild-type and transgenic lines were high (71.92-93.56 embryos g^{-1} FM) but the well-shaped embryo production was low (15.63-27.74 embryos g^{-1} FM). Number of total embryos of wild-type (CI07060) and transgenic lines showed a big variability but not significance, except *HEV2.1*::*HbERF-IXc5* (TS20A75). This line produced a largest number of total somatic embryos (257 per g of callus). These data showed a high number of abnormal embryos produced by both of untransformed line and transformed lines until 96.80% (TS20A45) (Table 14).

Table 14. Regeneration ability of the wild-type (CI07060 line) and independent transgenic callus lines overexpressing *HbERF-IXc4* and *HbERF-IXc5*.

Construct	Lines	Replication (no of RITA)	Total embryos (no g^{-1} FM)	Well-shaped embryos (WS) (no g^{-1} FM)	Abnormal embryos (%) (no g^{-1} FM)	Plantlets (P) (no g^{-1} FM)	Conversion (%) P/WS)
Wild-type	CI07060	16	71.92^{bc}	27.74^{a}	61.43%	11.92^{a}	43%
35S::HbERF-IXc4	TS17A24	5	97.04^{bc}	7.22^{a}	92.56%	0^{a}	0%
	TS17A35	7	200.12^{ab}	37.34^{ab}	81.34%	0.27^{a}	1%
	TS17A53	9	162.67^{abc}	32.39^{ab}	80.09%	0^{a}	0%
	TS17A61	13	5.07^{c}	0.65^{a}	87.18%	0.14^{a}	22%
	TS17A79	7	2.91^{c}	0.53^{a}	81.79%	0.13^{a}	25%
HEV2.1::HbERF-IXc4	TS18A37	14	52.80^{bc}	10.71^{a}	79.72%	8.32^{a}	78%
	TS18A09	9	112.11^{abc}	31.27^{ab}	72.11%	20.28^{a}	65%
	TS18A13	14	97.91^{bc}	38.54^{ab}	60.64%	20.56^{a}	53%
	TS18A20	11	38.66^{bc}	8.24^{a}	78.69%	0.72^{a}	9%
	TS18A69	13	16.04^{bc}	5.60^{a}	65.09%	3.45^{a}	62%
35S::HbERF-IXc5	TS19A46	12	32.22^{bc}	7.44^{a}	76.91%	4.60^{a}	62%
	TS19A59	5	12.83^{bc}	4.02^{a}	68.67%	1.34^{a}	33%
	TS19A90	13	189.96^{ab}	60.21^{ab}	68.30%	20.14^{a}	33%
	TS19A99	10	132.10^{abc}	14.49^{a}	89.03%	0.09^{a}	1%
HEV2.1::HbERF-IXc5	TS20A29	9	21.02^{bc}	9.20^{a}	56.23%	0^{a}	0%
	TS20A45	6	9.70^{bc}	0.31^{a}	96.80%	0^{a}	0%
	TS20A47	7	11.97^{bc}	2.29^{a}	80.87%	0.54^{a}	23%
	TS20A53	6	31.86^{bc}	5.6^{a}	82.42%	0^{a}	0%
	TS20A69	13	189.89^{ab}	87.83^{b}	53.75%	16.98^{a}	19%
	TS20A75	13	256.96^{a}	87.17^{b}	66.08%	27.39^{a}	31%

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter are not significantly different at the 0.05 probability level.

For number of well-shaped embryos, the statistical analysis showed that some lines from the candidate gene of *HbERF-IXc5* under the control of specific promoter *HEV2.1* (TS20A69 and TS20A75) gave a significant value compared to wild-type and produced high value of well-shaped embryos (87-88%). Only the well-shaped embryos had the ability to be converted into plantlets.

The data showed that there was a big variability of the conversion of total embryos into plantlets between wild-type and transgenic lines. The highest total plantlets were produced by *HEV2.1*::*HbERF-IXc5* (TS20A75). The average of the conversion of total embryos into plantlets with the highest percentage was achieved by construct gene *HEV2.1::HbERF-IXc4* (TS18). The results showed 78% of well-shaped embryos achieved their conversion into plantlet for TS18A37.

It was very interesting for *35S::HbERF-IXc4* (TS17) showed lowest value of regeneration capacity of transgenic line to induce somatic embryos. The percentages of abnormal embryos were very high (85%). It was correlated with the low of ability of embryos to be converted into plantlets. So this results suggests that a constitutive expression of *35S::HbERF-IXc4* was deleterious and reduced the ability to form somatic embryos and the subsequent ability to convert embryos into plantlets. On the other side, it was very interesting to observe and understand the same gene *HbERF-IXc4* but under different control of promoter specific *HEV2.1* (TS18). This construct had highest capability to convert well-shaped embryos into plantlets compared wild-type and other constructs. The results showed 78% of well-shaped embryos achieved their conversion into plantlet for TS18A37.

The data showed that there was a big variability of the conversion of total embryos into plantlets between wild-type and transgenic lines. The highest total plantlets were produced by *HEV2.1*::*HbERF-IXc5* (TS20A75). This data suggested that *HEV2.1::HbERF-IXc5* had better results than the others *in vitro* culture.

2. Contrasting plant regeneration capacity between transgenic lines

The average of regeneration capacity of transgenic lines were lower (28%) compared the wild-type (43%), but some transgenic lines have a higher of regeneration capacity from the construct of *HEV2.1::HbERF-IXc4* (TS18A09, TS18A13, TS18A37, TS18A69) and *35S::HbERF-IXc5* (TS19A46). Overall TS18 had the highest of regeneration capacity compared wild-type. The comparison of the average of regeneration ability between wild-type and transgenic lines showed *35SGFP+HEV2.1::HbERF-IXc4* (TS18) had a highest regeneration capacity (53%) from callus to plantlet (Table 15).

Table 15. The average of regeneration ability of the wild-type CI07060 line and independent transgenic callus lines overexpressing *HbERF-IXc4* and *HbERF-IXc5*.

Construct	Replication (no of RITA)	Total embryos (no g^{-1} FM)	Well-shaped embryos (WS) (no g^{-1} FM)	Plantlets (P) (no g-1 FM)	Conversion (% P/WS)
Wild-type	16	71.92[a]	27.74[a]	11.92[a]	43%
35S::HbERF-IXc4	41	93.56[a]	15.63[a]	0.11[a]	10%
HEV2.1::HbERF-IXc4	61	63.50[a]	18.87[a]	10.67[a]	53%
35S::HbERF-IXc5	40	91.78[a]	21.54[a]	5.23[a]	32%
HEV2.1::HbERF-IXc5	62	75.21[a]	27.55[a]	6.47[a]	21%

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter are not significantly different at the 0.05 probability level.

Based on Table 16, the construct of *35SGFP+HEV2.1::HbERF-IXc4* had a highest ratio plantlet/RITA among other constructs. The same gene (*HbERF-IXc4*) with different promoter (*35S CaMV*) showed the opposite effect. This construct obtained the lowest ratio of plantlet/RITA. Another candidate gene *HbERF-IXc5* with *35S CaMV* and *HEV2.1* promoter presented almost the same results of ratio plantlet/RITA. Finally, from twenty-nine GFP-positive lines established can produce sixteen lines regenerating plantlets. We maintained twelve line-developed plantlets based on acclimatization in greenhouse. Plantlets produced by some line regenerating plantlets showed a low of survival capability when they were transferred to greenhouse.

Table 16. The summary of somatic embryogenesis from transgenic lines.

Construct	RITA (No)	Cryopreserved transgenic line (No)	Tested line for regeneration (No)	Line regenerating plantlets (No)	Line-developed plantlets in greenhouse (No)	Plantlet (No)	Plantlet/ RITA (No)
Wild-type	16	-	-	1	1	191	11.94
35S::HbERF-IXc4	41	5	5	3	2	3	0.07
HEV2.1::HbERF-IXc4	61	7	7	5	5	670	10.98
35S::HbERF-IXc5	40	6	5	4	2	339	8.48
HEV2.1::HbERF-IXc5	62	11	11	4	3	610	9.84

3. Copy number of T-DNA in transgenic lines

Southern-blot molecular hybridization was performed using random primed ^{32}P radio-labelled probes corresponding *NPTII* gene and DNA samples from plants of 10 GFP-positive lines and one negative control (wild-type) (Figure 38 and Table 17). Genomic DNA was digested by the *Eco*RI restriction enzyme. This enzyme recognizes a unique site within the T-DNA; this allows counting the number of T-DNA copies since one band per T-DNA insertion can be observed. All these transgenic lines have 1 copy of the T-DNA except the wild-type (no insertion), TS17A79 (non-determined = nd) and transgenic lines TS18A69 and TS18A09, which have 2 and 3 copies, respectively. There was no band was detected in the non-transformed tissue ((wild-type) Cl07060) but bands were present in the transgenic lines.

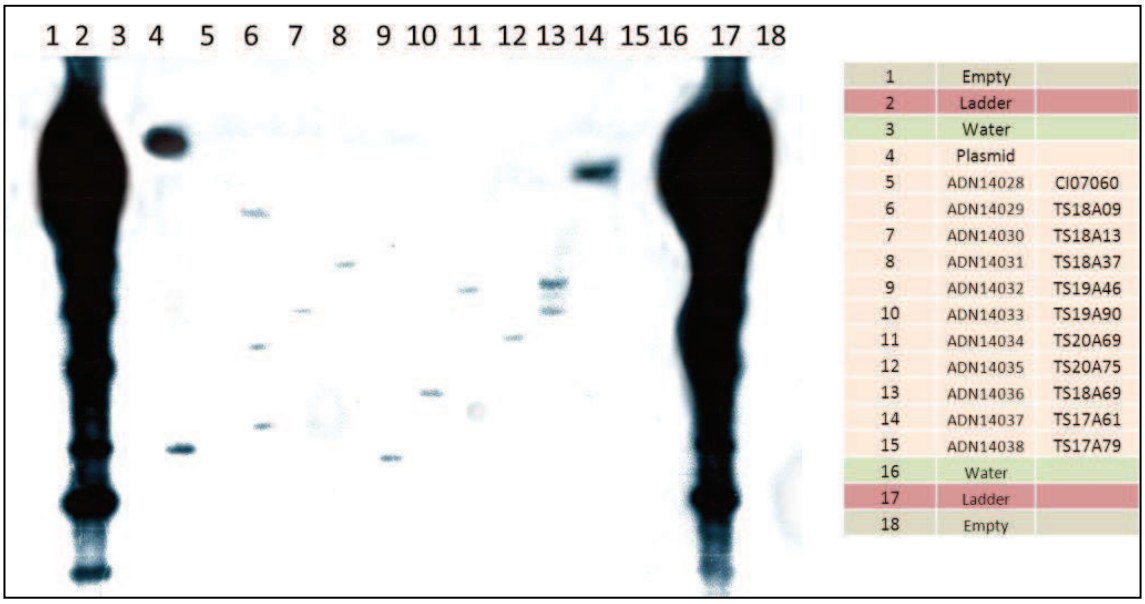

Figure 38. Southern-blot hybridization analysis of DNA. Genomic DNA samples of leaves were digested with *Eco*RI. The blot was hybridized with a ^{32}P radio-labelled probes corresponding to *NPTII* gene. Lane 1: empty, lane 2: Ladder (Exact Ladder DNA PreMix 2 log), lane 3: water, lane 4: plasmid (linearized pCamway *35S::HbERF-IXc4/c5*), lane 5: wild-type (CI07060), lane 6: TS18A09, lane 7: TS18A13, lane 8: TS18A37, lane 9: TS19A46, lane10: TS19A90, lane 11: TS20A75, lane 12: TS20A75, lane 13: TS18A69, lane 14: TS17A61, lane 15: TS17A79, lane 16: water, lane 17: Ladder (Exact Ladder DNA PreMix 2 log), lane 18: empty.

Table 17. Southern-blot molecular hybridization analysis of DNA from wild-type (CI07060) and transgenic lines using *NPTII* probe.

Construct	Transgenic line	T-DNA (No of copy)
Wild-type	CI07060	0
35S::HbERF-IXc4	TS17A61	1
	TS17A79	nd
HEV2.1::HbERF-IXc4	TS18A09	3
	TS18A13	1
	TS18A37	1
	TS18A69	2
35S::HbERF-IXc5	TS19A46	1
	TS19A90	1
HEV2.1::HbERF-IXc5	TS20A69	1
	TS20A75	1

nd= non-determined

4. Gene expression analysis of *HbERF-IXc4* and *HbERF-IXc5* lines by real-time RT-PCR

Real-time RT-PCR (qPCR) is a sensitive and precise method for quantifying gene expression; however, suitable reference genes are required. Several rules were applied in order to reduce the risk of errors in relative gene expression data. In this study, a systematic reference gene screening was first performed. Amplification of nine housekeeping genes was attempted in bark and leaf tissues from two-year-old wild-type (CI07060) and transgenic lines. *HbRH2b* was selected as internal reference gene for its stability in gene expression in mature trees and juvenile plants subjected to various treatments. The *HbRH2b* gene was amplified in each reaction plate in parallel with target genes.

This analysis by qPCR was initiated to know the level of relative transcript abundance, especially for *HbERF-IXc4* and *HbERF-IXc5* genes, which were over-expressed in transgenic plants. These transcription factors activate several target genes in relation to stress environment (Achard et al. 2006). Specific primers have already been designed for genes involved in ethylene biosynthesis and signalling pathways, and ROS-scavenging systems (Duan et al. 2010, Piyatrakul et al. 2014, Putranto et al. 2015a), which could be targeted by HbERF-IXc4 and HbERF-IXc5 transcription factors. For that reason, seven putative target genes were selected *HbPDF3*, *HbSUT3*, *HbETR2*, *HbERF-Xb1*, *HbPDF1*, *HbPDF2*, and *HbChit,* in addition to *HbERF-IXc4* and *HbERF-IXc5*. These genes were selected because their expression is regulated by stress and especially by ethylene. Ethylene response was accompanied by regulation of the transcript abundance of several genes (Duan et al. 2010). The ratio of relative transcript abundance was calculated between transgenic lines without treatment compared to wild-type (CI07060).

In bark, analysis of nine genes showed low relative transcript abundance except for *HbPDF3* gene. The expression level of *HbPDF3* gene was the highest level in wild-type. Two lines (TS18A13 and TS18A37) had significant difference of gene expression of *HbPDF3* compared to wild-type (Table 18). The relative transcript abundance of *HbERF-IXc4* and *HbERF-IXc5* for all lines were higher than wild-type. TS19A90 had highest of expression level of both genes and exhibited significant difference of *HbERF-IXc5* expression compared wild-type. Statistical analysis of effect of construct in bark showed there were significant differences in *HbPDF3* and *HbPDF2* genes but did not show discrepancy for the other genes. However, the mean value of relative transcript abundance of all construct of *HbERF-IXc4* and *HbERF-IXc5* genes were higher than wild-type. These results indicated that *HbERF-IXc4* and *HbERF-IXc5* genes were successfully over-expressed (Table 19).

In leaves, statistical analysis on the effect of lines showed there were no significant differences in expression of the nine genes, except for *HbChit1* gene. The other genes generally showed similar expression patterns in the expression levels in leaves. Line TS20A82 showed the highest expression level of *HbChit1* gene (6.98E-03) compared to wild-type and other transgenic lines. Based on the average of relative transcript, the expression level of *HbERF-IXc4* and *HbERF-IXc5* genes were relatively higher in transgenic lines compared to wild-type. The average of expression level from other genes showed diverse expression level in transgenic lines compared to wild-type and no significant differences (Table 20). Statistical analysis on the effect of construct in leaves showed that there were no effect on the relative transcript abundance between all of the construct, even though the average of relative transcript for *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* constructs indicated the highest expression level in *HbERF-IXc4* and *HbERF-IXc5* compared to wild-type (Table 21). The results suggested that relative transcript abundance of *HbERF-IXc4* and *HbERF-IXc5* genes maybe related with overexpression genes.

Table 18. Relative transcript abundance profile of nine genes in bark of wild-type (WT) and transgenic lines harbouring *HbERF-IXc4* or *HbERF-IXc5* genes under the control of *35SCaMV* and *HEV2.1* promoter.

Construct	Line	Genes								
		HbERF-IXc4	***HbERF-IXc5***	***HbPDF3***	***HbSUT3***	***HbETR2***	***HbERF-Xb1***	***HbPDF1***	***HbPDF2***	***HbChit1***
Wild-type	CI07060	118.0E-06[a]	3.3E-06[a]	407.8E-03[b]	31.3E-06[a]	6.5E-03[a]	237.6E-06[a]	2.9E-03[a]	3.7E-03[a]	7.3E-06[a]
HEV2.1::HbERF-IXc4	TS18A09	nd	8.5E-06[a]	112.3E-03[ab]	43.9E-06[a]	3.2E-03[a]	107.4E-06[a]	2.2E-03[a]	833.0E-06[a]	14.7E-06[a]
	TS18A13	517.0E-06[a]	nd	79.1E-03[a]	212.6E-06[a]	2.9E-03[a]	453.3E-06[a]	3.2E-03[a]	801.7E-06[a]	60.7E-06[a]
	TS18A37	nd	904.0E-06[ab]	40.6E-03[a]	126.4E-06[a]	2.9E-03[a]	46.5E-06[a]	3.4E-03[a]	682.0E-06[a]	14.6E-06[a]
35S::HbERF-IXc5	TS19A46	nd	80.0E-06[a]	310.0E-03[ab]	90.0E-06[a]	8.4E-03[a]	1.0E-03[a]	2.4E-03[a]	2.9E-03[a]	47.5E-06[a]
	TS19A90	5.5E-03[a]	7.2E-03[b]	99.6E-03[ab]	497.2E-06[a]	6.2E-03[a]	86.2E-06[a]	4.0E-03[a]	1.5E-03[a]	39.4E-06[a]
HEV2.1::HbERF-IXc5	TS20A69	3.7E-03[a]	509.0E-06[a]	103.7E-03[ab]	298.3E-06[a]	5.9E-03[a]	nd	2.1E-03[a]	1.8E-03[a]	100.9E-06[a]
	TS20A75	nd	10.0E-06[a]	175.8E-03[ab]	14.8E-06[a]	6.8E-03[a]	268.0E-06[a]	4.6E-03[a]	2.7E-03[a]	22.2E-06[a]
	TS20A82	589.0E-06[a]	2.1E-03[ab]	104.3E-03[ab]	1.4E-03[a]	5.1E-03[a]	107.0E-06[a]	3.4E-03[a]	2.6E-03[a]	1.9E-03[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level. nd = not determined

Table 19. Effect of constructs on relative transcript abundance profile of nine genes in bark of transgenic lines (*HbERF-IXc4* or *HbERF-IXc5* genes under the control of *35SCaMV* and *HEV2.1* promoter).

Construct	Genes								
	HbERF-IXc4	***HbERF-IXc5***	***HbPDF3***	***HbSUT3***	***HbETR2***	***HbERF-Xb1***	***HbPDF1***	***HbPDF2***	***HbChit1***
Wild-type	1.18E-04 [a]	3.30E-06 [a]	4.08E-01 [b]	3.13E-05 [a]	6.45E-03 [a]	2.38E-04 [a]	2.91E-03 [a]	3.70E-03 [b]	7.28E-06 [a]
HEV2.1::HbERF-IXc4	5.17E-04 [a]	4.56E-04 [a]	7.74E-02 [a]	1.28E-04 [a]	2.99E-03 [a]	2.02E-04 [a]	2.92E-03 [a]	7.72E-04 [a]	3.00E-05 [a]
35S::HbERF-IXc5	5.52E-03 [a]	3.64E-03 [a]	2.05E-01 [ab]	2.94E-04 [a]	7.31E-03 [a]	5.62E-04 [a]	3.20E-03 [a]	2.19E-03 [ab]	4.34E-05 [a]
HEV2.1::HbERF-IXc5	2.15E-03 [a]	8.58E-04 [a]	1.28E-01 [a]	5.63E-04 [a]	5.92E-03 [a]	2.68E-04 [a]	3.34E-03 [a]	2.38E-03 [ab]	6.65E-04 [a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Table 20. Effect of lines from relative transcript abundance profile of nine genes in leaves of transgenic lines (*HbERF-IXc4* or *HbERF-IXc5* genes under the control of *35S CaMV* and *HEV2.1* promoter).

Construct	Line	Genes								
		HbERF-IXc4	***HbERF-IXc5***	***HbPDF3***	***HbSUT3***	***HbETR2***	***HbERF-Xb1***	***HbPDF1***	***HbPDF2***	***HbChit1***
Wild-type	CI07060	8.83E-05[a]	2.38E-06[a]	2.99E-03[a]	1.65E-05[a]	1.95E-03[a]	1.03E-06[a]	7.30E-03[a]	4.52E-04[a]	2.70E-04[a]
HEV2.1::HbERF-IXc4	TS18A13	2.93E-04[a]	1.03E-05[a]	2.86E-03[a]	nd	2.00E-03[a]	6.50E-06[a]	3.21E-02[a]	5.36E-04[a]	7.93E-04[ab]
	TS18A37	1.61E-03[a]	nd	3.97E-03[a]	nd	7.76E-04[a]	nd	1.94E-02[a]	5.52E-04[a]	5.87E-04[ab]
35S::HbERF-IXc5	TS19A46	4.64E-05[a]	3.51E-05[a]	6.28E-03[a]	1.37E-05[a]	1.24E-03[a]	9.22E-07[a]	7.68E-03[a]	5.77E-04[a]	4.21E-04[a]
	TS19A59	3.34E-03[a]	2.76E-04[a]	3.83E-03[a]	8.58E-06[a]	6.13E-04[a]	3.25E-06[a]	7.67E-03[a]	3.07E-04[a]	3.41E-03[ab]
	TS19A90	1.48E-04[a]	2.50E-03[a]	5.86E-03[a]	1.66E-05[a]	6.42E-04[a]	2.01E-05[a]	6.37E-03[a]	4.45E-04[a]	3.74E-04[a]
HEV2.1::HbERF-IXc5	TS20A69	9.76E-04[a]	2.55E-04[a]	5.59E-03[a]	7.02E-06[a]	8.12E-04[a]	nd	1.64E-02[a]	3.60E-04[a]	1.72E-03[ab]
	TS20A75	1.31E-03[a]	4.16E-05[a]	4.73E-02[a]	2.24E-05[a]	1.19E-03[a]	4.45E-06[a]	7.77E-03[a]	8.24E-04[a]	3.87E-04[a]
	TS20A82	2.52E-03[a]	8.46E-05[a]	4.73E-03[a]	nd	2.28E-03[a]	3.88E-05[a]	1.75E-02[a]	6.37E-04[a]	6.98E-03[b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level. nd = not determined.

Table 21. Effect of constructs on relative transcript abundance profile of nine genes in leaves of transgenic lines (*HbERF-IXc4* or *HbERF-IXc5* genes under the control of *35S CaMV* and *HEV2.1* promoter).

Construct	Genes								
	HbERF-IXc4	***HbERF-IXc5***	***HbPDF3***	***HbSUT3***	***HbETR2***	***HbERF-Xb1***	***HbPDF1***	***HbPDF2***	***HbChit1***
Wild-type	8.83E-05[a]	2.38E-06[a]	2.99E-03[a]	1.65E-05[a]	1.95E-03[a]	1.03E-06[a]	7.30E-03[a]	4.52E-04[a]	2.70E-04[a]
HEV2.1::HbERF-IXc4	6.95E-04[a]	9.55E-06[a]	2.87E-03[a]	3.25E-05[a]	1.39E-03[a]	7.65E-06[a]	1.84E-02[a]	4.46E-04[a]	5.25E-04[a]
35S::HbERF-IXc5	1.18E-03[a]	9.38E-04[a]	5.32E-03[a]	1.30E-05[a]	8.30E-04[a]	8.07E-06[a]	7.24E-03[a]	4.43E-04[a]	1.40E-03[a]
HEV2.1::HbERF-IXc5	1.60E-03[a]	1.27E-04[a]	1.92E-02[a]	1.47E-05[a]	1.43E-03[a]	2.16E-05[a]	1.39E-02[a]	6.07E-04[a]	3.03E-03[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level. nd = not determined.

5. Monitoring growth and morphological parameters for one year after acclimatization

Somatic embryos were produced from wild-type (CI07060) and transgenic lines (TS17A24, TS17A35, TS17A53, TS17A61, TS17A79, TS18A37, TS18A09, TS18A13, TS18A20, TS18A69, TS19A46, TS19A59, TS19A90, TS19A99, TS20A29, TS20A45, TS20A47, TS20A53, TS20A69, TS20A2975). The conversion of embryos into plantlets and acclimatization step are illustrated in Figure 39. Plantlets were acclimatized in greenhouse at 28 °C with 60% relative humidity.

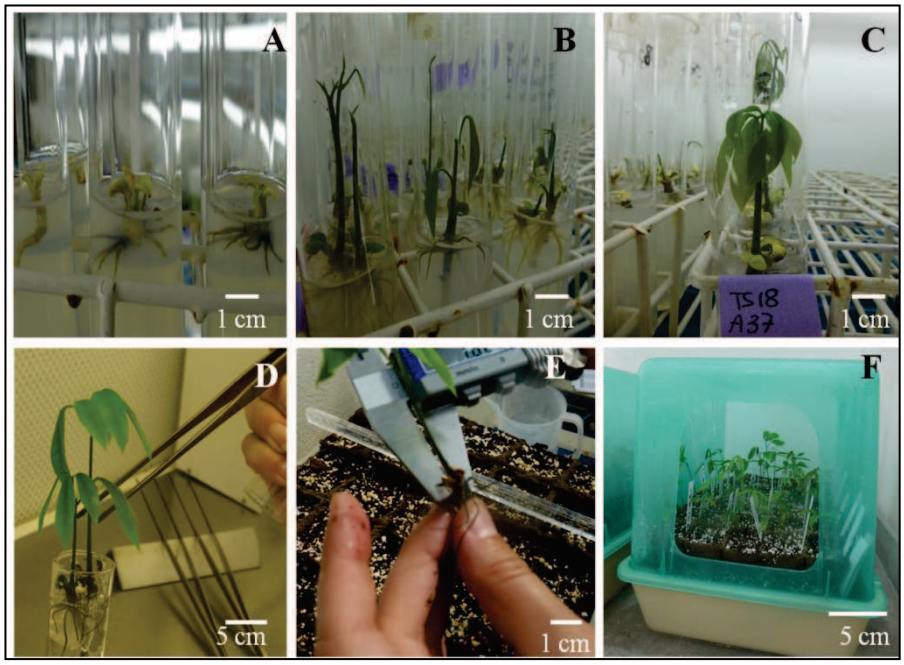

Figure 39. *In vitro* conversion of embryos into plantlets and plantlet acclimatization in greenhouse. (A) Germination after 1-2 weeks on DM3 medium. (B) Plantlets with taproot system after 2-4 weeks on DM3 medium. (C) Fully developed plantlets with leaves, taproot, and lateral roots after 4-8 weeks on DM3 medium. (D) Transfer of plantlet for acclimatization. (E) Measurement of plantlets before acclimatization. (F) First step of acclimatization of plantlets in greenhouse covered by tunnel.

The plants grew under controlled environmental conditions in greenhouse for 1 year. Plantlets were transferred from *in vitro* tubes to small pots (120 mL) and were covered by a special plastic box to maintain a high hygrometry (Figure 39F, Figure 40A). After 2 months of acclimatization, plants were transferred into bigger pots (2 L) to favour plant growth (Figure 40B). Several parameters were measured at different steps of development:

a. Plantlets before acclimatization (0 month): include height of root, diameter of root, height of stem, diameter of stem, number of leaves, number of leaflets, and number of lateral roots from *in vitro* plantlets.
b. 2 and 6-month-old plants after acclimatization: height of plant, diameter of stem, number of leaves, and number of leaflets (Figure 40C).
c. 12-month-old plant after acclimatization: diameter of stem, height of stem, number of leaves, number of leaflets, weight of leaves, weight of stem, weight of total root, and weight of the main root (Figure 40D and 41E).

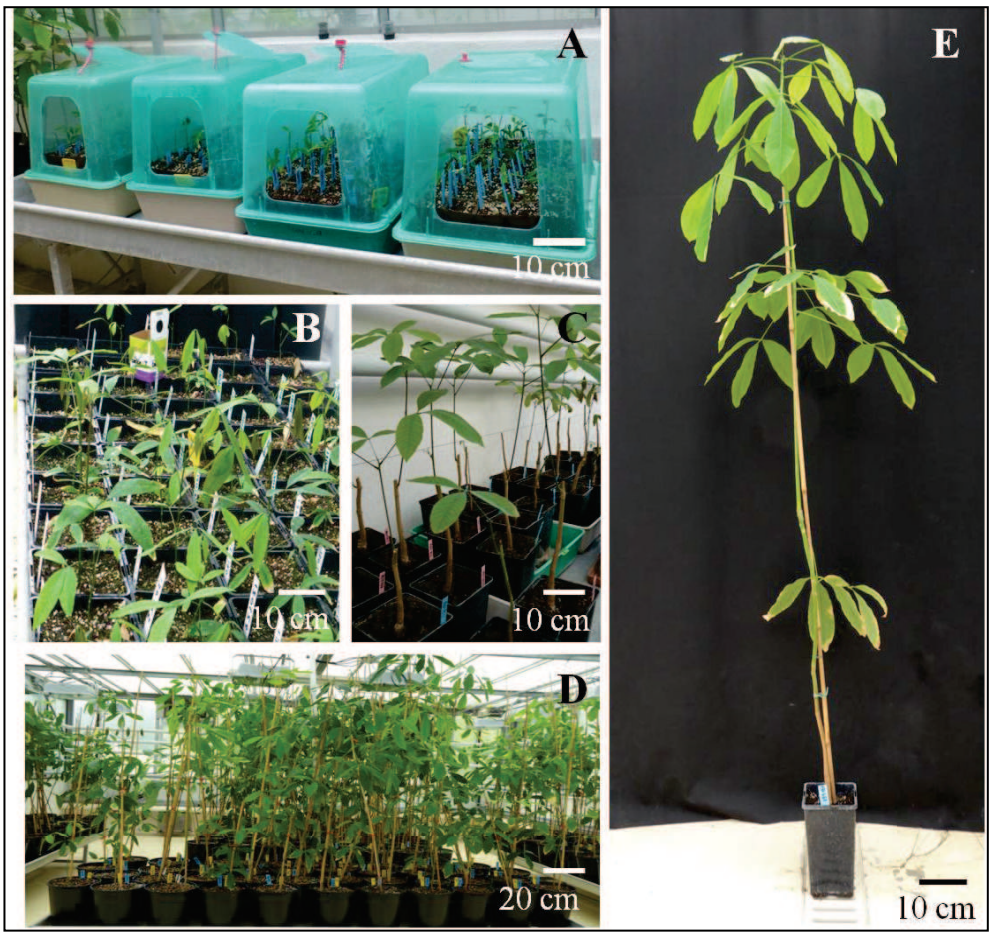

Figure 40. Acclimatization and plant growth in greenhouse. (A) The first transfer plantlets in the pot (120 mL). (B) The second transfer, 2-month-old plant in 2L pot. (C) 4-month-old plant. (D) 12-month-old plant. (D) The maintenance of transgenic rubber in the greenhouse for plants more than 12-month-old with bigger pots (5 L).

5.1. Survival rate of transgenic plants compared to wild-type for one year after transfer in greenhouse

Analysis on plant survival number before and after a 2-month acclimatization revealed significant differences between wild-type (CI07060) and some transgenic lines from the construct of *HEV2.1::HbERF-IXc4* (TS18A37, TS18A09, TS18A20, and TS18A69); *35S::HbERF-IXc5* (TS19A46); and *HEV2.1::HbERF-IXc5* (TS20A47). By contrast, there were no differences with construct for lines *HEV2.1::HbERF-IXc4* (TS18A13); *35S::HbERF-IXc5* (TS19A90); and *HEV2.1::HbERF-IXc5* (TS20A69) and TS20A75). A large proportion of plants died after 6 months of growth in greenhouse for WT (41%) and transgenic lines (17-83%). From statistical analysis, it showed there were not differences between wild-type (CI07060) and transgenic lines at month 6 and 12. There were 3 transgenic lines which have higher number of survival plants compared to other transgenic lines, TS18A13, TS20A69, and TS20A75 at month 12 (Figure 41).

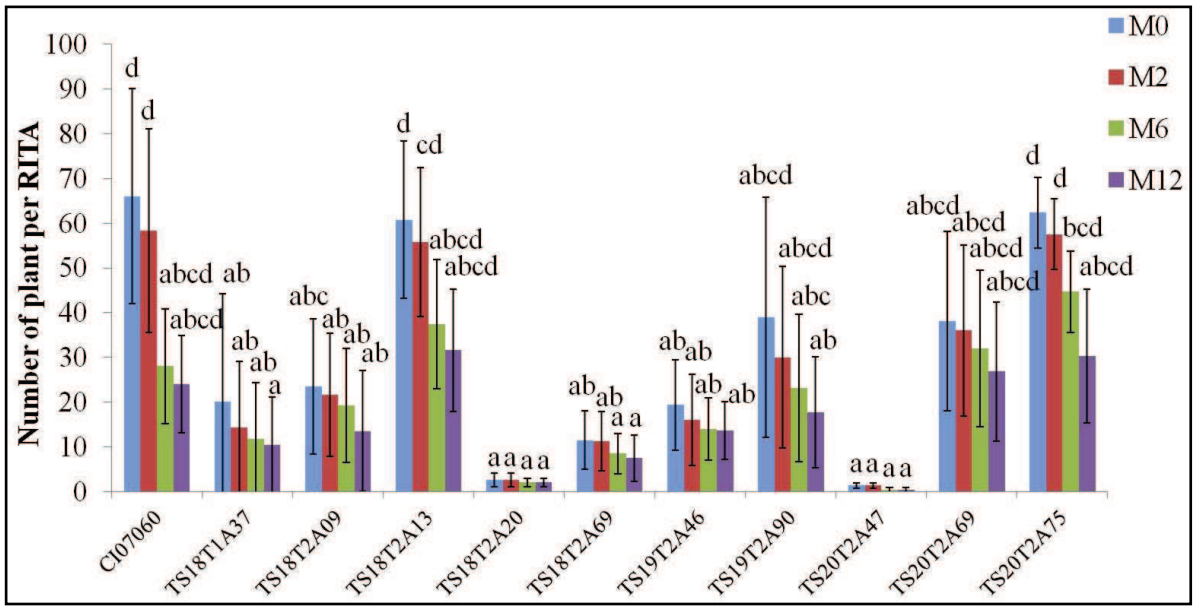

Figure 41. The diagram of mean of number of plants per RITA between the wild-type CI07060 line and independent transgenic callus lines overexpressing *HbERF-IXc4* and *HbERF-IXc5.* The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter are not significantly different at the 0.05 probability level.

Statistical analysis for the effect of construct on plant survival number showed a significant difference after 2 month of plant growth between constructs *HEV2.1::HbERF-IXc4* and *35S::HbERF-IXc5* and wild-type (Figure 42; Table 22). Based on F value of analysis of variance, there is a significant difference in plant growth at 0 month and 2 months after acclimatization (Table 22).

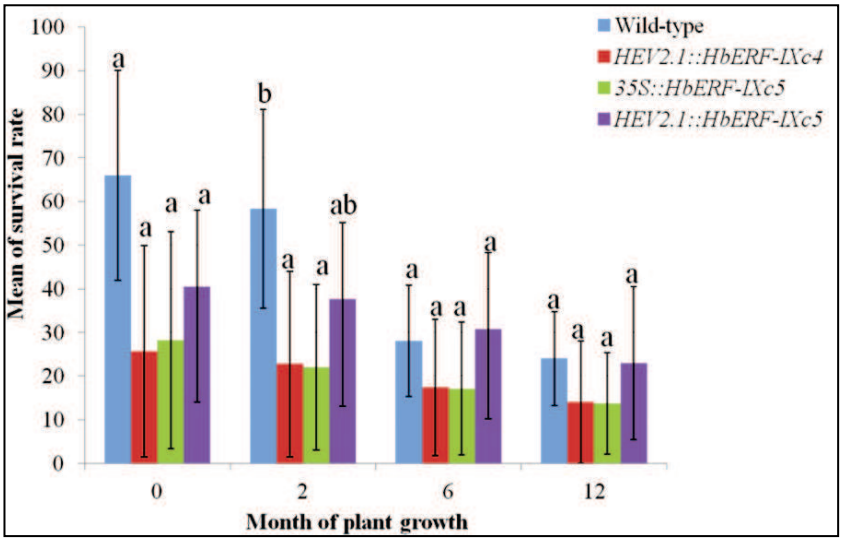

Figure 42. Diagram of the effect of construct to survival plant. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter are not significantly different at the 0.05 probability level.

Table 22. Effect of construct on mean of survival rate.

Construct	Month of plant growth			
	0	2	6	12
Wild-type	66.000 [a]	58.333 [b]	28.000 [a]	24.000 [a]
HEV2.1::HbERF-IXc4	25.654 [a]	22.731 [a]	17.385 [a]	14.000 [a]
35S::HbERF-IXc5	28.167 [a]	22.000 [a]	17.083 [a]	13.750 [a]
HEV2.1::HbERF-IXc5	40.467 [a]	37.667 [ab]	30.733 [a]	22.933 [a]

Analysis of survival rate described the number of plants per month divided by the number of plants at 0 month. This analysis showed the capabilities of survival rate of transgenic plants were higher compared to wild-type, except two transgenic lines from construct of *35S::HbERF-IXc5* (TS19A90) and *HEV2.1::HbERF-IXc5* (TS20A47). TS18A20 showed the highest values of survival rate (83%), but this data is not reliable because of the small number of plants at the 0 month (7 plantlets), and only 2 plants could not survive until 12 months of acclimatization. TS20A47 had the lowest survival rate (17%) because from 4 plantlets only 1 plantlet can survive until 12 months of acclimatization (Table 23).

Table 23. Mean of survival rate plants from 0 month (M0) until 12 months (M12).

Construct	Lines	M0/M0	M2/M0	M6/M0	M12/M0
Wild-type	CI07060	100%	88%	41%	35%
HEV2.1::HbERF-IXc4	TS18A37	100%	80%	60%	49%
	TS18A09	100%	93%	84%	40%
	TS18A13	100%	92%	62%	52%
	TS18A20	100%	100%	83%	83%
	TS18A69	100%	98%	75%	62%
35S::HbERF-IXc5	TS19A46	100%	79%	73%	71%
	TS19A90	100%	84%	56%	33%
HEV2.1::HbERF-IXc5	TS20A47	100%	100%	17%	17%
	TS20A69	100%	95%	82%	59%
	TS20A75	100%	92%	71%	48%

5.2. Analysis of height of plants from wild-type and various transgenic lines

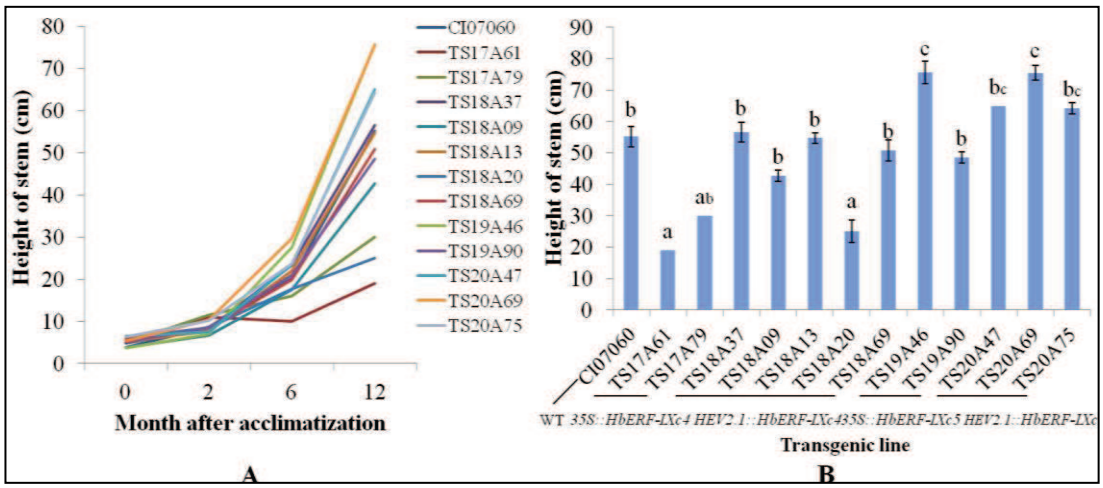

Figure 43. Analysis of height of plants from wild-type and various transgenic lines. (A) Evolution of plants height for 12-month-old (B) Height of 12-month-old plants. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Analysis on Figure 43A showed five transgenic lines had a higher height compared to wild-type. They were TS18A37, TS19A46, TS20A47, TS20A69, and TS20A75. Based on statistical analysis (Figure 43B) showed there were two transgenic lines TS19A46 and TS20A69 had significantly differences compared to wild-type. Figure 44 showed the transgenic lines could grow 1.5-2 times higher than wild-type.

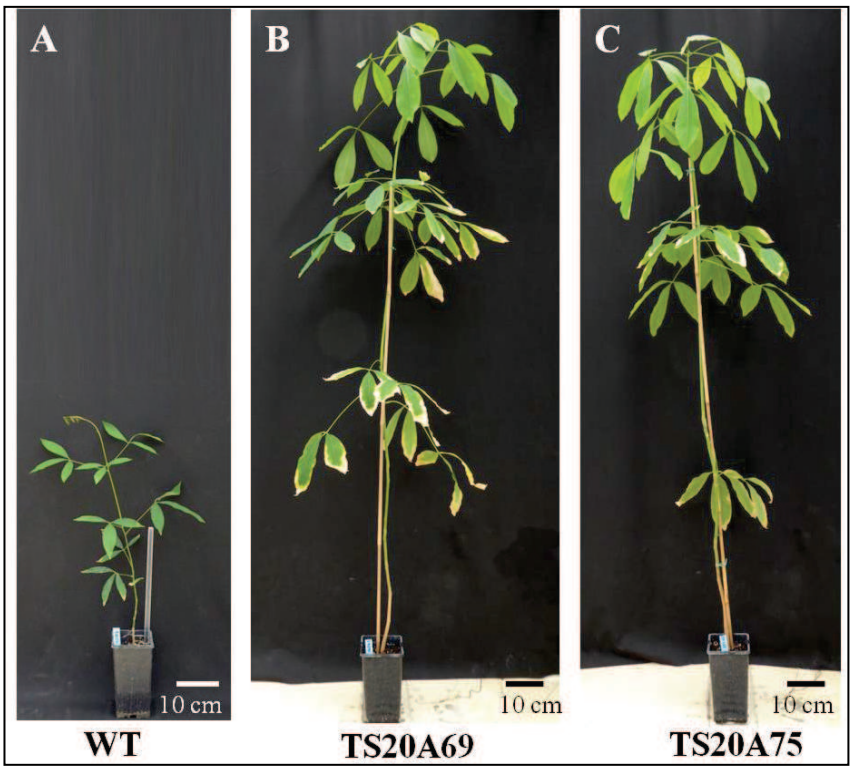

Figure 44. Twelve-month-old plants.

Analysis of plant height showed a significant difference in 0 month of plant before acclimatization between plants from constructs *HEV2.1::HbERF-IXc5* and wild-type. On the other hand, there is no significantly different in month 2, 6, and 12 of plant growth (Table 24).

Table 24. Effect of constructs on plant height.

Construct	Month after acclimatization			
	0	**2**	**6**	**12**
Wild-type	3.747 [a]	8.656 [ab]	19.789 [a]	54.547 [ab]
HEV2.1::HbERF-IXc4	5.044 [ab]	7.797 [a]	20.172 [a]	47.236 [a]
35S::HbERF-IXc5	4.591 [ab]	7.977[a]	23.517 [a]	58.767 [ab]
HEV2.1::HbERF-IXc5	5.821 [b]	10.239 [b]	26.695 [a]	69.859 [b]

5.3. Analysis of stem diameter of plants from wild-type and various transgenic lines

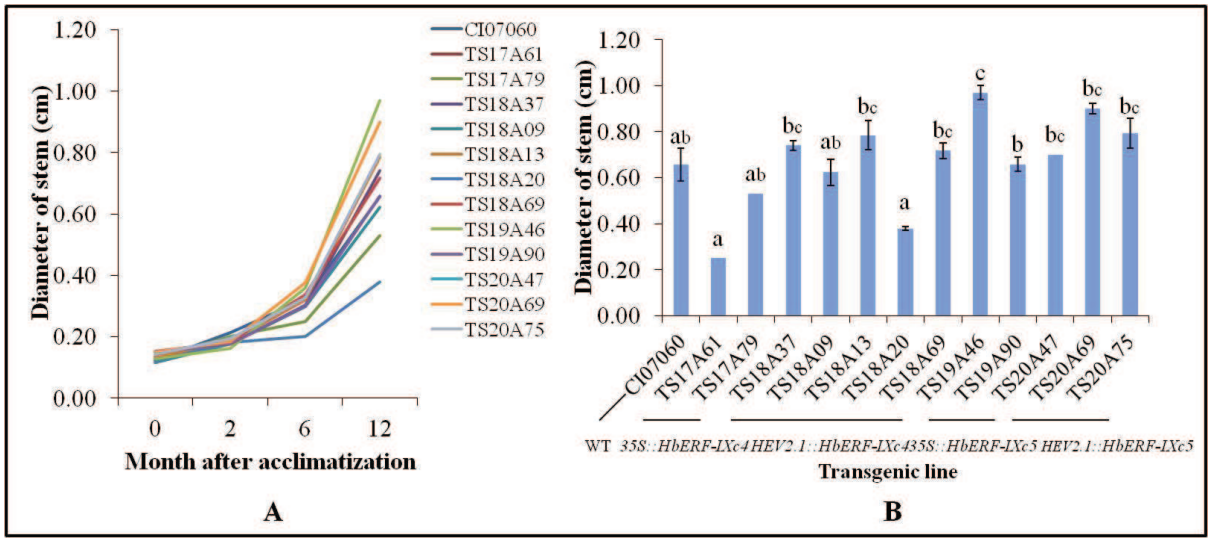

Figure 45. Analysis of stem diameter of plants from wild-type and various transgenic lines. (A) Evolution of plant stem diameter for 12-month-old (B) Stem diameter of 12-month-old plants. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

The mean of stem diameter values (Figure 45A) showed some transgenic lines (TS18A37, TS18A13, TS18A69, TS20A47, TS20A69, and TS20A75) had a greater stem diameter than wild-type. Statistical analysis (Figure 45B) showed no significant difference between wild-type and transgenic lines except the construct of *35S::HbERF-IXc5* (TS19A46). TS19A46 had highest average value of stem diameter of 12-month-old plants and more vigorous compared the others.

Based on statistical analysis, there is significantly different in diameter of plants between constructs *HEV2.1::HbERF-IXc4* and *35S::HbERF-IXc5* compared to wild-type at 2 months of plant growth. There is no significant difference in plant growth for 0, 6, and 12 months after acclimatization between wild-type and the various tested constructs (Table 25).

Table 25. Effect of constructs on plant stem.

Construct	Month after acclimatization			
	0	2	6	12
Wild-type	0.124 [a]	0.213 [b]	0.323 [a]	0.657 [a]
HEV2.1::HbERF-IXc4	0.132 [a]	0.179 [a]	0.314 [a]	0.715 [a]
35S::HbERF-IXc5	0.141 [a]	0.172[a]	0.323 [a]	0.775 [a]
HEV2.1::HbERF-IXc5	0.147 [a]	0.190 [ab]	0.352 [a]	0.847 [a]

5.4. Analysis of leaves and leaflets of plants from wild-type and various transgenic lines

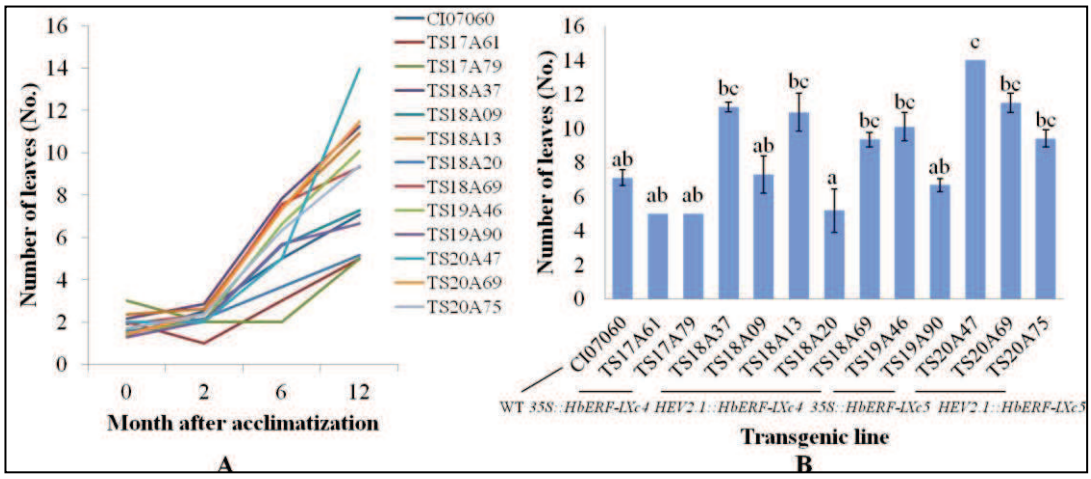

Figure 46. Analysis of leaves of plants from wild-type and various transgenic lines. (A) Evolution of number of plants leaves for 12-month-old (B) Number of leaves of 12-month-old plants. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

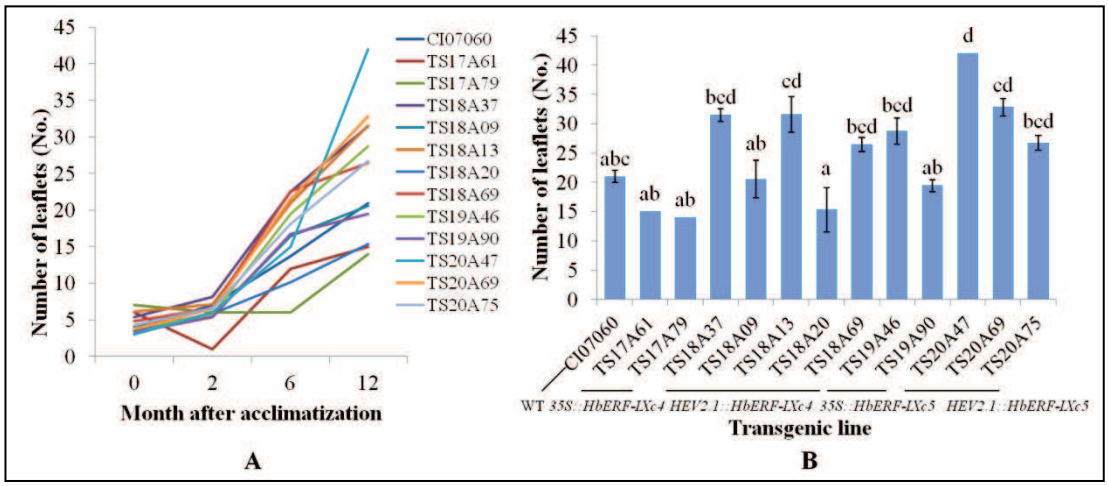

Figure 47. Analysis of leaflets of plants from wild-type and various transgenic lines. (A) Evolution of number of plants leaflets for 12-month-old (B) Number of leaflets of 12-month-old plants. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Number of leaves and leaflets from wild-type and transgenic lines tend to increase until the plants are 12-month-old, except TS17A61 which decreased the number of leaves and

leaflets in the 2nd month. Based on statistical analysis of leaves and leaflets, TS20A47 had the highest number of leaves and leaflets compared to wild-type (Figure 46 & 47). It was related with the lack of number of plants from this line.

Table 26. Effect of constructs on number of leaves.

Construct	Month after acclimatization			
	0	**2**	**6**	**12**
Wild-type	1.328 [a]	2.406 [a]	4.750 [a]	7.117 [a]
HEV2.1::HbERF-IXc4	1.939 [b]	2.404 [a]	6.425 [a]	8.847 [a]
35S::HbERF-IXc5	1.356 [a]	2.074 [a]	6.048 [a]	8.028 [a]
HEV2.1::HbERF-IXc5	1.590 [a]	2.385 [a]	6.741 [a]	10.229 [a]

Table 27. Effect of constructs on number of leaflets.

Construct	Month after acclimatization			
	0	**2**	**6**	**12**
Wild-type	3.489 [a]	6.739 [a]	12.989 [a]	12.050 [a]
HEV2.1::HbERF-IXc4	4.923 [b]	6.608 [a]	18.606 [a]	25.126 [a]
35S::HbERF-IXc5	3.428 [a]	5.574 [a]	17.798 [a]	23.186 [a]
HEV2.1::HbERF-IXc5	3.993 [a]	6.521 [a]	19.584 [a]	29.216 [a]

The statistical analysis showed there is no significant difference in the number of leaves and leaflets during the culture but in 0 month. There is a significant difference between construct *HEV2.1::HbERF-IXc4* and wild-type for 0 month (Table 26 and 27)

5.5. Analysis of weight of 12-month-old plants from wild-type and various transgenic lines

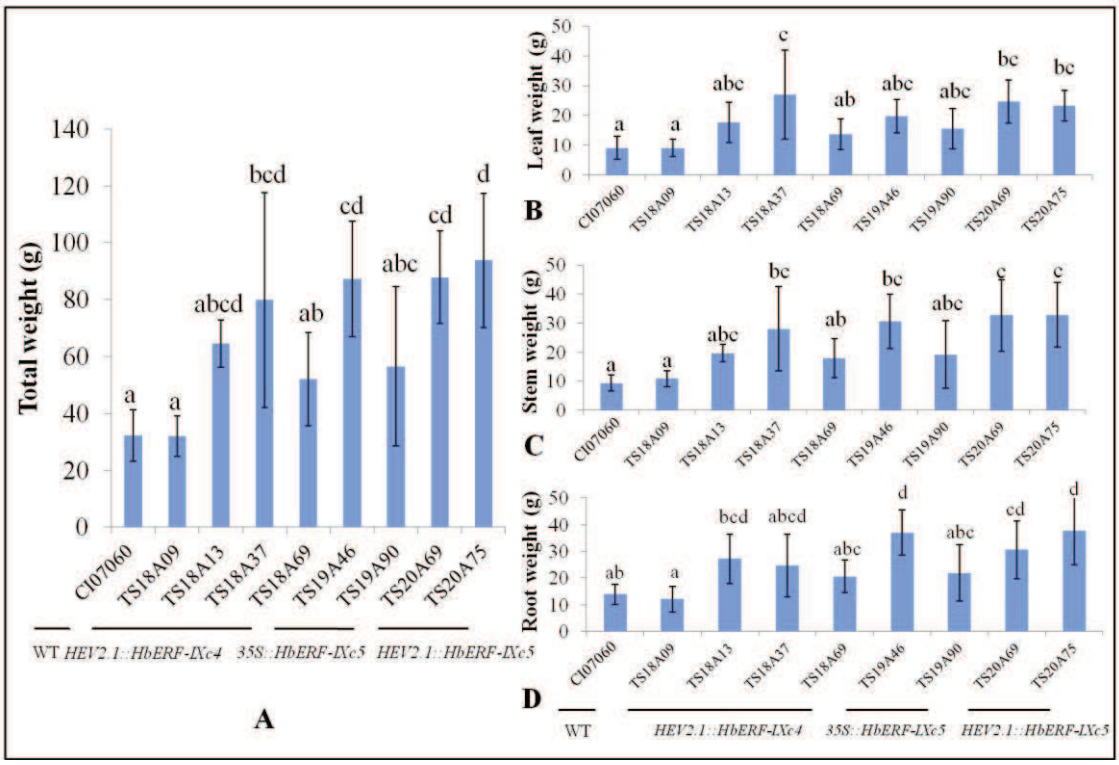

Figure 48. Analysis of (A) leaves weight, (B) stem weight, (C) total root weight, and (D) total plants weight of 12-month-old plants from wild-type and various transgenic lines. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Data of total plants weight was obtained from the sum of leaves weight, stem weight, and total roots weight. Statistical analysis of this data showed TS18A37, TS19A46, TS20A69, and TS20A75 had a significantly different with wild-type. These transgenic lines also showed a greater value of total plants weight compare to other (Figure 48A).

Statistical analysis of leaves weight of 12-month-old plants showed three transgenic lines (TS18A37, TS20A69, and TS20A75) had a significantly different compared to wild-type (Figure 48B). Analysis of stem weight showed four transgenic lines (TS18A37, TS19A46, TS20A69, and TS20A75) which significantly different with wild-type (Figure 48C), and analysis of total root weight showed three transgenic lines (TS19A46, TS20A69, and TS20A75) had a significantly different compared to wild-type (Figure 48D). These data suggest the construct of *HEV2.1::HbERF-IXc5* (TS20A69 and TS20A75) had a better growth and development plants compared to the wild-type.

Table 28. Effect of constructs on leaf weight (LW), stem weight (SW), total root weight (RW), and total plant weight of 12-month-old plants.

Construct	LW12	SW12	RW12	Total Plant Weight (g)
Wild-type	9.144 [a]	9.330 [a]	13.861 [a]	32.335 [a]
HEV2.1::HbERF-IXc4	16.785 [a]	19.038 [ab]	21.117 [ab]	56.940 [ab]
35S::HbERF-IXc5	17.666 [ab]	24.833 [bc]	29.439 [bc]	71.938 [bc]
HEV2.1::HbERF-IXc5	23.928 [b]	32.739 [c]	34.327 [c]	90.994 [c]

Weight of leaves, stem, total root and total plant from 12-month-old plants were statistically analysed. The results showed a significant difference in leaf weight between construct *HEV2.1::HbERF-IXc5* and wild-type. Weight of stem, total root and total plants were significantly different between constructs *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* compared to wild-type (Table 28).

5.6. Analysis of the root system in 12-month-old plants from wild-type and various transgenic lines

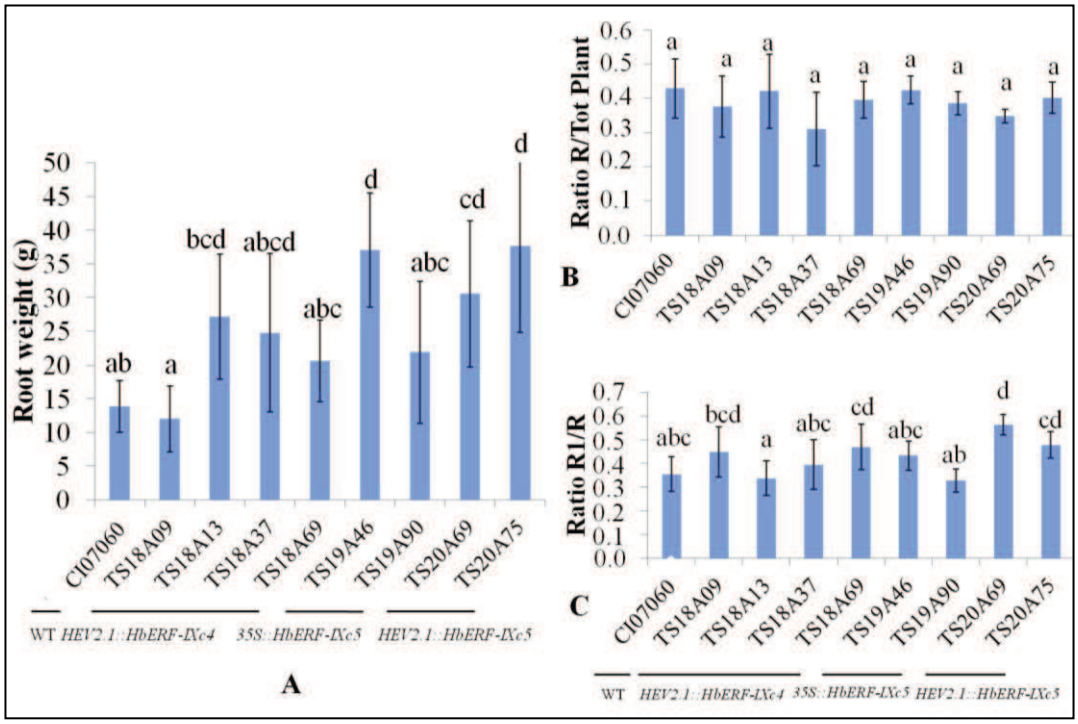

Figure 49. Analysis of (A) total root weight ratio, (B) Ratio R/total, and (C) Ratio R1/R plants from wild-type and various transgenic lines. The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Analysis on total weight root showed that there were three transgenic lines (TS19A46, TS20A69, and TS20A75) which significantly different with wild-type (Figure 49A), but from analysis on ratio root/total plant, the results showed there was no difference between wild-type and the others (Figure 49B). So, it means that the root system had a well-balanced development compared the whole plants. From the ratio R1/R, the construct of *HEV2.1::HbERF-IXc5* (TS20A69) had a significantly different value compare the wild-type (Figure 49C).

Table 29. Effect of constructs on total root weight (R), ratio root/tot plant and ratio R1/tot R plants.

Construct	LS means (RW12)	LS means (Ratio Root/tot plant)	LS means (Ratio R1/tot R)
Wild-type	13.861 [a]	0.434 [a]	0.360 [a]
HEV2.1::HbERF-IXc4	21.117 [ab]	0.377 [a]	0.415 [a]
35S::HbERF-IXc5	29.439 [bc]	0.409 [a]	0.386 [a]
HEV2.1::HbERF-IXc5	34.327 [c]	0.372 [a]	0.515 [b]

Table 29 showed the analysis of total root weight ratio, ratio R/tot plant, and ratio R1/R plants. There is no significant difference in ratio root/tot plant, but there is significant difference in total root weight ratio between constructs *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* compared to wild-type line. Analysis of ratio R1/tot R showed constructs *HEV2.1::HbERF-IXc5* have a significant difference to wild-type.

Table 30 showed a summary of morphological aspect for the different lines. TS20A69 showed higher performance compared to other lines.

Figure 50. Comparison roots of 12-month-old plants. (A) Total root of wild-type, (B) Taproot(R1) of wild-type, (C) Total root of TS19A46, (D) Taproot (R1) of TS19A46, (E) Total root of TS20A69, (F) Taproot (R1) of TS20A69, (G) Total root of TS20A75, (H) Taproot (R1) of TS20A75.

Table 30. Summary effect of overexpression of *HbERF-IXc4* or *HbERF-IXc5* on plant morphology. Notes: (blue) significantly different to controls with lower value, (orange) not significantly different to controls, (red) significantly different to controls with higher value and (white) no data.

Construct	Line	Height	Stem diameter	Leaves and leaflets	Total weight	Leaf weight	Stem weight	Root weight	Ratio R/tot plant	Ratio R1/tot R
35S::HbERF-IXc4	TS17A61									
	TS17A79									
HEV2.1::HbERF-IXc4	TS18A09									
	TS18A13									
	TS18A20									
	TS18A37									
	TS18A69									
35S::HbERF-IXc5	TS19A46									
	TS19A90									
HEV2.1::HbERF-IXc5	TS20A47									
	TS20A69									
	TS20A75									

6. Changes in some histological parameters

6.1. Analysis of quantitative and qualitative parameter from various wild-type and transgenic lines overexpressing *HbERF-IXc5*

A histo-cytological analysis study was undertaken to characterize the differences existing between wild-type (CI07060) and transgenic plants (TS19A46, TS19A90, TS20A69, and TS20A75 for *Hevea* clone PB260) overexpressing *HbERF-IXc5* under the control *35S CaMV* and *HEV2.1* promoter, respectively. The study was carried out on green stem, lignified stem, taproot (R1), and leaves on 12-month-old plants grown in greenhouse. Better

knowledge of the histo-cytology is required to examine tissue structures at the microscopic level in order to understand the physiological and anatomical functions between wild-type and transgenic plants.

6.1.1. Analysis of quantitative and qualitative parameters in leaves

Observations were carried out on two main parts of leaves: main nerve (counting of latex cells), and lamina (counting of latex cells, width measurement of cuticle, upper epidermis, palisade parenchyma, spongy parenchyma, lower epidermis, counting of palisade cell until 1-2 mm, and counting of stomata numbers) (Figure 51). Statistical analyses showed there was significantly higher number of latex cells in transgenic lines (TS19A46 and TS20A69) compared to wild-type. For lower epidermis, TS20A69 line showed significantly different values compared to wild-type and other transgenic lines. The width of lower epidermis of TS20A69 was thinner compared to others (Table 31).

Table 31. The effect of lines on mean value of width from different parts collected on leaf from wild-type and various transgenic lines overexpressing *HbERF-IXc5* and the number of palisade cells and stomata.

Construct	Line	Latex cell (No.) in main nerve	Lamina									
			Latex cell (No.)	Cuticle (µm)	Upper epidermis (µm)	Palisade parenchyma (µm)	Spongy parenchyma (µm)	Lower epidermis (µm)	Palisade cell for 1 mm (No.)	Palisade cell for 2 mm (No.)	Stomata (No.)
Wild-type	CI07060	50.50^{a}	4.50^{a}	1.62^{a}	7.25^{a}	41.66^{a}	49.05^{a}	8.17^{b}	69.67^{a}	148.67^{a}	4.33^{a}
35S::HbERF-IXc5	TS19A46	157.00^{c}	5.00^{a}	1.93^{a}	8.42^{a}	49.20^{a}	52.57^{a}	8.14^{b}	76.06^{a}	160.11^{a}	6.00^{a}
35S::HbERF-IXc5	TS19A90	37.00^{a}	3.00^{a}	1.69^{a}	8.26^{a}	45.03^{a}	52.57^{a}	7.52^{ab}	78.00^{a}	164.00^{a}	2.00^{a}
HEV2.1::HbERF-IXc5	TS20A69	113.00^{bc}	12.00^{a}	1.74^{a}	7.93^{a}	48.13^{a}	42.28^{a}	3.61^{a}	88.00^{a}	173.33^{a}	4.00^{a}
HEV2.1::HbERF-IXc5	TS20A75	84.00^{ab}	2.50^{a}	1.58^{a}	7.05^{a}	49.70^{a}	45.29^{a}	6.99^{ab}	76.39^{a}	160.17^{a}	6.33^{a}

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

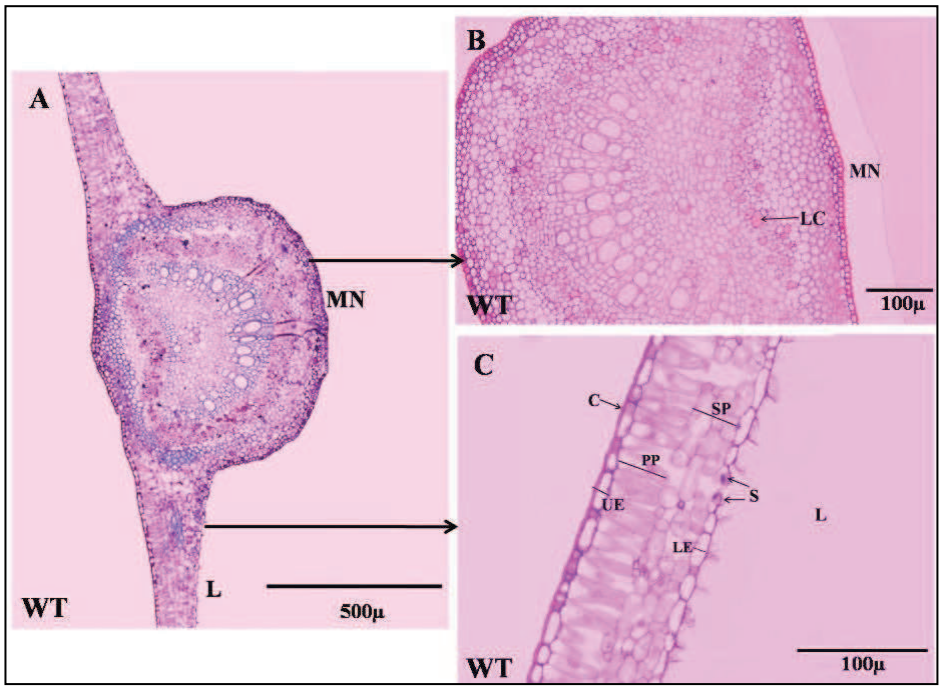

Figure 51. Histo-cytological description of leaf cross-section from WT plants of clone PB260. The histological sections were stained with Oil Red O. (A) Leaf section, (B) Main nerve, (C) Lamina. Cross-sections of leaf were annotated: MN. main nerve; LC. latex cell. C. cuticle; L. lamina; S. stomata; UP. upper epidermis; PP. palisade parenchyma; SP. spongy parenchyma; LE. lower epidermis.

Statistical analyses revealed a significant difference in the number of latex cells between the construct of *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* compared with wild-type. These data suggest that candidate gene of *HbERF-IXc5* had more capability for producing latex cells compared to wild-type (Table 32 and Figure 52).

Table 32. Effect of promoters driving the *HbERF-IXc5* gene on width mean value for different parts of leaves: number of palisade cells, and stomata.

Construct	Line	Latex cell (No.) in main nerve	Lamina								
			Latex cell (No.)	Cuticle (µm)	Upper epidermis (µm)	Palisade parenchyma (µm)	Spongy parenchyma (µm)	Lower epidermis (µm)	Palisade cell for 1 mm (No.)	Palisade cell for 2 mm (No.)	Stomata (No.)
Wild-type	CI07060	50.50[a]	4.50[a]	1.62[a]	7.25[a]	41.66[a]	49.05[a]	8.17[a]	69.67[a]	148.67 [a]	4.33[a]
35S::HbERF-IXc5	TS19A46	157.00[c]	5.00[a]	1.93[a]	8.42[a]	49.20[a]	52.57[a]	8.14[a]	76.06[a]	160.11[a]	6.00[a]
HEV2.1::HbERF-IXc5	TS20A75	84.00[b]	2.50[a]	1.58[a]	7.05[a]	49.70[a]	45.29[a]	6.99[a]	76.39[a]	160.17[a]	6.33[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

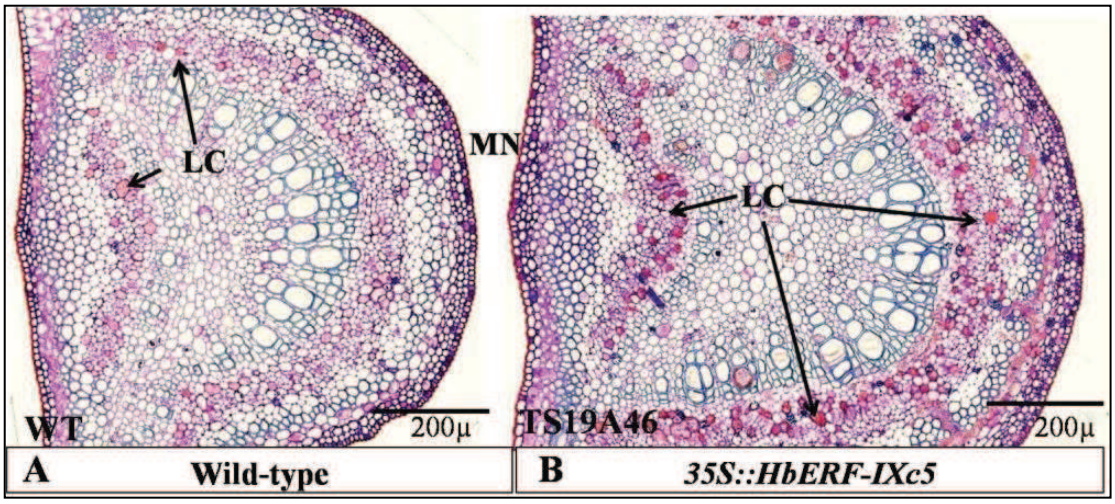

Figure 52. Localization of latex cells in leaves. (A) Wild-type (CI07060), (B) Transgenic line TS19A46. The histological sections were stained with Oil Red O. Cross-sections of leaf were annotated: MN. main nerve; LC. latex cells; WT. wild-type.

The qualitative analysis led to identify more starch reserves in main nerve compared to lamina. Starch was observed by staining periodic Acid-Schiff and Naphtol Blue Black (NBB) in pink-red colour. These dyes specifically stained soluble and storage proteins, like polysaccharides (starch) in violet. This histological analysis allowed identifying some compounds. Lines TS19A90 (*35S::HbERF-IXc5*) and TS20A69 (*HEV2.1::HbERF-IXc5*) had the highest starch content in main nerve (Table 33). Starch represents the most important carbohydrate used for nutrient. Starch accumulates mainly in the parenchyma and maybe related to growth in diameter and in length of the stem. Analysis of polyphenol showed that polyphenol was less produced in wild-type and transgenic lines. Polyphenol can be stained by Oil Red O in the dark blue.

Table 33. Evaluation of starch and polyphenol contents in leaves of plants from wild-type and transgenic lines. Notes: (-): absence; (+): rare; (++): 10─20 %; (+++): 20─50 %; (++++): >50 %.

Construct	Line	Starch		Polyphenol	
		Main nerve	Lamina	Main nerve	Lamina
Wild-type	CI07060	+	+	+	+
35S::HbERF-IXc5	TS19A46	++	+	+	+
35S::HbERF-IXc5	TS19A90	+++	+	++	+
HEV2.1::HbERF-IXc5	TS20A69	+++	+	+	-
HEV2.1::HbERF-IXc5	TS20A75	++	+	+	+

6.1.2. Analysis of quantitative and qualitative parameters of green stem

Width of bark, cambium, xylem, pith, and the percentage of pith have been measured and calculated, respectively in green stem (Figure 53). Transgenic line TS20A69 had wider cambium compared to wild-type and other transgenic lines. This result showed that TS20A69 had bigger the activity of cambium, because it might be due to transgene position effect. This effect probably influenced the cambium activity.

The width of pith of TS20A75 was significantly different compared to wild-type and others transgenic lines. The pith or medulla is a tissue in the stem of vascular plants which store and transport nutrients throughout the plant. The size of pith is probably related with the capability of plant to grow and develop. Statistical analysis showed that TS19A46 had significant difference in total radius of green stem. The averages of total radius of green stem were 3237 µm for TS19A46. There was not any difference in percentage of pith between wild-type and transgenic lines (Table 34).

Table 34. Effect of lines on mean value of width from parts collected on green stem from various wild-type and transgenic lines overexpressing *HbERF-IXc5*.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of green stem (µm)	Percentage of pith (%)
Wild-type	CI07060	293^{a}	25^{a}	790^{a}	844^{a}	1953^{a}	42.4^{a}
35S::HbERF-IXc5	TS19A46	538^{a}	27^{a}	1041^{a}	1631^{ab}	3237^{b}	50.1^{a}
35S::HbERF-IXc5	TS19A90	435^{a}	27^{a}	642^{a}	1246^{ab}	2349^{ab}	53.0^{a}
HEV2.1::HbERF-IXc5	TS20A69	435^{a}	62^{b}	1135^{a}	1280^{ab}	2911^{ab}	44.0^{a}
HEV2.1::HbERF-IXc5	TS20A75	397^{a}	32^{a}	572^{a}	1833^{b}	2834^{ab}	65.0^{a}

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

Statistical analysis of effect of construct used 3 replicates for wild-type and 3 replicates for each construct. Pith size increased dramatically in transgenic plants compared to wild-type (42.4% of total radius) and this difference become significant for construct *HEV2.1::HbERF-IXc5* (65.0%). Construct *35S::HbERF-IXc5* had a significant difference in bark. Construct *HEV2.1::HbERF-IXc5* had a significant difference in percentage of pith, and for both construct *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* had significant difference in radius of pith and total radius of green stem. These data suggested that both constructs promoted the capability of transgenic plants to grow more vigorously and became taller (Table 35).

Table 35. Effect of promoters driving the *HbERF-IXc5* gene on mean value of width from parts collected on green stem from wild-type (line CI07060) and various transgenic lines.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of green stem (µm)	Percentage of pith (%)
Wild-type	CI07060	293[a]	25[a]	790[a]	844[a]	1953[a]	42.4[a]
35S::HbERF-IXc5	TS19A46	538[b]	27[a]	1041[a]	1631[b]	3237[b]	50.1[ab]
HEV2.1::HbERF-IXc5	TS20A75	397[ab]	32[a]	572[a]	1833[b]	2834[b]	65.0[b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

Starch content was found in all parts of green stem section (bark, xylem, and pith) with almost the same percentage in wild-type and transgenic plants. Likewise, the content of polyphenol was found with lower percentage in all parts of tissue. Primary latex cell (PLC) and secondary laticifer (SL) was found in the leaves for all lines. It was interesting to notice the number of PLC and SL for TS19A46 (*35S::HbERF-IXc5*) was higher compared the other followed by TS20A75 (*HEV2.1::HbERF-IXc5*) (Table 36). Laticifer differentiation from vascular cambium in *Hevea* stems are good model for observing cell differentiation from the cambium. On the green stem, SL has not yet performed the ring of laticifer. The number of SL was high but they are still separated or in a group, not yet formed the anastomoses. Laticifer cells had thick cell wall, non-transparent, elastic cytoplasm, and stained in pink-red. Most of SL is distributed randomly (Figure 53).

Table 36. Evaluation of starch, polyphenol contents, primary latex cells (PLC), and secondary laticifer (SL) on green stem from various wild-type and transgenic lines. Notes: (-): absence; (+): rare; (++): 10─20 %; (+++): 20─50 %; (++++): >50 %.

Construct	Line	Starch			Polyphenol			PLC	SL
		Bark	Xylem	Pith	Bark	Xylem	Pith		
Wild-type	CI07060	+	+	+	+	+	-	+	+
35S::HbERF-IXc5	TS19A46	+	+	+	++	+	+	+++	+++
35S::HbERF-IXc5	TS19A90	+++	++	+++	++	+	++	+	+
HEV2.1::HbERF-IXc5	TS20A69	+++	++	++	+	+	+	+	+
HEV2.1::HbERF-IXc5	TS20A75	+	++	+	+	+	+	++	++

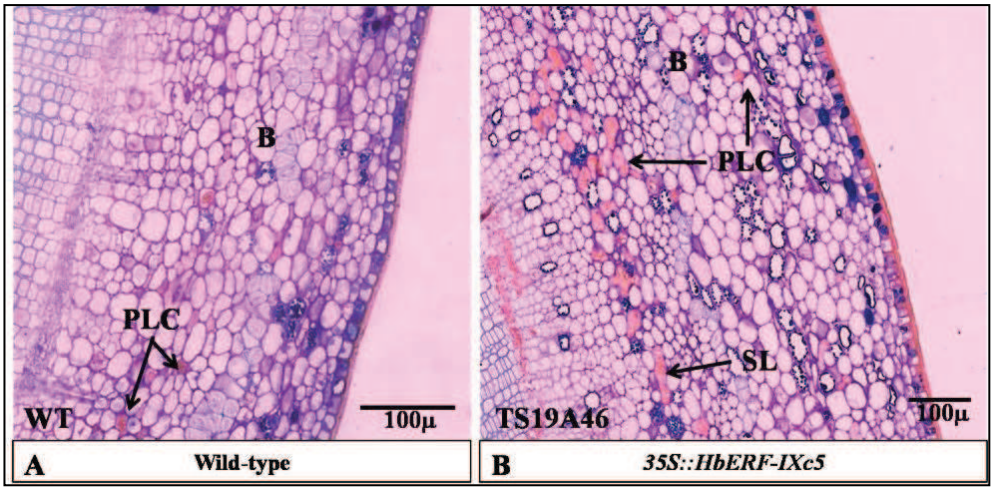

Figure 53. Histo-cytological descriptions of latex cells on green stem. (A) wild-type clone PB260, (B) transgenic line (TS19A46). The histological sections were stained with Oil Red O. Cross-sections of green stem were annotated: B. bark; PLC. primary latex cells; SL. secondary laticifer

6.1.3. Analysis of quantitative and qualitative parameter of lignified stem

The quantitative analyses of lignified stem covered the width of bark, cambium, xylem, pith, and the percentage of pith (Figure 54). There was no significant difference for all the measured parameters, except the width of xylem of TS19A46 (Table 37). The average width of xylem of TS19A46 was widest compared to other transgenic lines.

Table 37. Effect of lines on mean value of width from parts collected on lignified stem from various wild-type and transgenic lines overexpressing *HbERF-IXc5*.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of lignified stem (µm)	Percentage of pith (%)
Wild-type	CI07060	634[a]	67[a]	2165[a]	455[a]	3321[a]	13.9[a]
35S::HbERF-IXc5	TS19A46	861[a]	100[a]	3384[b]	475[a]	4819[a]	9.8[a]
35S::HbERF-IXc5	TS19A90	693[a]	81[a]	2237[ab]	406[a]	3417[a]	11.9[a]
HEV2.1::HbERF-IXc5	TS20A69	610[a]	96[a]	2317[ab]	408[a]	3431[a]	11.9[a]
HEV2.1::HbERF-IXc5	TS20A75	856[a]	101[a]	2864[ab]	466[a]	4287[a]	10.7[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

Based on statistical analysis, effect of construct used 2 replicates for wild-type and 3 replicates for each construct supported the statistical analysis of lines. The data showed that the width of xylem and total radius of lignified stem from construct of *35S::HbERF-IXc5* had significant difference compared to others transgenic lines (Table 38). This data suggested the width of xylem supposed correlated with the development of plants. Various shapes of pith on lignified stem were found from the observation (Figure 54).

Table 38. Effect of promoters driving the *HbERF-IXc5* gene on mean value of width from parts collected on lignified stem from wild-type (line CI07060) and transgenic lines.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of lignified stem (µm)	Percentage of pith (%)
Wild-type	CI07060	634[a]	67[a]	2165[a]	455[a]	3321[a]	13.9[a]
35S::HbERF-IXc5	TS19A46	861[a]	100[a]	3384[b]	475[a]	4819[b]	9.8[a]
HEV2.1::HbERF-IXc5	TS20A75	856[a]	101[a]	2864[ab]	466[a]	4287[ab]	10.7[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

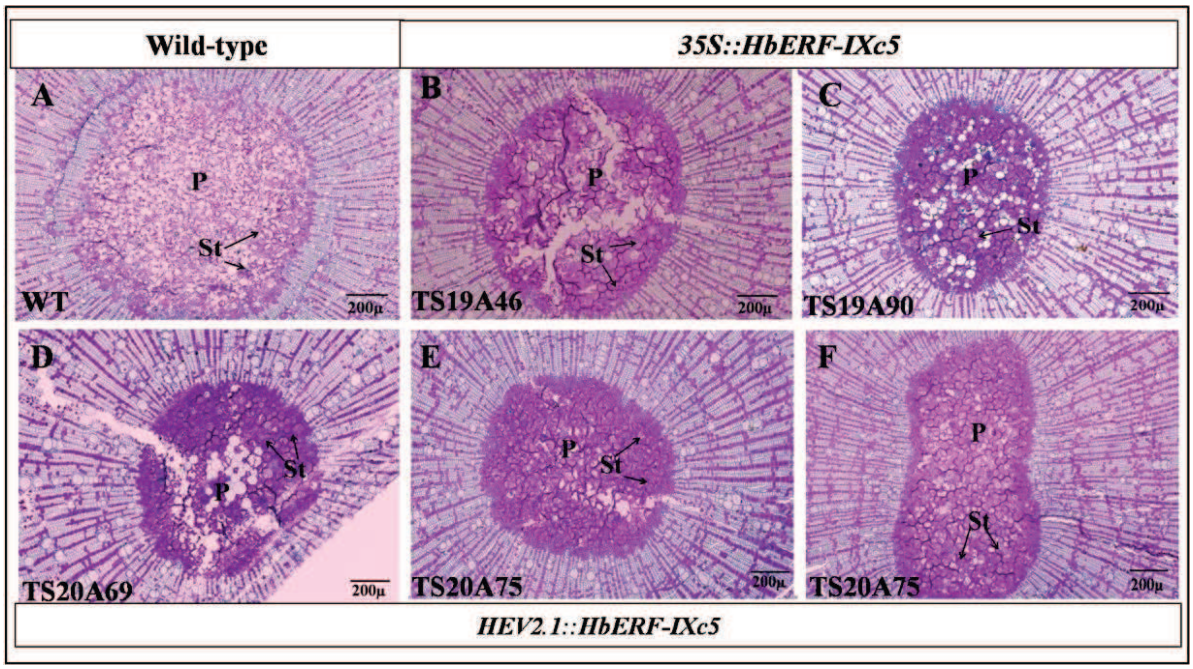

Figure 54. Pith shape in wild-type and transgenic lines. (A) wild-type, (B) TS19A46, (C) TS19A90, (D) TS20A69, (E) TS20A75, (F) TS20A75. The histological sections were stained with Schiff Naphthol Blue Black. Cross-sections of lignified stem were annotated: P. pith; St. starch; WT. wild-type.

Starch content was found in all parts of lignified stem section (bark, xylem, and pith) (Figure 55 and Table 38). Interestingly, numerous starch grains were found in xylem and pith, but less found in the bark. Starch reserves were more abundant in lignified stem of transgenic lines compared to the wild-type. On the contrary, polyphenol compounds were less abundant in bark, xylem, and pith, even polyphenol compounds were absent in some parts, like xylem and pith of wild-type (Figure 56).

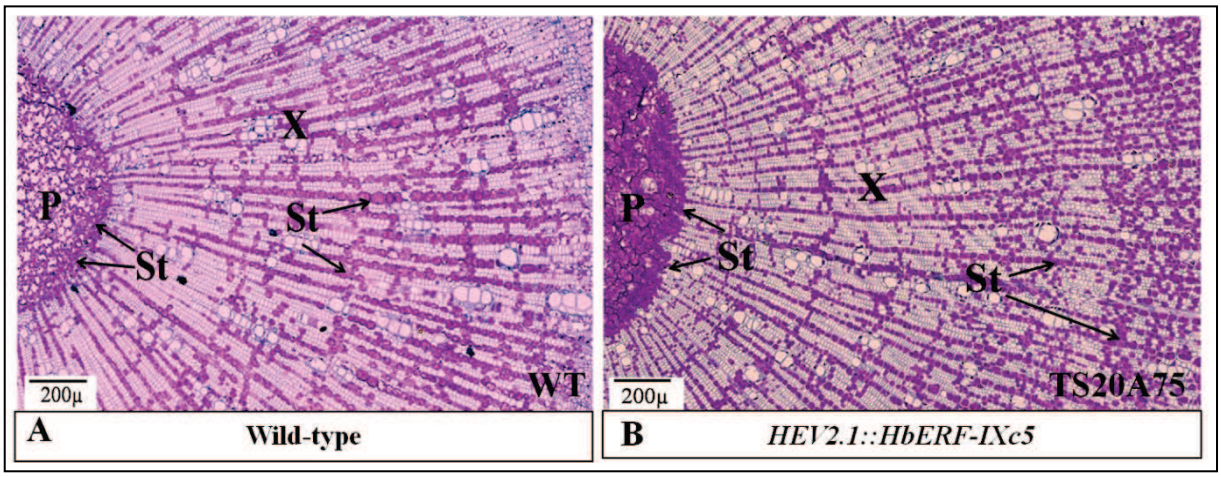

Figure 55. Content of starch of TS20A75. The histological sections were stained with Schiff Naphthol Blue Black. Cross-sections of lignified stem were annotated: X. xylem; P. pith; St. Starch.

Table 39. Evaluation of the presence of starch reserves, polyphenol compounds, and secondary laticifer (SL) in lignified stem from wild-type and transgenic lines. Notes: (-): absence; (+): rare; (++): 10─20 %; (+++): 20─50 %; (++++): >50 %.

Construct	Line	Starch			Polyphenol			SL
		Bark	Xylem	Pith	Bark	Xylem	Pith	
Wild-type	CI07060	+	+++	+++	+	-	-	+++
35S::HbERF-IXc5	TS19A46	+	++++	++++	++	+	+	++++
35S::HbERF-IXc5	TS19A90	+	++++	++++	+	+	+	++
HEV2.1::HbERF-IXc5	TS20A69	+	+++	++++	+	+	-	+
HEV2.1::HbERF-IXc5	TS20A75	+	++++	++++	+	+	+	++

There were a lot of secondary laticifers on lignified stem, especially from TS19A46 (*35S::HbERF-IXc5*) following the maturity and development of plant. The structural development of laticifers was formed through increased anastomoses and a balance in division and growth to become ring of laticifers. Cell division of cambium results in the formation of secondary phloem including secondary laticifers.

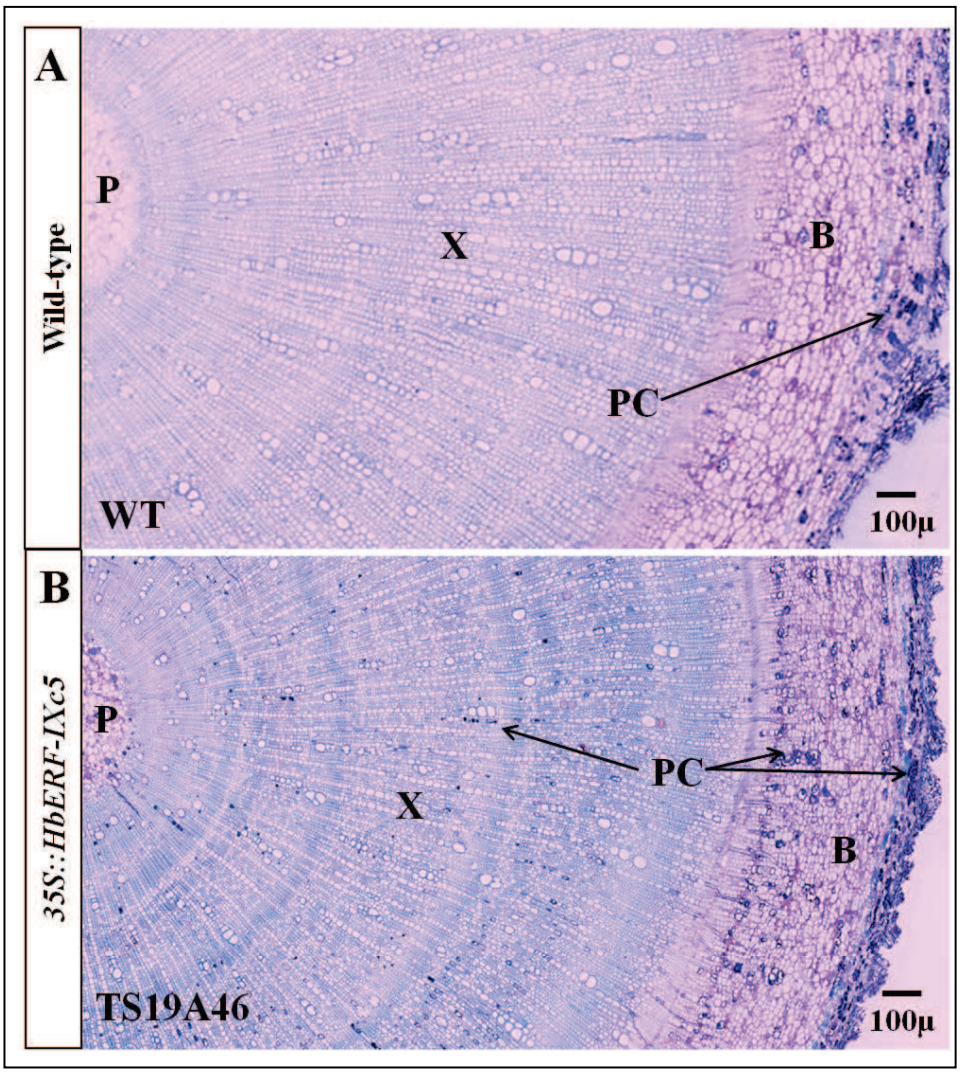

Figure 56. Comparison of phenolic compounds. (A) wild-type clone PB260, (B) transgenic line (TS19A46). The histological sections were stained with Oil Red O. Cross-sections of lignified stem were annotated: B. bark; X. xylem; P. pith; PC. phenolic compounds; WT. wild-type.

6.1.4. Analysis of quantitative and qualitative parameter of taproot

The analyses of quantitative of taproot (R1) covered the width of bark, cambium, xylem, pith, and the percentage of pith (Figure 57). Statistical analysis showed that there was significant difference of the width of cambium between TS20A69 and TS20A75 compared the wild-type and the other transgenic lines. The cross section of taproot was visualised on a section close from the cambium. The differences of cambium were supposedly influenced the physiological and metabolism activity in plants. Statistical analysis also showed significant difference in percentage of pith for line TS20A69 (Table 40).

Table 40 Effect of lines on mean value of width from parts collected on taproot (R1) from various wild-type and transgenic lines overexpressing *HbERF-IXc5*.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of taproot (µm)	Percentage of pith (%)
Wild-type	CI07060	463[a]	38[a]	2536[a]	687[a]	3724[a]	18.5[b]
35S::HbERF-IXc5	TS19A46	637[a]	28[a]	2842[a]	224[a]	3732[a]	5.5[ab]
35S::HbERF-IXc5	TS19A90	328[a]	31[a]	1352[a]	287[a]	1997[a]	14.4[ab]
HEV2.1::HbERF-IXc5	TS20A69	423[a]	75[b]	1832[a]	94[a]	2423[a]	3.9[a]
HEV2.1::HbERF-IXc5	TS20A75	570[a]	73[b]	3131[a]	469[a]	4242[a]	9.6[ab]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

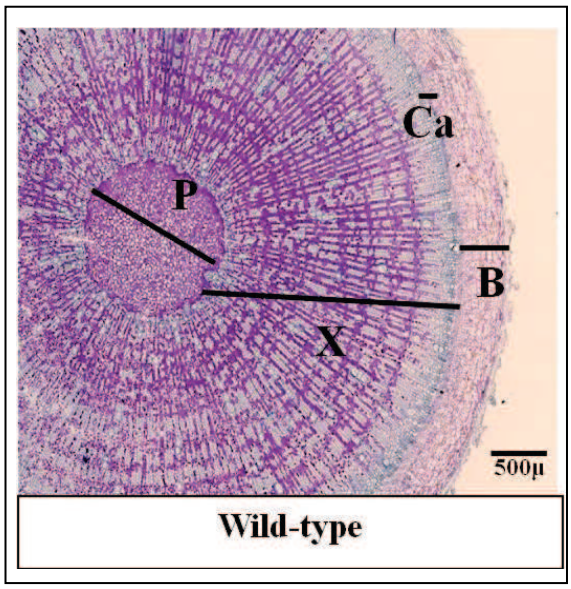

Figure 57. Histo-cytological analysis of taproot (R1) from WT plant for clone PB260. The histological sections were stained with Schiff Naphthol Blue Black and Oil Red O. Cross-sections of taproot1 (R1) were annotated: B. bark; Ca. cambium; X. xylem; P. Pith.

Statistical analysis of effect of construct used 2 replicates for wild-type and 3 replicates for each construct. Statistical analysis of construct showed that *HEV2.1::HbERF-IXc5* was significant difference of width of cambium compared to wild-type and *35S::HbERF-IXc5*. There were significant difference of percentage of pith from construct *35S::HbERF-IXc5* compared to wild-type. The percentage of pith of *35S::HbERF-IXc5* was lower (5.5%) (Table 41).

Table 41. Effect of promoters driving the *HbERF-IXc5* gene on mean value of width from parts collected on taproot (R1) from wild-type (line CI07060) and various transgenic lines.

Construct	Line	Bark (µm)	Cambium (µm)	Xylem (µm)	Pith (µm)	Total radius of taproot (µm)	Percentage of pith (%)
Wild-type	CI07060	463[a]	38[a]	2536[a]	687[a]	3724[a]	18.5[b]
35S::HbERF-IXc5	TS19A46	637[a]	28[a]	2842[a]	224[a]	3732[a]	5.5[a]
HEV2.1::HbERF-IXc5	TS20A75	570[a]	73[b]	3131[a]	469[a]	4242[a]	9.6[ab]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by Tukey (5%) test. Values with the same letter are not significantly different at the 0.05 probability level.

There was different of width of cambium between TS20A75 and wild-type (Figure 58). Cambium is a group of meristem cells with a thin layer tissue that is generated from procambium and promotes the secondary growth of xylem and phloem. Cambium has the most active cells. Cell division and differentiation in the cambium lead to the thickening of stems and roots and correlated the increasing of biomass (Miyashima et al. 2013). These data suggested the different value of parameters could influence the total biomass of plants.

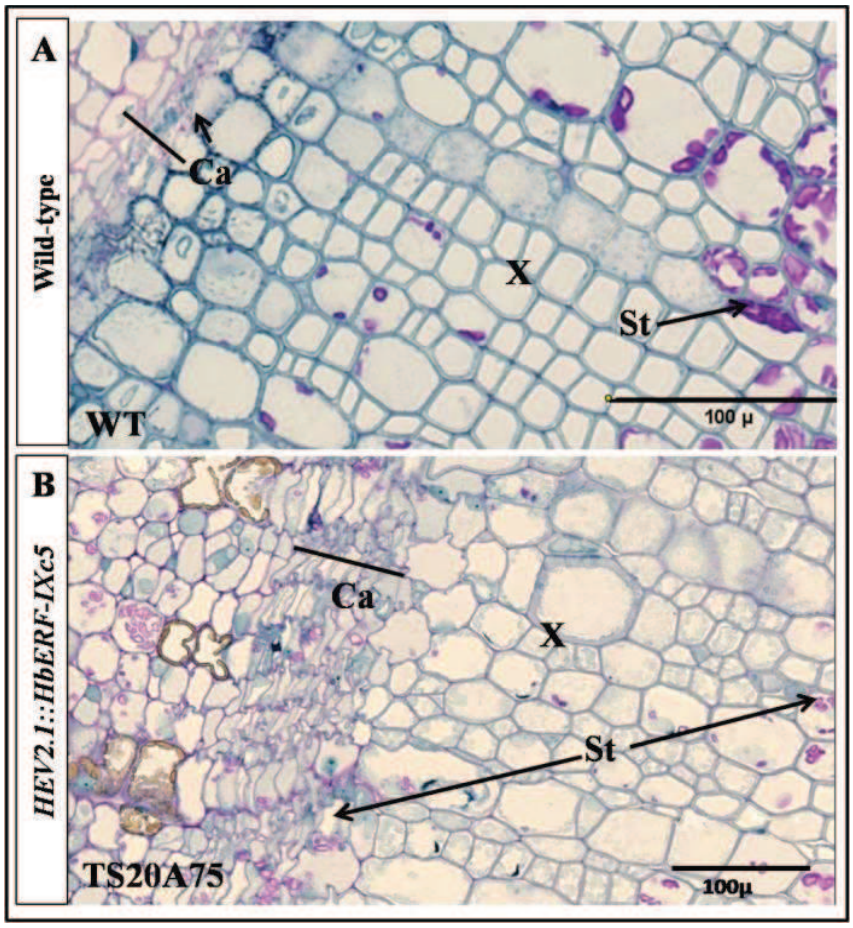

Figure 58. Comparison of cambium length between WT and transgenic line (TS20A75) from clone PB 260. The histological sections were stained with Schiff Naphthol Blue Black. Cross-sections of taproot (R1) were annotated: Ca. cambium; X. xylem; St. starch; WT. wild-type.

Taproot (R1) showed high starch accumulation in all parts section (bark, xylem, and pith) from both wild-type and transgenic lines (TS19A46, TS19A90, TS20A69, and

TS20A75), but lower accumulation of polyphenol compounds in all tissues except in bark (Table 42).

Table 42. Evaluation of the presence of starch, polyphenol, and secondary laticifer (SL) on taproot (R1) from various wild-type and transgenic lines**.** Notes: (-): absence; (+): rare; (++): 10─20 %; (+++): 20─50 %; (++++): >50 %.

Construct	Line	Starch			Polyphenol			SL
		Bark	Xylem	Pith	Bark	Xylem	Pith	
Wild-type	CI07060	+	+++	+++	++	+	+	+
35S::HbERF-IXc5	TS19A46	++	++++	++++	+++	+	+	+
35S::HbERF-IXc5	TS19A90	++++	++++	++++	+++	+	+	+
HEV2.1::HbERF-IXc5	TS20A69	+++	+++	+++	++	+	+	+
HEV2.1::HbERF-IXc5	TS20A75	++++	++++	+++++	++	+	+	+

Table 43. Summary effect of construct on plant anatomy. Notes: (orange) transgenic lines not significantly different to control and (red) significantly different to controls with higher value.

Construct	Line	Leaves		Green stem			Lignified stem			Taproot		
		Latex cell (No.)	Starch	Cambium (µm)	Xylem (µm)	Starch	Cambium (µm)	Xylem (µm)	Starch	Cambium (µm)	Xylem (µm)	Starch
35S::HbERF-IXc5	TS19A46											
	TS19A90											
HEV2.1::HbERF-IXc5	TS20A69											
	TS20A75											

The summary effect of lines in all histological aspect is shown in Table 43. So, we can conclude that TS20A69 had better performance in histological aspect compared with other lines.

7. Effect of abiotic and biotic stresses on plant ecophysiology and morphology

Several ecophysiological parameters were monitored after stress treatments in order to study the specific responses developed in transgenic lines harbouring *HbERF-IXc4* and *HbERF-IXc5* genes. Fv/Fm value, P. Index, and SPAD value were measured to know the effect of abiotic stress to plant physiology. In the other hand number of leaflets, abscission rate, and leaflets senescence were observed to know the effect of abiotic stress to plant morphology. The change in Fv/Fm was examined to determine the effect of different light intensity on the efficiency potential of photosystem II (PSII) photochemistry. Fv/Fm value reflects the maximum quantum efficiency of PSII has been widely used for early detection of stress in plant (Sharma et al. 2015). A decrease in Fv/Fm ratio indicates damage of thylakoid membranes, the lower damage of thylakoid membrane showed higher tolerance to stress (Kadir, Von Weihe and Al-Khatib 2007). Plant vitality could be characterized by performance index P. Index. P. Index was commonly used as a stress parameter, which shows the efficiency of the two photosystems (I and II). Data from P. Index along with Fv/Fm was used to identify the efficiency of photosystems and total chlorophyll content. SPAD is an indicator of the plant physiological status to evaluate the leaflets chlorophyll content (Kadir et al. 2007, Strasser, Tsimilli-Michael and Srivastava 2000).

Several observations of leaflets were recorded after stress treatments in order to study the specific responses developed in transgenic lines harbouring *HbERF-IXc4* and *HbERF-IXc5* lines. Various symptoms were exhibited by plants after stress from leaf senescence to leaf abscission through wilting, burned leaflet, combination of wilting and burned leaflets, and degradation of colours. Leaflet senescence is the process of aging in plants. Leaflets senescence involves a coordinated action at the cellular, tissue, organ, and organism levels and influenced by plant hormones (Lim, Kim and Nam 2007). Wilting leaflets is the

condition when plants have lost rigidity and the turgor pressure towards zero, as a result of water deficit in cells. The rate of water loss is greater than absorption of water in the plant. Burned leaflet is defined as a browning of plant tissues, including leaf margins and tips, and yellowing or darkening of veins which may lead to wilting and abscission of the leaflets.

7.1. Effect of cold stress on plant ecophysiology and morphology

In this study, various transgenic lines harbouring *HEV2.1::HbERF-IXc4*, *35S::HbERF-IXc5*, and *HEV2.1::HbERF-IXc5* were exposed to cold stress. (Cheng et al. 2013) revealed that the ERF1 play a key role on stress resistance phenotypes in ERF1 overexpression plants. Some studies showed the ERF1 enhanced tolerance to cold stress (Chinnusamy, Zhu and Sunkar 2010, Ma et al. 2014). Ecophysiology and morphology of plants were systematically investigated to learn the effect of cold stress in transgenic lines.

7.1.1. Effect of cold stress on plant ecophysiology

Figure 59 showed a decreasing value of Fv/Fm from day-0 until day-4 in all transgenic lines and control. These values continued to decrease until day-12 for TS18A09, TS18A13, TS19A90, and TS18A37 lines. In the other hand, Fv/Fm value of TS20A69, TS20A75, and control lines were increased until day-12 of treatment. Overall for TS20A75 showed the highest average value of Fv/Fm. High value of Fv/Fm indicated the high tolerance to cold stress (Kadir et al. 2007).

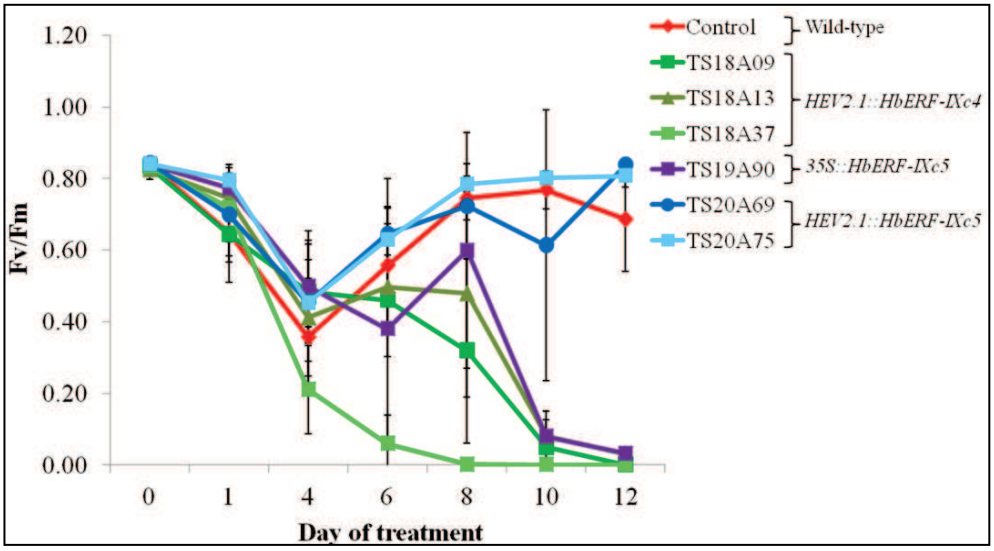

Figure 59. Effect of cold stress (10 °C) on Fv/Fm values for various WT and transgenic lines.

Statistical analysis of effect of constructs on Fv/Fm value showed a significant difference starting from day-8 until day-12 of treatment. Construct of *HEV2.1::HbERF-IXc5* showed higher average of Fv/Fm, even though it was no significant to control (Table 44). Statistical analysis of statistic in Table 45 showed that the Fv/Fm value was not significantly different from day-0 to day-4 but it was astonishing because from day-6 until day-12 the Fv/Fm value showed a significant difference. These data suggested cold stress can affect the potential of photosystem II efficiency. Fv/Fm reflects the prevailing photochemical status of the leaf (Roden, Egerton and Ball 1999).

Table 44. Effect of construct on Fv/Fm values in cold stress.

Construct	Day of treatment (No)						
	0	1	4	6	8	10	12
Wild-type	0.842[a]	0.649[a]	0.356[a]	0.556[a]	0.744[b]	0.768[b]	0.687[b]
HEV2.1::HbERF-IXc4	0.831[a]	0.703[a]	0.368[a]	0.419[a]	0.344[a]	0.059[a]	0.030[a]
35S::HbERF-IXc5	0.839[a]	0.774[a]	0.499[a]	0.380[a]	0.569[ab]	0.059[a]	0.021[a]
HEV2.1::HbERF-IXc5	0.842[a]	0.747[a]	0.453[a]	0.637[a]	0.754[b]	0.655[b]	0.815[b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Table 45. Analysis of variance of Fv/Fm value in cold stress. Data correspond to F values and P values.

Day of plant growth	DF	Sum of squares	Mean squares	F	Pr > F
0	3	0.001	0.000	1.436	0.248
1	3	0.062	0.021	2.258	0.097
4	3	0.118	0.039	1.614	0.202
6	3	0.423	0.141	3.547	0.025
8	3	1.189	0.396	8.956	0.000
10	3	2.595	0.865	22.410	< 0.0001
12	3	1.744	0.581	72.181	< 0.0001

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

The control had a lower P. Index at the beginning of observation before cold treatment. Figure 60 showed a decreased P. Index value in all transgenic lines start after treatment until day-4. These data described that the transgenic lines had a better photosynthetic activity of the plant and survival capability on cold stress condition. Two transgenic lines (TS20A69 and TS20A75) showed a higher P. Index compared to wild-type and other transgenic lines until day-8. The value of P. Index in some transgenic lines kept a very low data, but surprisingly the value of P. Index increased on day-12 after treatment, especially for TS20A69 and TS20A75. These data suggested that TS20A69 and TS20A75 had a mechanism of adaptation to cold stress.

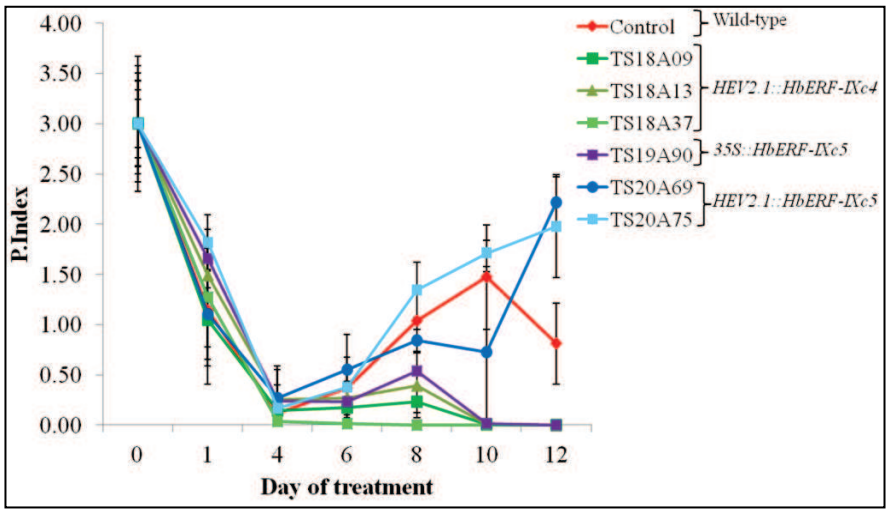

Figure 60. Effect of cold stress (10 °C) on P. Index for various WT and transgenic lines.

The interaction analysis in ANOVA showed there was a significant difference in day-0, 6, 10, and 12 of treatment. Construct *HEV2.1::HbERF-IXc5* always showed a higher value of P. Index compared to others (Table 46).

Table 46. Effect of constructs of P. Index in cold stress.

Construct	Day of treatment (No)						
	0	1	4	6	8	10	12
Wild-type	1.904 [a]	0.726 [a]	0.074 [a]	0.237 [ab]	0.661 [ab]	0.935 [ab]	0.515 [a]
HEV2.1::HbERF-IXc4	2.288 [ab]	0.953 [a]	0.108 [a]	0.154 [a]	0.240 [a]	0.006 [a]	0.001 [a]
35S::HbERF-IXc5	2.823 [b]	1.562 [b]	0.225 [a]	0.219 [ab]	0.505 [ab]	0.010 [a]	0.001 [a]
HEV2.1::HbERF-IXc5	2.676 [b]	1.283 [ab]	0.197 [a]	0.417 [b]	0.961 [b]	1.096 [b]	1.772 [b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Figure 61 showed all transgenic lines and control had a decreased chlorophyll content as describe in SPAD values during the treatment, except TS18A09 has a little increase in SPAD value on day-12. All constructs always showed a higher SPAD value compared to wild-type. The effect of constructs showed *HEV2.1::HbERF-IXc5* performed the highest value of SPAD compared control and the other construct (Table 47).

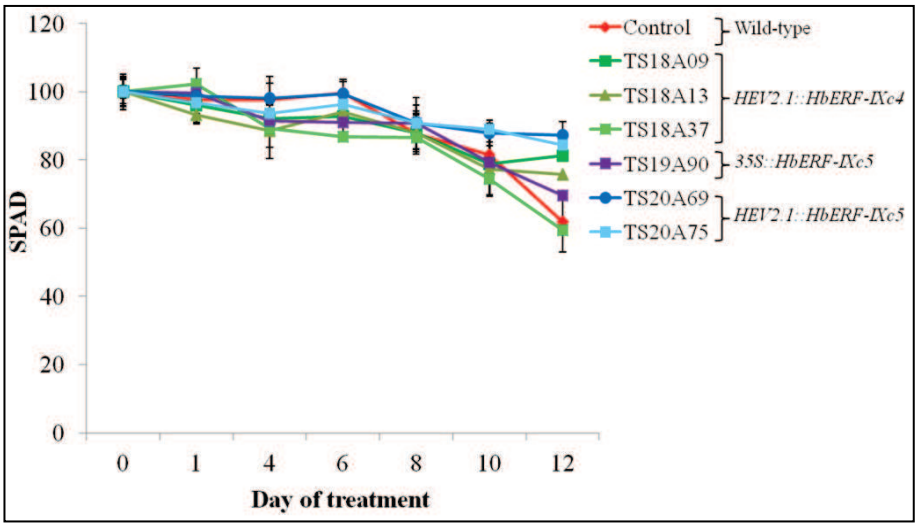

Figure 61. Effect of cold stress (10 °C) on chlorophyll content measured by SPAD instrument for various WT and transgenic lines.

Table 47. Effect of constructs on chlorophyll content measured by SPAD instrument of plants subjected to cold stress.

Construct	Day of treatment (No)						
	0	1	4	6	8	10	12
Wild-type	51.25 [a]	50.02 [a]	50.10 [a]	51.02 [a]	44.98 [a]	41.77 [a]	31.68 [a]
HEV2.1::HbERF-IXc4	60.42 [b]	58.66 [b]	54.88 [ab]	55.19 [a]	53.10 [ab]	46.75 [ab]	44.99 [ab]
35S::HbERF-IXc5	57.83 [b]	57.60 [b]	52.95 [ab]	52.65 [a]	52.57 [ab]	45.92 [ab]	40.20 [ab]
HEV2.1::HbERF-IXc5	62.70 [b]	61.29 [b]	60.10 [b]	61.33 [b]	56.96 [b]	55.46 [b]	53.63 [b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

7.1.2. Effect of cold stress on leaflet senescence

Total number of leaflets was measured to know the effect of cold stress on leaflet senescence. Figure 62 showed the mean numbers of leaflets were slowly decreased from day-0 until day-28. Based on statistical analysis, there was two transgenic lines (TS20A75 and TS18A13) that had a significant difference compared to wild-type. These transgenic lines also showed a higher number of leaflets compared to wild-type and other transgenic lines in the early until day-8 of treatment. Nevertheless, in the end of treatment (day-13) the highest number of leaflets were performed in TS18A09.

There was interesting data of observing the number of leaflets; some transgenic lines have recovery adaptability of cold stress with the growth of new shoots. TS20A75 had the highest adaptability with the highest growth of new shoots. It was shown by the increasing graph of the number of leaflets on day-28. In general, the ability of the transgenic plants against cold stress was better than the control plants (wild-type), especially for TS20A75. Some stress inducible genes have been over-expressed in transgenic lines produce a stress-tolerant phenotype of the plant (Shinozaki and Yamaguchi-Shinozaki 1996).

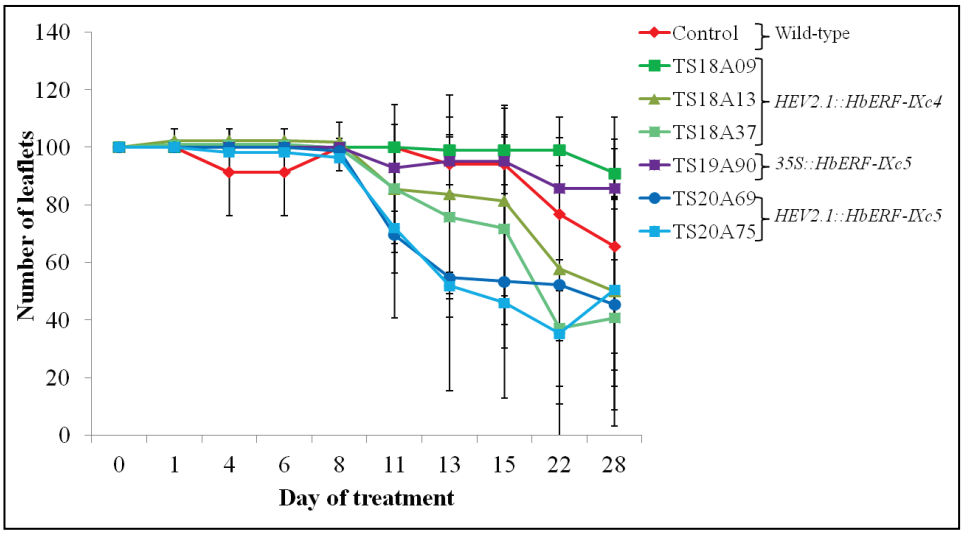

Figure 62. Effect of cold stress (10 °C) on mean number of leaflets value for various WT and transgenic lines.

Statistical analysis showed a significant difference at day-4 and day-6 after treatment for constructs *HEV2.1::HbERF-IXc4* and *HEV2.1::HbERF-IXc5* compared to wild-type and other constructs. Construct of *HEV2.1::HbERF-IXc4* showed the highest number of leaflets in day-0 until day-8, but from day-11 until day-22 after treatment the highest number of leaflets was performed by construct *HEV2.1::HbERF-IXc4* (Table 48). Effect of cold stress on plant morphology can be seen at Figure 63.

Table 48. Effect of constructs on number of leaflets in cold stress.

Construct	Day of treatment (No)									
	0	1	4	6	8	11	13	15	22	28
Wild-type	16.33 [a]	16.33 [a]	14.33 [a]	14.33 [a]	16.33 [a]	16.33 [a]	15.33 [a]	15.33 [a]	11.33 [a]	9.00 [a]
HEV2.1::HbERF-IXc4	30.67 [a]	31.11 [a]	31.11 [b]	31.11 [b]	31.00 [a]	28.33 [a]	27.22 [a]	26.56 [a]	21.22 [a]	19.67 [a]
35S::HbERF-IXc5	24.00 [a]	24.00 [a]	24.00 [ab]	24.00 [ab]	24.00 [a]	22.33 [a]	23.00 [a]	23.00 [a]	21.00 [a]	21.00 [a]
HEV2.1::HbERF-IXc5	32.50 [a]	32.50 [a]	32.17 [b]	32.17 [b]	31.67 [a]	23.00 [a]	17.00 [a]	15.67 [a]	13.33 [a]	15.17 [a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

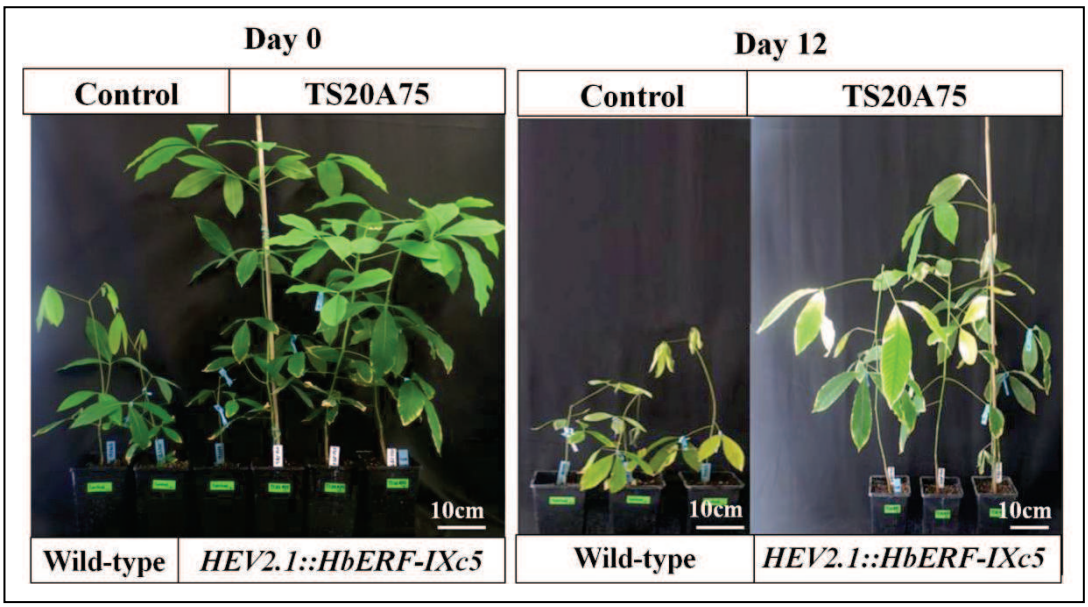

Figure 63. Plants morphology between control (wild-type) and line TS20A75 (*HEV2.1::HbERF-IXc5*).

7.2. Effect of salinity stress on plant physiology and morphology

Salinity is considered as the most important abiotic stress limiting crop production and affect plant growth. Plants are known to be able continuing survive under this stress by involving many mechanisms (de Lucena et al. 2012, Zhani et al. 2012). Salinity stress was responsible for decreased biosynthesis of chlorophyll and inefficiency of photosynthesis (Lichtenthaler et al. 2005). The influence of salinity on rubber and resin production was showed in some studies. Indicated plants grown under saline-irrigated condition may have higher rubber contents than plants with a plentiful non-saline water supply (Wadleigh, Gauch and Magistad 1946). Moderate salinity levels may increase rubber production even though total growth is reduced. Very high soil salinity concentration was reported decrease rubber content as well as growth (Hoffman and Heale 1987). Soil quality greatly sustains productivity of *H. brasiliensis*, which is the important source of natural latex production (Wongcharoen 2010).

This study was carried out to evaluate the impact of salinity stress by sodium chloride [500 mM] on the third day on the physiology and morphology parameters in transgenic plants of *H. brasiliensis*. The concentration of NaCl is used based on preliminary research.

7.2.1. Effect of salinity stress on plant physiology

The effect of salinity stress on Fv/Fm values showed that all data (except TS18A37) decreased from day-0 until day-22. The reduction of chlorophyll fluorescence is associated with the increased NaCl accumulation (Dionisio-Sese and Tobita 2000). The Fv/Fm of TS18A37 showed a decline value from day-19 until day-22. All data had higher value than control at the last day of treatment (day-22). Control had the lowest point compared to transgenic lines at day-22. TS19A90 and TS20A69 always showed stable and the highest value of Fv/Fm from day-0 until day-22 of treatment (Figure 64). These data suggested both of lines (TS19A90 and TS20A69) were more tolerant against salinity stress.

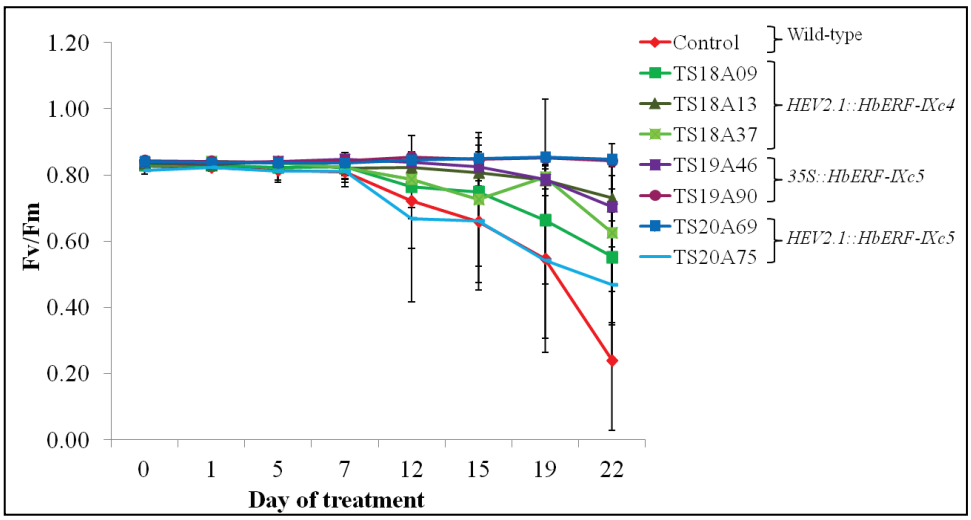

Figure 64. Effect of salinity stress on Fv/Fm value for various WT and transgenic lines.

Statistical analysis on the effect of constructs showed a significant difference during observation until day-22 except for day-12. Construct of *35S::HbERF-IXc5* showed the highest tolerance of the salinity stress (Table 49). In the other hand, control (wild-type) showed a lower value compared to others.

Table 49. Effect of constructs on Fv/Fm value during salinity stress.

Construct	Day of treatment (No)							
	0	**1**	**5**	**7**	**12**	**15**	**19**	**22**
Wild-type	0.827[a]	0.823[a]	0.813[a]	0.809[a]	0.721[a]	0.660[a]	0.547[a]	0.239[a]
HEV2.1::HbERF-IXc4	0.835[ab]	0.834[b]	0.833[ab]	0.822[ab]	0.791[a]	0.761[ab]	0.745[b]	0.645[b]
35S::HbERF-IXc5	0.841[b]	0.838[b]	0.839[b]	0.845[b]	0.847[a]	0.836[b]	0.819[b]	0.773[b]
HEV2.1::HbERF-IXc5	0.827[a]	0.830[ab]	0.823[ab]	0.824[ab]	0.757[a]	0.756[ab]	0.698[ab]	0.685[b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

All data on the line chart showed a decrease line from day-0 until day-22, except TS18A37. TS18A37 data value had increased until day-22. TS19A90 and TS20A69 showed a significant difference. Both of lines were also performed the highest P. Index value in all day of treatment (Figure 65).

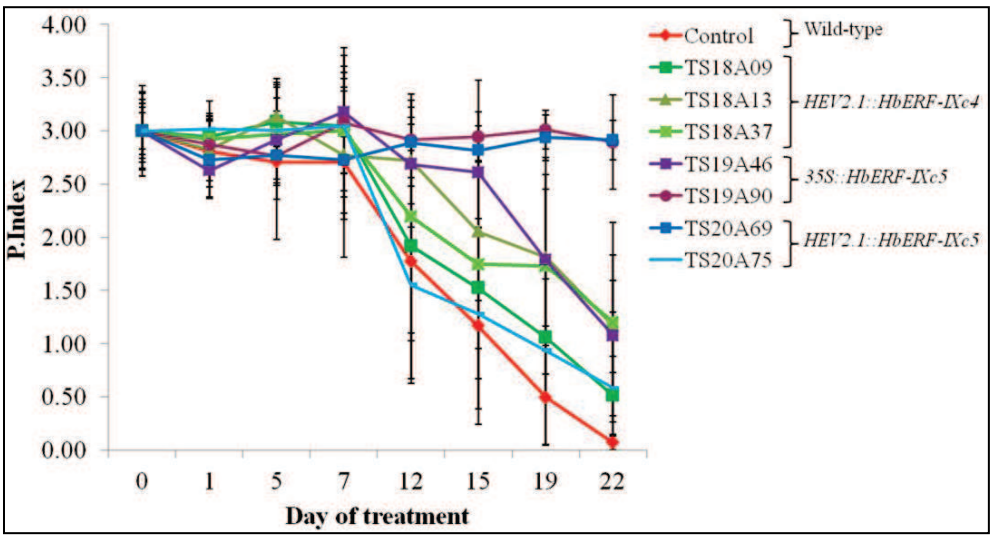

Figure 65. Effect of salinity stress on P. Index value for various WT and transgenic lines.

Table 50 showed there was a significant difference in day-0 until day-22 of treatment. Construct *35S::HbERF-IXc5* always showed a highest value of P. Index among others from day-0 until day-22. Control had the lowest value among others from day-5 until day-22.

Table 50. Effect of constructs on P. Index value in salinity stress.

Construct	Day of treatment (No)							
	0	1	5	7	12	15	19	22
Wild-type	2.23[a]	2.08[a]	2.01[a]	2.01[a]	1.32[a]	0.87[a]	0.37[a]	0.05[a]
HEV2.1::HbERF-IXc4	2.15[a]	2.08[a]	2.19[a]	2.11[a]	1.63[a]	1.27[a]	1.09[ab]	0.69[a]
35S::HbERF-IXc5	2.75[b]	2.53[b]	2.60[b]	2.87[b]	2.58[b]	2.56[b]	2.23[c]	1.87[b]
HEV2.1::HbERF-IXc5	2.41[ab]	2.28[ab]	2.30[ab]	2.29[a]	1.88[ab]	1.75[ab]	1.69[bc]	1.74[b]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Figure 66 showed all transgenic line and control had a decrease of SPAD value start from day-0 until day-22. TS19A90 and TS20A69 were showed a significant difference start from day-12 of treatment. Both of lines also performed the highest SPAD value during the treatment.

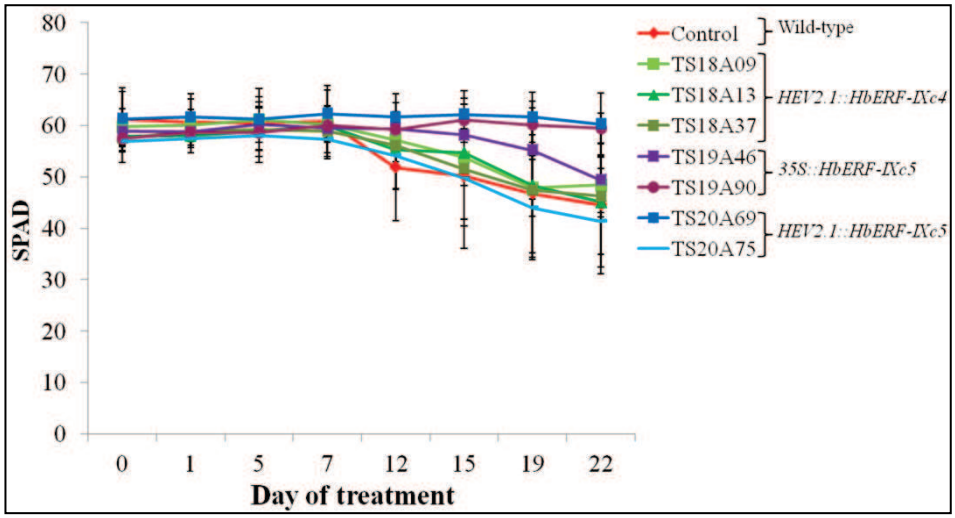

Figure 66. Effect of salinity stress on chlorophyll content measured by SPAD instrument for various WT and transgenic lines.

Statistical analysis showed a significant difference starting from day-12 to day-22 of treatment. Construct of *35S::HbERF-IXc5* had a significant difference in day-12 until day-19 of treatment (Table 51). Based on ecophysiological parameters, *35S::HbERF-IXc5* (TS19A90) was more tolerant than wild-type line to salinity stress.

Table 51. Effect of construct on chlorophyll content measured by SPAD instrument in salinity stress.

Construct	Day of treatment (No)							
	0	**1**	**5**	**7**	**12**	**15**	**19**	**22**
Wild-type	61.23[a]	60.69[a]	60.45[a]	60.84[a]	51.81[a]	50.13[a]	46.74[a]	44.57[a]
HEV2.1::HbERF-IXc4	58.41[a]	58.98[a]	59.75[a]	59.68[a]	56.19[ab]	53.35[ab]	47.87[a]	46.45[a]
35S::HbERF-IXc5	58.12[a]	58.69[a]	59.37[a]	59.76[a]	59.28[b]	59.6[b]	57.64[b]	52.18[a]
HEV2.1::HbERF-IXc5	59.09[a]	59.52[a]	59.64[a]	59.83[a]	57.89[ab]	55.95[ab]	52.79[ab]	54.40[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

7.2.2. Effect of salinity stress on number of leaflets

Salinity stress can decrease number of leaflets. Figure 67 below showed the numbers of leaflets were slowly decreased from day-0 until day-92. The decrease of leaflets numbers may be due to the accumulation of sodium chloride in the cell walls and cytoplasm of the older leaflets, and the capacity of vacuole sap to contain more salt. Decreasing the concentration of salt inside the cells which lead quick dead of cells (Munns 2002). TS18A13, TS19A90, and TS20A69 were performed the higher number of leaflets compared to control and other transgenic lines during treatment. Some transgenic lines (TS18A13, TS18A37, TS19A90, and TS20A69) developed the adaptation to salinity stress by growing new shoots.

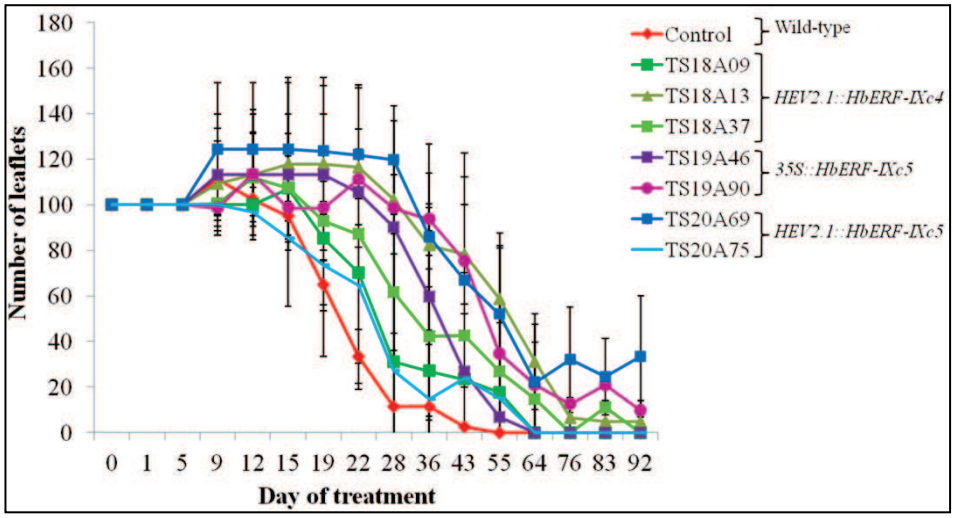

Figure 67. Effect of salinity stress on mean of leaflets for various WT and transgenic lines.

Table 52 showed a statistical analysis of effect promoters and genes in salinity stress. There was a significant difference start from day-0 until day-22 of observation. *HEV2.1::HbERF-IXc4* showed the highest number of leaflets in almost all day of observation. In the other hand, control (wild-type) performed the lowest number of leaflets. Leaf senescence is affected by a range of external parameters including salinity stress. Salinity stress could lead to premature senescence of adult leaves (Cramer and Nowak 1992). The first noticed symptoms of excessive salt are wilting plants and/or leaf "burn" or drying of the leaves, which are often caused by sodium and/or chloride toxicity. In the last day of observation there were no burned or wilting leaflets because the plants were fully abscission.

The observation of colour started at day-0 of NaCl treatment. There has been no changed in colour of leaflets since the beginning of observation but after day-12 of observation the colour of leaflets started showing yellow strips. The salinity stress could have caused the damage of the leaflets of colour pigment. The yellow colours of leaflets were correlated to lack photosynthetic activity (McCormac et al. 1997). Figure 68 showed plant morphology between control and transgenic plant with construct *HEV2.1::HbERF-IXc5.* Transgenic plant with construct *HEV2.1::HbERF-IXc5* also showed a better performance to adapt with salinity stress compared control.

Table 52. Effect of constructs on the number of leaflets during salinity stress.

Construct	Day of treatment (No)															
	0	**1**	**5**	**9**	**12**	**15**	**19**	**22**	**28**	**36**	**43**	**55**	**64**	**76**	**83**	**92**
Wild-type	20.38[a]	20.38[a]	20.38[a]	22.63[ab]	20.88[a]	19.38[a]	13.13[a]	6.75[a]	2.25[a]	2.25[a]	0.49[a]	0[a]	0[a]	0[a]	0[a]	0[a]
HEV2.1::HbERF-IXc4	28.08[a]	28.08[a]	28.08[a]	28.67[ab]	29.75[b]	29.75[b]	28.8[b]	24.75[b]	18.58[a]	14.3[a]	15[a]	10.08[a]	5.08[a]	0.67[a]	4.17[a]	4.17[a]
35S::HbERF-IXc5	21.13[a]	21.13[a]	21.13[a]	22[a]	23.38[ab]	22[a]	22[ab]	22.38[b]	19[a]	15[a]	9.13[a]	3.63[a]	1.88[a]	1.13[a]	1.88[a]	0.88[a]
HEV2.1::HbERF-IXc5	27.63[a]	27.63[a]	27.63[a]	30.38[b]	29.88[b]	28[ab]	26.25[ab]	24.75[b]	19.75[a]	13.25[a]	11.88[a]	8.88[a]	2.38[a]	4.38[a]	3.25[a]	4[a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

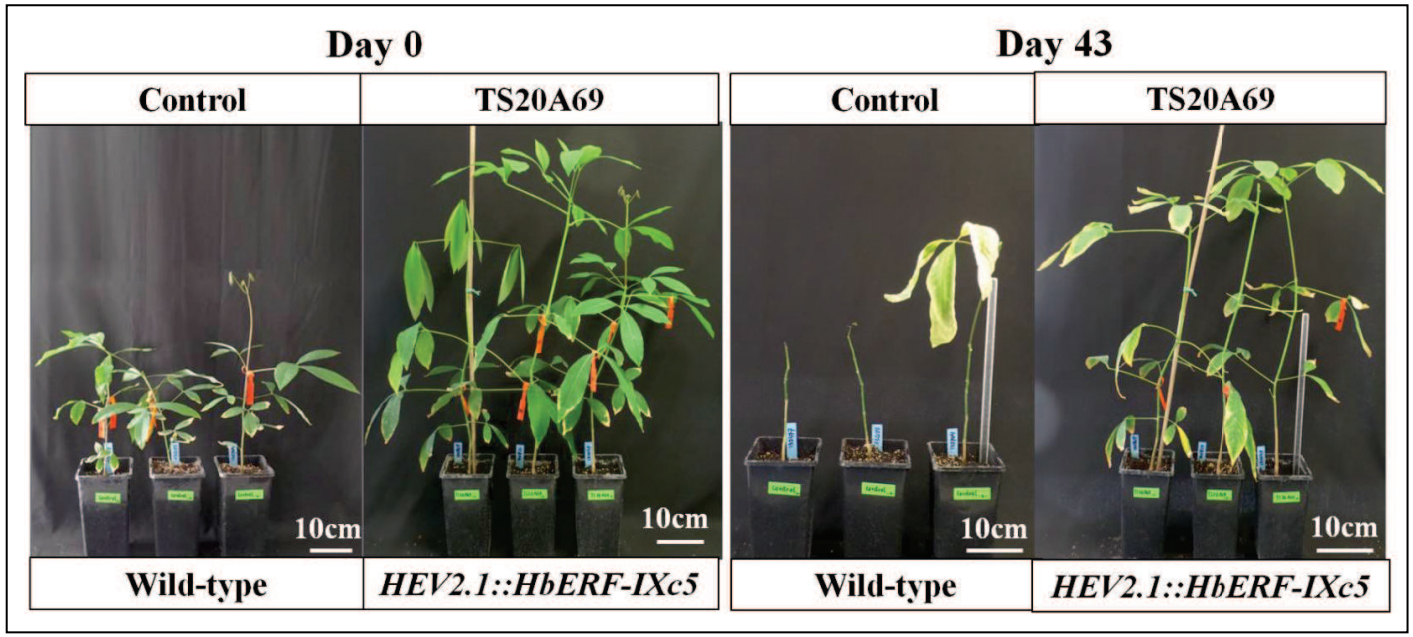

Figure 68. Plants morphology between control (wild-type) and lineTS20A69 (*HEV2.1::HbERF-IXc5*).

7.3. Effect of ethephon on plant leaflets

Ethephon can be used to stimulate latex production in *H. brasiliensis*. Part of the action of ethylene is mediated by the Ethylene Response Factors1 (ERF1) (Abeles et al. 1992, Pré et al. 2008, Reid and Wu 1992). A preliminary treatment of ethephon in various concentrations (0%, 1%, 2.5%, and 5%) was done. This preliminary study showed that ethephon with concentration 2.5% had the best effect to morphological changing in *Hevea* transgenic plants. Based on preliminary treatment, ethephon 2.5% was applied to various transgenic lines of *Hevea* harbouring by overexpressing with *35S CaMV* and *HEV2.1* promoter. Ethylene effects on leaf abscission, number of leaflets, abscission rate, senescence, and percentage of wilting-burning leaflets were analysed to know the effect of ethephon on plant morphology.

7.3.1. Effect of ethephon on number of leaflets and abscission rate

Treatment of ethephon 2.5% for six days provoked a leaf fall characterised by number of leaflets from day-0 until day-6. Figure 69 showed TS18A09 performed a slight increase of leaflets number from day-0 until day-3 of observations. The number of leaflets of TS18A09 started to decrease in day-3 toward day-6. From this observation, TS18A09 can be assumed as ethephon stress tolerance. In the other hand, TS18A37 and TS20A69 were performed a rapid decline of leaflets number. On day-6, number of leaflets dramatically decreased due to an abscission in all transgenic lines and control.

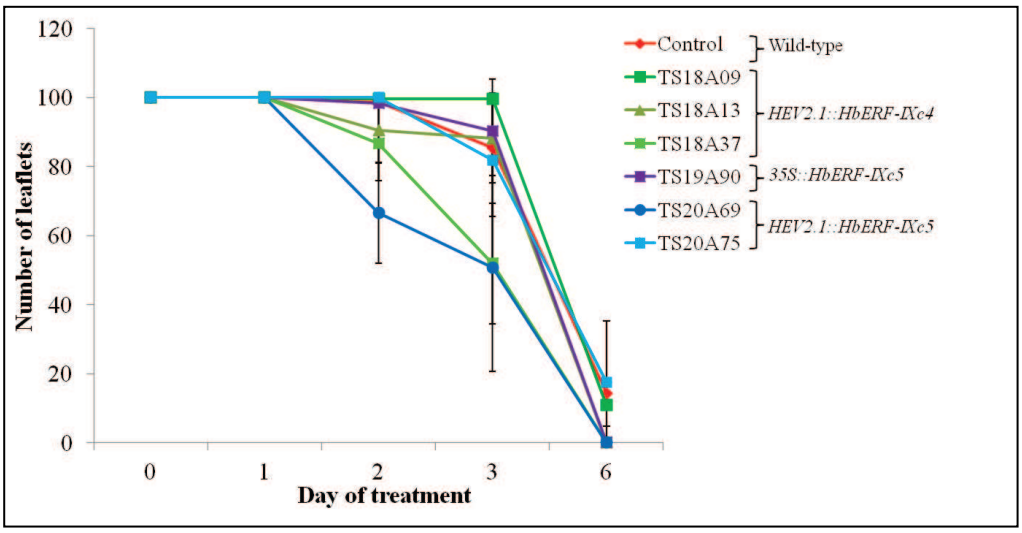

Figure 69. The effect of 2.5% ethephon on mean number of leaflets.

Treatment of ethephon 2.5% showed that the construct of *HEV2.1::HbERF-IXc4* had the highest mean number of leaflets since day-0 until day-3, but decreased drastically in day-6. At the end of the observation, construct of *HEV2.1::HbERF-IXc5* become the highest mean number of leaflets among other constructs. Control showed the lowest number of leaflets in day-0 until day-3 of observation. In the last day of observation (day-6), construct *35S::HbERF-IXc5* had the lowest number of leaflets (Table 53). Statistical analysis showed that there is a significant difference in the day-0 until day-3 observation, but no significant difference in the last day of treatment (Table 53). Treatment of ethephon 2.5% gave the great effect on abscission rate. TS18A09 performed the lowest and most stable abscission rate until day-6. From the results, TS18A09 can be assumed as ethephon stress tolerant.

Table 53. Effect of 2.5% ethephon on the number of leaflets of plants from various constructs.

Construct	Day of treatment (No)				
	0	1	2	3	6
Wild-type	17,000 [a]	17,000 [a]	16,833 [a]	14,833 [a]	0,333 [a]
HEV2.1::HbERF-IXc4	37,000 [c]	37,000 [c]	34,417 [b]	30,500 [b]	0,917 [a]
35S::HbERF-IXc5	21,333 [ab]	21,333 [ab]	21,000 [a]	19,333 [ab]	0,000 [a]
HEV2.1::HbERF-IXc5	31,667 [bc]	31,667 [bc]	25,500 [a]	20,667 [ab]	1,667 [a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

7.3.2. The effect of ethephon on leaflet senescence

Treatment of ethephon 2.5% caused leaflet senescence. At the end of observation, there were no leaflets from all lines due to abscission. Furthermore, ethephon treatment induced leaf physiological changes such as brown and white spots and yellowish leaf colour (Chen et al. 2010). Only leaflets from TS18A37 have a brown spot on the surface. The leaflets of ethephon 2.5% started changing the colour at day-1. The colour of leaflets was not only becoming a yellow, but also had brown and white spots (Figure 70 and 71).

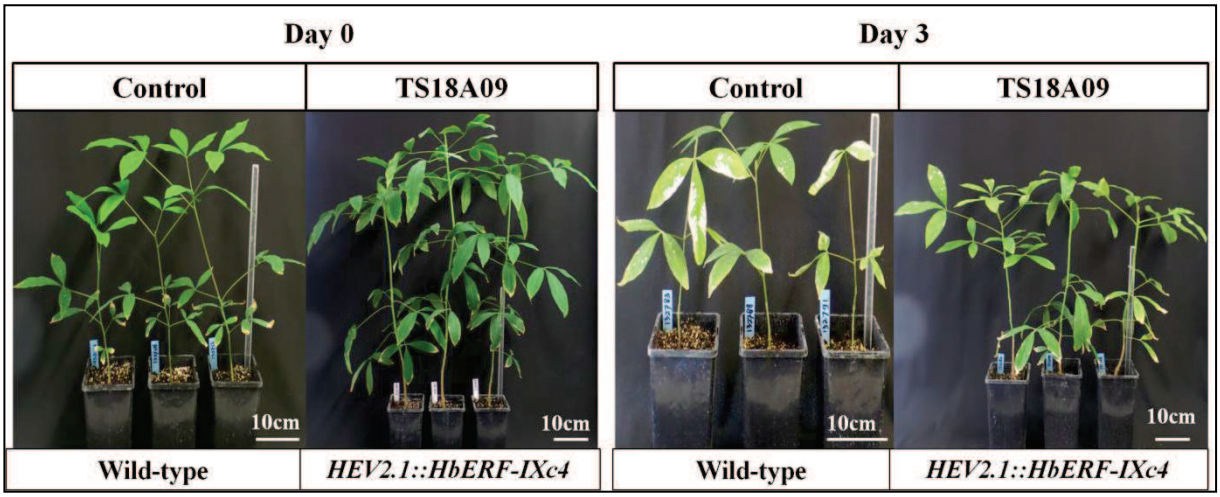

Figure 70. Plants morphology between control (wild-type) and line TS18A09 (*HEV2.1::HbERF-IXc4*).

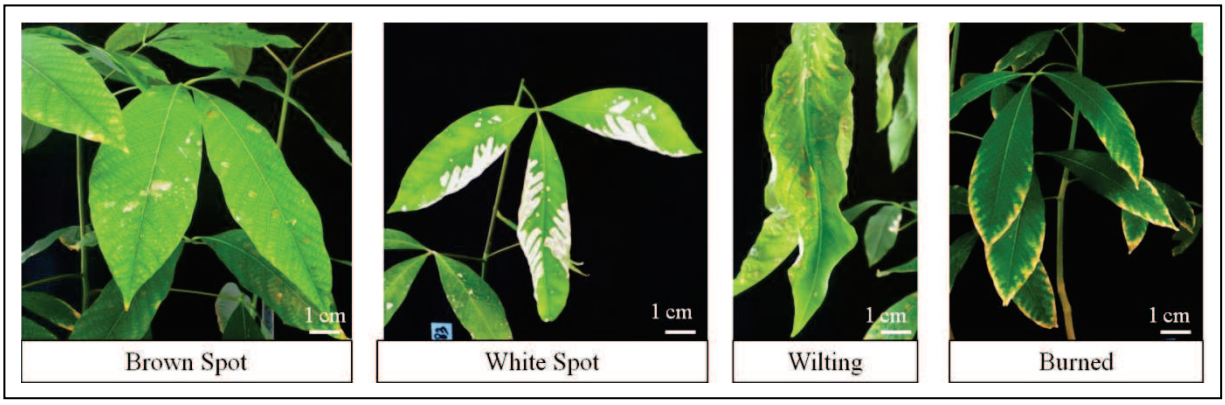

Figure 71. Leaf morphology which showed brown spot, white spot, wilting, and burned.

7.4. Effect of water deficit on plant physiology and morphology

Water deficit (drought) influences physiological and developmental changes at various levels. Expression of *ERF1* was rapidly and transiently induced by salt and dehydration treatments. *35S:ERF1* transgenic plants were more tolerant to drought, salt, and even heat stress. *ERF1* can bind to DRE elements in the promoters of drought-stress-responsive genes under drought stress (Cheng et al. 2013).

For this experiment, we monitored the effects of water deficit on ecophysiological parameter. Water deficit was induced by the methodology described in (Sanier et al. 2013). This method is based on the fraction of transpirable soil water (FTSW) (Sandras and Milroy 1996). Progressive water deficit influences many physiological processes such as transpiration, photosynthesis or leaf expansion. These physiological processes are inhibited when soil moisture available for transpiration decreases to values in the range of 40-50%, with a trend that appears to be consistent across a wide range of environments and genotypes. The available soil water was expressed as the fraction of transpirable soil water (FTSW) (Sandras and Milroy 1996). We developed a water deficit phenotyping platform, using FTSW as a soil moisture co-variable under controlled environmental conditions.

7.4.1. Effect of water deficit on FTSW and leaf senescence

The decline of FTSW value over time in the treatment of water deficit was shown in Figure 72. This figure showed water deficit treatment reduced the FTSW value for 14 days of observation. All of line including control showed a decrease of FTSW value from day-0 until day-11. TS19A90 performed a slow decrease of FTSW value from 1 to 0.816. In the other hand, TS18A13 showed a rapid decrease from 1 to 0.045 and had lower value than control (from 1 to 0.067). TS20A69 also performed lower value than control and tended to show a rapid decrease of FTSW value (from 1 to 0.043). In day-7 of observation, almost all transgenic lines were faced a moderate water deficit (FTSW value < 0.4). Whereas a high water deficit (FTSW value < 0.2) occurred at day-11 of observation (Sanier et al. 2013). The FTSW threshold indicates the timing of stomatal closure in response to soil water deficit (Sinclair and Ludlow 1986, Ray and Sinclair 1998).

Figure 73 showed the differences of plant morphology between TS19A90 (*35S::HbERF-IXc5*) and control (wild-type). TS19A90 had a best performance due to water deficit tolerance. The figure showed morphological changes of wild-type and TS19A90 (*35S::HbERF-IXc5*) at day-0 and day-14. In day-14 of observation, both of plants had already faced senescence.

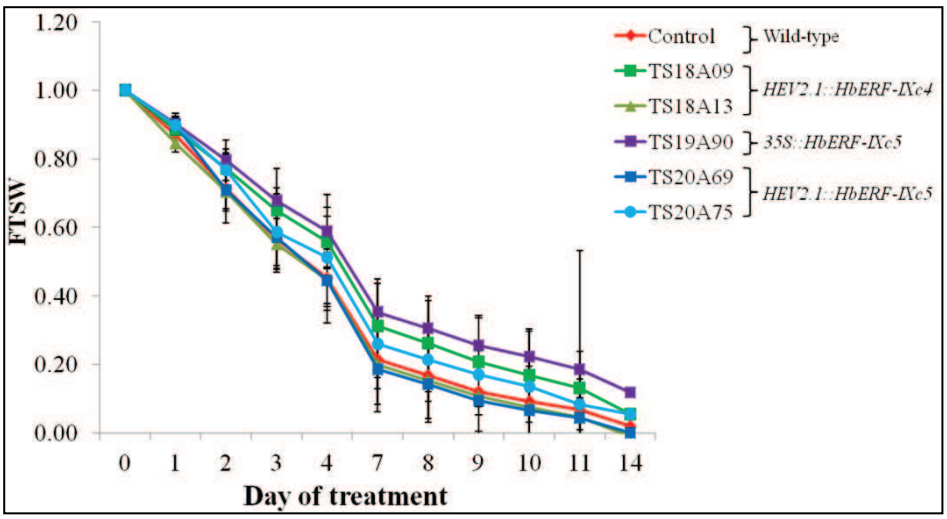

Figure 72. Diagram of effect of line in FTSW value in water stress.

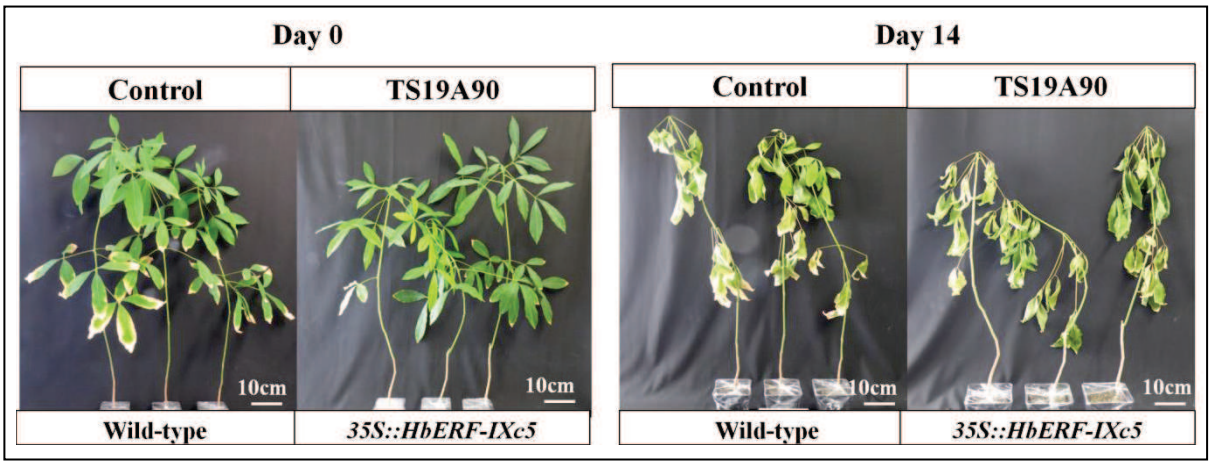

Figure 73. Morphology of plants between control (wild-type) and line TS19A90 (*35::HbERF-IXc5*).

Table 54. Effect of construct on FTSW value of water stress. Highlighting in green (1>FTSW>0.4), in blue (0.4>FTSW>0.2), in brown (FTSW<0.2).

Construct	Day of treatment (No)									
	1	2	3	4	7	8	9	10	11	14
Wild-type	0.870 [a]	0.713 [a]	0.567 [a]	0.452 [a]	0.214 [a]	0.168 [a]	0.120 [a]	0.092 [a]	0.067 [ab]	0.019 [a]
HEV2.1::HbERF-IXc4	0.866 [a]	0.738 [a]	0.601 [a]	0.503 [a]	0.255 [a]	0.207 [a]	0.157 [a]	0.121 [a]	0.088 [ab]	0.022 [a]
35S::HbERF-IXc5	0.903 [a]	0.796 [a]	0.679 [a]	0.588 [a]	0.352 [a]	0.306 [a]	0.255 [a]	0.223 [a]	0.186 [b]	0.117 [a]
HEV2.1::HbERF-IXc5	0.899 [a]	0.738 [a]	0.579 [a]	0.479 [a]	0.223 [a]	0.178 [a]	0.131 [a]	0.100 [a]	0.063 [a]	0.027 [a]

The data were analysed with XLSTAT software. Statistical analysis was performed with an ANOVA followed by the Tukey test. Values with the same letter were not significantly different at the 0.05 probability level.

Statistical analysis of effect of construct in FTSW values showed a significant difference between the two constructs *35S::HbERF-IXc5* and *HEV2.1::HbERF-IXc5* in day-11, but there is no significant difference between control and all construct (Table 54). All of constructs showed declined value from day-0 until day-14. Construct *35S::HbERF-IXc5* showed the highest tolerance of the water deficit stress, while control showed the lowest FTSW value. FTSW of 0.2 revealed a high-water stress in most of plant species and in

particular in rubber. Plants from all of constructs reached a value of FTSW lower than 0.2 in day-8 until day-9 except for plants from the line *35S::HbERF-IXc5* which reached the same level of stress after day-11.

8. Effect of cassicoline toxin on detached leaves from WT and transgenic plants

Corynespora Leaf Fall disease (CLFD) is one of the major leaf disease threatening rubber plantations caused by *Corynespora cassiicola*. A CIRAD team has developed a bioassay presently used to screen the resistance of *Hevea* segregating population to this disease. This bioassay consists of a toxin cassiicolin Cas1 and CCP filtrate produced by *Corynespora cassiicola*. *Corynespora cassiicola* produces a small phytotoxic protein named cassiicolin which was encoded by *Cas1* gene. This cassiicolin Cas1 toxin was used for bioassay on the leaflets. Filtrate CCP was obtained from liquid culture of *Corynespora cassiicola* isolates CCP, which is used for bioassay on leaflets. Both toxin treatment of *Corynespora cassiicola* behaves as necrotrophic agent in rubber tree causing yellow brown spot on the leaflets (Breton, Sanier and d' Auzac 2000, Déon et al. 2012).

The results of the biotic stress treatments are shown in Table 55. Plus (+) mark showing that the leaflets were affected by the toxin treatment. TS18A13, TS18A37, and TS19A90 have started showing a positive symptom in day-2 after treatment by Cas1. Meanwhile other lines (control (wild-type)), TS19A46, TS20A47, TS20A69, and TS20A75) have already shown a positive symptom since day-1 and the necrotic response getting bigger for the next day of the treatment. For CCP treatment, control (wild-type), TS19A90, TS20A47, TS20A69, and TS20A75 lines have already shown a positive symptom in day-1 after CCP treatment. Other lines (TS18A13, TS18A37, and TS19A46) showed a positive symptom in day-2 after CCP treatment.

Observation during 8 days suggested all leaflets from control (wild-type) and transgenic lines were susceptible again biotic stress (Cas1 and CCP) (Figure 74).

Table 55. Effect of line on Cas1 and CCP treatment of *Hevea brasiliensis* leaflets. Notes: (0) no response, (+) small response, (++) mild response, (+++) severe response.

Construct	Line	Treatment	Day of treatment (No)						
			1	2	4	5	6	7	8
Wild-type	Control	Cas1	+	+++	+++	+++	+++	+++	+++
		CCP	+	+++	+++	+++	+++	+++	+++
HEV2.1::HbERF-IXc4	TS18A13	Cas1	0	+++	+++	+++	+++	+++	+++
		CCP	0	+++	+++	+++	+++	+++	+++
	TS18A37	Cas1	0	+++	+++	+++	+++	+++	+++
		CCP	0	+++	+++	+++	+++	+++	+++
35S::HbERF-IXc5	TS19A46	Cas1	+	++	+++	+++	+++	+++	+++
		CCP	0	+++	+++	+++	+++	+++	+++
	TS19A90	Cas1	0	+++	+++	+++	+++	+++	+++
		CCP	+	+++	+++	+++	+++	+++	+++
HEV2.1::HbERF-IXc5	TS20A47	Cas1	++	+++	+++	+++	+++	+++	+++
		CCP	++	+++	+++	+++	+++	+++	+++
	TS20A69	Cas1	++	+++	+++	+++	+++	+++	+++
		CCP	++	+++	+++	+++	+++	+++	+++
	TS20A75	Cas1	+	+++	+++	+++	+++	+++	+++
		CCP	++	+++	+++	+++	+++	+++	+++

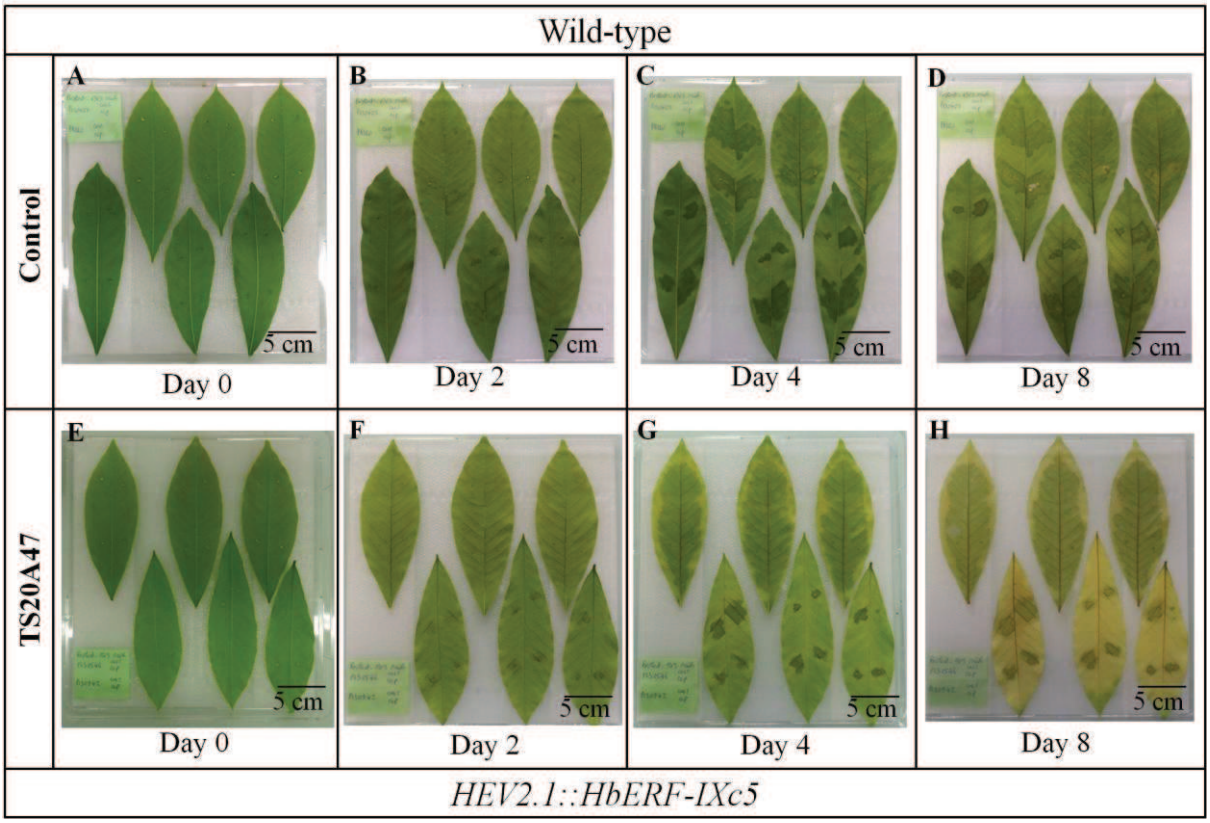

Figure 74. Symptom of leaflets after treatment with Cas1 and CCP during eight days.

Table 56 showed the summary effect of construct on abiotic and biotic stresses. From data bellow, we can conclude that construct *HEV2.1::HbERF-IXc5* performed better in all stresses that other lines.

Table 56. Summary effect of constructs on ecophysiological parameters monitored during abiotic and biotic stresses based on average value. Notes: (orange) not significantly different to control and (red) significantly higher value than control.

Construct	Abiotic stress										Biotic stress	Phenotype Description
	Cold				Salinity				Ethephon	Water		
	Fv/Fm	P.Index	SPAD	Leaflets	Fv/Fm	P.Index	SPAD	Leaflets				
HEV2.1::HbERF-IXc4												Wilting, burned, yellow, white spot, and brown spot
35S::HbERF-IXc5												
HEV2.1::HbERF-IXc5												

SECTION IV

GENERAL DISCUSSION

This study led to a successful functional analysis of *HbERF-IXc4* and *HbERF-IXc5* genes, which are involved in the regulation of laticifer metabolism and stress response in *Hevea brasiliensis.* We analysed their expression pattern and identified their biological function by overexpressing. Transgenic lines have been established. The overexpressing lines were identified by Southern blotting and real-time RT-PCR. The plant material performed higher vigour and better tolerance to abiotic stress.

A substantial number of transgenic plants did not survive to the transfer from *in vitro* condition to the greenhouse. The greenhouse condition has substantially lower relative humidity, higher light level and a septic environment that are stressful to the transgenic plants for acclimatization process (Hazarika 2003, Pavlović et al. 2010). The result of acclimatization process of transgenic plants in greenhouse showed an amazing result because these transgenic lines were successfully produced 1,622 transgenic plants with normal phenotype. Moreover, morphological and ecophysiological parameters were also analysed on 12-month-old plants in response to abiotic stress (cold, salinity, ethephon, and water) and biotic stress (cassicoline toxin). The four transgenic lines of the construct of *35S::HbERF-IXc5*, and *HEV2.1::HbERF-IXc5* (TS19A46, TS19A90, TS20A69, and TS20A75) which showed excellent growth and morphology continued to perform histology analysis.

This discussion was organized in five points:

1. The first successful functional analysis of transcription factors in *Hevea*,
2. The effect of promoters and candidate genes at different stages of genetic modification process,
3. The different putative function of *HbERF-IXc4* and *HbERF-IXc5*,
4. The putative role of *HbERF-IXc5* on plant development and stress response,
5. And the putative involvement of *HbERF-IXc5* in laticifer differentiation.

1. First successful functional analysis of transcription factors in *Hevea*

In order to meet the increasing rubber demand, it is important to identify and characterize the genes involved in agronomic traits, which are often under a polygenic control. Transcription factors are important regulators of a large number of target genes that act directly on metabolic pathways.

High-throughput characterization of gene function in rubber tree cannot be established by conventional reverse genetic technique such as insertional or chemical mutagenesis including T-DNA insertional mutagenesis or TILLING (Targeting Induced Local Lesions in Genomes). By contrast, over and down-expression of candidate genes can be developed in rubber tree by *Agrobacterium tumefaciens*-mediated genetic modification (Chen et al. 2012, Gebelin et al. 2013, McCallum et al. 2000, Uthup et al. 2011).

Genetic engineering is considered as a promising method for genetic improvement in order to enhance natural rubber production (Priya, Venkatachalam and Thulaseedharan 2006). It can be used to produce desirable agronomic traits quickly and efficiently (Arokiaraj et al. 2002). We choose genetic modification in our study because this method could modify the target trait without disrupting the other traits of a selected clone through recombination. (Birch 1997) conveyed in his review that the major technical challenge facing plant transformation biology is the development of methods and constructs to produce a high proportion of plants showing predictable transgene expression without collateral genetic damage (Birch 1997). However, this method is still difficult to apply in *H. brasiliensis* because it requires an efficient protocol to ensure the expression of candidate gene in transformed cell and also regenerate plants by *Agrobacterium tumefaciens*-mediated genetic transformation (Jayashree et al. 2003).

Several recent studies showed a breakthrough in *Hevea* genetic engineering. *Agrobacterium tumefaciens* has been used for genetic transformation of *H. brasiliensis*, and then transgenic plants *Agrobacterium*-mediated genetic transformation were produced (Arokiaraj and Wan 1991, Arokiaraj, Yeang and Cheong 1998). Montoro and coll. have studied the response of exogenous calcium on *Agrobacterium*-mediated gene transfer in *H. brasiliensis* friable calli (Montoro et al. 2000b). The Rubber Research Institute of Malaysia (RRIM) successfully developed an *Agrobacterium*-mediated anther callus genetic transformation procedure for *H. brasiliensis* cultivar GL1 with the cDNA encoding HANF (Human Atrial Natriuretic Factor) (Sunderasan et al. 2012). Furthermore, (Sobha et al. 2014) revealed the first report of multiple gene integration in *Hevea.* Integration of two genes manganese superoxide dismutase (*MnSOD*) for abiotic stress tolerance and 3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgr*I) gene for enhanced latex yield was successfully carried out in *Hevea* transgenic plants (Sobha et al. 2014). (Leclercq et al. 2012) reported *Hevea* transgenic plant lines overexpressing *HbCuZnSOD* gene showed more tolerance to water stress (Leclercq et al. 2012). Another study also successfully established the *Hevea* transgenic plants overexpressing *EcGSH1*, a gene involved in gluthatione biosynthesis. Transgenic lines which over expressed *EcGSH1* had a significant increase in thiol content in leaves, higher proline content, and higher glutathione reductase activity (Martin et al. 2015). All previous studies were essential for *Hevea* improvement programmes.

The recent development of transformation and regeneration system has allowed the introduction of useful genes in *H. brasiliensis* plants. Thanks to an efficient *Agrobacterium*-mediated genetic transformation procedure (Leclercq et al. 2012), functional analysis of candidate genes was attempted. The advantages of this procedure include maintaining the foreign gene at the same low copy number as that of the Ti plasmid in *Agrobacterium*, minimal rearrangement, could transfer of relatively large segment of DNA, and also high quality and fertility of transgenic plants (Gelvin 2003, Komari, Ishida and Hiei 2004).

Previous results showed that HbERF-IX was activator-type transcription factor. Several members of HbERF-IX could be a regulator of complex hormonal signalling pathways during latex production in rubber. Two *Hevea ERF*, *HbERF-IXc4* and *HbERF-IXc5*, are orthologs of *ERF1* gene from *Arabidopsis* and play a role in the response to stress (Duan et al. 2010, Putranto et al. 2015a). These genes could be a regulator at the crosstalk of ethylene and jasmonate signalling pathway in latex cells. In this study, transgenic lines harbouring two genes (*HbERF-IXc4* and *HbERF-IXc5*) were transformed under the control of *35S CaMV* and *HEV2.1* promoter. The role of gene regulatory regions (promoters) is important for understanding the regulation of gene expression in plants. Both of promoters have been demonstrated to drive a candidate gene in genetic transformation via *A. tumefaciens* (Blanc et al. 2006, Montoro et al. 2000b, Rattana et al. 2001). The *35S CaMV* promoter allows strong constitutive expression in all tissues, in the other hand *HEV2.1* promoter allows targeted expression in laticifer cells and in leaves (Montoro 2008). The practical application of functional analysis in plants often requires tissues-specific expression rather than expression in all parts of the plants. Our experiment conducted with four constructs described as: *35S*::*HbERF-IXc4, HEV2.1*::*HbERF-IXc4, 35S*::*HbERF-IXc5*, and *HEV2.1*::*HbERF-IXc5*. Based on these constructs, we set out to establish transgenic callus lines called T17, T18, T19, and T20.

Twenty-nine GFP-positive lines were established on paromomycin selection medium and 15 of them could produce plantlets. The plantlets were produced from wild-type (CI07060) and fifteen transgenic lines (TS17A35, TS17A61, TS17A79, TS18A37, TS18A09, TS18A13, TS18A20, TS18A69, TS19A46, TS19A59, TS19A90, TS19A99, TS20A47, TS20A69, and TS20A75). Of the 15 regenerate lines, 12 lines led to produce a sufficient number of plants for further phenotyping (TS17A61, TS17A79, TS18A09, TS18A13,

TS18A37, TS18A20, TS18A69, TS19A46, TS19A90, TS20A47, TS20A69, and TS20A75). Ten lines had a high level of plant acclimatization and were confirmed by Southern blot hybridization. Eight of these lines had only one T-DNA copy. This revealed that the genetic modification methodology did not affect too much the *Hevea* genome compared to other procedures using particle bombardment or too virulent *Agrobacterium* strains. For that reason, most of transgenic lines can be compared to each other without any strong effect of the copy number. Each line with one copy of T-DNA had a higher gene expression of their corresponding transgene.

Several lines showed a very low ability to form somatic embryos and transgenic plantlets and showed deleterious effect. Overexpression of transcription factors may also activate additional non-stress related genes that adversely affect the normal agronomic characteristics of a crop, producing deleterious effect on the phenotype and thus yield (Wang, Vinocur and Altman 2003). The strategy to solve these problems is raised the suitable condition to plant development and enhance the stress tolerance ability of transgenic plants.

The plantlets were acclimatized in greenhouse for one year. But not all plantlets that were produced from the establishing transgenic lines could survive when they were transferred to greenhouse. Two reasons could be related to this loss of regeneration ability. First, physiological changes induced by the candidate genes by itself. Second, the mutations related to the integration of T-DNA in the plant genome (Krysan, Young and Sussman 1999, Nakano et al. 2000, Wang et al. 2012). During this period, we observed the morphology of each transgenic plant. Not all plants that allowed full morphological, anatomical, and physiological characterization, only plants with sufficient number and competent to develop were analysed in different steps of development. Especially for two transgenic lines (TS17A61 and TS17A79) could not be analysed of morphological, anatomical, and physiological because they just only have limited number of plants, not sufficient for statistical analysis.

Study of transcription factor regulatory mechanism could provide specific information about gene expression changes underlying cellular and developmental responses to environment cues. Down-regulation or overexpression of transcription factor could assist us to determine the function and interconnectedness of individual transcription factor based on resulting cellular changes (Son et al. 2011). In some cases, the transgenic plants had pleiotropic effects on plant growth and physiology, which affect the production efficiency of recombinant proteins (Scotti and Cardi 2014). This pleiotropic effect could happen because a gene or multiple genes in some pathway affected more than one phenotype, regardless of whether the specific variants are shown to have cross-phenotype effect (Solovieff et al. 2013).

Several studies had been successful for analysis of the transcriptional regulation of some transcription factors. (Chen et al. 2011) analysed the *HbEREBP2I*, which involved in the regulation of jasmonate-mediated defence response in laticifers of rubber tree. Another researcher has been successfully identified the biological function of *HbERF1* by overexpressing this gene in *Arabidopsis* to develop transgenic lines (An et al. 2014). The result showed *HbERF1* was induced by ABA, MeJA, SA, and ethylene (ET), and it is a positive regulator of ET-responsive genes and drought tolerance in the rubber tree (An et al. 2014). The previous study showed that the *HbERF1*, *HbERF2*, *HbERF3*, and *HbRAV1* genes were induced by JA in bark during JA-induced laticifer differentiation. The *HbERF1*, *HbERF2*, and *HbERF3* genes were corresponded to *HbERF-VIIa3*, *HbERF-VIIa17* and *HbERF-VIIa1* with 99%, 98%, 99% homology (Duan et al. 2013, Piyatrakul et al. 2012).

The identification of two other ERF transcription factors (HbERF-IXc4 and HbERF-IXc5) had been studied. The results showed both of HbERF-IXc4 and HbERF-IXc5 play a role as transcription factor which correlated to latex production and also stress response in *H.*

brasiliensis (Duan et al. 2010, Putranto et al. 2015a). However, analysis of specific function for these transcription factors had not been done. This thesis was the first study which led to characterize the function of *HbERF-IXc4* and *HbERF-IXc5* using genetic modification strategy. The results of this study were expected to provide some new biological knowledge for better understanding of the effect of overexpression of these candidate genes, *HbERF-IXc4* and *HbERF-IXc5* and to provide deep understanding of the functional analysis of genes.

2. Effect of promoters and candidate genes at different stages of the genetic modification process

This study was conducted on four constructs combining two genes (*HbERF-IXc4* and *HbERF-IXc5*) and two promoters (*CaMV* and *HEV2.1*). Several changes were observed for these four constructs. Interestingly, some changes in terms of callus proliferation, embryo production, plant regeneration, and plant morphology can lead us to determine specific effects of promoters and studied genes.

2.1. Effects of promoters

Analysis the effects of promoters for each candidate genes had been done in this research. *HbERF-IXc4* gene has a lower callus proliferation was observed with the *35S CaMV* promoter compared to *HEV2.1.* For instance, *HbERF-IXc4* gene with *35S CaMV* need more subcultures than with *HEV2.1* to get a sufficient quantity of callus for further plant regeneration and callus cryopreservation. All lines could produce abundant yellow callus. At the beginning of culture, the callus appeared creamy in colour and then gradually became yellow or dark yellow. (Finer 1988) has been classified the cotyledon callus based on the colour as green, yellow, white, brown, and red. Only yellow callus could yield embryogenic subcultures (Finer 1988). Some studies verified that the constitutive promoter *35S CaMV* is a weak promoter for transgene expression in young olive somatic embryos. This promoter was more active in an organized tissue of mature alfalfa somatic embryos than in the less-organised tissues of young embryos (Pérez-Barranco et al. 2009, Tian et al. 2010).

The number of total embryos was similar for the two promoters (*35S CaMV* and *HEV2.1*). A high percentage of abnormal embryos were also recorded for all promoters. The rate of conversion of embryos into plantlets was lower for *35S CaMV* than *HEV2.1.* Finally, that led to produce a low quantity of plantlets for lines harbouring *35S*::*HbERF-IXc4* compared to lines harbouring *HEV2.1*::*HbERF-IXc4*. Because of this low quantity of plants especially for *35S*::*HbERF-IXc4* lines, promoter effects could not be analysed during plant development for the *HbERF-IXc4* gene.

Lines of *HbERF-IXc5* gene had a good callus proliferation, embryo, and plant regeneration capacity for both promoters. By contrast, promoter effects were observed during plant development. Histological analysis effectively characterized differences of specific tissue structure between wild-type and transgenic plants overexpressing *HbERF-IXc5* under two different promoters *35S CaMV* and *HEV2.1*. For the *35S CaMV* promoter, a larger number of latex cells and thicker xylem were measured in leaves and stem, respectively, compared to *HEV2.1.*

For the *HEV2.1* promoter, a thicker cambium was measured in plants (green stem and taproot) compared to *35S CaMV* promoter. Activation of cell division and differentiation in the cambium led to the thickening of stem and roots that related to the increasing of biomass (Miyashima et al. 2013). Cambium width of construct *HEV2.1::HbERF-IXc5* were threefold higher than construct *35S::HbERF-IXc5.* This data indicated *HEV2.1* play a role in promotes in differentiation of active cells in cambium. *HEV2.1* has been reported as being specifically

expressed in latex cells according to *in situ* hybridisation data (Montoro et al. 2008). Inner soft bark of *Hevea* contains productive and continuous latex vessels differentiated from vascular cambium. Latex vessels are more concentrated in the region near cambium (Premakumari and Panikkar 1992).

Application of abiotic stresses in this study showed that lines harbouring *HEV2.1* had a better response to cold stress compared to *35S CaMV* (it showed by the Fv/Fm value, P. Index, and SPAD value). Analysis of number of leaflets described that all promoters could raise the adaptability of transgenic plant against senescence due to cold stress.

In salinity stress analysis, average value of Fv/Fm and P. Index value showed *35S CaMV* promoter was higher than *HEV2.1*. The increase of Fv/Fm and P. Index was a consequence of salt-stressed plants. Fv/Fm described a maximum efficiency of light absorbed by the light-harvesting antennae of PSII. Plants exposed to salinity appear to have a more efficient PSII. Consequently, the long-term saline environment could produce an adaptation process in the plants.

Line under control of *35S CaMV* promoter could have delayed water deficit status revealed by FTSW value < 0.2. It was suggested that this promoter had better capability to avoid water deficit. Water deficit refers to ability of plant to maintain high water status even when water is scarce, for example by growing long roots to reach deep soil moisture, or reducing water loss by restricting the aperture of the stomata on leaf surfaces. In fact, stomata play a major role in plant adaptation to stress (Cominelli and Tonelli 2010).

2.2. Effects of candidate genes

Regeneration capacity and survival rate of the wild-type line were higher than in transgenic lines which over-expressed *HbERF-IXc4* and *HbERF-IXc5*. These data indicated that both genes had a negative effect on embryo regeneration and survival. Anyway, somatic embryogenesis capacity can be affected after genetic modification in rubber whatever the transgene. For *gusA* gene, somatic embryogenesis can be totally inhibited or enhanced by 10-fold after genetic modification (Blanc et al. 2006). By contrast, (Leclercq et al. 2010) reported that embryogenic capacity can be maintained for the majority of transgenic lines and slightly decreased for some others compared to wild-type after genetic modification with *35S::GFP*. Similarly, genetic modification with a transgene encoding the superoxide dismutase revealed a decrease in embryo production and plant regeneration (Leclercq et al. 2012).

For the *35S CaMV* promoter, both lower callus proliferation and smaller number of embryos were obtained for *HbERF-IXc4* gene. This suggests that *HbERF-IXc4* may affect dramatically callus multiplication and somatic embryogenesis. So, because we could not get any plants for *35S*::*HbERF-IXc4*, further effects of candidate genes can only be analysed for candidate genes under the control of *HEV2.1*.

Interestingly, a full comparison of *HbERF-IXc4* and *HbERF-IXc5* was successfully conducted for transgenes under the control of *HEV2.1*. The data showed that both genes (*HbERF-IXc4* and *HbERF-IXc5*) promoted a better growth in terms of plant height, stem diameter, and weight of aerial and root system, plants overexpressing *HbERF-IXc5* having a better performance than the ones with *HbERF-IXc4* (Table 57). The data result also showed the root system had developed well balanced with regard to the whole plant. Our data are contradictive with several studies on overexpression of *ERF1*, which revealed that adult *35S::ERF1* transgenic plants showed an extreme dwarf phenotype similar to that of the constitutive ethylene response mutant ctr1 and EIN3/EIL1-overexpressing transgenic plants. Recently, Mao and coll. reported also root growth inhibition by ethylene in overexpressing *ERF1* lines (Mao et al. 2016). Plants from several *ERF1*-expressing lines showed extreme

inhibition of cell enlargement and ultimately the plants wilted and died (Kieber et al. 1993, Chao et al. 1997, Solano et al. 1998). In *Arabidopsis*, *ERF1* was reported to control elongation of primary root by reducing cell elongation, but not reducing cell division. *ERF1* also induced the accumulation of auxin and ethylene-induced inhibition of root growth by binding to the promoter of Anthranilate Synthase α1. This analysis suggested that *HbERF-IXc4* and *HbERF-IXc5* may have a different effect to cell elongation and development compared to *ERF1*.

Plant response to stress is a pivotal fitness to survive because the natural environment is composed of a complex set of abiotic and biotic stresses. With regard to tolerance to abiotic stress, significant changes were observed for cold, salinity, ethephon, and water deficit stresses (Table 58). Lines *HEV2.1*::*HbERF-IXc4* could maintain some of their ecophysiological parameters like ethephon and salinity stress but overall the tolerance of *HEV2.1*::*HbERF-IXc4* to some stresses are lower than *HEV2.1*::*HbERF-IXc5*. This suggests a lower level of control of plant defence mechanism for *HbERF-IXc4* lines. Lines *35S*::*HbERF-IXc5* performed the best response to salinity and water deficit stress.

However, both of genes (*HbERF-IXc4* and *HbERF-IXc5*) could produce transgenic plants with a better response to salinity stress compared to control. The plant growth and development will be affected by salinity stress condition on the environment. Several traits of plants could be important parameters to determining salt tolerance ability of the plants, such as height of plant, shoot length, and root length. Genetic and environment could influence the gene expression. Salinity stress severely affected reduction of plant height, shoot elongation, shoot and root length. Plant growth under salinity stress will remain stunted compare to plant growth under normal condition (Shahid et al. 2011). Ashraf et al. (2003) reported that fresh and dry weights of roots, shoots along with shoot length were affected by salt stress (Ashraf, Arfan and Ahmad 2003). Shahid and coll. also reported that the highest salinity concentration exhibit drastic reduction of shoot length, root growth, and plant height. Lowest percentage of germination process also happened on plant under highest salinity concentration (Shahid et al. 2011). Reduction of plant's growth may occur due to toxic effect of Na^+ and Cl^- ions present in sodium chloride and low water potential in the rooting medium. Toxic effect of ions may also disturb the function of membrane (Grattan and Grieve 1998, Silvera et al. 2001). Low water potential around root leads reduction of cell elongation and cell division due to lower cell turgor (Greenway and Munns 1980). Salinity also affected chlorosis, necrosis, and senescence on leaves (Munns 2002).

The major signals involved in salinity stress are ethylene and ROS (Steffens 2014). Transcription factor from AP2/ERF family have been identified involved in the response of plant to salinity stress. Under high salinity concentration, *Tamarix hispida* exhibit the expression of ERF1 leading to stress tolerance (Wang et al. 2014). Transgenic tobacco plant exhibit higher expression of ERF1 because of NaCl treatment, indicate that ERF1 may be link to osmotic stress pathway (Huang et al. 2004). In responses to salinity stress, ERF1 will bind DRE-box in the promoter during adaptation to salinity stress (Achard et al. 2006). Overexpression of ERF1 in plants could increase plant tolerance to salt stress (Cheng et al. 2013). For ethephon treatment, lines *HEV2.1*::*HbERF-IXc4* showed a better response. (Arora 2005) reported overexpression of *ERF1* in an *ein3* background leads to constitutive activation of a subset of ethylene phenotypes in *Arabidopsis* (Arora 2005).

All these results above suggest that both of genes (*HbERF-IXc4* and *HbERF-IXc5*) had a similar function with *ERF1* in terms of stress response. Several recent reports showed that *ERF1* regulated biotic and abiotic stress response such as drought, salinity, and heat shock stress by binding different *cis*-element (DRE element or GCC box) in response to different stress signal (Lorenzo et al. 2003, Cheng et al. 2013, Kazan 2015). ERF1 in plant influenced by drought and salt stress has special characteristic. The size of stomatal aperture

becomes smaller, so it will reduce transpirational water loss (Cheng et al. 2013). (Duan 2011) has been reported that *HbERF-IXc5* functions like the *ERF1*. The response of *HbERF-IXc5* to the combined treatment of wounding, MeJA and ET was significantly multiplied (Duan 2011). Unfortunately, histological analyses were carried only for *HbERF-IXc5* gene, so no comparison was possible for the two candidate genes at this level.

The effect of *HbERF-IXc4* and *HbERF-IXc5* genes in stress response ability of transgenic plants was successfully conducted on several environmental stress treatments. The ability of plants species to tolerate cold stress was varying; it depends on gene expression to modify their physiology, metabolism, and growth (Chinnusamy et al. 2010). In cold stress response, *HbERF-IXc5* performed a better response than *HbERF-IXc4* under control *HEV2.1* promoter in parameters: Fv/Fm value, P. Index, and SPAD value. The maximum photochemical efficiency of the photosystem II (Fv/Fm) indicated particular responses of the genotypes. However, both of genes (*HbERF-IXc4* and *HbERF-IXc5*) had good ability to face senescence than control. It was described with the leaflets number, all transgenic lines showed more leaflets compared to the control. Similar with ERF1 function, HbERF-IXc5 might play a role to enhance the plants ability to cold stress response. ERF1 was directly regulates abiotic stress response genes (cold, salinity, and water deficit) in *Arabidopsis* (Hao et al. 2002, Cheng et al. 2013, Sakuma et al. 2002).

With regard to Fv/Fm and SPAD value, both of genes had a same capability in salinity stress tolerance, but *HbERF-IXc5* performed a better P. Index than *HbERF-IXc4*. The ratio of Fv /Fm is a direct measure of the "optimal quantum efficiency" of the plant (Genty, Briantais and Baker 1989). P.Index reflects the functionality of both photosystems I and II and gives quantitative information on the current state of plant performance under stress conditions (Strasser et al. 2004). In the other hand, the SPAD value can be used as an indicator of chlorophyll content that measure greenness based on optical responses (Kariya, Matsuzaki and Machida 1982). However, number of leaflets analysis revealed that transgenic plant with construct of *HEV2.1::HbERF-IXc5* also showed a better performance to adapt with salinity stress compared to control by growing a new shoot.

Leaf abscission was reflecting the effect of ethephon treatment. Ethephon or native ethylene was promoted in abscission by induction of hydrolytic enzyme synthesis and secretion of this enzyme into cell wall (Horton and Osborne 1967, Abeles 1969, Abeles and Leather 1971). Analysis of leaflets number and leaves morphology revealed that *HbERF-IXc4* gene had a great ability to face senescence. It might suggest that this gene could raise the ethephon tolerance in *Hevea* transgenic plants.

2.3. Main conclusions on the effects of promoters and candidate genes

The previous research showed that the promoter *35S CaMV* drives a strong GUS activity in embryogenic callus and plant vessels (Lagier 2004), but these results showed contrary result. This suggests that *HbERF-IXc4* has a negative effect on callus proliferation and plant regeneration when driven by this strong promoter.

HEV2.1::*HbERF-IXc4* and *HEV2.1*::*HbERF-IXc5* lines can regenerate a large number of plantlets with normal phenotype but with higher performance in terms of growth for root and aerial parts. Similarly, no obvious changes could be observed in plants from *35S*::*HbERF-IXc5* or *HEV2.1*::*HbERF-IXc5* lines. These observations led to conclude that there is no deleterious effect of this gene whatever the stages of development. *HbERF-IXc4* and *HbERF-IXc5* genes likely play different role especially in the control of different target genes. Indeed, HbERF-IXc4 only activates the promoter of the *SUT3* gene. HbERF-IXc4 has been identified as a binding factor to the promoter of the gene *HbSUT3* by simple hybrid

technique (Zhang 2014). This suggests involvement of HbERF-IXc4 in activating the sucrose loading in laticifers, which is a carbon source for the biosynthesis of polyisoprene chains.

Table 57. Summary effect of lines on morphology of plants. Red highlight is significant difference and blue highlight is not significant difference. R is total root weight and R1 is taproot.

Construct	Line	Height	Stem diameter	Leaves and leaflets	Total weight	Leaf weight	Stem weight	Root weight	Ratio R/tot plant	Ratio R1/tot R
Wild-type	CI07060									
35S::HbERF-IXc4	TS17A61									
	TS17A79									
HEV2.1::HbERF-IXc4	TS18A37									
	TS18A09									
	TS18A13									
	TS18A20									
	TS18A69									
35S::HbERF-IXc5	TS19A46									
	TS19A90									
HEV2.1::HbERF-IXc5	TS20A47									
	TS20A69									
	TS20A75									

Table 58. Summary effect of construct on abiotic and biotic stress based on average value. Red highlight is significant difference and blue highlight is not significant difference.

Construct	Abiotic stress										Biotic stress
	Cold				Salinity				Ethephon	Water	
	Fv/Fm	P. Index	SPAD	Leaflets	Fv/Fm	P. Index	SPAD	Leaflets			
Wild-type											
HEV2.1::HbERF-IXc4											
35S::HbERF-IXc5											
HEV2.1::HbERF-IXc5											

3. *HbERF-IXc4* and *HbERF-IXc5* are putatively orthologs to *ERF1* according to the phylogenetic analysis but might have additional functions

3.1. ERF1 phenotype in others species

ERF1 was suggested to be a key component for 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. ERF1 has been shown to confer resistance to several fungi. In addition, overexpression of *TERF1* and *JERF1* isolated in tomato improved transgenic rice and tobacco tolerance of osmotic stress (Huang et al. 2004, Zhang et al. 2004, Zhang et al. 2005). The activation of genes encoding *PLANT DEFENSIN1.2* (*PDF1.2*) is commonly observed in jasmonate-dependent defence responses (Brown et al. 2003, Penninckx et al. 1996). Constitutive overexpression of the *ERF1* gene activates the expression of several defence-related genes, including *PDF1.2*, *thionin2.1* (*Thi2.1*) and *basic-chitinase* (*ChiB*) in *Arabidopsis thaliana* (Berrocal-Lobo et al. 2002, Lorenzo et al. 2003).

3.2. Common effects between *ERF1* and *Hevea* putative orthologs genes

Although there is an opposite effect on plant development and no effect on the unique biotic system tested (*Corynespora cassiicola* filtrates), main common effects between *ERF1* and *Hevea* putative orthologs genes, *HbERF-IXc4* and *HbERF-IXc5*, are about the response to abiotic stress. Lines overexpressing *HbERF-IXc4* had a better ethephon tolerance, and for *HbERF-IXc5* had a better cold tolerance. Both of two genes have a better salinity tolerance.

Ethephon mediated several effects such as leaf senescence, chlorophyll content, and Fv/Fm value. Ethephon caused reduction of chlorophyll content, Fv/Fm, and leaf senescence in detached sweet potato leaves. The results of this study suggest that oxidative stress level elevated by ethephon plays an important role in the ethylene signalling leading to the changes of senescence-associated markers and leaf senescence. In oat, ethylene promoted damages on chloroplasts isolated from seedling primary leaves, and significantly reduced the chlorophyll content and PSI and PSII photosynthetic activities (Chen et al. 2010). The morphological characteristics showed leaves began to turn visible yellowing at day 2, and became almost completely yellow at day 3 after ethephon treatment.

ERF1 belongs to the group IX of ERF family, which is different than the group consisting CBF/DREB1 (CRT-binding factor/DRE-binding protein) in low-temperature signalling and DREB2 during osmotic stress. There are three type of CBF that involve in responses of cold stress that is CBF1, CBF2, and CBF3 (Chinnusamy, Zhu and Zhu 2007, Shinozaki et al. 2003). During cold stress, inducer of CBF expression (ICE1) is active. Our study did not cover the analysis of such mechanism. Previous study showed the transgenic tomato overexpressing CBF1 were showed higher photochemical efficiency of PSII (Fv/Fm) than in non-transformed plants under low temperature stress at low irradiance (Zhang et al. 2011). Nevertheless, (Cheng et al. 2013) reported that ERF1 plays also a key role on stress resistance, and some other studies showed that ERF1 enhanced tolerance to cold (Cheng et al. 2013).

Cold stress can take variety form of negative effect on morphology depending on the age of plant and frequency of cold stress exposure. Plant will reduce the leaf expansion, wilting, and chlorosis. In other cases, plant will undergo necrosis causing plant tissue will death. Cold stress also affects the reproductive development of plants and this has been seen

in rice plants at the time of anthesis (floral opening), which leads to sterility in flowers (Yadav 2010).

3.3. Specific effects of *HbERF-IXc4* and *HbERF-IXc5* genes compared to *ERF1*

By contrast with ERF1 lines in other species that produced dwarf material, overexpression of *HbERF-IXc4* and *HbERF-IXc5* genes in *Hevea* led to produce fast-growing plants with a vigorous root system. This outstanding phenotype is particularly highlighted for stem diameter and height, number of leaves, plant weigh especially for the root system.

The high performance of transgenic plants may be the cause or consequence of the large number of leaves and root system, which could lead to better carbon sequestration and nutrient up-taking by roots, respectively. High expression of the plant defence genes in transgenic lines may lead to a better physiological status accompanying this high plant performance.

Besides, transgenic *HbERF-IXc5* lines also revealed other specific features such as thicker cambium, higher number of latex cells, wider xylem, and more of starch content. This latter feature will be discussed below.

3.4. Conclusions

Prediction of orthology between *Hevea* genes, *HbERF-IXc4* and *HbERF-IXc5*, and *ERF1* is not totally supported by our characterization. These three genes revealed different phenotypes and activation of target genes. However, they are the closest genes in terms of sequence, regulation by ethylene and jasmonate, and induction of defence target genes. Co-linearity of gene position on the *Arabidopsis* and *Hevea* genome sequence will be a useful analysis to confirm their origin during evolution. Silencing of *HbERF-IXc4* and *HbERF-IXc5* genes will be also required for better understanding of their function. Finally, identification of the full set of target genes for *HbERF-IXc4* and *HbERF-IXc5* compared to *ERF1* will be necessary to describe the regulation of these genes.

4. *HbERF-IXc5* plays a putative role on plant development and stress response

Successful *in vitro* growth of somatic embryos from wild-type and other transgenic lines will proceed to acclimatization process. During acclimatization process, plants undergo alteration of morphological and physiological characteristics in order to maintain their survival rate. There are many plantlets die during this period. Observations of morphological parameters play a critical role to determine the success of acclimatization process. This parameter ensures survival rate and vigorous growth of the certain plant before it is used for other purposes (Chandra et al. 2010, Pospíšilová et al. 1999). Alteration of morphology during acclimatization plants will increase leaf thickness, leaf mesophyll will differentiate into palisade and spongy parenchyma, stomata density will decrease, and stomata will change from circular to ellipse. Other important changes are development of cuticle, epicuticular waxes, and increasing ability of stomata to stabilization of water status (Pospíšilová et al. 1999). The results showed there are three higher survival rates of transgenic lines compared others at month 12, *HEV2.1*::*HbERF-IXc4* (TS18A13) and *HEV2.1*::*HbERF-IXc5* (TS20A69, TS20A75).

In this study, morphological characteristic has been observed, height of stem, diameter of stem, number of leaflets, weight of 12-month-old plants, and analysis of root system. This parameter used to determine which lines have a greater growth performance than the other lines. Zhang et al. (2015) explained that greater growth performance by plant

can be identified by higher rate of growth and higher yield of biomass, but the result can be varying because of environment factor (Zhang, Serra and Helariutta 2015a).

Root system plays important role in plant development because major plant nutrition source is heterogeneously distributed in the soil. Because of that role, roots must grow into new regions of the soil to explore new sources water and mineral. The depth of rooting varies among species in similar conditions whether there is genetic control over root depth. Root system can be used as one parameter for measuring growth of the plant. A health root system is one factor to indicate a health of whole plant. Based on statement above we can conclude that plant with high rate of growth can be identified by its roots system (Day et al. 2010). Analysing total root weight, ratio root/total plant, and ratio taproot/total root can have used as a method to analyse overall plant growth performance.

There are quite high number of studies to explain the correlation between leaf and root system in grassland. Mathematically, correlation between spread of root be estimated up to 3 times of canopy spread. (Fort, Jouany and Cruz 2013) reported that increasing root length correlated with higher leaf area. Other study explained that correlation between leaf and root showed the strategy of plant to survive around stress environment condition (Fort et al. 2013, Day et al. 2010).

Leaves are important part of plant which the primary organs for carbon assimilation and transpiration in plants. Leaves can adapt their morphological due to response of environment changes, such as shape, structure, and the amount of the leaves. Number of leaves influences photosynthetic capacity of plants. Large number of leaves in plant will correlate with large surface area, therefore increasing the amount of light they can absorb. The amount of light will influence the rate of photosynthesis which correlates with production of starch (Ford 2014, Koester et al. 2014, Tao and Qichang 2015).

The availability of carbohydrates in the tissues engaged in latex synthesis (Tupy 1988). The sucrose content of latex is the result of carbohydrate loading to laticiferous cells. Sucrose is the main source for metabolism of latex. Several reports have indicated that rubber biosynthesis is strongly regulated by sucrose as a limiting factor (Moraes, Neto and Seeschaaf 1978, Tangpakdee et al. 1997). Catabolism of sucrose produce acetate molecule to initiate isoprene chain which led to production of latex. It showed that latex production highly significant correlated with sugar concentration (Chow et al. 2012, Priyadarshan 2011).

In our study, observation of starch content was measured in leaves, green stem, lignified stem, and taproot. The accumulation of starch content was more abundant in green stem and taproot (in bark, xylem and pith) and in lignified stem (in xylem and pith) of *HbERF-IXc5* transgenic lines compared to wild-type. More starch was found in main nerve of leaves. Starch is the major product of photosynthesis in leaves. In photosynthetic plants, chloroplast can produce sufficient ATP to support starch synthesis. The presence of starch could be an indirect indicator of photosynthetic activity (Geigenberger 2011, Zeeman, Smith and Smith 2007). Starch reserves in rubber tree were important to growth and latex regeneration when the demand exceeds supply from photosynthesis. The higher starch ability could sustain higher latex yield (Ketkakomol et al. 2014). Starch accumulation might have related to growth of diameter and length of stem. Starch was accumulated during the day and remobilized at night to support continued respiration, sucrose export, and growth. Starch was also identified as integrator in regulation of plant growth in the dark (Geiger and Servaites 1994, Sulpice et al. 2009). In rubber, tapping treatment could reduce the radial growth and increase the carbohydrate reserve in wood. The increase of carbohydrate reserves in response to carbohydrate diversion and stress was demonstrated that trees tend to adapt their reserve level to current needs (Gohet et al. 1996, Silpi et al. 2007, Silpi et al. 2006). Changes of light intensity/quality, day length, and abiotic stress could induce the fluctuation of carbon availability. The accumulation and remobilization of starch as a carbon reserve were

integrating changes in the balance between carbon supply and growth (Gibon et al. 2009, Stitt, Sulpice and Keurentjes 2010, Sulpice et al. 2009). In non-photosynthetic organs, starch synthesis regulated in response to fluctuations in the supply of sucrose from the leaves due to changes in the light/dark cycle, sink-source alterations, or developmental changes (Geigenberger and Stitt 2010, Tiessen et al. 2002).

5. Putative involvement of *HbERF-IXc5* in laticifer differentiation

Histological analysis has been only carried out on lines overexpressing *HbERF-IXc5* genes (Table 59). This analysis clearly showed that these lines have a wider cambium and a larger number of latex cells. Latex cells are differentiated from cambium therefore cambium activity is important for laticifer differentiation. Higher number of latex vessel rows was associated with higher rate of cambial activity (Premakumari et al. 1981).

Latex cells are numerous in main nerve of leaves, but also in lamina (trend but not significant in this tissue). Quantification of latex cells could be done only in main nerve of leaves and revealed a significant increase in latex cells for both promoters. Latex cells were more numerous in *35S::HbERF-IXc5* than in *HEV2.1::HbERF-IXc5* lines. In green stem, primary and secondary latex cells appeared more abundant for lines having the two kinds of promoters (*35S CaMV* and *HEV2.1*). Primary latex cells are differentiated from parenchymatous tissues when secondary latex cells come from cambium. Secondary latex cells anastomosed to create a network called laticifer. In lignified stem, mostly secondary latex cells were observed as laticifer rings both in wild-type and transgenic lines especially for *35S::HbERF-IXc5*. So, the larger number of latex cells in green stem did not lead to higher number of laticifers in more mature stems. This information must be carefully analysed because it will require additional observations and quantification. Nevertheless, quantification of histological samples in lignified stems is difficult because of the discontinuity of the laticifer rings. Analysis of discontinue laticifer rings revealed anyway no significant differences between wild-type and transgenic lines (data not shown).

HbERF-IXc5 might be involved in the molecular mechanism of latex differentiation. Wounding and application of methyl jasmonate were able to induce laticifer differentiation (Hao and Wu 2000, Tian et al. 2015). In secondary phloem of rubber tree, the secondary laticifer cells were differentiated from fusiform initials of vascular cambia. Secondary laticifer differentiation could be induced by environmental stress such as dehydration and mechanical wounding (Tian et al. 2015). Several hormones (jasmonate, ABA, and cytokinin) also play a crucial role in cell differentiation and division of vascular cambial in process of secondary laticifer differentiation in rubber tree (Mwange et al. 2005, Nieminen et al. 2008, Tian et al. 2015). The number of the secondary laticifers is closely related to the rubber productivity of *Hevea*. Intracellular concentration of latex inside the cells is characterized by dark region of the cell (Pickard 2008, Qian 1986., Tian et al. 2015). Laticifer differentiation can be influenced by exogenous jasmonic acid (JA). (Hao and Wu 2000) reported that both primary and secondary laticifers differentiation can be induced by JA. Secondary laticifers formed from vascular cambium because utilization of JA in young stem of *Hevea* (Hao and Wu 2000).

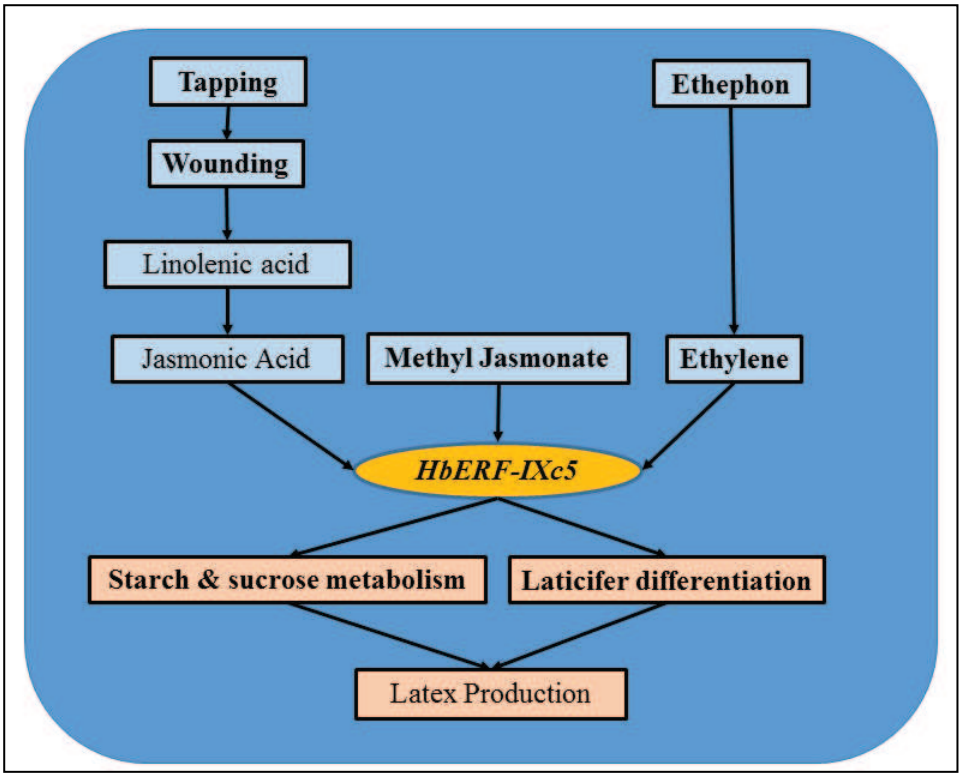

Figure 75. Activators of *HbERF-IXc5* gene expression and biological changes observed in transgenic plants overexpressing *HbERF-IXc5* transgene. In bold: factors that have been tested and observed.

HbERF-IXc5 is highly expressed in bark tissues (Piyatrakul et al. 2014). They are also activated in the latex from tapped or ethephon stimulated trees, and in leaves of plants grown under abiotic stress (Putranto et al. 2015b). A mix of ethylene and jasmonate, as ethylene gas or ethephon, and methyl jasmonate (MeJA) or wounding stress such as tapping or mechanical wounding known to induce the production of JA trigger a very strong expression of these genes in *Hevea*, as it was observed for *ERF1* ortholog in cotton (Champion et al. 2009). These results suggest that *HbERF-IXc5* plays an essential role in stress signalling. No major regulation of these genes occurs at the onset of TPD (Putranto et al. 2015b). Moreover, transactivation experiments of a reporter fused to a synthetic promoter gene containing *cis*-element GCC showed HbERF-IXc5 act as transcriptional activators (Putranto et al. 2015a). HbERF-IXc5 is involved in response to multiple harvesting stress (tapping, wounding, ethephon stimulation) and consequently to ethylene and jasmonate (Putranto et al. 2015b). For that reason, it can be considered as at the crosstalk of ethylene and jasmonate signalling pathways and responsible for the induction of genes related to the laticifer differentiation (Figure 75). Further identification of HbERF-IXc5 target genes will be necessary to identify candidate genes of this biological process of differentiation.

Table 59. Summary effect of construct on histology. Red highlight is significant difference and blue highlight is not significant difference.

Construct	Leaves		Green stem			Lignified stem			Taproot		
	Latex cell (No.)	Starch	Cambium (µm)	Xylem (µm)	Starch	Cambium (µm)	Xylem (µm)	Starch	Cambium (µm)	Xylem (µm)	Starch
Wild-type											
35S::HbERF-IXc5											
HEV2.1::HbERF-IXc5											

6. General Conclusions

This PhD thesis aimed at analysing the function of *HbERF-IXc4* and *HbERF-IXc5* genes, two putative *Hevea* orthologs of *ERF1* from *Arabidopsis*. *HbERF-IXc4* and *HbERF-IXc5* have been identified as putative regulators of the response to harvesting stress (tapping and ethephon stimulation). They were shown to be activators of the transcription of GCC *cis*-acting element of promoters by transactivation and to be nuclear protein by subcellular localization. Further characterization of *HbERF-IXc4* and *HbERF-IXc5* will require localization of their expression by RNA *in situ* hybridization and/or immune-localization. In addition, their promoter sequences have been cloned and have subjected to *in silico* analysis (Putranto et al. 2015a). Validation of this prediction might be carried out by promoter deletion analysis in fusion with the GFP reporter gene.

Functional analysis of *HbERF-IXc4* and *HbERF-IXc5* has been established in this thesis by overexpression in transgenic *Hevea* lines. This overexpression led to emphasize the effect of *HbERF-IXc4* and *HbERF-IXc5* (Figure 76-77). We observed a negative effect of a strong expression of *HbERF-IXc4* under the control of the *35S CaMV* promoter, but not for *HbERF-IXc5*, on callus proliferation and somatic embryo induction. Regenerated plants for *HEV2.1::HbERF-IXc4*, *35S::HbERF-IXc5*, and *HEV2.1::HbERF-IXc5* revealed a normal morphology of plants with fast-growing capacity, some changes in physiology (especially a better tolerance to some abiotic stress). Analysis in details of *HbERF-IXc5* lines also showed some changes in anatomy (cambium activity, number of latex cells, xylem and starch content). This successful analysis is however incomplete and cannot bring strict evidence about the role of *HbERF-IXc4* and *HbERF-IXc5* on important features such as the tolerance to stress and laticifer differentiation. Additional analyses are required in such a prospect.

First, the large number of transgenic plants with fast-growing capacity (1,622 plants) can also be a source of material to generate plant material for the characterization of the effect of aerial and root systems. A combination of rootstock and scion from wild-type and transgenic material may lead to understand if canopy and/or root systems are involved in the better growth of this material. Combined with ecophysiological parameter (Fv/Fm value, P. Index, and SPAD value) and histological analyses, this experimental design might lead to determine physiological mechanisms involved.

Second, silencing of *HbERF-IXc4* and *HbERF-IXc5* gene expression should be done to prove that phenotype emphasis in over-expressed lines is related to the identified features of gene function. Silencing is usually implemented by RNAi technology. However, artificial microRNA (amiR) has been successfully attempted in *Hevea* and could be the most adapted technique for silencing *HbERF-IXc4* and *HbERF-IXc5* in rubber (Rougier 2014).

Third, based on wild-type and transgenic lines (over-expressed and silenced lines), comparison of transcriptomes by RNA sequencing technology might be useful for identifying regulatory networks induced by *HbERF-IXc4* and *HbERF-IXc5*. Besides, more accurate technology might be developed to identify target genes for these two transcription factors. Today, two main technologies are available. The chromatin immunoprecipitation sequencing (ChIPseq) (Zhu et al. 2013) and Transient Assay Reporting Genome-wide Effects of Transcription factors (TARGET) (Bargmann et al. 2013) are two powerful techniques. CIRAD has just implemented some applications of the TARGET technology for the identification of target genes of some transcription factors related to the redox systems (Zhang 2014).

This plant material will not be able to be tested in field trial in the short term because of the public concern about GMOs. Anyway, these detailed characterizations may lead to a better understanding especially of the role of *HbERF-IXc5* on laticifer differentiation, which is a crucial biological question for natural rubber production, and identifying a set of genes

having a major role on plant defence and latex production for further applications on molecular breeding through the development of molecular markers from these genes, and development of a new stimulant for agronomical applications.

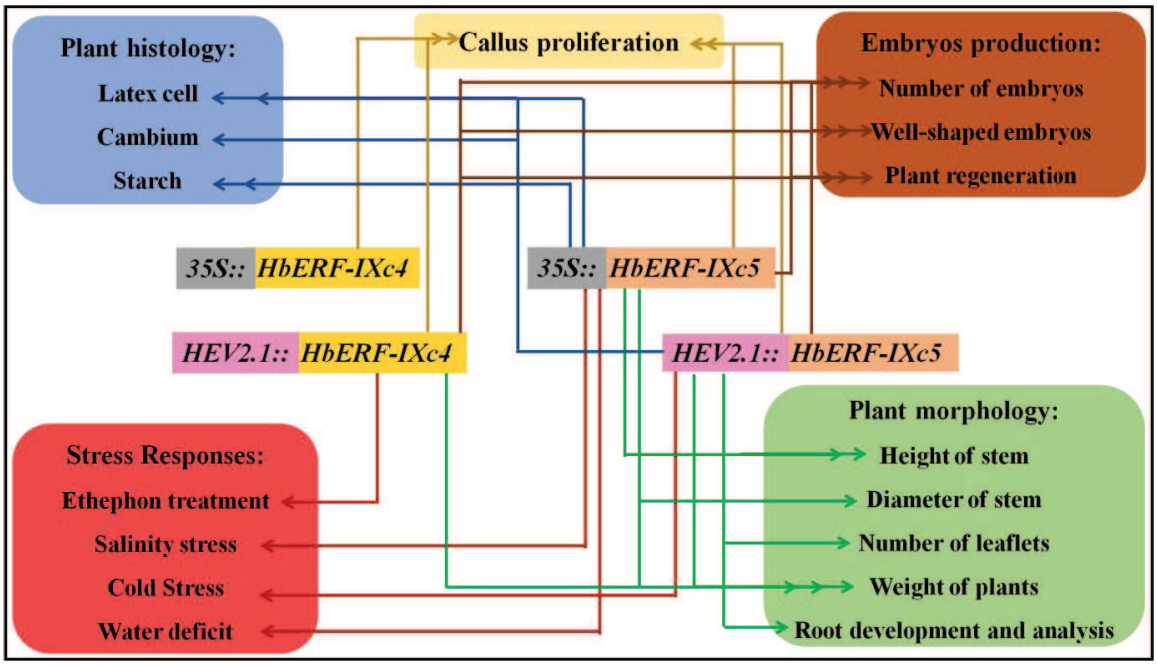

Figure 76. Summary of effects of an overexpression of *HbERF-IXc4* and *HbERF-IXc5* genes on callus proliferation, embryo production, plant morphology and stress responses of *Hevea brasiliensis* transgenic lines.

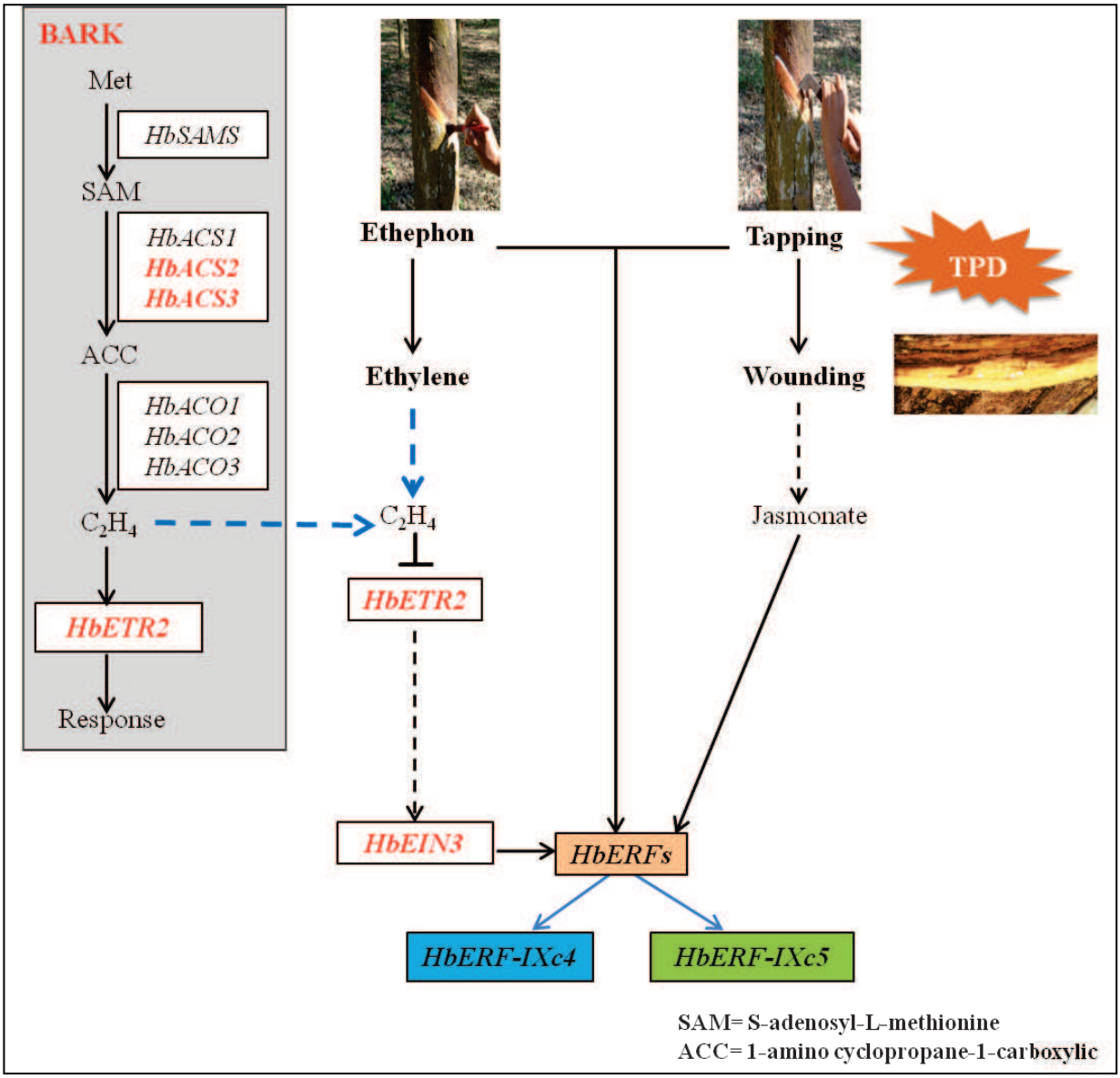

Figure 77. Summary of functional analysis of two putative genes *HbERF-IXc4* and *HbERF-IXc5*.

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