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TITRE DE LA THESE

Caractérisation des gènes AP2/ERF impliqués dans le développement chez *Hevea brasiliensis*

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Caractérisation des gènes AP2/ERF impliqués dans le développement chez *Hevea brasiliensis*

Résumé :

Hevea brasiliensis est une culture industrielle majeure pour la production de caoutchouc naturel (CN). La stimulation par l'éthéphon, un libérateur d'éthylène, est utilisée pour augmenter la production de latex en prolongeant son écoulement et en stimulant le métabolisme pour la régénération du latex. Cependant, le mécanisme d'action de l'éthylène n'est pas clairement élucidé chez l'hévéa. L'éthylène est un signal important qui régule le développement des plantes. Les facteurs de transcription AP2/ERF, et plus particulièrement les Ethylene Response Factors, jouent un rôle crucial dans le développement et la réponse aux stress biotiques et abiotiques chez les plantes. La production d'éthylène et sa signalisation sont aussi importantes en embryogenèse somatique et tout spécialement chez les espèces récalcitrantes à la culture *in vitro*.

Dans cette étude, le transcriptome de référence a été amélioré par addition des fragments de séquence d'ARN issus de tissus reproducteurs lors d'un nouvel assemblage. Les 30.342 contigs ont été annotés par la base de données Gene Ontology. L'analyse des facteurs de transcription a permis d'identifier 2.448 contigs qui ont été classés en 58 familles de facteurs de transcription. Six pourcents de ces facteurs de transcription correspondent aux membres de la superfamille des AP2/ERF. L'accumulation de transcrits des gènes AP2/ERF a été analysée au cours du processus d'embryogenèse somatique chez des lignées de cal avec différents potentiels de régénération et dans différents tissus végétatifs et reproducteurs. L'analyse de l'abondance relative de transcrits dans les différents tissus montre que les ERFs des groupes I, VII et VIII sont fortement présents à tous les stades de l'embryogenèse somatique et dans les tissus immatures et matures de fleurs males et femelle, d'embryons zygotiques, de feuilles, d'écorce et de latex. Quarante gènes AP2/ERF représentent des marqueurs d'expression génique pour le potentiel de régénération de plantes de lignées de cal à différents stades du processus d'embryogenèse somatique. Quatorze marqueurs d'expression génique permettent même de prédire la capacité de régénération dès le stade de multiplication du cal. Cinquante-neuf marqueurs d'expression géniques sont spécifiquement exprimés dans les différents tissus de l'hévéa, et plusieurs AP2/ERFs ont les transcrits fortement accumulés dans le latex. La plupart des marqueurs de l'expression génique du latex appartient aux ERF du groupe VII. Les ERFs de ce groupe ont un motif conservé en N-terminal (MCGGAII), lequel est impliqué dans la voie N-end rule. Les analyses de localisation subcellulaire et de transactivation suggèrent que ces gènes *HbERF-VII* codent pour des facteurs de transcription fonctionnels potentiellement impliqués dans la réponse à l'hypoxie dans le latex.

Mots clés : caoutchouc, éthylène, facteur de réponse à l'éthylène, hypoxie, latex, transcriptome, abondance relative de transcrit.

Characterization of the AP2/ERF genes involved in development of *Hevea brasiliensis*

Abstract:

Hevea brasiliensis is the major industrial crop for natural rubber (NR) production. Ethephon stimulation, an ethylene releaser, is used for increasing latex production by prolonging latex flow and stimulating the metabolism required for the latex regeneration. However, the mechanism of ethylene action is not clearly elucidated in this species. Ethylene is an important signal regulating the plant development. AP2/ERF transcription factors, and especially Ethylene-Response Factors, play a crucial role in plant development and response to biotic and abiotic stresses. Ethylene production and signalling are also important to somatic embryogenesis, especially for species that are recalcitrant in *in vitro* culture.

In this study, a comprehensive *Hevea* transcriptome was improved using additional RNA reads from reproductive tissues in a new assembly. The 30,342 contigs were annotated in the Gene Ontology database. The analysis of transcription factors led to 2,448 contigs being identified, which were classed in 58 transcription factor families. Six percent of the transcription factors corresponded to members from the AP2/ERF superfamily. The transcript accumulation of AP2/ERF genes was analyzed during somatic embryogenesis for callus lines with different regeneration potential and in various vegetative and reproductive tissue of *Hevea.* The relative transcript abundance were studied and showed that ERFs from group I, VII and VIII were abundant at all stages of the somatic embryogenesis as well as, in both immature and mature male and female flowers, zygotic embryos, leaf, bark and latex. Forty genes were identified as expression marker for callus with different plant regeneration potential regeneration capacity. Interestingly, fourteen expression marker genes were found that be able to predict the regeneration capacity of callus at proliferating calli, the early stage of somatic embryogenesis process. Fifty-nine expression marker genes were found in the various plant tissues. Several AP2/ERF genes were shown highly transcript accumulation in latex and were assigned as latex expression marker genes. Almost of latex expression marker genes belong to the ERF group VII. Base on conserved motif analysis showed this ERF group contained the conserved N-terminal motif (MCGGAII) involved in the N-end rule pathway. Subcellular localization and transactivation analyses suggested that *HbERF-VII* candidate genes encoded functional transcription factors.

Key words: ERF, ethylene, *HbERF-VII*, hypoxia, latex, transcriptome, relative transcript abundance, rubber.

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LIST OF ABBREVIATIONS

1-MCP	1-Methyl Cyclopropane
ACC	1-Aminocyclopropane-1-Carboxylic-acid
ACO	ACC Oxidase
ACS	ACC Synthase
AFLP	Amplified Fragment Length Polymorphism
ANRPC	Association of Natural Rubber Producing Countries
ANT	AINTEGUMENTA
AOS	Allene Oxide Synthase
AP2	APETALA2
cDNA	Complementary Deoxyribonucleic Acid
ChiB	Basic-Chitinase
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
COI1	Coronatine Insensitive 1
Cp	Crossing point
DNA	Deoxyribonucleic Acid
DRE	Dehydration-Responsive Element
DREB	Dehydration Responsive Element Binding Proteins
EAR	ERF-associated amphiphilic repression motif
EIL1	ETHYLENE INSENSITIVE3-LIKE 1
EIN3	ETHYLENE INSENSITIVE3
EMG	Expression marker genes
EMSA	Electrophoretic Mobility Shift Assays
EREBP	Ethylene-Responsive Element Binding Proteins
EREBP	Ethylene-Responsive Element Binding Proteins
ERF	Ethylene Responsive Factors
EST	Expressed Sequence Tags
ET	Ethylene
FDP	Farnesyl Diphosphate
FDS	Farnesyl Diphosphate Synthase
GFP	Green Fluorescent Protein
GS	Glutamine Synthetase
HbSOD	*Hevea brasiliensis* Superoxide Dismutase
HCN	Cyanide
HEL	Hevein-like protein
HMGR	Hydroxy-3-methylglutaryl coenzyme A reductase
HMGS	Hydroxy-3-methylglutaryl coenzyme A synthase
HRE	HYPOXIA RESPONSIVE
INF	Friable callus induction medium

IPP	Isopentenyl diphosphate (isopentenyl pyrophosphate)
IRSG	International Rubber Study Group
JA	Jasmonic acid
JAI1/JIN1	JASMONATE-INSENSITIVE1
JAR1	Jasmonate resistant 1
JAZ	Jasmonate ZIM-domain
LD	Latex Diagnosis
LOX	Lipoxygenase
LST	Large-scale trials
LUC	LUCIFERASE
MAS	Markers-Assisted Selection
MAT	Met Adenosyl Transferase
MeJA	Methyl Jasmonate
MEP	plastidic 2-C-methyl-D-erythritol 4-phosphate
MiR	MicroRNA
MM	Maintenance medium
mRNA	Messenger RNA
MS	Murashige and Skoog medium
MSE	Maintained somatic embryogenesis
MTA	5'-methylthioadenosine
MVA	Mevalonate
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDF1.2	PLANT DEFENSIN1.2
PLT1	PLETHORA
PR genes	Pathogenesis Related genes
PSE	Primary somatic embryogenesis
Q-PCR	Quantitative real time PCR
QTL	Quantitative Trait Locus
RAP2	RELATED TO AP2 2
RAPD	Random Amplification of Polymorphic DNA
REF	Rubber Elongation Factor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RRIT	Rubber Research Instutute of Thailand
RT-PCR	Reverse Transcription PCR
RuT	Rubber Transferase
S/2 d2	Half spiral cut tapped once in two days
S/2 d3	Half spiral cut tapped once in three days
SALB	South American Leaf Blight

SAM	S-adenosyl-L-methionine
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SRPP	Small Rubber Particle Protein
SSE	Indirect Secondary Somatic Embryogenesis
SSH	Suppression Subtractive Hybridization
SSRs	Simple Sequence Repeats
SST	Small-scale trials
SUT	Sucrose Transporter
TP	Turgor pressure
TPD	Tapping Panel Dryness
TSC	Total Solid Content
V-PPase	Vacuolar H(+)-Pyrophosphatase
W	Wounding
βCAS	Beta-Cyanoalanine Synthase

General Introduction

1. *Hevea brasiliensis* and natural rubber industry

1.1 Botany

The major industrial crop for natural rubber (NR) production is rubber tree (*Hevea brasiliensis* Muell. Arg.), a member of the *Euphorbiaceae* family. Rubber tree has 2n=36 chromosomes and behaves as a diploid. The species is a perennial plant and allogamous (cross-pollinaion). The floral morphology is monoecious, with lateral inflorescences (branched panicles) bearing basal staminate flowers and pistillate flowers which appear at the proximal end. The leaf are trifoliate (Clément-Demange et al. 2007).

1.2 The global production consumption and exportation of NR

Despite rubber tree is native to the Amazon rainforest (Schultes 1970), the production and consumption of NR increased considerably during the period. Presently, about 93% of NR world production is produced in Asia, the following continents are Africa (4-5%) and Latin America (2.5-3%). During the year 2012, world production and consumption of NR reached 11.38 and 10.92 million metric tons, respectively, with a surplus of 460,000 metric tons according to the International Rubber Study Group (ISRG) projections (Figure 1). Thailand had the highest level of NR production in the world. The leaders of global NR exportation included in 4 countries, Thailand was the largest exporter with 35% of world's NR exportation followed by Indonesia 31%, Malaysia 16% and Malaysia 13% (Figure 2).

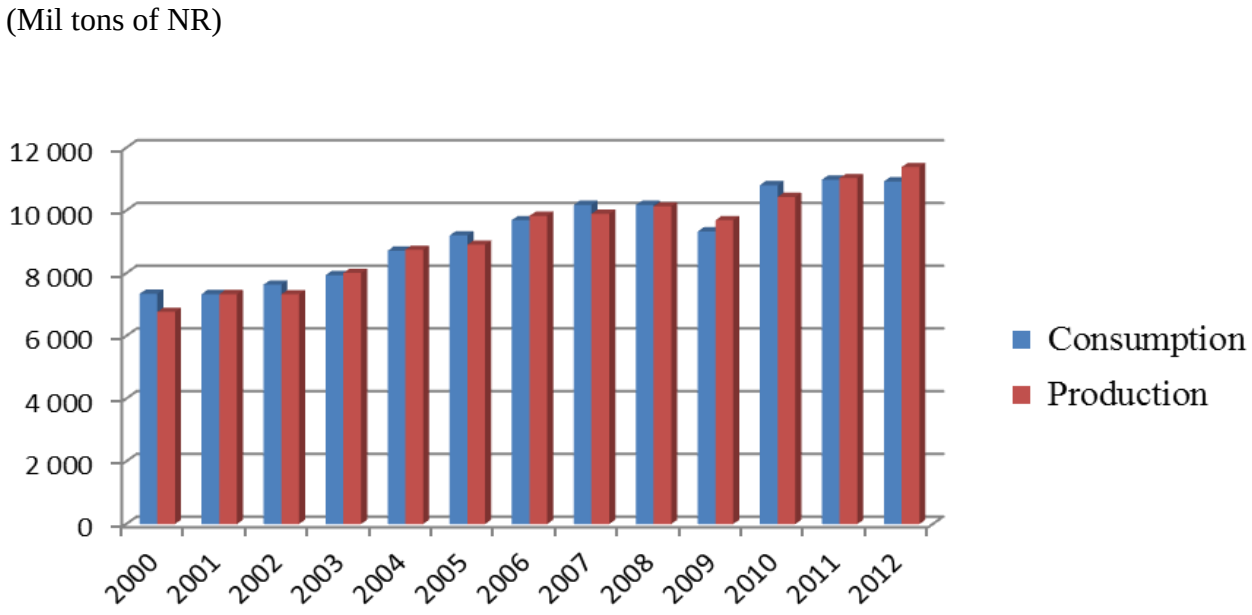

Figure 1. Global natural rubber production and consumption (Source: IRSG)

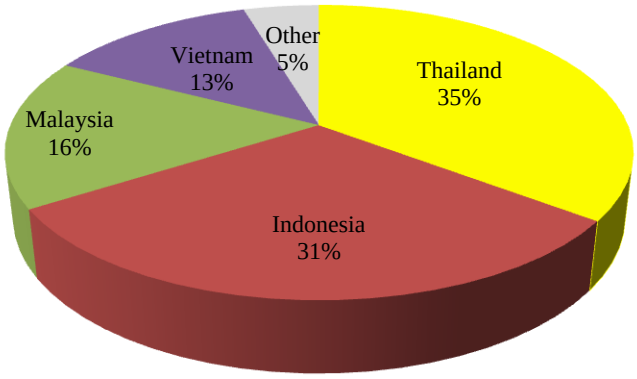

Figure 2. Gross exports of natural rubber from main rubber producing countries in 2012 (Source: IRSG)

1.3 The NR product and export situation of Thailand

Thailand has been the world's largest producer and exporter of natural rubber since 1991. In the end of 2012, the NR production and exportation are reached to 3.8 and 3.1 million tons respectively (Figure 3). In addition, rubber was the first agricultural product export value in Thailand (39.9%). The main export markets were China accounting for 52% (1.6 million tons), Malaysia at 11%, Japan at 9%, South Korea and EU at 6% and USA at 5% (Figure 4).

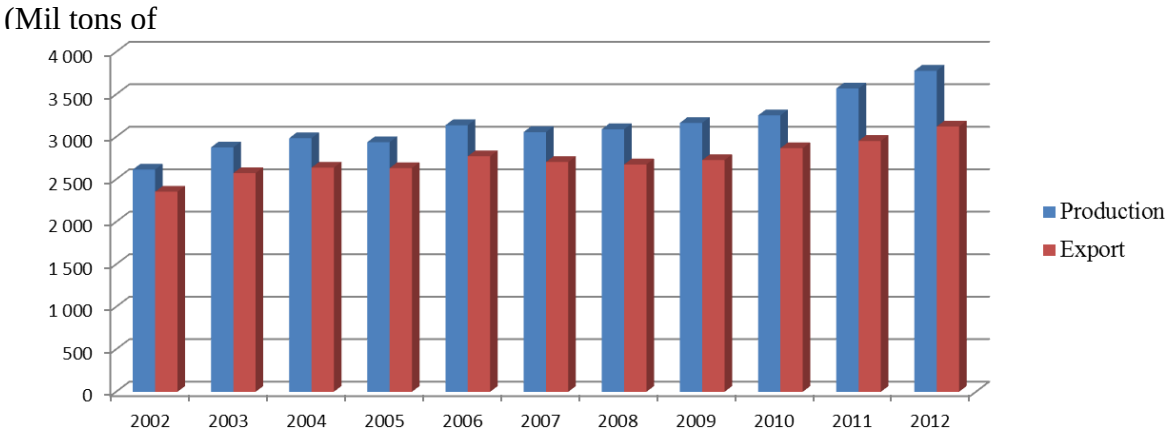

Figure 3. Nature rubber production and exportation of Thailand in 2002 – 2012 (Source: Rubber Research Institute of Thailand)

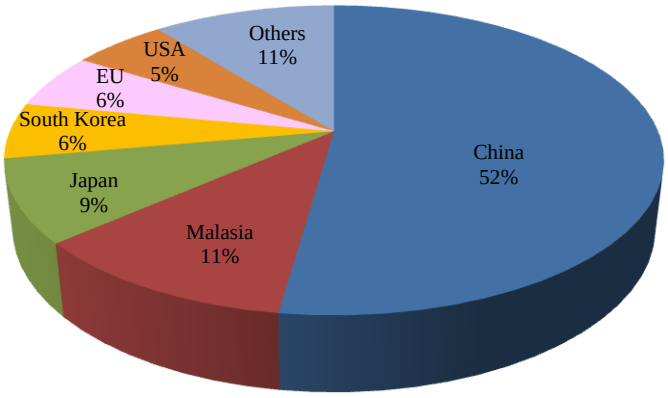

Figure 4. Exports of nature rubber by destinations from Thailand in 2012 (Source: Rubber Research Institute of Thailand)

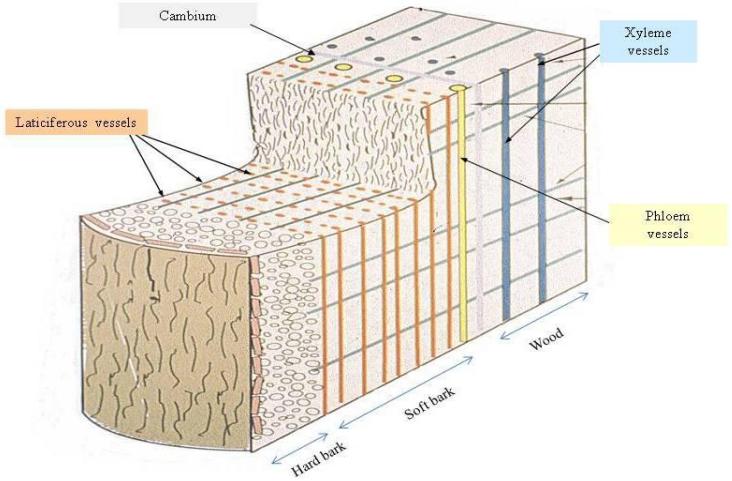

Figure 5. Anatomy of bark at the tapping cut in *Hevea brasiliensis*

2. Regulation of latex production in *Hevea brasiliensis*

2.1 The laticifer system

Hevea laticifers are periodically emitted from the cambium as single cells and found in the secondary phloem of the trunk (de Faÿ, Hébant and Jacob 1989). The laticifers are formed from chains of adjacent cells that are arranged in parallel rings. Between the laticifers in each ring, there are anastomose which develop a laticifers network structure in the soft bark of the rubber tree (Sando et al. 2009) (Figure 5). The number of laticifers is one of the most important factors influencing rubber yield (Gomez 1982). Laticifer ring differentiation from vascular cambium in *Hevea* clones is genetically controlled and is also influenced by environmental conditions. In addition, the level of latex exploitation correlates with the formation of laticifer rings. The comparison between non-harvested trees and harvested trees showed a 2-3 times higher number of laticifer rings in harvested tree in the same period in the area of bark from the latex flowed during latex harvesting (Hao and Wu 2000).

2.2 NR biosynthesis

Latex is essentially the cytoplasm of laticifers or latex vessels (d' Auzac and Jacob 1989). Detailed microscopic observation of the laticifers in *H. brasiliensis* shows that latex contains three main particulate components: rubber particles, lutoid particles and Frey-Wyssling complex which are present in major amounts, in addition to typical components such as nuclei, mitochondria and ribosomes (Ohya and Koyama 2001). In fact, the rubber particles constitute 25 - 45% of the volume of the fresh *Hevea* latex (de Faÿ et al. 1989). The chemical composition of natural rubber is *cis*-polyisoprene, which consists of sequential condensation of isopentenyl diphosphate (IPP) units. The long chains of IPP are produced through the mevalonate (MVA) pathway, the isoprenoid biosynthesis pathway (Kekwick 1989). Thus sugars are utilized as the main source of carbon for rubber formation. However, the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway been considered a possible alternative route to the synthesis of IPP for *cis*-polyisoprene (rubber) biosynthesis since its more recent discovery in plants. The supply of IPP for *cis*-polyisoprene from the MEP pathway is related to carotenoid production in latex (Chow et al. 2012) (Figure 6).

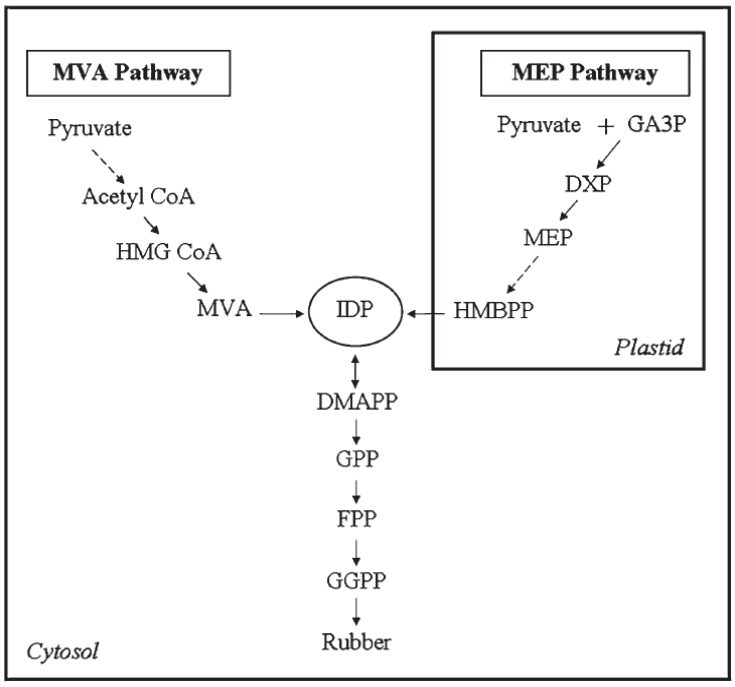

Figure 6. Biosynthesis of natural rubber in *Hevea brasiliensis*. IDP for the biosynthesis of rubber may be contributed by the MVA and MEP pathways. Broken arrows indicate multiple pathway steps. (Figure adapted from Chow *et al*. 2007).

2.3 Harvesting systems

2.3.1 Tapping

Rubber tree has an initial growth phase varying generally from 5 to 7 years, depending on climate, soil conditions, and management. Trees are tapped when their trunks attain 50 cm in girth (Clément-Demange et al. 2007). Traditionally, the rubber tree is tapped with S/2 d2 (i.e. half spiral cut tapped once in two days) or tapping once in three days (S/2 d3) harvesting system with or without stimulation by the application of ethephon as recommended in Thailand (http://www.rubberthai.com). Upon bark wounding or deliberate tapping, the latex expelled from the laticifers (de Faÿ et al. 1989), until coagulation of the rubber particles blocks the latex flow. The availability metabolism of sugar (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 one tapping was estimated to be around 72 hr (Serres et al. 1994).

2.3.2 Ethephon stimulation

The most extensively studied hormone is ethylene which stimulates the latex production. Ethylene significantly activates the regenerating metabolism within the laticifer (Pujade-Renaud et al. 1994) and prolongs latex flow (Coupe and Chrestin 1989). The stimulation is applied as ethephon (ethylene-releaser compound). Bark treatment with ethephon allows 1.5–2 fold increase of latex production (Pujade-Renaud et al. 1994). For yield optimisation, the frequency of stimulation and the concentration of stimulant should be modulated to the clone, tree age, and tapping system (Njukeng et al. 2011).

2.3.3 Limiting factor of latex yield

The latex yield is limited by several factors. At the level of the latex producing tissues, two factors; latex flow and latex regeneration between two consecutive tappings are considered as main limiting factors (Chrestin, Gidrol and Kush 1997). First, the duration of the latex flow, which determines the volume of latex collected at each tapping. The duration of the flow is limited by the coagulation, leading to the plugging of the severed extremity of latex vessels (d'Auzac, 1989). This phenomenon is caused by lutoids and the contents of Frey-Wyssling particles which contain coagulating factors (cations, organic acids, positively charged protein and hydrolyatic enzymes) (Jacob et al. 1989). Turgor pressure (TP) in the laticifers is directly responsible for latex flow at tapping, as high as 10–14 atmospheres before sunrise. Water plays a key role in latex flow after tapping; all the ecophysiological factors that affect water balance and water flux in the tree influence latex flow and coagulation (Pakianathan, Harridas and and d' Auzac 1989). Second, the latex regeneration between two consecutive tappings is related to the cellular metabolism of the laticifer system and to the ecophysiological functioning of the tree. The sufficient time (around 72 hr) for the reconstitution of the cell material lost during tapping is required for full latex regeneration (Serres et al. 1994). This corresponds to the net synthesis of about 50 g of dry rubber and 1.2 g of protein. In addition, NR regeneration mainly depends on the availability and metabolism of sugar (Tupý 1989). Thus, a very intense metabolic activity is required, in particular energy-generating catabolic pathways like glycolysis (Jacob, 1970), as well as anabolic processes allowing reconstitution of the intracellular components. In this context, nitrogen metabolism involved in protein and nucleic acid synthesis takes a prominent part (Pujade-Renaud et al. 1994).

3. Genetic improvement of rubber tree

3.1 Breeding programme

Breeding of rubber tree, like for many other tree crops, is a long-term process. Large-scale cultivation of new *Hevea* clones can only be reached after 20–25 years of field experiments on large areas (Webster and Baulkwill 1989). Breeding of rubber usually consists of three main steps: seedling evaluation trials (SET) using 100 to 400 progenies, small-scale trials (SST), and large-scale trials (LST) including the evaluation of latex production.

Although Thai breeding program has been started in 1960, 80% of rubber plantations were planted with the clone RRIM 600 from Malaysia. So far, new high-yielding clones and resistance to leaf diseases were selected and progressively planted in Thailand. In the 1990s, the government promoted the development of rubber cultivation in marginal area (North-East Thailand). The RRIT has tried to developed new clones adapted to this drought area. More recently, the demand for rubber wood justified the selection and the plantation of new latex timber clones.

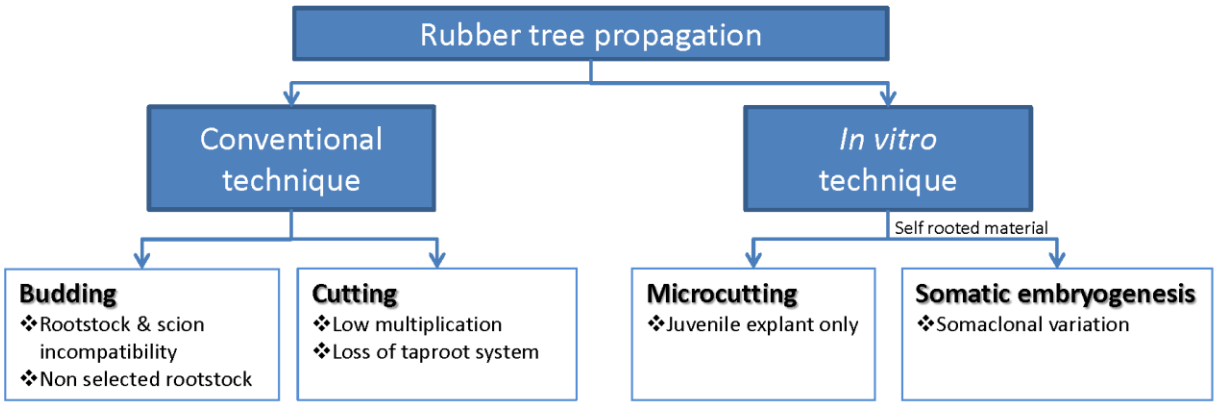

Figure 7. The choice for rubber tree propagation

3.2 Clonal propagation

H. brasiliensis is a heterogamous species that requires vegetative propagation of selected material. As soon as *Hevea* was used as crop in plantations, several conventional propagation methods have been evaluated, and more recently in the 1970s, the *in vitro* techniques (Figure 7). As it has never been possible to expand the propagation of self-rooted *Hevea* clones by cuttings on an industrial level, to date, conventional budding is used for *Hevea* propagation (Clément-Demange et al. 2007). Conventional budding method uses bud as scions on un-selected seedling rootstocks. Thereby, the best clones cannot show the optimal latex yield potential. This limitation factor is related to: (1) the loss of juvenility linked to lower vigor, (2) un-selected rootstock material, which maintains high heterogeneity between budded trees, (3) and the graft, which can be a barrier to nutrient and water supplies when anarchic vessels are formed.

The micro-cutting was developed from juvenile seedling material and rejuvenated clonal material with grafting on young seedling or soma-plant (Carron, Enjalric and Deschamps 1984). Even if this technic offers a strong advantage for true-to-type multiplication, however, the capacity of that technique therefore depends on the juvenility of the material treated. Its propagation capacities are limited.

Hevea somatic embryogenesis was first developed in China and Malaysia, using the anther wall as initial mother tissue explants (Carron M.P. 1989). Paranjothy (1975) obtained the first *Hevea* somatic embryos (PARANJOTHY and GHANDIMATHI 1975). Successful plantlet formation and acclimatization were achieved for *Hevea* clones Haiken 1, Haiken 2, and SCATC 88/13 (Wang et al. 1980). The production of somatic embryogenesis for clones RRIM600 and GL1 were reported afterwards (Clément-Demange et al. 2007). The protocols developed for *Hevea* regeneration were published by various research groups.

At CIRAD, somatic embryogenesis procedures have been developed using the inner integument of immature seed. The development of somatic embryos is obtain through four successive phases: (1) callogenesis, (2) differentiation of embryos, (3) multiplication of embryos, and (4) germination of embryos and development into plantlets (Carron and Enjalric 1982, Carron et al. 1984). After several years of in-depth research, two somatic embryogenesis

procedures have been proposed for *Hevea*: primary somatic embryogenesis and maintained somatic embryogenesis.

Primary somatic embryogenesis (PSE), refer to a short-term process was previously described by Carron et al. and consisted of 6 phase (initiation of callogenesis; generally producing a compact callus, embryogenesis expression; differentiation of undifferentiated cells into embryogenic cell, proembryo development, maturation of somatic embryos, germination of somatic embryos, and plantlet development) (Carron et al. 1995). *In vitro* plantlets produced by PSE are usually of good quality with better growth and latex production than budded clones (Carron et al. 2009). However, primary calli are subject to browning due to high ethylene and carbon dioxide release which leads to a low callus multiplication rate and restricted to a few clones (PB260 and RRIM703) (Auboiron, Carron and Michaux-Ferrière 1990).

Maintained somatic embryogenesis (MSE) is based on long-term maintenance of both the proliferation and regeneration of friable calli (Carron, Lardet and Dea 1998). The fragments of inner integument were induced a friable callus (friable callus induction medium; INF). Four to eight fortnight-long subcultures were usually required to initiate friable callus formation. Then, the friable callus aggregates were cultured on maintenance medium (MM) for callus proliferation. Calli were subcultured regularly every two weeks. Until calli gave rise to, they were induced to embryos development and conversion into plantlets. In addition, long−term maintenance of friable embryogenic calli lead to loss of callus regeneration competence in rubber tree (Blanc et al. 2006) and increase the risk of somaclonal variations as it was observed in some other species (Lardet L 2006). According to the limitation of MSE, cryopreservation technique, a long-term storage in liquid nitrogen, is used to overcome the problems.

Cryopreservation technique was developed for friable embryogenic callus lines of *Hevea brasiliensis.* The technique included a callus pre-culture on maintenance medium containing 1 mM $CaCl_2$ prior to cryopreservation to promote post-thaw callus growth recovery. Application of this cryopreservation technique on a sample of 39 callus lines, showed a high percentage of post-thaw lines growth recovery and subsequent maintenance of embryogenic and regeneration competences (Lardet et al. 2007).

The alternative procedure have been developed according to cope with the disadvantage of previous procedures, this new procedure was call indirect Secondary Somatic Embryogenesis (SSE) (Lardet et al. 2009). PSE consisting of a first phase of callogenesis (Figure 8a), embryogenesis expression and embryo development was used to provide somatic embryos (Figure 8a). Secondly, the embryo-derived embryogenic callus was developed using MSE procedure until the friable callus had stabilized rate of proliferation (Figure 8b). Thirdly the cryopreservation of friable calli has been incorporated into the process to limit tissue proliferation and, thereby, the risks linked to somaclonal variation (Figure 8c). Fourthly, embryo development and plant regeneration were induced from friable callus after thawing treatments (Figure 8d).

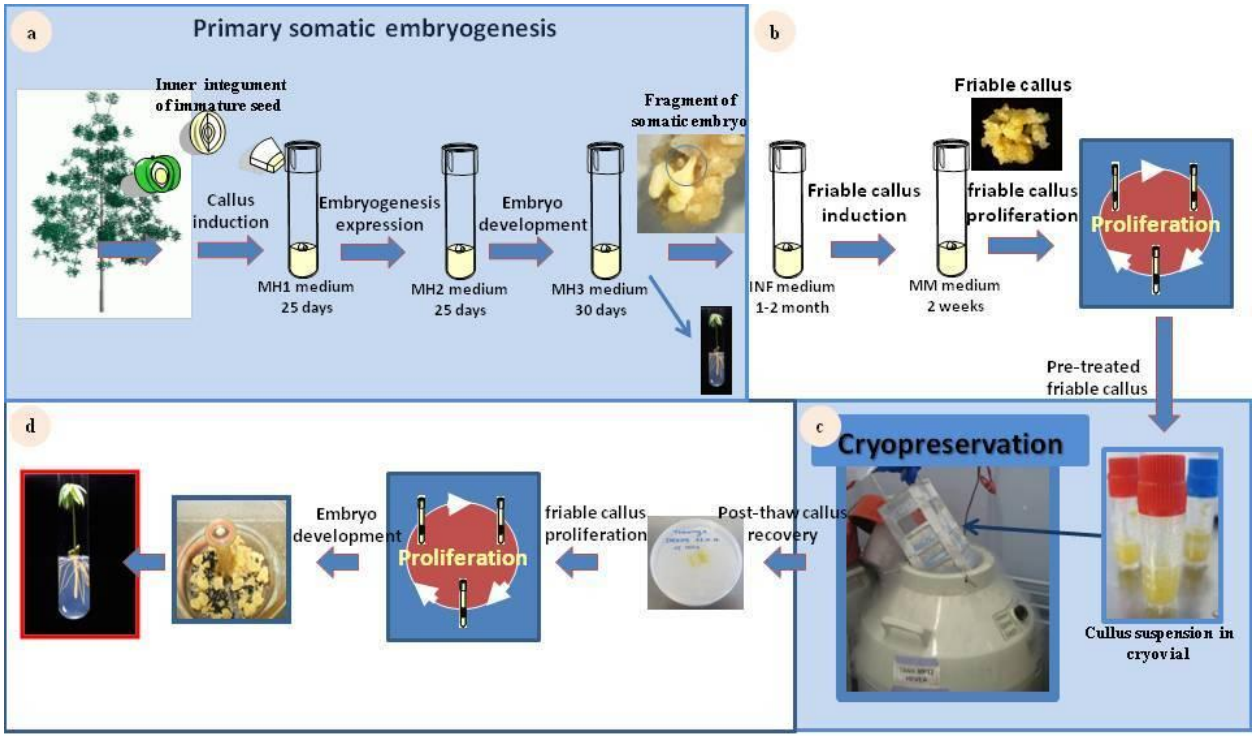

Figure 8. Indirect secondary somatic embryogenesis in *Hevea brasiliensis* process in four steps. (a) Primary somatic embryogenesis using the inner integument of immature seeds, (b) Establishment of friable callus line, (c) cryopreservation and (d) plant regeneration methods have been described in several papers (extracted from reference: Plant Cell Reports (Lardet et al.,2007), J.Rubb.Res (Lardet *et al*., 2009)).

4. Ethylene biosynthesis and signalling in plants

Ethylene biosynthetic pathway was first described by Yang (Figure 9). First, *S*-adenosylmethionine (*S*-AdoMet) is synthesized by SAM synthetase from the methionine. Then *S*-AdoMet is converted to ACC by ACC synthase (ACS). In addition to ACC, ACC synthase (ACS) also produces 5'-methylthioadenosine (MTA) in this reaction, which is then converted to methionine by using a modified methionine. Thereby, ethylene can be synthesized continuously without lacking methionine substrate. The activity of ACS determines the rate of ethylene production, Finally, ACC is oxidized by ACC oxidase to form ethylene, CO_2, and cyanide, which is detoxified to β-cyanoalanine by β-cyanoalanine synthase (β-CAS) in order to prevent toxicity of accumulated cyanide (Yang and Hoffman 1984, Wang, Li and Ecker 2002).

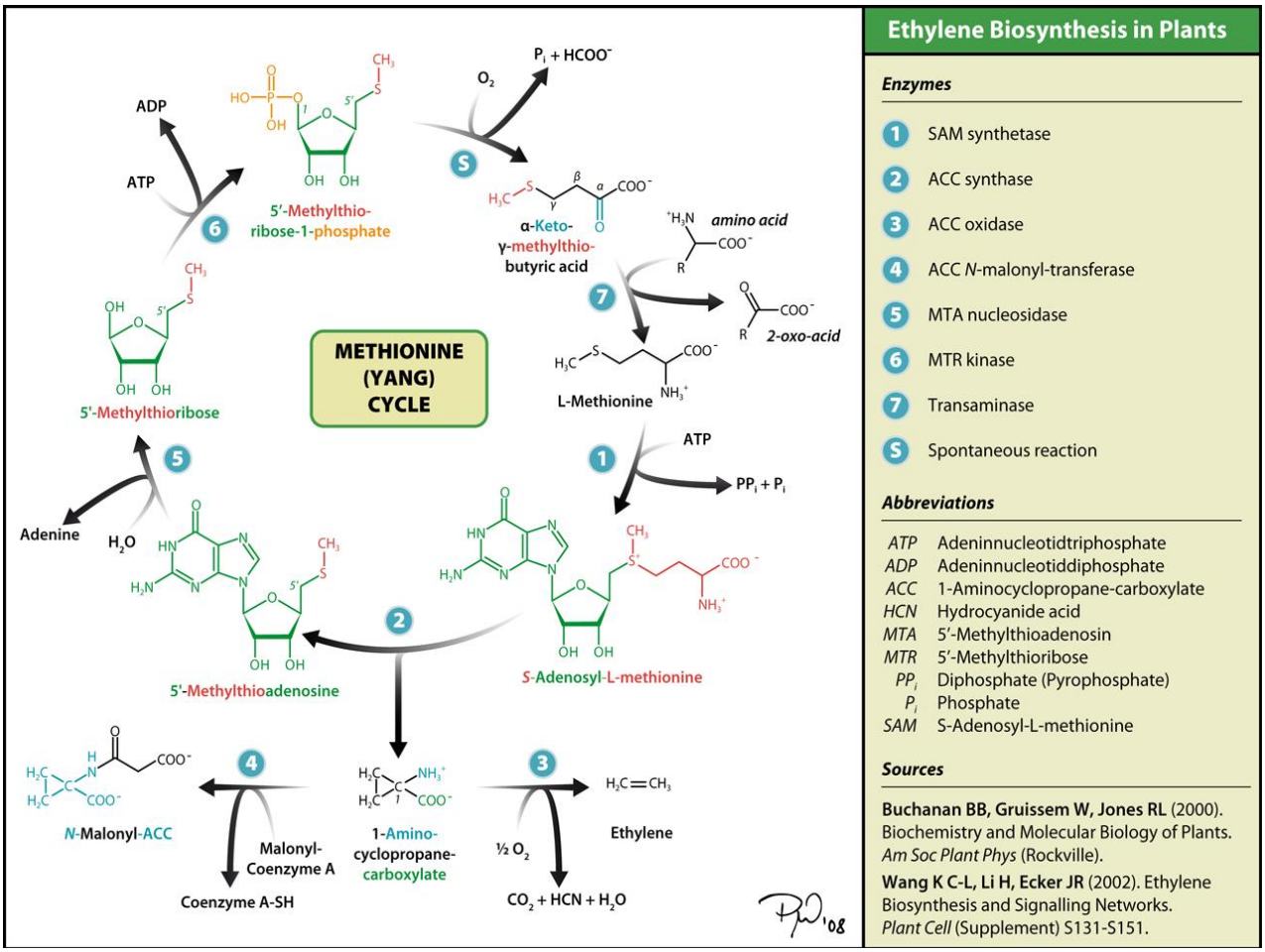

Figure 9. Biosynthetic pathway and regulation of ethylene (Figure adapted from Yang *et al.*,1984).

Ethylene is perceived by a family of five membrane-localized receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) which were identified in *Arabidopsis* (Chang et al., 1993; Hua et al., 1995). The ethylene receptors contain three trans-membrane domains and a conserved histidine kinase domain. Only ETR1, ETR2, and EIN4 contain a receiver domain at the C-terminal part. Ethylene binding occurs at the N-terminal trans-membrane domain of the receptors. Ethylene receptors are negative regulators of the ethylene response. In the absence of ethylene signal, ethylene receptors activate a Raf-like kinase, CTR1, and CTR1is in an active state that serves to repress ethylene responses. Conversely, binding of ethylene inactivates the receptors, thereby inactivating CTR1 allowing EIN2 to function as a positive regulator of the ethylene pathway. EIN2 initiates a transcriptional cascade involving the EIN3/EIL transcription factors. EIN3 binds to the promoter of ERF1 gene and stimulate its transcription in order to activate ethylene response genes (Wang et al. 2002, CHEN, ETHERIDGE and SCHALLER 2005, Guo and Ecker 2004) (Figure 10).

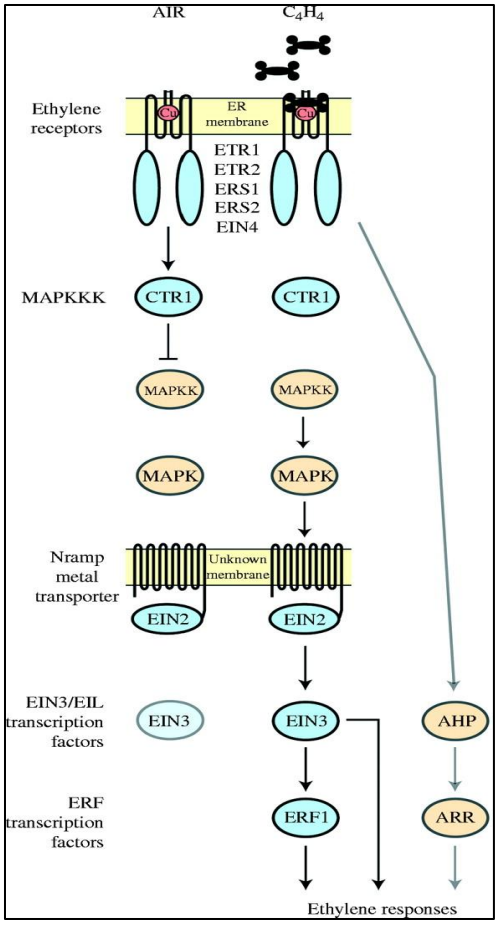

Figure 10. Ethylene signal transduction (Figure adapted from Chen *et al.*,2005)

5. AP2/ERF superfamily

The AP2/ERF is one of the largest superfamily of transcription factors in plants (Wessler 2005). AP2/ERF transcription factors regulate to biological processes including development such as ovule development, floral organ growth, hormones responses, adaptation to biotic and abiotic stresses (Elliott et al. 1996), (Zarei et al. 2011, Cheng et al. 2013, Lorenzo et al. 2003, Mizoi, Shinozaki and Yamaguchi-Shinozaki 2012). Members of the AP2/ERF superfamily contain at least one AP2 domain, which is responsible for DNA binding to the GCC-box (AGCCGCC) (Ohme-Takagi and Shinshi 1995) or to a dehydration response element (DRE: TACCGACAT) containing the C-repeat(Ito et al. 2006). This conserved domain consists of about 60-amino acid (Ohme-Takagi and Shinshi 1995).

The AP2 domain of about 58-62 amino acid residues consists of 2 conserved sequence blocks. The first block, referred to as the YRG element, consists of 19–22 amino acids, is highly basic and contains the conserved YRG amino acid motif. The second block, referred to as the RAYD element, is 42–43 amino acids in length and contains a highly conserved 18-amino acid

core region that is predicted to form an amphipathic α-helix (Okamuro et al. 1997). The AP2/ERF superfamily has been classified based on the number of AP2 domains. The AP2 family contains proteins with a double, tandem repeated, AP2 domain (Okamuro et al. 1997). The ERF family genes code for transcription factors with a single AP2 domain. Lastly, the RAV family encodes proteins possessing a single AP2 domain plus an additional B3 domain (Kagaya, Ohmiya and Hattori 1999). There are some differences of classification. Nakano's classification method was compared with Sakuma's method, both of them using a sequence comparison of complete AP2 domain (Table 1). Sakuma *et al*. described five subfamilies including AP2, RAV, Dehydration Responsive Element Binding Proteins (DREB), Ethylene-Responsive Element Binding Proteins (EREBP), also called the Ethylene-Response Factors (ERF) family whiles, Nakano *et al*. classified these proteins in only three major families: AP2, ERF and RAV (Sakuma et al. 2002, Nakano et al. 2006).

Table 1 Correspondence between Nakano's and Sakuma's classification methods for AP2/ERF superfamily genes. Total for each family is shown in bold. (from Nakano *et al*.,2006).

Nakano *et al*. (2006)			**Sakuma *et al*. (2002)**		
Classification	**Group**	**No.**	**Classification**	**Subgroup**	**No**
AP2 family		**18**	AP2 subfamily		**17**
ERF family		**122**	DREB, ERF subfamily		**121**
Groups I to IV	Ia		DREB subfamily	A-6	
	IIa				
	IIb			A-5	
	IIc				
	IIIa			A-4	
	IIIb				
	IIIc			A-1	
	IIId			A-4	
	IIIe				
	IVa			A-2	
	IVb				
	IVb			A-3	
Groups V to X	Va		ERF subfamily	B-6	
	Vb				
	VI			B5	
	VII			B2	
	VIII			B1	
	Ixa				
	Ixb			B3	
	Ixc				
	X			B4	
Groups VI-L and Xb-L	VI-L			B6	
	Xb-L			B6	
At4g13040		**1**	AL079349		**1**
RAV family		**6**	RAV subfamily		**6**
Total		**147**	Total		**145**

5.1 AP2 family

AP2 family play a key roles in participate in the regulation of developmental processes, e.g. flower development, spikelet meristem determinacy, leaf epidermal cell identity, and embryo development (Riechmann and Meyerowitz 1998). The AP2 family can be further classified into two groups: the APETALA2 (AP2) and the AINTEGUMENTA (ANT). The AP2 group included TOE3 (At5g67180), TOE1 (At2g28550), TOE2 (At5g60120), SMZ (At3g54990), and SNZ (At2g39250) and AP2 (At4g36920) while, the ANT group included AtBBM/AIL2 (At5g17430), PLETHORA1(PLT1)/AIL3 (At3g20840), PLETHORA2 (PLT2)/AIL4 (At1g51190), AIL1(At1g72570), AIL5 (At5g57390), AIL6 (At5g10510) and AIL7 (At5g65510) (Shigyo, Hasebe and Ito 2006, Nole-Wilson, Tranby and Krizek 2005) (Table 2).

Table 2. Gene list for the AP2 family.

Family	AGI ID	Known Gene Name(s)	AP2 domain
AP2	At4g37750	ANT/DRG/CKC/CKC1	Double domain
	At4g36920	AP2/FLO2/FL1	Double domain
	At1g72570		Double domain
	At5g65510	AIL7/PLT7	Double domain
	At3g20840	PLT1: PLETHORA 1	Double domain
	At5g57390	AIL5/CHO1/EMK/PLT5	Double domain
	At1g51190	PLT2	Double domain
	At1g79700		Double domain
	At5g10510	AIL6/PLT3	Double domain
	At5g17430	AtBBM	Double domain
	At1g16060	ADAP	Double domain
	At5g67180	TOE3	Double domain
	At2g28550	RAP2.7/TOE1	Double domain
	At3g54320	WRI1/ASML1	Double domain
	At2g41710		Single domain
	At2g39250	SNZ	Single domain
	At3g54990	SMZ	Single domain
	At5g60120	TOE2	Single domain

AP2 genes function as key developmental regulators in reproductive and vegetative organs (Riechmann and Meyerowitz 1998). *AP2* is a class of floral homeotic gene that plays a central role in the establishment of the floral meristem, the specification of floral organ identity in Arabidopsis (Bowman, Smyth and Meyerowitz 1989, Kunst et al. 1989, Jofuku et al. 1994). Moreover, AGAMOUS, the class C floral homeotic gene is regulated negatively by AP2

(Drews, Bowman and Meyerowitz 1991). ANT group involved in lateral organ development by controlling cell number and growth (Jofuku et al. 1994, Shigyo et al. 2006, Elliott et al. 1996, Mizukami and Fischer 2000). ANT group including *ANT* and *AINTEGUMENTA-like* (*AIL*) also known as *PLETHORA* (*PLT*) contain eight genes and are high sequence similarity within AP2 domain (Nole-Wilson et al. 2005). *ANT* regulates cell division in integuments and is necessary for floral organ growth. In addition, *ANT* controls plant organ cell number and organ size thorough out shoot development (Mizukami and Fischer 2000). *AIL6/PLT3*, *AIL5/PLT5* and *AIL7/PLT7* are regulators of shoot phyllotaxis control (Prasad et al. 2011). AIL6/PLT3 regulate floral organ size and involve in both proliferation and differentiation in flowers (Krizek and Eaddy 2012). Members of the AIL/PLT gene family also regulate meristem development. PLT1 and PLT2, control establishment of the stem cell niche during embryonic pattern formation (Aida et al. 2004). PLT protein dosage regulate distinct cellular responses in root. High levels of PLT activty promote stem cell identity and maintenance, whiles lower levels promote mitotic activity of stem cell daughters (Galinha et al. 2007). *ANT, AIL6/PLT3* and *AIL7/PLT7* have distinct functions within the meristem but are required for continuous shoot apical meristem function (Mudunkothge and Krizek 2012). Furthermore, *ANT* and *AIL6/PLT3* have partially redundant roles in several aspects of shoot and flower development, including lateral organ growth, floral meristem initiation and floral patterning (Krizek 2009). BABY BOOM (BBM) activates a complex network that associated with cell proliferation and growth, leading primarily to somatic embryogenesis, but also to organogenesis and callus formation (Passarinho et al. 2008).

5.2 ERF family

ERF proteins consist of one AP2 domain and several specific motifs. ERF group I to IV correspond to the DREBs of the Sakuma classification, which are involved in cold-stress- and osmotic-stress-responsive gene expression in *Arabidopsis.* This transcription factors group is specifically to bind to DRE/CRT, a *cis*-acting element that is involved in gene expression responsive to drought and low temperature stress. ERF group V to X are the DNA-binding specificity of ERF proteins, involved in ethylene-responsive gene expression and specifically bind to the GCC box (Sakuma et al. 2002, Yamamoto, Suzuki and Shinshi 1999).

ERF group I functions in stress responses, dehydration, high salinity and heat, but their target genes are different from those of DREB1s/CBFs and DREB2s (Mizoi et al. 2012). ERF group II are considered as active repressors of transcription. RAP2.1 is categorized in this group, which are contain ERF-associated amphiphilic repression (EAR) motif (Ohta et al. 2001). Overexpression of these genes results in reduced expression of DREB1/CBF and DREB2 target genes under cold and dehydration respectively (Ohta et al. 2001, Dong and and Liu 2010), ERF group III correspond to the DREB1/CBF involved in the cold tolerance are characterized by several hydrophobic clusters. The representative genes in this group, DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 are induced by low-temperature stress but not by dehydration or high salinity (Liu et al. 1998). ERF group IV correspond to DREB2 involved in the drought tolerance. Expression of the *DREB2A* and *DREB2B* genes is induced by dehydration, high-salt stress and heat shock.(Liu et al. 1998, Nakashima et al. 2000, Sakuma et al. 2006). The other ERF groups are more related to the response to biotic and abiotic stress. Interestingly, group VII regulate hypoxia-responsive genes. The gene member of this group, HYPOXIA RESPONSIVE1 and 2 (HRE1 and HRE2) and RELATED TO AP2 2 (RAP2.2) have been shown to enhance plant responses to hypoxia or anoxia. According to the N-end rule pathway of

targeted proteolysis associates the fate of a protein substrate with the identity of its N-terminus (Fukao and Xiong 2013, Gibbs et al. 2011, Licausi et al. 2010). ERF group VIII, EAR motif has also been discovered in the gene member of this group (Ohta et al. 2001, Nakano et al. 2006). However, members of ERF repressors show differential function. AtERF4 was shown to be a negative regulator in the expression of ethylene, jasmonate and ABA-responsive gene. In addition, AtERF7 was shown to play an important role in ABA response in plants (Yang et al. 2005, Song et al. 2005). Champion *et al*., reported that ERF from group IX in cotton are involved in jasmonate (JA), ethylene (ET) and pathogen responses (Champion et al. 2009).ERF are at the crosstalk of several signalling pathways. ORA59, a member of the ERF transcription factor family in Arabidopsis, integrates JA and ethylene signal. By doing so, ORA59 controls the expression of a subset of JA- and ethylene-dependent genes, including PDF1.2. Expression of these genes in response to these signals, or after perception of certain pathogens, depends on ORA59, and no other transcription factor, including ERF1 or AtERF2, can bypass the requirement for ORA59 (Pré et al. 2008).

5.3 RAV family

The RAV family proteins contain two distinct DNA-binding domain conserved, AP2 and B3, both of which are uniquely found in plants (Kagaya and and Hattori 2009, Kagaya et al. 1999). The B3 DNA binding domain is shared among various plant-specific transcription factors, including factors involved in auxin-regulated and abscisic acid–regulated transcription (Yamasaki et al. 2004). Expression of RAV1 and its homologues is closely associated with leaf maturation and senescence (Woo et al. 2010).

6. Towards the characterization of the ethylene biosynthesis and signalling in Hevea brasiliensis

6.1 Background

In *Hevea brasiliensis*, ethylene is a major stimulating factor of natural rubber production. Ethylene biosynthetic pathway has been described (Kuswanhadi 2006). ACS and ACO are encoded by multifamily genes of at least three genes in *Hevea*. Analysis of their transcript abundance in response to harvesting stress (wounding, tapping, ethylene) revealed two ethylene production systems: one constitutive which down-regulated by stress and one induced by these stresses. With regard to the ethylene transduction, several genes have been isolated and characterized (Duan et al. 2010), and more recently most of members of the ERF transcription factor family were identified (Duan et al. 2013). The AP2/ERF superfamily consists of 22, 115, 4 members for the AP2, ERF and RAV families, respectively.

6.2 Objectives of this thesis

In this thesis, the objective is to characterize *AP2/ERF* genes that could play important role during *Hevea* development and in some specific tissues such as laticifers. The strategy was as follows (Figure 11):

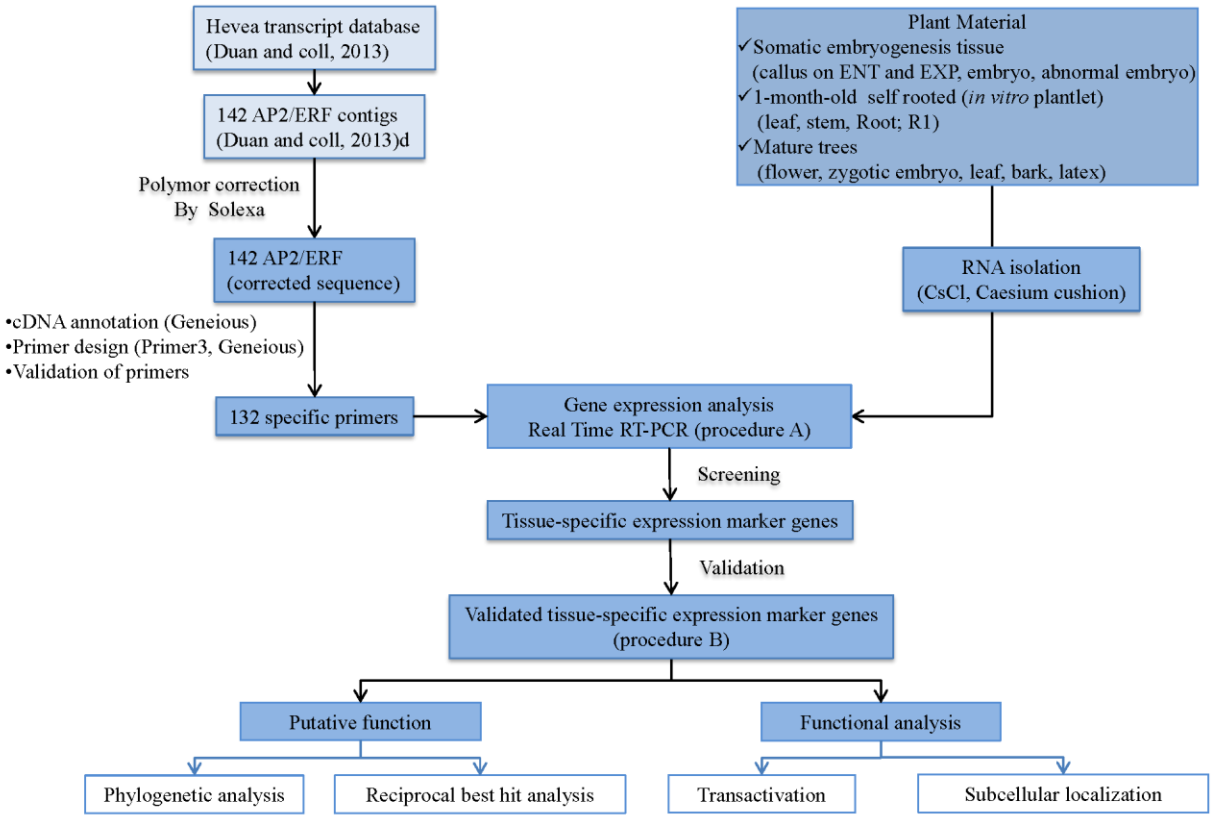

Figure 11. General strategy to characterize *AP2/ERF* genes involved in *Hevea* development through the identification of tissue-specific expression marker genes, prediction of their putative function and function analysis of these transcript transcription factors

- First, *AP2/ERF* expression marker genes (EMG) were identified in various tissues from somatic embryogenesis and *Hevea* plantlets and trees. From 152 AP2/ERF sequences, 132 specific couples of primers were designed. A first gene expression screening was done with these 132 couples of primers by real-time RT-PCR (Figure 12).
- Second, putative functions were searched using phylogenetic analysis with sequence from *Arabidopsis* and *Oryza sativa* to which functions have been demonstrated (Figure 13).
- Third, EMGs were validated by a new gene expression analysis and sequencing of amplicons (Figure 14).
- Finally, the function of EMG as transcription has been validated by subcellular localization and transactivation experiments (Figure 15).

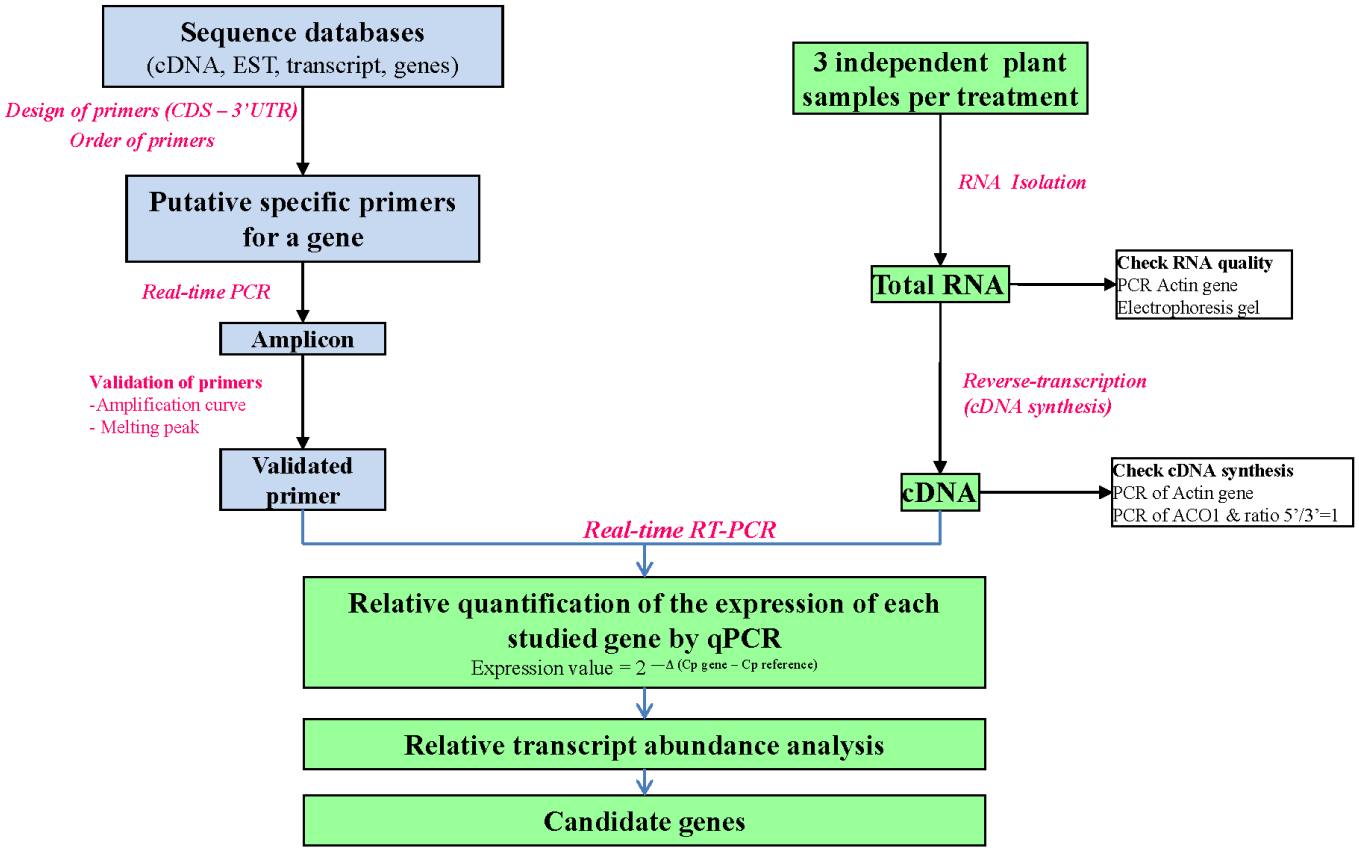

Figure 12. Real-time RT-PCR: Steps for large screening of gene expression using real-time PCR Light Cycler 480 (Roche).

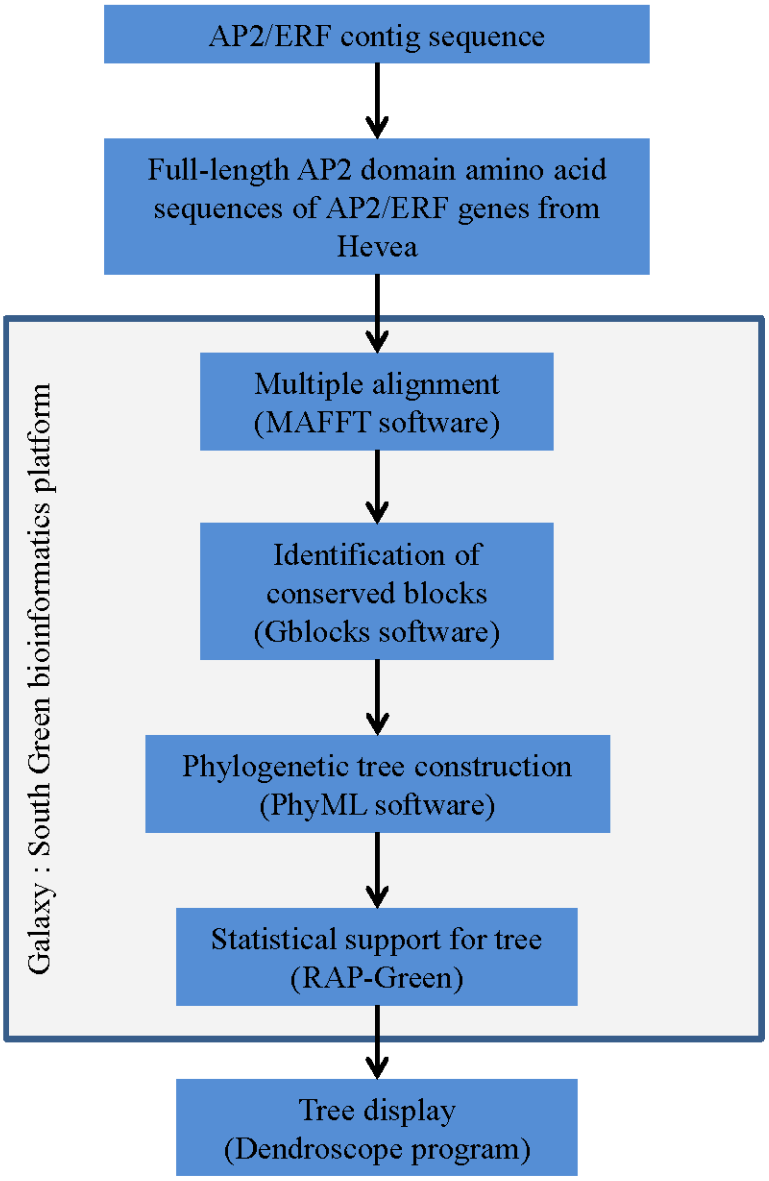

Figure 13. Phylogenetic analysis of AP2/ERF deduced amino acid sequence using Galaxy workflow and Dendroscope program for the visualization of phylogenetic tress.

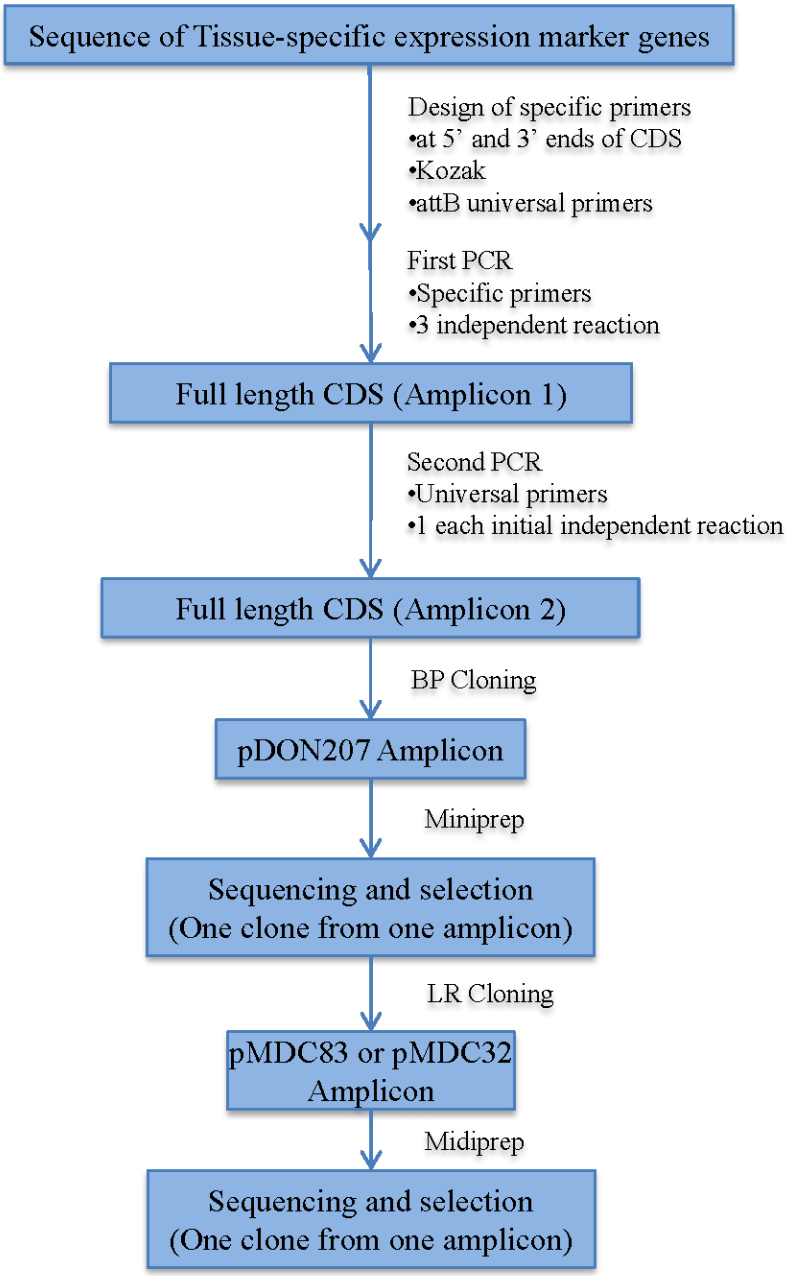

Figure 14. Procedure to test the functionality of putative transcription factors (validated tissue-specific *ERF* expression marker genes). This procedure includes the cloning of full length CDS in gateway vectors for transactivation and subcellular localization experiments.

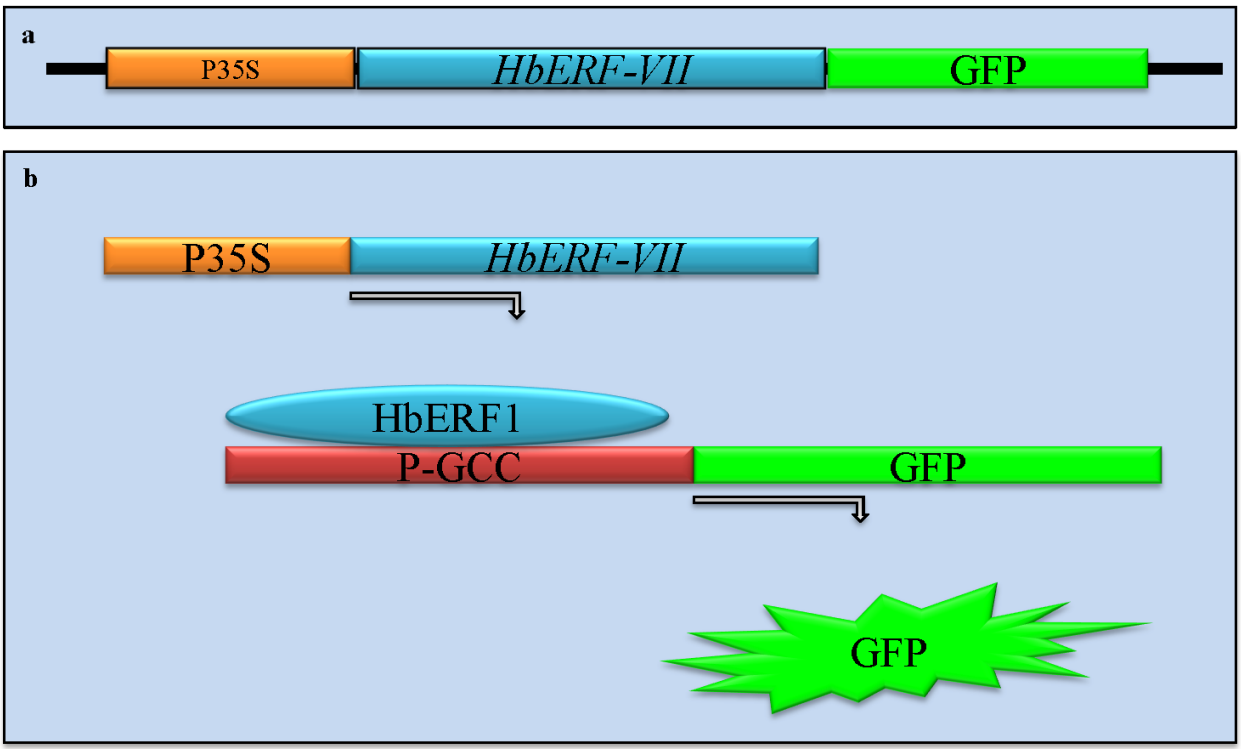

Figure 15. Construct for subcellular localization (a) and transactivation of synthetic GCC-box containing promoter (b).

7. References

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CHAPITRE 1

Article intitulé “Some ethylene biosynthesis and AP2/ERF genes reveal a specific pattern of expression during somatic embryogenesis in *Hevea brasiliensis*”

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Some ethylene biosynthesis and AP2/ERF genes reveal a specific pattern of expression during somatic embryogenesis in *Hevea brasiliensis*

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Abstract

Background

Ethylene production and signalling play an important role in somatic embryogenesis, especially for species that are recalcitrant in *in vitro* culture. The AP2/ERF superfamily has been identified and classified in *Hevea brasiliensis*. This superfamily includes the ERFs involved in response to ethylene. The relative transcript abundance of ethylene biosynthesis genes and of AP2/ERF genes was analysed during somatic embryogenesis for callus lines with different regeneration potential, in order to identify genes regulated during that process.

Results

The analysis of relative transcript abundance was carried out by real-time RT-PCR for 142 genes. The transcripts of ERFs from group I, VII and VIII were abundant at all stages of the somatic embryogenesis process. Forty genetic expression markers for callus regeneration capacity were identified. Fourteen markers were found for proliferating calli and 35 markers for calli at the end of the embryogenesis induction phase. Sixteen markers discriminated between normal and abnormal embryos and, lastly, there were 36 markers of conversion into plantlets. A phylogenetic analysis comparing the sequences of the AP2 domains of *Hevea* and *Arabidopsis* genes enabled us to predict the function of 13 expression marker genes.

Conclusions

This first characterization of the AP2/ERF superfamily in *Hevea* revealed dramatic regulation of the expression of AP2/ERF genes during the somatic embryogenesis process. The gene expression markers of proliferating callus capacity to regenerate plants by somatic embryogenesis should make it possible to predict callus lines suitable to be used for multiplication. Further functional characterization of these markers opens up prospects for discovering specific AP2/ERF functions in the *Hevea* species for which somatic embryogenesis is difficult.

Keywords

Gene expression, Plant hormone, Plant regeneration, Recalcitrant, Rubber, Signalling, Transcription factor

Background

Understanding the molecular mechanisms controlling somatic embryogenesis is crucial, be it biologically or for applications. Indeed, propagating cultivated plants by somatic embryogenesis still remains limited by the efficiency of the procedures in some recalcitrant species or genotypes. Genetic predeterminism of recalcitrance in some woody and herbaceous perennial species has been linked to their response to wounding and their phenolic compound content [1]. The production of ethylene, carbon dioxide and free radicals lies behind the induction of cell defence mechanisms, leading to the oxidation of phenol compounds, the breakdown of cell walls and membrane peroxidation [2]. Adding growth regulators, and the stress induced by *in vitro* culture, play a leading role in such mechanisms [3]. These phenomena result in the differentiation of undifferentiated cells and in tissue browning, which lead to a loss in embryogenic capacity [2,4].

The negative effect of ethylene on somatic embryogenesis has been known for a long time [5]. Some super-embryogenic explant cultures of *Medicago truncatula* revealed the repression of numerous genes, including those involved in ethylene biosynthesis and signalling [6]. In alfalfa, loss of embryogenic capacity following thidiazuron application is linked to the induction of an ethylene biosynthesis gene [7]. Ethylene also induces some stress factors conducive to the acquisition of somatic embryogenesis capacities [8,9], and embryo maturation [10]. Abscisic acid and methyl jasmonate are regulators of ethylene biosynthesis during somatic embryogenesis in *Medicago sativa* [11]. Some members of the ETHYLENE RESPONSE FACTOR (ERF) family are involved in response to *in vitro* stress and in the regulation of developmental processes. In *Medicago truncatula*, the *SOMATIC EMBRYO RELATED FACTOR 1* gene (*MtSERF1*) is induced by ethylene and may act under the influence of WUSCHEL (WUS), whose fixation sites are found on the *SERF1* gene promoter [12]. ENHANCER OF SHOOT REGENERATION1 (ESR1) of *Arabidopsis* is induced by cytokinins to regulate the start of shoot regeneration [13]. DORNROSCHEN (DRN)/ESR1 plays a role in meristem and organ development and consequently in shoot regeneration [14]. ESR2 expression confers "cytokinin-independent shoot regeneration" through transcriptional regulation of the *CUP-SHAPED COTYLEDON 1* gene (*CUC1*) [15]. When interacting with other transcription factors, the induction of CALLUS EXPRESSING FACTOR1 (CEF1) through stress would appear to disrupt auxin/cytokinin homeostasis in *Nicotiana tabacum*. Of the numerous other transcription factors involved in embryogenesis and organ development ([16] for a review), several members of the APETALA2 family (AP2) play a major role [17]. Like ERFs, this family belongs to the AP2/ERF superfamily. For example, BABY BOOM (BBM) is known for its role in cell proliferation and morphogenesis during embryogenesis [18]. AINTEGUMENTA (ANT) is involved in ovule development and in the growth of floral organs [19]. WRINKLED (WRl1) is involved in regulating storage metabolism in seeds [20,21]. The ANT subfamily includes several members called ANT-like genes or AILs. The initiation of post-embryonic shoot organs takes place in the shoot apical meristem, also involving several members of the AP2 family (ANT, AIL6/PLT3 and AIL7/PLT7) [22].

Hevea brasiliensis is a particularly difficult species which has led to numerous micropropagation studies [23]. This cross-fertilizing species is cloned by budding in the absence of any other efficient vegetative propagation techniques for own-rooted plants. Two somatic embryogenesis methods have been developed for *Hevea* ([23-25] for a review). The first is somatic embryogenesis on primary callus (SEP) obtained from fragments of the internal integument of immature seeds or anthers [26-28]. This method is effective for around twenty *Hevea* clones following numerous culture medium and atmosphere studies [25,29]. However, primary calli are subject to browning due to high ethylene and carbon dioxide release, which leads to a low callus multiplication rate [30]. *In vitro* plantlets produced by SEP are usually of good quality with better growth and latex production than budded clones [31]. A second method was developed from friable callus maintained over the long term with a view to large-scale multiplication. This method has evolved with the use of fragments of embryos derived from SEP in order to rapidly establish embryogenic friable callus lines [29,32]. Lastly, cryopreservation of friable calli has been incorporated into the process to limit tissue proliferation and, thereby, the risks linked to somaclonal variation [33]. However, this indirect secondary somatic embryogenesis process is restricted to just a few clones and the embryo and plant regeneration capacity is variable. The absence of plant regeneration potential in some friable callus lines has been linked to early vacuolization of cells in the embryogenic globules [34]. Some transcriptional changes have also been reported for certain *Hevea* callus lines with different embryogenic potentials [34].

A study on the role of ethylene in stimulating latex production led to the characterization of ethylene biosynthesis and signalling genes and more recently to the identification of the different members of the AP2/ERF superfamily [35-38]. The AP2/ERF superfamily contains 173 members, of which 142 are classed in families and groups [35]. The AP2 family contains 20 genes organized in two subfamilies (8 ANT and 12 AP2 genes). The ERF family is divided into 10 groups comprising a total of 115 genes. Lastly, there remains the RAV family with four genes and the soloists with 3 genes. Based on this knowledge, our study set out to gain a clearer understanding of the regulation of ethylene biosynthesis and signalling genes, including ERFs, along with the members of the AP2 and RAV families during the somatic embryogenesis process in *Hevea*. The relative transcript abundance analysis for these genes was carried out by real-time RT-PCR on friable callus lines with different embryogenic potential [33], on normal and abnormal somatic embryos, and on different young plant tissues. The gene expression profiles showed that several genes were markers of embryogenic potential for the lines in proliferating calli and in calli induced by embryogenesis, and also markers of somatic embryo quality. Prediction of the function of those marker genes provides a dynamic understanding of the somatic embryogenesis process.

Results

Morphogenetic potential of callus lines with different somatic embryogenesis capacities

The secondary indirect somatic embryogenesis process is described in Figure 1 (cf. Materials and Methods). Primary somatic embryogenesis is induced from inner integument of immature seed as maternal tissue (Figure 2 A). Compact callus-bearing mature somatic embryos were obtained after three subcultures (Figure 2 B). Friable callus lines were established from fragments of somatic embryo. The proliferating friable calli grown on the ENT medium were fairly comparable whatever their embryogenic capacity (Figure 2 D). After four weeks' culture on EXP, the calli of the non-embryogenic (Figure 2 J) and embryogenic (Figure 2 H) lines were more hydric and whitish than those of the regenerant line (Figure 2 E). Callus growth was slowed during embryo regeneration in the RITA culture system on DEV medium. The calli turned brown in all the lines (Figure 2 F, I and K). Browning occurred to the benefit of embryo formation for embryogenic lines (Figure 2 F and I). The normal embryos had an embryonic body and two well-developed cotyledons (Figure 2 L). The abnormal types of embryos were most numerous: for example, some embryos with a single cotyledon (Figure 2 M), or with malformed cotyledons (Figure 2 N), and a double embryonic body (Figure 2 O) were found. Plantlets derived from normal embryos developed a taproot and a lateral root system, and a stem with leaves within a month (Figure 2 G).

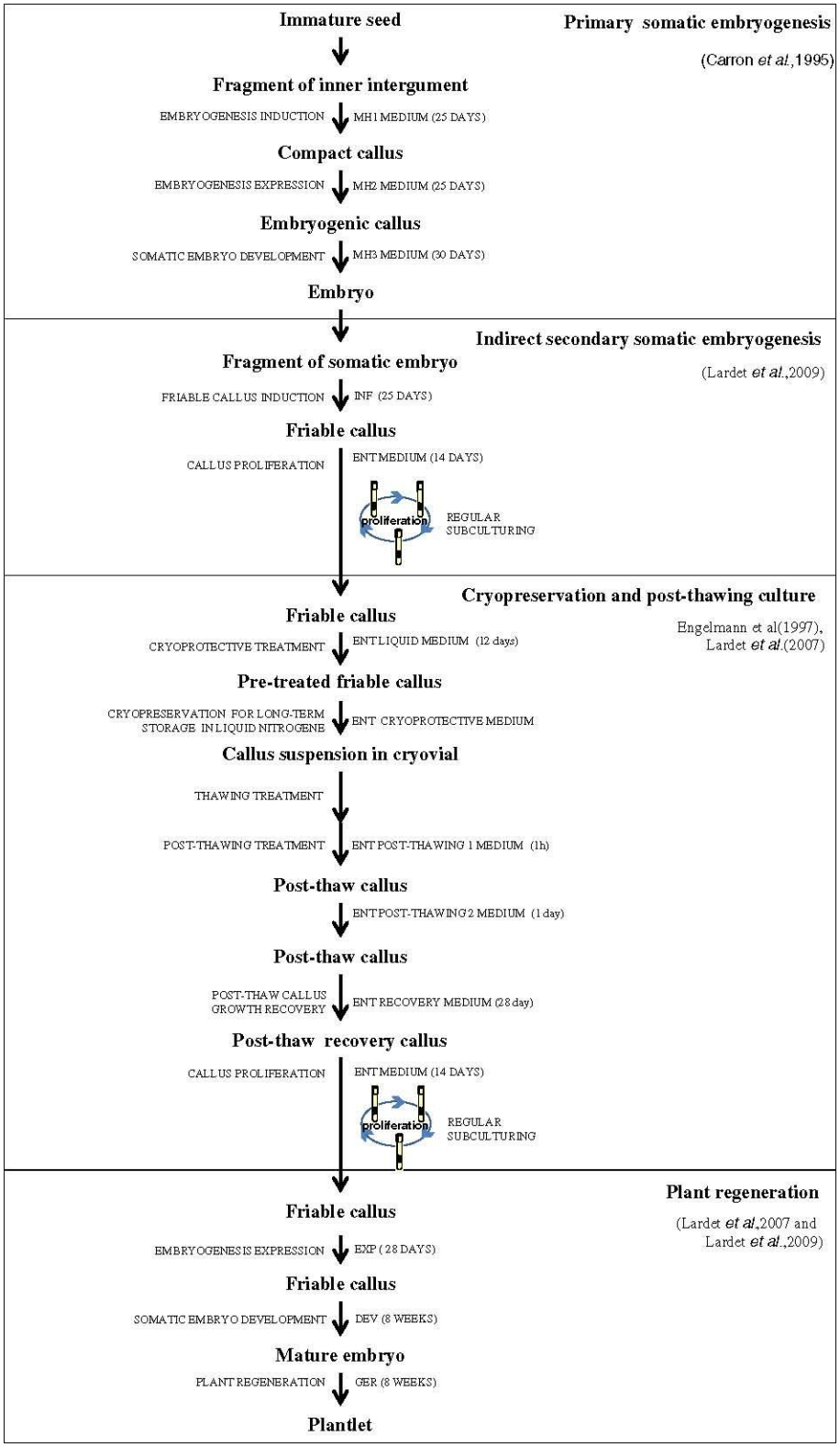

Figure 1 Plant regeneration process for *Hevea brasiliensis*. Primary somatic embryogenesis using the inner integument of immature seeds, indirect secondary somatic embryogenesis, cryopreservation and plant regeneration methods have been described in several papers [24,29,33,39].

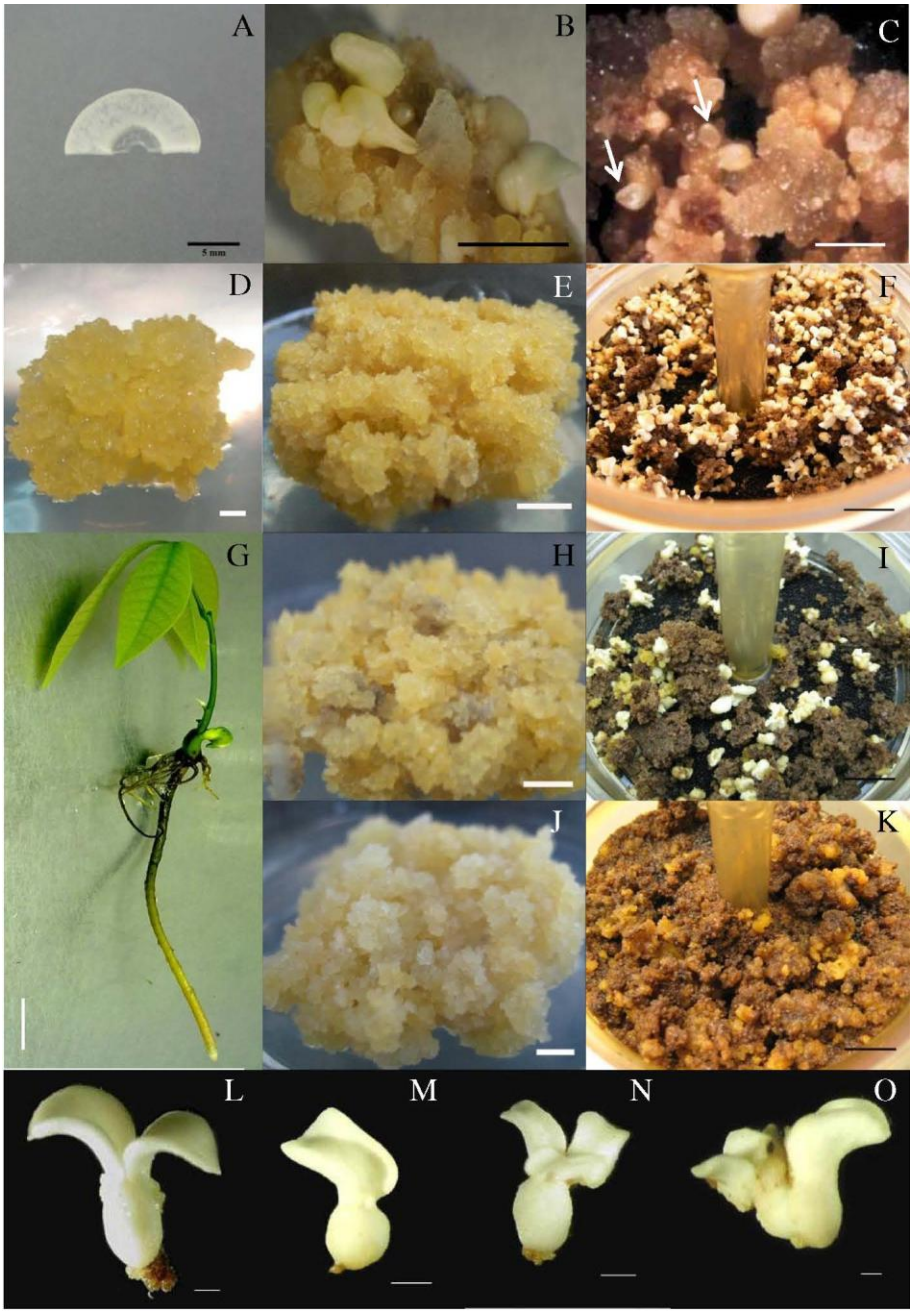

Figure 2 Morphology of callus with different embryogenic capacities and somatic embryos. (**A**) Inner integument of immature seed (scale bar = 5 mm). (**B**) Compact callus on somatic embryo development medium (MH3) from primary somatic embryogenesis (bar = 5 mm). (**C**) Embryogenic callus bearing pro-embryo structures (white arrow) on somatic embryo development medium (DEV) (bar = 5 mm). (**D**) Regenerant line callus on embryogenesis induction medium (ENT) (bar = 1 mm). Calli were from three types of lines on somatic embryogenesis expression medium (EXP): (**E**) regenerant line callus, (**H**) embryogenic and non-regenerant line and (**J**) non-embryogenic line on somatic embryogenesis expression medium (EXP) (bar = 5 mm). Friable callus on embryo development medium (DEV): (**F**) from regenerant line callus, (**I**) embryogenic and non-regenerant line and (**K**) non-embryogenic line (bar = 10 mm). Various types of somatic embryo: (**L**) normal cotyledonated embryo, and (**M, N, O**) abnormal somatic embryos (bar = 1 mm). (**G**) Plantlet from a normal somatic embryo (bar = 10 mm).

The morphogenetic capacities of three friable callus lines were tested up to somatic embryo conversion into plantlets (Table 1). The non-embryogenic line CI04115 (NE) did not produce any somatic embryos. Line CI04106, called embryogenic (E), produced 19.5 somatic embryos per gram of callus, most of which were abnormal (93.54%). Only the regenerant line CI07060 (R) produced a large number of total somatic embryos (590 per gram of callus). The proportion of abnormal embryos was very high for the embryogenic and regenerant lines, at 93.54% and 82.71% respectively. Out of 102 normal embryos derived from the regenerant line transferred to germination medium, 53 developed into plantlets.

Table 1 Morphogenetic capacities of three callus lines. The non-embryogenic line (CI 04115) cannot regenerate any embryos, the embryogenic line (CI 04106) can only produce a few embryos but cannot regenerate any plantlets, and the regenerant line (CI 07 060) can produce a large number of embryos and regenerate plantlets

Embryogenic capacity	Line	Embryo					Plantlet from normal embryo	
		Total	Normal		Abnormal			
		(No/g^{-1} cal)	(No/g^{-1} cal)	(%)	(No/g^{-1} cal)	(%)	(No/g^{-1} cal)	(%)
Non-embryogenic	CI04115	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	-	0.00 ± 0.00 [a]	-	-	-
Embryogenic	CI04106	19.50 ± 19.13 [a]	0.65 ± 1.17 [a]	3.33	18.24 ± 17.37 [a]	93.54	0.00 ± 0.00 [a]	-
Regenerant	CI07060	591.00 ± 52.66 [b]	102.20 ± 57.75 [b]	17.28	488.80 ± 65.02 [b]	82.71	53.40 ± 36.81 [b]	52.25

Identification of 14 marker genes of somatic embryogenesis capacities during callus proliferation

The relative transcript abundance for ethylene biosynthesis and signalling genes, and for the AP2 and RAV families, was analysed in proliferating calli on ENT medium (Figure 3). Some high levels were found for several genes in each family of genes, except for the RAVs (Figure 3 A). The ERFs from groups I, VII and VIII amounted to 8, 15 and 10 genes respectively with levels over 1 in relative value compared to the transcript abundance of the internal control RH2b. The comparison between lines with different embryogenic potential revealed that 14 genes were differentially expressed (Figure 3 B). The relative transcript abundance was lower in the calli of the R line than in the E and NE lines for 11 genes, of which three were involved in ethylene biosynthesis and perception (*HbSAMS, HbACS2, HbETR1*), four were from the ERF family (*HbERF-Ia1, HbERF-VI2, HbERF-VIIa4, HbERF-VIIIa3*), and 4 were from the AP2 family (*HbAP2-1, HbAP2-5, HbAP2-12, HbAP2-18*). The transcripts of the *HbAP2-11* gene were greatly accumulated in the embryogenic line. Lastly, the transcripts of the *HbERF-VIIa23* gene were less abundant in the non-embryogenic line, while, conversely, those of the *HbRAV-4* gene were accumulated more in the calli of that line.

Gene		Callus on ENT medium					
		A			B		
		NE	E	R	NE	E	R
Ethylene biosynthetic & transduction pathways	*HbSAMS*	1,12E-01 *	2,14E-01 *	8,55E-08 *	1,12E-01 *	2,14E-01 *	8,55E-08 *
	HbACS1	1,35E-01 *	5,10E-02 *	3,00E-02 *	1,35E-01	5,10E-02	3,00E-02
	HbACS2	9,72E-01 *	6,97E-01 *	7,36E-08 *	9,72E-01	6,97E-01	7,36E-08 *
	HbACS3	1,33E+00 *	5,10E+00 *	2,94E+00 *	1,33E+00	5,10E+00	2,94E+00
	HbACO1	1,57E-08 *	3,40E-08 *	3,38E-08 *	1,57E-08	3,40E-08	3,38E-08
	HbACO2	1,66E-01 *	3,77E-01 *	1,69E-01 *	1,66E-01	3,77E-01	1,69E-01
	HbACO3	7,14E-08 *	8,34E-08 *	7,42E-08 *	7,14E-08	8,34E-08	7,42E-08
	HbETR1	5,12E-01 *	4,47E-01 *	1,80E-01 *	5,12E-01 *	4,47E-01 *	1,80E-01 *
	HbETR2	1,79E+00 *	2,92E+00 *	1,94E+00 *	1,79E+00	2,92E+00	1,94E+00
	HbEIN2	3,32E-01 *	7,62E-01 *	1,76E-01 *	3,32E-01	7,62E-01	1,76E-01
HbERF-I	*HbERF-Ia1*	9,14E-03 *	1,90E-02 *	2,84E-08 *	9,14E-03 *	1,90E-02 *	2,84E-08 *
	HbERF-Ib1	2,12E+00 *	3,96E+00 *	6,27E-01 *	2,12E+00	3,96E+00	6,27E-01
	HbERF-Ib2	8,59E-01 *	9,68E-01 *	4,16E-01 *	8,59E-01	9,68E-01	4,16E-01
	HbERF-Ib3	7,05E-01 *	3,00E+00 *	1,72E+00 *	7,05E-01	3,00E+00	1,72E+00
	HbERF-Ib4	1,29E+00 *	8,09E-01 *	3,71E-01 *	1,29E+00	8,09E-01	3,71E-01
	HbERF-Ib5	1,84E+00 *	2,34E+00 *	1,87E+00 *	1,84E+00	2,34E+00	1,82E+00
	HbERF-Ib6	1,62E+01 *	2,59E+01 *	2,28E+00 *	1,62E+01	2,59E+01	2,28E+00
	HbERF-Ib7	6,30E+00 *	8,20E+00 *	1,87E+00 *	6,30E+00	8,20E+00	1,83E+00
	HbERF-Ib8	1,00E+01 *	8,38E+00 *	3,38E+00 *	1,00E+01	8,38E+00	3,38E+00
	HbERF-Ib9	2,59E+00 *	3,47E+00 *	3,41E+00 *	2,59E+00	3,47E+00	3,41E+00
	HbERF-Ib10	1,31E-01 *	2,23E-02 *	3,67E-02 *	1,51E-01	2,25E-02	3,67E-02
	HbERF-Ib11	1,84E-02 *	2,07E-02 *	2,32E-02 *	1,84E-02	2,07E-02	2,32E-02
HbERF-II	*HbERF-IIa1*	1,71E-02 *	2,38E-02 *	4,55E-08 *	1,71E-02	2,38E-02	4,55E-08
	HbERF-IIa3	2,46E+00 *	3,45E+00 *	3,17E+00 *	2,46E+00	3,45E+00	3,17E+00
	HbERF-IIb1	1,62E-01 *	1,60E-01 *	1,44E-01 *	1,62E-01	1,60E-01	1,44E-01
	HbERF-IIb2	9,88E-02 *	1,90E-01 *	5,90E-02 *	9,88E-02	1,90E-01	5,90E-02
	HbERF-IIb4	1,01E-02 *	7,15E-03 *	3,03E-08 *	1,01E-02	7,15E-03	3,03E-08
	HbERF-IIb5	2,85E-01 *	4,47E-01 *	1,36E-01 *	2,85E-01	4,47E-01	1,36E-01
HbERF-III	*HbERF-IIIa1*	9,01E-01 *	6,59E-01 *	1,22E+00 *	9,01E-01	6,59E-01	1,22E+00
	HbERF-IIIb1	2,21E-02 *	1,69E-02 *	8,16E-03 *	2,21E-02	1,69E-02	8,16E-03
	HbERF-IIIb2	4,15E-01 *	3,72E-02 *	5,66E-02 *	4,15E-01	3,72E-02	5,66E-02
	HbERF-IIIc1	5,79E-01 *	8,68E-01 *	6,87E-01 *	5,79E-01	8,68E-01	6,87E-01
	HbERF-IIId2	8,01E-08 *	8,19E-04 *	2,00E-02 *	8,01E-08	8,19E-04	2,00E-02
	HbERF-IIId3	9,71E-08 *	4,75E-02 *	3,24E-02 *	9,71E-08	4,75E-02	3,34E-02
	HbERF-IIIe1	1,25E-02 *	6,86E-08 *	8,23E-08 *	1,25E-02	6,86E-08	8,23E-08
	HbERF-IIIe2	3,37E-02 *	7,74E-02 *	2,37E-02 *	3,37E-02	7,74E-02	2,37E-02
	HbERF-IIIe3	1,04E-02 *	1,94E-02 *	9,68E-08 *	1,04E-02	1,94E-02	9,68E-08
	HbERF-IIIe4	2,09E-01 *	1,30E+00 *	9,89E-02 *	2,09E-01	1,06E+00	9,89E-02
HbERF-IV	*HbERF-IVa1*	1,06E+00 *	5,43E-01 *	1,54E-01 *	1,06E+00	5,43E-01	1,54E-01
	HbERF-IVa2	1,07E+00 *	4,96E-01 *	1,46E-01 *	1,07E+00	4,96E-01	1,46E-01
	HbERF-IVa3	1,22E-01 *	1,37E-01 *	8,23E-02 *	1,22E-01	1,37E-01	8,23E-02
HbERF-V	*HbERF-Va1*	6,40E-01 *	1,97E+00 *	9,42E-01 *	6,40E-01	1,97E+00	9,42E-01
	HbERF-Va2	7,24E-01 *	1,83E+00 *	1,31E+00 *	7,24E-01	1,80E+00	1,31E+00
	HbERF-Vb1	3,54E-02 *	3,59E-02 *	1,26E-02 *	3,54E-02	3,59E-02	1,26E-02
	HbERF-Vb2	3,70E-02 *	6,11E-02 *	7,89E-02 *	3,70E-02	6,11E-02	7,89E-02
	HbERF-Vb3	8,06E-08 *	4,41E-08 *	1,40E-02 *	8,06E-08	4,41E-08	1,40E-02
HbERF-VI	*HbERF-VI1*	7,84E-02 *	1,09E-01 *	9,59E-02 *	7,84E-02	1,09E-01	9,59E-02
	HbERF-VI2	1,06E+01 *	1,83E-01 *	1,10E-02 *	1,06E+01	1,83E-01	1,10E-02 *
	HbERF-VI3	7,89E-02 *	7,11E-02 *	5,09E-02 *	7,89E-02	7,11E-02	5,09E-02
	HbERF-VI4	1,66E-02 *	1,21E-02 *	4,49E-08 *	1,66E-02	1,21E-02	4,49E-08
	HbERF-VI5	7,51E-01 *	5,73E-01 *	2,57E-01 *	7,51E-01	5,73E-01	2,57E-01
	HbERF-VI-L1	8,02E-01 *	9,80E-01 *	3,61E-01 *	8,02E-01	9,80E-01	3,61E-01
	HbERF-VI-L2	6,29E-01 *	8,43E-01 *	7,04E-01 *	6,29E-01	8,43E-01	7,04E-01
	HbERF-VI-L3	1,05E+00 *	1,54E+00 *	6,02E-01 *	1,05E+00	1,34E+00	6,02E-01
	HbERF-VI-L4	3,22E-01 *	1,15E+00 *	1,94E-01 *	3,22E-01	1,15E+00	1,94E-01
	HbERF-VI-L5	4,53E-01 *	1,47E+00 *	4,38E-02 *	4,53E-01	1,47E+00	4,38E-02
	HbERF-VI-L6	1,07E-01 *	6,22E-01 *	4,66E-01 *	1,07E-01	6,22E-01	4,66E-01
HbERF-VII	*HbERF-VIIa1*	7,26E+00 *	8,58E+00 *	3,22E+00 *	7,26E+00	8,58E+00	3,22E+00
	HbERF-VIIa2	6,34E+00 *	8,22E+00 *	2,55E+00 *	6,34E+00	8,22E+00	2,55E+00
	HbERF-VIIa3	3,85E-01 *	1,05E+00 *	1,33E-01 *	3,85E-01 *	1,05E+00 *	1,33E-01 *
	HbERF-VIIa4	1,61E-01 *	1,54E+00 *	3,01E-01 *	1,61E-01	1,54E+00	3,01E-01 *
	HbERF-VIIa5	2,90E-02 *	6,99E-02 *	3,74E-02 *	2,90E-02	6,99E-02	3,74E-02
	HbERF-VIIa6	2,97E+01 *	1,12E+02 *	3,06E+01 *	2,97E+01	1,12E+02	3,06E+01
	HbERF-VIIa7	1,23E+01 *	4,01E+01 *	2,02E+01 *	1,23E+01	4,01E+01	2,02E+01
	HbERF-VIIa9	2,30E+00 *	2,26E+00 *	1,73E+00 *	2,30E+00	2,26E+00	1,75E+00
	HbERF-VIIa10	5,45E+00 *	1,12E+01 *	7,53E+00 *	5,45E+00	1,12E+01	7,55E+00
	HbERF-VIIa11	1,06E+00 *	2,29E-01 *	1,00E+00 *	1,06E+00	7,29E-01	1,00E+00
	HbERF-VIIa12	1,79E+01 *	2,66E+01 *	4,19E+01 *	1,79E+01	2,66E+01	4,19E+01
	HbERF-VIIa13	2,26E+00 *	4,43E+00 *	5,97E+00 *	2,26E+00	4,43E+00	5,97E+00
	HbERF-VIIa15	2,82E+00 *	3,90E+00 *	2,01E+00 *	2,82E+00	3,90E+00	2,01E+00
	HbERF-VIIa17	3,72E-01 *	6,50E-01 *	1,20E+00 *	3,72E-01	6,50E-01	1,20E+00
	HbERF-VIIa18	2,53E-08 *	1,26E-02 *	1,78E-02 *	2,53E-08	1,26E-02	1,78E-02
	HbERF-VIIa19	9,22E+00 *	1,59E+01 *	2,85E+00 *	9,22E+00	1,59E+01	2,85E+00
	HbERF-VIIa20	3,98E-08 *	8,95E-08 *	2,88E-02 *	3,98E-08	8,95E-08	2,88E-02
	HbERF-VIIa21	4,00E-01 *	2,83E+00 *	3,67E+00 *	4,00E-01	2,83E+00	3,67E+00
	HbERF-VIIa22	3,61E-02 *	1,13E-01 *	2,27E-01 *	3,61E-02	1,13E-01	2,27E-01
	HbERF-VIIa23	1,35E-01 *	7,13E-01 *	5,68E-01 *	1,35E-01 *	7,13E-01	5,68E-01 *
HbERF-VIII	*HbERF-VIIIa1*	1,31E+00 *	1,63E+00 *	3,30E-01 *	1,31E+00	1,63E+00	3,30E-01 *
	HbERF-VIIIa2	6,99E-01 *	9,44E-01 *	3,61E-01 *	6,99E-01	9,44E-01	3,61E-01
	HbERF-VIIIa3	1,14E+00 *	1,76E+00 *	1,70E-01 *	1,14E+00 *	1,76E+00 *	1,70E-01 *
	HbERF-VIIIa4	3,99E+00 *	1,50E+00 *	8,75E-01 *	3,99E+00	1,50E+00	8,75E-01
	HbERF-VIIIa5	5,97E-01 *	5,13E-01 *	4,42E-01 *	5,97E-01	5,13E-01	4,42E-01
	HbERF-VIIIa6	1,79E+00 *	1,74E+00 *	1,24E+00 *	1,79E+00	1,74E+00	1,24E+00
	HbERF-VIIIa7	3,67E+00 *	6,21E+00 *	2,83E+00 *	3,67E+00	6,21E+00	2,83E+00
	HbERF-VIIIa8	3,36E+00 *	4,87E+00 *	4,76E+00 *	3,36E+00	4,87E+00	4,76E+00
	HbERF-VIIIa9	6,76E+00 *	8,62E+00 *	4,55E+00 *	6,76E+00	8,62E+00	4,55E+00
	HbERF-VIIIa10	8,07E-01 *	1,58E+00 *	1,24E+00 *	8,07E-01	1,58E+00	1,24E+00
	HbERF-VIIIa11	6,90E-01 *	1,13E+00 *	7,50E-01 *	6,90E-01	1,13E+00	7,50E-01
	HbERF-VIIIa12	3,02E-01 *	5,14E-01 *	2,35E-01 *	3,02E-01	5,14E-01	2,35E-01
	HbERF-VIIIa13	1,45E+00 *	3,01E+00 *	1,15E+00 *	1,45E+00	3,01E+00	1,15E+00
	HbERF-VIIIa14	3,37E-01 *	3,00E-01 *	6,69E-01 *	3,37E-01	3,00E-01	6,69E-01
	HbERF-VIIIb1	7,57E-01 *	7,27E-01 *	6,66E-01 *	7,57E-01	7,27E-01	6,66E-01
HbERF-IX	*HbERF-IXa1*	3,14E-01 *	3,23E-01 *	2,98E-01 *	3,14E-01	3,25E-01	2,96E-01
	HbERF-IXa2	4,34E-01 *	7,63E-01 *	1,92E+00 *	4,34E-01	7,63E-01	1,92E+00
	HbERF-IXa3	3,47E-01 *	6,26E-01 *	8,69E-01 *	3,47E-01	6,26E-01	8,69E-01
	HbERF-IXa4	7,89E-02 *	1,44E-01 *	1,90E-01 *	7,89E-02	1,44E-01	1,92E-01
	HbERF-IXb1	2,89E-01 *	2,39E-01 *	3,23E-01 *	2,89E-01	2,39E-01	3,25E-01
	HbERF-IXb2	1,30E-02 *	6,83E-08 *	4,08E-08 *	1,30E-02	6,83E-08	4,08E-08
	HbERF-IXb3	3,31E-01 *	2,22E-01 *	7,87E-02 *	3,31E-01	2,22E-01	7,87E-02
	HbERF-IXb4	8,21E-01 *	8,01E-01 *	2,06E+00 *	8,21E-01	8,01E-01	2,06E+00
	HbERF-IXb5	1,29E+00 *	1,81E+00 *	8,69E-01 *	1,29E+00	1,81E+00	8,69E-01
	HbERF-IXb6	6,89E-01 *	9,62E-01 *	2,78E-01 *	6,89E-01	9,62E-01	2,78E-01
	HbERF-IXb7	2,84E-02 *	3,19E-02 *	1,48E-02 *	2,84E-02	3,19E-02	1,48E-02
	HbERF-IXb8	8,46E-02 *	6,60E-02 *	3,71E-02 *	8,46E-02	6,60E-02	3,71E-02
	HbERF-IXc1	9,87E-02 *	3,33E-01 *	1,76E-01 *	9,87E-02	3,33E-01	1,76E-01
	HbERF-IXc3	8,30E-08 *	1,43E-02 *	2,50E-02 *	8,30E-08	1,45E-02	2,50E-02
	HbERF-IXc4	1,56E-01 *	2,09E-02 *	1,33E-01 *	1,56E-01	2,09E-02	1,33E-01
	HbERF-IXc5	2,80E-02 *	5,15E-02 *	3,46E-02 *	2,80E-02	5,15E-02	3,46E-02
	HbERF-IXc6	3,63E-04 *	1,64E-08 *	2,85E-08 *	3,63E-04	1,64E-08	2,85E-08
	HbERF-IXc7	2,34E-08 *	1,78E-02 *	1,62E-02 *	2,34E-08	1,78E-02	1,62E-02
HbERF-X	*HbERF-Xa1*	2,49E+00 *	3,12E+00 *	1,23E+00 *	2,49E+00	3,12E+00	1,23E+00
	HbERF-Xa2	1,59E-01 *	1,24E-01 *	1,83E-01 *	1,59E-01	1,24E-01	1,83E-01
	HbERF-Xa3	4,21E+00 *	7,07E+00 *	3,10E+00 *	4,21E+00	7,07E+00	3,10E+00
	HbERF-Xa4	3,06E-01 *	6,79E-02 *	3,30E-02 *	3,06E-01	6,79E-02	3,30E-02
	HbERF-Xa5	2,89E-02 *	2,46E-02 *	3,63E-02 *	2,89E-02	2,46E-02	3,63E-02
	HbERF-Xa6	6,04E-01 *	5,42E-01 *	4,36E-01 *	6,04E-01	5,42E-01	4,36E-01
	HbERF-Xa8	2,19E-02 *	6,60E-02 *	4,94E-02 *	2,19E-02	6,60E-02	4,94E-02
	HbERF-Xb1	5,67E-08 *	8,21E-08 *	9,74E-08 *	5,67E-08	8,21E-08	9,74E-08
HbAP2	*HbAP2-1*	3,35E-01 *	4,39E-01 *	3,02E-01 *	3,35E-01	4,39E-01 *	3,02E-01 *
	HbAP2-2	4,03E-02 *	2,63E-02 *	1,21E-02 *	4,03E-02 *	2,63E-02 *	1,21E-02 *
	HbAP2-3	1,34E+01 *	1,26E+01 *	1,08E+01 *	1,34E+01	1,26E+01	1,08E+01
	HbAP2-4	3,31E-08 *	1,27E-02 *	2,24E-02 *	3,31E-08	1,27E-02	2,24E-02
	HbAP2-5	4,12E-01 *	3,47E-01 *	3,52E-02 *	4,12E-01 *	3,47E-01 *	3,52E-02 *
	HbAP2-6	1,05E-01 *	7,63E-02 *	9,15E-02 *	1,05E-01	7,63E-02	9,15E-02
	HbAP2-7	6,73E-02 *	1,70E-02 *	2,99E-02 *	6,73E-02	1,70E-02	2,99E-02
	HbAP2-8	3,00E-01 *	8,40E-02 *	7,94E-02 *	3,00E-01	8,40E-02	7,94E-02
	HbAP2-9	6,49E+00 *	7,67E+00 *	1,08E+01 *	6,49E+00	7,67E+00	1,08E+01
	HbAP2-10	3,87E-02 *	8,60E-02 *	3,55E-02 *	3,87E-02	8,60E-02	3,55E-02
	HbAP2-11	3,10E-02 *	8,12E-02 *	1,59E-02 *	3,10E-02 *	8,12E-02 *	1,59E-02 *
	HbAP2-12	1,31E-01 *	1,44E-01 *	1,44E-02 *	1,31E-01	1,44E-01 *	1,44E-02 *
	HbAP2-13	1,09E-01 *	5,68E-02 *	1,00E-02 *	1,09E-01	5,68E-02	1,00E-02
	HbAP2-15	5,02E-01 *	1,33E-01 *	1,20E-01 *	5,02E-01	1,33E-01	1,20E-01
	HbAP2-16	1,03E+00 *	8,57E-01 *	1,31E+00 *	1,03E+00	8,57E-01	1,31E+00
	HbAP2-17	6,95E-08 *	3,72E-08 *	1,29E-08 *	6,95E-08	3,72E-08	1,29E-08
	HbAP2-18	2,27E+00 *	1,06E+00 *	1,74E+00 *	2,27E+00	1,06E+00	1,74E+00 *
	HbAP2-19	5,09E-02 *	1,36E-01 *	3,74E-02 *	5,09E-02	1,36E-01	3,74E-02
	HbAP2-20	2,95E+00 *	1,94E+00 *	1,83E+00 *	2,95E+00	1,94E+00	1,83E+00
HbRAV	*HbRAV-1*	1,09E-08 *	1,14E-08 *	9,41E-04 *	1,09E-08	1,14E-08	9,41E-04
	HbRAV-2	1,82E-08 *	3,18E-08 *	1,79E-02 *	1,82E-08	3,18E-08	1,79E-02
	HbRAV-3	2,75E-08 *	1,59E-08 *	1,01E-08 *	2,75E-08	1,59E-08	1,01E-08
	HbRAV-4	2,44E-01 *	5,45E-02 *	3,98E-02 *	2,44E-01	5,45E-02 *	3,98E-02 *

Figure 3 Expression profile of 142 genes involved in the ethylene biosynthesis and signalling pathways for callus with different embryogenic capacities grown for 2 weeks on embryogenesis induction medium (ENT). Calli were from three types of lines: (NE) non-embryogenic line (CI04115), (E) embryogenic and non-regenerant line (CI04106), and (R) regenerant line (CI07060). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundances of three biological replicates. (Figure 3 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥1, 10^{-1}, 10^{-2}, 10^{-3} and ≤10^{-4} from dark to light green. (Figure 3 **B**) Values of relative transcript abundance in callus of the various lines were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

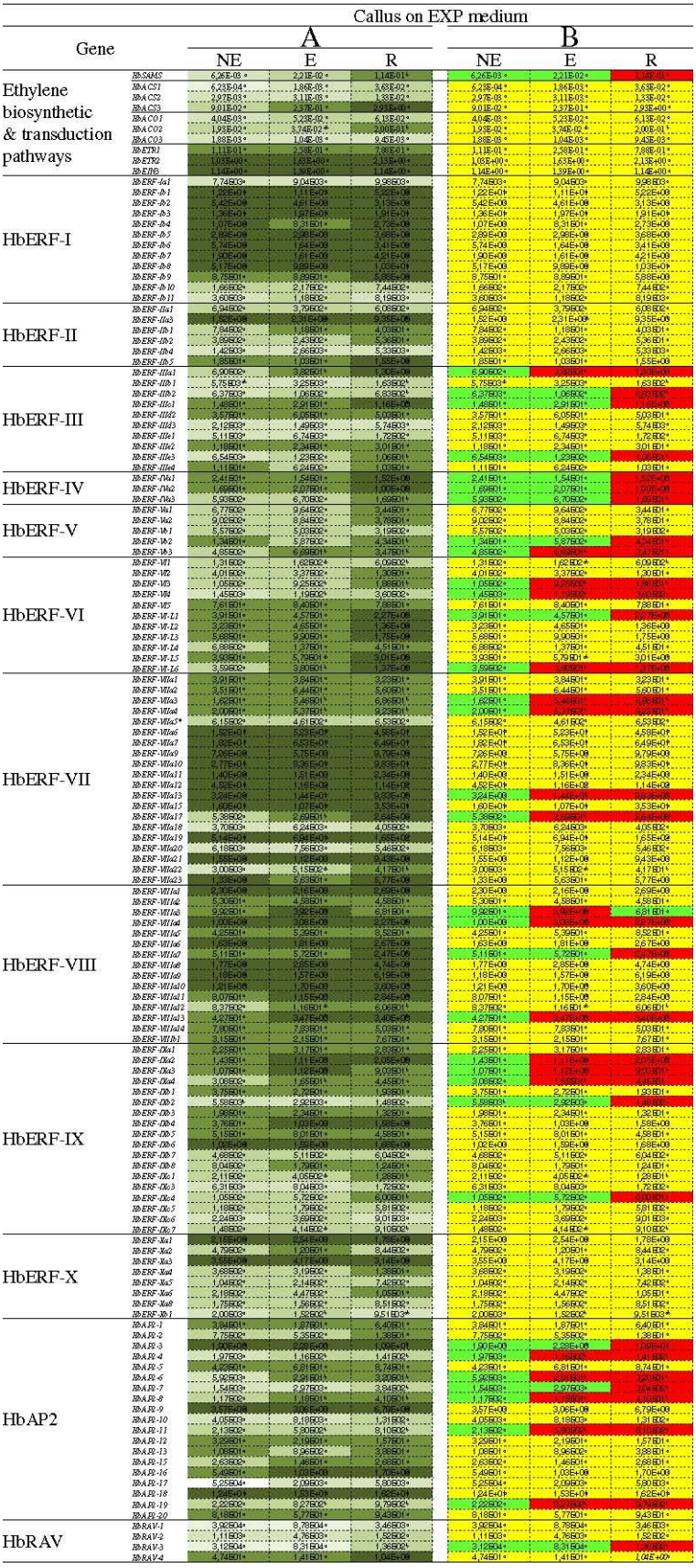

Figure 4 Expression profile of 142 genes involved in the ethylene biosynthesis and signalling pathways for callus with different embryogenic capacities grown for 4 weeks on somatic embryogenesis expression medium (EXP). Calli were from three types of lines: (NE) non-embryogenic line (CI04115), (E) embryogenic and non-regenerant line (CI04106), and (R) regenerant line (CI07060). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 4 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green. (Figure 4 **B**) Values of relative transcript abundance in callus of the various lines were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

Identification of 35 marker genes of somatic embryogenesis capacities during somatic embryogenesis expression/induction

The calli transferred to a somatic embryogenesis induction medium also strongly expressed a large number of the genes studied for virtually all the families and groups, except for the ERF-V group (Figure 4 A). As previously, most of the members of groups I (9 genes), VII (12 genes) and VIII (10 genes) of the ERF family had a high transcript level. The differential expression between calli of the different lines was significant for 35 genes (Figure 4 B). Of those genes, 15 had higher transcript accumulation in the regenerant line than in the NE and E lines: 1 ethylene biosynthesis gene (*HbSAMS*), 11 genes of the ERF family (*HbERF-IIIb2, HbERF-IIIc1, HbERF-IIIe3, HbERF-IVa1,HbERF-IVa2, HbERF-IVa3, HbERF-Vb2, HbERF-VI-L1, HbERF-VIIIa7, HbERF-IXb2, HbERF-IXc4*), 2 genes of the AP2 family (*HbAP2-3, HbAP2-7*) and 1 gene of the RAV family (*HbRAV-3*). Transcripts of the *HbERF-VIIIa3* gene were accumulated in the calli of the embryogenic line compared to the other two lines. Lastly, 19 genes comprising 14 ERFs (*HbERF-IIIa1, HbERF-Vb3, HbERF-VI3, HbERF-VI4, HbERF-VI-L6, HbERF-VIIa3, HbERF-VIIa4, HbERF-VIIa13, HbERF-VIIa17, HbERF-VIIIa4, HbERF-VIIIa13, HbERF-IXa2, HbERF-IXa3, HbERF-IXa4*) and 5 AP2s (*HbAP2-4, HbAP2-6, HbAP2-8, HbAP2-11, HbAP2-19*) had a lower transcript level in the non-embryogenic line than in the others.

Sixteen genes were differentially expressed in normal and abnormal somatic embryos

The normal and abnormal somatic embryos produced by the regenerant line revealed a differential expression profile solely for certain members of the ERF family (Figure 5). The gene transcripts belonging to each of the families and groups were highly accumulated, except for the RAV family (Figure 5 A). Most of the members of the ERF-I, ERF-VII and ERF-VIII groups were highly expressed in the two types of embryos. The transcript abundance was lower in the normal embryos than in the abnormal embryos for 12 genes belonging to the ERF family (*HbERF-Ia1, HbERF-Ib1, HbERF-Ib3, HbERF-Ib6, HbERF-Ib7, HbERF-Ib8, HbERF-IIb5, HbERF-IVa2, HbERF-VIIIa11, HbERF-IXb4, HbERF-IXb8, HbERF-Xb1*) (Figure 5 B). Conversely, the transcripts of 4 genes were accumulated in the normal embryos (*HbERF-VI1, HbERF-VIIa1, HbERF-VIIa3, HbERF-VIIa4*).

Evolution of transcript abundance during the process of somatic embryogenesis and conversion into plantlets

An overall analysis of the somatic embryogenesis process for the regenerant line using proliferating callus up to the conversion of normal embryos into plantlets showed that the expression of numerous genes was highly modulated (Figure 6). The transcripts of numerous ethylene biosynthesis and transduction genes, and some ERFs from groups I, VII and VIII and of the AP2 family, were highly accumulated (Figure 6 A). Thirty-six genes from all the families or groups revealed differential accumulation during the embryogenesis process, except for the ERFs from group X (Figure 6 B). Several gene expression profiles were observed. Eleven markers were activated right from the somatic embryogenesis induction phase (*HbSAMS, HbACO1, HbEIN3, HbERF-IIIe2, HbERF-VIIa9, HbERF-VIIa23, HbERF-VIIIa3, HbERF-IXb2, HbAP2-5, HbAP2-11, HbAP2-13*). The transcripts of eight genes

(*HbERF-Ib5*, *HbERF-IIa3*, *HbERF-IIIa1*, *HbERF-VIIa1*, *HbERF-VIIIa14*, *HbERF-IXa3*, *HbAP2-1*, *HbAP2-3*) were very abundant in embryogenic tissues, whereas they were significantly less abundant in the tissues of the plantlet. A transient accumulation was seen for two genes (HbERF-Vb2, HbERF-Vb3) in calli induced in embryogenesis on EXP medium, for six genes (HbERF-VIIa3, HbERF-VIIa4, HbERF-VIIa17, HbERF-VIIIa3, HbAP2-2, HbAP2-6) in somatic embryos and for two genes (HbERF-IVa1, HbERF-VIIa18) in both calli on EXP medium and in embryos. The transcripts of three genes (HbERF-IXb2, HbERF-IXb6, HbERF-IXb8) were preferentially accumulated in leaves and, in the case of HbERF-IXc3, in both somatic embryos and leaves. Lastly, the relative transcript abundance was high in all tissues except in embryos, where it decreased significantly for the *HbAP2-16* gene, and except in calli, embryos and roots for *HbRAV4*.

Gene		Somatic embryo			
		A		B	
		Normal	Abnormal	Normal	Abnormal
Ethylene biosynthetic & transduction pathways	*HbSAM5*	1,75E-01 a	2,21E-01 a	1,75E-01 a	2,21E-01 a
	HbACS1	1,37E-01 a	7,71E-01 a	1,37E-01 a	7,71E-01 a
	HbACS2	1,15E-01 a	3,25E-01 a	1,15E-01 a	3,25E-01 a
	HbACS3	2,94E-01 a	1,15E+00 a	2,94E-01 a	1,15E+00 a
	HbACO1	4,37E-01 a	1,11E-01 a	4,37E-01 a	1,11E-01 a
	HbACO2	5,01E+00 a	3,94E+00 a	5,01E+00 a	3,94E+00 a
	HbACO3	1,17E-01 a	3,96E-01 a	1,17E-01 a	3,96E-01 a
	HbETR1	1,47E+00 a	7,45E-01 a	1,47E+00 a	7,45E-01 a
	HbETR2	1,95E+00 a	6,31E-01 a	1,95E+00 a	6,31E-01 a
	HbETR3	6,79E-01 a	6,93E-01 a	6,79E-01 a	6,93E-01 a
HbERF-I	*HbERF-Ia1*	2,88E-03 a	1,13E-02 a	2,88E-03 a	1,13E-02 a
	HbERF-Ib1	2,56E-01 a	1,06E+00 a	2,56E-01 a	1,06E+00 a
	HbERF-Ib2	1,33E-01 a	5,16E-01 a	1,33E-01 a	5,16E-01 a
	HbERF-Ib3	2,04E-01 a	1,46E+00 a	2,04E-01 a	1,46E+00 a
	HbERF-Ib4	2,88E-01 a	4,47E-01 a	2,88E-01 a	4,47E-01 a
	HbERF-Ib5	1,81E+00 a	3,21E+00 a	1,81E+00 a	3,21E+00 a
	HbERF-Ib6	2,03E+00 a	1,15E+01 b	2,03E+00 a	1,15E+01 b
	HbERF-Ib7	2,36E+00 a	1,27E+01 b	2,36E+00 a	1,27E+01 b
	HbERF-Ib8	2,92E+00 a	8,27E+00 a	2,92E+00 a	8,27E+00 a
	HbERF-Ib9	1,08E-01 a	2,16E+00 a	1,08E+01 a	2,16E+00 a
	HbERF-Ib10	8,36E-02 a	3,25E-02 a	8,36E-02 a	3,25E-02 a
	HbERF-Ib11	6,80E-02 a	2,12E-02 a	6,80E-02 a	2,12E-02 a
HbERF-II	*HbERF-IIa1*	3,70E-03 a	4,55E-03 a	3,70E-03 a	4,55E-03 a
	HbERF-IIa3	4,87E+00 a	6,64E+00 a	4,87E+00 a	6,64E+00 a
	HbERF-IIb1	3,20E-01 a	8,26E-01 a	3,20E-01 a	8,26E-01 a
	HbERF-IIb2	3,62E-02 a	1,01E-01 a	3,62E-02 a	1,17E+00 a
	HbERF-IIb4	1,32E-02 a	5,04E-03 a	1,32E-02 a	5,04E-03 a
	HbERF-IIb5	8,03E-02 a	8,37E-01 a	8,03E-02 a	8,37E-01 a
HbERF-III	*HbERF-IIIa1*	3,72E+00 a	3,66E+00 a	3,72E+00	3,66E+00 a
	HbERF-IIIb1	3,14E-02 a	7,35E-02 a	3,14E-02 a	7,35E-02 a
	HbERF-IIIb2	1,04E-02 a	5,26E-01 a	1,04E-02 a	5,26E-01 a
	HbERF-IIIc1	1,08E-01 a	2,15E+00 a	1,20E+00 a	2,15E+00 a
	HbERF-IIId2	1,76E-02 a	1,43E-02 a	1,76E-02 a	1,43E-02 a
	HbERF-IIId3	8,06E-03 a	5,48E-03 a	8,06E-03 a	5,48E-03 a
	HbERF-IIIe1	3,71E-01 a	1,88E-01 a	3,71E-01 a	1,88E-01 a
	HbERF-IIIe2	1,44E+00 a	7,60E-01 a	1,44E+00 a	7,60E-01 a
	HbERF-IIIe3	1,08E-01 a	5,22E-02 a	1,08E-01 a	5,22E-02 a
	HbERF-IIIe4	8,34E-02 a	1,45E-01 a	8,34E-02 a	1,45E-01 a
HbERF-IV	*HbERF-IVa1*	9,39E-01 a	2,95E+00 a	9,39E-01 a	2,95E+00 a
	HbERF-IVa2	1,25E+00 a	3,85E+00 a	1,25E+00 a	3,85E+00 a
	HbERF-IVa3	1,19E-01 a	1,45E-01 a	1,19E-01 a	1,45E-01 a
HbERF-V	*HbERF-Va1*	3,57E-01 a	1,96E-01 a	3,57E-01 a	1,96E-01 a
	HbERF-Va2	4,55E-01 a	1,59E-01 a	4,55E-01 a	1,59E-01 a
	HbERF-Vb1	9,39E-03 a	1,34E-02 a	9,39E-03 a	1,34E-02 a
	HbERF-Vb2	3,37E-02 a	2,22E-02 a	3,37E-02 a	2,22E-02 a
	HbERF-Vb3	2,44E-02 a	2,39E-02 a	2,44E-02 a	2,39E-02 a
HbERF-VI	*HbERF-VI1*	3,12E-01 b	1,03E-01 a	3,12E-01 b	1,03E-01 a
	HbERF-VI2	2,73E-02 a	1,01E-01 a	2,73E-02 a	1,01E-01 a
	HbERF-VI3	4,10E-01 a	1,64E-01 a	4,10E-01 a	1,64E-01 a
	HbERF-VI4	8,71E-03 a	8,15E-03 a	8,71E-03 a	8,15E-03 a
	HbERF-VI5	2,08E+00 a	1,30E+00 a	2,08E+00 a	1,30E+00 a
	HbERF-VI-L1	6,63E-01 a	4,70E-01 a	6,63E-01 a	4,70E-01 a
	HbERF-VI-L2	1,31E+00 a	6,41E-01 a	1,31E+00 a	6,41E-01 a
	HbERF-VI-L3	7,83E-01 a	5,80E-01 a	7,83E-01 a	5,80E-01 a
	HbERF-VI-L4	9,14E-02 a	5,80E-02 a	9,14E-02 a	5,80E-02 a
	HbERF-VI-L5	5,14E-01 a	1,39E+00 a	5,14E-01 a	1,39E+00 a
	HbERF-VI-L6	4,11E-01 a	2,13E-01 a	4,11E-01 a	2,13E-01 a
HbERF-VII	*HbERF-VIIa1*	6,94E-01 b	1,90E-01 a	6,94E-01 b	1,90E-01 a
	HbERF-VIIa2	7,65E-01 a	3,13E-01 a	7,65E-01 a	3,13E-01 a
	HbERF-VIIa3	4,92E+00 b	6,96E-01 a	4,92E+00 b	6,96E-01 a
	HbERF-VIIa4	3,23E+00 b	9,86E-01 a	3,23E+00 b	9,86E-01 a
	*HbERF-VIIa5**	6,92E-02 a	4,16E-02 a	6,92E-02 a	4,16E-02 a
	HbERF-VIIa6	2,90E+01 a	3,11E+01 a	2,90E+01 a	3,11E+01 a
	HbERF-VIIa7	2,34E+01 a	3,14E+01 a	2,34E+01 a	3,14E+01 a
	HbERF-VIIa9	3,43E+00 a	4,06E+00 a	3,43E+00 a	4,06E+00 a
	HbERF-VIIa10	2,30E+01 a	1,54E+01 a	2,30E+01 a	1,54E+01 a
	HbERF-VIIa11	8,46E-01 a	8,25E-01 a	8,46E-01 a	8,25E-01 a
	HbERF-VIIa12	4,08E+01 a	3,30E+01 a	4,08E+01 a	3,30E+01 a
	HbERF-VIIa13	8,92E+00 a	4,78E+00 a	8,92E+00 a	4,78E+00 a
	HbERF-VIIa15	7,80E+00 a	4,94E+00 a	7,80E+00 a	4,94E+00 a
	HbERF-VIIa17	3,25E+01 a	1,14E+01 a	3,25E+01 a	1,14E+01 a
	HbERF-VIIa18	3,39E+02 a	3,86E+02 a	3,39E+02 a	3,86E+02 a
	HbERF-VIIa19	7,76E+01 a	2,06E+01 a	7,76E+01 a	2,06E+01 a
	HbERF-VIIa20	2,03E+01 a	1,16E+01 a	2,03E+01 a	1,16E+01 a
	HbERF-VIIa21	2,64E+01 a	2,24E+01 a	2,64E+01 a	2,24E+01 a
	HbERF-VIIa22	3,23E+00 a	1,95E+00 a	3,23E+00 a	1,95E+00 a
	HbERF-VIIa23	1,19E+01 a	1,02E+01 a	1,19E+01 a	1,02E+01 a
HbERF-VIII	*HbERF-VIIIa1*	1,69E+00 a	1,12E+00 a	1,69E+00 a	1,12E+00 a
	HbERF-VIIIa2	3,65E-01 a	2,08E-01 a	3,65E-01 a	2,08E-01 a
	HbERF-VIIIa3	2,07E+00 a	1,07E+00 a	2,07E+00 a	1,07E+00 a
	HbERF-VIIIa4	2,35E+00 a	1,35E+00 a	2,35E+00 a	1,35E+00 a
	HbERF-VIIIa5	6,46E-01 a	3,63E-01 a	6,46E-01 a	3,63E-01 a
	HbERF-VIIIa6	1,62E+00 a	1,05E+00 a	1,62E+00 a	1,05E+00 a
	HbERF-VIIIa7	1,25E+00 a	2,61E+00 a	1,25E+00 a	2,61E+00 a
	HbERF-VIIIa8	2,47E+00 a	7,54E+00 a	2,47E+00 a	7,54E+00 a
	HbERF-VIIIa9	2,84E+00 a	4,75E+00 a	2,84E+00 a	4,75E+00 a
	HbERF-VIIIa10	1,61E+00 a	9,13E+00 a	1,61E+00 a	9,13E+00 a
	HbERF-VIIIa11	9,06E-01 a	6,17E+00 a	9,06E-01 a	6,17E+00 a
	HbERF-VIIIa12	9,72E-01 a	1,11E+00 a	9,72E-01 a	1,11E+00 a
	HbERF-VIIIa13	2,85E+01 a	8,65E+00 a	2,85E+01 a	8,65E+00 a
	HbERF-VIIIa14	5,98E-01 a	4,80E-01 a	5,98E-01 a	4,80E-01 a
	HbERF-VIIIb1	1,47E-02 a	1,16E-01 a	1,47E-02 a	1,16E-01 a
HbERF-IX	*HbERF-IXa1*	2,27E-01 a	7,40E-01 a	2,27E-01 a	7,40E-01 a
	HbERF-IXa2	7,66E+00 a	5,13E+00 a	7,66E+00 a	5,13E+00 a
	HbERF-IXa3	4,88E-01 a	2,78E+00 a	4,88E-01 a	2,78E+00 a
	HbERF-IXa4	2,26E+00 a	1,30E+00 a	2,26E+00 a	1,30E+00 a
	HbERF-IXb1	7,41E-01 a	1,39E-01 a	7,41E-01 a	1,39E-01 a
	HbERF-IXb2	3,32E-02 a	1,49E-02 a	3,32E-02 a	1,49E-02 a
	HbERF-IXb3	1,18E-01 a	6,06E-01 a	1,18E-01 a	6,06E-01 a
	HbERF-IXb4	1,11E+00 a	6,64E+00 b	1,11E+00 a	6,64E+00 b
	HbERF-IXb5	5,47E-01 a	1,62E+00 a	5,47E-01 a	1,62E+00 a
	HbERF-IXb6	4,69E-01 a	2,70E+00 a	4,69E-01 a	2,70E+00 a
	HbERF-IXb7	2,58E-02 a	1,72E-01 a	2,58E-02 a	1,72E-01 a
	HbERF-IXb8	3,20E-02 a	4,71E-01 a	3,20E-02 a	4,71E-01 a
	HbERF-IXc1	2,83E-01 a	1,61E-01 a	2,85E-01 a	1,61E-01 a
	HbERF-IXc3	1,24E-01 a	6,13E-01 a	1,24E-01 a	6,13E-01 a
	HbERF-IXc4	1,79E-01 a	1,54E-01 a	1,79E-01 a	1,54E-01 a
	HbERF-IXc5	3,57E-02 a	5,97E-02 a	3,57E-02 a	5,97E-02 a
	HbERF-IXc6	9,19E-03 a	4,58E-03 a	9,19E-03 a	4,58E-03 a
	HbERF-IXc7	4,10E-01 a	4,68E-02 a	4,10E-01 a	4,68E-02 a
HbERF-X	*HbERF-Xa1*	5,45E-01 a	3,40E-01 a	5,45E-01 a	3,40E-01 a
	HbERF-Xa2	1,40E-01 a	7,66E-02 a	1,40E-01 a	7,66E-02 a
	HbERF-Xa3	9,23E-01 a	5,45E-01 a	9,23E-01 a	5,45E-01 a
	HbERF-Xa4	1,59E-02 a	3,44E-02 a	1,59E-02 a	3,44E-02 a
	HbERF-Xa5	3,04E-01 a	1,76E-01 a	3,04E-01 a	1,76E-01 a
	HbERF-Xa6	3,19E+00 a	4,95E+00 a	3,19E+00 a	4,95E+00 a
	HbERF-Xa8	7,73E-02 a	1,94E-01 a	7,73E-02 a	1,94E-01 a
	HbERF-Xb1	2,40E-03 a	1,34E-01 a	2,40E-03 a	1,34E-01 a
HbAP2	*HbAP2-1*	6,07E-02 a	7,28E-02 a	6,07E-02 a	7,28E-02 a
	HbAP2-2	6,64E-01 a	4,20E-01 a	6,64E-01 a	4,20E-01 a
	HbAP2-3	7,21E+00 a	1,09E+01 a	7,21E+00 a	1,09E+01 a
	HbAP2-4	1,73E-02 a	3,39E-02 a	1,73E-02	3,39E-02 a
	HbAP2-5	3,15E-01 a	2,45E-01 a	3,15E-01 a	2,45E-01 a
	HbAP2-6	6,91E+00 a	9,70E+00 a	6,91E+00 a	9,70E+00 a
	HbAP2-7	8,70E-02 a	5,71E-02 a	8,70E-02 a	5,71E-02 a
	HbAP2-8	2,41E-01 a	2,65E-01 a	2,41E-01 a	2,65E-01 a
	HbAP2-9	1,46E+00 a	4,95E+00 a	1,46E+00 a	4,95E+00 a
	HbAP2-10	1,09E+00 a	2,74E+00 a	1,09E+00 a	2,74E+00 a
	HbAP2-11	2,11E-01 a	1,20E-01 a	2,11E-01 a	1,20E-01 a
	HbAP2-12	3,37E-02 a	2,35E-02 a	3,37E-02 a	2,35E-02 a
	HbAP2-13	1,37E+00 a	9,83E-01 a	1,37E+00 a	9,83E-01 a
	HbAP2-15	3,09E-01 a	5,14E-01 a	3,09E-01 a	5,14E-01 a
	HbAP2-16	1,21E-01 a	3,34E-01 a	1,21E-01 a	3,34E-01 a
	HbAP2-17	3,99E-03 a	1,05E-02 a	3,99E-03 a	1,05E-02 a
	HbAP2-18	2,65E+00 a	4,04E+00 a	2,65E+00 a	4,04E+00 a
	HbAP2-19	3,14E-01 a	3,27E-01 a	3,14E-01 a	3,27E-01 a
	HbAP2-20	1,11E+00 a	3,33E+00 a	1,11E+00 a	3,33E+00 a
HbRAV	*HbRAV-1*	6,89E-03 a	1,38E-03 a	6,89E-03 a	1,36E-03 a
	HbRAV-2	6,30E-03 a	6,30E-03 a	6,30E-03 a	6,30E-03 a
	HbRAV-3	1,47E-02 a	1,00E-02 a	1,47E-02 a	1,00E-02 a
	HbRAV-4	7,74E-02 a	1,02E-01 a	7,74E-02 a	1,02E-01 a

Figure 5 Expression profile of 142 genes involved in the ethylene biosynthesis and signalling pathways for normal and abnormal embryos from regenerant line (CI07060) callus on embryo development medium (DEV). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 5 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green . (Figure 5 **B**) Values of relative transcript abundance in normal and abnormal embryos were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

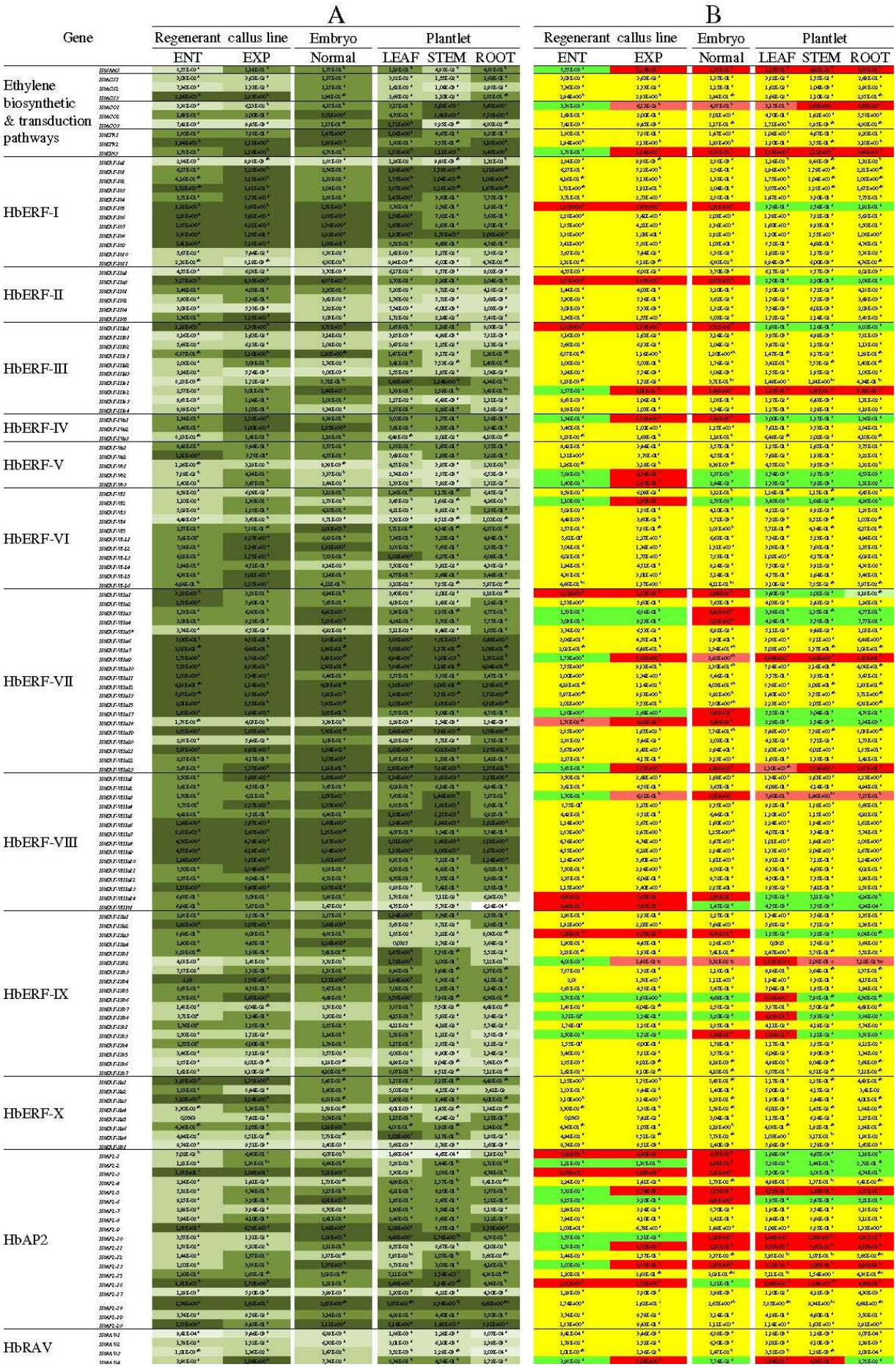
A
B
Gene
Regenerant callus line
Embryo
Plantlet
ENT
EXP
Normal
LEAF
STEM
ROOT
Ethylene biosynthetic & transduction pathways
HbERF-I
HbERF-II
HbERF-III
HbERF-IV
HbERF-V
HbERF-VI
HbERF-VII
HbERF-VIII
HbERF-IX
HbERF-X
HbAP2
HbRAV

Figure 6 Expression profile of 142 genes involved in the ethylene biosynthesis and signalling pathways for regenerant line (CI07060) callus on embryogenesis induction medium (ENT) and somatic embryogenesis expression medium (EXP), embryos and 3 plantlet organs: (L) Leaf, (S) Stem, (R) Root. The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 6 A) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green. (Figure 6 B) Values of relative transcript abundance were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

Identification of putative functions for somatic embryogenesis marker genes

Of the fifty-six genes differentially expressed during the somatic embryogenesis process, forty were regulated in the same way when the regenerant line was compared with the other two non-embryogenic or embryogenic lines, and when the normal embryos were compared with the abnormal embryos (Table 2). The ratios between the relative transcript abundance of the regenerant line and the other two lines showed that the markers tended to be under-expressed in the proliferating calli of the regenerant line (from 0.01 to 0.4) and over-expressed in the calli induced in embryogenesis on EXP medium (from 2 to 43 times). Of the sixteen embryo expression markers, twelve were under-expressed and four were over-expressed

role of these gene expression markers was first predicted by a reciprocal best hit analysis (BLASTX) of the transcript sequences (Table 2). Roles were mostly found in the response to biotic and abiotic stress, and more precisely in tolerance of dehydration, salinity and cold. The genes of the AP2 and RAV families, along with a small proportion of ERFs, played a role in developmental processes (embryogenesis and flower development). In order to find *Hevea* potential orthologs to *Arabidopsis* genes functionally described in the literature, the deduced amino acid sequences of the AP2 domain were analysed for the AP2 family and the different ERF groups (I, III, IV, VI, X) making up the marker genes in *Hevea* (Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4, Additional file 5: Figure S5 and Additional file 6: Figure S6). The summary of the phylogenetic tree analyses can be found in Table 2. Thirteen of the *Hevea* marker genes proved to be potential orthologs of sixteen *Arabidopsis* genes including some co-orthologs: *HbERF-Ia1* with *ERF53*, *HbERF-IIIc1* with *AtCBF/DREB (CBF3/DREB1A, CBF2/DREB1C, CBF1/DREB1B, CBF4/DREB1D, DDF1, DDF2)*, *HbERF-IIIe3* with *TINY*, *HbERF-IVa1* and *HbERF-IVa2* with *DREB2A*, *HbERF-VI1* and *HbERF-VI2* with *CRF2/TMO3*, *HbERF-Xb1* with *RRTF1*, *HbAP2-1* with *BBM*, *HbAP2-3* with two *AINTEGUMENTA-Like* genes (*AIL7/PLT7* and *AIL6/PLT3*), *HbAP2-7* with *AINTEGUMENTA*, and *HbAP2-12* and *HbAP18* with *APETALA2*.

Table 2 Identification of putative functions for somatic embryogenesis marker genes based on the reciprocical best hit analysis (BLASTX) using full cDNA sequences, and then co-orthology relationships, inferred by phylogeny, using the deduced amino acid sequences of the AP2 domain for each gene (see Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4, Additional file 5: figure S5 and Additional file 6: Figure S6). The ratios between the relative transcript abundance of the regenerant and non-embryogenic lines (R/NE), the regenerant and embryogenic lines (R/E), of the normal and abnormal embryos (Nor/Ab) are presented for only significant down-regulated genes (< 1) and up-regulated (> 1)

Gene	Callus on ENT		Callus on EXP		Embryo	Reciprocal best hit analysis				Phylogenetic analysis			
	R/NE	R/E	R/NE	R/E	Nor/Ab	Homology gene	Putative function	Species	Reference	Orthologous gene	Accession No	Putative Function	Reference
HbSAMS	0.08	0.04	18.20	5.16		SAMS	Precursor for ethylene biosynthesis	*Eucalyptus*	[40,41]	.			
HbACS2	0.01	0.01				ACS	ACS synthase	*Ricinus*	[41,42]	.			
HbETR1	0.35	0.40				HbETR	Signal transduction	*Hevea*	[41,42]	.			
HbERF-Ia1	0.31	0.15			0.26	DREB	Freezing & dehydration tolerance	*Glycine*	[43-45]	ERF53	At2g20880	Regulates drought-responsive gene expression	[46]
HbERF-Ib1					0.24	DREB	Freezing & dehydration tolerance	*Populus*	[43-45]	.	At4g39780	Unknown	
HbERF-Ib3					0.14	DREB	Freezing & dehydration tolerance	*Populus*	[43-45]	.	At4g39780	Unknown	
HbERF-Ib6					0.18	DREB1p	Dwarfed phenotypes,freezing & dehydration tolerance	*Hevea*	[44]	.			
HbERF-Ib7					0.19	DREB1p	Dwarfed phenotypes,freezing & dehydration tolerance	*Hevea*	[44]	.			
HbERF-Ib8					0.35	DREB1p	Dwarfed phenotypes,freezing & dehydration tolerance	*Hevea*	[44]	.			
HbERF-IIb5					0.09	TINY	Affects plant height, hypocotyl elongation, and fertility	*Populus*	[47,48]	.	At1g44830	Unknown	

HbERF-IIIa1	18.80	3.40		TINY	Affects plant height, hypocotyl elongation, and fertility	*Populus*	[47,48]	.	At1g01250	Unknown	
HbERF-IIIb2	10.70	6.44		ERF025	Control of ethylene-responsive transcription genes	*Glycine*	[49]	.	At1g63040	Unknown	
HbERF-IIIc1	7.84	3.99		CRT/DRE	Heat stress tolerance	*Hevea*	[50,51]	CBF3/DREB1A	At4g25480	Response to low temperature, abscisic acid	[52]
								CBF2/DREB1C	At4g25470	Response to low temperature, abscisic acid,	[53]
								CBF1/DREB1B	At4g25490	Response to low temperature, abscisic acid	[52]
								CBF4/DREB1D	At5g51990	Response to drought stress and abscisic acid	[54]
								DDF2	At1g63030	Regulates in GA biosynthesis and stress tolerance	[55]
								DDF1	At1g12610	Regulates in GA biosynthesis and stress tolerance	[55]
HbERF-IIIe3	16.20	8.62		TINY	Affects plant height, hypocotyl elongation, and fertility	*Populus*	[47,48]	TINY	At5g25810	Suppresses cell proliferation and exhibits pleiotropic effects	[47]
HbERF-IVa1	6.31	9.87		DREB2	Enhances drought stress tolerance	*Populus*	[50,51]	DREB2A	At5g05410	Drought-responsive gene expression	[50]
HbERF-IVa2	5.92	4.83	0.33	DREB2C	Heat stress tolerance	*Ricinus*	[51]				
HbERF-IVa3	2.85	2.52		DREB2B	Water deprivation stimulus	*Arabidopsis*	[56]	.			
HbERF-Vb2	3.24	7.39		RAP2.2	Response to hypoxic stress	*Arabidopsis*	[57]	.			
HbERF-VI1			3.03	CRF2		*Vitis*	[58]	CRF2/TMO3	At4g23750	Related to root	[58,59]

HbERF-VI2	0.10	0.06				CRF2	Development of embryos and response to cytokin	*Arabidopsis*	[58]			initiation at later embryonic stages
HbERF-VI-L1			5.81	4.97		ERF	Control of ethylene-responsive transcription genes	*Ricinus*	[60,61]	.		
HbERF-VIIa1					3.65	AP2/ERF	Response to biotic and abiotic stress conditions	*Populus*	[45]	.		
HbERF-VIIa3			4.23		7.07	AP2/ERF	Response to biotic and abiotic stress conditions	*Populus*	[45]	.		
HbERF-VIIa4	0.22	0.21	4.62		5.29	AP2/ERF	Response to biotic and abiotic stress conditions	*Populus*	[45]	.		
HbERF-VIIa17			49.10	9.81		ERF-2	Control of ethylene-responsive transcription genes	*Gossypium*	[60,61]	.		
HbERF-VIIIa3	0.15	0.10		017		ERF3	Control of ethylene-responsive transcription genes	*Arabidopsis*	[62]	.		
HbERF-VIIIa7			4.83	4.32		ATERF-4	Modulates ethylene and abscisic acid responses	*Arabidopsis*	[62]	.		
HbERF-VIIIa11					0.16	ATERF-4	Modulates ethylene and abscisic acid responses	*Arabidopsis*	[62]	.		
HbERF-IXb2			2.65	5.07		ERF	Control of ethylene-responsive transcription genes	*Olimarabidopsis*	[60,61]		At5g07580	Unknown
HbERF-IXb4					0.17	ERF	Control of ethylene-responsive transcription genes	*Ricinus*	[60,61]	.		
HbERF-IXb8					0.07	ERF1	Drought, salt and freezing tolerances	*Malus*	[60,61]	.	At5g51190	Unknown
HbERF-IXc4			57.10	10.50		ERF1	Drought, salt and freezing tolerances	*Ricinus*	[60,61]	.		

HbERF-Xb1					0.02	AP2/ERF	Response to biotic and abiotic stress conditions	*Populus*	[45]	RRTF1	At4g34410	Regulates redox homeostasis related to photosynthetic stress	[63]
HbAP2-1	0.14	0.11				ANT	Regulates up-regulation of genes establishing organ polarity and those specifying organ identity	*Ricinus*	[19,64]	BBM	At5g17430	Promotes cell proliferation and morphogenesis during embryogenesis	[18,65]
HbAP2-3			5.74	4.78		BBM	Cell proliferation and morphogenesis during embryogenesis	*Ricinus*	[18]	AIL7/PLT7	At5g65510	Regulates radial pattern formation process of a shoot apical meristem.	[66]
										AIL6/PLT3	At5g10510	Regulation of floral meristem growth	[67]
HbAP2-5	0.13	0.10				AP2/ERF	Response to biotic and abiotic stress conditions	*Ricinus*	[45]	.	At2g41710	Unknown	
HbAP2-7			24.90	12.90		AIL	Related to floral development	*Vitis*	[64]	ANT/DRG/CKC/CKC1	At4g37750	Regulates up—regulation of genes establishing organ polarity and those specifying organ identity	[19,64]
HbAP2-12	0.13	0.10				AP2	Meristem maintenance and cell differentiation	*Arabidopsis*	[68,69]	AP2/FLO2/FL1	At4g36920	Meristem maintenance and cell differentiation	[68,69]
HbAP2-18	0.22	0.16				AP2		*Vitis*					
HbRAV-3			43.60	16.40		RAV1	Leaf maturation and senescence	*Ricinus*	[70]	.			

Discussion

Factors contributing to the loss of embryogenic capacity

Although callus browning occurs late during embryo development in the normal process, early browning in proliferating calli contributes to a loss of their embryogenic capacity by promoting the differentiation of active cells (meristematic and embryogenic) [25]. In *Hevea*, such browning has been linked to a strong accumulation of oxidized polyphenols in cells and to ethylene production [4,30,71]. This study corroborates those findings with the activation of ethylene biosynthesis and signalling genes in proliferating calli of low embryogenic or non-embryogenic lines (Figure 3), whereas this only occurred after induction in embryogenesis on EXP medium for the regenerant line. The gene expression markers of plant regeneration capacity found in proliferating calli belonged to groups VI, VII and VIII of the ERFs (*HbERF-VI2, HbERF-VIIa4, HbERF-VIIIa3*), which are factors of response to hormonal signals (ethylene, jasmonate, etc.). Other factors generally linked to development were also activated in proliferating calli from the embryogenic or non-embryogenic lines. Four genes from the AP2 family can be noted: *HbAP2-1, HbAP2-5, HbAP2-12, HbAP2-18*. The phylogenetic analysis between the *Hevea* and *Arabidopsis* families led to the prediction of several orthologs. *HbAP2-1* and *HbAP2-12/HbAP2-18* were found to be potential orthologs of *Arabidopsis BBM* and *AP2* genes, respectively [18,68]. *BBM* is preferentially expressed in developing embryos and seeds [18]. Its ectopic expression in *Arabidopsis*, *Brassica* and *Nicotiana* has led to the spontaneous formation of somatic embryos and cotyledon-like structures in seedlings [18,72]. However, such ectopic expression gives rise to pleiotropic phenotypes such as neoplastic growth, regeneration of plants on a hormone-free medium, and an alteration of leaf and flower morphology. The role of BBM in promoting cell proliferation and morphogenesis during embryogenesis seems to be confirmed for other species such as *Brassica napus* and *Elaeis guineensis* [17,73]. The *AP2* gene is involved in the control of *Arabidopsis* flower and seed development [68]. This gene is known to be expressed in non-floral organs, such as leaves and stems, and may play a general role in controlling *Arabidopsis* development. Nevertheless, early activation of these genes, especially *HbAP2-12* and *HbAP2-18*, in proliferating *Hevea* callus might be not appropriate since it is not conducive to further induction of somatic embryogenesis.

Change occurring during somatic embryogenesis induction

Somatic embryogenesis is triggered by reducing the concentration of growth regulators in the culture medium of *Hevea* calli. This helps to slow down callus growth to the benefit of embryo formation [74]. Embryogenesis induction is also accompanied by callus browning. In this study, that transition was linked to changes in gene expression. Several ethylene biosynthesis genes, such as *HbACS3* and *HbACO2*, were highly transcribed in the calli before and after embryogenesis induction. When calli were transferred to the EXP medium, transcripts of the *SAMS* gene accumulated dramatically. That gene is also a marker that differentiated between the regenerant line and the other two low- or non-embryogenic lines, be it for the callus proliferation phase or during somatic embryogenesis induction. *SAMS* catalyses the formation of S-adenosyl methionine, which is a substrate for the ethylene and polyamine biosynthesis pathways. The latter would seem to play a decisive role in the somatic embryogenesis of *Hevea* [75]. However, the induction of several ERFs indicates the establishment of ethylene signalling. Four ERFs (*HbERF-IVa1*, *HbERF-Vb2*, *HbERF-Vb3*, *HbERF-VI2*) and the *HbRAV4* gene were transiently induced in calli on the EXP culture medium for somatic embryogenesis induction (Figure 6). At that stage of the process, the comparison between the regenerant line and the low- or non-embryogenic lines also revealed strong induction of 13 ERFs (*HbERF-IIIa1, HbERF-*

IIIb2, *HbERF-IIIc1*, *HbERF-IIIe3*, *HbERF-IVa1*, *HbERF-IVa2*, *HbERF-IVa3*, *HbERF-Vb2*, *HbERF-VI-L1*, *HbERF-VIIa17*, *HbERF-VIIIa7*, *HbERF-IXb2*, *HbERF-IXc4*), 2 AP2 (*HbAP2-3*, *HbAP2-7*) and the *RAV3* gene (Figure 4 or Table 2).

Several of these gene expression markers were predicted to be orthologs of *Arabidopsis* genes with characterized functions. *HbERF-IIIc1* is orthologous to several *Arabidopsis DREB1s* from group 1 induced by cold [52,53]. *HbERF-IIIe3* is the putative ortholog of *TINY* known to be activated by drought, cold, ethylene and, to a lesser degree, methyl jasmonate [47]. The semi-dominant *tiny* mutation causes a reduction in plant height, and affects hypocotyl elongation and fertility. *TINY* might play a role in communication between biotic and abiotic stress signalling pathways. *HbERF-IVa1* and *HbERF-IVa2* are two potential orthologs of *DREB2A* involved in drought-responsive gene expression [50]. *HbERF-IVa1* was transitionally induced in calli placed on somatic embryogenesis induction medium, and both *HbERF-IVa1* and *HbERF-IVa2* were good markers of the regeneration potential compared with the low-embryogenic or non-embryogenic line. These two genes could therefore be very good gene expression markers but would also seem to play a key role in the somatic embryogenesis process. The transcripts of *HbERF-VI1* were accumulated in the normal embryos while *HbERF-VI2* showed under-expression in the proliferating calli of the regenerant line. These genes are putative orthologs to *CRF2*, which has been previously described as *TARGET OF MP3 (TMO3)*. The *TMO* gene is targeted by the auxin-dependent transcription factor MONOPTEROS (MP), which is a regulatory signal in embryonic root specification [59]. *AP2* genes are generally transcribed in multiple tissues during development. AILs also play a role in the specification of meristematic or division-competent states [76]. *HbAP2-3* is the putative ortholog of two *Arabidopsis* genes, *AIL7/PLT7* [66] and *AIL6/PLT3*, involved in floral meristem growth [67]. *HbAP2-7* would seem to be orthologous to the *ANT* gene [19]. ANT regulates cell proliferation and organ growth by maintaining the meristematic competence of cells during organogenesis [77]. More recently, it was shown to be promoting the initiation and growth of lateral organ primordia, and organ polarity [64]. Consequently, its very high expression in regenerant *Hevea* callus lines, compared with non-embryogenic and embryogenic lines, was in line with its role in embryo development. With regard to RAV, this family is regulated by ethylene [78] and brassinosteroids [79]. RAVs are involved in the response to biotic and abiotic stress [80]. The increase in *HbRAV4* transcripts during somatic embryogenesis induction is in accordance with dramatic changes provoked by ethylene.

Control of development and of somatic embryo quality

The ontogenesis of somatic embryos involves an embryo growth phase, followed by the formation of apical meristem and roots, along with the procambial bundles, and lastly the accumulation of reserves needed for germination [81]. Somatic embryos gradually become dehydrated to acquire the quiescent state [82]. The analysis of *AP2/ERF* gene expression carried out at the end of the somatic embryo maturation phase revealed that the relative transcript abundance was very high for a large number of genes involved in ethylene biosynthesis and signalling, along with several *AP2* genes, including *HbAP2-3*, *HbAP2-6*, *HbAP2-9*, *HbAP2-10*, *HbAP2-13*, *HbAP2-18* and *HbAP2-20* (Figure 5). The potential roles previously described for HbAP2-3 (orthologous to AIL6 and AIL7) and for HbAP2-18 (orthologous to APETALA2) highlight the importance of these genes. All the 16 marker genes discriminating between the normal and abnormal embryos belonged to the ERF family. The abnormal embryos accumulated the transcripts of 6 ERFs belonging to group I (DREB subfamily [50]) more than the normal embryos did. The abundance of the transcripts of another DREB gene, HbERF-IVa2 orthologous to DREB2A, in the normal embryos is worth noting. This suggests differential

regulation between the two types of embryos for the ERFs involved in the response to dehydration, salinity and cold.

Conclusion

Of the 132 *AP2/ERF* genes studied, 40 were expression markers linked to the different stages of the somatic embryogenesis process in *Hevea*. With the identification of 11 very early markers, it was possible to predict the regeneration potential of proliferating callus lines, which opens up prospects for their application in selecting lines of interest for large-scale propagation. The phylogenetic analysis made it possible to predict more precisely the function of certain genes characterized already in *Arabidopsis*. The functions of 9 markers suggested that the regulation of hormone and stress signals play just as important a role in somatic embryogenesis as the genes involved in morphogenesis regulation. In addition to these marker genes, another *ERF* gene expressed during somatic embryogenesis is a potential ortholog of CRF (CYTOKININ RESPONSE FACTOR) encoded by *HbERF-VI1 or HbERF-VI2* [58]. An in-depth functional characterization of these markers should lead to a better understanding of somatic embryogenesis and explain the loss of embryogenic capacity, and embryo abnormality. Genetic variability in these genes could also be studied to determine whether allelic variations can be used in breeding programmes to select *Hevea* clones not only for agronomic traits but also for their responsiveness to somatic embryogenesis. The AP2/ERF superfamily could thus play a major role for several other biological functions in *Hevea*. Firstly, rubber production is stimulated by applying ethephon. In cases of over-tapping *in situ* coagulation of rubber particles leads to production losses: this is tapping panel dryness. Secondly, the *ERF1*, *ERF2*, *ERF3* and *RAV1* genes, corresponding to *HbERF-VIIa1*, *HbERF-VIIa3*, *HbERF-VIIa17* and *HbRAV1* [35], are induced at the same time as secondary laticifer differentiation [83]. Studying this superfamily in *Hevea* thus provides some new biological knowledge.

Methods

Plant material

The plant regeneration procedure from somatic embryogenesis consists of four steps described in Figure 1. Firstly, primary somatic embryogenesis was carried out using the inner integument of immature seeds from the *Hevea* clone PB 260 [24]. Secondly, friable callus lines were established from fragments of somatic embryos [29]. Thirdly, callus lines were cryopreserved for long-term storage and to avoid long-term subculturing [33,39]. Fourthly, embryo development and plant regeneration were induced from friable callus after thawing treatments [24].

In this study, three callus lines with different embryogenic capacity were previously identified from cryopreserved material [33]. The non-embryogenic callus line CI04115 does not have the ability to produce somatic embryos. The embryogenic but non-regenerant callus line CI04106 can produce a few somatic embryos but cannot regenerate plantlets. The regenerant callus line CI07060 can regenerate embryos and plantlets. These cryopreserved callus lines were thawed by immersing the cryovials in a warm water-bath maintained at 37°C for 2 min. ENT medium was prepared with the basic MH macro and microelements supplemented with 9 mM $CaCl_2$, 234 mM sucrose, 30 µM $AgNO_3$, 1.34 µM BAP, 1.34 µM 3,4-D, 0.5 µM ABA and 2.3 $g.L^{-1}$ Phytagel [24]. The content of the cryovials was placed for 1 h in Petri dishes containing 25 mL of ENT post-thawing medium, which is a modified ENT medium with 1 M sucrose and 1 mM $CaCl_2$ [33]. Calli were then transferred for 1 day to fresh ENT post-thawing 2 medium supplemented

with 0.5 M sucrose, after which they were transferred again to ENT recovery medium containing a normal sucrose concentration (234 mM) and 1 mM $CaCl_2$. After callus growth recovery in Petri dishes, friable callus aggregates were isolated and transferred to test tubes containing ENT medium. Calli were grown in the dark at 27°C and subcultured regularly every two weeks on fresh ENT medium.

For embryo induction, one gram of friable callus proliferating on ENT medium was transferred to a 250-mL bottle containing 50 mL of expression medium (EXP). EXP was a modified ENT medium supplemented with 9 mM $CaCl_2$, 58.5 mM sucrose, 175.5 mM maltose, 0.44 µM BAP, 0.44 µM 3,4-D and 0.5 µM ABA. This culture was incubated in the dark at 27°C for 4 weeks. Development of somatic embryos was obtained by transferring callus from one bottle to a temporary immersion system containing 200 mL of liquid DEV medium (RITA®, CIRAD, France). There were two successive subcultures of 4 weeks with 1 min of immersion per day in DEV medium, which had the same composition. The DEV medium was a modified ENT medium supplemented with 3 mM $CaCl_2$ and without plant growth regulators. After 8 weeks of culture, mature embryos with a well-formed embryonic axis and cotyledons (normal) were transferred to glass tubes containing a germination medium (GER), which contained MS macro-elements, MH micro-elements and vitamins, and 234 mM sucrose, semi-solidified with 7 g/L Agar. Embryos were cultured under a light intensity of 60 µmol m^{-2} s^{-1} with a 12 h day/12 h dark photoperiod for embryo conversion into plantlets. Abnormal embryos were counted and discarded at the end of DEV2.

Total RNA isolation

For the callus lines with different embryogenic capacities, callus was collected at the end of the ENT and EXP cultures. For the regenerant callus line, normal and abnormal embryos were collected at the end of DEV2. Leaves, stems and roots were collected from one-month-old plantlets. All samples were frozen in liquid nitrogen and stored in the freezer at -80°C pending total RNA extraction. Total RNAs were isolated using the caesium chloride cushion method adapted from Sambrook and coll. [84] by Duan and coll. [38]. One gram of fresh matter was ground and transferred to a tube containing 30 mL of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone and 1% ß-mercapto-ethanol. After homogenization, tubes were kept on ice and then centrifuged at 10,000 g at 4°C for 30 min. The supernatant was transferred to a new tube containing 8 mL of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 89,705 g at 20°C for 20 h. The supernatant and caesium cushion were discarded whilst the RNA pellet was washed with 70% ethanol. After 30 min of air drying, the pellet was dissolved in 200 µL of sterile water. Although DNA could not cross the caesium cushion for this centrifugation condition, DNA contamination was checked by PCR amplification using primers of the Actin gene including the intron sequence. RNAs were conserved at -80°C.

Primer design and analysis of transcript abundances by real-time RT-PCR

Several rules were applied in order to reduce the risk of error in relative gene expression data. The integrity of total RNA was checked by electrophoresis. Primers were designed at the 3' side of each sequence in order to reduce the risk of error due to short cDNA synthesis using the Primer 3 module of Geneious (Biomatters Ltd., New Zealand). Real-time PCR amplification and the fusion curve were carried out using a mix of cDNAs in order to check the specificity of each pair of primers. Primer sequences are listed in Additional file 7: Table S1.

cDNAs were synthesized from 2 µg of total RNA to the final 20 µL reaction mixture using a RevertAidTM M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Full-length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA using primers at the cDNA ends. Quantitative gene expression analysis was finally carried out by real-time RT-PCR using a Light Cycler 480 (Roche, Switzerland). Real-time PCR reaction mixtures consisted of 2 µL RT product cDNA, 0.6 µL of 5 µM of each primer, and 3 µL 2 × SYBR green PCR master mix (LightCycler® 480 SYBR Green I Master, Roche Applied Sciences) in a 6-µL volume. PCR cycling conditions comprised one denaturation cycle at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 60°C for 15 s, and 72°C for 20s). Expression analysis was performed in a 384-well plate. Samples were loaded using an automation workstation (Biomek NX, Beckman Coulter).

Real-time PCR was carried out for eleven housekeeping genes in order to select the most stable gene as the internal control for all the compared tissues (HbelF1Aa, HbUBC4, HbUBC2b, HbYLS8, HbRH2b, HbRH8, HbUBC2a, HbalphaTub, Hb40S, HbUbi, HbActin) (Additional file 8: Table S2). *HbRH2b* was selected as the best reference gene due to its stability in tissues from various stages of somatic embryogenesis. The homogeneity of the *HbRH2b* gene Cp confirmed that it could be used as an internal reference gene (Table 3). The *HbRH2b* gene was amplified in each reaction plate in parallel with target genes. The transcript abundance level for each gene was relatively quantified by normalization with the transcript abundance of the reference *HbRH2b* gene. Relative transcript abundance took into account primer efficiencies. All the normalized ratios corresponding to transcript accumulation were calculated automatically by Light Cycler Software version 1.5.0 provided by the manufacturer using the following calculation: Normalized Ratio = Efficiency $^{-\Delta(Cp\ target-Cp\ RH2b)}$.

Table 3 Comparison of Cp values, standard deviation and coefficient of variance for gene expression analysis by real-time RT-PCR of 11 housekeeping genes in 11 tissues from various stages of somatic embryogenesis

Gene	Cp Mean	Standard deviation	Coefficient of variance
HbelF1Aa	23.64	1.22	0.052
HbUBC4	28.31	2.62	0.093
HbUBC2b	21.70	1.73	0.08
HbYLS8	23.23	1.24	0.053
HbRH2b	22.69	1.01	0.045
HbRH8	25.04	1.21	0.048
HbUBC2a	24.93	1.40	0.056
HbalphaTUB	26.87	1.23	0.046
Hb40S	29.12	1.92	0.066
HbUbi	33.56	1.61	0.048
HbActin	22.88	1.47	0.064

Statistical data analyses

Each callus line was maintained in three biological replicates. Morphological data were recorded and calculated per gram of callus transferred to EXP medium. Statistical analysis was performed with an ANOVA followed by a Student Newman-Keuls test. Values with the same letter did not differ significantly at the 0.05 probability level in Table 1.

Real-time PCR reactions were set up with three biological replications. Statistical analysis was performed with an ANOVA after logarithmic transformation of raw data. The ANOVA was followed by a Student Newman-Keuls test when values of relative transcript abundances were compared for each stage of the somatic embryogenesis process between the three callus lines with different embryogenic capacity (regenerant, embryogenic and non-embryogenic lines), the two types of embryos (normal and abnormal), and the tissues from the normal somatic embryogenesis process from callus to plantlets (regenerant callus on ENT and EXP media, normal embryo, leaf, stem and root). Values with the same letter did not differ significantly at the 0.05 probability level.

In Table 2, the level of expression was calculated as the ratio between the relative transcript abundances in callus of the regenerant line/embryogenic line, the regenerant line/non-embryogenic line grown on ENT and EXP media, and in normal/abnormal somatic embryos. It was considered as an up-regulation with a ratio >1.0, and a down-regulation with a ratio <1.0. The statistical analysis was carried out from the logarithm of raw data using the two-tailed probability values of the *t* test. The ratio of the relative transcript abundances with a p-value $\leq$ 0.05 was adopted as significant for down or up-regulation. Only significant data are discussed in the manuscript.

Phylogenetic analysis of the AP2 domain from AP2/ERF marker genes

A multiple alignment analysis was performed on full-length AP2 domain sequences from *Hevea* and *Arabidopsis* for the AP2, ERF (groups III, IV, V, VII, VIII, X) and RAV families (Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3, Additional file 4: Figure S4, Additional file 5: figure S5 and Additional file 6: Figure S6). The full AP2-domain sequences derived from *Hevea* and *Arabidopsis* AP2-domain proteins of around 60 amino acids were then aligned using MUSCLE software [85,86], which uses a progressive multiple alignment method. The alignment was curated by Gblocks software [87], searching for at least 10-amino-acid-long conserved blocks, and the block with 57 amino acids was extracted. This block of 57 amino acids was used to construct the phylogenetic tree using PhyML software [88], which implements a maximum likelihood tree reconstruction method, using the LG+gamma model, starting from a BioNJ tree [89]. A RAP-Green analysis was performed from the initial PhyML tree to improve gene function inferences and predict gene duplications in phylogenetic trees [90]. The final tree was drawn and displayed with the Archaeoptheryx program, and rooted on the branch separating the RAV family from the rest of the tree. Branch supports were computed using the aLRT-SHlike method [91].

Authors' contributions

FD and FM established the various friable callus lines for this experiment from cryopreserved material. PP induced somatic embryogenesis and regenerated both embryos and plantlets, and then carried out the total RNA isolation and cDNA synthesis from samples. PP and RP designed the primers from the AP2/ERF genes. MR and JL supervised the real-time RT-PCR analysis on the Roche platform. LL supervised the tissue culture experiments. JFD advised on the strategy and supervised the phylogenetic analyses. PP and PM drafted the manuscript. PM coordinated this research work. All the authors read and approved the final manuscript.

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Additional files

Additional_file_1 as PDF

Additional file 1: Figure S1 Phylogenetic tree of the AP2 family. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_2 as PDF

Additional file 2: Figure S2 Phylogenetic tree of ERF group I. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_3 as PDF

Additional file 3: Figure S3 Phylogenetic tree of ERF group III. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_4 as PDF

Additional file 4: Figure S4 Phylogenetic tree of ERF group IV. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_5 as PDF

Additional file 5: Figure S5 Phylogenetic tree of ERF group VI. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_6 as PDF

Additional file 6: Figure S6 Phylogenetic tree of ERF group X. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were

aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. *Hevea* somatic embryogenesis marker genes are indicated in bold letters.

Additional_file_7 as XLSX

Additional file 7: Table S1 List of primer sequences for 132 AP2/ERF genes from H. brasiliensis clone PB 260

Additional_file_8 as XLSX

Additional file 8: Table S2 Comparison Cp value of callus with various embryogenic capacities grown on embryogenesis induction medium (ENT) and somatic embryogenesis expression medium (EXP){Calli are from three types of lines: (NE) non-embryogenic line (CI04115), (E) embryogenic and non-regenerant line (CI04106), and (R) regenerant line (CI07060)},normal and abnormal embryo and 3 organ of plantlet : (L) Leaf, (S) Stem, (R) Root in 11 house keeping genes.

Appendix

In chapter 2, we improved the RNA database and corrected the contig sequences using genomic scaffold sequences. The number of AP2/ERF was reduced from 142 to 114. In the previous work in chapter 1, primer couples were designed for 132 contigs. Then, the specificity of qPCR amplicons was checked only for expression marker genes.

In this appendix, gene expression profile was shown only for the 94 instead of 132 AP2/ERF genes previously studied. The 94 genes were confirmed in chapter 2.

Appendix

Gene		Callus on ENT medium					
		A			B		
		NE	E	R	NE	E	R
Ethylene biosynthetic & transduction pathways	HbSAMS	1,12E-01[b]	2,14E-01[b]	8,55E-03[a]	1,12E-01[b]	2,14E-01[b]	8,55E-03[a]
	HbACS1	1,35E-01[a]	5,10E-02[a]	3,03E-02[a]	1,35E-01	5,10E-02	3,03E-02
	HbACS2	9,72E-01[b]	6,73E-01[b]	7,36E-03[a]	9,72E-01[b]	6,73E-01[b]	7,36E-03[a]
	HbACS3	1,33E+00[a]	3,19E+00[a]	2,94E+00[a]	1,33E+00	3,19E+00	2,94E+00
	HbACO1	1,57E-03[a]	3,40E-03[a]	3,38E-03[a]	1,57E-03	3,40E-03	3,38E-03
	HbACO2	1,66E-01[a]	5,77E-01[a]	1,69E-01[a]	1,66E-01	5,77E-01	1,69E-01
	HbACO3	7,14E-03[a]	8,34E-03[a]	7,42E-03[a]	7,14E-03	8,34E-03	7,42E-03
	HbETR1	5,12E-01[b]	4,47E-01[b]	1,80E-01[a]	5,12E-01[b]	4,47E-01[b]	1,80E-01[a]
	HbETR2	1,79E+00[a]	2,92E+00[a]	1,94E+00[a]	1,79E+00	2,92E+00	1,94E+00
	HbEIN3	7,32E-01[a]	7,62E-01[a]	1,78E-01[a]	7,32E-01	7,62E-01	1,78E-01
HbERF-I	HbERF-Ia1	9,14E-03[b]	1,90E-02[c]	2,84E-03[a]	9,14E-03[b]	1,90E-02[c]	2,84E-03[a]
	HbERF-Ib2	8,59E-01[a]	9,68E-01[a]	4,16E-01[a]	8,59E-01	9,68E-01	4,16E-01
	HbERF-Ib4	1,29E+00[a]	8,09E-01[a]	3,71E-01[a]	1,29E+00	8,09E-01	3,71E-01
	HbERF-Ib5	1,84E+00[a]	2,34E+00[a]	1,82E+00[a]	1,84E+00	2,34E+00	1,82E+00
	HbERF-Ib7	6,30E+00[a]	8,20E+00[a]	1,85E+00[a]	6,30E+00	8,20E+00	1,85E+00
	HbERF-Ib9	2,59E+00[a]	3,47E+00[a]	3,41E+00[a]	2,59E+00	3,47E+00	3,41E+00
	HbERF-Ib11	1,84E-02[a]	2,07E-02[a]	2,32E-02[a]	1,84E-02	2,07E-02	2,32E-02
HbERF-II	HbERF-IIa1	1,71E-02[a]	2,38E-02[a]	4,55E-03[a]	1,71E-02	2,38E-02	4,55E-03
	HbERF-IIa3	2,46E+00[a]	3,45E+00[a]	3,17E+00[a]	2,46E+00	3,45E+00	3,17E+00
	HbERF-IIb1	1,62E-01[a]	1,60E-01[a]	1,44E-01[a]	1,62E-01	1,60E-01	1,44E-01
	HbERF-IIb2	9,88E-02[a]	1,90E-01[a]	5,90E-02[a]	9,88E-02	1,90E-01	5,90E-02
	HbERF-IIb4	1,01E-02[a]	7,15E-03[a]	3,03E-03[a]	1,01E-02	7,15E-03	3,03E-03
HbERF-III	HbERF-IIIa1	9,01E-01[a]	6,59E-01[a]	1,22E+00[a]	9,01E-01	6,59E-01	1,22E+00
	HbERF-IIIb1	2,21E-02[a]	1,69E-02[a]	8,16E-03[a]	2,21E-02	1,69E-02	8,16E-03
	HbERF-IIIb2	4,13E-01[a]	3,72E-02[a]	5,66E-02[a]	4,13E-01	3,72E-02	5,66E-02
	HbERF-IIIc1	5,79E-01[a]	8,68E-01[a]	6,87E-01[a]	5,79E-01	8,68E-01	6,87E-01
	HbERF-IIId2	8,01E-03[a]	8,19E-04[a]	2,06E-02[a]	8,01E-03	8,19E-04	2,06E-02
	HbERF-IIId3	9,71E-03[a]	4,73E-02[a]	3,24E-02[a]	9,71E-03	4,73E-02	3,24E-02
	HbERF-IIIe1	1,23E-02[a]	6,86E-03[a]	8,23E-03[a]	1,23E-02	6,86E-03	8,23E-03
	HbERF-IIIe2	3,37E-02[a]	7,74E-02[a]	2,57E-02[a]	3,37E-02	7,74E-02	2,57E-02
	HbERF-IIIe3	1,04E-02[a]	1,94E-02[a]	9,68E-03[a]	1,04E-02	1,94E-02	9,68E-03
	HbERF-IIIe4	2,09E-01[a]	1,08E+00[a]	9,89E-02[a]	2,09E-01	1,08E+00	9,89E-02
HbERF-IV	HbERF-IVa2	1,07E+00[a]	4,96E-01[a]	3,46E-01[a]	1,07E+00	4,96E-01	3,46E-01
HbERF-V	HbERF-Va2	7,24E-01[a]	1,30E+00[a]	1,31E+00[a]	7,24E-01	1,30E+00	1,31E+00
	HbERF-Vb1	3,34E-02[a]	5,59E-02[a]	1,26E-02[a]	3,34E-02	5,59E-02	1,26E-02
	HbERF-Vb2	3,70E-02[a]	6,11E-02[a]	7,89E-02[a]	3,70E-02	6,11E-02	7,89E-02
	HbERF-Vb3	8,08E-03[a]	4,41E-03[a]	1,40E-02[a]	8,08E-03	4,41E-03	1,40E-02
HbERF-VI	HbERF-VI1	7,84E-02[a]	1,09E-01[a]	9,59E-02[a]	7,84E-02	1,09E-01	9,59E-02
	HbERF-VI2	1,08E-01[b]	1,83E-01[b]	1,10E-02[a]	1,08E-01[b]	1,83E-01[b]	1,10E-02[a]
	HbERF-VI3	7,89E-02[a]	7,11E-02[a]	5,09E-02[a]	7,89E-02	7,11E-02	5,09E-02
	HbERF-VI4	1,66E-02[a]	1,21E-02[a]	4,49E-03[a]	1,66E-02	1,21E-02	4,49E-03
	HbERF-VI5	7,51E-01[a]	3,73E-01[a]	2,57E-01[a]	7,51E-01	3,73E-01	2,57E-01
	HbERF-VI-L3	1,05E+00[a]	1,34E+00[a]	6,02E-01[a]	1,05E+00	1,34E+00	6,02E-01
	HbERF-VI-L4	3,22E-01[a]	1,15E+00[a]	1,94E-01[a]	3,22E-01	1,15E+00	1,94E-01
	HbERF-VI-L6	1,07E-01[a]	6,22E-01[a]	4,66E-01[a]	1,07E-01	6,22E-01	4,66E-01
HbERF-VII	HbERF-VIIa1	7,26E+00[a]	8,58E+00[a]	3,22E+00[a]	7,26E+00	8,58E+00	3,22E+00
	HbERF-VIIa4	1,41E+00[b]	1,44E+00[b]	3,03E-01[a]	1,41E+00[b]	1,44E+00[b]	3,03E-01[a]
	HbERF-VIIa7	1,23E+01[a]	4,01E+01[a]	2,02E+01[a]	1,23E+01	4,01E+01	2,02E+01
	HbERF-VIIa12	1,79E+01[a]	2,66E+01[a]	4,19E+01[a]	1,79E+01	2,66E+01	4,19E+01
	HbERF-VIIa17	3,72E-01[a]	6,50E-01[a]	1,20E+00[a]	3,72E-01	6,50E-01	1,20E+00
	HbERF-VIIa20	3,98E-03[a]	8,95E-03[a]	2,88E-02[a]	3,98E-03	8,95E-03	2,88E-02
HbERF-VIII	HbERF-VIIIa3	1,14E+00[b]	1,76E+00[b]	1,70E-01[a]	1,14E+00[b]	1,76E+00[b]	1,70E-01[a]
	HbERF-VIIIa4	3,99E+00[a]	1,50E+00[a]	8,75E-01[a]	3,99E+00	1,50E+00	8,75E-01
	HbERF-VIIIa5	5,97E-01[a]	5,13E-01[a]	4,42E-01[a]	5,97E-01	5,13E-01	4,42E-01
	HbERF-VIIIa8	3,36E+00[a]	4,87E+00[a]	4,76E+00[a]	3,36E+00	4,87E+00	4,76E+00
	HbERF-VIIIa9	6,76E+00[a]	8,62E+00[a]	4,55E+00[a]	6,76E+00	8,62E+00	4,55E+00
	HbERF-VIIIa10	8,07E-01[a]	1,58E+00[a]	1,24E+00[a]	8,07E-01	1,58E+00	1,24E+00
	HbERF-VIIIa12	3,02E-01[a]	5,14E-01[a]	2,35E-01[a]	3,02E-01	5,14E-01	2,35E-01
	HbERF-VIIIa13	1,45E+00[a]	3,01E+00[a]	1,15E+00[a]	1,45E+00	3,01E+00	1,15E+00
	HbERF-VIIIa14	3,37E-01[a]	3,00E-01[a]	6,68E-01[a]	3,37E-01	3,00E-01	6,68E-01
	HbERF-VIIIb1	7,57E-01[a]	7,27E-01[a]	6,66E-01[a]	7,57E-01	7,27E-01	6,66E-01
HbERF-IX	HbERF-IXa2	4,34E-01[a]	7,63E-01[a]	1,92E+00[a]	4,34E-01	7,63E-01	1,92E+00
	HbERF-IXa3	3,47E-01[a]	6,28E-01[a]	8,69E-01[a]	3,47E-01	6,28E-01	8,69E-01
	HbERF-IXb1	2,89E-01[a]	2,39E-01[a]	3,23E-01[a]	2,89E-01	2,39E-01	3,23E-01
	HbERF-IXb2	1,30E-02[a]	6,83E-03[a]	4,08E-03[a]	1,30E-02	6,83E-03	4,08E-03
	HbERF-IXb3	3,31E-01[a]	2,22E-01[a]	7,87E-02[a]	3,31E-01	2,22E-01	7,87E-02
	HbERF-IXc1	9,87E-02[a]	3,33E-01[a]	1,76E-01[a]	9,87E-02	3,33E-01	1,76E-01
	HbERF-IXc4	1,56E-01[a]	2,09E-02[a]	1,55E-01[a]	1,56E-01	2,09E-02	1,55E-01
	HbERF-IXc5	2,80E-02[a]	5,15E-02[a]	3,46E-02[a]	2,80E-02	5,15E-02	3,46E-02
	HbERF-IXc6	3,63E-04[a]	1,64E-03[a]	2,85E-03[a]	3,63E-04	1,64E-03	2,85E-03
HbERF-X	HbERF-Xa1	2,49E+00[a]	3,12E+00[a]	1,25E+00[a]	2,49E+00	3,12E+00	1,25E+00
	HbERF-Xa2	1,59E-01[a]	1,24E-01[a]	1,83E-01[a]	1,59E-01	1,24E-01	1,83E-01
	HbERF-Xa4	3,06E-01[a]	6,79E-02[a]	3,30E-02[a]	3,06E-01	6,79E-02	3,30E-02
	HbERF-Xa6	6,04E-01[a]	5,42E-01[a]	4,36E-01[a]	6,04E-01	5,42E-01	4,36E-01
	HbERF-Xa8	2,19E-02[a]	6,60E-02[a]	4,94E-02[a]	2,19E-02	6,60E-02	4,94E-02
	HbERF-Xb1	5,67E-03[a]	8,21E-03[a]	9,74E-03[a]	5,67E-03	8,21E-03	9,74E-03
HbAP2	HbAP2-1	3,72E-01[b]	4,79E-01[b]	5,02E-02[a]	3,72E-01[b]	4,79E-01[b]	5,02E-02[a]
	HbAP2-3	1,34E+01[a]	1,26E+01[a]	1,08E+01[a]	1,34E+01	1,26E+01	1,08E+01
	HbAP2-5	4,12E-01[b]	5,57E-01[b]	5,32E-02[a]	4,12E-01[b]	5,57E-01[b]	5,32E-02[a]
	HbAP2-6	1,06E-01[a]	7,65E-02[a]	9,15E-02[a]	1,06E-01	7,65E-02	9,15E-02
	HbAP2-7	6,73E-02[a]	1,70E-02[a]	2,99E-02[a]	6,73E-02	1,70E-02	2,99E-02
	HbAP2-8	3,00E-01[a]	8,40E-02[a]	7,94E-02[a]	3,00E-01	8,40E-02	7,94E-02
	HbAP2-9	6,49E+00[a]	7,67E+00[a]	1,03E+01[a]	6,49E+00	7,67E+00	1,03E+01
	HbAP2-10	3,87E-02[a]	8,60E-02[a]	3,55E-02[a]	3,87E-02	8,60E-02	3,55E-02
	HbAP2-11	3,10E-02[a]	8,12E-02[b]	1,58E-02[a]	3,10E-02[a]	8,12E-02[b]	1,58E-02[a]
	HbAP2-12	1,10E-01[b]	1,44E-01[b]	1,44E-02[a]	1,10E-01[b]	1,44E-01[b]	1,44E-02[a]
	HbAP2-13	1,09E-01[a]	5,68E-02[a]	1,00E-02[a]	1,09E-01	5,68E-02	1,00E-02
	HbAP2-15	5,02E-01[a]	1,53E-01[a]	1,20E-01[a]	5,02E-01	1,53E-01	1,20E-01
	HbAP2-16	1,03E+00[a]	8,57E-01[a]	1,31E+00[a]	1,03E+00	8,57E-01	1,31E+00
	HbAP2-17	6,93E-03[a]	3,72E-03[a]	1,29E-03[a]	6,93E-03	3,72E-03	1,29E-03
	HbAP2-18	8,07E+00[b]	1,08E+01[b]	1,74E+00[a]	8,07E+00[b]	1,08E+01[b]	1,74E+00[a]
HbRAV	HbRAV-2	1,82E-03[a]	3,18E-03[a]	1,79E-02[a]	1,82E-03	3,18E-03	1,79E-02
	HbRAV-3	2,73E-03[a]	1,59E-03[a]	1,01E-03[a]	2,73E-03	1,59E-03	1,01E-03
	HbRAV-4	2,44E-01[b]	5,45E-02[a]	3,98E-02[a]	2,44E-01[b]	5,45E-02[a]	3,98E-02[a]

Figure 3 Expression profile of 94 genes involved in the ethylene biosynthesis and signalling pathways for callus with different embryogenic capacities grown for 2 weeks on embryogenesis induction medium (ENT). Calli were from three types of lines: (NE) non-embryogenic line (CI04115), (E) embryogenic and non-regenerant line (CI04106), and (R) regenerant line (CI07060). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundances of three biological replicates. (Figure 3 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green. (Figure 3 **B**) Values of relative transcript abundance in callus of the various lines were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

Gene		Callus on EXP medium					
		A			B		
		NE	E	R	NE	E	R
Ethylene biosynthetic & transduction pathways	HbSAMS	6,26E-03 a	2,21E-02 a	1,14E-01 b	6,26E-03 a	2,21E-02 a	1,14E-01 b
	HbACS1	6,23E-04 a	1,86E-03 a	3,63E-02 a	6,23E-04 a	1,86E-03 a	3,63E-02 a
	HbACS2	2,97E-03 a	3,11E-03 a	1,33E-02 a	2,97E-03 a	3,11E-03 a	1,33E-02 a
	HbACS3	9,01E-02 a	2,37E-01 a	2,93E+00 a	9,01E-02 a	2,37E-01 a	2,93E+00 a
	HbACO1	4,04E-03 a	5,23E-02 a	6,13E-02 a	4,04E-03 a	5,23E-02 a	6,13E-02 a
	HbACO2	1,93E-02 a	3,74E-02 ab	2,00E-01 b	1,93E-02 a	3,74E-02 ab	2,00E-01 b
	HbACO3	1,88E-03 a	1,04E-03 a	9,45E-03 a	1,88E-03 a	1,04E-03 a	9,45E-03 a
	HbETR1	1,11E-01 a	2,58E-01 a	7,88E-01 a	1,11E-01 a	2,58E-01 a	7,88E-01 a
	HbETR2	1,03E+00 a	1,63E+00 a	2,13E+00 a	1,03E+00 a	1,63E+00 a	2,13E+00 a
	HbEIN3	1,14E+00 a	1,39E+00 a	1,14E+00 a	1,14E+00 a	1,39E+00 a	1,14E+00 a
HbERF-I	HbERF-Ia1	7,74E-03 a	9,04E-03 a	9,98E-03 a	7,74E-03 a	9,04E-03 a	9,98E-03 a
	HbERF-Ib2	5,42E+00 a	4,61E+00 a	3,13E+00 a	5,42E+00 a	4,61E+00 a	3,13E+00 a
	HbERF-Ib4	1,07E+00 a	8,31E-01 a	2,73E+00 a	1,07E+00 a	8,31E-01 a	2,73E+00 a
	HbERF-Ib5	2,89E+00 a	2,98E+00 a	3,68E+00 a	2,89E+00 a	2,98E+00 a	3,68E+00 a
	HbERF-Ib7	1,90E+00 a	1,61E+00 a	4,21E+00 a	1,90E+00 a	1,61E+00 a	4,21E+00 a
	HbERF-Ib9	8,75E-01 a	8,89E-01 a	5,88E+00 a	8,75E-01 a	8,89E-01 a	5,88E+00 a
	HbERF-Ib11	3,60E-03 a	1,18E-02 a	8,19E-03 a	3,60E-03 a	1,18E-02 a	8,19E-03 a
HbERF-II	HbERF-IIa1	6,94E-02 a	3,79E-02 a	6,08E-02 a	6,94E-02 a	3,79E-02 a	6,08E-02 a
	HbERF-IIa3	1,52E+00 a	2,31E+00 ab	9,35E+00 b	1,52E+00 a	2,31E+00 ab	9,35E+00 b
	HbERF-IIb1	7,84E-02 a	1,18E-01 a	4,03E-01 a	7,84E-02 a	1,18E-01 a	4,03E-01 a
	HbERF-IIb2	3,89E-02 a	2,43E-02 a	5,36E-01 a	3,89E-02 a	2,43E-02 a	5,36E-01 a
	HbERF-IIb4	1,42E-03 a	2,66E-03 a	5,33E-03 a	1,42E-03 a	2,66E-03 a	5,33E-03 a
HbERF-III	HbERF-IIIa1	6,90E-02 a	3,82E-01 b	3,30E+00 c	6,90E-02 a	3,82E-01 b	3,30E+00 c
	HbERF-IIIb1	5,75E-03 ab	3,25E-03 a	1,63E-02 b	5,75E-03 ab	3,25E-03 a	1,63E-02 b
	HbERF-IIIb2	6,37E-03 a	1,06E-02 a	6,83E-02 b	6,37E-03 a	1,06E-02 a	6,83E-02 b
	HbERF-IIIc1	1,48E-01 a	2,91E-01 a	1,16E+00 b	1,48E-01 a	2,91E-01 a	1,16E+00 b
	HbERF-IIId2	3,57E-01 a	6,05E-01 a	5,03E-01 a	3,57E-01 a	6,05E-01 a	5,03E-01 a
	HbERF-IIId3	2,12E-03 a	1,49E-03 a	5,74E-03 a	2,12E-03 a	1,49E-03 a	5,74E-03 a
	HbERF-IIIe1	5,11E-03 a	6,74E-03 a	1,72E-02 a	5,11E-03 a	6,74E-03 a	1,72E-02 a
	HbERF-IIIe2	1,18E-01 a	2,34E-01 a	3,01E-01 a	1,18E-01 a	2,34E-01 a	3,01E-01 a
	HbERF-IIIe3	6,54E-03 a	1,23E-02 a	1,06E-01 b	6,54E-03 a	1,23E-02 a	1,06E-01 b
	HbERF-IIIe4	1,11E-01 a	6,24E-02 a	1,03E-01 a	1,11E-01 a	6,24E-02 a	1,03E-01 a
HbERF-IV	HbERF-IVa2	1,69E-01 a	2,07E-01 a	1,00E+00 b	1,69E-01 a	2,07E-01 a	1,00E+00 b
HbERF-V	HbERF-Va2	9,02E-02 a	8,84E-02 a	3,78E-01 a	9,02E-02 a	8,84E-02 a	3,78E-01 a
	HbERF-Vb1	5,57E-02 a	5,03E-02 a	3,19E-02 a	5,57E-02 a	5,03E-02 a	3,19E-02 a
	HbERF-Vb2	1,34E-01 a	5,87E-02 a	4,34E-01 b	1,34E-01 a	5,87E-02 a	4,34E-01 b
	HbERF-Vb3	4,85E-02 a	6,69E-01 b	3,47E-01 b	4,85E-02 a	6,69E-01 b	3,47E-01 b
HbERF-VI	HbERF-VI1	1,31E-02 a	1,62E-02 ab	6,09E-02 b	1,31E-02 a	1,62E-02 ab	6,09E-02 b
	HbERF-VI2	4,01E-02 a	3,37E-02 a	1,30E-01 a	4,01E-02 a	3,37E-02 a	1,30E-01 a
	HbERF-VI3	1,05E-02 a	9,25E-02 b	1,88E-01 b	1,05E-02 a	9,25E-02 b	1,88E-01 b
	HbERF-VI4	1,45E-03 a	1,19E-02 b	3,60E-02 c	1,45E-03 a	1,19E-02 b	3,60E-02 c
	HbERF-VI5	7,61E-01 a	8,40E-01 a	7,88E-01 a	7,61E-01 a	8,40E-01 a	7,88E-01 a
	HbERF-VI-L3	5,68E-01 a	9,90E-01 a	1,75E+00 a	5,68E-01 a	9,90E-01 a	1,75E+00 a
	HbERF-VI-L4	6,88E-02 a	1,37E-01 a	4,51E-01 a	6,88E-02 a	1,37E-01 a	4,51E-01 a
	HbERF-VI-L6	3,59E-02 a	3,80E-01 b	1,37E+00 b	3,59E-02 a	3,80E-01 b	1,37E+00 b
HbERF-VII	HbERF-VIIa1	3,91E-01 a	3,84E-01 a	3,23E-01 a	3,91E-01 a	3,84E-01 a	3,23E-01 a
	HbERF-VIIa4	2,00E-01 a	5,37E-01 b	9,23E-01 b	2,00E-01 a	5,37E-01 b	9,23E-01 b
	HbERF-VIIa7	1,82E+01 a	6,53E+01 a	6,49E+01 a	1,82E+01 a	6,53E+01 a	6,49E+01 a
	HbERF-VIIa12	4,52E+01 a	1,16E+02 a	1,14E+02 a	4,52E+01 a	1,16E+02 a	1,14E+02 a
	HbERF-VIIa17	5,38E-02 a	2,69E-01 b	2,64E+00 c	5,38E-02 a	2,69E-01 b	2,64E+00 c
	HbERF-VIIa20	6,18E-03 a	7,56E-03 a	5,46E-02 a	6,18E-03 a	7,56E-03 a	5,46E-02 a
HbERF-VIII	HbERF-VIIIa3	9,92E-01 a	3,92E+00 b	6,81E-01 a	9,92E-01 a	3,92E+00 b	6,81E-01 a
	HbERF-VIIIa4	1,00E+00 a	3,08E+00 b	2,27E+00 b	1,00E+00 a	3,08E+00 b	2,27E+00 b
	HbERF-VIIIa5	4,25E-01 a	5,39E-01 a	8,52E-01 a	4,25E-01 a	5,39E-01 a	8,52E-01 a
	HbERF-VIIIa8	1,77E+00 a	2,85E+00 a	4,74E+00 a	1,77E+00 a	2,85E+00 a	4,74E+00 a
	HbERF-VIIIa9	1,18E+00 a	1,57E+00 a	6,19E+00 a	1,18E+00 a	1,57E+00 a	6,19E+00 a
	HbERF-VIIIa10	1,21E+00 a	1,70E+00 a	3,60E+00 a	1,21E+00 a	1,70E+00 a	3,60E+00 a
	HbERF-VIIIa12	8,37E-02 a	1,16E-01 ab	6,06E-01 b	8,37E-02 a	1,16E-01 ab	6,06E-01 b
	HbERF-VIIIa13	4,27E-01 a	3,47E+00 b	3,40E+00 b	4,27E-01 a	3,47E+00 b	3,40E+00 b
	HbERF-VIIIa14	7,80E-01 a	7,83E-01 a	5,03E-01 a	7,80E-01 a	7,83E-01 a	5,03E-01 a
	HbERF-VIIIb1	3,15E-01 a	2,15E-01 a	7,67E-01 a	3,15E-01 a	2,15E-01 a	7,67E-01 a
HbERF-IX	HbERF-IXa2	1,43E-01 a	1,11E+00 b	2,05E+00 b	1,43E-01 a	1,11E+00 b	2,05E+00 b
	HbERF-IXa3	1,07E-01 a	1,12E+00 b	9,03E-01 b	1,07E-01 a	1,12E+00 b	9,03E-01 b
	HbERF-IXb1	3,75E-01 a	2,72E-01 a	1,93E-01 a	3,75E-01 a	2,72E-01 a	1,93E-01 a
	HbERF-IXb2	5,58E-03 b	2,92E-03 a	1,48E-02 c	5,58E-03 b	2,92E-03 a	1,48E-02 c
	HbERF-IXb3	1,98E-01 a	2,34E-01 a	1,32E-01 a	1,98E-01 a	2,34E-01 a	1,32E-01 a
	HbERF-IXc1	2,11E-02 a	4,05E-02 ab	1,28E-01 b	2,11E-02 a	4,05E-02 ab	1,28E-01 b
	HbERF-IXc4	1,05E-02 a	5,72E-02 a	6,00E-01 b	1,05E-02 a	5,72E-02 a	6,00E-01 b
	HbERF-IXc5	1,18E-02 a	1,79E-02 a	5,81E-02 a	1,18E-02 a	1,79E-02 a	5,81E-02 a
	HbERF-IXc6	2,24E-03 a	3,69E-02 a	9,01E-03 a	2,24E-03 a	3,69E-02 a	9,01E-03 a
HbERF-X	HbERF-Xa1	2,15E+00 a	2,54E+00 a	1,78E+00 a	2,15E+00 a	2,54E+00 a	1,78E+00 a
	HbERF-Xa2	4,79E-02 a	1,20E-01 a	8,44E-02 a	4,79E-02 a	1,20E-01 a	8,44E-02 a
	HbERF-Xa4	3,68E-02 a	3,19E-02 a	1,38E-01 a	3,68E-02 a	3,19E-02 a	1,38E-01 a
	HbERF-Xa6	2,18E-02 a	4,47E-02 a	1,05E-01 a	2,18E-02 a	4,47E-02 a	1,05E-01 a
	HbERF-Xa8	1,75E-02 a	1,56E-02 a	8,51E-02 a	1,75E-02 a	1,56E-02 a	8,51E-02 a
	HbERF-Xb1	2,00E-03 a	1,52E-02 b	9,51E-03 ab	2,00E-03 a	1,52E-02 b	9,51E-03 ab
HbAP2	HbAP2-1	3,84E-01 a	1,87E-01 a	6,40E-01 a	3,84E-01 a	1,87E-01 a	6,40E-01 a
	HbAP2-3	1,90E+00 a	2,28E+00 a	1,09E+01 b	1,90E+00 a	2,28E+00 a	1,09E+01 b
	HbAP2-5	4,23E-01 a	6,81E-01 a	8,74E-01 a	4,23E-01 a	6,81E-01 a	8,74E-01 a
	HbAP2-6	5,92E-03 a	2,91E-01 b	3,20E-01 b	5,92E-03 a	2,91E-01 b	3,20E-01 b
	HbAP2-7	1,54E-03 a	2,97E-03 a	3,84E-02 b	1,54E-03 a	2,97E-03 a	3,84E-02 b
	HbAP2-8	1,17E-02 a	1,18E-01 b	4,10E-01 b	1,17E-02 a	1,18E-01 b	4,10E-01 b
	HbAP2-9	3,57E+00 a	3,06E+00 a	6,79E+00 a	3,57E+00 a	3,06E+00 a	6,79E+00 a
	HbAP2-10	4,05E-03 a	8,18E-03 a	1,31E-02 a	4,05E-03 a	8,18E-03 a	1,31E-02 a
	HbAP2-11	2,13E-02 a	5,80E-02 b	8,10E-02 b	2,13E-02 a	5,80E-02 b	8,10E-02 b
	HbAP2-12	3,29E-01 a	2,19E-01 a	1,57E-01 a	3,29E-01 a	2,19E-01 a	1,57E-01 a
	HbAP2-13	1,08E-01 a	8,96E-02 a	3,88E-01 a	1,08E-01 a	8,96E-02 a	3,88E-01 a
	HbAP2-15	2,63E-02 a	1,46E-01 a	2,68E-01 a	2,63E-02 a	1,46E-01 a	2,68E-01 a
	HbAP2-16	5,49E-01 a	1,03E+00 a	1,70E+00 a	5,49E-01 a	1,03E+00 a	1,70E+00 a
	HbAP2-17	5,25E-04 a	2,09E-03 a	5,80E-03 a	5,25E-04 a	2,09E-03 a	5,80E-03 a
	HbAP2-18	1,24E+01 a	1,53E+01 a	1,62E+01 a	1,24E+01 a	1,53E+01 a	1,62E+01 a
HbRAV	HbRAV-2	1,11E-03 a	4,76E-03 a	1,52E-02 a	1,11E-03 a	4,76E-03 a	1,52E-02 a
	HbRAV-3	3,12E-04 a	8,31E-04 a	1,36E-02 b	3,12E-04 a	8,31E-04 a	1,36E-02 b
	HbRAV-4	4,74E-01 a	1,41E-01 a	1,04E+00 a	4,74E-01 a	1,41E-01 a	1,04E+00 a

Figure 4 Expression profile of 94 genes involved in the ethylene biosynthesis and signalling pathways for callus with different embryogenic capacities grown for 4 weeks on somatic embryogenesis expression medium (EXP). Calli were from three types of lines: (NE) non-embryogenic line (CI04115), (E) embryogenic and non-regenerant line (CI04106), and (R) regenerant line (CI07060). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 4 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green. (Figure 4 **B**) Values of relative transcript abundance in callus of the various lines were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

Gene		Somatic embryo			
		A		B	
		Normal	Abnormal	Normal	Abnormal
Ethylene biosynthetic & transduction pathways	HbSAMS	1,75E-01 a	2,21E-01 a	1,75E-01 a	2,21E-01 a
	HbACS1	1,37E-01 a	7,71E-01 a	1,37E-01 a	7,71E-01 a
	HbACS2	1,15E-01 a	3,25E-01 a	1,15E-01 a	3,25E-01 a
	HbACS3	2,94E-01 a	1,13E+00 a	2,94E-01 a	1,13E+00 a
	HbACO1	4,37E-01 a	1,11E-01 a	4,37E-01 a	1,11E-01 a
	HbACO2	5,01E+00 a	5,94E+00 a	5,01E+00 a	5,94E+00 a
	HbACO3	1,17E-01 a	3,96E-01 a	1,17E-01 a	3,96E-01 a
	HbETR1	1,47E+00 a	7,45E-01 a	1,47E+00 a	7,45E-01 a
	HbETR2	1,93E+00 a	6,31E-01 a	1,93E+00 a	6,31E-01 a
	HbEIN3	6,78E-01 a	6,93E-01 a	6,78E-01 a	6,93E-01 a
HbERF-I	HbERF-Ia1	2,88E-03 a	1,13E-02 b	2,88E-03 a	1,13E-02 b
	HbERF-Ib2	1,33E-01 a	5,16E-01 a	1,33E-01 a	5,16E-01 a
	HbERF-Ib4	2,88E-01 a	4,47E-01 a	2,88E-01 a	4,47E-01 a
	HbERF-Ib5	1,31E+00 a	3,21E+00 a	1,31E+00 a	3,21E+00 a
	HbERF-Ib7	2,36E+00 a	1,27E+01 b	2,36E+00 a	1,27E+01 b
	HbERF-Ib9	1,03E+01 a	2,16E+00 a	1,03E+01 a	2,16E+00 a
	HbERF-Ib11	6,80E-02 a	2,12E-02 a	6,80E-02 a	2,12E-02 a
HbERF-II	HbERF-IIa1	3,70E-03 a	4,55E-03 a	3,70E-03 a	4,55E-03 a
	HbERF-IIa3	4,87E+00 a	6,64E+00 a	4,87E+00 a	6,64E+00 a
	HbERF-IIb1	3,20E-01 a	8,26E-01 a	3,20E-01 a	8,26E-01 a
	HbERF-IIb2	3,62E-02 a	1,17E+00 a	3,62E-02 a	1,17E+00 a
	HbERF-IIb4	1,32E-02 a	5,04E-03 a	1,32E-02 a	5,04E-03 a
HbERF-III	HbERF-IIIa1	3,72E+00 a	3,66E+00 a	3,72E+00	3,66E+00 a
	HbERF-IIIb1	3,14E-02 a	7,35E-02 a	3,14E-02 a	7,35E-02 a
	HbERF-IIIb2	1,04E-02 a	5,26E-01 a	1,04E-02 a	5,26E-01 a
	HbERF-IIIc1	1,20E+00 a	2,15E+00 a	1,20E+00 a	2,15E+00 a
	HbERF-IIId2	1,76E-02 a	1,43E-02 a	1,76E-02 a	1,43E-02 a
	HbERF-IIId3	8,06E-03 a	5,48E-03 a	8,06E-03 a	5,48E-03 a
	HbERF-IIIe1	3,71E-01 a	1,88E-01 a	3,71E-01 a	1,88E-01 a
	HbERF-IIIe2	1,44E+00 a	7,60E-01 a	1,44E+00 a	7,60E-01 a
	HbERF-IIIe3	1,08E-01 a	5,22E-02 a	1,08E-01 a	5,22E-02 a
	HbERF-IIIe4	8,34E-02 a	1,45E-01 a	8,34E-02 a	1,45E-01 a
HbERF-IV	HbERF-IVa2	1,25E+00 a	3,85E+00 b	1,25E+00 a	3,85E+00 b
HbERF-V	HbERF-Va2	4,55E-01 a	1,59E-01 a	4,55E-01 a	1,59E-01 a
	HbERF-Vb1	9,39E-03 a	1,54E-02 a	9,39E-03 a	1,54E-02 a
	HbERF-Vb2	3,37E-02 a	2,22E-02 a	3,37E-02 a	2,22E-02 a
	HbERF-Vb3	2,44E-02 a	2,39E-02 a	2,44E-02 a	2,39E-02 a
HbERF-VI	HbERF-VI1	3,12E-01 b	1,03E-01 a	3,12E-01 b	1,03E-01 a
	HbERF-VI2	2,73E-02 a	1,01E-01 a	2,73E-02 a	1,01E-01 a
	HbERF-VI3	4,10E-01 a	1,64E-01 a	4,10E-01 a	1,64E-01 a
	HbERF-VI4	8,71E-03 a	8,15E-03 a	8,71E-03 a	8,15E-03 a
	HbERF-VI5	2,08E+00 a	1,30E+00 a	2,08E+00 a	1,30E+00 a
	HbERF-VI-L3	7,83E-01 a	5,80E-01 a	7,83E-01 a	5,80E-01 a
	HbERF-VI-L4	9,14E-02 a	5,80E-02 a	9,14E-02 a	5,80E-02 a
	HbERF-VI-L6	4,11E-01 a	2,13E-01 a	4,11E-01 a	2,13E-01 a
HbERF-VII	HbERF-VIIa1	6,94E-01 b	1,90E-01 a	6,94E-01 b	1,90E-01 a
	HbERF-VIIa4	5,23E+00 b	9,89E-01 a	5,23E+00 b	9,89E-01 a
	HbERF-VIIa7	2,34E+01 a	3,14E+01 a	2,34E+01 a	3,14E+01 a
	HbERF-VIIa12	4,03E+01 a	3,30E+01 a	4,03E+01 a	3,30E+01 a
	HbERF-VIIa17	3,25E+01 a	1,14E+01 a	3,25E+01 a	1,14E+01 a
	HbERF-VIIa20	2,03E-01 a	1,16E-01 a	2,03E-01 a	1,16E-01 a
HbERF-VIII	HbERF-VIIIa3	2,07E+00 a	1,07E+00 a	2,07E+00 a	1,07E+00 a
	HbERF-VIIIa4	2,35E+00 a	1,35E+00 a	2,35E+00 a	1,35E+00 a
	HbERF-VIIIa5	6,46E-01 a	3,63E-01 a	6,46E-01 a	3,63E-01 a
	HbERF-VIIIa8	2,47E+00 a	7,54E+00 a	2,47E+00 a	7,54E+00 a
	HbERF-VIIIa9	2,84E+00 a	4,75E+00 a	2,84E+00 a	4,75E+00 a
	HbERF-VIIIa10	1,61E+00 a	9,13E+00 a	1,61E+00 a	9,13E+00 a
	HbERF-VIIIa12	9,72E-01 a	1,11E+00 a	9,72E-01 a	1,11E+00 a
	HbERF-VIIIa13	2,85E+01 a	8,65E+00 a	2,85E+01 a	8,65E+00 a
	HbERF-VIIIa14	5,98E-01 a	4,80E-01 a	5,98E-01 a	4,80E-01 a
	HbERF-VIIIb1	1,47E-02 a	1,16E-01 a	1,47E-02 a	1,16E-01 a
HbERF-IX	HbERF-IXa2	7,66E+00 a	5,13E+00 a	7,66E+00 a	5,13E+00 a
	HbERF-IXa3	4,88E-01 a	2,78E+00 a	4,88E-01 a	2,78E+00 a
	HbERF-IXb1	7,41E-01 a	1,39E-01 a	7,41E-01 a	1,39E-01 a
	HbERF-IXb2	3,32E-02 a	1,49E-02 a	3,32E-02 a	1,49E-02 a
	HbERF-IXb3	1,18E-01 a	6,08E-01 a	1,18E-01 a	6,08E-01 a
	HbERF-IXc1	2,85E-01 a	3,61E-01 a	2,85E-01 a	3,61E-01 a
	HbERF-IXc4	1,79E-01 a	1,54E-01 a	1,79E-01 a	1,54E-01 a
	HbERF-IXc5	3,57E-02 a	5,97E-02 a	3,57E-02 a	5,97E-02 a
	HbERF-IXc6	9,19E-03 a	4,58E-03 a	9,19E-03 a	4,58E-03 a
HbERF-X	HbERF-Xa1	5,45E-01 a	3,49E-01 a	5,45E-01 a	3,49E-01 a
	HbERF-Xa2	1,40E-01 a	7,66E-02 a	1,40E-01 a	7,66E-02 a
	HbERF-Xa4	1,59E-02 a	3,44E-02 a	1,59E-02 a	3,44E-02 a
	HbERF-Xa6	2,19E+00 a	4,95E+00 a	2,19E+00 a	4,95E+00 a
	HbERF-Xa8	7,73E-02 a	1,94E-01 a	7,73E-02 a	1,94E-01 a
	HbERF-Xb1	2,40E-03 a	1,34E-01 b	2,40E-03 a	1,34E-01 b
HbAP2	HbAP2-1	6,07E-02 a	7,28E-02 a	6,07E-02 a	7,28E-02 a
	HbAP2-3	7,21E+00 a	1,09E+01 a	7,21E+00 a	1,09E+01 a
	HbAP2-5	3,15E-01 a	2,45E-01 a	3,15E-01 a	2,45E-01 a
	HbAP2-6	6,91E+00 a	9,70E+00 a	6,91E+00 a	9,70E+00 a
	HbAP2-7	8,70E-02 a	5,71E-02 a	8,70E-02 a	5,71E-02 a
	HbAP2-8	2,41E-01 a	2,65E-01 a	2,41E-01 a	2,65E-01 a
	HbAP2-9	1,49E+00 a	4,93E+00 a	1,49E+00 a	4,93E+00 a
	HbAP2-10	1,09E+00 a	2,74E+00 a	1,09E+00 a	2,74E+00 a
	HbAP2-11	2,11E-01 a	1,20E-01 a	2,11E-01 a	1,20E-01 a
	HbAP2-12	3,37E-02 a	2,35E-02 a	3,37E-02 a	2,35E-02 a
	HbAP2-13	1,37E+00 a	9,83E-01 a	1,37E+00 a	9,83E-01 a
	HbAP2-15	3,09E-01 a	5,14E-01 a	3,09E-01 a	5,14E-01 a
	HbAP2-16	1,21E-01 a	3,34E-01 a	1,21E-01 a	3,34E-01 a
	HbAP2-17	3,99E-03 a	1,05E-02 a	3,99E-03 a	1,05E-02 a
	HbAP2-18	2,65E+00 a	4,04E+00 a	2,65E+00 a	4,04E+00 a
HbRAV	HbRAV-2	6,30E-03 a	6,30E-03 a	6,30E-03 a	6,30E-03 a
	HbRAV-3	1,47E-02 a	1,00E-02 a	1,47E-02 a	1,00E-02 a
	HbRAV-4	7,74E-02 a	1,02E-01 a	7,74E-02 a	1,02E-01 a

Figure 5 Expression profile of 94 genes involved in the ethylene biosynthesis and signalling pathways for normal and abnormal embryos from regenerant line (CI07060) callus on embryo development medium (DEV). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 5 **A**) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green . (Figure 5 **B**) Values of relative transcript abundance in normal and abnormal embryos were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

Gene		A						B					
		Regenerant callus line		Embryo	Plantlet			Regenerant callus line		Embryo	Plantlet		
		ENT	EXP	Normal	LEAF	STEM	ROOT	ENT	EXP	Normal	LEAF	STEM	ROOT
Ethylene biosynthetic & transduction pathways	HbSAMS	8,55E-03^{a}	1,14E-01^{b}	1,75E-01^{b}	1,19E-01^{b}	4,93E-02^{b}	4,88E-01^{b}	8,55E-03^{a}	1,14E-01^{b}	1,75E-01^{b}	1,19E-01^{b}	4,93E-02^{b}	4,88E-01^{b}
	HbACS1	3,03E-02^{a}	3,63E-02^{a}	1,37E-01^{a}	3,82E-02^{a}	1,55E-02^{a}	2,69E-01^{a}	3,03E-02^{a}	3,63E-02^{a}	1,37E-01^{a}	3,82E-02^{a}	1,55E-02^{a}	2,69E-01^{a}
	HbACS2	7,36E-03^{a}	1,33E-02^{a}	1,15E-01^{a}	1,62E-02^{a}	1,04E-03^{a}	2,98E-02^{a}	7,36E-03^{a}	1,33E-02^{a}	1,15E-01^{a}	1,62E-02^{a}	1,04E-03^{a}	2,98E-02^{a}
	HbACS3	2,94E+00^{b}	2,93E+00^{b}	2,94E-01^{ab}	2,66E-02^{a}	1,10E-02^{a}	2,85E-01^{ab}	2,94E+00^{b}	2,93E+00^{b}	2,94E-01^{ab}	2,66E-02^{a}	1,10E-02^{a}	2,85E-01^{ab}
	HbACO1	3,38E-03^{a}	6,13E-02^{b}	4,37E-01^{b}	3,17E-01^{b}	2,69E+00^{c}	5,60E+00^{c}	3,38E-03^{a}	6,13E-02^{b}	4,37E-01^{b}	3,17E-01^{b}	2,69E+00^{c}	5,60E+00^{c}
	HbACO2	1,69E-01^{a}	2,00E-01^{a}	5,01E+00^{a}	4,70E-01^{a}	1,42E+00^{a}	5,58E+00^{a}	1,69E-01^{a}	2,00E-01^{a}	5,01E+00^{a}	4,70E-01^{a}	1,42E+00^{a}	5,58E+00^{a}
	HbACO3	7,42E-03^{a}	9,45E-03^{a}	1,17E-01^{ab}	1,71E+00^{b}	8,95E-03^{ab}	4,80E-02^{ab}	7,42E-03^{a}	9,45E-03^{a}	1,17E-01^{ab}	1,71E+00^{b}	8,95E-03^{ab}	4,80E-02^{ab}
	HbETR1	1,80E-01^{a}	7,88E-01^{a}	1,47E+00^{a}	1,06E+00^{a}	4,67E-01^{a}	9,20E-01^{a}	1,80E-01^{a}	7,88E-01^{a}	1,47E+00^{a}	1,06E+00^{a}	4,67E-01^{a}	9,20E-01^{a}
	HbETR2	1,94E+00^{b}	2,13E+00^{b}	1,93E+00^{b}	1,80E-01^{a}	3,55E-01^{ab}	3,10E+00^{b}	1,94E+00^{b}	2,13E+00^{b}	1,93E+00^{b}	1,80E-01^{a}	3,55E-01^{ab}	3,10E+00^{b}
	HbEIN3	1,78E-01^{a}	1,14E+00^{b}	6,78E-01^{b}	1,53E+00^{b}	1,11E+00^{b}	3,46E+00^{b}	1,78E-01^{a}	1,14E+00^{b}	6,78E-01^{b}	1,53E+00^{b}	1,11E+00^{b}	3,46E+00^{b}
HbERF-I	HbERF-Ia1	2,84E-03^{a}	9,98E-03^{ab}	2,88E-03^{a}	1,26E-02^{b}	9,48E-03^{ab}	1,32E-02^{b}	2,84E-03^{a}	9,98E-03^{ab}	2,88E-03^{a}	1,26E-02^{b}	9,48E-03^{ab}	1,32E-02^{b}
	HbERF-Ib2	4,16E-01^{ab}	3,13E+00^{b}	1,33E-01^{a}	1,75E+00^{b}	1,04E+00^{ab}	1,06E+00^{ab}	4,16E-01^{ab}	3,13E+00^{b}	1,33E-01^{a}	1,75E+00^{b}	1,04E+00^{ab}	1,06E+00^{ab}
	HbERF-Ib4	3,71E-01^{a}	2,73E+00^{a}	2,88E-01^{a}	1,47E+00^{a}	3,80E-01^{a}	7,75E-01^{a}	3,71E-01^{a}	2,73E+00^{a}	2,88E-01^{a}	1,47E+00^{a}	3,80E-01^{a}	7,75E-01^{a}
	HbERF-Ib5	1,82E+00^{b}	3,68E+00^{b}	1,31E+00^{b}	3,76E-01^{a}	2,56E-01^{a}	1,92E-01^{a}	1,82E+00^{b}	3,68E+00^{b}	1,31E+00^{b}	3,76E-01^{a}	2,56E-01^{a}	1,92E-01^{a}
	HbERF-Ib7	1,85E+00^{a}	4,21E+00^{a}	2,36E+00^{a}	1,60E+00^{a}	9,93E-01^{a}	8,50E-01^{a}	1,85E+00^{a}	4,21E+00^{a}	2,36E+00^{a}	1,60E+00^{a}	9,93E-01^{a}	8,50E-01^{a}
	HbERF-Ib9	3,41E+00^{a}	5,88E+00^{a}	1,03E+01^{a}	8,52E-01^{a}	4,69E-01^{a}	4,76E-01^{a}	3,41E+00^{a}	5,88E+00^{a}	1,03E+01^{a}	8,52E-01^{a}	4,69E-01^{a}	4,76E-01^{a}
	HbERF-Ib11	2,32E-02^{ab}	8,19E-03^{ab}	6,80E-02^{b}	9,94E-03^{ab}	6,00E-03^{a}	4,76E-02^{ab}	2,32E-02^{ab}	8,19E-03^{ab}	6,80E-02^{b}	9,94E-03^{ab}	6,00E-03^{a}	4,76E-02^{ab}
HbERF-II	HbERF-IIa1	4,55E-03^{a}	6,08E-02^{a}	3,70E-03^{a}	6,17E-02^{a}	8,57E-03^{a}	9,02E-03^{a}	4,55E-03^{a}	6,08E-02^{a}	3,70E-03^{a}	6,17E-02^{a}	8,57E-03^{a}	9,02E-03^{a}
	HbERF-IIa3	3,17E+00^{b}	9,35E+00^{b}	4,87E+00^{b}	2,70E-01^{a}	2,20E-01^{a}	2,04E-01^{a}	3,17E+00^{b}	9,35E+00^{b}	4,87E+00^{b}	2,70E-01^{a}	2,20E-01^{a}	2,04E-01^{a}
	HbERF-IIb1	1,44E-01^{a}	4,03E-01^{a}	3,20E-01^{a}	5,20E-02^{a}	8,72E-02^{a}	4,18E-02^{a}	1,44E-01^{a}	4,03E-01^{a}	3,20E-01^{a}	5,20E-02^{a}	8,72E-02^{a}	4,18E-02^{a}
	HbERF-IIb2	5,90E-02^{a}	5,36E-01^{a}	3,62E-02^{a}	1,76E-02^{a}	5,72E-03^{a}	3,69E-03^{a}	5,90E-02^{a}	5,36E-01^{a}	3,62E-02^{a}	1,76E-02^{a}	5,72E-03^{a}	3,69E-03^{a}
	HbERF-IIb4	3,03E-03^{a}	5,33E-03^{a}	1,32E-02^{a}	7,54E-03^{a}	6,01E-03^{a}	2,03E-03^{a}	3,03E-03^{a}	5,33E-03^{a}	1,32E-02^{a}	7,54E-03^{a}	6,01E-03^{a}	2,03E-03^{a}
HbERF-III	HbERF-IIIa1	1,22E+00^{b}	1,30E+00^{b}	3,72E+00^{b}	1,68E-01^{a}	1,16E-01^{a}	9,00E-02^{a}	1,22E+00^{b}	1,30E+00^{b}	3,72E+00^{b}	1,68E-01^{a}	1,16E-01^{a}	9,00E-02^{a}
	HbERF-IIIb1	8,16E-03^{a}	1,63E-02^{a}	3,14E-02^{a}	3,95E-03^{a}	4,99E-03^{a}	7,81E-03^{a}	8,16E-03^{a}	1,63E-02^{a}	3,14E-02^{a}	3,95E-03^{a}	4,99E-03^{a}	7,81E-03^{a}
	HbERF-IIIb2	5,66E-02^{a}	6,83E-02^{a}	1,04E-02^{a}	3,47E-02^{a}	1,25E-02^{a}	1,13E-02^{a}	5,66E-02^{a}	6,83E-02^{a}	1,04E-02^{a}	3,47E-02^{a}	1,25E-02^{a}	1,13E-02^{a}
	HbERF-IIIc1	6,87E-01^{ab}	1,16E+00^{b}	1,20E+00^{ab}	1,47E-01^{ab}	9,17E-02^{a}	1,29E-01^{ab}	6,87E-01^{ab}	1,16E+00^{b}	1,20E+00^{ab}	1,47E-01^{ab}	9,17E-02^{a}	1,29E-01^{ab}
	HbERF-IIId2	2,06E-02^{a}	5,03E-01^{b}	1,76E-02^{a}	3,41E-01^{b}	7,53E-02^{ab}	1,40E-01^{ab}	2,06E-02^{a}	5,03E-01^{b}	1,76E-02^{a}	3,41E-01^{b}	7,53E-02^{ab}	1,40E-01^{ab}
	HbERF-IIId3	3,24E-02^{a}	5,74E-03^{a}	8,06E-03^{a}	1,55E-02^{a}	1,95E-02^{a}	1,06E-02^{a}	3,24E-02^{a}	5,74E-03^{a}	8,06E-03^{a}	1,55E-02^{a}	1,95E-02^{a}	1,06E-02^{a}
	HbERF-IIIe1	8,23E-03^{ab}	1,72E-02^{a}	3,71E-01^{bc}	1,49E+00^{c}	1,24E+00^{bc}	4,34E-01^{bc}	8,23E-03^{ab}	1,72E-02^{a}	3,71E-01^{bc}	1,49E+00^{c}	1,24E+00^{bc}	4,34E-01^{bc}
	HbERF-IIIe2	2,57E-02^{a}	3,01E-01^{bc}	1,44E+00^{c}	1,33E-01^{b}	2,59E-01^{bc}	3,43E-01^{bc}	2,57E-02^{a}	3,01E-01^{bc}	1,44E+00^{c}	1,33E-01^{b}	2,59E-01^{bc}	3,43E-01^{bc}
	HbERF-IIIe3	9,68E-03^{a}	1,06E-01^{a}	1,08E-01^{a}	1,27E-02^{a}	6,43E-03^{a}	1,31E-02^{a}	9,68E-03^{a}	1,06E-01^{a}	1,08E-01^{a}	1,27E-02^{a}	6,43E-03^{a}	1,31E-02^{a}
	HbERF-IIIe4	9,89E-02^{a}	1,03E-01^{a}	8,34E-02^{a}	1,27E-01^{a}	2,29E-01^{a}	8,19E-02^{a}	9,89E-02^{a}	1,03E-01^{a}	8,34E-02^{a}	1,27E-01^{a}	2,29E-01^{a}	8,19E-02^{a}
HbERF-IV	HbERF-IVa2	3,46E-01^{a}	1,00E+00^{a}	1,25E+00^{a}	7,61E-01^{a}	3,53E-01^{a}	2,84E-01^{a}	3,46E-01^{a}	1,00E+00^{a}	1,25E+00^{a}	7,61E-01^{a}	3,53E-01^{a}	2,84E-01^{a}
HbERF-V	HbERF-Va2	1,31E+00^{a}	3,78E-01^{a}	4,55E-01^{a}	7,69E-02^{a}	1,93E-01^{a}	3,22E-01^{a}	1,31E+00^{a}	3,78E-01^{a}	4,55E-01^{a}	7,69E-02^{a}	1,93E-01^{a}	3,22E-01^{a}
	HbERF-Vb1	1,26E-02^{ab}	3,19E-02^{b}	9,39E-03^{ab}	4,55E-02^{b}	3,95E-03^{a}	2,32E-02^{b}	1,26E-02^{ab}	3,19E-02^{b}	9,39E-03^{ab}	4,55E-02^{b}	3,95E-03^{a}	2,32E-02^{b}
	HbERF-Vb2	7,89E-02^{b}	4,34E-01^{c}	3,37E-02^{b}	2,74E-03^{a}	2,87E-03^{a}	6,53E-03^{a}	7,89E-02^{b}	4,34E-01^{c}	3,37E-02^{b}	2,74E-03^{a}	2,87E-03^{a}	6,53E-03^{a}
	HbERF-Vb3	1,40E-02^{a}	3,47E-01^{b}	2,44E-02^{a}	1,33E-02^{a}	7,92E-03^{a}	1,31E-02^{a}	1,40E-02^{a}	3,47E-01^{b}	2,44E-02^{a}	1,33E-02^{a}	7,92E-03^{a}	1,31E-02^{a}
HbERF-VI	HbERF-VI1	9,59E-02^{a}	6,09E-02^{a}	3,12E-01^{b}	1,26E-01^{ab}	1,15E-01^{ab}	6,45E-02^{a}	9,59E-02^{a}	6,09E-02^{a}	3,12E-01^{b}	1,26E-01^{ab}	1,15E-01^{ab}	6,45E-02^{a}
	HbERF-VI2	1,10E-02^{a}	1,30E-01^{c}	2,73E-02^{b}	3,45E-02^{b}	1,66E-02^{ab}	4,26E-02^{b}	1,10E-02^{a}	1,30E-01^{c}	2,73E-02^{b}	3,45E-02^{b}	1,66E-02^{ab}	4,26E-02^{b}
	HbERF-VI3	5,09E-02^{a}	1,88E-01^{a}	4,10E-01^{a}	4,21E-02^{a}	9,92E-02^{a}	1,28E-01^{a}	5,09E-02^{a}	1,88E-01^{a}	4,10E-01^{a}	4,21E-02^{a}	9,92E-02^{a}	1,28E-01^{a}
	HbERF-VI4	4,49E-03^{a}	3,60E-02^{b}	8,71E-03^{a}	7,32E-03^{a}	9,51E-03^{ab}	1,00E-02^{ab}	4,49E-03^{a}	3,60E-02^{b}	8,71E-03^{a}	7,32E-03^{a}	9,51E-03^{ab}	1,00E-02^{ab}
	HbERF-VI5	2,57E-01^{a}	7,88E-01^{ab}	2,08E+00^{b}	7,71E-01^{ab}	4,34E-01^{ab}	6,27E-01^{ab}	2,57E-01^{a}	7,88E-01^{ab}	2,08E+00^{b}	7,71E-01^{ab}	4,34E-01^{ab}	6,27E-01^{ab}
	HbERF-VI-L3	6,02E-01^{a}	1,75E+00^{a}	7,83E-01^{a}	1,02E+00^{a}	6,27E-01^{a}	6,09E-01^{a}	6,02E-01^{a}	1,75E+00^{a}	7,83E-01^{a}	1,02E+00^{a}	6,27E-01^{a}	6,09E-01^{a}
	HbERF-VI-L4	1,94E-01^{a}	4,51E-01^{a}	9,14E-02^{a}	7,50E-02^{a}	3,91E-02^{a}	4,38E-02^{a}	1,94E-01^{a}	4,51E-01^{a}	9,14E-02^{a}	7,50E-02^{a}	3,91E-02^{a}	4,38E-02^{a}
	HbERF-VI-L6	4,66E-01^{bc}	1,37E+00^{c}	4,11E-01^{bc}	3,10E-02^{a}	7,85E-02^{ab}	5,97E-02^{ab}	4,66E-01^{bc}	1,37E+00^{c}	4,11E-01^{bc}	3,10E-02^{a}	7,85E-02^{ab}	5,97E-02^{ab}
HbERF-VII	HbERF-VIIa1	3,22E+00^{b}	3,23E-01^{b}	6,94E-01^{b}	3,40E-02^{a}	2,01E-02^{a}	9,19E-02^{ab}	3,22E+00^{b}	3,23E-01^{b}	6,94E-01^{b}	3,40E-02^{a}	2,01E-02^{a}	9,19E-02^{ab}
	HbERF-VIIa4	3,03E-01^{a}	9,23E-01^{a}	5,23E+00^{b}	4,24E-01^{a}	3,78E-01^{a}	7,77E-01^{a}	3,03E-01^{a}	9,23E-01^{a}	5,23E+00^{b}	4,24E-01^{a}	3,78E-01^{a}	7,77E-01^{a}
	HbERF-VIIa7	2,02E+01^{ab}	6,49E+01^{b}	2,34E+01^{ab}	5,03E+00^{a}	1,37E+01^{ab}	1,09E+01^{ab}	2,02E+01^{ab}	6,49E+01^{b}	2,34E+01^{ab}	5,03E+00^{a}	1,37E+01^{ab}	1,09E+01^{ab}
	HbERF-VIIa12	4,19E+01^{ab}	1,14E+02^{b}	4,03E+01^{ab}	8,39E+00^{a}	2,09E+01^{ab}	1,93E+01^{ab}	4,19E+01^{ab}	1,14E+02^{b}	4,03E+01^{ab}	8,39E+00^{a}	2,09E+01^{ab}	1,93E+01^{ab}
	HbERF-VIIa17	1,20E+00^{a}	2,64E+00^{a}	3,25E+01^{b}	5,55E-01^{a}	3,04E-01^{a}	4,78E-01^{a}	1,20E+00^{a}	2,64E+00^{a}	3,25E+01^{b}	5,55E-01^{a}	3,04E-01^{a}	4,78E-01^{a}
	HbERF-VIIa20	2,88E-02^{a}	5,46E-02^{a}	2,03E-01^{a}	4,23E-02^{a}	5,71E-02^{a}	1,73E-01^{a}	2,88E-02^{a}	5,46E-02^{a}	2,03E-01^{a}	4,23E-02^{a}	5,71E-02^{a}	1,73E-01^{a}
HbERF-VIII	HbERF-VIIIa3	1,70E-01^{a}	6,81E-01^{b}	2,07E+00^{c}	7,60E-01^{b}	1,46E+00^{bc}	7,27E-01^{b}	1,70E-01^{a}	6,81E-01^{b}	2,07E+00^{c}	7,60E-01^{b}	1,46E+00^{bc}	7,27E-01^{b}
	HbERF-VIIIa4	8,75E-01^{a}	2,27E+00^{a}	2,35E+00^{a}	9,82E-01^{a}	1,89E+00^{a}	8,66E-01^{a}	8,75E-01^{a}	2,27E+00^{a}	2,35E+00^{a}	9,82E-01^{a}	1,89E+00^{a}	8,66E-01^{a}
	HbERF-VIIIa5	4,42E-01^{a}	8,52E-01^{a}	6,46E-01^{a}	1,20E+00^{a}	1,15E+00^{a}	6,92E-01^{a}	4,42E-01^{a}	8,52E-01^{a}	6,46E-01^{a}	1,20E+00^{a}	1,15E+00^{a}	6,92E-01^{a}
	HbERF-VIIIa8	4,76E+00^{a}	4,74E+00^{a}	2,47E+00^{a}	1,01E+00^{a}	1,46E+00^{a}	2,08E+00^{a}	4,76E+00^{a}	4,74E+00^{a}	2,47E+00^{a}	1,01E+00^{a}	1,46E+00^{a}	2,08E+00^{a}
	HbERF-VIIIa9	4,55E+00^{a}	6,19E+00^{a}	2,84E+00^{a}	1,33E+00^{a}	2,06E+00^{a}	2,67E+00^{a}	4,55E+00^{a}	6,19E+00^{a}	2,84E+00^{a}	1,33E+00^{a}	2,06E+00^{a}	2,67E+00^{a}
	HbERF-VIIIa10	1,24E+00^{a}	3,60E+00^{a}	1,61E+00^{a}	9,91E-01^{a}	7,12E-01^{a}	1,24E+00^{a}	1,24E+00^{a}	3,60E+00^{a}	1,61E+00^{a}	9,91E-01^{a}	7,12E-01^{a}	1,24E+00^{a}
	HbERF-VIIIa12	2,35E-01^{a}	6,06E-01^{a}	9,72E-01^{a}	4,50E-01^{a}	5,35E-01^{a}	2,99E-01^{a}	2,35E-01^{a}	6,06E-01^{a}	9,72E-01^{a}	4,50E-01^{a}	5,35E-01^{a}	2,99E-01^{a}
	HbERF-VIIIa13	1,15E+00^{a}	3,40E+00^{a}	2,85E+01^{a}	8,93E-02^{a}	7,41E-01^{a}	2,58E-01^{a}	1,15E+00^{a}	3,40E+00^{a}	2,85E+01^{a}	8,93E-02^{a}	7,41E-01^{a}	2,58E-01^{a}
	HbERF-VIIIa14	6,68E-01^{c}	5,03E-01^{c}	5,98E-01^{c}	1,78E-02^{a}	7,11E-02^{b}	6,26E-02^{b}	6,68E-01^{c}	5,03E-01^{c}	5,98E-01^{c}	1,78E-02^{a}	7,11E-02^{b}	6,26E-02^{b}
	HbERF-VIIIb1	6,66E-01^{b}	7,67E-01^{b}	1,47E-02^{a}	4,75E-03^{a}	5,78E-03^{a}	6,24E-04^{a}	6,66E-01^{b}	7,67E-01^{b}	1,47E-02^{a}	4,75E-03^{a}	5,78E-03^{a}	6,24E-04^{a}
HbERF-IX	HbERF-IXa2	1,92E+00^{a}	2,05E+00^{a}	7,66E+00^{a}	5,68E-02^{a}	8,71E-02^{a}	2,39E-01^{a}	1,92E+00^{a}	2,05E+00^{a}	7,66E+00^{a}	5,68E-02^{a}	8,71E-02^{a}	2,39E-01^{a}
	HbERF-IXa3	8,69E-01^{b}	9,03E-01^{b}	4,88E-01^{b}	1,85E-02^{a}	3,22E-02^{a}	9,04E-02^{ab}	8,69E-01^{b}	9,03E-01^{b}	4,88E-01^{b}	1,85E-02^{a}	3,22E-02^{a}	9,04E-02^{ab}
	HbERF-IXb1	3,23E-01^{ab}	1,93E-01^{a}	7,41E-01^{ab}	2,47E+00^{b}	5,78E-01^{ab}	5,52E-02^{a}	3,23E-01^{ab}	1,93E-01^{a}	7,41E-01^{ab}	2,47E+00^{b}	5,78E-01^{ab}	5,52E-02^{a}
	HbERF-IXb2	4,08E-03^{a}	1,48E-02^{b}	3,32E-02^{b}	1,72E+00^{d}	2,08E-01^{c}	7,12E-02^{bc}	4,08E-03^{a}	1,48E-02^{b}	3,32E-02^{b}	1,72E+00^{d}	2,08E-01^{c}	7,12E-02^{bc}
	HbERF-IXb3	7,87E-02^{a}	1,32E-01^{a}	1,18E-01^{a}	9,96E-01^{b}	3,64E-01^{ab}	2,37E-01^{ab}	7,87E-02^{a}	1,32E-01^{a}	1,18E-01^{a}	9,96E-01^{b}	3,64E-01^{ab}	2,37E-01^{ab}
	HbERF-IXc1	1,76E-01^{a}	1,28E-01^{a}	2,85E-01^{a}	4,11E-02^{a}	4,19E-02^{a}	5,74E-02^{a}	1,76E-01^{a}	1,28E-01^{a}	2,85E-01^{a}	4,11E-02^{a}	4,19E-02^{a}	5,74E-02^{a}
	HbERF-IXc4	1,55E-01^{a}	6,00E-01^{a}	1,79E-01^{a}	1,17E-01^{a}	2,85E-02^{a}	9,12E-02^{a}	1,55E-01^{a}	6,00E-01^{a}	1,79E-01^{a}	1,17E-01^{a}	2,85E-02^{a}	9,12E-02^{a}
	HbERF-IXc5	3,46E-02^{a}	5,81E-02^{a}	3,57E-02^{a}	6,06E-02^{a}	9,90E-03^{a}	1,34E-02^{a}	3,46E-02^{a}	5,81E-02^{a}	3,57E-02^{a}	6,06E-02^{a}	9,90E-03^{a}	1,34E-02^{a}
	HbERF-IXc6	2,85E-03^{a}	9,01E-03^{ab}	9,19E-03^{ab}	4,99E-02^{b}	9,04E-03^{ab}	7,64E-03^{ab}	2,85E-03^{a}	9,01E-03^{ab}	9,19E-03^{ab}	4,99E-02^{b}	9,04E-03^{ab}	7,64E-03^{ab}
HbERF-X	HbERF-Xa1	1,25E+00^{b}	1,78E+00^{b}	5,45E-01^{ab}	1,17E-01^{a}	2,25E-01^{ab}	4,43E-01^{ab}	1,25E+00^{b}	1,78E+00^{b}	5,45E-01^{ab}	1,17E-01^{a}	2,25E-01^{ab}	4,43E-01^{ab}
	HbERF-Xa2	1,83E-01^{a}	8,44E-02^{a}	1,40E-01^{a}	5,00E-02^{a}	4,15E-02^{a}	3,41E-02	1,83E-01^{a}	8,44E-02^{a}	1,40E-01^{a}	5,00E-02^{a}	4,15E-02^{a}	3,41E-02
	HbERF-Xa4	3,30E-02^{ab}	1,38E-01^{b}	1,59E-02^{ab}	6,01E-03^{a}	1,45E-02^{ab}	1,84E-02^{ab}	3,30E-02^{ab}	1,38E-01^{b}	1,59E-02^{ab}	6,01E-03^{a}	1,45E-02^{ab}	1,84E-02^{ab}
	HbERF-Xa6	4,36E-01^{ab}	1,05E-01^{a}	2,19E+00^{b}	4,08E-01^{ab}	1,92E-01^{ab}	2,24E-01^{ab}	4,36E-01^{ab}	1,05E-01^{a}	2,19E+00^{b}	4,08E-01^{ab}	1,92E-01^{ab}	2,24E-01^{ab}
	HbERF-Xa8	4,94E-02^{a}	8,51E-02^{ab}	7,73E-02^{ab}	1,03E+00^{c}	3,17E-01^{bc}	1,98E-02^{a}	4,94E-02^{a}	8,51E-02^{ab}	7,73E-02^{ab}	1,03E+00^{c}	3,17E-01^{bc}	1,98E-02^{a}
	HbERF-Xb1	9,74E-03^{a}	9,51E-03^{a}	2,40E-03^{a}	5,66E-03^{a}	2,79E-03^{a}	1,60E-03^{a}	9,74E-03^{a}	9,51E-03^{a}	2,40E-03^{a}	5,66E-03^{a}	2,79E-03^{a}	1,60E-03^{a}
HbAP2	HbAP2-1	5,02E-02^{b}	6,40E-01^{c}	6,07E-02^{b}	1,66E-04^{a}	4,65E-04^{a}	1,19E-02^{b}	5,02E-02^{b}	6,40E-01^{c}	6,07E-02^{b}	1,66E-04^{a}	4,65E-04^{a}	1,19E-02^{b}
	HbAP2-3	1,08E+01^{c}	1,09E+01^{c}	7,21E+00^{c}	5,30E-02^{a}	2,08E-01^{b}	6,74E-01^{b}	1,08E+01^{c}	1,09E+01^{c}	7,21E+00^{c}	5,30E-02^{a}	2,08E-01^{b}	6,74E-01^{b}
	HbAP2-5	5,32E-02^{a}	8,74E-01^{b}	3,15E-01^{b}	6,21E-01^{b}	9,20E-01^{b}	5,37E-01^{b}	5,32E-02^{a}	8,74E-01^{b}	3,15E-01^{b}	6,21E-01^{b}	9,20E-01^{b}	5,37E-01^{b}
	HbAP2-6	9,15E-02^{a}	3,20E-01^{a}	6,91E+00^{b}	1,95E-01^{a}	2,47E-01^{a}	5,21E-01^{a}	9,15E-02^{a}	3,20E-01^{a}	6,91E+00^{b}	1,95E-01^{a}	2,47E-01^{a}	5,21E-01^{a}
	HbAP2-7	2,99E-02^{a}	3,84E-02^{a}	8,70E-02^{a}	1,90E-01^{a}	3,54E-01^{a}	8,18E-02^{a}	2,99E-02^{a}	3,84E-02^{a}	8,70E-02^{a}	1,90E-01^{a}	3,54E-01^{a}	8,18E-02^{a}
	HbAP2-8	7,94E-02^{a}	4,10E-01^{a}	2,41E-01^{a}	2,46E-01^{a}	8,66E-01^{a}	3,12E-01^{a}	7,94E-02^{a}	4,10E-01^{a}	2,41E-01^{a}	2,46E-01^{a}	8,66E-01^{a}	3,12E-01^{a}
	HbAP2-9	1,03E+01^{a}	6,79E+00^{a}	1,49E+00^{a}	1,09E+00^{a}	8,85E-01^{a}	1,33E+00^{a}	1,03E+01^{a}	6,79E+00^{a}	1,49E+00^{a}	1,09E+00^{a}	8,85E-01^{a}	1,33E+00^{a}
	HbAP2-10	3,55E-02^{a}	1,31E-02^{a}	1,09E+00^{b}	4,49E+00^{c}	1,76E+00^{bc}	6,58E-01^{b}	3,55E-02^{a}	1,31E-02^{a}	1,09E+00^{b}	4,49E+00^{c}	1,76E+00^{bc}	6,58E-01^{b}
	HbAP2-11	1,58E-02^{a}	8,10E-02^{b}	2,11E-01^{b}	9,55E-02^{b}	8,67E-02^{b}	6,10E-02^{b}	1,58E-02^{a}	8,10E-02^{b}	2,11E-01^{b}	9,55E-02^{b}	8,67E-02^{b}	6,10E-02^{b}
	HbAP2-12	1,44E-02^{a}	1,57E-01^{c}	3,37E-02^{ab}	5,95E-02^{bc}	1,07E-01^{bc}	5,66E-02^{abc}	1,44E-02^{a}	1,57E-01^{c}	3,37E-02^{ab}	5,95E-02^{bc}	1,07E-01^{bc}	5,66E-02^{abc}
	HbAP2-13	1,00E-02^{a}	3,88E-01^{b}	1,37E+00^{b}	8,73E-02^{b}	3,03E-01^{b}	4,16E-01^{b}	1,00E-02^{a}	3,88E-01^{b}	1,37E+00^{b}	8,73E-02^{b}	3,03E-01^{b}	4,16E-01^{b}
	HbAP2-15	1,20E-01^{a}	2,68E-01^{ab}	3,09E-01^{abc}	7,11E-01^{bc}	1,54E+00^{c}	4,38E-01^{abc}	1,20E-01^{a}	2,68E-01^{ab}	3,09E-01^{abc}	7,11E-01^{bc}	1,54E+00^{c}	4,38E-01^{abc}
	HbAP2-16	1,31E+00^{b}	1,70E+00^{b}	1,21E-01^{a}	3,49E+00^{b}	1,24E+00^{ab}	4,34E-01^{b}	1,31E+00^{b}	1,70E+00^{b}	1,21E-01^{a}	3,49E+00^{b}	1,24E+00^{ab}	4,34E-01^{b}
	HbAP2-17	1,29E-03^{a}	5,80E-03^{a}	3,99E-03^{a}	1,20E-02^{a}	4,22E-03^{a}	4,30E-03^{a}	1,29E-03^{a}	5,80E-03^{a}	3,99E-03^{a}	1,20E-02^{a}	4,22E-03^{a}	4,30E-03^{a}
	HbAP2-18	1,74E+00^{a}	1,62E+01^{c}	2,65E+00^{ab}	5,85E+00^{abc}	9,34E+00^{bc}	6,64E+00^{abc}	1,74E+00^{a}	1,62E+01^{c}	2,65E+00^{ab}	5,85E+00^{abc}	9,34E+00^{bc}	6,64E+00^{abc}
HbRAV	HbRAV-2	1,79E-02^{a}	1,52E-02^{a}	6,30E-03^{a}	3,01E-03^{a}	6,10E-03^{a}	2,39E-03^{a}	1,79E-02^{a}	1,52E-02^{a}	6,30E-03^{a}	3,01E-03^{a}	6,10E-03^{a}	2,39E-03^{a}
	HbRAV-3	1,01E-03^{ab}	1,36E-02^{b}	1,47E-02^{b}	3,52E-03^{b}	1,29E-03^{ab}	2,03E-04^{a}	1,01E-03^{ab}	1,36E-02^{b}	1,47E-02^{b}	3,52E-03^{b}	1,29E-03^{ab}	2,03E-04^{a}
	HbRAV-4	3,98E-02^{a}	1,04E+00^{b}	7,74E-02^{a}	8,34E-01^{b}	4,54E-01^{b}	2,72E-02^{a}	3,98E-02^{a}	1,04E+00^{b}	7,74E-02^{a}	8,34E-01^{b}	4,54E-01^{b}	2,72E-02^{a}

Figure 6 Expression profile of 94 genes involved in the ethylene biosynthesis and signalling pathways for regenerant line (CI07060) callus on embryogenesis induction medium (ENT) and somatic embryogenesis expression medium (EXP), embryos and 3 plantlet organs: (L) Leaf, (S) Stem, (R) Root. The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundance of three biological replicates. (Figure 6 A) Heat map representation of the expression profile was used for values ranging as follows ≥ 1, 10^{-1}, 10^{-2}, 10^{-3} and $\leq 10^{-4}$ from dark to light green. (Figure 6 B) Values of relative transcript abundance were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman–Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

CHAPITRE 2

Article intituled “Sequence and expression analyses of Ethylene Response Factor from group VII in *Hevea brasiliensis* suggest a regulation of low oxygen stress in latex cells”

Sequence and expression analyses of Ethylene Response Factor from group VII in *Hevea brasiliensis* suggest a regulation of low oxygen stress in latex cells

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Abstract

Background: The AP2/ERF superfamily encodes transcription factors that play a key role in plant development and responses to abiotic and biotic stress. In *Hevea brasiliensis*, *ERF* genes have been identified by RNA sequencing. This study set out to validate the number of *HbERF* genes, and identifying ERF genes involved in the regulation of latex cell metabolism.

Results: A comprehensive *Hevea* transcriptome was improved using additional RNA reads from reproductive tissues. The 30,342 contigs were annotated in the Gene Ontology database and were assigned to 3 main categories: component, molecular function and biological process. The analysis of transcription factors led to 2,448 contigs being identified, which were classed in 58 transcription factor families. AP2/ERF superfamily is the third more represented compared with other transcription factor families. Comparison with genomic scaffolds led to estimate 114 *AP2/ERF* genes in *Hevea brasiliensis*. Based on a phylogenetic analysis, functions were predicted for 26 *HbERF* genes. A relative transcript abundance analysis was performed by real-time RT-PCR in various tissues. Transcripts of ERFs from group I and VIII are very abundant in all tissues when those of group VII are highly accumulated in latex cells. Seven of the thirty-five ERF expression marker genes were highly expressed in latex. *HbERF-VII* genes showed the conserved N-terminal motif (MCGGAII) involved in the N-end rule pathway. Subcellular localization and transactivation analyses suggested that *HbERF-VII* candidate genes encoded functional transcription factors.

Conclusions: A comprehensive transcriptome for the *Hevea* clone PB 260 was completed with RNA from reproductive tissues. This transcriptome was annotated, particularly the transcription factors. Phylogenetic analyses led to identify orthologues to HbERF genes. Several tissue-specific expression marker genes revealed that ERFs might be involved in some developmental or adaptation mechanisms in *Hevea*. Sequence and transcript abundance analyses of *HbERF* group VII suggest that they were likely to be associated with the response to low oxygen in latex and could play an important role in the regulation of latex production.

Keywords: AP2/ERF, ERF, ethylene, genome, hypoxia, transcription factor, transcriptome, RNAseq, rubber

Background

Transcription factors (TFs) activate or repress the transcription of genes. The regulation of gene expression can be constitutive, tissue-specific or induced in response to environmental stimuli [1]. Plants are sessile organisms, which develop different mechanisms to protect themselves against aggressors, but also to be adapted in various environments. For that, plants are subjected to exogenous and endogenous signals, such as hormones. The gaseous plant hormone ethylene is

reported to play an active role in a wide range of developmental and adaptation processes [2-4]. Studies on ethylene signalling have revealed a linear transduction pathway that leads to the activation of transcriptional regulators belonging to the Ethylene Response Factor (ERF) type. The Ethylene Response Factor (ERF) is one of the most important families of transcription factors and plays a key role in hormone, sugar and redox signalling in a context of abiotic and biotic stress [5]. ERFs have one AP2 domain, which is involved in DNA binding. This domain is about 60 amino acid residues which recognize GCC or DRE boxes in the promoter sequence of their target genes. Based on this conserved domain, ERFs were classed in ten groups by Nakano [6] or several subclasses by Sakuma [7]. The ERF family belongs to the AP2/ERF superfamily, which has been described for several species. Among the few woody plant species studied [8-10], only one subtropical crop, *Hevea brasiliensis*, has been studied [11].

Hevea is the only commercial source of natural rubber. Natural rubber is synthesized in the cytoplasm of laticifers, which are periodically emitted from the cambium [12]. A laticifer is a cellular network created by anastomosis of latex cells, which is embedded in phloem tissues. The latex is collected by tapping the soft bark tissues. Ethephon, an ethylene releaser, is applied to the surface of the tapping panel to stimulate latex production. For that reason, ethylene biosynthetic and signalling pathways, as well as ethylene-responsive genes, have been intensively studied in *Hevea* [13-18]. Based on NGS sequencing of five tissue-type libraries (latex, bark, leaf, root, somatic embryogenic tissues), 173 AP2 domain-containing transcripts have been identified in *Hevea*, of which 142 have a full-length AP2 domain [11]. In *Hevea*, the ERF transcription factor family consists of 115 members divided into ten main groups. The three groups VII, VIII and IX account for more than 50% of HbERF. The expression of some *HbERF* genes has been associated with somatic embryogenesis [19], jasmonic acid-induced laticifer differentiation [20], and abiotic stress [21, 22]. According to Duan, the three *HbERF* genes induced upon laticifer differentiation correspond to three members of group VII (HbERF-VIIa3, HbERF-VIIa17 and HbERF-VIIa1) [11].

This paper first set out to better estimate the number of *HbAP2/ERF* genes by completing the *Hevea* clone PB 260 transcriptome by sequencing additional tissues and annotating functions, in particular transcription factors on the one hand, and comparing these RNA sequences with genomic on the other hand. A reproductive tissue-type library (immature and mature male and female flowers, zygotic embryos) was sequenced and re-assembled with reads from previously sequenced libraries (leaf, bark, latex, root, somatic embryogenesis tissues) using the same bioinformatics pipeline published by Duan [11]. Then, a gene expression analysis was performed in various tissues using real-time RT-PCR in order to identify *AP2/ERF* genes potentially involved in the regulation of latex production. Highly expressed in latex, the functionality of HbERF-VII was tested by subcellular localization and transactivation of an artificial GCC-box containing promoter. Both functional and gene expression analyses of HbERF-VIIs suggested their involvement in controlling the low oxygen response in rubber-producing cells subjected to recurrent stress and metabolic reactivation.

Results

Functional annotation and classification of a comprehensive *Hevea* transcriptome

Transcript sequences were previously produced from several tissue-type libraries (somatic embryogenic tissues, leaf, bark, latex and root) by the pyrosequencing GS-FLX 454 technique in order to have the most complete representation of the transcriptome of the *Hevea* clone PB 260 [11]. We supplemented this reference transcriptome with RNA sequences from a reproductive tissue-type library (immature and mature male and female flowers, zygotic embryos). All reads generated for these six libraries were re-assembled using the automatic pipeline described by Duan and coll. [11]. A total of 3,525,203 reads were cleaned and assembled in 86,941 contigs (Supplementary Data Table 1). From these contigs, 30,342 were then annotated in the Gene Ontology (GO) database, and 30,312 were classed using BLASTX (Supplementary Data Table 2). All contigs were categorized in 42 functional groups and classed in three ontologies: cellular component, molecular function and biological process (Figure 1). The largest number of contigs was assigned to the biological process, which contained six major subcategories (biological regulation, cellular process, establishment of localization, localization, metabolic process, and regulation of biological process). This was followed by the cellular component, which contained five subcategories (cell, cell part, macromolecular complex, organelle and organelle part). The minority category was the molecular function, which contained two major subcategories (binding and catalytic activity).

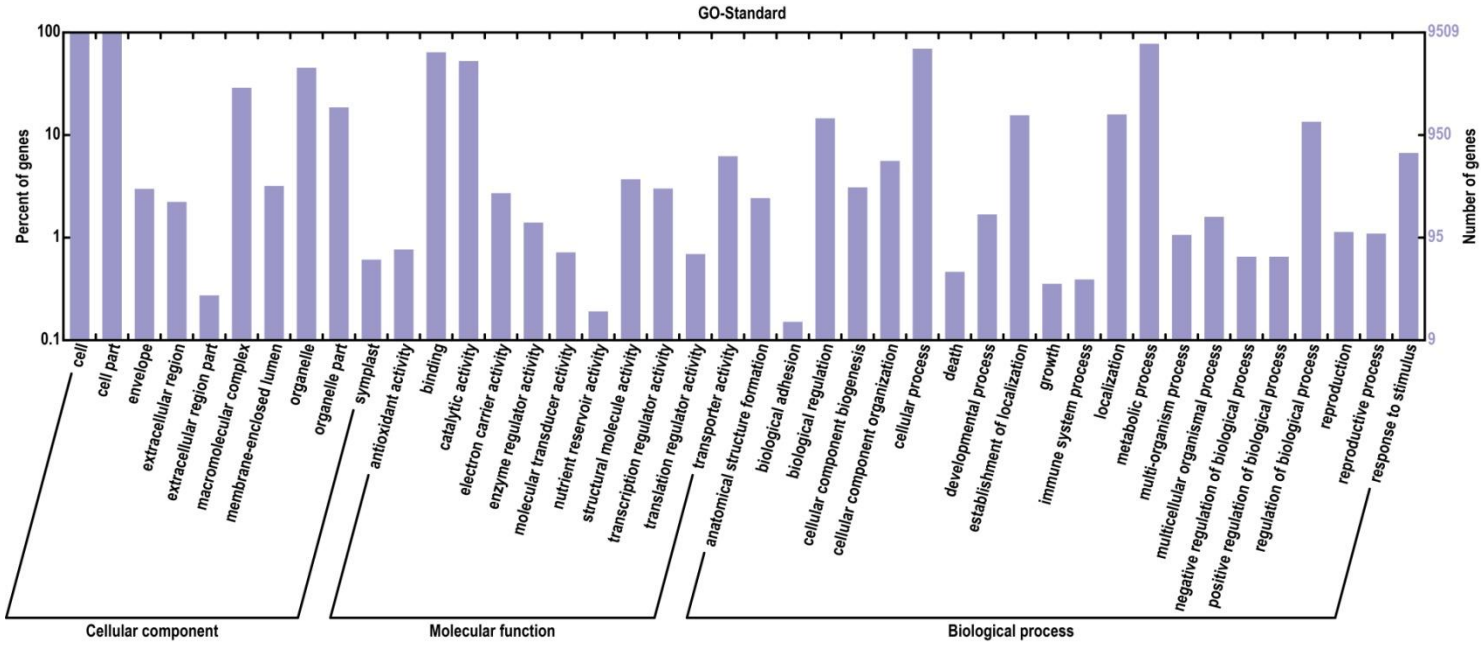


Figure 1. Gene Ontology classification of *Hevea brasiliensis* in various tissue types.

The transcription regulator activity belonging to the molecular function category accounted for 2,448 contigs classed in 58 transcription factor families (Supplementary Data Table 3). Thirteen transcription factor families counted for more than 3% of contigs (75 contigs) related to transcription factors (Figure 2). Among them, AP2/ERF, MYB, bHLH were the three largest families with at least 6% of contigs. The search of AP2 domain-containing genes in the genome database from the CATAS/BIG project showed 114 *AP2/ERF* genes found in this new transcriptome database except the soloists. This revealed a better prediction than the Duan's database counting 142 genes including 28 miss-assembled contigs [11] (Supplementary Data Table 4). Interestingly, 109, 4 and 1 of the genomic scaffolds harboured 1, 2 and 3 genes, respectively. The number of members of the *Hevea* AP2/ERF superfamily was compared with six other species (Table 1). *Hevea brasiliensis* showed the smallest number of *AP2/ERF* genes (114), followed by *Prunus persica* (131), *Arabidopsis thaliana* (147), *Vitis vinifera* (149), *Oryza sativa* (180) and *Populus trichocarpa* (202). This variation in gene number is mostly due to the number of gene from the ERF family, some ERF groups being highly duplicated such as groups II, III and IX.

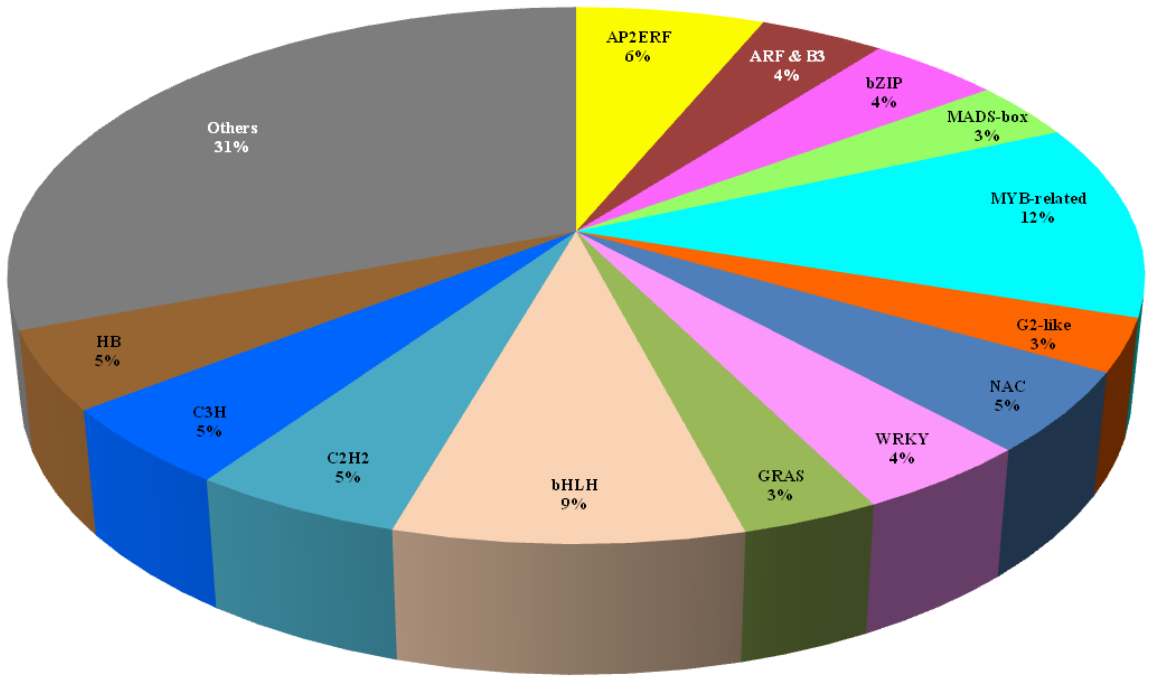


Figure 2. Transcription factor family distribution and percentage in *Hevea brasiliensis.*

Table 1. Number of AP2/ERF genes in plant species according to genomic analyses: *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum*, *Hevea brasiliensis*, *Populus trichocarpa*, *Prunus persica*, and *Vitis vinifera*. ERF family has been classed according to the Nakano's method. ND: not determined.

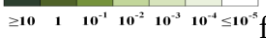
Plant species		*Arabidopsis*	*Oryza*	*Solanum*	*Hevea*	*Populus*	*Prunus*	*Vitis*
Family	Group							
ERF	I	10	9	ND	9	5	6	5
	II	15	16	ND	11	20	9	8
	III	23	27	ND	13	35	23	22
	IV	9	6	ND	5	6	7	5
	V	5	8	19	7	10	11	11
	VI	8	6	4	6	11	3	5
	VI-L	4	3	3	3	4	4	2
	VII	5	15	5	6	6	6	3
	VIII	15	15	9	11	17	10	11
	IX	17	18	28	12	42	19	40
	X	8	12	9	8	9	6	10
	Xb-L	3	10	0	0	4	0	0
	Subtotal	122	145	-	87	169	104	122
AP2		18	29	18	20	26	21	20
RAV		6	5	3	3	6	5	6
Soloist		1	1	ND	0	1	1	1
Total		147	180	-	114	202	131	149

Relative transcript abundance in various tissues

The relative transcript abundance of 66 ERFs was analysed in various tissues (both immature and mature male and female flowers, zygotic embryos, leaf, bark and latex) (Figure 3). A high relative transcript abundance (value greater than one, compared with the reference gene *HbRH2b* showed stable in all tested tissues) was observed in all tissues for ERF members from groups I (4/7 genes), II (4/5 genes), VII (5/6 genes) and VIII (6/10 genes) (Figure 3A). In any case, a high

relative transcript accumulation has been found for a few members of other ERF groups (*ERF-IIIe01*, *HbERF-VI05*, *HbERF-IXa02*, *HbERF-IXb02*, *HbERF-IXb03* and *HbERF-IXc01*).

HbERF		Relative transcript abundance																			
		A									B										
		Flower				Seed		Leaf	Bark	Latex	Flower				Embryo	Cotyledon	Leaf	Bark	Latex		
Group	Member	Im-male	M-male	Im-female	M-female	Embryo	Cotyledon				Im-male	M-male	Im-female	M-female							
I	HbERF-Ia01	5.93E-02 ab	1.12E-02 ab	2.73E-02 ab	1.65E-02 ab	1.64E-02 ab	7.39E-03 a	8.36E-02 b	1.06E-02 a	1.90E-02 ab	5.93E-02 ab	1.12E-02 ab	2.73E-02 ab	1.65E-02 ab	1.64E-02 ab	7.39E-03 a	8.36E-02 b	1.06E-02 a	1.90E-02 ab		
	HbERF-Ib02	9.74E-01 ab	1.56E-01 a	4.32E+00 b	2.58E-01 ab	1.85E-01 ab	1.65E-01 a	1.54E+00 b	7.85E-01 ab	5.46E-01 ab	9.74E-01 ab	1.56E-01 a	4.32E+00 b	2.58E-01 ab	1.85E-01 a	1.65E-01 a	1.54E+00 b	7.85E-01 ab	5.46E-01 ab		
	HbERF-Ib04	8.50E-01 cd	5.46E-02 a	8.39E-01 cd	6.77E-01 cd	2.53E-01 b	3.87E-01 bc	8.24E-01 cd	3.09E+00 d	1.75E+00 d	8.50E-01 cd	5.46E-02 a	8.39E-01 cd	6.77E-01 cd	2.53E-01 b	3.87E-01 bc	8.24E-01 cd	3.09E+00 d	1.75E+00 d		
	HbERF-Ib05	5.63E+00 f	6.98E-01 ab	1.83E+00 b	9.21E-01 abc	1.26E+00 ab	3.24E+00 bc	1.97E+00 bc	2.53E+00 bc	3.73E-01 a	5.63E+00 c	6.98E-01 ab	1.83E+00 bc	9.21E-01 abc	1.26E+00 abc	3.24E+00 bc	1.97E+00 bc	2.53E+00 bc	3.73E-01 a		
	HbERF-Ib07	3.17E+00 f	1.02E+00 ab	2.39E+00 bc	7.98E-01 ab	1.29E+00 bc	1.60E+00 bc	2.98E+00 c	2.16E+00 bc	3.86E-01 a	3.17E+00 c	1.02E+00 ab	2.39E+00 bc	7.98E-01 ab	1.29E+00 bc	1.60E+00 bc	2.98E+00 c	2.16E+00 bc	3.86E-01 a		
	HbERF-Ib09	2.60E-02 ab	3.58E-02 b	3.94E-02 ab	1.89E-02 ab	6.17E-03 ab	5.49E-03 ab	4.27E-03 ab	1.10E-02 ab	5.81E-03 a	2.60E-02 ab	3.58E-02 b	3.94E-02 ab	1.89E-02 ab	6.17E-03 ab	5.49E-03 ab	4.27E-03 ab	1.10E-02 ab	5.81E-03 a		
	HbERF-Ib11	6.77E-03 b	3.59E-02 c	4.39E-03 b	2.54E-03 b	4.64E-03 b	1.56E-03 ab	3.23E-03 b	7.97E-04 a	9.10E-05 a	6.77E-03 b	3.59E-02 c	4.39E-03 b	2.54E-03 b	4.64E-03 b	1.56E-03 ab	3.23E-03 b	7.97E-04 a	9.10E-05 a		
II	HbERF-IIa01	1.28E-02 ab	5.80E-03 ab	6.73E-03 ab	2.22E-03 a	2.89E-02 bc	1.32E-01 c	3.61E-03 a	1.17E-02 ab	1.71E-02 ab	1.28E-02 ab	5.80E-03 ab	6.73E-03 ab	2.22E-03 a	2.89E-02 bc	1.32E-01 c	3.61E-03 a	1.17E-02 ab	1.71E-02 ab		
	HbERF-IIa03	9.32E-01 b	2.43E+00 b	1.57E+00 b	8.08E-01 b	2.37E+00 b	1.46E+00 b	1.58E-01 a	1.86E+00 b	1.07E+01 c	9.32E-01 b	2.43E+00 b	1.57E+00 b	8.08E-01 b	2.37E+00 b	1.46E+00 b	1.58E-01 a	1.86E+00 b	1.07E+01 c		
	HbERF-IIb01	2.13E+00 c	1.80E-01 b	1.84E+00 c	1.73E+00 c	3.58E-02 a	3.12E-02 a	6.99E+00 d	1.03E-01 b	3.27E-02 a	2.13E+00 c	1.80E-01 b	1.84E+00 c	1.73E+00 c	3.58E-02 a	3.12E-02 a	6.99E+00 d	1.03E-01 b	3.27E-02 a		
	HbERF-IIb02	6.08E-01 d	3.64E-02 c	5.53E-01 d	5.51E-01 d	1.60E-03 a	2.61E-02 c	2.08E+00 e	1.56E-02 c	3.98E-03 b	6.08E-01 d	3.64E-02 c	5.53E-01 d	5.51E-01 d	1.60E-03 a	2.61E-02 c	2.08E+00 e	1.56E-02 c	3.98E-03 b		
	HbERF-IIb04	2.60E-03 a	2.48E-03 a	1.27E-03 a	1.11E-03 a	4.35E-03 a	4.49E-03 a	3.10E-03 a	5.59E-02 b	1.81E+00 c	2.60E-03 a	2.48E-03 a	1.27E-03 a	1.11E-03 a	4.35E-03 a	4.49E-03 a	3.10E-03 a	5.59E-02 b	1.81E+00 c		
III	HbERF-IIIa01	1.19E-02 a	1.19E-02 a	6.28E-02 a	7.77E-03 a	2.49E-02 a	1.54E-02 a	1.44E-02 a	5.53E-02 a	1.71E-02 a	1.19E-02 a	1.19E-02 a	6.28E-02 a	7.77E-03 a	2.49E-02 a	1.54E-02 a	1.44E-02 a	5.53E-02 a	1.71E-02 a		
	HbERF-IIIb01	1.88E-02 cd	5.77E-03 bc	2.03E-02 cd	1.24E-02 cd	2.52E-02 cd	1.12E-02 cd	5.24E-02 d	2.51E-03 b	5.25E-04 a	1.88E-02 cd	5.77E-03 bc	2.03E-02 cd	1.24E-02 cd	2.52E-02 cd	1.12E-02 cd	5.24E-02 d	2.51E-03 b	5.25E-04 a		
	HbERF-IIIb02	8.50E-03 abc	2.35E-03 ab	1.69E-02 bc	5.55E-03 abc	7.11E-03 abc	1.81E-03 a	8.18E-03 abc	1.94E-02 c	5.69E-03 abc	8.50E-03 abc	2.35E-03 ab	1.69E-02 bc	5.55E-03 abc	7.11E-03 abc	1.81E-03 a	8.18E-03 abc	1.94E-02 c	5.69E-03 abc		
	HbERF-IIIc01	1.23E-01 bc	1.82E-02 ab	1.27E-01 bc	2.94E-02 abc	2.55E-02 abc	1.12E-01 bc	2.04E-01 c	1.09E-02 a	1.25E-01 bc	1.23E-01 bc	1.82E-02 ab	1.27E-01 bc	2.94E-02 abc	2.55E-02 abc	1.12E-01 bc	2.04E-01 c	1.09E-02 a	1.25E-01 bc		
	HbERF-IIId01	4.70E-02 bc	3.60E-02 bc	7.17E-02 c	5.90E-02 c	9.91E-03 a	2.09E-02 ab	1.26E-01 b	6.09E-03 a	8.40E-03 a	4.70E-02 bc	3.60E-02 bc	7.17E-02 c	5.90E-02 c	9.91E-03 a	2.09E-02 ab	1.26E-01 c	6.09E-03 a	8.40E-03 a		
	HbERF-IIId03	6.71E-04 ab	1.00E-05 a	2.36E-02 c	2.22E-02 c	1.81E-03 bc	7.83E-04 ab	1.78E-02 c	2.09E-02 c	3.00E-04 ab	6.71E-04 ab	1.00E-05 a	2.36E-02 c	2.22E-02 c	1.81E-03 bc	7.83E-04 ab	1.78E-02 c	2.09E-02 c	3.00E-04 ab		
	HbERF-IIIe01	1.90E-03 ab	1.62E-03 a	8.96E-03 b	1.74E-02 bc	1.84E-01 cd	2.41E-02 bc	1.19E+00 e	2.30E-01 cd	4.93E-03 b	1.90E-03 ab	1.62E-03 a	8.96E-03 b	1.74E-02 bc	1.84E-01 cd	2.41E-02 bc	1.19E+00 d	2.30E-01 cd	4.93E-03 b		
	HbERF-IIIe02	7.37E-02 c	1.76E-04 a	1.16E-01 c	7.26E-02 c	4.95E-01 c	3.38E-01 c	9.20E-04 b	2.08E-02 c	2.35E-03 b	7.37E-02 c	1.76E-04 a	1.16E-01 c	7.26E-02 c	4.95E-01 c	3.38E-01 c	9.20E-04 b	2.08E-02 c	2.35E-03 b		
	HbERF-IIIe03	2.72E-02 a	1.07E-01 a	2.34E-02 a	4.44E-03 a	2.00E-01 a	1.60E-02 a	7.89E-02 a	1.53E-02 a	4.24E-02 a	2.72E-02 a	1.07E-01 a	2.34E-02 a	4.44E-03 a	2.00E-01 a	1.60E-02 a	7.89E-02 a	1.53E-02 a	4.24E-02 a		
	HbERF-IIIe04	5.13E-02 d	1.92E-02 c	1.16E-01 d	7.27E-02 d	5.11E-02 d	4.86E-03 b	1.14E-01 d	6.52E-02 d	6.74E-04 a	5.13E-02 d	1.92E-02 c	1.16E-01 d	7.27E-02 d	5.11E-02 d	4.86E-03 b	1.14E-01 d	6.52E-02 d	6.74E-04 a		
IV	HbERF-IVa02	3.17E-01 b	1.08E-01 a	3.02E-01 b	1.40E-01 ab	2.28E+00 c	1.90E+00 c	6.38E-02 a	1.16E+00 c	9.77E-01 c	3.17E-01 b	1.08E-01 a	3.02E-01 b	1.40E-01 ab	2.28E+00 c	1.90E+00 c	6.38E-02 a	1.16E+00 c	9.77E-01 c		
	HbERF-IVa03	3.15E-02 b	3.37E-03 a	2.02E-02 b	2.05E-02 b	3.44E-02 b	3.59E-02 b	5.56E-02 b	3.03E-02 b	1.07E-01 c	3.15E-02 b	3.37E-03 a	2.02E-02 b	2.05E-02 b	3.44E-02 b	3.59E-02 b	5.56E-02 b	3.03E-02 b	1.07E-01 c		
V	HbERF-Va02	4.05E-02 cd	2.23E-02 cd	1.12E-01 d	5.88E-02 d	1.10E-02 bc	4.95E-03 ab	3.43E-03 a	1.29E-01 d	9.23E-02 d	4.05E-02 cd	2.23E-02 cd	1.12E-01 d	5.88E-02 d	1.10E-02 bc	4.95E-03 ab	3.43E-03 a	1.29E-01 d	9.23E-02 d		
	HbERF-Vb01	1.47E-02 a	5.62E-04 a	3.92E-03 a	2.23E-03 a	1.64E-03 a	2.96E-04 a	1.16E-02 a	5.91E-03 a	6.45E-04 a	1.47E-02 a	5.62E-04 a	3.92E-03 a	2.23E-03 a	1.64E-03 a	2.96E-04 a	1.16E-02 a	5.91E-03 a	6.45E-04 a		
	HbERF-Vb02	2.12E-03 ab	4.41E-04 a	1.13E-03 a	8.30E-04 a	3.72E-03 ab	5.68E-04 a	1.31E-04 a	2.72E-03 ab	1.79E-02 b	2.12E-03 ab	4.41E-04 a	1.13E-03 a	8.30E-04 a	3.72E-03 ab	5.68E-04 a	1.31E-04 a	2.72E-03 ab	1.79E-02 b		
VI	HbERF-VI01	3.06E-02 ef	8.84E-04 a	1.83E-02 de	1.02E-02 cd	5.94E-02 f	2.57E-02 ef	2.50E-03 b	5.10E-03 bc	4.51E-03 bc	3.06E-02 ef	8.84E-04 a	1.83E-02 de	1.02E-02 cd	5.94E-02 f	2.57E-02 ef	2.50E-03 b	5.10E-03 bc	4.51E-03 bc		
	HbERF-VI02	7.07E-03 a	9.36E-04 a	3.60E-03 a	2.13E-03 a	2.08E-02 a	4.88E-03 a	9.02E-04 a	2.73E-03 a	1.09E-03 a	7.07E-03 a	9.36E-04 a	3.60E-03 a	2.13E-03 a	2.08E-02 a	4.88E-03 a	9.02E-04 a	2.73E-03 a	1.09E-03 a		
	HbERF-VI03	1.36E-02 b	3.03E-02 bc	5.34E-02 c	2.95E-02 bcd	4.33E-01 g	8.03E-02 d	3.07E-03 a	2.58E-02 bcd	2.15E-02 bc	1.36E-02 b	3.03E-02 bc	5.34E-02 c	2.95E-02 bc	4.33E-01 g	8.03E-02 c	3.07E-03 a	2.58E-02 bc	2.15E-02 bc		
	HbERF-VI04	2.47E-01 d	1.19E-01 cd	4.71E-02 bc	1.12E-01 cd	4.95E-02 bc	2.38E-02 b	1.72E-02 b	1.70E-02 b	3.64E-03 a	2.47E-01 d	1.19E-01 cd	4.71E-02 bc	1.12E-01 cd	4.95E-02 bc	2.38E-02 b	1.72E-02 b	1.70E-02 b	3.64E-03 a		
	HbERF-VI05	7.82E-01 bc	3.17E-01 b	5.43E-01 b	7.14E-01 b	2.10E+00 f	1.82E+00 e	7.42E-01 bc	2.17E+00 e	3.82E-02 a	7.82E-01 bc	3.17E-01 b	5.43E-01 b	7.14E-01 b	2.10E+00 c	1.82E+00 c	7.42E-01 bc	2.17E+00 c	3.82E-02 a		
VI-L	HbERF-VI-L03	2.29E-01 f	1.37E-02 a	1.38E-01 bc	8.74E-02 b	1.12E+00 i	3.49E-01 cde	2.65E-01 cd	4.72E-01 cde	9.62E-01 de	2.29E-01 c	1.37E-02 a	1.38E-01 bc	8.74E-02 b	1.12E+00 e	3.49E-01 cde	2.65E-01 cd	4.72E-01 cde	9.62E-01 de		
	HbERF-VI-L04	1.34E-01 cdef	9.41E-03 a	7.03E-02 bcd	9.05E-02 bcd	4.39E-02 bc	3.20E-02 b	2.57E-01 de	2.85E-01 de	3.40E-01 e	1.34E-01 cde	9.41E-03 a	7.03E-02 bcd	9.05E-02 bcd	4.39E-02 bc	3.20E-02 b	2.57E-01 de	2.85E-01 de	3.40E-01 e		
	HbERF-VI-L06	7.51E-02 b	6.08E-02 b	6.68E-02 b	7.94E-02 b	7.14E-02 b	4.54E-02 b	3.09E-02 b	8.35E-02 b	5.40E-03 a	7.51E-02 b	6.08E-02 b	6.68E-02 b	7.94E-02 b	7.14E-02 b	4.54E-02 b	3.09E-02 b	8.35E-02 b	5.40E-03 a		
VII	HbERF-VIIa01	1.02E-01 c	1.26E-01 b	3.65E-02 a	6.44E-02 ab	1.44E+00 f	1.11E+00 e	1.60E-01 c	5.33E-01 c	1.13E-01 c	1.02E-01 b	1.26E-01 b	3.65E-02 a	6.44E-02 ab	1.44E+00 f	1.11E+00 d	1.60E-01 b	5.33E-01 c	1.13E-01 b		
	HbERF-VIIa04	1.51E+00 hi	1.95E-01 a	9.57E-01 b	1.56E+00 b	8.30E+00 i	3.28E+00 de	2.45E+00 e	5.63E+00 f	2.80E+01 g	1.51E+00 bc	1.95E-01 a	9.57E-01 b	1.56E+00 bc	8.30E+00 f	3.28E+00 de	2.45E+00 cd	5.63E+00 ef	2.80E+01 g		
	HbERF-VIIa07	2.53E+00 ab	1.19E+00 ab	2.52E+00 ab	9.48E-01 ab	3.99E+00 b	2.23E+00 ab	5.05E-01 a	3.72E+00 b	2.09E+01 c	2.53E+00 ab	1.19E+00 ab	2.52E+00 ab	9.48E-01 ab	3.99E+00 b	2.23E+00 ab	5.05E-01 a	3.72E+00 b	2.09E+01 c		
	HbERF-VIIa12	2.42E+00 ab	1.72E+00 ab	5.84E+00 c	1.93E+00 ab	7.92E+00 c	4.30E+00 bc	9.66E-01 a	1.73E+01 d	3.11E+01 d	2.42E+00 ab	1.72E+00 ab	5.84E+00 c	1.93E+00 ab	7.92E+00 c	4.30E+00 bc	9.66E-01 a	1.73E+01 d	3.11E+01 d		
	HbERF-VIIa17	2.50E+00 ab	6.08E-01 a	2.12E+01 cde	3.24E+00 b	4.08E+01 fg	1.22E+01 e	2.80E-01 a	7.93E+01 g	7.22E+00 c	2.50E+01 de	6.08E-01 a	2.12E+01 cde	3.24E+00 b	4.08E+01 ef	1.22E+01 cd	2.80E-01 a	7.93E+01 f	7.22E+00 c		
	HbERF-VIIa20	1.24E-01 c	1.66E-02 b	6.20E-01 d	6.63E-02 b	6.23E-02 b	7.34E-02 bc	7.10E-04 a	4.27E-01 d	6.10E-01 d	1.24E-01 c	1.66E-02 b	6.20E-01 d	6.63E-02 b	6.23E-02 bc	7.34E-02 bc	7.10E-04 a	4.27E-01 d	6.10E-01 d		
VIII	HbERF-VIIIa03	6.01E-01 ab	6.74E-01 ab	5.92E-01 ab	4.27E-01 ab	1.88E-01 a	3.25E-01 a	2.48E+00 c	1.57E+00 b	6.43E-01 ab	6.01E-01 ab	6.74E-01 ab	5.92E-01 ab	4.27E-01 ab	1.88E-01 a	3.25E-01 a	2.48E+00 c	1.57E+00 b	6.43E-01 ab		
	HbERF-VIIIa04	7.66E-01 cd	1.41E+00 de	1.13E+00 cd	8.06E-01 cd	3.75E-01 b	1.85E-01 a	5.37E-01 bc	2.37E+00 ef	2.83E+00 f	7.66E-01 cd	1.41E+00 de	1.13E+00 cd	8.06E-01 cd	3.75E-01 b	1.85E-01 a	5.37E-01 bc	2.37E+00 ef	2.83E+00 f		
	HbERF-VIIIa05	7.80E-02 ab	1.30E-01 bc	1.94E-01 bcd	8.52E-02 ab	3.67E-02 a	3.72E-02 a	8.61E-02 ab	2.45E-01 cd	5.41E-01 d	7.80E-02 ab	1.30E-01 bc	1.94E-01 bcd	8.52E-02 ab	3.67E-02 a	3.72E-02 a	8.61E-02 ab	2.45E-01 cd	5.41E-01 d		
	HbERF-VIIIa08	2.21E+00 c	8.34E-01 b	2.68E+00 c	3.48E+00 c	6.31E-01 b	9.05E-01 b	3.22E+00 c	1.92E+00 c	2.45E-01 a	2.21E+00 c	8.34E-01 b	2.68E+00 c	3.48E+00 c	6.31E-01 b	9.05E-01 b	3.22E+00 c	1.92E+00 c	2.45E-01 a		
	HbERF-VIIIa09	2.31E+00 abc	8.91E-01 a	3.22E+00 bc	3.44E+00 abc	2.32E+00 abc	2.46E+00 abc	4.98E+00 d	8.35E-01 b	1.39E+00 bc	2.31E+00 abc	8.91E-01 a	3.22E+00 bc	3.44E+00 abc	2.32E+00 abc	2.46E+00 abc	4.98E+00 d	8.35E-01 b	1.39E+00 bc		
	HbERF-VIIIa10	1.81E+00 cd	1.86E-01 ab	2.69E+00 d	1.32E+00 cd	7.34E-01 bcd	9.61E-01 cd	2.66E+00 d	4.53E-01 bc	1.29E-01 a	1.81E+00 cd	1.86E-01 ab	2.69E+00 d	1.32E+00 cd	7.34E-01 bcd	9.61E-01 cd	2.66E+00 d	4.53E-01 bc	1.29E-01 a		
	HbERF-VIIIa12	1.79E-01 ab	6.32E-02 a	4.43E-01 bc	2.26E-01 ab	1.74E-01 ab	1.81E-01 ab	1.67E-01 ab	8.96E-01 c	4.41E-01 bc	1.79E-01 ab	6.32E-02 a	4.43E-01 bc	2.26E-01 ab	1.74E-01 ab	1.81E-01 ab	1.67E-01 ab	8.96E-01 c	4.41E-01 bc		
	HbERF-VIIIa13	4.11E-01 bc	1.54E-02 a	9.06E-01 bcd	2.18E-01 b	2.02E+00 g	4.37E-01 bc	2.82E-02 a	9.82E-01 cd	2.41E-02 a	4.11E-01 bc	1.54E-02 a	9.06E-01 bcd	2.18E-01 b	2.02E+00 d	4.37E-01 bc	2.82E-02 a	9.82E-01 cd	2.41E-02 a		
	HbERF-VIIIa14	1.38E-01 a	2.60E-02 a	2.15E-01 a	3.50E-02 a	1.08E-01 a	8.82E-02 a	3.14E-02 a	3.40E-01 b	5.10E-02 a	1.38E-01 a	2.60E-02 a	2.15E-01 a	3.50E-02 a	1.08E-01 a	8.82E-02 a	3.14E-02 a	3.40E-01 b	5.10E-02 a		
	HbERF-VIIIb01	1.65E-03 a	8.02E-04 a	5.78E-03 a	1.69E-03 a	5.95E-04 a	6.40E-04 a	2.74E-03 a	6.11E-03 a	1.76E-03 a	1.65E-03 a	8.02E-04 a	5.78E-03 a	1.69E-03 a	5.95E-04 a	6.40E-04 a	2.74E-03 a	6.11E-03 a	1.76E-03 a		
IX	HbERF-IXa02	4.57E-01 c	5.63E-02 b	2.52E+00 f	5.39E-01 c	3.25E-02 b	8.04E-03 a	5.61E-02 b	5.72E-01 c	8.97E-02 b	4.57E-01 c	5.63E-02 b	2.52E+00 d	5.39E-01 c	3.25E-02 b	8.04E-03 a	5.61E-02 b	5.72E-01 c	8.97E-02 b		
	HbERF-IXa03	1.65E-01 bc	3.43E-02 b	4.03E-01 c	2.60E-01 c	3.48E-02 b	4.71E-02 b	7.41E-02 b	2.98E-01 c	2.11E-03 a	1.65E-01 bc	3.43E-02 b	4.03E-01 c	2.60E-01 c	3.48E-02 b	4.71E-02 b	7.41E-02 b	2.98E-01 c	2.11E-03 a		
	HbERF-IXb01	1.31E-01 cd	3.59E-02 bc	1.22E-01 bcd	9.25E-02 bcd	6.63E-02 bc	1.31E-01 cd	2.76E-01 d	2.96E-02 b	7.29E-03 a	1.31E-01 cd	3.59E-02 bc	1.22E-01 bcd	9.25E-02 bcd	6.63E-02 bc	1.31E-01 cd	2.76E-01 d	2.96E-02 b	7.29E-03 a		
	HbERF-IXb02	6.67E-02 ab	5.57E-02 ab	1.39E-01 ab	1.04E-01 ab	2.14E-02 ab	1.41E-02 a	1.39E-01 ab	1.11E-02 ab	6.85E-03 a	6.67E-02 ab	5.57E-02 ab	1.39E-01 ab	1.04E-01 ab	2.14E-02 ab	1.41E-02 a	1.39E-01 ab	1.11E-02 ab	6.85E-03 a		
	HbERF-IXb03	8.09E-01 d	1.58E-01 c	5.47E-01 cd	3.51E-01 cd	9.60E-03 b	2.07E-02 b	1.19E+00 f	1.81E-02 b	1.67E-03 a	8.09E-01 d	1.58E-01 c	5.47E-01 cd	3.51E-01 cd	9.60E-03 b	2.07E-02 b	1.19E+00 d	1.81E-02 b	1.67E-03 a		
	HbERF-IXc01	4.75E-01 bc	1.26E-01 b	3.41E-01 bc	3.23E-02 a	2.15E-02 a	6.19E-02 a	1.04E-02 a	1.32E+00 e	7.13E-03 a	4.75E-01 bc	1.26E-01 b	3.41E-01 bc	3.23E-02 a	2.15E-02 a	6.19E-02 a	1.04E-02 a	1.32E+00 e	7.13E-03 a		
	HbERF-IXc04	1.61E-02 bcd	1.21E-02 abc	1.11E-01 e	3.12E-02 cde	8.12E-03 ab	6.68E-03 ab	3.60E-03 a	8.07E-01 f	5.34E-02 de	1.61E-02 bcd	1.21E-02 abc	1.11E-01 e	3.12E-02 cde	8.12E-03 ab	6.68E-03 ab	3.60E-03 a	8.07E-01 f	5.34E-02 de		
	HbERF-IXc05	2.29E-02 bcd	7.40E-03 ab	1.14E-01 d	4.28E-02 bcd	1.15E-02 abc	2.96E-02 abc	3.91E-03 a	8.50E-01 f	6.19E-02 cd	2.29E-02 bcd	7.40E-03 ab	1.14E-01 d	4.28E-02 bcd	1.15E-02 abc	2.96E-02 abc	3.91E-03 a	8.50E-01 f	6.19E-02 cd		
	HbERF-IXc06	3.05E-02 d	2.06E-03 b	1.42E-01 e	8.57E-03 c	5.36E-04 a	2.31E-04 a	1.30E-02 c	1.41E-01 e	4.02E-04 a	3.05E-02 d	2.06E-03 b	1.42E-01 e	8.57E-03 c	5.36E-04 a	2.31E-04 a	1.30E-02 c	1.41E-01 e	4.02E-04 a		
X	HbERF-Xa01	3.62E-01 d	4.09E-01 d	3.53E-01 d	8.98E-02 c	1.06E-01 c	1.16E-01 c	3.05E-03 a	1.28E-01 c	2.14E-02 b	3.62E-01 d	4.09E-01 d	3.53E-01 d	8.98E-02 c	1.06E-01 c	1.16E-01 c	3.05E-03 a	1.28E-01 c	2.14E-02 b		
	HbERF-Xa02	3.58E-03 ab	1.07E-02 ab	2.24E-01 d	1.52E-03 ab	4.95E-02 c	6.74E-04 a	2.02E-03 ab	8.51E-03 b	1.20E-02 b	3.58E-03 ab	1.07E-02 ab	2.24E-01 d	1.52E-03 ab	4.95E-02 c	6.74E-04 a	2.02E-03 ab	8.51E-03 b	1.20E-02 b		
	HbERF-Xa04	2.89E-03 a	3.45E-02 b	1.76E-02 b	3.77E-03 a	5.05E-02 b	2.86E-02 b	2.27E-03 a	5.06E-02 b	1.27E-01 c	2.89E-03 a	3.45E-02 b	1.76E-02 b	3.77E-03 a	5.05E-02 b	2.86E-02 b	2.27E-03 a	5.06E-02 b	1.27E-01 c		
	HbERF-Xa06	8.17E-02 b	2.66E-01 b	1.41E-01 b	7.87E-02 b	2.25E-01 b	1.61E-01 b	1.87E-03 a	2.27E-01 b	9.47E-02 b	8.17E-02 b	2.66E-01 b	1.41E-01 b	7.87E-02 b	2.25E-01 b	1.61E-01 b	1.87E-03 a	2.27E-01 b	9.47E-02 b		
	HbERF-Xa08	1.74E-02 c	8.86E-03 bc	2.93E-02 c	1.49E-02 c	2.21E-02 c	2.51E-02 c	1.31E-02 c	4.62E-03 ab	1.75E-03 a	1.74E-02 c	8.86E-03 bc	2.93E-02 c	1.49E-02 c	2.21E-02 c	2.51E-02 c	1.31E-02 c	4.62E-03 ab	1.75E-03 a		
	HbERF-Xb01	4.35E-02 d	8.89E-03 c	8.58E-02 de	1.84E-01 e	1.41E-04 a	2.18E-03 b	3.58E-01 f	9.87E-04 b	4.95E-04 ab	4.35E-02 d	8.89E-03 c	8.58E-02 de	1.84E-01 e	1.41E-04 a	2.18E-03 b	3.58E-01 f	9.87E-04 b	4.95E-04 ab		

Figure 3. Relative transcript abundance profile of 107 *HbERF* genes for various tissue types (both immature and mature male and female flowers, zygotic embryos, leaf, bark and latex). The relative transcript abundances were measured by real-time RT-PCR. Values are the means of the relative transcript abundances of three biological replicates. (Figure 3A) heat map representation of the expression profile was used for values ranging as follows  from dark to light green. (Figure 3B) Values of relative transcript abundances in various tissue types were analysed with XLSTAT software after log transformation. The statistical analysis was performed with an ANOVA followed by the Student Newman-Keuls test. Values with significantly high relative transcript abundances shown in red and significantly low relative transcript abundances shown in green. The non-significant genes are shown in yellow.

According to the statistical analysis, 35 *HbERF* expression marker genes showed a significant higher or lower relative transcript abundance level in one tissue compared with other tissues (Figure 3B). In mature male flowers, 6 genes (*HbERF-Ib04, HbERF-IIIe02, HbERF-IVa03, HbERF-VI01, HbERF-VI-L03* and *HbERF-VI-L04*) showed a significant lower transcript accumulation and 1 gene (*HbER-Ib11*) a higher relative transcript accumulation compared with other tissues. In immature female flowers, 2 genes (*HbERF-IXa02, HbERF-Xa02*) showed a specific high transcript accumulation. In zygotic embryos, 3 expression marker genes were identified. Only the *HbERF-VIIa01* gene showed high relative transcript abundance in both the embryo body and cotyledon tissues. Two other genes showed embryo body tissue-specific relative transcript abundance: low abundance for *HbERF-IIb02* and high abundance for *HbERF-VI03*. The relative transcript abundance of *HbERF-IXa02* was specifically lower in the cotyledon than in the other tissues. Seven ERF genes were identified as leaf expression marker genes. The relative transcript abundance was lower for 5 genes (*HbERF-IIa03, HbERF-VI03, HbERF-VIIa20, HbERF-Xa01* and *HbERF-Xa06*) compared with the other tissues. High relative transcript abundance in leaf was recorded for 2 genes (*HbERF-IIb01, HbERF-IIb02*). Three *ERF* genes (*HbERF-VIIa17, HbERF-IXc4* and *HbERF-IXc5*) showed a higher relative transcript abundance in bark compared with the other tissues. Interestingly, the largest number of expression marker genes (16 ERFs) was found in latex. Seven genes (*HbERF-IIa03, HbERF-IIb04, HbERF-IVa03, HbERF-VIIa04, HbERF-VIIa07, HbERF-VIIIa04* and *HbERF-Xa04*) and nine genes (*HbERF-IIIb01, HbERF-IIIe04, HbERF-VI04, HbERF-VI05, HbERF-VI-L06, HbERF-VIIIa08, HbERF-IXa03, HbERF-IXb01* and *HbERF-IXb03*) revealed a higher and lower relative transcript abundance, respectively. Two additional expression marker genes showed a higher (*HbERF-VIIa12*) or lower (*HbERF-Ib11*) relative transcript abundance in both bark and latex compared with other tissues.

Identification of putative functions for *HbERF* genes

The phylogenetic tree was constructed using the deduced amino acid sequences of the AP2 domain in order to identify *Hevea* potential orthologs to *Arabidopsis* proteins using RAP-Green analysis (Supplementary Figures 1-11; Supplementary Data Tables 5-6). Among the 87 *HbERF* genes, functions could be predicted for 25 genes (Supplementary Data Table 6). Fifteen of the 35 expression marker genes showed significant orthology parameters with 11 *Arabidopsis* genes as follows: *HbERF-IIb02* with *ORA47, HbERF-IIIe02* with *TINY, HbERF-VI01* with *CRF2/TMO3, HbERF-VI-L03* with *CRF10, HbERF-VIIa07* and *HbERF-VIIa12* with *RAP2.12, HbERF-VIIa17* and *HbERF-VIIa20* with *AtEBP/RAP2.3/ERF72, HbERF-VIIIa04* with *AtERF3, HbERF-IXa03* with *AtERF1, HbERF-IXb03* with *ERF5, HbERF-IXc04* and *HbERF-IXc05* with *ERF1, HbERF-Xa01* and *HbERF-Xa02* with *RAP2.6L*, and finally *HbERF-Xa06* with *ERF110* (Table 2). The putative functions of these ortholog genes were related to the response to biotic and abiotic stress, hypoxia, regulation of cell proliferation or root initiation, and hormone signalling.

Table 2. Identification of putative functions for expression marker genes based on a phylogenetic tree analysis with *Arabidopsis thaliana* using the deduced amino acid sequences of the AP2 domain for each gene (Supplementary Figure1-10; Supplementary Data Tables 5-6)

Gene	Transcript accumulation	Phylogenetic analysis			
		Orthologous gene	Acc.No.	Putative function	Reference
HbERF-Ib04	Low in mature male flower	-	At4g39780-Ib	-	-
HbERF-Ib11	High in mature male flower	-	At4g13620-Ib	-	-
HbERF-IIa03	High in latex	-	-	-	-
HbERF-IIb01	High in leaf	-	-	-	-
HbERF-IIb02	High in leaf	-	At1g19210-IIb	-	-
		ORA47	At1g74930-IIb	Biotic and abiotic stress	[88]
HbERF-IIb04	High in latex	-	-	-	-
HbERF-IIIb01	Low in latex	-	At1g63040-IIIb	-	-
HbERF-IIIe02	Low in mature male flower	TINY	At5g25810-IIIe	Suppressed cell proliferation and exhibited pleiotropic effects	[89]
HbERF-IIIe04	Low in latex	-	At4g32800-IIIe	-	-
HbERF-Iva03	High in latex	-	-	-	-
HbERF-VI01	Low in mature male flower	CRF2/TMO3	At4g23750-VI	Related to root initiation at later embryonic stages	[47, 90]
HbERF-VI03	High in zygotic embryo	-	-	-	-
HbERF-VI04	Low in latex				
HbERF-VI05	Low in latex	-	-	-	-
HbERF-VI-L03	Low in mature male flower	CRF10	At1g68550.1-VI-L	Cytokinin signalling pathway	[91]
HbERF-VI-L04	Low in mature male flower	-	-	-	-
HbERF-VI-L06	Low in latex	-	-	-	-
HbERF-VIIa01	High in zygotic embryo and cotyledon	-	-	-	-
HbERF-VIIa04	High in latex	-	-	-	-
HbERF-VIIa07	High in latex	RAP2.12	At1g53910	Activate gene expression for hypoxia	[51, 63]
HbERF-VIIa12	High in bark and latex				
HbERF-VIIa17	High in bark	AtEBP/ RAP2.3/ ERF72	At3g16770-VIIa	Jasmonate and/or ethylene	[55]
HbERF-VIIa20	Low in leaf				
HbERF-VIIIa04	High bark and latex	AtERF3	At1g50640-VIIIa	Repressors of GCC box-dependent transcription	[92]
HbERF-VIIIa08	Low in latex	-	-	-	-
HbERF-IXa02	High in immature female flower, low in cotyledon	-	-	-	-
HbERF-IXa03	Low in latex	AtERF1	At4g17500-IXc	Activators of GCC box–dependent transcription	[92]
HbERF-IXb01	Low in latex	-	-	-	-
HbERF-IXb03	Low in latex	ERF5	At5g47230-IXc	Activators of GCC box–dependent transcription	[92]
HbERF-IXc04	High in bark	ERF1	At3g23240_Ixc	Integrates signals from ethylene and jasmonate pathways in plant defense	
HbERF-IXc05	High in bark				
HbERF-Xa01	Low in leaf	Rap2.6L	At5g13330 -Xa	Expression drought-responsive gene	-
HbERF-Xa02	High in immature female flower				[93]
HbERF-Xa04	High in latex	-	-	-	-
HbERF-Xa06	Low in leaf	ERF110	At5g50080-Xa	Regulation of bolting time	[94]

Given the involvement of some ERFs from the group VII in the regulation hypoxia-responsive genes and the identification of some new functions in *Oryza species*, an additional phylogenetic analysis was carried out using the 6 *Hevea*, 15 *Oryza sativa* and 2 *Oryza nivara* members (Supplementary Figure 11). This phylogenetic analysis showed that genes *HbERF-VIIa07* and *HbERF-VIIa12* were found to be orthologs to the *OsEREBP1*. Finally, a phylogenetic analysis was carried out by comparing AP2 domain-deduced amino acid sequences from five dicots (*Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera*, *Malus×domestica* and *Hevea brasiliensis*) and two monocots (*Oryza sativa* and *Zea mays*) (Figure 4). The differentiation of members from ERF group VII clearly occurred before the speciation between dicot and monocot species because ERF-VIIs from all species were distributed in several clades [23]. Only one clade was early differentiated to generate genes involved in the tolerance to submergence in rice.

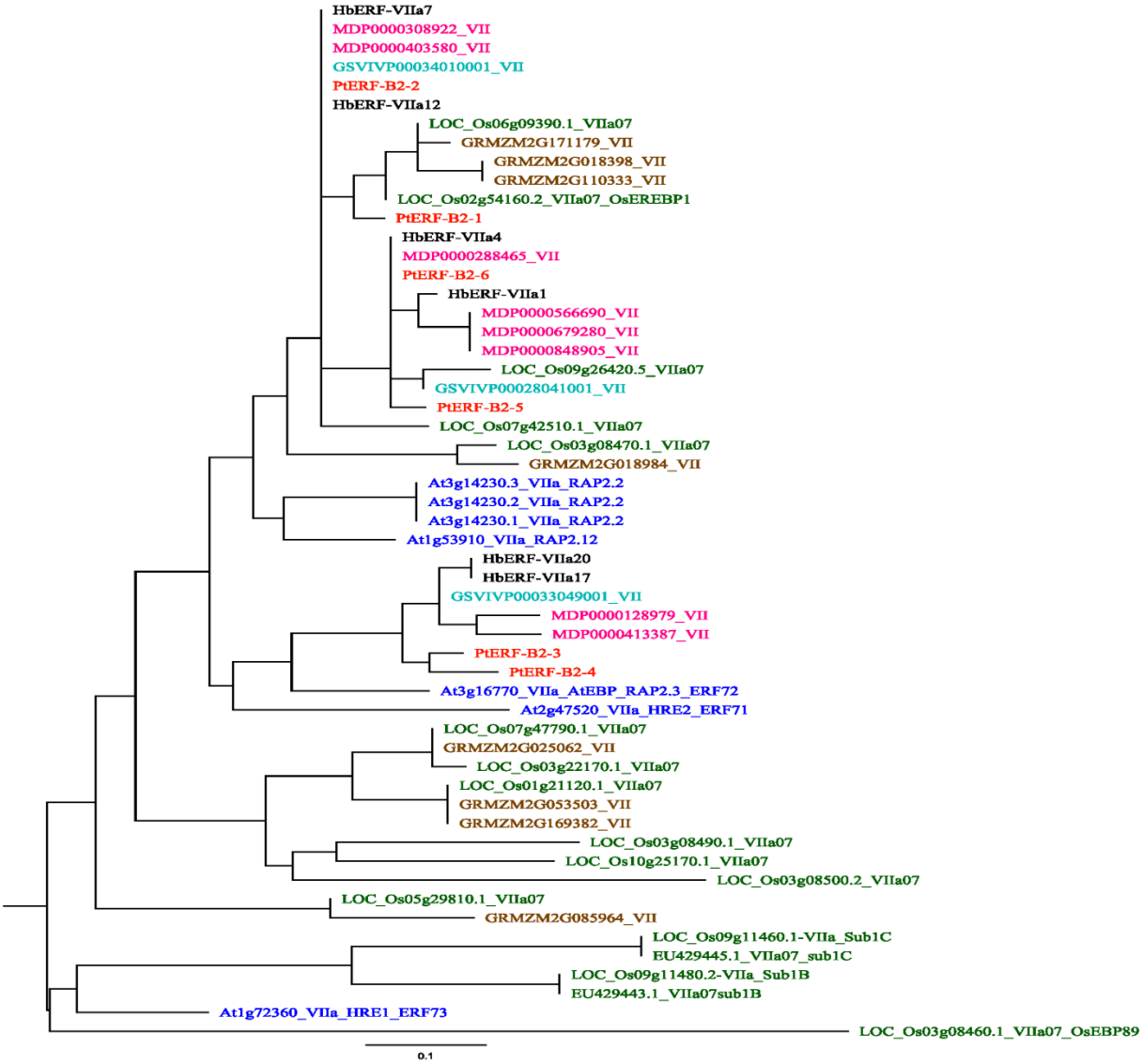


Figure 4. Phylogenetic tree illustrating the relatedness of group VII ERFs among plant species. The amino acid sequences of the AP2 domain were aligned using Muscle, and the phylogenetic tree was constructed using PhyML. The 7 plant species were: *Arabidopsis thaliana* (At2g47520,At3g16770, At1g72360, At1g53910, At3g14230.1,At3g14230.2, At3g14230.3); *Populus trichocarpa* (PtERF-B2-1, PtERF-B2-2, PtERF-B2-3, PtERF-B2-4, PtERF-B2-5, PtERF-B2-6) as named by Zhuang *et al*. (2008); *Vitis vinifera* (GSVIVP00034010001, GSVIVP00033049001, GSVIVP00028041001); *Oryza sativa* (Os02g54160.2, Os06g09390.1, Os03g08500.2, Os01g21120.1, Os03g22170.1, Os07g42510.1, Os07g47790.1, Os10g25170.1, Os03g08460.1, Os03g08490.1, Os09g26420.5, Os03g08470.1, Os05g29810.1, Os09g11460.1 and Os09g11480.2) as named by Nakano *et al*.(2006); *Orysa nivara* (EU429443.1submergence-1B,EU429445.1submergence-1C) as named by Fukao *et al*. (2009); *M.× domestica* (MDP0000848905, MDP0000679280, MDP0000566690, MDP0000413387, MDP0000403580, MDP0000308922, MDP0000288465 and MDP0000128979); *Zea mays* (GRMZM2G171179, GRMZM2G169382, GRMZM2G110333, GRMZM2G085964, GRMZM2G053503, GRMZM2G025062, GRMZM2G018984 and GRMZM2G018398) and *Hevea brasiliensis* (*HbERF-VIIa1, HbERF-VIIa4, HbERF-VIIa7, HbERF-VIIa12, HbERF-VIIa17* and *HbERF-VIIa20).*

Gene structure and *in silico* promoter analysis of *HbERF-VIIs*

Gene structure of the 6 HbERF-VIIs was analysed using genomic scaffold sequences from the *Hevea* clone CATAS 7-33-97. These scaffolds revealed that the *ERF-VII* genes possessed 2 exons and at least 1 intron (Figure 5). The first exon was shorter (174-287 bp) than the second exon (568-968 bp), which contained the AP2 domain. Two genes (*HbERF-VIIa07* and *HbERF-VIIa12*) contained 2 introns, the second intron being located in the 3'UTR sequence. These two genes showed a large first intron (1472 to 1848 bp) compared with other genes (90-142 bp), and a short second intron (129-135 bp).

In order to better understand the regulation of these *ERFs*, an *in silico* analysis of the 2000 bp upstream sequence ATG codon of these scaffolds was carried out using PLACE and Plant Care ([24, 25]; Supplementary Table 7). Forty-one *cis*-acting elements were selected for their putative role in the response to tissue specificity, hormones, sugar /starch and stress (Table 4). According to the selected regulatory groups, 11 *cis*-acting elements were observed. E-box, POLLEN1 and RAV1-A were found for all of the studied genes. Interestingly, CAT-box, meristem specific *cis*-acting elements only had a high frequency (27 copies) in *HbERF-VIIa1*. L1 box (L1 layer specific) and XYL (regulating secondary xylem development *cis*-acting element) only existed in *HbERF-VIIa17*. Thirteen *cis*-acting elements of six hormone responses (jasmonic acid, ethylene, abscisic acid, auxin, gibberellin and salicylic acid) were observed. Three *cis*-acting elements were found individually, TCA-element in *HbERF-VIIa20*, GCC-box in *HbERF-VIIa1*and JERE in *HbERF-VIIa17*. For sugar and starch responses, eight *cis*-acting elements (A-box, amylase box, CGACG element, CMSRE-1, OsBP-5, SRE, TATCCA element and TATCCAY motif) were found. The *cis*-acting elements in this regulatory function were

distributed over the studied genes except *HbERF-VIIa7*. The majority of the upstream elements were categorized in stress-related responses. Eight *cis*-acting elements (ARE, DRE/CRT, LTRE, CNGTTR-motif, CANNTG-motif, TC-rich repeats, W-box and WUN-motif) were discovered. CANNTG-motif and TC-rich repeats were found for all of the studied genes, while the WUN-motif was specifically present in *HbERF-VIIa04*.

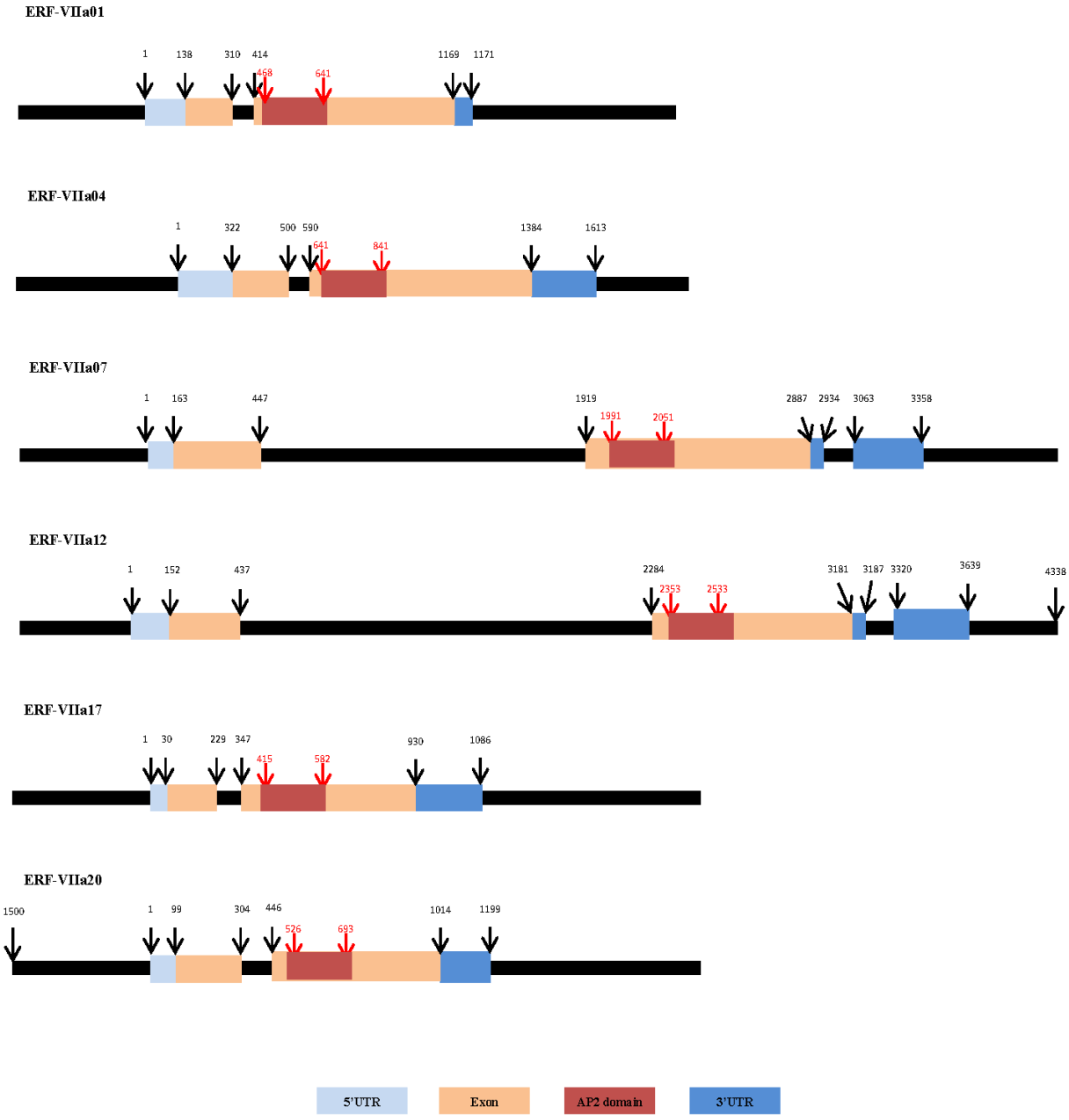


Figure 5. Gene structure of the 6 *HbERF-VII* genes.

Table 3. *Cis*-acting regulatory elements identified in *HbERF-VIIs* promoter region using -2000 bp region upstream ATG (start codon) by PLACE and PlantCare.

Regulatory group	*Cis*-acting element	Function	Number of *cis*-acting elements					
			HbERF-VIIa01	HbERF-VIIa04	HbERF-VIIa07	HbERF-VIIa12	HbERF-VIIa17	HbERF-VIIa20
Tissue-specific expression	(CA)n element	Embryo and endosperm-specific	-	-	-	1	1	2
	CAT-box	Meristem expression	27	1	-	-	-	-
	DRE2 core	Embryogenesis	-	-	-	-	-	1
	E-box	Embryo and endosperm-specific	10	18	14	6	20	20
	L1 box	Layer L1 protoderm of organ primordia	-	-	-	-	1	-
	motif I	Root specific	-	-	1	-	1	-
	POLLEN1	Pollen specific expression	4	9	3	7	13	12
	RAV1-A	Tissue-specific expression	4	1	4	2	9	3
	RAV1-B	Tissue-specific expression	-	2	1	1	2	-
	TTAATGG-motif	Central cells-specific expression	-	1	1	-	1	-
	XYL	Regulating secondary xylem development	-	-	-	-	1	-
Hormone response	ABRE	ABA signalling	-	-	-	-	7	2
	DPBF-1 and 2	ABA signalling and embryo-specification	4	-	-	1	6	2
	ARF	Auxin signalling	1	2	1	1	1	-
	TGA-element	Auxin signalling	1	-	-	-	1	-
	GARE-motif	Gibberellin signalling	-	-	-	1	1	2
	TATCCAC box	Gibberellin response	-	2	-	-	2	-
	TCA-element	Salicylic acid signalling	-	-	-	-	-	2
	ERE	Primary ethylene signalling	-	1	1	-	-	-
	GCC-box	Ethylene signalling	1	-	-	-	-	-
	DRE/CRT	Ethylene signalling	3	4	-	-	1	2
	CGTCA-motif	Jasmonate signalling	-	-	-	-	3	4
	JERE	Jasmonate signalling	-	-	-	-	1	-
	T/G-box	Jasmonate signalling	2	1	-	-	1	-
	TGACG-motif	Jasmonate signalling	1	-	-	-	3	4
Sugar and starch response	A-box	Sugar repression	-	-	-	2	-	2
	amylase box	Sugar starvation	1	1	-	-	2	-
	CGACG element	Sugar starvation	-	1	-	-	-	2
	CMSRE-1	Sucrose-inducible expression	-	1	-	-	-	-
	OsBP-5	Starch synthase.	1	1	-	-	-	-
	SRE	Sugar repression	1	2	-	1	3	-
	TATCCA element	Mediate sugar and hormone regulation	3	4	-	-	7	1
	TATCCAY motif	Sugar repression	1	3	-	-	3	-
Stress-related response	ARE	Oxidative stress response	-	1	-	1	1	4
	ANAERO	Anaerobic gene regulation	1	5	-	2	4	3
	LTRE	Low temperature response	-	-	-	1	-	1
	CNGTTR-motif	MYB recognition site responses in stress and development	-	2	2	2	12	6
	CANNTG-motif	MYC recognition site responses in stress and development	10	20	14	6	20	20
	TC-rich repeats	Defence and stress responsiveness	2	3	2	1	2	1
	W-box	Multiple responses in stress and development	13	35	-	12	31	21
	WUN-motif	Wound-response element	-	1	-	-	--	-

Functionality of HbERF-VIIs

A search for the conserved motifs outside the DNA-binding AP2 domain of the HbERF-VII deduced amino acid sequences was carried out using the MEME program [26], by comparison with *Arabidopsis* motifs (Figure 6; Supplementary Table 8). Interestingly, HbERF-VII proteins located in the same clades of the phylogenetic tree showed a similar pattern of motif distribution. Most of the *Arabidopsis* motifs (CMVII-1, CMVII-3, CMVII-4, CMVII-5, CMVII-6 and CMVII-7) existed in the HbERF-VIIs, except CMVII-2 and CMVII-8. One *Hevea*-specific motif (HbCMVII) with an unknown function was found in HbERF-VIIa07 and HbERF-VIIa12.

In order to validate the functionality of HbERF-VIIs as transcription factors, a subcellular localization experiment was carried out using HbERF coding sequences/GFP translational fusion into pMDC83 (Figure 7). Transient expression into BY-2 tobacco protoplasts revealed GFP activity of the fusion protein in the nucleus for each tested HbERF, in contrast with a pMDC83 empty control plasmid. Moreover, the GFP reporter gene under the control of a synthetic promoter harbouring the GCC box *cis*-acting element was transactivated by the three HbERF-VII candidates (Figure 8). The effector constructs and the reporter constructs driven either by a GCC-rich synthetic promoter (GCC) or a synthetic promoter containing a mutated GCC motif (mGCC::TCCTCC) were co-transformed with 35S::HbERF-VII constructs into BY2 tobacco protoplasts. GFP activity was quantified by flow cytometry for at least 100 protoplasts. The ratio of GFP activities between GCC::GFP and mGCC::GFP constructs revealed the capacity of HbERF-VII to activate (ratio > 1) or repress (ratio < 1) the GCC promoter. All the three ERF-VIIs showed a ratio higher than 1 but only HbERF-VIIa07 could significantly be considered as activators.

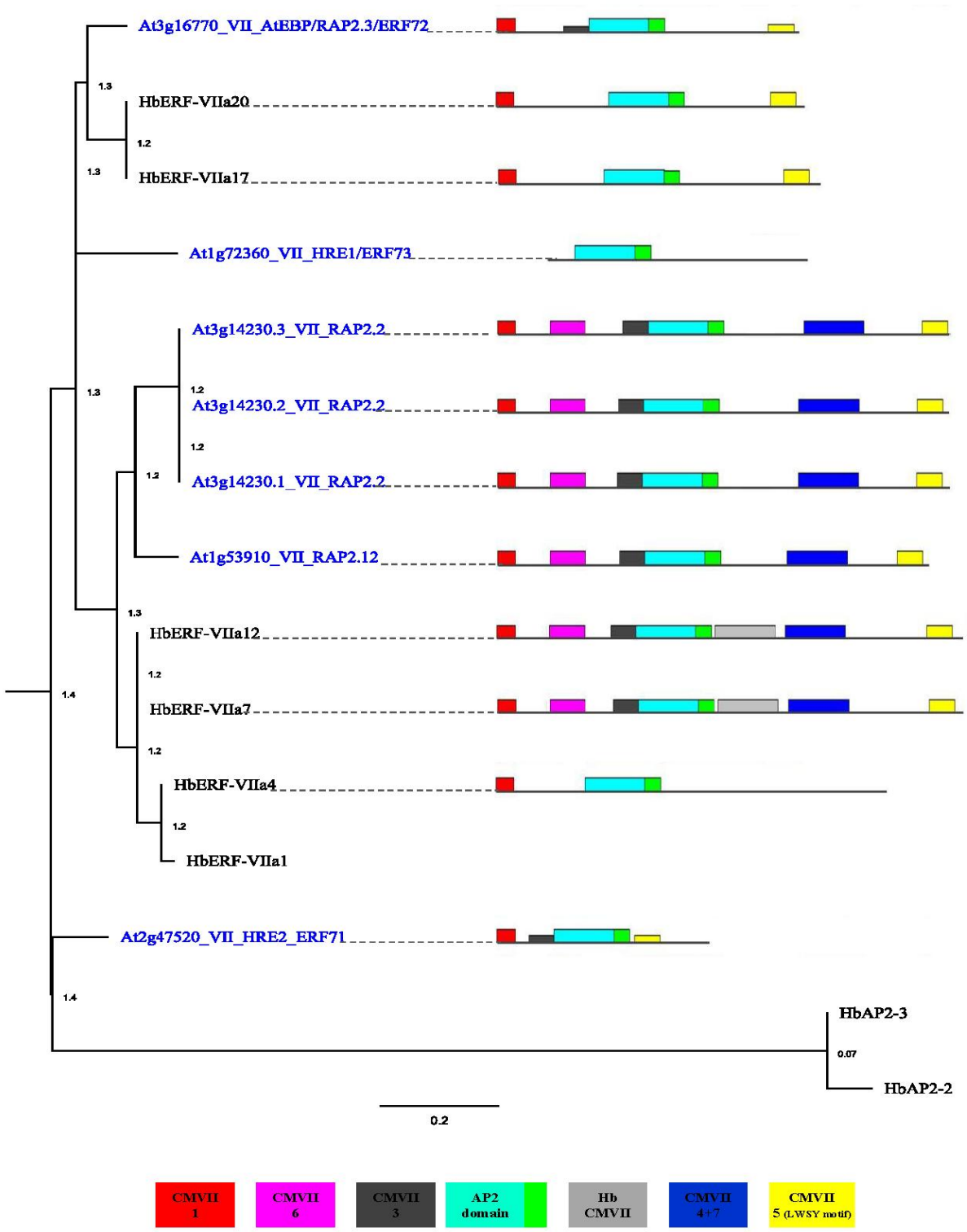


Figure 6. Phylogenetic tree and a schematic diagram of the conserved motif of group VII ERFs among *Arabidopsis thaliana* and *Hevea brasiliensis.*

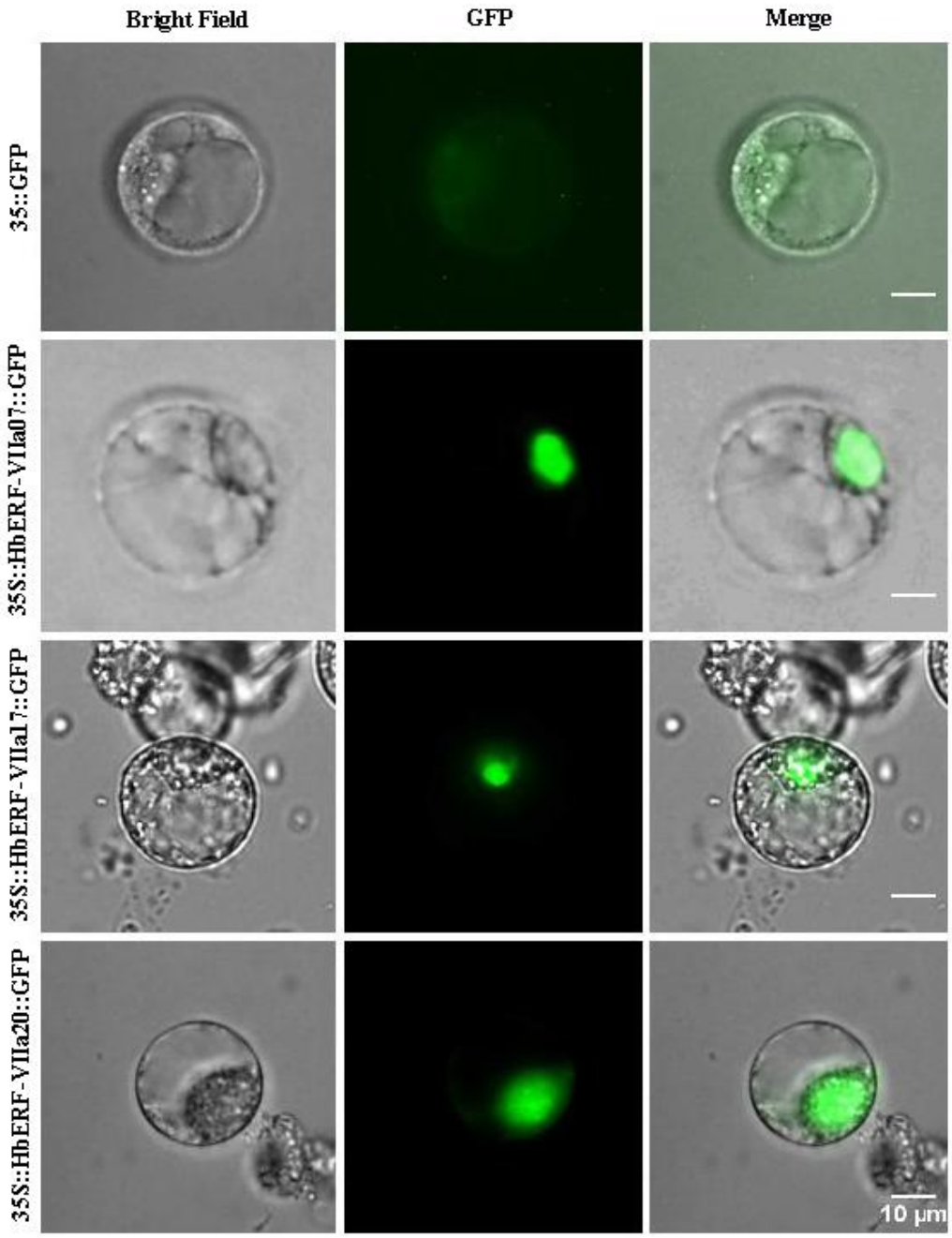


Figure 7. Subcellular localization of HbERF-VIIs. Tree HbERF-VII GFP fusion proteins (HbERF-VIIa7, HbERF-VIIa17 and HbERF-VIIa20) were transiently expressed in protoplasts from BY-2 tobacco cells under the control of the 35S promoter. Subcellular localization was analysed by confocal laser scanning microscopy. The merged pictures of the green fluorescence channel (middle panels) and the corresponding bright field (left panels) are shown (right panels). Control cells expressing fluorescence absence are shown in the top panel. The scale bar indicates 10 µm.

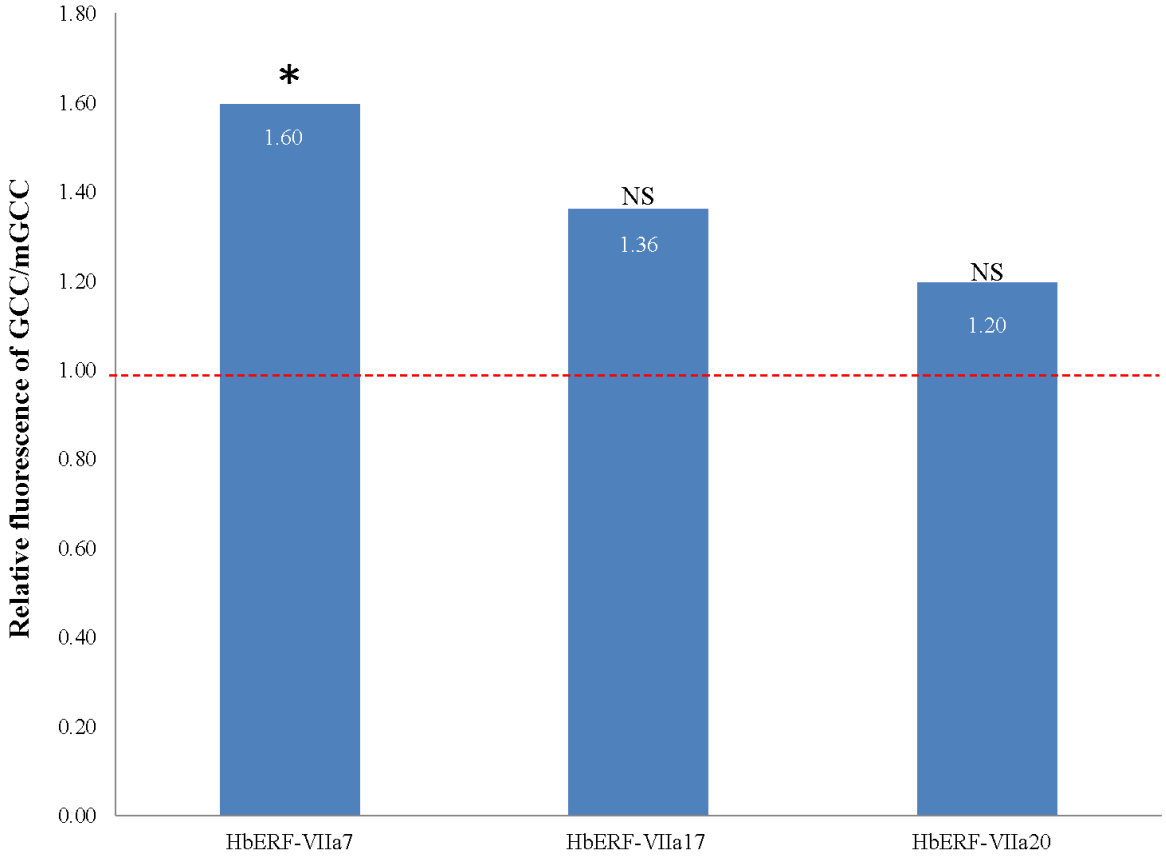


Figure 8. Transactivation of the synthetic GCC-box and mGCC containing promoters by HbERF-VIIa7, HbERF-VIIa17 and HbERF-VIIa20 proteins. Transient expression in BY-2 tobacco protoplasts co-transformed by pMDC32 harbouring the effector construct (ERF candidate genes under the control of the CaMV 35S promoter) and the reporter constructs, 4 x GCC::GFP or 4 x mGCC::GFP, respectively. The fluorescence activity was measured by flow cytometry for three independent biological replicates. The ratio of fluorescence between the constructs with functional GCC and mutated mGCC boxes revealed that ERF candidates are activators (ratio > 1) or repressors (ratio < 1). (*) indicates significant difference for the Student test ($p<0.01$).

Discussion

Towards a comprehensive *Hevea* transcriptome

Over the last decade, several studies were conducted in *Hevea brasiliensis* to identify ESTs from latex [27, 28], especially those involved in rubber biosynthesis [29, 30], and also TPD-related genes [31]. More recently, several high-throughput analyses have led to the identification of microRNAs [32, 33], transcriptomes [11, 34-36], and genomes [37] using NGS technologies. All the transcriptome analyses provided a partial overview of the *Hevea* transcriptome since RNAs from only a few specific tissues have been sequenced, such as shoot apex [34] and leaf [37] from clone RRIM 600, along with leaf and latex from CATAS 7-33-97 [35]. This latter work by Xia and coll. also revealed incomplete sequences for the ERF family, with partial sequences unfit for classification [11]. Duan and coll. reported having developed a comprehensive transcriptome using several tissues (root, bark, latex, leaf, and tissues from somatic embryogenesis) from plants growing under different environmental conditions [11]. The present study improved this previous transcriptome by adding RNA sequences from reproductive tissues (immature and mature male and female flowers, zygotic embryos) and by performing gene annotation. Fifty-eight transcription factor families were identified accounting for 2448 contigs (8.07% of predicted genes). Another study based on the *Hevea* genome sequence showed 5978 transcription factors (8.5% of gene models) classed in 50 families [37]. This number of transcription factors is very high (2.5 to 6 times) compared with other species and even with our own annotation. This large number could be explained by an overestimation. For instance, based on repeat motif identification, Rahman and coll. found 139 *AP2* genes, 246 *ERF* genes and 25 *RAV* genes. The presence of the AP2 domain in three transcription factor families (AP2, ERF, RAV), and the B3 domains in two other families (RAV, B3) probably led to one contig being annotated in more than one family carrying the same specific domain. The careful annotation carried out in our work is likely to provide a more accurate estimation of transcription factors and the ERF family in particular. As in our annotation, 58 transcription factor families were commonly found from 49 species [38]. The number of transcription factors ranged from 1291 to 3546 genes, with, for instance, 2201 genes for *Manihot esculenta*, a *Euphorbiaceae* close to *Hevea brasiliensis*, and 2585 genes in *Populus trichocarpa* in woody species.

The present study led to a better estimation of *AP2/ERF* genes for the *Hevea brasiliensis* clone PB 260. Supplemented with RNA sequences from reproductive tissues, the present transcriptome database contains a lower number of *AP2/ERF* genes (114 instead of 142 found in the Duan's paper) closed to the genomic information provided by the Chinese *Hevea* Genome Project on the *Hevea* clone CATAS 7-33-97. Although the first assembly of RNA reads was carried out with 2,387,930 reads from GS-FLX 454 sequencing, the addition of 643,113 reads from the reproductive tissues in this study led to a significant improvement of sequence quality comparable with genomic data (Supplementary data Table 1). This reveals the limitation of some transcriptome analyses and the necessity to refer to genomic data. The ten

additional genes found in the genome of the Chinese clone CATAS 7-33-97 revealed either a duplication of some genes to favour adaptation to the Hainan province conditions, or that the *Hevea* clone PB 260 transcriptome requires to be completed by other tissues such as cambium and xylem, which are absent in the present work. The decrease in gene number between the Duan and coll. and the present reports is mostly due to the reduction in gene number for the ERF groups VII (from 23 to 6), VIII (from 15 to 11) and IX (from 19 to 12).

Some HbERFs could play an important role in latex

Several members of three ERF groups (I, VII, VIII) are highly expressed in latex. In total, sixteen latex expression marker genes with higher or lower transcript abundance compared with other tissues were found in all ERF groups except the group V. Seven highly expressed *ERFs* in latex, not significantly different with other tissues, belong to the group II, IV, VII and VIII. HbERF-II and HbERF-VIIIa deduced proteins contain the repressor EAR motif. Their high expression in normal condition suggests a certain negative control of ethylene response especially in latex by HbERF-IIa03 and HbERF-IIb04, and in leaf by HbERF-IIb01 and HbERF-IIb02. Members of the group VIII are known to drive the response to jasmonate. This hormone is supposed to play a crucial role in the response to tapping and in particular to mechanical wounding of bark. The latex expression marker gene *HbERF-VIIIa04* had AtERF3, a repressor of abiotic stress response and an inducer of cell death, as ortholog.

The HbERF-IVs correspond to the DREB2 in the Sakuma's classification. These genes are generally involved in the response to dehydration. The role of HbERF-IVa03 in the response to water stress is consistent since laticifers are subjected to recurrent osmotic stress after tapping and consequent latex flow. The group VII was described as regulator of the hypoxia-responsive genes, which will be developed in the next part of discussion. For the group X, one member was described as a master regulator of the redox potential (RRTF1) [39]. In *Hevea*, *HbERF-Xb1* is ortholog to *RRTF1*but not an expression marker gene. By contrast, the marker *HbERF-Xa04* did not have known function.

With regard to the nine latex expression marker genes with very low relative transcript abundance, two belong to the group III. This group corresponds to the CBF/DREB1 in the Sakuma's classification. In addition to a low expression of the *HbERF-III* genes, this group III counts a small number of genes in *Hevea* compared with other species. This could explain the low adaptation of this subtropical species to cold. Indeed, genetic analysis showed that a duplication of *CBF* genes was related to the tolerance to low temperature of *Arabidopsis*, tomato [40], maize [41], and *Eucalyptus* [42]. In wheat and barley, the Frost resistance-2 (Fr-2) locus is coincident with a cluster of more than 12 *CBF* genes [43]. In *Eucalyptus*, 14 of the 17 *CBF* genes are located in a cluster on chromosome 1 [44]. QTLs of cold tolerance have been linked to *CBF* genes in *Arabidopsis* [45], and *Eucalyptus nitens* [46]. Three other latex expression marker genes belong to the ERF group VI and VI-like, which are involved in the response to cytokinin [47]. Finally, two latex expression marker genes belonging to the group

IX had orthologs with functions related to vascular cell division (HbERF-IXa03/AtERF99) [48], and programmed cell death (HbERF-IXb03/AtERF102) [49].

HbERF-VII genes could play a role in hypoxia response

The ERFs from group VII are known to regulate hypoxia-responsive genes. ERF-VII proteins govern the response to low oxygen in plants by post-translational regulation. The regulation of these transcription factors has been characterized in depth for AtERF75/RAP2.2, AtEBP/RAP2.3, AtERF74/RAP2.12, AtERF71/HRE2 and AtERF73/HRE1 in *Arabidopsis* [50-53]. AtEBP also confers resistance to hydrogen peroxide and heat treatments [54], and this protein was shown to interact with ACBP4 [55]. The stability of all these proteins is enabled by binding to the Acyl CoA binding proteins (ACBP). Under hypoxia, ERF-VII proteins are dissociated and then activate hypoxia-responsive genes. When normoxia is recovered, the N-end rule pathway is involved in the proteosomal degradation of ERF-VII [56]. The N-terminal MCGGAII motif of ERF-VII proteins is targeted by the N-end rule pathway associated with the proteosomal degradation pathway [56, 57]. The presence of the N-terminal MCGGAII motif in all the identified HbERF-VII deduced proteins, as in other species, suggests similar post-translational regulation in *Hevea*.

Most species have 3 to 8 *ERF-VII* genes: 5 for *Arabidopsis thaliana* [6], 6 for *Populus trichocarpa* [9], 3 for *Vitis vinifera* [8], 8 for *Malus* × *domestica* [58], 4 for *Solanum lycopersicon* [59], and 6 for *Hevea brasiliensis* (present work). Only rice had a larger number (15) of genes [6]. Interestingly, phylogenetic analysis of ERF group VII proteins revealed that *Oryza sativa* had one additional clade with three genes (*OsSUB1A*, *OsSUB1B*, *OsSUB1C*) [60]. Interestingly, *OsSUB1A* is involved in the tolerance to submergence [61, 62]. Thus, ERF-VIIs are pivotal regulators of responses to flooding and low oxygen [63]. These ERF-VII proteins have also been shown to be regulated by oxidative stress [64, 65]. The divergence of these genes clearly led to a specific adaptation of rice to submergence by orchestrating various acclimatization responses [66], by creating a rice-specific group consisting of Sub1 and Snorkel proteins [23]. By contrast, the high expression of ERF-VIIs in *Hevea brasiliensis* with a small number of genes suggests a transcriptional-based adaptation in latex cells.

Some hypoxia-responsive genes targeted by ERF-VIIs identified in *Arabidopsis* were also present in latex. An analysis of the gene expression pattern revealed that *Arabidopsis* RAP2.2 controls the induction of genes involved in sugar metabolism and fermentation pathway enzymes [67]. Alcohol dehydrogenase (ADH), non-symbiotic haemoglobin (HB1), SUCROSE SYNTHASE1 (SUS1) and SUCROSE SYNTHASE4 (SUS4) are considered as good markers of anaerobic response [23]. In order to overcome insufficient production of ATP by mitochondrial respiration, the catabolism of soluble sugars and starch can lead to an adjustment of the energy crisis by maintaining ATP production and NAD^+ regeneration [50, 63, 67]. Fermentation of pyruvate to ethanol by pyruvate decarboxylase and ADH plays a central role in hypoxia response. *ADH* expression is actively induced by RAP2.12 in *Arabidopsis* [68].

In *Hevea*, *ADH*, *SUS1*, *SUS4* and *HB1* were transcribed in all tissues and especially in latex, with the *ADH* presence of two contigs (CL1Contig11114, CL1536Contig2), *HB1* presence of one contig (CL2139Contig4), and *SUS1* presence of four contigs (CL1Contig20718, CL33Contig19, CL33Contig3 and CL33Contig8) (Supplementary Table 9). Their corresponding enzyme activities had also been recorded in latex for long [69]. The presence of *cis*-acting elements involved in anaerobic response (ANAERO) and the sugar or starch content (amylase box, CGACG, BP-5, TATCCAY) in the promoter sequence of four *HbERF-VII* genes also revealed potential transcriptional regulation by the metabolism.

Response to low oxygen concentration in latex and regulation of the rubber production

Sucrose is the source of carbon and energy for the biosynthesis of natural rubber. AcetylCoA generated by glycolysis is used by the mevalonate pathway to produce isopentenyl pyrophosphate (IPP). Then, IPP is the precursor for elongation of the polyisoprene chain in the rubber particles. Latex regeneration after tapping requires a lot of energy from glycolysis. A hypoxic condition was suggested in laticifers since the fermentative pyruvate metabolism is the main route of sugar degradation in latex cytosol [70]. The intermediate product, pyruvate, can be used by both aerobic and anaerobic pathways to generate acetyl CoA [69]. In addition, oxygen consumption was observed for two latex-specific organelles, in the lutoids by peroxidase and NADH-quinone reductase, and the Frey-Wyssling particles by *o*-diphenoloxidase [69]. Lutoidic NAD(P)H oxidase generates toxic forms of oxygen such as superoxide anions, which are involved in lipid peroxidation of lutoids. Coagulant factors are released from the damaged lutoids and lead to the aggregation of rubber particles [71]. This *in situ* coagulation of rubber particles reduces the latex flow after tapping. This physiological syndrome is called Tapping Panel Dryness (TPD). In TPD-affected trees, the consumption of oxygen by NADH-Cytochrome-c-oxidoreductase from lutoids was particularly high [72]. According to these authors, the NADH-dependent consumption of oxygen was inhibited by superoxide dismutase activity [73].

The involvement of oxygen in the latex metabolism and TPD syndrome and the tolerance of rubber trees to wounding suggest that some HbERF-VIIs might play an important role in latex production. Indeed, three (*HbERF-VIIa04*, *HbERF-VIIa07*, *HbERF*-VIIa12) of the six *HbERF- VIIs* identified in this study as expression marker genes are highly regulated in latex and are orthologs to AtEBP/RAP2.3 and AtERF74/RAP2.12. Another *HbERF-VII* gene, *HbERF-VIIa17* ortholog genes to *AtEBP*, might play a role in the response to the accumulation of reactive oxygen species generated during latex regeneration. In addition, three *HbERF* genes induced upon laticifer differentiation [20], correspond to three members of group VII (HbERF-VIIa3, HbERF-VIIa17 and HbERF-VIIa1) according to Duan and coll. [11]. In the present work, we showed that HbERF-VIIa3 corresponds to the new HbERF-VIIa4. Further characterization of the *HbERF-VII* genes and their target genes should lead to the identification of new functions in *Hevea brasiliensis*.

Material and methods

Plant material

For the *Hevea* transcriptome analysis, plant material of clone PB 260 was grown according to the conditions described in Duan and coll. [11]. Reproductive tissue-type samples (immature and mature male and female flowers, zygotic embryos) were supplemented in this study as plant material. Immature and mature male and female flowers were collected at the Chachoengsao rubber research centre from 15-year-old trees. Zygotic embryos were collected at IRRI's Sembawa Centre from 5-year-old trees. For the relative transcript abundance analysis, plant material was the same as for the transcriptome analysis, except latex and bark, which were harvested from 5-year-old trees without ethylene treatment and collected at IRRI's Sembawa Centre.

Total RNA isolation

All samples were frozen in liquid nitrogen and stored in the freezer at -80°C pending total RNA extraction. Total RNAs were isolated using the caesium chloride cushion method adapted from Sambrook and coll. [74] by Duan and coll. [75]. One gram of fresh matter was ground and transferred to a tube containing 30 mL of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone and 1% ß-mercapto-ethanol. After homogenization, tubes were kept on ice and then centrifuged at 10,000 g at 4°C for 30 min. The supernatant was transferred to a new tube containing 8 mL of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 89,705 g at 20°C for 20 h. The supernatant and caesium cushion were discarded whilst the RNA pellet was washed with 70% ethanol. After 30 min of air drying, the pellet was dissolved in 200 µL of sterile water. Although DNA could not cross the caesium cushion for this centrifugation condition, DNA contamination was checked by PCR amplification using primers of the Actin gene including the intron sequence. RNAs were stored at -80°C.

Sequencing technique and contig assembly

Total RNA samples of each reproductive tissue were pooled together. Single-stranded cDNA was synthesised from pooled RNA samples. Pyrosequencing was carried out using a GS FLX (Roche / 454) Titanium run (Roche Applied Science) by the GATC-Biotech company in Germany. A 454 sequencing half-run (200 Mbp) generated more than 500,000 reads with an average read length of 400 bp for each library according to the manufacturer. Reads were analysed using the ESTtik tool (Expressed Sequence Tag Treatment and investigation kit) [76] modified for the analysis of 454 data items. Reads were first cleaned to avoid mis-assembly by discarding sequences that were both lower than 120 bp and of low complexity. We then discarded non-coding reads by comparing the reads against the fRNAdb database using the Megablast algorithm with an e-value cutoff of 1e-20 [77]. More than 400,000 cleaned reads were obtained for each library. Reads were then assembled in contigs using the TGICL program

[78], integrated in the ESTtik pipeline (Figure 1). The removal of poor end regions of reads and the computation of overlaps between reads was done using the default parameters of the CAP3 program (best hit cut-off for difference -b = 20; best hit for clipping –c = 12) [79]. Clustering was carried out for reads with an overlap of at least 60 bp and 94% identity between reads. The second step was an assembly of reads from each cluster with greater stringency: the length of sequence overlap was then 60 bp with 95% identity between reads. The transcript sequence database consisted of contigs.

The annotation of contigs was processed using Orfpredictor to obtain the longest ORFs leading to an Interproscan annotation. Then, all contigs were aligned to the NCBI non-redundant protein database using BLASTX to obtain an xml output format. Lastly, we treated the Interproscan and BLASTX results as the input files of the Blast2Go program and generated the output file listing the GO number for each annotated contig. By using this file, combining their GO databases (component.ontology, function.ontology, and process.ontology) downloaded from the GO website, we generated the figure using our own perl program.

Primer design and analysis of transcript abundances by real-time RT-PCR

Several rules were applied in order to reduce the risk of error in relative gene expression data. The integrity of total RNA was checked by electrophoresis. Primers were designed at the 3' side of each sequence in order to reduce the risk of error due to short cDNA synthesis using the Primer 3 module of Geneious (Biomatters Ltd., New Zealand). Real-time PCR amplification and the fusion curve were carried out using a mix of cDNAs in order to check the specificity of each pair of primers. Primer sequences are listed in Supplementary Table 10.

cDNAs were synthesized from 2 µg of total RNA to the final 20 µL reaction mixture using a RevertAidTM M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Full-length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA using primers at the cDNA ends. Quantitative gene expression analysis was finally carried out by real-time RT-PCR using a Light Cycler 480 (Roche, Switzerland). Real-time PCR reaction mixtures consisted of 2 µL RT product cDNA, 0.6 µL of 5 µM of each primer, and 3 µL 2 × SYBR green PCR master mix (LightCycler® 480 SYBR Green I Master, Roche Applied Sciences) in a 6-µL volume. PCR cycling conditions comprised one denaturation cycle at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 60°C for 15 s, and 72°C for 20s). Expression analysis was carried out in a 384-well plate. Samples were loaded using an automation workstation (Biomek NX, Beckman Coulter).

Real-time PCR was carried out for eleven housekeeping genes in order to select the most stable gene as the internal control for all the compared tissues (*HbelF1Aa, HbUBC4, HbUBC2b, HbYLS8, HbRH2b, HbRH8, HbUBC2a, HbalphaTub, Hb40S, HbUbi, HbActin*) (Supplementary Table 10). *HbRH2b* was selected as the best reference gene due to its stability

in tissues from immature and mature male and female flowers, zygotic embryos, latex and bark. The homogeneity of the *HbRH2b* gene Cp confirmed that it could be used as an internal reference gene (Supplementary Table 11). The *HbRH2b* gene was amplified in each reaction plate in parallel with target genes. The transcript abundance level for each gene was relatively quantified by normalization with the transcript abundance of the reference *HbRH2b* gene. Relative transcript abundance took into account primer efficiencies. All the normalized ratios corresponding to transcript accumulation were calculated automatically by Light Cycler Software version 1.5.0 provided by the manufacturer using the following calculation: Normalized Ratio = Efficiency $^{-\Delta(Cp\ target-Cp\ RH2b)}$.

Statistical data analyses

Real-time PCR reactions were set up with three biological replications. Statistical analysis was performed with an ANOVA after logarithmic transformation of raw data. The ANOVA was followed by a Student Newman-Keuls test when the values of relative transcript abundances were compared for immature and mature flowers, zygotic embryos, latex and bark. Values with the same letter did not differ significantly at the 0.05 probability level.

Phylogenetic analysis of the AP2 domain from ERF marker genes

A multiple alignment analysis was performed on full-length AP2 domain sequences from *Hevea* and *Arabidopsis* for the ERF (groups I, II, III, V, VI, VI-L, VII, VIII, IX and X) and from five dicots (*Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera*, *Malus×domestica* and *Hevea brasiliensis*) and two monocots (*Oryza sativa* and *Zea mays*) (Supplementary Figures 1-11, Figure 4).The full AP2-domain sequences derived from *Hevea*, *Arabidopsis*, *Populus*, *Vitis*, *Malus×domestica*, *Oryza* and *Zea mays* AP2-domain proteins of around 60 amino acids were then aligned using MUSCLE software [80, 81] which uses a progressive multiple alignment method. The alignment was curated by Gblocks software [82], searching for at least 10-amino-acid-long conserved blocks, and the block with 57 amino acids was extracted. This block of 57 amino acids was used to construct the phylogenetic tree using PhyML software [83], which implements a maximum likelihood tree reconstruction method, using the LG+gamma model, starting from a BioNJ tree [84]. A RAP-Green analysis was performed from the initial PhyML tree to improve gene function inferences and predict gene duplications in phylogenetic trees [85]. The final tree was drawn and displayed with the Archaeoptheryx program, and rooted on the branch separating the RAV family from the rest of the tree. Branch supports were computed using the aLRT-SHlike method [86].

***Cis*-acting element analysis and determination of conserved motifs**

In silico promoter analysis was searched again in the PLACE database [25] and The Plant CARE database [24]. The *Hevea* genomic scaffold was provided for the *HbERF-VIIa7*, *HbERF-VIIa17* and *HbERF-VIIa20* genes by the CATAS-BIG *Hevea* Genome Project coordinated by Prof Chaorong Tang and Prof Songnian Hu. A 2000 bp sequence upstream from

the start codon was scanned for the presence of putative *cis*-acting regulatory elements using the database associated search tools. The number of copies for each *cis*-acting element was then counted.

Conserved motifs were investigated by multiple alignment analyses using ClustalW and MEME version 3.0 [26].

Subcellular localization and transcriptional activity tests by transient expression in a single cell system

GFP C-terminal fusions were obtained with *HbERF-VIIa7, HbERF-VIIa17* and *HbERF-VIIa20* and used for tobacco protoplast BY-2 transfection according to [87]. The subcellular location of the fluorescence was determined after 20 hours using a DM4500 microscope (Leica).

A synthetic reporter construct (4XGCC-GFP) was used [59]. Effector constructs were generated by fusing the 35S promoter to the CDS of the genes (*HbERF-VIIa7, HbERF-VIIa17* and *HbERF-VIIa20*). For transient assays, tobacco (*Nicotiana tabacum*) BY-2 protoplasts were co-transformed with reporter and effector constructs [87]. Transformation assays were performed in three independent replicates. After 16 h, GFP expression was analysed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences) on a flow cytometry platform (MRI). Data were analysed using Flowing software. For each sample, 100–400 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponded to the average fluorescence intensity of the cell population after subtraction of autofluorescence determined with non-transformed BY-2 protoplasts. The data were normalized using an experiment with protoplasts transformed with the reporter vector in combination with the vector used as the effector plasmid, but lacking the *HbERF* coding sequence.

List of abbreviations used

AIL: AIntegumenta-Like; ANT: AINTEGUMENTA; AP2: APETALA2; CBF: Cold responsive element binding factor; DREB: Drought Responsive Element Binding protein; ERF: Ethylene Responsive Factor; PCR: Polymerase Chain Reaction; RAP2: Related to APETALA2; RAV: Related to ABI3/VP1; RT: Reverse transcriptase.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

MS carried out contig assembly and generated the transcript sequence database. MY carried out GO annotations. PP and KT prepared the flower samples. PP and RP carried out the phylogenetic analyses, RNA isolation and design of primers. PP performed real-time RT-PCR analyses. PP, FD and JP carried out subcellular localization and transactivation experiments. PP and PM planned the experiments. PP, MY, JP and PM participated in drafting the manuscript. All the authors read and approved the final manuscript.

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Supplementary data

Supplementary Figure 1. Phylogenetic tree of ERF group I. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 2. Phylogenetic tree of ERF group II. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 3. Phylogenetic tree of ERF group III. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 4. Phylogenetic tree of ERF group IV. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 5. Phylogenetic tree of ERF group VI. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 6. Phylogenetic tree of ERF group VI-L. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 7. Phylogenetic tree of ERF group VII. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 8. Phylogenetic tree of ERF group VIII. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 9. Phylogenetic tree of ERF group IX. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 10. Phylogenetic tree of ERF group X. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Arabidopsis* (blue letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model. Expression marker genes are indicated in bold letters.

Supplementary Figure 11. Phylogenetic analysis of ERF group VII using the 6 *Hevea brasiliensis*, 15 *Oryza sativa* and 2 *Oryza nivara* members. The deduced amino acid sequences of the AP2 domain from *Hevea* (black letter) and *Oryza sativa* and *Oryza nivara* (green letter) were aligned using Muscle, and the phylogenetic tree was constructed using PhyML with an LG+T model.

Discussion générale

1. Enrichment of the RNAseq database for the improvement of the AP2/ERF sequences

Duan *et al.* identified 142 AP2/ERFs according to full length of AP2 domain from the *Hevea* clone PB 260 transcriptome, which were produced from somatic embryogenic, leaf, bark, latex and root tissues. A new transcriptome was supplemented with a reproductive tissue-type library (immature and mature male and female flowers, zygotic embryos). The present work led to identify 114 contigs, which were confirmed by analysis of genomic scaffold from the *Hevea* clone CATAS 7-33-97. The absent of some tissues in our libraries such as cambium and xylem could explain the lack for some ERF genes. Another explanation of the difference in AP2/ERF gene number between transcriptome and genome analysis could come from the difference of clone. The clone CATAS 7-33-97 is adapted to cold winter in the Hainan province. We can note one additional gene for the ERF group III (correspond to DREB1/CBF) and three genes for the group IV (DREB2). These two groups are known to be involved in cold and drought tolerance.

2. Identification of AP2/ERF with putative important function in *Hevea*

Among the AP2/ERF family, some members were identified as expression marker genes (EMG), highly expressed in *Hevea* tissues and with putative function related to latex production based on phylogenetic and bibliographical analyses. We are aware that probably not all EMG have important function in *Hevea*. Moreover, some other AP2/ERF members, which are not EMG, could also have important function because the screen based on gene expression allowed identifying only transcriptional regulations. Given post-transcriptional regulation play also an important role in the regulation of AP2/ERF, we have to pay attention on all levels of regulation.

2.1 Transcriptional and post-transcriptional regulation

Transcriptional regulation in non-stressed condition

AP2/ERF superfamily is involved in the control of primary and secondary metabolism, growth and developmental programs (Licausi, Ohme-Takagi and Perata 2013a) AP2/ERF transcription factors have been identified and investigated in several plants, including Arabidopsis, rice (Nakano et al. 2006, Sharoni et al. 2011), grape (*Vitis vinifera*) (Licausi et al. 2010a), poplar (*Populus tricocarpa*) (Zhuang et al. 2008), wheat (*Triticum aestivum*) (Zhuang et al. 2011), tomato (*Solanum lycopersicon*) (Pirrello et al. 2012), soybean (*Glycine max*) (Zhang et al. 2008) and rubber tree (*Hevea brasiliensis*) (Duan et al. 2013). In *Hevea*, 142 AP2/ERF genes consisting of 20 *AP2s*, 115 *ERFs*, 4 *RAVs* and 3 *Soloists* were identified (Duan et al. 2013). In this study, *AP2/ERF* genes were observed in various tissue such as somatic embryogenesis-derived tissue, vegetative tissue (leaf, bark and latex), reproductive tissue (flower and seed) under non stress conditions, 52 genes showed high or low relative transcript abundance significantly in specific tissues and were assigned as tissue-specific EMG. Thereby, *AP2/ERF*

genes displayed a regulation in somatic embryogenesis, flower development and latex production in *Hevea*. Licausi *et al*. reported that during the ripening of grape, 31 *ERF* genes were up-regulated at least 4 fold in the fruit skin tissue. Although grape is a non-climacteric fruit which ripen without ethylene, ethylene still plays a crucial role during development and ripening processes such as anthocyanin accumulation and alcohol dehydrogenase expression (Licausi et al. 2010a). Sixteen tomato *ERF* genes preferentially expressed in reproductive tissues and the other 5 *ERFs* expressed in vegetative tissues (Pirrello et al. 2012). *AP2* and *RAV* genes are the main actors in the determination of cell fate and development from meristems to fully developed organs. Six out of 20 *HbAP2* genes are EMG involved in somatic embryogenesis, fruit and flower development. *HbAP2-1*, *HbAP2-3* and *HbAP2-7* are orthologous to *BBM*, *AIL6* or *AIL7* and *ANT* respectively. *BABY BOOM* (*BBM*) plays important roles in cell proliferation and embryogenesis in *Arabidopsis thaliana* (*AtBBM*) and *Brassica napus* (*BnBBM*). Constitutive expression of *GmBBM1* in Arabidopsis induced somatic embryos on vegetative organs and other pleiotropic effects on vegetative organ development (El Ouakfaoui et al. 2010). Several members of the *AINTEGUMENTA-LIKE/PLETHORA* (*AIL/PLT*) transcription factor family, including *AINTEGUMENTA* (*ANT*) and *AIL6/PLT3*, are important regulators of cell proliferation in flowers. The *ant ail6* double mutants revealed that these two transcription factors regulate both proliferation and differentiation in flowers (Krizek and Eaddy 2012, Krizek 2003). In addition, *ANT* is required for ovule initiation and development. This gene regulates organ initiation and organ size by maintaining the proliferative potential of organ primordial (Klucher et al. 1996).

Post-transcriptional regulation

Although this present study focused on transcriptional regulation, we are aware that AP2/ERF can be regulated at the post-transcriptional level. For instance, alternative splicing, control of the protein stability, acetylation, nitrosylation have been shown in the literature (for review (Licausi et al. 2013a)). A high transcript accumulation was observed in all studied tissues for almost all members of HbERF group I, VII and VIII. This constitutive expression of some *HbERF* genes led us to consider a post-transcriptional regulation to control the activity of these proteins according to developmental and environmental responses.

Many members of the groups II, III and IX of *Vitis vinifera* and group VII of *Arabidopsis* were highly expressed in all the tissues examined. The relatively high expression of members, under non stress conditions, may represent a default defence system, which was already present before the pathogen attack or the environmental stress (Licausi et al. 2010a). DREB2s belong to *ERF-IVa* containing a Ser/Thr-rich region (a putative phosphorylation site) involved in post-translational modification. A deletion of a Ser/Thr-rich motif, led to a constitutively active form of AtDREB2A (Sakuma et al. 2006). For example, overexpression of *Arabidopsis AtDREB2A* and rice *OsDREB2A* did not activate downstream genes under normal growth conditions, suggesting that AtDREB2A and OsDREB2A require post-translational modification for activation (Dubouzet et al. 2003, Liu et al. 1998). The overexpression of *TaAIDFa* (wheat DREB gene), which encodes a protein lacking a Ser/Thr-rich region, activated transcription of *RD29A*, *COR15A*, and *ERD10* in transgenic *Arabidopsis* plants. Therefore, the putative phosphorylation sites on these DREB-2 type proteins are possible motifs for negative regulation (Xu et al. 2008).

DREB2s were also reported to be post-transcriptionally regulated by alternative splicing like *HvDRF1* in barley (Xue and Loveridge 2004), *Wdreb2* in wheat (Egawa et al. 2006), *ZmDREB2A* in maize (Qin et al. 2007), and the SIERF2 in tomato (Pirrello et al. 2012) and *OsDREB2B* in rice (Matsukura et al. 2010). These genes have two types of transcripts: first is an

inactive transcript with a remaining intron which generates s a stop codon before the DNA-binding domain, and second is an active transcript that can encode a full-length protein. This post-translational regulation was observed in ERF group VII.

The plant homeostatic response to hypoxic condition operate via N-end rule pathway (Sasidharan and Mustroph 2011). Under normal condition, ERF-VII transcription factors are targeted by proteolysis at N-terminal, which possessed the N degron. An N degron contains a destabilizing residue (initiating motif Met-Cys), which is created through specific proteolytic cleavage and led to protein degradation (Licausi, Pucciariello and Perata 2013b). Gibbs et al. reported that plants lacking components of the N-end rule pathway constitutively express core hypoxia-response genes and are more tolerant of hypoxic stress (Gibbs et al. 2011). In Arabidopsis, the *RAP2.2* gene is a member of ERF-VII and was constitutively expressed in roots. Its overexpression resulted in improved the hypoxia stress tolerance in plant (Hinz et al. 2010).

MicroRNAs (miRNAs) play a key function in responses to abiotic stress at post-transcription level and were recently reported for the regulation of AP2/ERF. Several microRNAs were predicted to inhibit *Hevea* AP2/ERF transcripts (Duan et al. 2013). Although inhibition mostly involved a transcript cleavage, the inhibition of translation was predicted for nine genes (*HbERF-Ib5, HbERF-IIa1, HbERF-VI5, HbERF-VIIa4, HbERF-VIIIa7, HbERF-IXc3, HbERF-IXc5, HbERF-Xa1, HbSoloist3*). Further degradome analysis should lead to the validation of this type of post-transcriptional regulation (pers. Comm. Julie Leclercq).

2.2 Identification of functions for some *HbAP2/ERF*

For expression marker gene, we could predict function for some *HbAP2/ERF* genes based on the analysis of their orthologous genes in *Arabidopsis* (Table 3). Fifty-two genes were identified as EMG from the characterization AP2*/ERF* genes which were differentially expressed during somatic embryogenesis process and in various tissues of mature tree. Twenty genes were orthologous to *Arabidopsis* genes. Their putative functions could be predicted. Anyway, we are aware that some EMG without orthologous genes in *Arabidopsis* could have specific function in *Hevea*. For instance, *HbERF1, HbERF2, HbERF3* and *HbRAV1* genes were suggested to be induced by JA in bark during JA-induced laticifer differentiation (Wu et al. 2010). These tree *ERF* genes correspond in our classification to *HbERFVIIa4, HbERF-VIIa17* and *HbERF-VIIa1*. Finally, most of EMG should not be directly involved in some functions but could be used as expression marker to predict the embryogenic capacity in callus under the early stage of development.

Table 3. List of EMG (Expression Marker Genes) identified during the somatic embryogenesis process, and in the *Hevea* tissues.

Gene	Expression marker gene		Phylogenetic analysis			
	Somatic embryo process	Mature tree	Orthologous gene	Acc. No.	Putative Function	Reference
HbAP2-1	R-ENT	-	BBM	At5g17430	Promote cell proliferation and morphogenesis during embryogenesis	(Boutilier et al. 2002, Passarinho et al. 2008)
HbAP2-3	R-EXP	-	AIL7/PLT7	At5g65510	Regulate radial pattern formation process of a shoot apical meristem.	(Prasad et al. 2011)
		-	AIL6/PLT3	At5g10510	Regulation of floral meristem growth	(Krizek and Eaddy 2012)
HbAP2-5	R-ENT	-	-	At2g41710	Unknown	-
HbAP2-7	R-EXP	-	ANT/DRG/CKC/CKC1	At4g37750	Regulate up- regulate genes establishing organ polarity and those specifying organ identity	(Elliott et al. 1996, Nole-Wilson, Tranby and Krizek 2005)
HbAP2-12	R-ENT	-	AP2/FLO2/FL1	At4g36920	Meristem maintenance and cell differentiation	(Jofuku et al. 1994, Würschum, Groß-Hardt and Laux 2006)
HbAP2-18	R-ENT	-				
HbERF-Ia1	R-ENT, Nor-em	-	ERF53	At2g20880	Regulate drought-responsive gene expression	(Cheng et al. 2012)
HbERF-Ib4	-	Mature male flower	-	At4g39780	-	-
HbERF-Ib7	Nor-em		-	-	-	-
HbERF-Ib11	-	Mature male flower	-	-	-	-
HbERF-IIa3	-	Latex	-	-	-	-
HbERF-IIb1	-	Leaf	-	-	-	-
HbERF-IIb2	-	Leaf	-	At1g19210	Unknown	-
HbERF-IIb4		Latex	-	-	-	-
HbERF-IIIa1	Nor-em	-	-	At1g01250	Unknown	-
HbERF-IIIb1		Latex	-	At1g63040	Unknown	-
HbERF-IIIb2	R-EXP	-	-	At1g63040	Unknown	-
HbERF-IIIc1	*R-EXP*	-	CBF4/DREB1D	At5g51990	Response to drought stress and abscisic acid	(Haake et al. 2002)
HbERF-IIIe2	-	Mature male flower	TINY	At5g25810	Suppressed cell proliferation and exhibited pleiotropic effects	(Wilson et al. 1996)
HbERF-IIIe3	R-EXP		TINY	At1g77200		
HbERF-IIIe4	-	Latex	-	At4g32800	Unknown	-
HbERF-IVa2	R-EXP, Nor-em		*, DREB2A*	At5g05410	Multiole abiotic stress response	(Lata and Prasad 2011)
HbERF-IVa3	R-EXP	Latex	-	-	-	-
HbERF-Vb2	Leaf	-	-	-	-	-
HbERF-VI1	Nor-em	Mature male flower	-	-	-	-
HbERF-VI2	R-ENT		CRF2/TMO3	At4g23750	Relate to root initiation at later embryonic stages	(Rashotte et al. 2006, Schlereth et al. 2010)
HbERF-VI3	-	Zygotic embryo	-	-	-	-
HbERF-VI4			-	-	-	-
HbERF-VI5	-	Latex	-	-	-	-
HbERF-VI-L3	-	Mature male flower				

HbERF-VI-L4	-	Mature male flower	-	-	-	-
HbERF-VI-L6	-	Latex	-	-	-	-
HbERF-VIIa1	Nor-em	Zygotic embryo and cotyledon	-	-	-	-
HbERF-VIIa4	R-ENT,	Latex	-	-	-	-
HbERF-VIIa7	-	Latex	RAP2.12	At1g53910	Low oxygen sensing	(Bailey-Serres et al. 2012)
HbERF-VIIa12	-	Bark and latex				
HbERF-VIIa17	R-EXP	Bark	AtEBP/RAP2.3/	At3g16770	Jasmonate and/or ethylene	(Li, Xiao and Chye 2008)
HbERF-VIIa20	-	Leaf				
HbERF-VIIIa3	R-ENT, R-EXP		-	-	-	-
HbERF-VIIIa8	-	Latex	-	-	-	-
HbERF-IXa2	-	Immature female flower, cotyledon	-	-	-	-
HbERF-IXa3	-	Latex	AtERF1	At4g17500	Activators of GCC box–dependent transcription	(Fujimoto et al. 2000)
HbERF-IXb1	-	Latex	-	-	-	-
HbERF-IXb2	R-EXP			At5g07580	Unknown	-
HbERF-IXb3	-	Latex	ERF5	At5g47230	Activators of GCC box–dependent transcription	(Fujimoto et al. 2000)
HbERF-IXc4	R-EXP	High in bark				
HbERF-IXc5	-	High in bark				
HbERF-Xa1	-	Low in leaf	Rap2.6L	At5g13330	Expression drought-responsive gene	(Krishnaswamy et al. 2011)
HbERF-Xa2	-	Immature female flower				
HbERF-Xa4	-	Latex	-	-	-	-
HbERF-Xa6	-	Leaf	ERF110	At5g50080	Regulation of bolting time	(Zhu et al. 2013)
HbRAV-3	R-EXP	-	-	-	-	-

For highly expressed of *AP2/ERF* genes, even if they are not considered as EMG specifically expressed in one tissue or at a certain stage of development, the high level of expression led us to suggest their involvement in some regulation. HbERFs from group VII had high expression in latex and HbERF from group I and VIII were highly expressed in all tissues.

A characteristic feature of ERF-VIIs is a MCGGAI(I/L) motif at the N-terminal (Nakano et al. 2006) associated with the N-end rule pathway of targeted proteolysis. The removal of the N-terminal methionine by methionine aminopeptidase (MAP) leaves the cysteine residue exposed and destabilized. Under normal oxygen concentration condition (normoxia), the N-terminal cysteine is oxidized by oxygen or nitric oxide. Oxidized cysteine induced the addition of an arginyl residue at the N-terminus and promoted polyubiquitination, which led to the degradation of ERF-VII (Licausi 2013, Licausi et al. 2013b). The *Arabidopsis* ERF-VII have been reported to be regulated by hypoxia or anoxia responses, consisting of *HYPOXIA RESPONSIVE1* and *2* (*HRE1* and *HRE2*), *RELATED TO AP2.2* (*AP2.2*) and *RELATED TO AP2.12* (*RAP2.12*). Overexpression of *RAP2.2* provided in improved plant survival under hypoxia and was induced by ethylene. In addition, *RAP2.2* affects only part of the low-oxygen response, particularly the induction of genes encoding sugar metabolism and fermentation pathway enzymes, as well as ethylene biosynthesis genes (Hinz et al. 2010). Over-expression of *HRE1* showed an improved tolerance to anoxia. *HRE1* might play a negative regulatory role in modulating ethylene responses (Yang et al. 2011, Licausi et al. 2010b).

ERFs from group II and VIII contained EAR motif, which are repressors of transcription (Ohta et al. 2001, Yang et al. 2005). These ERF repressors can induce cell death (Ogata et al. 2013). The multi-functional co-repressor, TOPLESS (TPL) and TPL-related (TPR) interact directly with the EAR motif of ERF-VIIIs. The interaction of TPL/TPR and transcription factors has been co-opted multiple times to modulate gene expression in diverse biological pathways, including hormone signalling, stress responses, and the control of flowering time (Causier et al. 2012). *AtERF3*, *AtERF4*, *AtERF7*, *AtERF10*, *AtERF11*, *LEP* and *ESR1/DRN* belong to ERF-VIII. Nevertheless, these genes showed differential expressions with different roles during plant growth and development; and in response to environmental stress. For instance, *AtERF4* and *AtERF7* play an important role in ABA responses (Yang et al. 2005, Song et al. 2005), Overexpression of *AtERF7* increases ABA sensitivity in guard cells (Song et al. 2005). *LEP* is involved in cell division activity in the marginal meristem and function in leafy petiole development as well as inflorescence branching (van der Graaff et al. 2000). *ESR1/DRN* is involved in petiole development and embryo patterning and cotyledon development (Chandler et al. 2007). Overexpression of genes encoding EAR motif-containing proteins led to repression of DREB1A/CBF3 or DREB2A and their target genes are up-regulated (Maruyama et al. 2004, Mizoi, Shinozaki and Yamaguchi-Shinozaki 2012). The EAR motif is found in all HbERF-II and HbERF-VIII. The high expression of *HbERF-VIII* genes in all *Hevea* tissues suggests post-transcriptional regulation of other ERFs through their role of repressors.

Two genes in ERF group I are well known the function (WOUND INDUCED DEDIFFERENTIATION 1 (WIND1) and RAP2.4). WIND1 is associated with the control of wound-induced cell dedifferentiation in Arabidopsis. These gene is rapidly induced at the wound site, and promotes cell dedifferentiation, lead to cell proliferation to form a mass of pluripotent cells termed callus (Iwase et al. 2011). *RAP2.4* is highly expressed in stems and roots and is differentially induced in response to cold, dehydration and osmotic stress. Moreover this gene

was suggested that play a role in the regulation of water homeostasis (Rae, Lao and Kavanagh 2011). Given we do not have any ortholog genes in *Hevea*, we should further characterize *HbERF-I* genes highly express in *Hevea*.

Finally, some ERF groups have very important function that are related to the latex metabolism such as the response to jasmonate (group IX), to hypoxia (group VII). Group IX is known to be involved in JA induced secondary metabolism, 3 (*HbERF-IXa3*, *HbERF-IXb1* and *HbERF-IXb3)* among 7 EMGs of *HbERF-IX* were identified as latex EMG based on their significant low transcript level. Contrastingly, *HbERF-IXc4* and *HbERF-IXc5* had high transcript accumulation in bark and were assigned to bark EMG. In *Hevea*, 12 members belong to ERF group IX and were classified into 3 subgroups. The subgroup IXc (*HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6*) contains putative orthologous genes to the *Arabidopsis ERF1* genes (Duan et al. Submitted). The expression of *ERF1* can be activated rapidly by ET or jasmonate (JA) and can be activated synergistically by both hormones (Lorenzo et al. 2003). In response to biotic stress, ERF1 bound to GCC boxes but not DRE elements; conversely, under abiotic stress, ERF1 was exhibited specific binding to DRE elements (Cheng et al. 2013). Duan *et al.* studied the relative transcript abundance in bark tissue of juvenile plants for all *HbERF-IX* group genes in response to mechanical wounding (W), methyl jasmonate (MeJA) and ethylene (ET) in order to check the specificity of gene induction in response to these factors. *HbERF-IXb2* was significantly down-regulated by W x MeJA x ET treatment. Contrastingly, the *HbERF-IXc4*, *HbERF-IXc5* and *HbERF-IXc6* genes were dramatically increased by these combined treatments (Duan et al. Submitted).

3. Functional analysis of AP2/ERFs with putative important function

Some *AP2/ERF* genes could be putatively involved in the control of latex production. Sucrose is carbon source for rubber biosynthesis. Besides the aerobic pathway generating ATP and acetyl CoA, anaerobic pathway exists and could be control by HbERF-VIIs. According to Jacob and coll., mitochondria are almost absent in the latex collected during tapping of mature laticiferous vessels (Jacob, Prévôt and Kekwick 1989, Tupý and Resing 1968). In addition, the large quantity of CO_2 present mainly in the cytosol serum is produced by decarboxylase activities. Very low oxygen concentration appears to be present in collected latex and serum. All this information led to consider that the rubber synthesis is mainly performed by an anaerobic metabolism (Jacob et al. 1989, Tupý and Resing 1968). Both of bark and latex tissues are likely to be prone to hypoxia. Bark of trees is known to have low oxygen concentration. Ethylene was shown to be an efficient stimulant of latex yield by increase latex metabolism. For instance, ethylene activates the expression of sucrose transporter genes (Dusotoit-Coucaud et al. 2009). Metabolic activities are also known to consume oxygen and generate reactive oxygen species. Interestingly, expression marker genes from group VII are likely to be activator of hypoxia-responsive genes (*HbADH*, *HbSUS1*, etc.). The deep characterization of HbERF-VII should clarify their involvement in latex production. Hypoxia stress and/or ethylene treatment could be investigated. The regulation of expression marker genes, hypoxia responsive genes (*ADH*, *SUS4*

and *PDC1*) and sucrose transporters (*HbSUT1A* and *HbSUt2A*) upon both anaerobic and aerobic conditions should lead to a better understanding of latex production.

Rubber biosynthesis pathway belongs to the secondary metabolism. For that reason, we are thinking that ERF from group IX could play an important role in the control of latex metabolism. *HbERF-IXc4* and *HbERF-IXc5* are dramatically overexpressed by the combination of ethylene and MeJA treatments (Duan et al. Submitted). Further characterization of the genes will require the identification of their regulons (target genes) by CHIP-seq, and functional analysis of these genes in transgenic lines. Overexpression and silencing of candidate genes have been initiated. For instance, transgenic plants overexpressing *HbERF-IXc4* and *HbERF-IXc5* have been regenerated (PhD, Retno Lestari). In order to avoid dwarf phenotypes usually observed with ERF transformants, the *HEV2.1* latex-specific promoter have been used to drive the expression of these genes into the laticifeous cells and in the leaves.

We also propose to consider the use of inducible promoter to control the expression of the target, and to silence the gene expression using RNAi, amiRNA or CREST technologies. Based on our experience, we propose to study the following candidate *HbERF-VIIa07, HbERF-VIIa17, HbERF-VIIa20, HbERF-IXc4* and *HbERF-IXc5.*

4. Use of expression marker genes

4.1 Application of expression marker genes for somatic embryogenesis

The process of callus multiplication and plant regeneration is very long. The variability of callus lines for embryogenic capacity hampers the commercial development of this technology. The prediction of the embryogenic capacity at the early state of the embryogenesis process, especially during the callus proliferation, will be helpful. The validation of the EMG found in ENT culture (*HbSAMS, HbACS2, HbETR1, HbERF-Ia1, HbERF-VI2, HbERF-VIIa4, HbERF-VIIIa3, HbAP2-1, HbAP2-5, HbAP2-12* and *HbAP2-18*) could be carried out for lines from clones available in our laboratory (clones RRIM 600, Reyan 88-13, etc.). Then validated markers could be used for screening the new established lines for any new clones.

The characterization of expression marker genes could be also implemented to understand their role in somatic embryogenesis and maybe able to elucidate why some clones are recalcitrant to somatic embryogenesis. Identification of putative functions for somatic embryogenesis marker genes based on phylogenetic analysis enabled us to predict the function of some markers. Some *Hevea* markers have orthologous genes in *Arabidopsis* with important function in somatic embryogenesis: HbERF-IIIe3 with TINY, HbERF-VI2 with TMO3, HbAP2-1 with BBM, HbAP2-3 with AIL7/AIL6, HbAP2-7 with ANT, and HbAP2-12 and HbAP2-18 with AP2. So these markers should be tested to validate the prediction of regeneration potential

of proliferating callus lines. This opens up prospects for their application in selecting lines of interest for large-scale propagation.

4.2 Application of EMG for ethylene response in latex production

Verification of latex and/or bark EMGs: eighteen latex and/or bark EMGs were identified in response to harvesting stress in clone PB 260 which was described as high yielding clone This clone has an active metabolism but responds poorly to ethylene stimulation. These set of EMG could be examined with clone PB 217, which is a low yielding clone with a lower metabolic activity, but responds highly to ethylene stimulation in order to observe the relative transcript abundance using real-time PCR in differential yielding clone and between stimulated and non-stimulated trees by ethylene.

Molecular genetic markers: some *ERF* expression marker genes could be used to develop molecular genetic markers of the capacity of clone to respond to ethylene (latex yield) if sequence polymorphism can be detected. The molecular genetic markers such as microsatellites (SSR) or single nucleotide polymorphism (SNP) marker could be generated from the eighteen latex and/or bark EMGs. The analysis of polymorphic loci could be done in several clones according to *Hevea* genomic database publication. The marker genes would be used for the construction of an integrated parental linkage map of *H. brasiliensis* base on an F1 mapping population and be able to identify the quantitative trait loci (QTL) maps.

5. Conclusion

Hevea brasiliensis is the major industrial crop for natural rubber (NR) production. The indigenousness of *Hevea* is South America and become an important industrial crop in Asia. At present, Thailand is the world's largest natural rubber producer. Budding is used for propagation since rubber tree is a heterogamous species. *In vitro* propagation techniques have been developed to clone various varietal types such as self-rooted, rootstock and rejuvenated plant material. CIRAD developed 'indirect Secondary Somatic Embryogenesis' (SSE) and cryopreservation technique for somatic embryogenesis procedure. Besides the improvement of propagation techniques, a lot of efforts have been conducted on latex and the whole plant physiology to better understand the mechanisms involved in rubber yield. Natural rubber (*cis*-1, 4-polyisoprene) is synthesized into rubber particles of laticifer. The laticifers are formed from chains of adjacent cells that are arranged in rings parallel to the vascular cambium. Between the laticifers in each ring, there are anastomoses and develop a laticifer network structure in the soft bark of the rubber tree. Biosynthesis of NR is affected by various plant hormones. In addition, the MVA pathway was proposed for isoprenoid biosynthesis but more recently, transcriptome studies have suggested the involvement of the MEP pathway. Both pathways coexist in laticifers and need sucrose as a precursor. Ethylene stimulation is known to increase latex flow, and acceleration of

sucrose metabolism. Nevertheless, the mechanism of ethylene action is not completely elucidated, especially in molecular aspect. AP2/ERF transcription factors regulate biological processes including development, hormones responses, adaptation to biotic and abiotic stresses. Our research attempt to identify among the AP2/ERF transcription factors superfamily the members that regulate biological process including development, hormones responses, and adaptation to biotic and abiotic stresses.

The main outputs of the PhD thesis are summarised in Figure 16:

- This study improved the transcriptome by supplementing RNA sequences with reproductive tissues. The contig were annotated in the Gene Ontology database and were classed using BLASTX. Fifty-eight transcription factor families were discovered on the transcriptome and the AP2/ERF transcription factor superfamily, the third largest family, was characterized. The genome database from *Hevea* clone CATAS 7-33-97 allowed identifying accurately the number of *HbAP2/ERF* genes. One hundred fourteen *HbAP2/ERFs* were identified.
- The relative transcript abundance of ethylene biosynthesis and *AP2/ERF* genes was analysed during the 4 steps of somatic embryogenesis for 3 callus lines with different plant regeneration potential in order to identify genes differentially regulated during that process. The expression marker genes found in proliferating callus will be useful for predicting the embryogenic capacity of callus, which is a long process and time-consuming. The relative transcript abundance analysis was also performed for *ERFs* in vegetative and reproductive tissues. High relative transcript abundance was observed in all tissues for almost all *ERFs* from group I, II, VII and VIII including somatic embryogenic tissues.
- According to sequence and bibliographical analyses, *ERF-II* and *ERF-VIII* are referred to repressor and *ERF-VIIs* are known to regulate hypoxia-responsive genes. The sequence analysis was carried out on *HbERF-VII* for better understand the regulation of *HbERFs*. The *in silico* promoter analysis showed that the studied genes contain *cis*-acting regulatory elements which regulate anaerobic genes such as ANAERO and ARE. Furthermore, conserve motif outside AP2 domain analysis confirm the involvement of hypoxis in *HbERF-VII.*

Following this present report, further characterizations have to be implemented to study:

- Consistency of expression marker genes identified in clone PB 260 and to predict the embryogenic capacity in various *Hevea* clones,
- Allelic variations of latex expression markers in order to develop functional molecular genetic markers that could be useful for breeding programmes,
- Regulation of metabolic activities in latex such as the regulation under hypoxia, the role of ERF repressors, etc. in order to better understand the regulation of latex production and in particular natural rubber biosynthesis. For instance, functional analysis of some candidate genes (*HbERF-VIIa7, HbERF-VIIa12, HbERF-VIIa17* or *HbERF-VIIa20*) by transgenesis will be useful to confirm the function of these genes

in the metabolic regulation under hypoxia. In addition, we could consider looking for their target genes either by co-expression analysis or better by CHIP-sequencing.

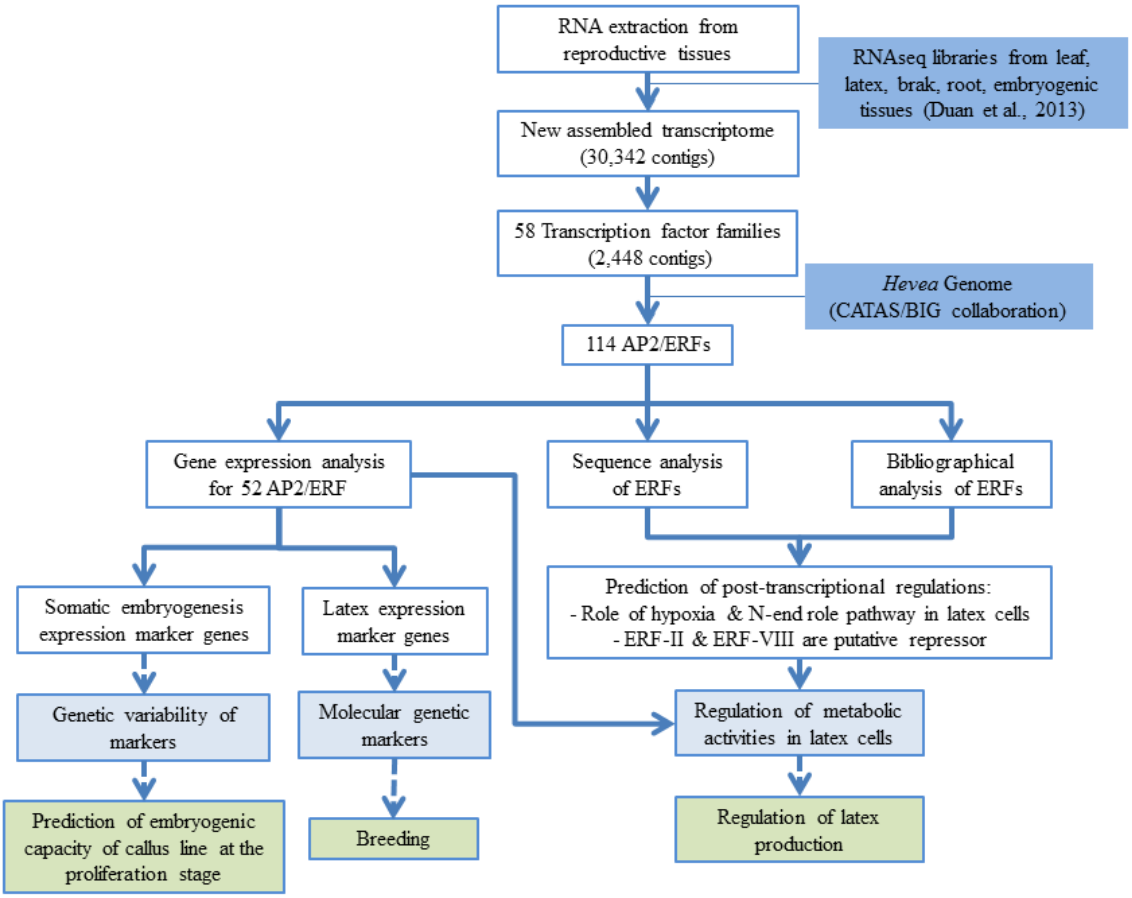

Figure 16. Summary of main outputs of this PhD thesis.

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