Structure and expression profile of the sucrose synthase gene family in the rubber tree: indicative of roles in stress response and sucrose utilization in the laticifers

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Sucrose synthase (Sus, EC 2.4.1.13) is widely recognized as a key enzyme in sucrose metabolism in plants. However, nothing is known about this gene family in *Hevea brasiliensis* (para rubber tree). Here, we identified six *Sus* genes in *H. brasiliensis* that comprise the entire Sus family in this species. Analysis of the gene structure and phylogeny of the *Sus* genes demonstrates evolutionary conservation in the Sus families across *Hevea* and other plant species. The expression of *Sus* genes was investigated via Solexa sequencing and quantitative PCR in various tissues, at various phases of leaf development, and under abiotic stresses and ethylene treatment. The *Sus* genes exhibited distinct but partially redundant expression profiles. Each tissue has one abundant *Sus* isoform, with *HbSus3, 4* and *5* being the predominant isoforms in latex (cytoplasm of rubber-producing laticifers), bark and root, respectively. *HbSus1* and *6* were barely expressed in any tissue examined. In mature leaves (source), all *HbSus* genes were expressed at low levels, but *HbSus3* and *4* were abundantly expressed in immature leaves (sink). Low temperature and drought treatments conspicuously induced *HbSus5* expression in root and leaf, suggesting a role in stress responses. *HbSus2* and *3* transcripts were decreased by ethylene treatment, consistent with the reduced sucrose-synthesizing activity of Sus enzymes in the latex in response to ethylene stimulation. Our results are beneficial to further determination of functions for the *Sus* genes in *Hevea* trees, especially roles in regulating latex regeneration.

Introduction

Sucrose is the major form of photoassimilate, and is transferred from source leaves to various sink tissues [1]. Once unloaded into recipient sink cells, sucrose is cleaved into hexoses by sucrose synthase (Sus, EC 2.4.1.13) or invertase (Inv, EC 3.2.1.26) for use in cellular metabolism, biosynthesis, storage and signaling [2–5]. Sus catalyzes the reversible conversion of sucrose

and UDP into UDP-glucose and fructose, and plays important roles in the regulation of carbon partitioning into various sink tissues or organs [6–9]. For instance, Sus activity has been repeatedly reported to correlate well with the sink strength of various starch-storing organs, including potato tubers (*Solanum tuberosum*), carrot roots (*Daucus carota*), maize kernels

Abbreviations
Inv, invertase; Sus, sucrose synthase; SUT, sucrose transporter.

(*Zea mays*) and pea embryos (*Pisum sativum*) [6,7,10–12]. In cotton (*Gossypium hirsutum*) and poplar (*Populus trichocarpa*), Sus is proposed to be a key regulator of sink strength, and plays crucial roles in cellulose synthesis and secondary cell-wall formation [13–15]. In addition, Sus activity is implicated in many other important metabolic processes, such as phloem loading [9,10], environmental stresses response [16,17] and nitrogen fixation [16–19].

In many plant species examined to date, Sus isoforms are encoded by a small multi-gene family. With the sequencing of the genomes in many plants, more and more Sus gene families have been identified. For example, in the model plant *Arabidopsis thaliana*, the Sus family has six distinct members grouped into three groups [20]. Rice (*Oryza sativa*) also has six *Sus* genes dispersed throughout the genome, of which four are expressed in a tissue- and stage-specific manner [21]. In poplar, there are seven Sus family members, three of which were preferentially expressed in the stem xylem [22]. In cotton, there are also seven Sus family members (of which one appears to be a pseudogene), most of which were differentially expressed in a wide range of tissues, and showed development-dependent expression profiles in cotton fiber cells [23]. In other plant species, such as carrot, maize, *Lotus japonicus* and *Citrus unshiu*, *Sus* genes have also been shown to be expressed in tissue-specific and/or development-dependent patterns [15,24–26]. In all cases, distinct expression patterns are observed for the various isoforms in the respective Sus families, implying that each Sus isoform may have evolved a specialized function in a given species. Although *Sus* genes in a few plant species such as *Arabidopsis*, rice (*Oryza sativa*) and cotton have been extensively studied, the *Sus* genes in rubber tree (*Hevea brasiliensis* Muell. Arg.) have not.

Natural rubber (*cis*–1,4-polyisoprene) is an important raw material that is widely used in various industries, and the sole commercial source of natural rubber is the rubber tree, a perennial tropical tree [27]. Rubber is synthesized and stored in the cytoplasm (latex) of highly specialized cells called laticifers that are differentiated from the cambium and arranged in rings [27,28]. Rubber harvesting is performed by making an incision in the bark every 2–3 days to sever the laticifer rings, a process that is called tapping [27]. After tapping, several tens to a few hundred milliliters of latex per tree are expelled from the laticifers and harvested, and, for sustainable rubber production, sufficient new latex must be regenerated before the next tapping. Sucrose is the precursor molecule of rubber biosynthesis and latex regeneration, and therefore the laticifers in the trunk bark of regularly tapped

rubber trees represent a strong sucrose sink [29]. Understanding the mechanisms of sucrose transport and metabolism in the laticifers is of fundamental importance for improving *Hevea* productivity [27]. Six sucrose transporter (SUT) genes related to sucrose transport have been cloned in rubber tree, of which *HbSUT3* (*HbSUT1B*) has been identified as the key member responsible for sucrose loading into laticifers [30–32]. Sucrose catabolism in the latex is the first step of sucrose entry into the rubber biosynthesis pathway, and is mainly accomplished by the activity of Inv and retarded by the synthetic activity of Sus [33,34]. In addition, regulation of the enzyme activity of Inv and Sus is reported to be highly correlated with the enhanced latex metabolism and improved rubber productivity after application of Ethrel (2–chloroethyl-phosponic acid, an ethylene releaser) to the trunk bark [34]. Determination of the effect of Ethrel treatment on *Inv* or *Sus* gene expressions is useful to further unravel the molecular mechanisms underlying ethylene-stimulated rubber production.

In the present work, we describe the identification and characterization of six *Sus* genes in *Hevea* tree. This study mainly focused on gene identification, genomic structure, phylogeny and expression patterns of the *Hevea Sus* gene family in various tissues, in response to various treatments, and at various phases of leaf development. The roles of the *Hevea Sus* genes in latex metabolism of rubber tree are also discussed. The results obtained from this study are beneficial for the performance of further studies to obtain a comprehensive understanding of the physiological roles of each *Hevea Sus* gene in regulating latex regeneration and other important biological processes.

Results

Cloning, sequence and structure analysis of the *HbSus* family

In order to identify the potential Sus homologs in rubber tree, using the mRNA sequences of *Populus* and *Arabidopsis Sus* genes as queries, a large collection of ESTs of *H. brasiliensis* were searched as described in Experimental procedures, leading to assembly of six contigs as putative *Sus* genes. The six putative *Hevea Sus* genes were named *HbSus1–6*, and their full-length cDNAs were cloned by PCR amplification. The deduced proteins encoded by these putative *HbSus* genes contain 806–904 amino acids (predicted molecular mass 92.5–102.7 kDa) with isoelectric points of 5.75–7.21 (Table S1), similar to the molecular features of Sus isozymes

from other plant species. Additionally, all the HbSus amino acid sequences share a conserved Ser residue in the N–terminal region (Fig. 1), which has been reported to be phosphorylated by Ser/Thr protein kinase in maize [35,36]. Furthermore, using the Interproscan algorithm (http://www.ebi.ac.uk/interpro/), two conserved domains (Sus and glucosyl transferase) typical of Sus proteins were also identified in all the deduced HbSus proteins (data not shown). Together, these findings indicate that these isolated genes encode various isozymes

of Sus in *H. brasileinsis*. Multiple sequence alignment using the DNAMAN algorithm (http://www.lynnon.com/) revealed high levels of similarities between the coding sequences of *HbSus1–6*, with the highest percentage of nucleotide and amino acid sequence identity found between *HbSus3* and *HbSus4* (94.5% and 97.1%, respectively), followed closely by *HbSus2* and *HbSus5* (93.9% and 94.5%), and then by *HbSus1* and *HbSus6* (74.7% and 74.2%) (Table 1). HbSus1 and HbSus6 are much less closely related to the four other HbSus
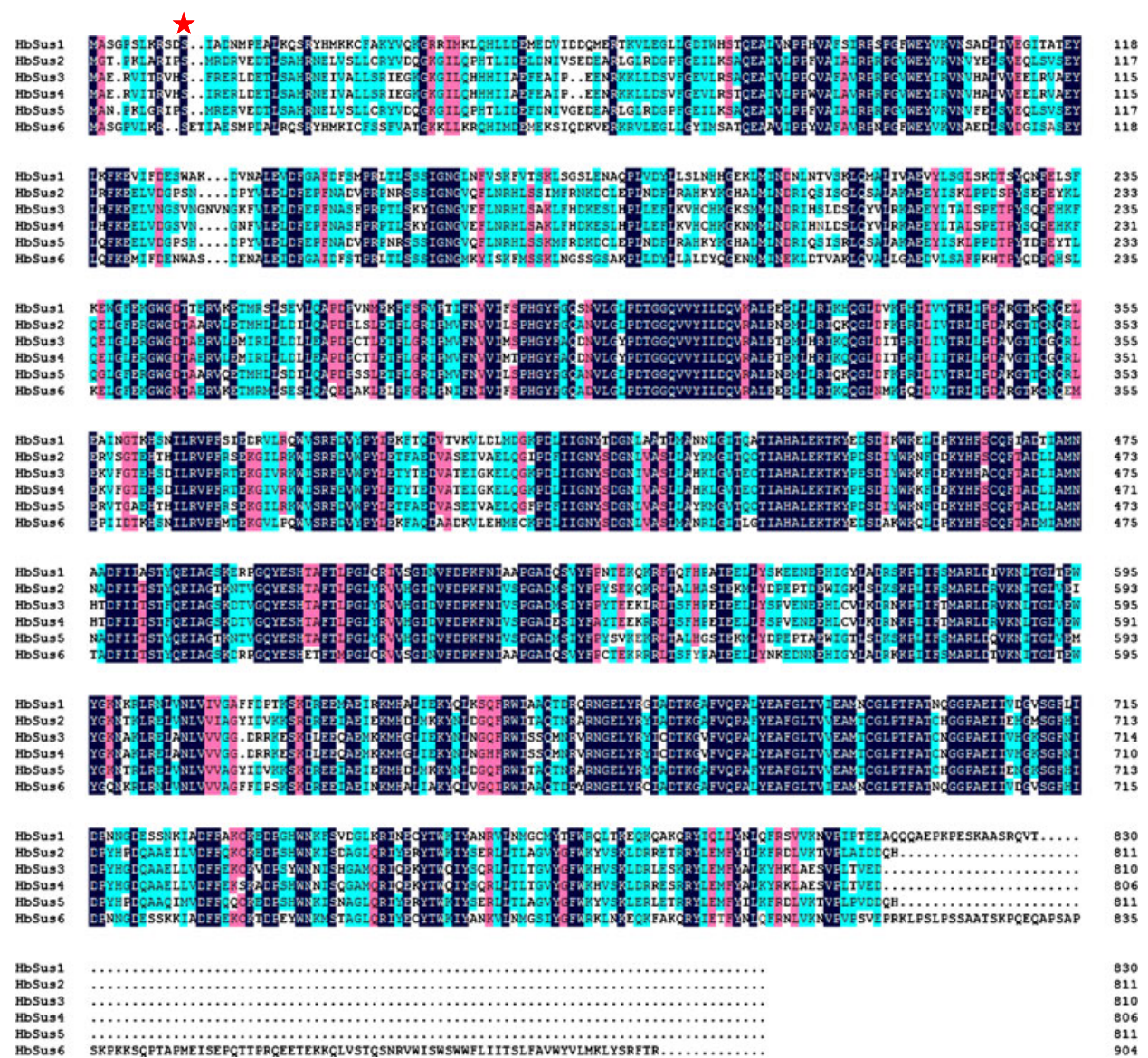
Fig. 1. Multiple sequence alignment for the predicted amino acid sequences of the six *Hevea Sus* genes (accession numbers: *HbSus1*, KC492043; *HbSus2*, KC492044; *HbSus3*, KC492045; *HbSus4*, KC492046; *HbSus5*, KC492047; *HbSus6*, KC492048). Sequence alignment was performed using DNAMAN 6.0 software (http://www.lynnon.com/). Identical amino acids are shaded and gaps are indicated by dots. The predicted conserved serine residue for phosphorylation by Ser/Thr protein kinase is indicated by a red star.

**Table 1.** Identity matrix for the six *HbSus* gene coding sequences and predicted protein sequences.

	Nucleotide identity					
	HbSus1 (%)	*HbSus2* (%)	*HbSus3* (%)	*HbSus4* (%)	*HbSus5* (%)	*HbSus6* (%)
Amino acid identity						
HbSus1	–	60.6	60.0	59.6	60.8	74.7
HbSus2	57.1	–	69.5	69.5	93.9	61.4
HbSus3	55.4	71.6	–	94.5	69.4	60.8
HbSus4	55.3	71.2	97.1	–	69.2	61.0
HbSus5	57.3	94.5	71.1	70.5	–	61.8
HbSus6	74.2	57.8	55.8	56.0	58.7	–

isoforms (HbSus2–5), with < 60% amino acid identity. By contrast, HbSus2–5 showed more than 70% amino acid identity between each other.

The predicted exon/intron structure between the start and stop codons of the *HbSus* genes was analyzed by comparing the cDNA sequences with PCR fragments amplified from genomic DNA, revealing structures similar to those reported for Sus homologs of other plants [23]. The six *HbSus* genes had 13 or 15 exons (Fig. S1), with conserved exon number and exon length. Introns, although different in size, appeared at equivalent positions and were flanked by typical GT/AG boundaries, except for the 7th intron of *HbSus1* and the 8th intron of *HbSus6*, which were spliced at unusual GC/AG splicing sites. The *HbSus* genes differ with respect to intron loss events. For example, *HbSus1* lacks the first intron that exists in other *HbSus* genes, resulting in formation of a larger exon 1. In addition, *HbSus6* has a longer exon 15, and an additional 3′ exon that is not observed among the other *HbSus* genes. As a consequence, the HbSus6 protein has a much higher molecular mass (102.7 kDa) than those calculated for other HbSus peptides (92.5–94.2 kDa) (Table S1).

Phylogenetic analysis of HbSus proteins and other plant Sus homologs

In order to establish the phylogenetic relationships among *Sus* gene families between *Hevea* and other plant species, including the six *Hevea* Sus isoforms, a total of 38 plant Sus amino acid sequences, representing eight species (*H. brasiliensis*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Arabidopsis thaliana*, *Populus trichocarpa* and *Ricinus communis*), were aligned and used to construct an unrooted tree using the neighbor-joining method in MEGA 4.0 software [37]. The robustness of the phylogram's topology was also determined by a bootstrap analysis (1000 replicates). As shown in Fig. 2, the 38 plant Sus
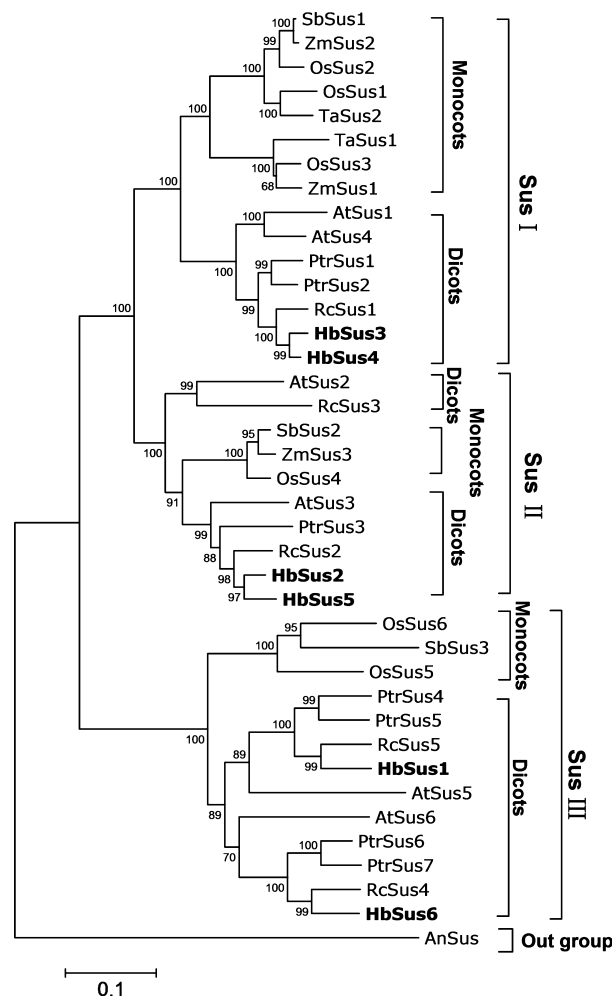

Fig. 2. Phylogenetic analysis of the HbSus proteins and other plant Sus homologs. Unrooted phylogenetic tree of plant Sus proteins constructed using the neighbor-joining method with MEGA 4.0 program [37]. Isozymes and the corresponding plant species are: *H. brasiliensis*, HbSus1–6 (this study, highlighted in bold); *Sorghum bicolor*, SbSus1–3; *Zea mays*, ZmSus1–3; *Oryza sativa*, OsSus1–6; *Triticum aestivum*, TaSus1 and 2; *Arabidopsis thaliana*, AtSus1–6; *Populus trichocarpa*, PtSus1–7; *Ricinus communis*, RcSus1–5. The Sus protein of the filamentous cyanobacterium *Anabaena variabilis* is used as an outgroup.

proteins are clustered into three major groups with high bootstrap values (100%), named Sus I, II and III. In addition, Sus proteins in the Sus I group may be further classified into two distinct sub-groups, consisting exclusively of dicot Sus proteins and monocot Sus proteins, respectively. The six *Hevea* Sus isoforms were evenly separated between the three Sus groups: HbSus3 and 4 in the dicot sub-group of Sus I, HbSus 2 and 5 in Sus II, and HbSus1 and 6 in Sus III, consistent with the exon/intron organization pattern and nucleotide/amino acid sequence identity (Table 1 and Fig. S1). The HbSus isoforms are more closely related to their putative Sus orthologs from *Ricinus communis*, which belongs to the same family of Euphorbiaceae as *H. brasiliensis*, reflecting consistency in the evolution of Sus isoforms and plant lineages.

Expression analysis of *HbSus* genes in *Hevea* tissues

In order to understand the potential functions of specific isoforms of Sus in *Hevea*, the tissue-specific expression of *HbSus* genes was examined in seven *Hevea* tissues, including latex, bark, leaf, root, seed,

and female and male flowers, using Solexa sequencing and quantitative PCR. For sequencing-based expressional analyses, cDNA libraries for samples of the seven tissues were subjected to Solexa sequencing, and a total of 344.9 million ESTs (~ 100 nucleotides long), i.e. a mean of 49.3 million ESTs for each tissue, were generated (Table S2). The EST counts of each gene were expressed as ESTs per million ESTs, and this value was used as a measurement to compare relative gene expression among *Hevea* tissues or *Sus* genes. As shown in Fig. 3A, high variations in the relative expression levels were observed between various *Hevea* tissues for a single *HbSus* gene and between *HbSus* genes for a single *Hevea* tissue. Transcripts of *HbSus1* and *HbSus6* were barely detectable in almost all tissues examined, while those of other *HbSus* genes were detectable in a wide range of tissues and showed distinct but partially overlapping expression patterns. Comparison of the transcripts of all *HbSus* genes in each single tissue revealed predominant *HbSus* isoforms for various tissues: *HbSus3* in latex and male flower, *HbSus4* in bark, female flower and seed, and *HbSus5* in root and leaf. Furthermore, quantitative PCR was performed on three abundant *HbSus* genes
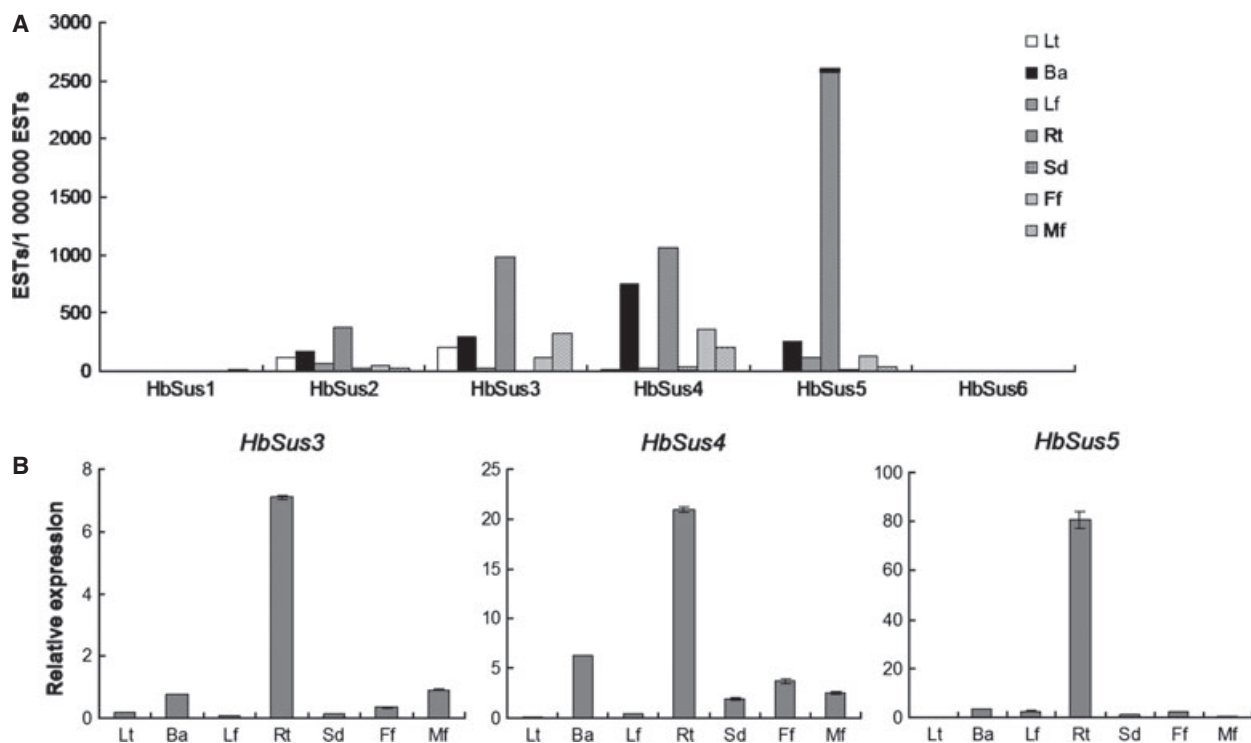
Fig. 3. Expressional analyses of the *HbSus* genes in various *Hevea* tissues. (A) EST levels of the six *HbSus* genes in various tissues, including latex (Lt), bark (Ba), leaf (Lf), root (Rt), seed (Sd), female flower (Ff) and male flower (Mf), examined by Solexa sequencing. (B) Relative expression of *HbSus3–5* in the same tissues as in (A) by quantitative PCR. Values for quantitative PCR are means ± SD of three replicates.

(*HbSus3–5*) to validate their tissue expression patterns, and their expression profiles were found to be very similar to those observed by sequencing-based expressional analyses, both with respect to relative expression of respective *HbSus* isoforms among various tissues or different *HbSus* isoforms in a single tissue (Fig. 3B).

To obtain further information on the importance of *Sus* genes in the development of *Hevea* leaves, the expression levels of *HbSus* genes were examined by Solexa sequencing in the leaves of 1-year-old bud-grafted plants at four progressive developmental stages (bronze, color change, pale-green and mature). For each stage of leaf development, 33.9–48.7 million ESTs of ~ 100 bp in length were generated (Table S2). As shown in Fig. 4A, the six *HbSus* genes may be expressionally categorized into four distinct groups: (a) *HbSus1* and especially *HbSus6*, transcripts of which were found at low or very low levels at all stages of leaf development; (b) *HbSus2* and 5, transcripts of which increased gradually with leaf development, peaking at the mature stage; (c) *HbSus3*, transcripts of which increased continuously during the first three stages of leaf development, then decreased abruptly and were barely detectable at the mature stage; (d) *HbSus4*, transcripts of which peaked at the initial stage of leaf development (bronze), and then decreased rapidly during leaf development to a low level at the mature stage. Quantitative PCR expressional analyses were performed on *HbSus3–5* (Fig. 4B), and again their expression patterns were similar to those from sequencing-based expression analysis.

Expression analyses of *HbSus* genes in response to Ethrel treatment

To understand the roles of *HbSus* genes in Ethrel-stimulated latex metabolism, *HbSus* transcript levels were analyzed in latex after 3, 12 and 24 h of Ethrel treatment using the Solexa sequencing method (Table S2). The results showed that, although the transcript levels of *HbSus1*, *HbSus5* and *HbSus6* were still low or even undetectable in latex after different hours of Ethrel treatments, a significant repression was observed for those of *HbSus2–4* (Fig. 5A). Compared to the expression levels in latex without Ethrel treatment, expression of *HbSus2–4* was decreased by 2.3-, 2.6- and 3.5-fold, respectively, after 24 h of treatment. Quantitative PCR analyses performed on *HbSus2–4* revealed similar expression changes after Ethrel treatment (Fig. 5B). The decrease in *HbSus* expression in response to Ethrel treatment is consistent with the weakened enzymatic activity of Sus proteins in latex after Ethrel treatment [34]. Further, the down-regulation of *HbSus3* expression in latex by wounding (Fig. 5C) may also involve the participation of ethyl-
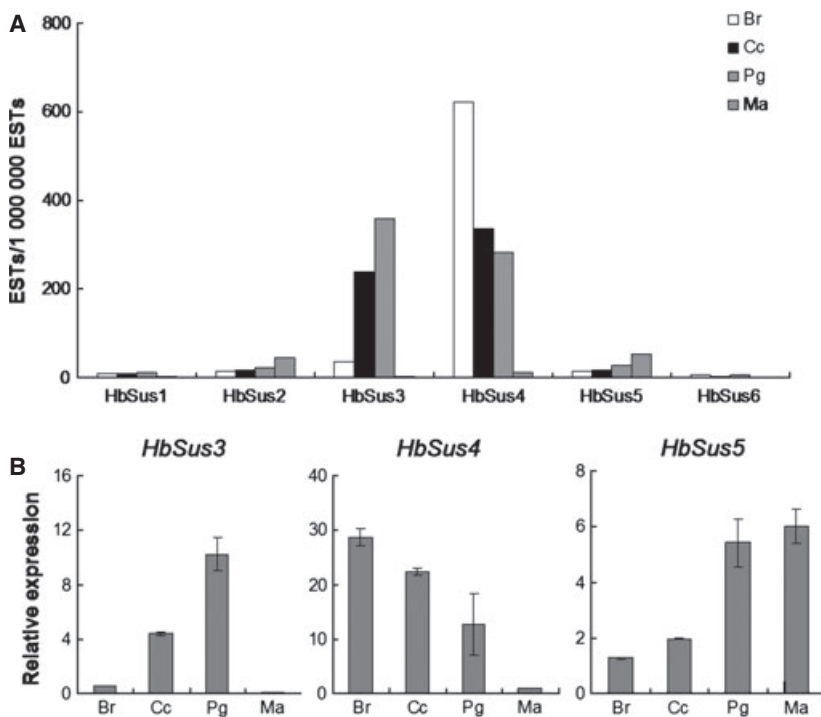
Fig. 4. Expression analyses of the *HbSus* genes at various developmental stages of *Hevea* leaves. (A) EST levels of the six *HbSus* genes at four progressive stages of leaf development, i.e. bronze (Br), color change (Cc), pale-green (Pg) and mature (Ma), examined by Solexa sequencing. (B) Relative expression of *HbSus3–5* in the same tissues as in (A) by quantitative PCR. Values for quantitative PCR are means ± SD of three replicates.
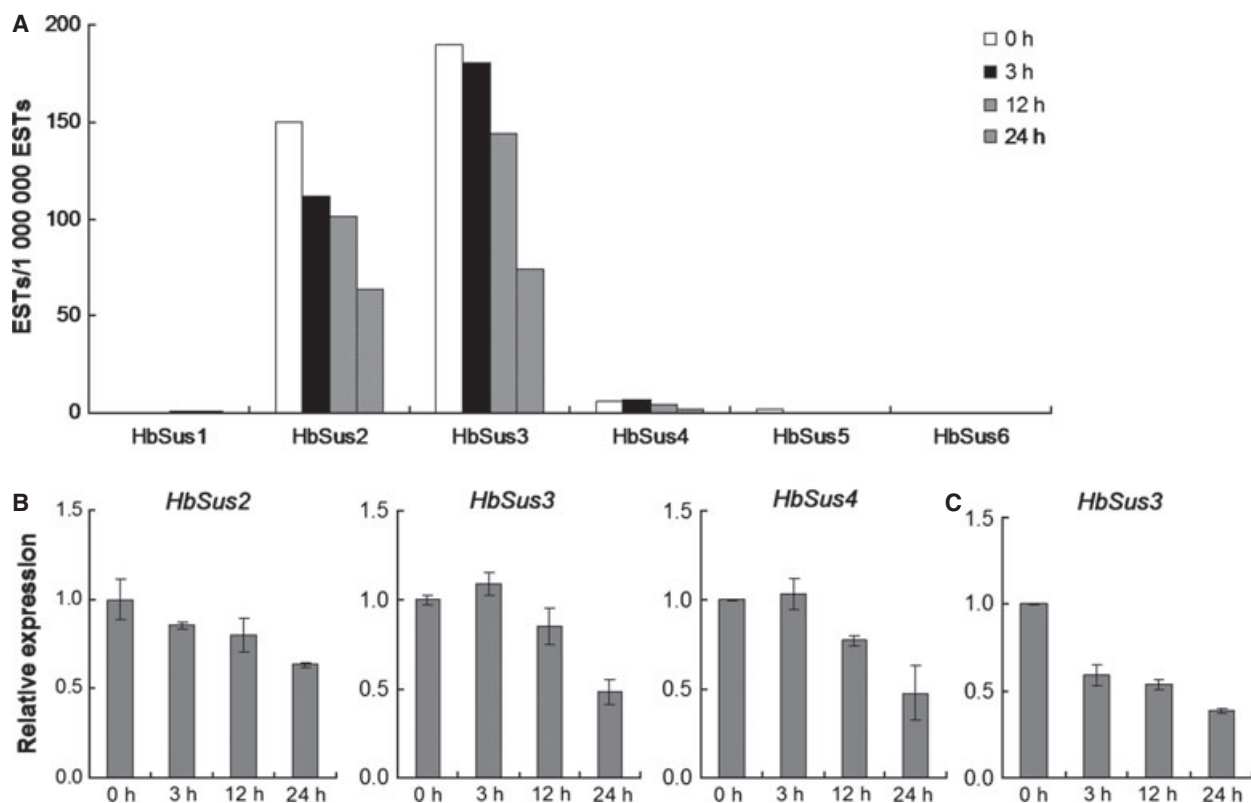
Fig. 5. Expression of the six *HbSus* genes in latex during Ethrel treatment. (A) Change of EST levels by Solexa sequencing for the six *HbSus* genes after Ethrel treatment. (B) Expression kinetics assessed by quantitative PCR for *HbSus2–4* under the same treatment as (A). (C) *HbSus3* expression in latex response to wounding assessed by quantitative PCR. Values for quantitative PCR are means ± SD of three replicates.

ene, as the effect of wounding includes release of 'wound ethylene' [38].

Expression analyses of *HbSus* genes in response to cold, heat and drought treatments

Five-month-old tissue-cultured *Hevea* plants derived from secondary somatic embryogenesis [39] were subjected to three stress treatments, i.e. cold (5 °C), heat (40 °C) and simulated drought by polyethylene glycol solutions for various durations. Leaves, bark and roots were collected after each treatment time, and RNA samples were prepared for quantitative PCR examination of the expression of the predominant *Sus* isoforms in the various tissues (Fig. 6).

In leaf, the effect of stress treatments on expression of the leaf-predominant isoform *HbSus5* (Fig. 3) was examined (Fig. 6A). *HbSus5* mRNA levels were significantly induced (11.2-fold) after 3 h of cold treatment, and decreased thereafter but maintained a high level of up-regulation compared to untreated samples. During the 24 h heat treatment, *HbSus5* expression showed

regular fluctuations: it first decreased markedly at 3 h, then increased significantly at 12 h, and finally decreased abruptly at 24 h. For the drought treatment, an overall gradual increase of *HbSus5* transcripts was observed during the first 4 days of treatment, with a 9.3-fold increase at the 4th day.

In bark, the effect of stress treatments on expression of the bark-predominant isoform *HbSus4* (Fig. 3) was investigated (Fig. 6B). The expression of *HbSus4* was induced slightly by cold stress, with a 1.5-fold induction after 12 h of treatment. In contrast, *HbSus4* mRNA levels decreased progressively after heat and drought treatments, showing 5.6- and 2.8-fold decreases, respectively, at the end of treatment.

In root, the effect of stress treatments on expression of the root-predominant isoform *HbSus5* (Fig. 3) was assessed (Fig. 6C). *HbSus5* transcript levels were induced quickly by cold stress, showing a 24.6-fold induction after 3 h treatment and further increases thereafter. However, an obvious decrease was observed in *HbSus5* expression after heat treatment, with a 4.6-fold decrease after 12 h of treatment, remaining stable
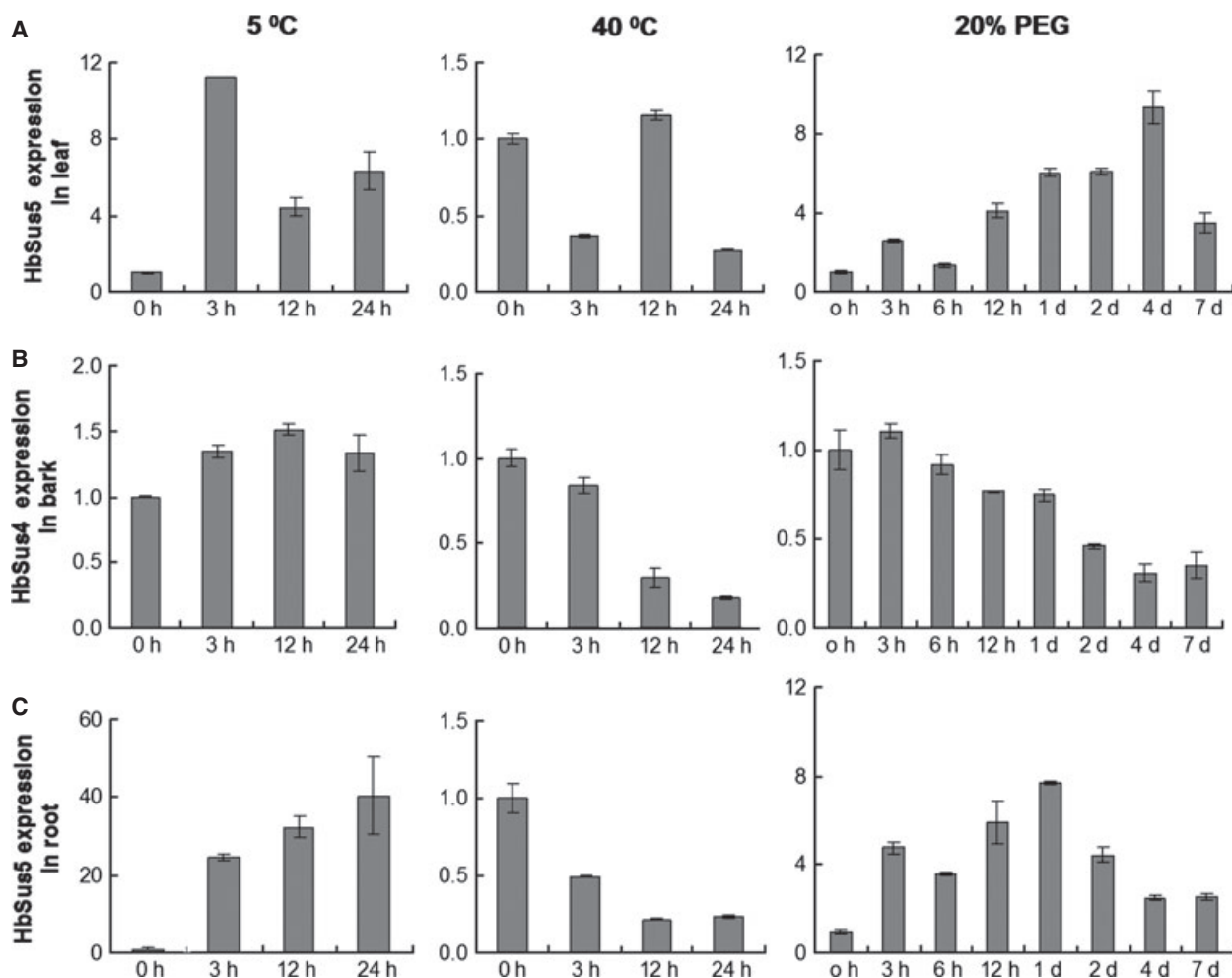
Fig. 6. Expression assessed by quantitative PCR of the predominant *HbSus* isoforms in response to stress treatments (cold, heat and drought) in various *Hevea* plant tissues. (A) Expression of *HbSus5* in leaf in response to stress treatments. (B) Expression of *HbSus4* in bark in response to stress treatments. (C) Expression of *HbSus5* in root in response to stress treatments. Values are means ± SD of three or four replicates.

thereafter. During the 7-day period of drought treatment, *HbSus5* expression revealed two-phase expression kinetics. The first phase comprises overall up-regulation from 0 h to 1 day, peaking at 1 day at 7.7-fold induction, followed by a second phase involving a 3.0-fold decrease from 1 to 7 days.

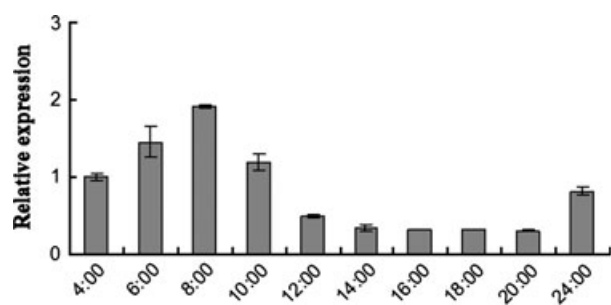## Discussion

Functional divergence of *HbSus* genes

Functional diversity after gene duplication through changes in expression patterns and/or protein properties is an important evolutionary driving force to allow organisms to differentiate new organs or increase fitness to new environments [40,41]. To date, no *Sus*

genes have been cloned and studied in rubber tree, although the activity and biochemical kinetics of partially purified Sus in the latex have been reported [34]. The present study describes the cloning and systematic analysis of the expression patterns for six isoforms of the *HbSus* gene family.

Previous studies have shown that Sus activity is highly correlated with sink strength [6,7,10–12]. In this study, we also demonstrated that the overall expression of *HbSus* genes is much higher in sink tissues, such as root, bark, latex and flower, than in the source tissue (mature leaf) (Fig. 3A). However, when examined in detail, no two *HbSus* genes share identical expression patterns, implying obvious functional divergence among the six *HbSus* genes (Figs 4–7). Although no *Sus* genes are exclusively expressed in a

single tissue, each tissue tends to have one abundant *Sus* isoform (Fig. 3). For example, the most abundantly expressed *Sus* isoforms in root, bark and latex are *HbSus5*, *4* and *3*, respectively, implying predominant roles in the respective tissues. Most *HbSus* genes are expressed at low or very low levels in mature leaves (source leaf), whereas *HbSus3* and *4* are highly expressed in immature leaves (sink leaf) (Fig. 4). This finding is consistent with the idea that *Sus* genes are expressed mainly in non-photosynthetic sink tissues [42]. Similar results have been obtained for the Sus family of rice, all six *Sus* genes of which are much more highly expressed in elongating flag leaves wrapped in leaf sheaths (sink leaf) than in fully expanded flag leaves (source leaf) [21]. However, in *Populus*, the transcript abundance for all *Sus* members was higher in mature leaves than in young ones [22]. *HbSus4* transcripts show a pattern of progressive down-regulation with leaf development, with a 52-fold reduction from the bronze to the mature stage, which suggests that *HbSus4* plays a major role in immature leaves (Fig. 4). In contrast, although *HbSus2* and *5* are expressed at low levels in bronze-stage leaves, their transcripts increase gradually with leaf development, and are the most abundant *Sus* isoforms in mature leaves (Fig. 4A), which implies a role in the metabolism of sink leaves. This implication is corroborated by the patterns of diurnal expression of *HbSus5* in mature leaves, which exhibits obvious changes in transcript abundance over the course of a day (Fig. 7), suggesting a role in supplying energy for phloem loading [9,43]. The marked induction of *HbSus5* expression in the leaves and roots of *Hevea* plants under low temperature and drought treatments suggests a role for *HbSus5* in abiotic stresses (Fig. 6). The induction of *Sus* gene expression in response to dehydration and cold treatment has been well documented for *Arabidopsis Sus* genes *AtSUS1* and *3* [20], and barley (*Hordeum vulgare*) *Sus* genes *HvSs1* and *3* [44]. The induced expression of *Sus* genes highlights their contribution to meeting the increased glycolytic demand during conditions of abiotic stresses [45].

Functional expression of *HbSus* genes in heterogeneous biological systems will be beneficial to further characterizing the discrepancies of these *Sus* genes. To this end, one latex-abundant *Sus* isoform (*HbSus2*) was tentatively expressed in *Escherichia coli* (Fig. S2). However, *E. coli* cells successfully over-expressing HbSus2 proteins did not show obvious sucrose-cleaving or sucrose-synthesizing activities, indicating the existence of post-translational modifications that cannot be fulfilled in prokaryotic systems [46] but are essential for the functionality of HbSus proteins. Therefore, expression of these *Sus* genes in eukaryotic systems, e.g. yeast, is necessary for such experiments.

Evolutionary conservation across Sus homologs of plants

Comprehensive analysis of phylogenetic tree and exon/intron gene structures between Sus isoforms in plants allows us to conclude that evolutionary conservation exists in the Sus family of the plant kingdom. First, all plants examined to date have a small, multi-gene family that comprises at least three *Sus* genes. Interestingly, a number of plant species with available genome sequences, including *Arabidopsis* [20], rice [21] and *Brachypodium distachyon* [44], have six *Sus* genes. In this study, six *Sus* isoforms were identified in *H. brasiliensis* (Figs 1 and 2 and Table S1), and all appear to be functional as their transcripts were detected in at least one tissue examined (Figs 4 and 5). As a very high number of *Hevea* ESTs (see Experimental procedures) were used for Sus searching, the six *Sus* genes are believed to represent the entire Sus family in *Hevea* tree. This prediction is supported by Sus searching against the recently released *Hevea* draft genome sequence [47]. Although seven Sus isoforms have been identified in cotton, one isoform (*GaSus2*) appears to be a pseudogene [23]. Up to now, the only species that has more than six functional *Sus* genes is Populus trichocarpa, with seven ones to be exact. [22]. Second, in each plant species, the Sus isoforms may be divided into three major groups (Sus I, II and III), with at least one member for each group, according to the phylogenetic tree and molecular structure analysis of their sequences [21,23,48]. The six *Hevea* Sus isoforms are equally separated into the three Sus groups (Fig. 2): *HbSus3* and *4* in Sus I, *HbSus2* and *5* in Sus II, and *HbSus1* and *6* in Sus III. Based on the predicted order of evolution for the cotton Sus family

Fig. 7. Diurnal changes of *HbSus5* transcripts assessed by quantitative PCR in mature *Hevea* leaves. Values are means ± SD of four replicates.

[23], *HbSus1* and *HbSus6* may be evolutionarily older than the other four *Sus* genes (*HbSus2–5*) (Fig. 2). Third, the exon/intron structures of the *Sus* family are highly conserved in plants. Previous comparative analysis of the *Sus* homologs revealed conservation in terms of both the number and position of introns in several distantly related dicot and monocot plants [23]. The number and position of introns were also highly conserved in the six *HbSus* genes. When examined in detail, the exon/intron structures of the six *HbSus* genes may be further classified into four types (Fig. S1): *HbSus3* and *HbSus4*, *HbSus2* and *HbSus5*, *HbSus1* and *HbSus6*. In addition, the evolutionary conservation of the *Sus* genes in plants is reflected in the patterns of expression for the early evolved Sus isoforms. These *Sus* isoforms tend to be expressed at low levels in most tissues and are not responsive to exogenous treatments, e.g. in the case of *AtSUS6* in *Arabidopsis* [20], *Sus5* and *6* in rice [21], *HvSs4* in barley [44], *GaSus7* in cotton [23], and *HbSus1* and *6* in *Hevea* (this study).

### The roles of Sus in latex metabolism of *Hevea* tree

In regularly tapped rubber trees, the rubber-producing laticifers represent an active sink for sucrose, and sucrose catabolism in latex is the first step leading to rubber synthesis and forms an important point of control for rubber production [27]. Physio-biochemical studies reveal that sucrose catabolism in the latex is mainly accomplished by a neutral/alkaline type of Inv [33]. However, the role of Sus should not be ignored. In the latex of regularly tapped trees, the enzyme activity of Sus functions mainly in the direction of sucrose synthesis, and therefore acts as a counteracting factor in sucrose catabolism [34]. Of the six *HbSus* genes identified in this study, only *HbSus2* and *HbSus3* show substantial transcript abundance in the latex (Fig. 3), suggesting major roles for these isoforms in the overall enzyme activity of Sus in this tissue. After Ethrel treatment, the latex transcripts of both *Sus* genes decreased progressively with the duration of treatment (Fig. 5), consistent with the reduced Sus synthesis activity in the latex [34], indicating the significance of transcriptional regulation on the activity of Sus enzymes. Direct correlation of transcript abundance of *Sus* genes with their functions has also been reported in other plants [6,11,14,16,19]. However, protein phosphorylation and other types of post-translational modifications do affect the subcellular localization and functions of some Sus enzymes [49]. The physiological significance of Sus in the latex is not

clear, but the existing evidence suggests a role in preventing excess sugar consumption in the laticifers. In rubber production, excessive artificial interventions, such as over-stimulation with Ethrel and intensive tapping, often result in the occurrence of tapping panel dryness [50,51]. Tapping panel dryness is a complex physiological disorder that finally causes complete stoppage of latex flow, and nutritional deficit is proposed to be an early causative signal. It remains to be determined whether the occurrence of tapping panel dryness is related to a weakened role of Sus as the valve to repress sugar over-consumption. In addition, the transcript levels of *HbSus3* in the latex correlated well with the yield of rubber cultivars/clones or individual rubber trees of the same cultivar/clone (Fig. 8), further complicating understanding of the physiological roles of Sus in latex metabolism.

Experimental procedures

Plant materials

Unless otherwise noted, Reyan7–33–97 (synonym for CATAS7–33–97 or RY3–33–97), Reyan8–79 (synonym for CATAS8–79 or RY8–79) and PR107 rubber trees (*H. brasiliensis*) selected for this study were cultivated at the experimental plantation of the Rubber Research Institute of the Chinese Academy of Tropical Agricultural Sciences (Danzhou, Hainan, China). These trees were regularly tapped for latex collection in a half spiral pattern, every 3 days, without Ethrel stimulation. To study the tissue-specific expression of *HbSus* genes, all tissues except roots were collected for RNA extraction from 10-year-old mature Reyan7–33–97 trees that had been tapped for the last 2 years. These mature trees were treated with Ethrel to
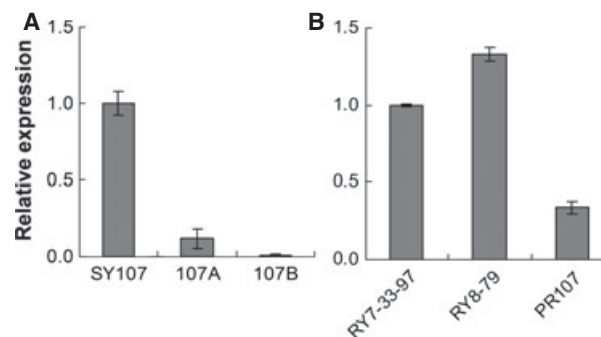
Fig. 8. Relative expression by quantitative PCR of *HbSus3* in latex of various rubber trees or clones that differ in terms of yield. (A) Expression of *HbSus3* among PR107 trees of different yield (SY107 > 107A > 107B). (B) Expression of *HbSus3* among clones of different yield (RY8–79 > RY7–33–97 > PR107). Values for quantitative PCR are means ± SD of three replicates.

examine the effect of Ethrel on *HbSus* gene expression. To ensure genetic homogeneity, the roots used for tissue expression studies were harvested from 5-month-old self-rooted juvenile Reyan7–33–97 plants derived from secondary somatic embryogenesis (Hua *et al.* 2010), as the rubber trees being used for rubber production were propagated by grafting axillary buds of elite clones onto seedlings. The self-rooted juvenile plants were used to examine the effect of various stress treatments on *HbSus* gene expression. Mature Reyan7–33–97 virgin (untapped) trees were used to examine the effect of wounding on *HbSus* gene expression. To investigate the expression of *HbSus* genes at various stages of leaf development, leaves of four progressive stages (bronze, color change, pale-green and mature) were collected for RNA extraction from 1-year-old grafted RY7–33–97 plants cultivated at the *Hevea* Germplasm Repository of the Rubber Research Institute of the Chinese Academy of Tropical Agricultural Sciences. To compare the relative expression of *HbSus3* among the various rubber clones, 20 trees were selected from each of the three clones (PR107, RY8–79 and Reyan7–33–97) and latex was collected for RNA extraction. The previously described 34-year-old PR107 trees at the Yunnan rubber plantations were used to compare the relative expression of *HbSus3* among rubber trees or clones producing different yields [52].

Ethrel, wounding and stress treatments

Ethrel (2-chloroethyl- 11 phosponic acid, an ethylene releaser) and wounding treatments and latex collection for RNA extraction were performed as previously described [30]. For stress treatments, 1-month-old tissue-cultured juvenile Reyan7–33–97 plants were transferred from test tubes to a sand bed for hardening. Two months after hardening, tissue-cultured plants were transferred into Hoagland's solution [53] for another two months of growth under semi-controlled environmental conditions in a naturally lit greenhouse with a temperature range of 22.0–35.0 °C and a relative humidity range of 60–90%, and then subjected to various treatments. During the period of hydroponics, the Hoagland's solution was changed every week, and aerated every 2 days for 8 h. For the low-temperature stress, tissue-cultured plants were transferred to conical flasks containing Hoagland's solution placed in a growth chamber at 5 °C under continuous white light, and incubated for 0, 3, 12 and 24 h. For the high-temperature stress, the manipulations were similar to those for the low-temperature stress, except that the temperature was adjusted to 40 °C, and the relative humidity was maintained at 80%. For the drought stress, the tissue-cultured plants were transferred into basic Hoagland's solution containing 20% PEG6000, and incubated for various durations (0, 3, 6 and 12 h, and 1, 2, 4 and 7 days). For all stresses, leaf, bark and root were sampled for RNA extraction from stressed plants at each time

point, and samples from unstressed plants were used as controls.

DNA and RNA extraction

Genomic DNA was isolated from the leaves using the cetyltrimethylammonium bromide method [54]. Total RNA was extracted from latex using the method described by Tang *et al.* [55], and from tissues other than latex as described previously [30]. RNA samples were treated with DNase I (Takara Biotechnology (Dalian) Co., Ltd) to eliminate trace contaminants of genomic DNA. The integrity of the RNA and DNA samples was checked by agarose gel electrophoresis, and the concentration and purity were examined by scanning using a spectrophotometer (Alpha Spec, ProteinSimple) at 230, 260, 270 and 280 nm [56].

Cloning of *Hevea* sucrose synthase genes

To identify the Sus homologs in *H. brasiliensis*, *Populus* [22] and *Arabidopsis* [20] Sus genes were used as queries to search a large collection of Sanger, 454 and Solexa ESTs of *H. brasiliensis* (YJ Fang and CR Tang, unpublished data; Table S2) by BLAST searching, and contigs were assembled as putative *Sus* genes. Based on the sequences of the resulting contigs, multiple pairs of primers were designed and used to amplify the cDNA and genomic DNA of putative *Hevea* Sus genes. The PCR products were cloned into the pMD18–T cloning vector (TaKaRa Biotechnology), and then transformed into *E. coli* cells (DH5α) for sequencing. The obtained sequences were submitted to the National Center for Biotechnology Information database for BLAST searching and other bioinformatics analysis.

Expression in *E. coli*

For expression of *HbSus2* in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany), its ORF was isolated by PCR using primers 5′-CGCGTCGACTCATGGGAACTCCCAAGTTGGCTAGAATCCCCA-3′ (forward) and 5′-CCGCTCGAGTTAATGTTGGTCGTCAATTGCCAAAGGAA-3′ (reverse). The PCR products were first cloned into the pMD18–T vector (TaKaRa), and then sub-cloned into expression vector pET28a(+) (Novagen) at the *Sal*I and *Xho*I sites. The transformed bacteria carrying the expression vectors were grown to mid-log phase at 37 °C, and then protein biosynthesis was induced using 1.0 mM isopropyl thio-β–D–galactoside at 20 °C for 1, 3 and 5 h. Expression of HbSus2 recombinant protein was monitored by SDS/PAGE using 10% polyacrylamide gels [57]. Bacterial cells were pelleted, and suspended in a one-twentieth culture volume of citrate phosphate buffer (pH 6.0). The cell suspensions were sonicated using a Branson digital sonifier (Danbury, CT, USA) for 10 min at 40% setting, and then used to investigate

Table 2. Gene-specific primers used for quantitative PCR analysis of *HbSus* gene expression.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
HbSus2	AAGTACGTTTCCAAACTTGACCG	AAATACTCACCAAATCACGGAAC
HbSus3	GCTGAATCTGTTCCTCTAACTGT	TATTCTTCACAACTCCGAAACTT
HbSus4	TGGTGTATATGGCTTCTGGAAAC	GTTAGAGGAACTGATTCAGCCAA
HbSus5	TATTGATATGATCAAGAGGCAGC	GGCTTTGCATAAGAAAAGTTTCA

sucrose cleavage and sucrose synthesis activities as described previously [34].

Phylogenetic and gene structure analyses

The sequence data used in this study were obtained using the keyword 'sucrose synthase' and performing a query search in GenBank using the known Sus gene sequences from *Hevea*, *Populus* and *Arabidopsis*. Phylogenetic and molecular evolutionary analysis was performed using MEGA version 4.0 [37], and the neighbor-joining method was used to build phylogenetic trees [37]. Bootstrap analysis was performed using 1000 replicates. Exon/intron structures of the target Sus genes were analyzed by comparing the cDNA sequences and their genomic DNA sequences using the web server GSDS [58].

Expressional analysis based on Solexa sequencing

RNA samples were prepared from various *Hevea* samples, including seven tissues (latex, bark, leaf, root, seed, female flower and male flower), leaves of four developmental stages (bronze, color change, pale-green and mature), and latex collected at 0, 3, 12 and 24 h after Ethrel stimulation. Approximately 6 μg RNA representing each group was used for Solexa sequencing as described previously [59] on an Illumina Genome Analyzer (San Diego, CA, USA) at Beijing Institute of Genomics, Chinese Academy of Sciences. The raw data were filtered to remove adaptor reads, low-quality reads, repeat sequence reads and reads of copy number 1, yielding a dataset consisting of more than 640 million high-quality clean reads (Table S2). The trimmed reads were then mapped onto respective *HbSus* cDNAs using BWA software [60]. The EST counts of each gene were expressed as ESTs per million ESTs, and this value was used as a measure to compare relative gene expression across samples.

Real-time quantitative RT–PCR

To verify the data obtained by Solexa sequencing, real-time quantitative RT-PCR (quantitative PCR) was performed on the *HbSus* genes as described previously [61]. The RNA samples used for quantitative PCR assays were the same as those used for the Solexa sequencing. The reaction was performed using the Light Cycler 2.0 system (Roche Diagnostics, Penzberg, Germany) using SYBR Green premix kit

(TaKaRa) according to the manufacturer's instructions. The specificity of primers designed for the *HbSus* genes (Table 2) was confirmed by separating the products on agarose gels and sequencing after the PCR reaction. It is worth noting that, due to the high sequence similarity between the coding sequences of some *HbSus* genes, the reverse primers were designed within the 3′ UTRs of these genes to guarantee the specificity of primers. The *Hevea YLS8* gene was used as an internal control (forward primer: 5′-CCTCGTC GTCATCCGATTC-3′, reverse primer: 5′-CAGGCACCTCA GTGATGTC-3′) [61].

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Exon/intron structural organization of the six *HbSus* genes.
Fig. S2. Expression of *HbSus2* in *E. coli*.
Table S1. Information for the six *HbSus* genes and their predicted proteins.
Table S2. Information for Illumina sequencing data of *Hevea* tissues.