Sucrose importation into laticifers of *Hevea brasiliensis*, in relation to ethylene stimulation of latex production

Anaïs Dusotoit-Coucaud1, Nicole Brunel1, Panida Kongsawadworakul2, Unchera Viboonjun2, André Lacointe1, Jean-Louis Julien1, Hervé Chrestin23 and Soulaïman Sakr14,*

1UMR 547 PIAF, INRA, Université Blaise Pascal, 24 avenue des Landais, 63177 Aubière Cedex, France, 2Department of Plant Science, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand, 3Institut de Recherche pour le Developpement (IRD), UR 060 CLIFA/CEFE-CNRS, 1919 route de Mende, F34293, Montpellier Cedex 5, France and 4Agrocampus Ouest, Centre d’Angers, UMR SAGAH, IFR QUASAV 149, 2 rue le Nôtre, 49045 Angers Cedex, France

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Background and Aims The major economic product of *Hevea brasiliensis* is a rubber-containing cytoplasm (latex), which flows out of laticifers (latex cells) when the bark is tapped. The latex yield is stimulated by ethylene. Sucrose, the unique precursor of rubber synthesis, must cross the plasma membrane through specific sucrose transporters before being metabolized in the laticifers. The relative importance of sucrose transporters in determining latex yield is unknown. Here, the effects of ethylene (by application of Ethrel†) on sucrose transporter gene expression in the inner bark tissues and latex cells of *H. brasiliensis* are described.

Methods Experiments, including cloning sucrose transporters, real time RT-PCR and *in situ* hybridization, were carried out on virgin (untapped) trees, treated or untreated with the latex yield stimulant Ethrel.

Key Results Seven putative full-length cDNAs of sucrose transporters were cloned from a latex-specific cDNA library. These transporters belong to all SUT (sucrose transporter) groups and differ by their basal gene expression in latex and inner soft bark, with a predominance of *HbSUT1A* and *HbSUT1B*. Of these sucrose transporters, only *HbSUT1A* and *HbSUT2A* are distinctly increased by ethylene. Moreover, this increase was shown to be specific to laticifers and to ethylene application.

Conclusion The data and all previous information on sucrose transport show that *HbSUT1A* and *HbSUT2A* are related to the increase in sucrose import into laticifers, required for the stimulation of latex yield by ethylene in virgin trees.

Key words: *Hevea brasiliensis*, laticifers, latex production, ethylene, sucrose transporters.

INTRODUCTION

Natural rubber (cis-polyisoprene) is the main economic product of *Hevea brasiliensis* and is widely used industrially. cis-Polyisoprene synthesis takes place in the cytoplasm of highly specialized cells, known as laticifers (latex cells), and is their main metabolic function. Because sucrose is the unique precursor for rubber synthesis, its import into the laticifers may be an important limiting factor in latex production.

Laticifers are periodically differentiated from the cambium and arranged in an isolated network in the inner bark of *H. brasiliensis* (Hébant and de Fay, 1980; de Fay and Jacob, 1989). The cytoplasm of laticifers, known as latex, is expelled when the bark is wounded or deliberately regularly cut (tapped) to obtain the latex (Gomez, 1976; Gomez and Moir, 1979). Rubber particles represent 30–50 % of the latex in volume and 90 % in dry weight, and therefore laticifers have to regenerate their cytoplasm between two consecutive tapings. Much research has shown that rubber synthesis is brought about through the mevalonate-dependent metabolic pathway (Hepper and Audley, 1969), but more recently, transcriptome studies have suggested that synthesis could also follow a mevalonate-independent pathway (Ko et al., 2003). Both pathways coexist in laticifers and need sucrose as a precursor (d’Auzac, 1964; Chow et al., 2007). In addition, sucrose contributes, together with polyols and potassium, to the generation of the high turgor pressure (0.9–1.5 MPa) in the laticifers and their surrounding cells in the soft bark (liber), which is important for latex flow (Buttery and Boatman, 1966). Moreover, the cytosolic sucrose concentration is used to evaluate metabolic activity of laticifers (Jacob et al., 1988, 1989).

Laticifers are regarded as a strong sink for sucrose and need to be adequately supplied exogenously with sucrose to meet their high carbon and energy demands (Tupy, 1973; Eschbach et al., 1986; Silpi et al., 2007). Based on a few circumstantial observations, active transport across the plasma membrane of laticifers had been hypothesized as the main route of sugar uptake. Ultrastructural studies on mature laticifers showed that they have no plasmodesmata and are therefore apoplastically isolated from adjacent cells (Hébant, 1981; de Fay and Jacob, 1989). Electrophysiological research carried out on isolated laticifers and protoplasts indicates that sucrose is able to depolarize the plasma membrane and this process is sensitive to H+/ATPase inhibitors (Bouteau et al., 1991, 1992, 1999), suggesting the existence of an energypedependent, H+/sucrose symporter in the plasma membrane. However, there are no reports, based on molecular evidence, of the presence of such sucrose transporters in the laticifers.
Sucrose transporter (SUT) cDNAs have been isolated mainly from herbaceous plants, but also from some woody plants (Lemoine, 2000; Sauer, 2007). Generally, these sucrose transporters belong to the major facilitative superfamily (MFS; Marger and Saier, 1993) and are encoded by a multigenic family. Their activity was reported to be finely controlled (Sakr et al., 1997; Chiou and Bush, 1998; Roblin et al., 1998; Vaughn et al., 2002; Schneidereit et al., 2008) and modulated by exogenous (Juergensen et al., 2003; Meyer et al., 2004; Decourteix et al., 2006) as well as endogenous signals such as sucrose (Chiou and Bush, 1998) and hormones (Chincinska et al., 2008). Phylogenetic analyses have shown that sucrose transporter proteins fall into three independent phylogenetic clades, called SUT1, SUT4 and SUT2 (Barker et al., 2000; Weise et al., 2000; Barth et al., 2003).

Sucrose transporters play a key role in many physiological processes. This is true both for source and for sink organs. For example, ArSUC5, specifically expressed in the endosperm, is required for the supply of sucrose to seeds during early stages of development (Baud et al., 2005). Based on comparison of expression patterns of three sucrose transporters of the grape berry (VvSUC11, VvSUC12 and VvSUC27), Davies et al. (1999) suggested that only two sucrose transporters (VvSUC11, VvSUC12) might have a significant role in ripening-related sucrose import. Inhibition of LeSUT2 in tomato by antisense expression resulted in reduction of the number of fruit and the number of seed fertilized per fruit. This phenotype is due to the inability of these organs to download sucrose from phloem, and supports the central role of this sucrose transporter in fruit and seed development (Hackel et al., 2006). JrsUT1 (from Juglans regia sucrose transporter), isolated from xylem parenchyma cells, is involved in sucrose retrieval from the xylem vessel after freezing and thawing (Decourteix et al., 2006) and in providing sucrose to bursting vegetative buds (Decourteix et al., 2008). Although sucrose is the predominant soluble sugar in H. brasiliensis latex and is required for rubber synthesis and for latex cell cytoplasm regeneration between tappings, no study has yet focused on the involvement of sucrose transporters in these processes.

As ethylene was shown to be an efficient stimulant of latex yield (Abraham et al., 1968; d’Auzac and Ribaillier 1969), Ethrel® and ethylene gas are commonly used to increase rubber yield in plantations worldwide. This stimulation may occur in several ways: through an increase in sugar metabolism (Tupy, 1973; Tupy and Primot, 1976; Silpi et al., 2006) and the consecutive increase in energy (ATP) availability (Amalou et al., 1992), by increased sugar import (Lacroute et al., 1985) and by a hyperpolarization of the laticifer’s plasma membrane (Bouteau et al., 1992), suggesting a possible stimulation of H⁺-ATPase plasma membrane by ethylene application, activity of which is required for a plasma membrane H⁺-symporting sucrose transporter.

Rubber biosynthesis is the major metabolic feature of laticifers, and requires exclusively sucrose as precursor. These cells are a metabolically active sink but are apoplastically isolated from neighbouring cells. Therefore, to meet adequately their substantial carbon and energy needs, they have to import sucrose through hypothetical plasma membrane sucrose/H⁺ symporters. Our hypothesis is that these kinds of transporters (sucrose/H⁺ symporters) may be localized on the plasma membrane of laticifers and are positively regulated by ethylene application to increase sucrose import, required for the stimulation of latex production by ethylene. The present study was carried out on unexploited (virgin) trees treated or untreated with the latex yield stimulant Ethrel. Mature virgin trees were used to analyse independently the direct effects of ethylene per se upon a first tapping, and then upon a second tapping to study the combined effects of ethylene and of the metabolic changes due to the previous tapping (latex regeneration demand) on sucrose transporter gene expression. In addition, study was made of the clone PB217, which is characterized by medium rubber production and metabolic activity without ethylene stimulation, but with high latex yield when treated with ethylene (Gohet, 1996; Gohet et al., 1997, 2003).

Among seven sucrose transporter cDNAs identified, the relative transcript level of two isoforms (HbSUT1A and HbSUT2A) is found to be increased by ethylene application. Further characterization showed that they were induced specifically by ethylene. In addition, their localization in the inner bark tissues and especially in the latex cells was confirmed by in situ hybridization. The data show that these two isoforms could play an essential role in sucrose import into laticifers of virgin trees, necessary for ethylene-stimulated latex production.

MATERIALS AND METHODS

Plant material

Latex and bark samples were collected from the trunk of mature virgin rubber trees (about 8 years old) of the PB217 clone in the Bongo/SAPH plantation (Côte d’Ivoire). Latex samples were collected as described by Pujade-Renaud et al. (1994). Briefly, after discarding the first 20 drops, the latex samples were collected as a mixture of 2 mL from three trees per treatment in an equal volume of 2× fixation buffer. The samples were immediately deep-frozen in liquid nitrogen and stored at −80°C.

Back-cutted trees (cut 6 months earlier) grown in a controlled environment chamber at CPN (Composants Naturels, Michelin Group, Clermont-Ferrand, France) were used to analyse the gene expression patterns of the sucrose transporter in different organs (mature leaves, bark and xylem) and for in situ hybridization (stems). These young trees were grown with 12 h daylight of 40 μmol m⁻² s⁻¹ photosynthetically active radiation, at 27–30°C, with 70 ± 10 % relative humidity, and at 25°C, with 90 % relative humidity (night). Each pot of 160 L, filled with potting mix, was connected to a watering system and sprayed to maintain high atmosphere and soil moisture.

Field experiments of ethylene treatments

Seven batches of three homogeneous (in growth) mature virgin trees were selected as described by Pujade-Renaud et al. (1994) and Sookmark et al. (2002): two as a control (treated with palm oil only) and five treated with 1 mL of 2.5 % Ethrel® emulsion (approx. 70 mm ethephon) in crude palm oil on a 1-cm-wide, slightly scraped bark band and just beneath the next tapping cut, for 4, 8, 16, 24 and 40 h,
respective, before the first sampling. To avoid the influence of possible weather variations, in all experiments the trees were opened (tapped for the first time) on a half spiral with a tapping knife, and the latex and the bark samples were collected for analysis on the same day and at the same time. The homogenous depth (approx 1 mm from the cambium) of tapping was tested with a sharp calibrated punch.

After a 3-d rest (without further stimulation), latex and bark samples from the same trees were collected again and analysed as the second tapping (Tap2).

**Hormones and other bark treatments**

As the relative transcript level of sucrose transporters was reported to be regulated by hormonal treatments such as gibberellins (Chincinska et al., 2008), ethylene (Chincinska et al., 2008), auxin (Harms et al., 1994) and cytokinin (Harms et al., 1994; Ehness and Roitsch, 1997), we wanted to check whether various hormone or wounding treatments could induce any effect on latificer SUT expression. To do this, a protocol similar to that for ethylene application was used. Briefly, two batches were used as controls (treated with 1 g palm oil containing 0.05 % Tween 20), and four others were treated with 1 g of 2 % jasmonic acid (JA), 1 % salicylic acid (SA), 1.5 % 4-chlorophenoxyacetic acid (CPA, auxin analog) and 2 % abscissic acid (ABA) palm oil emulsion with 0.05 % Tween 20, and a last batch was mechanically wounded by sealing 15 nails up to the cambium (Nails), on an approx. 1-cm-wide, slightly scraped bark band, just beneath the next tapping cut, for 4, 8 and 16 h, respectively, before the first tapping. The latex and bark samples were collected on the same day and analysed as the first tapping (Tap1) in the same way as for the Ethrel experiment. After a 3-d rest, samples from the batches of the 4-h-treated trees were collected again and analysed as the second tapping (Tap2).

**Molecular cloning of sucrose transporter (HbSUTs) full-length cDNA**

Four latex and bark sucrose transporter probes were obtained by polymerase chain reaction (PCR) directly on latex or bark cDNA libraries, kindly provided by the team of Dr P. Kongsawadworakful, Unchera Viboonjun and H. Chrestin (Mahidol University, Bangkok, Thailand). For this purpose, degenerate primers were designed from conserved regions of published plant sucrose transporter cDNAs (EMBL data library). One primer couple was used for each SUT group: SUT1 group [primer SUT1F (5′-TCCTTTGTTGTTGGGGCCCA-3′) and primer SUT1AR (5′-TCCTTTGTTGTTGGGGCCCA-3′)], SUT2 group [primer SUT2F (5′-GACTTCTCAGTTCAAAC-3′) and primer SUT2BR (5′-GCTTTGGAAAGGCCTTGTAAT-3′)], SUT3 group [primer SUT3F (5′-CAAGATTTTTGGGCATGAGTAT-3′) and primer SUT3BR (5′-CTTCTTTACGCTATGCCCC-3′)], SUT4 group [primer SUT4F (5′-GCTTCTTTACGCTATGCCCC-3′) and primer SUT4R (5′-GCTTCTTTACGCTATGCCCC-3′)]. Amplifications were carried out in a thermal cycler (PTC-200; MJ Research, Watertown, MA, USA) using standard protocols (DeCourteix et al., 2006). The different amplified fragments were cloned and sequenced, and then used as SUT homologous probes to screen the latex cDNA libraries under low-stringency conditions. Seventeen clones were obtained and fully sequenced. Their nucleotide sequence alignment showed that they corresponded to seven different isoforms, referred to as HbSUT1A, HbSUT1B, HbSUT2A, HbSUT2B, HbSUT2C, HbSUT4 and HbSUT5 (H. brasiliensis sucrose transporters). In addition, the sequences of five of them (HbSUT1A, HbSUT2A, HbSUT2B, HbSUT4 and HbSUT5) were identical to H. brasiliensis SUT sequences, concomitantly registered in the EMBL/GenBank/DDBJ under accession numbers DQ985466, DQ985467, DQ985465, EF067355 and EF067333, respectively (Yang et al., 2006, direct submission). The HbSUT1B and HbSUT2C sequences were registered in the EMBL/GenBank/DDBJ under accession numbers AM492537 and AM491808, respectively.

**RNA extraction**

Total RNAs were extracted from latex as described by Pujade-Renaud et al. (1994, 1997) and developed by Sookmark et al. (2002). Total inner bark RNA extraction from mature trees used the caesium chloride cushion method, adapted from Sambrook et al. (1989), using approx. 2 g of inner bark ground under liquid nitrogen.

Deep-frozen leaves, bark and xylem samples collected from back-cutted trees were ground in liquid nitrogen and total RNAs were extracted using CTAB extraction buffer (Chang et al., 1993) and then treated with RNase-free RQ1 DNase (Promega, Madison, WI, USA). mRNAs were spectro-photometrically quantified and checked by agarose gel electrophoresis.

**Specific primer for real-time RT-PCR**

Primer couples specific to each isoform were designed for real-time RT-PCR: HbSUT1A [primer SUT1AF (5′-CAGCTTCTTTGTTGGGGCCCA-3′) and primer SUT1AR (5′-TGCACATCTTATGACATCAGTCCA-3′)], HbSUT1B [primer SUT1BF (5′-GACGTCTTTGTTGGGGCCCA-3′) and primer SUT1BR (5′-CCTCATTGGCCATGATGCCCA-3′)], HbSUT2A [primer SUT2AF (5′-GGTTTCTTATGCTTTAAGCGTAAC-3′) and primer SUT2AR (5′-TGATAAAAGCCTACTCTTTTACATCA-3′)], HbSUT2B [primer SUT2BF (5′-GCTTCTTTGTTGGGGCCCA-3′) and primer SUT2BR (5′-GCTTCTTTGTTGGGGCCCA-3′)], HbSUT2C [primer SUT2CF (5′-TCCTTTGAAGCCTTCCGTAAT-3′) and primer SUT2CR (5′-CTTCTTTGAAGCCTTCCGTAAT-3′)], HbSUT4 [primer SUT4F (5′-GACTTCTTTGTTGGGGCCCA-3′) and primer SUT4R (5′-CTTCTTTGAAGCCTTCCGTAAT-3′)] and primer SUT5R (5′-GCTTCTTTGTTGGGGCCCA-3′) and primer SUT5R (5′-ATGCTTCTTTGTTGGGGCCCA-3′). The specificity of each primer pair was tested by semi-quantitative and real-time RT-PCR on each full-length cDNA cloned in pGEM-T easy vector (Promega). PCR reactions were performed as described below for real-time RT-PCR.

Besides sucrose transporters, the expression pattern of the glutamine synthetase gene (HbGS) was monitored by using the specific primer pair HbGS_F (5′-GCTGCGATCAACATTAGTGG-3′) and HbGS_R (5′-CAACGCCCCACATAAGAAGTG-3′). HbGS has been reported to be a good marker of the ethylene effect on gene expression in latex (Pujade-Renaud et al., 1994, 1997). For bark tissue, the expression pattern of the ethylene-induced gene ACC oxidase (1-aminoacyclopropane-
1-carboxylate oxidase) was determined as a positive control of the response to ethylene (Kongsawadwokkul et al., 2004). The specific primer pair used was: HbAccOx_F (5’-ATGGACACAGTTGAGGATGAC-3’) and HbAccOx_R (5’-AGGTGGCGAGGAGAAGG-3’).

Quantification of sucrose transporter transcripts by real-time RT-PCR

After treatment with DNase I, 2 μg of total RNA was used as the template in the first-strand cDNA synthesis reaction, according to the manufacturer’s instructions (SuperScript-III, Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using the generated cDNA as target template, a fluorescent dye SYBR-Green and an iCycler system (Bio-Rad, Hercules, CA, USA). cDNA encoding actin was used as the internal control gene. PCR reactions were performed as follows: 5 min at 95 °C for denaturation, 20 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C for amplification, and 10 min at 72 °C for final extension. ΔCt was calculated from the formula ΔCt = Ct(treated sample) − Ct(control sample). The normalized expression ratio (Qr) was calculated using the comparative Ct method, with the formula: Qr = E−ΔΔCt (E = efficiency of the primer couple, ΔΔCt = ΔCtsucrose transporter − ΔCtactin). This method makes it possible to visualize the increase (Qr > 1) and decrease (Qr < 1) of genes (up- and downregulation, respectively). The efficiency of each primer pair was previously evaluated and found to be between 1.75 and 1.85. To compare the relative abundance of transcripts in different organs, expression was calculated as given in the Bio-Rad real-time PCR Application Guide.

Statistical analysis

As empirical errors in Qr increased with Qr values, consistent with the above exponential formulation, statistical procedures were performed on log-transformed data. Two samplings, each consisting of two independent plants, were used to estimate an unbiased biological error (with two degrees of freedom) for comparison of means using Student’s t-test. Results were considered to be statistically different at P < 0.01.

In situ hybridization

Tissue preparation was done according to Brunel et al. (2002). Transverse sections, 12 μm thick, were cut with a rotary microtome, mounted on SuperFrost Plus slides (Fisher Scientific, Elancourt, France) and dried at 42 °C for 2 d. Paraffin was removed via two baths (10 min each) in Histoclear II. Slides were progressively rehydrated in an ethanol series and then washed for 5 min in DEPC water and twice in 1× phosphate-buffered saline (PBS). Slides were treated with proteinase K at 10 μg ml−1 in 1× PBS at 37 °C for 15 min and then incubated in glycerine at 0-2% in 1× PBS at room temperature for 2 min. After two washes in 1× PBS, slides were fixed in 4% paraformaldehyde (in 1× PBS) for 10 min, rinsed in 1× PBS and then acetylated using 0.5% acetic anhydride in 0-1 m triethanolamine for 10 min and rinsed in 1× PBS. Before hybridization, sections were dehydrated in an ethanol series. Sections were hybridized with equal concentrations (1-5 ng μL−1) of either sense or antisense probes in 1× Denhart’s, 10% dextran sulfate, 1 mg ml−1 tRNA, 50% formamide, 1× salts (300 mM NaCl, 10 mM Tris–HCl, pH 6.8, 10 mM NaH2PO4, 10 mM Na2HPO4 and 5 mM EDTA), and incubated at 50 °C overnight in a humid chamber. Sections were washed twice in 2× saline sodium citrate (SSC), six times in 0.1× SSC for 15 min at 42 °C each and twice in 1× PBS for 10 min at room temperature. Detection of digoxigenin (DIG)-labelled probes used an antidigoxigenin alkaline phosphatase conjugate (Poupart et al., 2001). After suitable colour development, the reaction was stopped by rinsing in water and the sections were dried and mounted in Eukitt. Sections were observed under a Zeiss Axioplan 2 microscope and with an AxioCam HR camera (Zeiss) with AxioVision digital imaging software.

RESULTS

Latex production

Application of Ethrel® under the tapping-cut significantly stimulated latex yield (Fig. 1), especially for the first tapping (Tap1). In comparison with the control of Tap1, latex production increased strongly by 16 h and reached its maximum value 40 h after treatment (27-fold more than in the control). The standard deviation for the marked increase in yield was very large, suggesting substantial heterogeneity in the response between trees. Nevertheless, the treatment differed significantly from the control. For the second tapping (Tap2), latex production increased only weakly at 4 h (three-fold) and remained constant until 40 h after treatment.

Cloning cDNA of putative sucrose transporters from latex

Degenerated oligonucleotide primers from conserved regions of each group of sucrose transporters (SUT1, SUT2 and SUT4) were used and allowed cloning of four different amplification products from latex and bark libraries. These four probes were used for screening a latex cDNA library, which led to the identification of seven full-length cDNA-encoding putative sucrose transporters. Based on their sequence homologies, two isoforms (HbSUT1A and

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**FIG. 1.** Latex yield of virgin PB217 trees after ethylene stimulation. Batches of three homogenous mature virgin trees were treated with 5 % Ethrel® (ethylene) 4, 8, 16, 24 or 40 h before the first simultaneous tapping (Tap1). After 3 d without any further treatment, the same trees were tapped again (Tap2). C1 and C2 represent two batches of three trees, which were un-stimulated as controls. Latex yield is expressed in grams of fresh latex per tree per tapping. Bars represent the biological standard deviation.
HbSUT1B) were found to belong to the SUT1 group, three (HbSUT2A, HbSUT2B and HbSUT2C) to the SUT2 group and two others (HbSUT4 and HbSUT5) to the SUT4 group.

Further investigations showed that HbSUT1A, HbSUT2A, HbSUT2B, HbSUT4 and HbSUT5 were identical to those concomitantly registered by Yang et al. (direct submission, 2006) in the EMBL/GenBank/DDBJ under accession numbers DQ985466, DQ985467, DQ985465, EF067335 and EF067333, respectively. The new HbSUT1B and HbSUT2C full-length nucleotide sequences were registered in the EMBL/GenBank/DDBJ under accession numbers AM492537 and AM491808, respectively.

All these putative transporters belonged to the MFS and shared main characteristics with the previously cloned sucrose transporters. Their nucleotide sequence contained an open reading frame ranging from 1.5 to 1.9 kb that encoded 500 to 633 amino-acid polypeptides. In addition, their predicted amino-acid sequences exhibited highest identity to those of Ricinus communis (about 83%) and Manihot esculenta (about 90%), both of which belong, like H. brasiliensis, to the Euphorbiaceae family. According to the proposed classification of sucrose transporters (Sauer, 2007), these were divided into three groups (Fig. 2; accession numbers of the sequences presented are given in the Appendix).

Basal expression of putative sucrose transporters in latex and bark of untreated virgin trees

Because 3' untranslated region (UTR) sequences are the most divergent regions within isoforms (Lerchl et al., 1995; Duval

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**Fig. 2.** Phylogenetic tree of plant sucrose transporters. Confirmed or predicted plant sucrose transporter sequences from public databases (EMBL/GenBank/DDBJ) were used to construct a phylogenetic tree. Deduced protein sequences were aligned using MUltiple Sequence Comparison by Log-Expectation (http://www.ebi.ac.uk/Tools/muscle/). The tree was constructed using the program PhyML (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phylm) and Treeview. Internal labels indicate bootstrap values (100 bootstraps).
first and second tappings (Fig. 5), which validated the ability of the ethylene bark treatment to regulate gene expression in the laticifers, the relative transcript level of HbGS expression in the laticifers, the relative transcript level of HbGS was determined by real-time RT-PCR in latex and bark, using actin expression as control.

Fig. 3. Specificity of the different primers and basal expression of different sucrose transporters in latex and bark of virgin trees. (A) Specificity, and (B) basal expression of different HbSUT isoforms in latex and (C) in bark. The relative expression of HbSUTs was determined by real-time RT-PCR in bark and latex, using actin expression as control.

To confirm that ethylene treatment of bark stimulated gene expression in the laticifers, the relative transcript level of HbGS was investigated as a positive ethylene-responsive gene in these cells (Pujade-Renaud et al., 1994, 1997). HbGS increased in the latex cells, starting within 16 h of ethylene treatment, with a maximum (18-fold) after 24 h (Tap1, Fig. 4A). This response was prolonged for the second tapping, regardless of when the ethylene treatment was applied, with a maximum (ten-fold expression compared with the control) at 8 and 16 h (Tap2, Fig. 4A). As shown in Fig. 4, the effect of ethylene treatment not only differed between the sucrose transporter isoforms, but also between the first (Tap1) and the second (Tap2) tapping. The transcripts of HbSUT1A (Fig. 4B) and, to a lesser extent, of HbSUT2A (Fig. 4C) were found to be the most responsive to ethylene treatment.

No significant effect was observed at the Tap1 within the first 8 h following ethylene treatment. At 16 h, the transcripts of HbSUT1A and HbSUT2A increased 16- and six-fold, respectively (Fig. 4B, C). This accumulation was abrupt and transient for HbSUT1A, whereas it was progressive and maximum after 40 h for HbSUT2A. Transcripts of HbSUT2B and HbSUT2C were very slightly and transiently increased by ethylene. No significant ethylene effect was detected for HbSUT4 and HbSUT5 (Fig. 4D). Of all these sucrose transporter isoforms, HbSUT1B was the only isoform for which expression was decreased by ethylene stimulation after 24 h treatment (Fig. 4B).

In contrast to Tap1, there was hardly any regulation of the sucrose transporter transcripts during Tap2. However, a slight and transient induction was observed for HbSUT1A between 8 and 16 h after ethylene treatment (Fig. 4B–D).

Ethylene effect on expression profiles of the putative sucrose transporters in the inner bark

First, ethylene treatment was confirmed to induce an early (4 h) and strong (up to 200-fold) accumulation of ACC oxidase transcripts (Kongsawadworakul et al., 2004) during the first and second tappings (Fig. 5), which validated the ability of the ethylene bark treatment to regulate gene expression in the soft inner bark.

At Tap1, most of the sucrose transporter transcripts either remained constant or decreased (Fig. 5B–D). Only the HbSUT2B isoform was weakly and transiently increased at 40 h (Fig. 5C). During Tap2, HbSUT1A, HbSUT2B and HbSUT4 were transiently increased with a maximum around 16 h (Fig. 5C, D). The increase in transcripts was strongest...
for the HbSUT1A isoform, which was markedly increased at 4 h, and reached a plateau between 8 and 40 h (Fig. 5B).

Effect of hormones and wounding on expression of HbSUT1A, HbSUT2A and HbSUT2B in the latex

As HbSUT1A, HbSUT1B and HbSUT2A were found to be significantly regulated by ethylene, the effects of various hormonal and wounding treatments applied to bark on their respective expression in latex were also investigated. For HbSUT2A, no significant increase was observed, regardless of the treatment, whereas HbSUT1A exhibited an early, transient and low stimulation in the presence of SA and CPA (Fig. 6A, C). CPA and, to a lesser extent, the other treatments (especially JA) were found to induce a significant accumulation of HbSUT1B at Tap1 (from 8 h after treatment) and Tap2 (Fig. 6B).

HbSUT1A, HbSUT1B and HbSUT2A expression in different organs

HbSUT1A, HbSUT1B and HbSUT2A gene expression analysis was carried out by real-time PCR, using RNAs from leaves, bark and xylem of young back-cutted trees. These three transporters were detectable to varying degrees in the source and sink organs (Fig. 7). HbSUT1A was more highly expressed in sink organs (xylem and bark) than in leaves. HbSUT1B was predominantly expressed in leaves and bark, but relatively weakly in xylem. A similar pattern but of a reduced magnitude was found for HbSUT2A.

Fig. 4. Transcript accumulation of glutamine synthetase and different sucrose transporters after ethylene stimulation in virgin tree latex. Relative transcript levels of glutamine synthetase (A), and sucrose transporters of SUT1 group (B), SUT2 group (C) and SUT4 group (D) were monitored in latex of ethylene-treated virgin trees. Latex mRNA from mature virgin trees (PB217) was used for real-time RT-PCR. Q_r was obtained by the E_{-18}^2D method. Bars represent the technical standard deviation.

Fig. 5. Transcript accumulation of ACC oxidase and different sucrose transporters after ethylene stimulation in bark of mature virgin trees. Relative transcript levels of ACC oxidase (A) and sucrose transporters of SUT1 group (B), SUT2 group (C) and SUT4 group (D) were monitored in bark of ethylene-treated virgin trees. Bark mRNA samples from virgin mature trees (PB217) were used for real-time RT-PCR. Q_r was obtained by the E_{-18}^2D method. Bars represent the technical standard deviation.
which exhibit strong sequence identity, and the second was quite HbSUT2A-specific. As shown in Fig. 8A and B, young stems of H. brasiliensis contain some isolated laticifers exclusively localized in the phloem region, near cambial cells.

A typical colouration (violet staining) was only detected with the antisense probe (Fig. 8E, F, I, J), whereas no signal was observed with the sense probe (Fig. 8C, D, G, H), suggesting that the colouration indicates the presence of transcripts of HbSUT1s or HbSUT2A.

With regard to HbSUT1 mRNAs, colouration was found in inner bark tissue, more precisely in young phloem, laticifers and the cambial zone. No colouration was found in xylem tissue, implying limited or no expression of these transporters. Similar data were found for HbSUT2A, for which a positive hybridization signal was also mainly confined to the cambial zone and the phloem tissue. However, a weaker colouration than for the HbSUT1 probe was observed in the fusing young latex cells.

**DISCUSSION**

Due to their active metabolism in the synthesis of rubber, laticifers are a strong sink and require substantial and rapid sucrose import to meet their carbon and energy demands. Ethrel® is often used as latex yield stimulant (Tupy, 1973). A series of field and molecular experiments were conducted, for the first time, to assess if sucrose transporters are involved in the ethylene-induced increase in latex yield. These experiments were performed with mature virgin trees, at the very first tapping, to study the direct effect of ethylene, and then upon a second tapping. The PB217 rubber clone was used because it responds well to Ethrel yield stimulation.

**Laticifers contain transcripts of many putative sucrose transporters**

It is normally very difficult to access the pure cytoplasm of one single cell type. This is not the case for laticifers, from which the out-flowing cytoplasm (latex) can be collected by simple bark tapping. Moreover, the presence of polysomes and mRNA in the expelled latex (Coupé et al., 1977; Kush et al., 1990; Pujade-Renaud et al., 1994; Ko et al., 2003; Chow et al., 2007) makes it possible to study the expression of any gene expressed in this kind of cell. Therefore, to identify the sucrose transporters expressed in laticifers, a latex-specific cDNA library was screened using four PCR-amplified homologous probes. Seven putative sucrose transporter isoforms were cloned from these cells, indicating that sucrose transporters are also encoded by a multigenic family in H. brasiliensis, as is the case for many other species. For example, nine sucrose transporter isoforms were identified from Arabidopsis thaliana (Sauer and Stolz, 1994; Barker et al., 2000; Gottwald et al., 2000; Ludwig et al., 2000; Meyer et al., 2000, 2004; Sauer et al., 2004), three from tobacco (Burkle et al., 1998; Ward et al., 1998; Lemoine et al., 1999), three from tomato (Barker et al., 2000; Weise et al., 2000), four from Vitis vinifera (Davies et al., 1999; Ageorges et al., 2000), two from citrus (Li et al., 2003) and three from Oryza sativa (Scofield et al., 2007; Sun et al., 2008).
Based on their sequence homology, these sucrose transporters were found to be closely related to previously identified sucrose transporters from herbaceous as well as woody species (Lemoine, 2000; Sauer, 2007). The predicted amino-acid sequences of *Hevea* SUTs exhibited greatest identity (more than 83%) with those of *Ricinus communis* and *Manihot esculenta*, which also belong to the Euphorbiaceae family. In addition, these sucrose transporters belong to all previously characterized groups of sucrose transporters, with two isoforms (HbSUT1A and HbSUT1B) in the SUT1 group, three (HbSUT2A, HbSUT2B and HbSUT2C) in the SUT2 group and two (HbSUT4 and HbSUT5) in the SUT4 group.

Analysis of their basal transcript expression showed that these sucrose transporters were expressed to varying degrees in the latex, with greater expression of the SUT1 group (HbSUT1A and HbSUT1B) isoforms (Fig. 3B, C). Two isoforms of the SUT2 group (HbSUT2A and HbSUT2A) exhibited very low expression, while expression of the other SUT2 (HbSUT2C) and of the two SUT4 isoforms could barely be detected. On the basis of this transcript accumulation pattern, HbSUT1A and HbSUT1B may be considered as the main sucrose transporters involved in sucrose importation into the laticifers. Such a physiological role was supported by the in situ hybridization data, showing that these two transporters were expressed in the inner soft bark (liber) of young stems, including the young laticifers (Fig. 8).

To our knowledge, the presence of many isoforms of sucrose transporters in one single type of cell has only been reported once: this was for sieve elements with three isoforms (LeSUT1, LeSUT2 and LeSUT4) proposed to be involved in sucrose retrieval from the apoplastic compartment (Barker et al., 2000; Weise et al., 2000; Kühn, 2003). Laticifers would therefore be expected to function as highly active sites of sucrose absorption.

**Ethylene-induced increase in HbSUT1A and HbSUT2A transcripts parallels the ethylene-induced stimulation in latex yield**

Much evidence in the literature indicates that an increase in sucrose transporter transcripts is often associated with a higher activity of the corresponding transporters, suggesting a major transcriptional regulation of these proteins (Sakr et al., 1993, 1997; Chiou and Bush, 1998; Lemoine et al., 1999; Decourteix et al., 2006, 2008). Hence, as ethylene-stimulated latex production has been reported to occur through an increase in sugar metabolism (Tupy, 1973; Tupy and Primot, 1976; Silpi et al., 2006), sugar influx (Lacrotte et al., 1985) and hyperpolarization of the plasma membrane of laticifers (Boutau et al., 1999), an increased sucrose influx should be partly linked to an ethylene-induced stimulation of, at least, some of the putative cloned sucrose transporters. This should be particularly true for laticifers, as they are a very active sink cell type completely devoid of any starch reserve (Tupy, 1989).

In accordance with previously published data (Pujade-Renaud et al., 1994), ethylene treatment below the tapping cut led to an
increase in \(HbGS\) expression in laticifers (Fig. 4A). This result confirmed that the ethylene signal was perceived by these cells. The expression pattern of the seven identified sucrose transporters was thus monitored on the same latex samples, using highly specific primer couples, designed from the UTR of their cDNAs (Fig. 3A). Only two transporters (\(HbSUT1A, HbSUT2A\)) were found to be, to varying degrees, significantly stimulated by ethylene. This stimulation was time-dependent as it peaked at 24 and 48 h after treatment for \(HbSUT1A\) and \(HbSUT2A\), respectively. Note that \(HbSUT1B\), which has the highest expression of any sucrose transporter isoform in the latex of untreated virgin trees, was significantly decreased when treated with ethylene. Under the same experimental conditions, the other isoforms of sucrose transporters were almost insensitive to ethylene. Taken together, these data suggest that the ethylene effect cannot be extended to all sucrose transporters initially cloned from the latex, and that the ethylene-induced sucrose influx into the laticifers (Lacrotte et al., 1985) may be due, at least in part, to the ethylene stimulation of \(HbSUT1A\) and \(HbSUT2A\). Moreover, this ethylene-induced transcript accumulation of \(HbSUT1A\) and \(HbSUT2A\) was correlated with the ethylene-stimulated latex production (Fig. 1). This potential role of \(HbSUT1A\) and \(HbSUT2A\) in ethylene-induced stimulation of latex production in virgin trees could be related to the fact that none of the sucrose transporters was found to be greatly increased during Tap2 (Fig. 4), which caused much less latex production (Fig. 1).

The ethylene effect is tissue-specific

As ethylene applied to the bark tapping area induced a pronounced increase in some SUTs in the laticifers, we verified whether a similar effect could be found in the surrounding inner liber tissues. In contrast to laticifers, no sucrose transporter was found to be significantly regulated by ethylene treatment on the first tapping (Fig. 5B–E). This lack of an ethylene effect could not be due to a failure of bark cells to perceive the ethylene signal (Fig. 5A), as ethylene did induce a marked stimulation of an ACC oxidase gene expression, which has been reported to behave as a positive marker of the response of bark tissues to ethylene treatment (Kongsawadworakul et al., 2004). These data suggested that the expression of \(HbSUT1A\) and \(HbSUT2A\) induced by ethylene was limited to the laticifers, which are devoted to rubber synthesis and, thereby, latex production. A tissue-specific regulation was recently reported for \(JrSUT1\), a putative plasma membrane-localized sucrose transporter, isolated from walnut tree xylem (Decourteix et al., 2006). Analysis of its expression showed that \(JrSUT1\) was induced by freezing and thawing in xylem tissue but not in bark, indicating the occurrence of differential regulation mechanisms between these tissues.

Laticifers are considered to be apoplastic sinks which import sucrose from the phloem. The efficiency of phloem unloading is strongly related to the sink strength, which is defined as the capacity of cells to attract photoassimilates (Ho, 1988). This sink strength closely depends on the abundance and activity of plasma membrane transporters. Moreover, a close relationship between the transcript changes and the activity of sucrose transporters has been reported in some species (Chiou and Bush, 1998; Decourteix et al., 2006, 2008). As ethylene induces a stimulation of two sucrose transporters in latex without having any effect on those of bark, this situation might reflect a diversion of carbon assimilates (sucrose) in favour of laticifers, where latex production is strongly stimulated by ethylene. This scenario emphasizes coordinated regulation between different sink organs of the stem.

**Stimulation of HbSUT1A and HbSUT2A in latex is ethylene-specific**

The transcript abundance of sucrose transporters in plants has been reported to be regulated by exogenous (Sak et al., 1993; Matsukura et al., 2000; Meyer et al., 2004; Decourteix et al., 2006) and endogenous (Ehness and Roitsch, 1997; Chiou and Bush, 1998; Ward et al., 1998; Matsukura et al., 2000; Li et al., 2003) stimuli. We have shown here that the ethylene effect was restricted to two sucrose transporters (\(HbSUT1A\) and \(HbSUT2A\)) and correlated with ethylene-induced stimulation production. It is noteworthy that ethylene decreased \(HbSUT1B\) expression (Fig. 4A) but it was significantly increased by most treatments, especially JA and CPA (an auxin-like chemical; Fig. 6B).

With regard to \(HbSUT1A\) and \(HbSUT2A\), none of the hormonal treatments induced a stimulation of their respective transcript during Tap1 (Fig. 6B, C). In addition, as wounding (nail sealing, without latex exudation) did not lead to upregulation of either \(HbSUT1A\) or \(HbSUT2A\), it can be hypothesized that either their response to ethylene is not due to a cross-talk between the ethylene and wounding pathway, or that the concentration of endogenous ethylene, probably induced by wounding in the rubber tree bark, might be too low to induce pronounced regulation of these two genes, as compared with the application of exogenous ethylene. This differential response of sucrose transporters means that laticifers are able to adapt the transcript amount of their sugar transporters to different environmental or developmental conditions.

The present study provides insight into the physiological role that sucrose transporters play in sucrose import to laticifers, in relation to ethylene-induced stimulation of latex production. We have described the potential involvement of two sucrose transporters, \(HbSUT1A\) and \(HbSUT2A\), in this process. Indeed, \(HbSUT1A\) and \(HbSUT2B\) were increased by ethylene and this effect was positively correlated with ethylene stimulation of latex production. Moreover, this ethylene effect is specific to laticifers, in which cis-polysoprene synthesis takes place. To elucidate how the expression of these transporters is stimulated by ethylene, their respective promoters will be isolated in future experiments to determine whether they harbour an ethylene-specific cis-element. Because latex yield is the most important trait in breeding programmes for \(H. brasiliensis\), and rubber synthesis depends on sucrose importation in laticifers, the above two sucrose transporters may constitute a useful molecular tool to underpin latex yield in this plant. Thus, single nucleotide polymorphisms (SNPs) and microsatellites will be looked for in these SUT cDNA clones to map them in the *Hevea* gene maps (Low et al., 1996; Guen et al., 2004) and verify if they may co-localize with latex yield and/or growth quantitative traits.
loci. This would be of major help in rubber breeding programmes.

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LITERATURE CITED


**APPENDIX**

Accession numbers of sucrose transporter sequences presented in Fig. 2: AbSUT1 (Asarina barclaiana; AAF04294), AgSUT3 (Apium graveolens; ABB89051), AmSUT1 (Alonsoa meridionalis; AAF04295), AtSUC1 (Arabidopsis thaliana; At1g71880), AtSUC2 (Arabidopsis thaliana; At1g22710), AtSUC3 (Arabidopsis thaliana; At2g02860), AtSUC4 (Arabidopsis thaliana; At1g09960), AtSUC5 (Arabidopsis thaliana; At1g71890), AtSUC6 (Arabidopsis thaliana; At5g43610), AtSUC7 (Arabidopsis thaliana; At5g06170), BoSUC1 (Brassica oleracea; AAL58071), BoSUC2 (Brassica oleracea; AAL58072), CsSUT2 (Citrus sinensis; AAM29153), DgSUT4 (Datisca glomerata; CAG70682), DcSUT1 (Daucus carota; BAA89448), EeSUCx (Euphorbia esula; AAF65765), EuSUT2 (Eucommia ulmoides; AAX49396), HbSUT1A (Hevea brasiliensis; AM492537), HbSUT2A (Hevea brasiliensis; ABJ51934), HbSUT2B (Hevea brasiliensis; ABJ51932), HbSUT2C (Hevea brasiliensis; AM491808), HbSUT4 (Hevea brasiliensis; EF067335), HbSUT5 (Hevea brasiliensis; ABK60189), HvSUT1 (Hordeum vulgare; CAB75882), HvSUT2 (Hordeum vulgare; CAB75881), JrSUT1 (Juglans regia; AAU11810), LeSUT2 (Lycopersicum esculentum; AAG12987), LeSUT4 (Lycopersicum esculentum; AAG09270), LjSUT4 (Lotus japonicus; CAD61275), MdSUT1 (Malus domestica; AAR17700), MeSUT2 (Manihot esculenta; ABA08445), MeSUT4 (Manihot esculenta; ABA08443), NiSUT1 (Nicotiana tabacum; X82276), NiSUT3 (Nicotiana tabacum; AAD34610), OsSUT1 (Oryza sativa; AAP90181), OsSUT2 (Oryza sativa; AAN15219), OsSUT3 (Oryza sativa; BAB68368), OsSUT4 (Oryza sativa; BAC67164), OsSUC4 (Oryza sativa; Q2QL11), OsSUT5 (Oryza sativa; BAC67165), PmSUC1 (Plantago major; CAI59565), PmSUC2 (Plantago major; X75764), PmSUC3 (Plantago major; CAD58887), PtSUT1-1 (Populus tremula × Populus tremuloides; CAJ33718), PsSUT1 (Pisum sativum; AAD41024), RcsCR1 (Ricinus communis; CAA83436), SdSUT2 (Solanum demissum; Q03411), ShSUT1 (Saccharum hybridum; AAV41028), SoSUT1 (Spinacea oleracea; Q03411), StSUT1 (Spinacea oleracea; CAI48915), StSUT4 (Solania tuberosum; AAG25923), TaSUT1A (Triticum aestivum; AAM13408), TaSUT1B (Triticum aestivum; AAM13409), TaSUT1D (Triticum aestivum; AAM13410), TaSUT4 (Triticum aestivum; AAM13410), VISUCx (Vicia faba; CAB07811), VvSUCy (Vitis vinifera; AAL32020), VvSUC1 (Vitis vinifera; AAP08329), VvSUC12 (Vitis vinifera; AAP08330), VvSUC27 (Vitis vinifera; AAF08331), ZmSUT1 (Zea mays; BAA83501), ZmSUT2 (Zea mays; AAS91375), ZmSUT4 (Zea mays; AAT51689).