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Research paper

Ethylene response factors regulate expression of HbSUT3, the sucrose influx carrier in laticifers of *Hevea brasiliensis*

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Natural rubber is an important industrial raw material and is commercially produced by rubber trees (*Hevea brasiliensis*). The sucrose transporter HbSUT3 plays an essential role in rubber production. Its expression in latex (cytoplasm of rubber-producing laticifers) is induced by bark treatment with Ethrel, an ethylene releaser, and the inducing effect correlates well with Ethrel-stimulated rubber yield increase. However, the mechanisms of ethylene induction on *HbSUT3* expression are not known. Here, five *Ethylene Response Factor* (*ERF*) genes were identified from the cDNA library of *Hevea* latex by yeast one-hybrid screening with the promoter of *HbSUT3* gene as bait. As revealed in a tobacco (*Nicotiana tabacum*) protoplast transient expression system, these HbERFs were mainly localized in the nucleus and four of them exhibited apparent transactivation activity. Of the five *HbERF* genes, *HbERF-IXc4* was the most frequently screened in yeast one-hybrid, accounting for 65% of the *ERF* clones obtained. Moreover, among the five *HbERF*s, *HbERF-IXc4* showed the strongest transactivation capacity when expressed in tobacco protoplast, the highest transcript abundance in latex and a close expressional correlation with its target gene, *HbSUT3*, in response to the Ethrel treatment. Taken together, our results indicate that ERFs, especially HbERF-IXc4, are critically involved in the activation of *HbSUT3* expression in latex after Ethrel treatment on *Hevea* bark, and thus the stimulated latex yield.

Keywords: ethylene signaling, gene expression, rubber biosynthesis, sucrose transporter, transcription factor.

Introduction

Natural rubber (*cis*-1, 4-polyisoprene), an important industrial raw material, is commercially harvested from a tropical tree species, *Hevea brasiliensis* (para rubber tree), by way of tapping, viz. cutting periodically (commonly every 3 days) the bark of tree trunk, and hence severing the rings of rubber-producing laticifers wherein. The rubber production can be stimulated by ethylene, which has resulted in the routine application of Ethrel (2-chloroethyl phosphonic acid), an ethylene releaser, on trunk bark in rubber plantations (Zhu and Zhang 2009). A vast number of physiological and molecular studies have demonstrated that Ethrel application accelerates the supply and metabolism of sucrose in laticifers (Dusotoit-coucaud et al. 2009, Liu et al. 2015) and prolongs the time of latex flow (Tungngoen et al. 2009), and thus increases the latex yield of rubber trees (Zhu and Zhang 2009).

Rubber biosynthesis begins with the sucrose as precursor molecule, and covers >20 enzymatic reactions in laticifers (Sando et al. 2008). Due to the symplasmic isolation of laticifers from the neighboring cells/tissues, a sucrose transporter (SUT) has long been hypothesized for sucrose loading into laticifers, and to determine the carbon source for natural rubber biosynthesis and rubber yield (Tupý 1985). Studies on *SUT* genes in *Hevea* tree were first conducted independently in two research groups and a total of six distinct *SUT* genes have been cloned and characterized (Jianghua et al. 2007,

Dusotoit-coucaud et al. 2009, Dusotoit-coucaud et al. 2010, Tang et al. 2010). Among the six *Hevea* SUTs, HbSUT3 (=HbSUT1B) is identified as the responsible SUT member in sucrose loading into laticifers in regularly tapped *Hevea* trees (Tang et al. 2010). Both *HbSUT3* expression in latex and latex production are stimulated by ethylene treatment, and more importantly, the latex yield correlates significantly with the stimulating effect of ethylene on *HbSUT3* expression (Dusotoit-coucaud et al. 2010, Tang et al. 2010). A recent study reports that *HbSUT3* expression correlates positively with the latex yield of *Hevea* varieties and its expression in seedling bark can serve as an indicator in the early selection for high-yielding clones in *Hevea* breeding programs (Klaewklad et al. 2017). However, the molecular mechanisms by which ethylene generates inducement on *HbSUT3* expression in laticifers are still not known.

Plant SUTs are membrane-localized, energy-dependent proton/sucrose symporters, and contain 12 conserved transmembrane α-helices separated by a central cytoplasmic loop (Sauer 2007). They play a central role in the regulation of source-to-sink sucrose transport that determines plant growth and development (Durand et al. 2018). Based on the involvement in different sucrose translocation steps, SUTs can be classified into three types: plasma membrane efflux carriers, plasma membrane influx carriers and tonoplast carriers (Lemoine 2000). HbSUT3 is a high sucrose affinity transporter and belongs to plasma membrane influx carriers (Tang et al. 2010). Its *Arabidopsis* ortholog, AtSUC2, is highly expressed and involved in phloem sucrose loading in leaves, stems and roots (Truernit and Sauer 1995, Durand et al. 2018).

Expressions of sugar transporter genes are regulated by different transcription factors that vary with plant species and experimental conditions, and are implicated widely in plant growth and development. The companion cell-specific expression of *AtSUC2* is determined by conserved cis-regulatory elements for DNA-binding-with-one finger and homeo-domain-leucine-zipper transcription factors (Schneidereit et al. 2008). Abscisic acid (ABA)-responsive transcription factor MdAREB2 directly activates the expression of amylase and sugar transporter genes to promote soluble sugar accumulation in apple (Ma et al. 2017). Rice transcription factor OsDOF11 modulates the expression of *OsSUT1* and two Sugars Will Eventually be Exported Transporters (SWEET) genes, *OsSWEET11* and *OsSWEET14* (Wu et al. 2018). HbEIN3/EILs regulate the expression of two sugar transporters, which is predicted by a genomic-wide scanning the binding site of these transcription factors, G-box, in the promoters of rubber tree genes (Wang et al. 2019). Cotton fiber elongation requires sucrose transport into expanding fibers, via regulating the expression of SUT gene *GhSWEET12* by the transcription factor GhMYB 212 (Sun et al. 2019). Pollen tube growth is modulated via a MYB transcription factor MdMYB39L, which functions by regulating the expression of a sugar transporter MdSTP13a in apple (Li et al. 2020*b*). A WRKY transcription factor PuWRKY31 induces *PuSWEET15* transcription, which affects the accumulation of sucrose in pear fruit (Li et al. 2020*a*).

Here, five genes encoding ERF transcription factors were identified from *H. brasiliensis* latex by yeast one-hybrid system with the promoter of *HbSUT3* as bait. We show that these ERFs, especially one of them, HbERF-IXc4, play a critical role in ethylene induction on HbSUT3 expression, and thus the ethylene-stimulated latex production in *Hevea* trees. To our knowledge, this is the first report of direct involvement of ERFs in regulating sugar transporter genes in response to ethylene treatment.

Materials and methods

Plant material

Rubber tree clone Reyan7-33-97 was cultivated on the plantation of Chinese Academy of Tropical Agricultural Sciences (Danzhou, Hainan province, China). They were 10 years old and regularly tapped for latex in a half spiral pattern every 3 days (S/2, d/3). Different tissues, viz. leaf, seed, latex, bark, root, female and male flowers, were sampled from three individual trees, kept in liquid nitrogen and brought to the lab for immediate RNA isolation or stored at −80 °C. To study the expression patterns of *HbSUT3* and *ERF* transcription factor genes in latex after the ethylene treatment, 1.5% (w/w) Ethrel in 1% (w/w) carboxyl methyl cellulose (CMC) was applied to the trunk bark by spreading just above the tapping panel. The latex was sampled by tapping after 3, 12 and 24 h of Ethrel treatment, as described by Pujade-Renaud et al. (1994). For each time point, three batches of five trees were used. Another three batches of five trees were treated with 1% CMC as a control. Total RNA was extracted from latex samples pooled from five trees, respectively, as described (Tang et al. 2007).

Yeast one-hybrid screening

To identify the transcription factors that regulate *HbSUT3* expression, a fragment of *HbSUT3* promoter (HbSUT3p) was used as bait for yeast one-hybrid screening. The *HbSUT3* promoter fragment employed was cloned by a modified adaptor-PCR in our previous work (Xin et al. 2012), being 2028 base-pairs long upstream of the initiation codon. HbSUT3p was amplified with the primers of 5′- TCGA GCTCTGGGAGCAACCATTACATCAAAATAGAA-3′ (forward) and 5′-AGGTCGACGGGAGTTGGTGGTGAGAAGAGTGTG-3′ (reverse), digested with the restriction enzymes of SacI and SalI and cloned into the SacI–SalI cleaved bait vector pAbAi (TaKaRa, Beijing, China) by a ligation reaction with T4 DNA ligase. A normalized latex cDNA library was produced from 0.1 μg latex mRNA as previously reported (Zhang et al. 2014). The mRNA sample used was isolated from an equal ratio mixture of total RNA from latex samples collected at four different time points

(0, 3, 12 and 24 h) after Ethrel treatment. Yeast one-hybrid library was screened following the user manuals of Yeast One-Hybrid System-Matchmaker Gold kit (TaKaRa).

Identification of ERF transcription factors in silico

To annotate the genes screened by yeast one hybrid, their sequences were analyzed firstly by BLAST in the NCBI database (https://www.ncbi.nlm.nih.gov/) and *Hevea* genome database (http://hevea.catas.cn/home/index) (Tang et al. 2016). Then, conserved domains of HbERFs annotated by BLAST were identified by a Pfam search in the EMBL-EBI database (http://pfam.xfam.org/search). Annotated HbERFs were validated lastly by phylogenetic analysis with characterized *Arabidopsis* AP2/ERF proteins. Phylogenetic tree was constructed by the Neighbor-Joining method in Mega-X, of which the bootstrap method was tested using 1000 replicates. The AtERF amino acid sequences were downloaded from UniProt database (https://www.uniprot.org/). To study the function of these HbERFs, their sequences were analyzed by BLAST and matched to the *Hevea* AP2/ERF superfamily reported (Duan et al. 2013).

Transcript abundance analysis by real-time PCR

Several rules were applied for ensuring the accuracy of the analysis. The integrity of total RNA samples after DNase I digested was checked by electrophoresis. The cDNA for detecting gene expression was synthesized from 1 μg latex total RNA with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Shanghai, China). The primers of *HbERFs* for real-time PCR were designed close to 3′ side of gene coding sequences (Table S1 available as Supplementary data at *Tree Physiology* Online), and the primers of *HbSUT3* and *HbUBC4* for real-time PCR was as previously reported by Tang et al. (2010) and Li et al. (2011), respectively. Each sample was tested with three repeats at the same time for real-time PCR.

Real-time PCR was performed using SYBR Premix Ex Taq™ kit (TaKaRa) on a LightCycler 2.0 system (Roche, Shanghai, China). The PCR procedure was performed as follows: pre-denaturation at 94 °C for 30 s; 45 amplification cycles of denaturation at 94 °C for 5 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s; melting curves plotted from 72 °C to 94 °C with a heating rate of 0.2 °C s^{-1}.

The amplifying specificity of primers was double-checked by Sanger sequencing of the PCR product. A new pair of primers was designed for replacement if the PCR product was found to be mingled. The amplification efficiency of each primer pair was evaluated via the same PCR procedure but with gradient diluted latex cDNA as template. The relative abundance of transcripts was calculated according to standard curve method (Morrison et al. 1998). *Hevea* ubiquitin-protein ligase gene *HbUBC4* was used as a suitable internal reference as reported (Li et al. 2011).

Construction of plant expression vectors

Vector construction was carried out by a Gateway cloning strategy (Suzuki et al. 2005) following the user manuals of Gateway® BP Clonase® II Enzyme mix and Gateway® LR Clonase® Enzyme mix (Invitrogen, Thermo Fisher Scientific). The open reading frames of each *HbERF* gene with and without stop codon were amplified with specific pairs of primers containing attB adaptors. The PCR products were inserted into a donor vector pDonr207 by a BP recombination reaction and then each *HbERF* was transferred into a destination vector by LR recombination reactions (pDonr207 was a gift from Yihua Zhou, Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijing, China). Expressions of these *HbERFs* were driven by a CaMV 35S promoter in destination vectors, pEaleygate 104, pMDC83 and pMDC32, which were ordered from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Ohio, USA). All the sequences of these *HbERF*-amplifying primers were listed in Table S2 available as Supplementary data at *Tree Physiology* Online. For subcellular localization analysis of these HbERFs, green and yellow fluorescent proteins (GFP and YFP) were fused at their C-terminus in pMDC83 and N-terminus in pEaleygate 104 vectors, respectively.

Synthetic fragments containing four repeats of GCC-box (GCCGCC) and mutant GCC-box (TCCTCC), as an autoactivation control, were inserted into pMDC107 (ABRC), respectively, for the detection of each HbERF's activity. These vectors that have a GFP reporter driven by the GCC box or mutant GCC box, named, respectively, as pMDC107-GCC or pMDC107-mGCC, were constructed previously (Piyatrakul et al. 2014). The vectors of pMDC107-GCC or pMDC107-mGCC and HbERF expression vectors were co-transformed into tobacco protoplasts for transactivation activity detection.

Subcellular localization and transactivation detection in tobacco BY-2 cells

Tobacco suspension cell line (*Nicotiana tabacum* L. cv Bright Yellow2, BY-2) was preserved and cultured by the research group of Cellular & Molecular Biology of Stress Responses in Tropical Woody Species (BURST) in French Agricultural Research Centre for International Development (CIRAD). The procedures for BY-2 culturing, protoplast preparation and transfection were as described (Nagata et al. 1992).

To determine the subcellular localization of HbERFs, the protoplasts were transfected with recombinant pEarleyGate104 and pMDC83 vectors harboring the HbERFs fused with YFP and GFP, respectively. The transfected protoplasts were observed under fluorescence microscopy (Leica DM6000 B, Germany) and photographed by Hamamatsu camera (Japan). To detect the transactivation activity of each HbERFs, the protoplasts were co-transfected with the pMDC32 vectors inserted with an HbERF and pMDC107-GCC or pMDC107-mGCC. The GFP signal of

Table 1. *HbSUT3* promoter-binding ERF transcription factors identified from yeast one-hybrid screening

Gene	No. of clones	Amino acid residues	Annotation by NCBI BLAST	*Hevea* genome (Tang et al. 2016) BLAST		*Hevea* AP2/ERF database (Piyatrakul et al. 2014) BLAST	
				Gene ID	Identity	Best hit	Identity
HbERF-IXa2	3	267	ERF	scaffold0206_1492583	100.0%	*HbERF-IXa2*	99.5%
HbERF-IXa3	1	284	ERF	scaffold0668_444732	100.0%	*HbERF-IXa3*	99.4%
HbERF-IXc4	17	219	ERF	scaffold1015_293137	99.7%	*HbERF-IXc4*	98.6%
HbERF-IXc7	4	134	ERF	scaffold0095_1888719	99.6%	*HbERF-IXa2*	72.6%
HbERF-IXc8	1	147	ERF	scaffold5750_395	99.5%	*HbERF-IXa2*	75.0%
Other genes	95	–	Known or unknown proteins other than genuine interacting transcription factors (Table S6 available as Supplementary data at *Tree Physiology* Online)				

transfected protoplasts was detected on a flow cytometry (FACS Calibur II, BD, USA).

Results

Cloning of transcription factors interacting with HbSUT3 promoter

The cDNA library generated from the latex mRNA of rubber tree after Ethrel treatment was screened by yeast one-hybrid with a ~2 kb promoter fragment of *HbSUT3* as a bait. Our screening generated 121 yeast candidate clones. After subcloning, sequencing and BLAST searching in NCBI and *Hevea* genomics database (http://hevea.catas.cn/home/index) (Tang et al. 2016), 26 clones represented five distinct *Hevea* ERF transcription factors, named, respectively, as *HbERF-IXa2*, *HbERF-IXa3*, *HbERF-IXc4*, *HbERF-IXc7* and *HbERF-IXc8* as guided by subsequent blasting and phylogenetic analysis, while the remaining 95 were annotated as known or unknown proteins other than genuine interacting transcription factors (Table 1,Table S6 available as Supplementary data at *Tree Physiology* Online). Among the five *HbERF*s, *HbERF-IXc4* was most frequently identified, with 17 clones in the library screening, which may represent the most abundant transcription factor binding to the *HbSUT3* promoter (Table 1). Rapid amplification of cDNA ends (RACE) technology was then used to obtain the full coding sequences of these *HbERF*s, which were predicted to encode proteins of 147 to 267 amino acid residues (Table S3 available as Supplementary data at *Tree Physiology* Online). The interactions between the five HbERFs with full open reading frames and the promoter of HbSUT3 were retested as positive by transforming the HbERF preys into the HbSUT3 promoter bait strain (Figure S1 available as Supplementary data at *Tree Physiology* Online). All five HbERFs belonged to the ERF family of Apetala 2/Ethylene Responsive Factor (AP2/ERF) superfamily, containing only one AP2 domain as revealed by searching HbERF amino acid sequences in EMBL-EBI database (https://www.ebi.ac.uk/Tools/hmmer/).

A phylogenetic tree constructed with the five HbERFs and the entire *Arabidopsis thaliana* AP2/ERF superfamily revealed that the five HbERFs were clustered in a single clade of ERF family with eight AtERFs (Figure 1). A BLAST search against a comprehensive *Hevea* AP2/ERF superfamily database of 114 genes (Piyatrakul et al. 2014) revealed that three of the five *HbERF* genes had >98% nucleotide identities, respectively, with *HbERF-IXc4, HbERF-IXa2* and *HbERF-IXa3* in the database, whereas the two remaining had homologs of <80% sequence identity. *HbERF-IXc4* shows higher expression in bark compared with other *Hevea* tissues (Duan et al. 2013, Piyatrakul et al. 2014).

ERF transcription factors transcriptionally regulate the expression of their target genes through AP2 domain binding to a cis-regulatory element, GCC-box, on the promoters (Allen et al. 1998, Franco-Zorrilla et al. 2014). We checked the sequence of *HbSUT3* promoter and found a GCC-box in −30 bp position upstream of the initiation codon and downstream of transcription start site (Table S5, Figure S2 available as Supplementary data at *Tree Physiology* Online), justifying the obtaining of five HbERFs in our yeast one-hybrid screening with the *HbSUT3* promoter as a bait (Table 1).

Subcellular localization and transactivation of HbERFs

The protoplast transient expression system of tobacco BY-2 suspension cells was used to determine the subcellular localization and transactivation activity of the five HbERF transcription factors. For subcellular localization assay, the HbERFs were fused with the GFPs at their C-terminal in the pMDC83 vector, and with the YFPs fused at the N-terminal in the pEarleyGate 104 vector. As shown in Figure 2, HbERF-IXc4, HbERF-IXc7 and HbERF-IXc8 were localized mainly in the nucleus and partially in cytoplasm, while HbERF-IXa2 and HbERF-IXa3 were exclusively localized in the nucleus (Figure 2).

To detect the transactivation activity of the five HbERFs, initiator and reporter vectors were constructed and co-transfected into tobacco protoplasts. In the initiator vectors of pMDC32-HbERFs, expressions of the HbERFs were driven by a double 35S promoter (Figure 3A). In the reporter vector of pMDC107-GCC, expression of a GFP reporter was driven by four GCC box repeats, whereas the vector of pMDC107-mGCC was used as a self-activation control

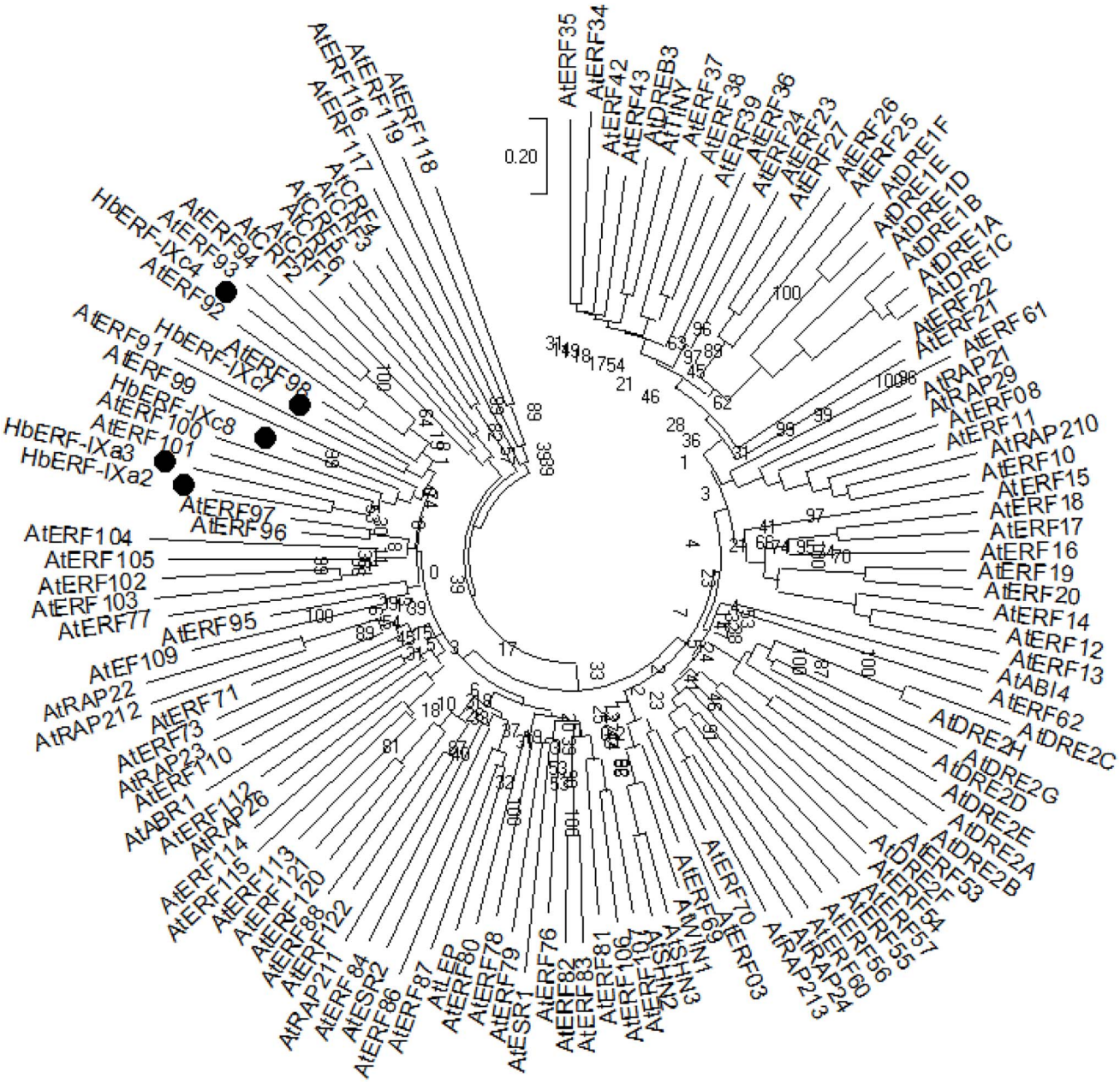

Figure 1. Phylogenetic tree of five *Hevea* ERFs and the entire *Arabidopsis* AP2/ERF superfamily. Five *Hevea* ERFs (HbERFs) are highlighted with a round black dot placed after each member in the tree.

substituted with four mutant GCC box repeats (Figure 3A). Two groups of protoplasts were transfected by pMDC32-HbERFs plus pMDC107-GCC and pMDC32-HbERFs plus pMDC107-mGCC, respectively. Taking the blank vector pMDC32 as the background control in the same experiment, the average absolute value of green fluorescence intensity generated from these two groups was detected by flow cytometry as 'pMDC32-XX + GCC' and 'pMDC32-XX + mGCC' ('XX' can be any of the five HbERFs or empty), respectively. Therefore, the relative transactivation activity of a certain HbERF can be calculated by the following formula: (pMDC32-HbERF + GCC)/(pMDC32-HbERF + mGCC) − (pMDC32 + GCC)/(pMDC32 + mGCC) (Piyatrakul et al. 2014). Of the five HbERFs, four (HbERF-IXa2, HbERF-IXa3, HbERF-IXc4 and HbERF-IXc8) showed obvious transactivation activity, with HbERF-IXc4 being the strongest (Figure 3B, Table S7 available as Supplementary data at *Tree Physiology* Online). Interestingly, the remainder, HbERF-IXc7, seemed to be a repressor of reporter activity.

Expressions of HbSUT3 and HbERFs in rubber tree

To study the tissue-specific expression patterns of *HbSUT3* and the five *HbERFs*, transcripts were investigated by real time PCR (qPCR) in seven *Hevea* tissues, viz. seed, female flower, male

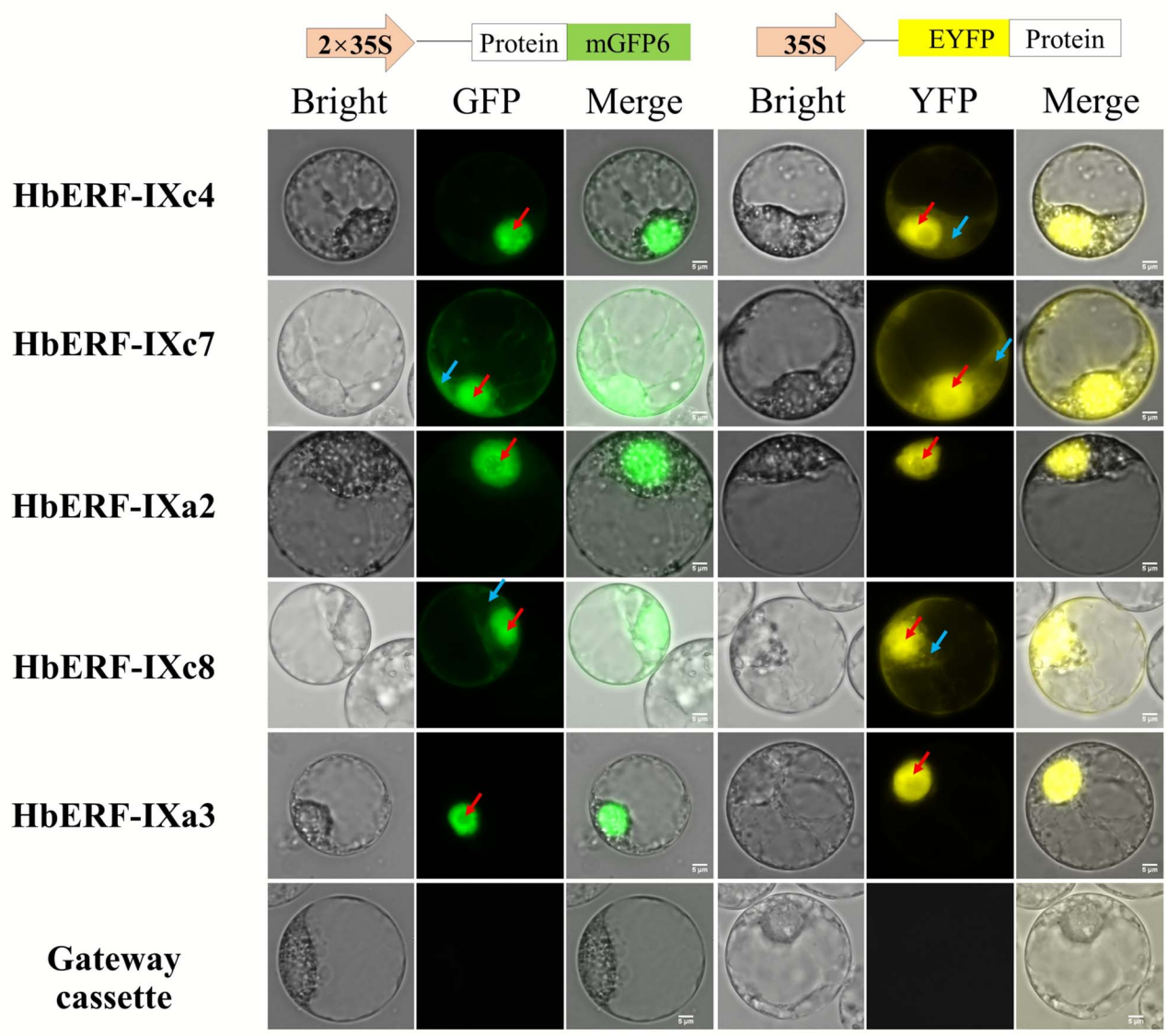

Figure 2. Subcellular localization of HbERFs in tobacco protoplasts. The five HbERF proteins were fused to GFP and YFP in the C-terminus and N-terminus, respectively. The chimeric genes driven by 35S promoter were transiently expressed in BY-2 tobacco protoplast. Subcellular localization was then analyzed by fluorescence microscopy. The bright fields (bright), the corresponding fluorescence channels (GFP or YFP) and the merged pictures are shown. Control cells expressing gateway cassette alone are shown in the bottom panel. The red arrows indicate localization in the nucleus and blue arrows indicate localization in cytoplasm. Bars = 5 μm.

flower, leaf, trunk bark and latex. As shown in Figure 4, *HbERF-IXc7* and *HbERF-IXc8* were expressed at very low levels in all the tissues examined. *HbERF-IXa3* expressed at high levels in the most of the tissues explored, but at very low level in latex. *HbERF-IXc4* and *HbERF-IXa2* were expressed at moderate levels in most of the tissues examined. Among the five *HbERFs*, *HbERF-IXc4* was the predominant isoform expressed in latex, the expression of which was significantly higher than that of the other four *HbERFs* together (Figure 4). *HbSUT3*, the putative target of the five *HbERFs*, expressed at high levels in female flower and root, moderate in seed, male flower, leaf and latex, but very low levels in trunk bark. It is worth noting that latex (cytoplasm of laticifers) is harvested from trunk bark where the laticifers reside. In this regard, the expression of *HbSUT3* has been found to be significantly enriched in laticifers of trunk bark.

To further probe the regulatory roles of HbERF transcription factors in *HbSUT3* expression, the expressions of *HbSUT3* and five *HbERF*s in latex after Ethrel bark treatment were examined by qPCR. As shown in Figure 5, expressions of *HbSUT3* and the five *HbERFs* were all upregulated significantly by the Ethrel treatment. The expression of *HbSUT3* in response to Ethrel treatment was consistent with its crucial role in ethylene-stimulated latex production in rubber tree (Dusotoit-coucaud et al. 2010, Tang et al. 2010). Among the five *HbERFs*, *HbERF-IXc4* not only represented the most abundant ERF isoform in latex after the Ethrel treatment, but also exhibited a time course pattern

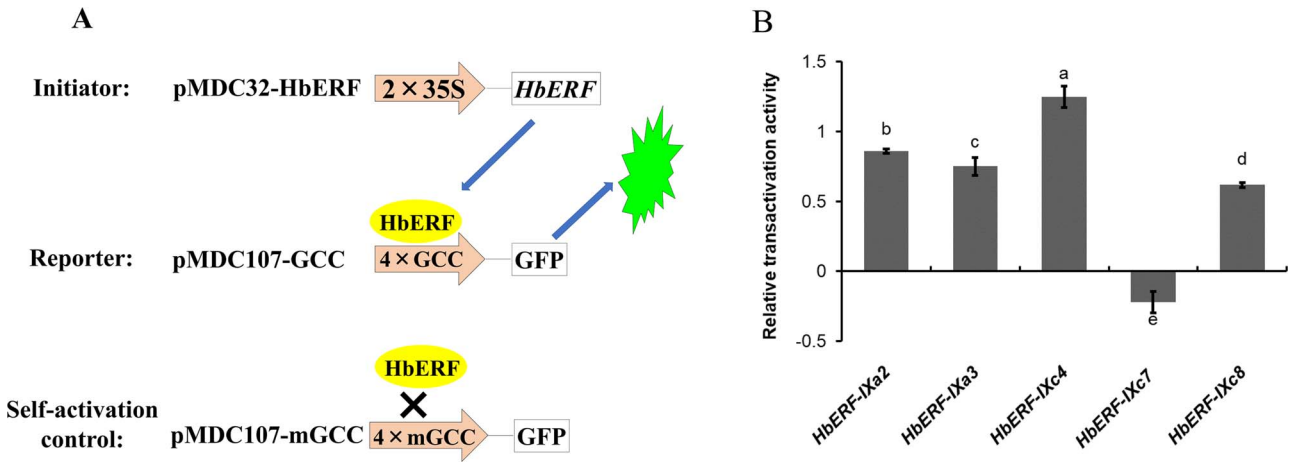

Figure 3. Transactivation activity detection of five *HbERF*s. (A). Expression cassette in the constructs for transactivation activity detection. (B). Relative transactivation activity of HbERFs. Error bars represent the standard deviation of three independent experiment repeats.

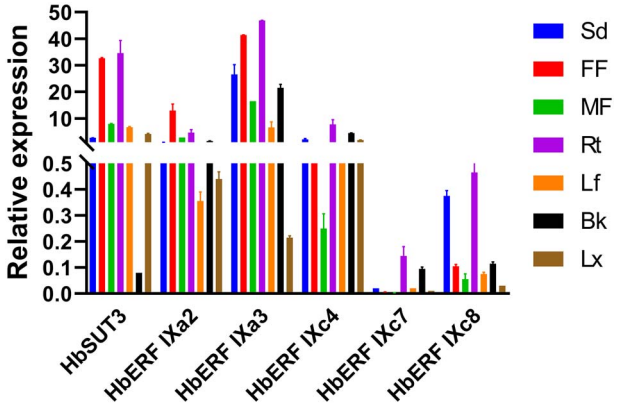

Figure 4. Expressions of *HbSUT3* and *HbERF*s in seven tissues of rubber tree. Transcript levels of *HbSUT3* and *HbERF*s were measured by real-time PCR and normalized by a reference gene *HbUBC4*. Results are given as the mean of three independent experiments. Error bars represent the standard deviation of three independent experiment repeats. Sd, seed; FF, female flower; MF, male flower; Rt, root; Lf, leaf; Bk, trunk bark; Lx, latex.

of expression similar to *HbSUT3* (Figure 5). Similar results of the five *HbERF* expression patterns were observed in Solexa transcriptome data (Table S4 available as Supplementary data at *Tree Physiology* Online) (Xiao et al. 2014).

Discussion

The five HbERFs belong to plant ERF subfamily

The AP2/ERF superfamily members have emerged as key transcriptional regulators of biological processes related to plant growth and development, as well as various stress responses for survival, in which they also respond to multiple hormones, including gibberellins, cytokinins, brassinosteroids, methyl jasmonate, abscisic acid and ethylene (Xie et al. 2019). AP2/ERFs are characterized by containing at least one AP2/ERF DNA binding domain with 40 – 70 conserved amino acids (Sakuma et al. 2002). AP2/ERF superfamily proteins can be classified into three major families defined according to the type of conserved domains contained, with AP2 family proteins containing two repeated AP2/ERF domains, related to ABA Insensitive 3/Viviparous 1 (RAV) family proteins containing a AP2/ERF domain and a B3 DNA binding domain, and ERF family containing a single AP2/ERF domain, respectively (Nakano et al. 2006). The ERF family can be further divided into 12 groups (groups I to X, VI-L and Xb-L), of which groups I to IV and V to X correspond to the Dehydration Responsive Element Binding protein/C-repeat Binding Factor (DREB/CBF) subfamily and ERF subfamily, respectively (Nakano et al. 2006).

The five HbERFs identified by the yeast one-hybrid screening contained a single AP2/ERF domain, therefore belonging to the ERF family under the AP2/ERF superfamily. Phylogenetic analysis revealed the clustering of the five HbERFs with eight AtERFs (AtERF91, AtERF92, AtERF93, AtERF94, AtERF98, AtERF99, AtERF100 and AtERF101) (Figure 1), all of which belong to group IX within the ERF subfamily (Nakano et al. 2006). The ERF subfamily proteins bind to a cis-acting DNA regulatory element, the Ethylene-Response Element (ERE), with AGCCGCC core sequence (GCC-box), and involve in conferring resistance to disease and abiotic stresses (Nakano et al. 2006, Franco-Zorrilla et al. 2014). Some *Arabidopsis* ERFs (not from group IX) can additionally bind to the cis element of DREBs or C-Repeat Element (DRE/CRT) with A/GCCGAC core sequence on stress-responsive genes, and confer resistance to drought, cold and heat abiotic stresses (Xie et al. 2019). However, the DRE/CRT element is not present in the promoter of *HbSUT3*, where only a GCC-box was identified (Table S5 available as Supplementary data at *Tree Physiology* Online). A BLAST search against a comprehensive *Hevea* AP2/ERF superfamily database further reveals the clustering of the five HbERFs are in group IX under the *Hevea* ERF subfamily (Table 1). Three group IX HbERFs, i.e., HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6, have

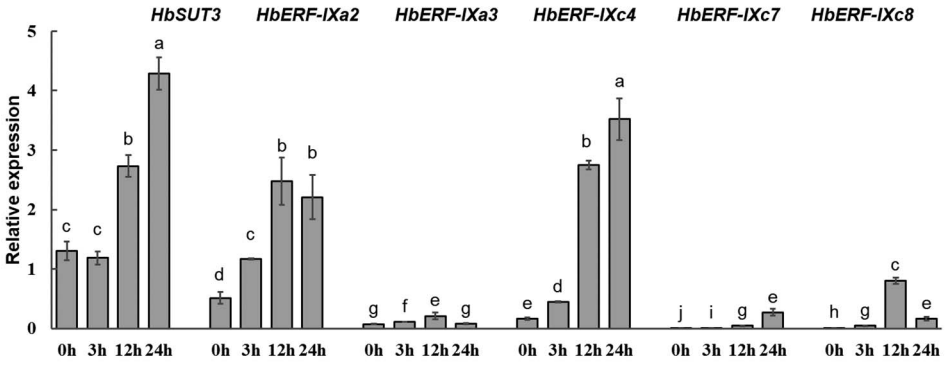

Figure 5. Effect of Ethrel treatment on expressions of *HbSUT3* and *HbERF*s in latex. Transcript levels of *HbSUT3* and the five *HbERF*s were measured by real-time PCR and normalized by a reference gene *HbUBC4*. Results are given as the mean of three independent experiments. Different labels on the top of columns mean significant difference (P-value < 0.05). Error bars represent the standard deviation of three independent experiment repeats.

been confirmed containing transactivation activity by binding to the synthetic GCC-box promoters (Putranto et al. 2015). Transactivation activity detection experiments in this study revealed that the five HbERFs bound to GCC-box and activated/repressed the transcription of the GFP reporter gene (Figure 3).

HbERFs are implicated in biological processes related to rubber production

The ethylene biosynthesis and signaling pathways have been studied in rubber tree for the main reason of understanding ethylene stimulation on latex production (Kuswanhadi et al. 2010). Several biological processes related to rubber production can be stimulated by ethylene treatment in laticifers, such as sucrose loading (Dusotoit-coucaud et al. 2010, Tang et al. 2010), water uptake (Tungngoen et al. 2009) and synthesis of nitrogen assimilation enzymes (Pujade-Renaud et al. 1994). The key regulators in the ethylene signaling pathway, EIN3/EILs, were predicted to target 41 genes involved in rubber biosynthesis and latex flow (Wang et al. 2019). A large number of HbERFs correlate to the special response of rubber trees to ethylene stimulation and harvesting stress (Putranto et al. 2015). A total of 87 *ERFs* were identified and clustered into 11 groups from rubber tree transcriptome and genomic scaffolds (Duan et al. 2013, Piyatrakul et al. 2014). Transcripts of HbERFs from groups I to VIII are abundant in all the tissues examined and transcripts of HbERFs from group VII are highly accumulated in laticifers (Piyatrakul et al. 2012, Piyatrakul et al. 2014).

HbERF group IX is suggested to be implicated in the crosstalk between ethylene and jasmonate signaling pathways influencing latex production and laticifer differentiation (Duan et al. 2013, Putranto et al. 2015). The transcripts of three genes of group IX, namely HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6, were dramatically accumulated in latex after a treatment combining wounding/jasmonate and ethylene suggesting that these members could be essential integrators of complex hormonal signaling pathways in *Hevea* (Putranto et al. 2015). The jasmonate signaling pathway is also involved in laticifer differentiation that consists of one of the determining factors affecting rubber production in rubber trees (Deng et al. 2018). Interestingly, the overexpression of *HbERF-IXc5* enhances plant growth and abiotic stress tolerance, and affects laticifer differentiation in transgenic rubber tree lines (Lestari et al. 2018). The five HbERFs identified in this study were defined to ERF group IX (Table 1). Several HbERF-IX members have been previously confirmed as activator-type transcription factors (Putranto et al. 2015). In this study, four of five HbERFs were revealed to be transcription activators, while the remainder, HbERF-IXc7, seems to act as a repressor (Figure 3).

HbERF-IXc4 is actively involved in ethylene-stimulated HbSUT3 expression in latex

The five *HbERFs* were identified by yeast one-hybrid screening with the *HbSUT3* promoter as bait. They are mainly localized in nucleus and four of them, HbERF-IXa2, HbERF-IXa3, HbERF-IXc4 and HbERF-IXc8, are activator-type ERF transcription factors. Four pieces of evidence bolster the roles of HbERF-IXc4 as a critical transcriptional activator of *HbSUT3* expression in latex. First, only ERF-type transcription factors are identified in the yeast one-hybrid screening, and more importantly, HbERF-IXc4 is the most frequently identified ERF, accounting for >65% of the total ERF clones (Table 1). Second, HbERF-IXc4 exhibits much stronger transactivation activity than any other HbERFs examined when assayed in a tobacco protoplast transactivation system (Figure 3). Third, *HbERF-IXc4* is the most abundant ERF isoform among these five *HbERFs* in latex whether or not exposed to Ethrel stimulation (Figure 4, Table S4 available as Supplementary data at *Tree Physiology* Online). Fourth, dynamic expression of *HbERF-IXc4* is most similar to that of *HbSUT3* in latex after Ethrel treatment, although expressions of the other

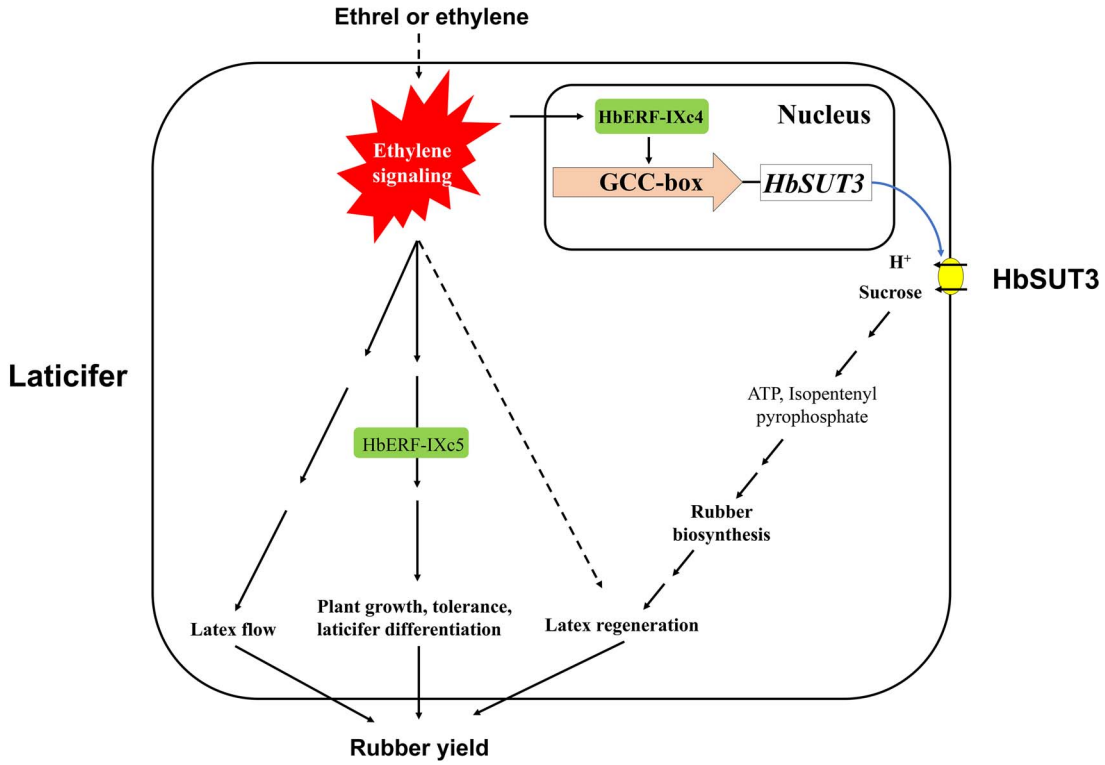

Figure 6. A schematic model of ethylene response factors HbERF-IXc4 (this study) and HbERF-IXc5 (Lestari et al. 2018) in ethylene stimulation of rubber yield in *Hevea brasiliensis*.

four *HbERFs* are bolstered as well (Figure 5). Taken together, our results indicate that *HbSUT3*'s expression is regulated by HbERFs, mainly the HbERF-IXc4 in latex after Ethrel treatment. Our work unravels a new way of ethylene regulation on *SUT* gene expression in higher plants. Considering the key roles of HbSUT3 in the process of sucrose loading in laticifers and ethylene-stimulated rubber production (Tang et al. 2010), we speculate that HbERF-IXc4 plays an important role in enhancing latex production in response to Ethrel stimulation (Figure 6).

Conflict of interest

The authors declare no competing financial interests.

Authors' contributions

Y.Z. performed most of the experiments, analyzed the data and drafted the manuscript. L.X. constructed the bait vector for yeast one-hybrid screening. J.Y. and J.Q. participated in library construction and field experiments. J.P. and P.M. supervised the experiments of subcellular localization and transactivation activity detection. Y.F. participated in the bioinformatics analysis. C.T. conceived the study and coordinated the project. C.T. and P.M. edited the manuscript, and all authors approved the manuscript.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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