รายงานวิจัยฉบับสมบูรณ์

โครงการ การระบุและศึกษาลักษณะของยีนที่ถูกควบคุมโดยเอธิลีน และเกี่ยวข้องกับผลผลิตของยางพารา

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พฤษภาคม 2553

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

การเพิ่มผลผลิตของน้ำยางพาราทำได้โดยการใช้อีเธรลซึ่งเป็นสารเคมีเร่งน้ำยางทาที่บริเวณเปลือกลำต้นใกล้รอยกรีดของต้นยางพารา อีเธรลสามารถปลดปล่อยแก๊ซเอธิลีนซึ่งทำให้เกิดการเปลี่ยนแปลงทั้งทางด้านสรีรวิทยาและกระบวนการเมแทบอลิซึมภายในเซลล์ท่อน้ำยาง งานวิจัยนี้ใช้เทคนิค Suppression Subtractive Hybridization (SSH) ในการเปรียบเทียบการแสดงออกของยีนที่ระดับ transcriptome ของเซลล์ท่อน้ำยางและเปลือกลำต้นระหว่างต้นยางพาราที่ไม่ได้ถูกกระตุ้นและต้นยางพาราที่ถูกกระตุ้นด้วยเอธิลีน เพื่อทำความเข้าใจเกี่ยวกับชีววิทยาระดับโมเลกุลของกระบวนการเพิ่มผลผลิตของน้ำยางหลังกระตุ้นด้วยสารเคมีเร่งน้ำยาง จากผลการวิเคราะห์ยีนที่มีการแสดงออกที่ต่างกัน พบว่าสามารถแบ่งยีนดังกล่าวออกได้เป็น 4 กลุ่ม คือ known function, unknown function with information, unknown และ no hit จากการวิเคราะห์การแสดงออกของยีนโดยวิธี SSH และ macroarray พบว่ายีนจำนวนมากในเซลล์ท่อน้ำยางมีการแสดงออกลดลงเมื่อต้นยางถูกกระตุ้นด้วยเอธิลีน โดยในการทดลองได้เลือกศึกษายีนที่มีการแสดงออกสูงขึ้นหรือต่ำลงอย่างมีนัยสำคัญ ยีน *Abscisic Acid Stress Ripening* (*ASR*) จำนวน 2 ยีน คือ *ASR2* และ *ASR3* มีการแสดงออกที่จำเพาะต่อเซลล์ท่อน้ำยางและเปลือกลำต้นตามลำดับ โดยทั้ง 2 ยีนมีการแสดงออกลดลงเมื่อต้นยางพาราถูกกระตุ้นด้วยเอธิลีน ในขณะที่ยีน *sucrose transporter* มีการแสดงออกเพิ่มขึ้น ดังนั้นโปรตีน ASR อาจทำหน้าที่เป็น negative regulator ของยีน *sucrose transporter* ยีน *1-aminocyclopropane-1-carboxylic oxidase* ที่เกี่ยวข้องกับการสังเคราะห์เอธิลีนมีการแสดงออกเพิ่มขึ้นในเปลือกลำต้นบ่งชี้ถึงการเกิด autocatalytic production ของเอธิลีน ยีน *lipoxygenase* ที่เกี่ยวข้องกับการสังเคราะห์จัสโมเนทมีการแสดงออกเพิ่มขึ้นในเปลือกลำต้น ซึ่งสนับสนุนการทำงานร่วมกันของเอธิลีนและจัสโมเนทในการควบคุมการแสดงออกของยีน การแสดงออกที่ลดลงของยีน non-phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) อาจเป็นไปได้ว่าเซลล์เลือกที่จะใช้เอนไซม์ในกระบวนการที่ให้พลังงานมากกว่าโดยเพิ่มการแสดงออกของยีน phosphorylating *GAPDH* การแสดงออกของยีน *(1-4)-beta-mannan endohydrolase* ลดลงอาจส่งผลให้ผนังเซลล์ของเซลล์ท่อน้ำยางแข็งแรงขึ้นเนื่องจากเอนไซม์นี้ทำหน้าที่ย่อยสลายผนังเซลล์ ยีน *b-keto acyl reductase* มีการแสดงออกลดลงอาจส่งผลให้เซลล์ท่อน้ำยางสร้างกรดไขมันลดลง ซึ่งสอดคล้องกับการแสดงออกที่ลดลงของ*ยีน cis-prenyltransferase* ที่เกี่ยวข้องกับการสังเคราะห์ polyisoprene *unknown* gene (singleton no. 353) และ *no similarity* gene (singleton no. 823) ซึ่งมีการแสดงออกเพิ่มขึ้น อาจเป็นยีนที่มีความจำเพาะต่อต้นยาง ดังนั้นจึงควรที่จะทำการโคลน full-length cDNAs และศึกษาหน้าที่ของยีนดังกล่าว ความรู้ทั้งหมดที่ได้จากการศึกษานี้ช่วยทำให้เข้าใจกลไกของเอธิลีนในการเพิ่มผลผลิตของน้ำยางมากขึ้น และควรมีการศึกษาเพิ่มเติมต่อไป

คำสำคัญ: เปลือกลำต้น เอธิลีน *Hevea brasiliensis* น้ำยางพารา ต้นยางพารา Suppression Subtractive Hybridization

Abstract

Rubber tree latex yield can be improved through bark stimulation with Ethylene. Ethrel®, an ethylene releaser, induces marked changes in the physiology and metabolism of the latex cells. In this study, the Suppression Subtractive Hybridization (SSH) technique was performed to compare the *Hevea* latex and bark transcriptomes between control and Ethrel® stimulated trees. After Expressed Sequence Tag (EST) sequencing, the differentially enriched cDNA fragments were analyzed and classified into four groups including known function, unknown function with information, unknown and no hit. The SSH and macroarray analysis confirmed that the transcript levels of numerous genes in latex cells decreased after the ethylene treatment. Several candidate genes which were significantly up- or down-regulated by ethylene were selected for further analysis. *Abscisic Acid Stress Ripening* (*ASR*) genes, *ASR2* in latex and *ASR3* in bark, were specifically down-regulated thus proposed to act as the negative regulator and participate in the up-regulation of some plasmalemma sucrose transporter genes in response to bark ethylene treatment. The up-regulation of *1-aminocyclopropane-1-carboxylic oxidase* involved in ethylene biosynthesis confirmed autocatalytic ethylene production. Up-regulation of *lipoxygenase* gene involved in jasmonate biosynthesis confirmed the hormones crosstalk between ethylene and jasmonate in plants. The down-regulation of the non-phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene may lead to more energy production via the activity of the phosphorylating *GAPDH*. The down-regulation of the *(1-4)-beta-mannan endohydrolase* gene by ethylene may reduce the depolymerization of the cell wall polysaccharides leading to the strengthening of the cell wall of the laticifers. The lower expression of *b-keto acyl reductase* in stimulated trees may lead to the reduction of fatty acid biosynthesis in laticifers which is corresponded to the lower expression of *cis-prenyltransferase* gene involved in polyisoprene synthesis. The *unknown* gene (singleton no. 353) and the *no similarity* gene (singleton no. 823) were up-regulated by ethylene. The relevant information of these two genes might represent *Hevea* specific genes. Therefore, their full-length cDNAs should be cloned and characterized. The knowledge gained from this study gives further insight into the understanding of ethylene effect in the increase in latex yield and further investigations needs to be performed.

Keywords: Bark, Etylene, *Hevea brasiliensis*, Latex, Rubber tree, Suppression Subtractive Hybridization

Executive Summary

Latex yield of rubber tree (*Hevea brasiliensis*) can be improved through bark stimulation with Ethrel®, an ethylene releaser. Ethrel® induces marked changes in the physiology and metabolism of the latex cells. The effects of ethylene to increase latex yield have been extensively investigated for its physiological role in the laticifers. However, the molecular events of ethylene action in the latex cells and inner soft bark tissues of mature rubber tree have not been extensively studied. To obtain new integrated basic knowledge about the effects of ethylene on the molecular physiology of rubber tree, Suppression Subtractive Hybridization (SSH) technique was performed to compare the *Hevea* latex and bark transcriptomes between control and Ethrel® stimulated trees.

A total of 2,258 ESTs and 2,231 ESTs were sequenced from latex and bark SSH cDNA libraries, respectively. Subsequently, macroarray analysis was performed to confirm the differential expression of all unique genes in latex SSH cDNA libraries. All genes showing significant differences in expression level between control and stimulated trees were classified into six groups; early down-regulated, transiently down-regulated, late down-regulated, early up-regulated, transiently up-regulated and late up-regulated by ethylene. The more promising candidate genes from this statistical analysis are under investigation.

Among 2,258 sequenced ESTs from control and Ethrel®-stimulated latex SSH cDNA libraries, 43 ESTs identified as two isoforms of Abscisic acid Stress Ripening (*ASR*) gene, *ASR1* and *ASR2*, represented one of the highest percentage of the ESTs of a given class of gene. Moreover, a total of 53 ESTs identified as another isoform of *ASR* gene, *ASR3*, represented as high percentage in bark control SSH library only. *ASR2* and *ASR3* genes appear to be specific for the latex cells and inner soft bark, respectively. Based on recent reports on ASR proteins function, a new hypothesis for the ASRs function in the latex cells was proposed.

Among 2,231 sequenced ESTs from control and Ethrel®-stimulated inner soft bark SSH libraries, 55 ESTs, grouped in four contigs, identified as at least two isoforms of lipoxygenase (*LOX*) gene, represented the highest percentage of the ESTs of a given class of gene. These genes were present only in the ethylene-treated bark SSH library indicating probable up-regulation of these genes in response to ethylene. Results from semi-quantitative RT-PCR confirmed that the expression of these *LOX* gene isoforms was very low or undetectable in control trees, but were transiently markedly induced in Ethrel®-treated rubber tree bark only. Neither any basal expression nor ethylene induced overexpression of *LOX* could be detected in the latex, indicating that expression and ethylene induction of these *LOX* genes were tissue (bark) specific. These results confirm the well-known crosstalk between the ethylene and jasmonate pathways in plant. Given the importance of the jasmonate pathway in the regulation of cascade genes expression that may lead to increase rubber yield, full-length cDNA of *LOX* gene was screened from Ethrel®-treated bark cDNA library of rubber tree for further characterization.

Through real time quantitative PCR, *cis*-prenyltransferase and ACC oxidase genes were confirmed to be down-regulated and up-regulated by ethylene, respectively. The lower expression of *cis-prenyltransferase* gene which is involved in polyisoprenoid biosynthesis after ethylene stimulation may be due to the decrease in rubber biosynthesis efficiency (Chrestin, personal communication). In addition, the up-regulation of *1-aminocyclopropane-1-carboxylic oxidase* (*ACC oxidase*) gene involved in ethylene biosynthesis confirmed autocatalytic ethylene production.

Six candidate genes in response to ethylene were selected from the macroarray analysis. The down-regulation of the non-phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene may lead to more energy production via the activity of the phosphorylating *GAPDH*. The down-regulation of the *(1-4)-beta-mannan endohydrolase* gene by ethylene may reduce the depolymerization of the cell wall polysaccharides leading to the strengthening of the cell wall of the laticifers. The lower expression of *b-keto acyl reductase* in stimulated trees may lead to the reduction of fatty acid biosynthesis in laticifers which is corresponded to the lower expression of *cis-prenyltransferase* gene involved in polyisoprenoid biosynthesis. The *unknown* gene (singleton no. 353) and the *no similarity* gene (singleton no. 823) were up-regulated by ethylene. The relevant information of these two genes might represent *Hevea* specific genes. Therefore, their full-length cDNAs should be cloned and characterized.

Objective

1. To obtain new integrated basic knowledge about the effects of ethylene on the molecular physiology of the rubber tree latex producing tissues

2. To identify and characterize the ethylene regulated genes which might be potentially involved in latex yield, especially at the level of the latex producing tissues

3. To propose expression marker(s) of rubber high yield potential

4. Through a better understanding of the molecular mechanisms of ethylene action on rubber yield, the ultimate output of such project will be rubber yield improvement. This will help in maintaining the natural rubber competitiveness, compared to the petroleum-derived synthetic elastomers, and therefore guarantee, on the long term, a sustainable profitable exploitation of rubber, especially in Thailand

Materials and Methods

1. Latex and bark sample collection

Latex and bark samples were collected from 2-year-half-spiral-tapped trees of the PB 217 clone which had been selected for their medium homogeneous growth and yield. The trees were kept unstimulated for 4 months and left untapped for 10 days before treatments and sampling. Seven batches of 3 trees were set up: two as a control (unstimulated) and 5 others were treated on a 1 cm wide lightly scraped bark band, just beneath S/2 tapping cut, with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping. The latex and the bark samples were collected on the same day and analyzed at the 1st tapping after eventual treatment.

Latex collection were performed according to the method described elsewhere (Pujade-Renaud *et al*., 1997). After discarding the first 20 drops, the latex samples were collected as a mix of 2 ml each from 3 trees per treatment in an equal volume of the 2X fixation buffer (50 mM Tris-HCl, pH 9, containing 150 mM LiCl, 5 mM EDTA, and 5% SDS). The samples were immediately deep-frozen in liquid nitrogen then stored at –80°C before RNA extraction.

Using a stainless steel cork borer, pieces of bark, up to the cambium, at 5 cm below the tapping cut were punched out. As for latex, each sample consisted of a mix of 3 trees per treatment. The 2-2.5 mm of inner soft bark, including the cambium area, were quickly peeled off with a scalpel and immediately deep-frozen in liquid nitrogen. The samples were stored at -80°C. The bark total RNA was extracted according to the cesium chloride cushion method, adapted from Sambrook *et al*. (1989).

2. RNA Extraction

The procedure for total RNA isolation from latex was derived from the method described by Pujade-Renaud *et al*. (1994). After thawing, most of the rubber was discarded by centrifugation at 14,000xg for 30 min at 18°C. The white fraction recovered was deproteinized through three times of 1 v phenol/ chloroform/ isoamyl alcohol (25/24/1, v/v/v), and once with 1 v chloroform/ isoamyl alcohol (24/1, v/v). RNA precipitation was performed overnight in 2M LiCl at 4°C and followed by centrifugation at 10,000xg for 30 min at 4°C then resuspended in 400 µl of DEPC-H_2O. Two additional purifications with 1 v of phenol/ chloroform/ isoamyl alcohol and 1 v chloroform/ isoamyl alcohol were performed. Afterwards, the RNA was precipitated overnight at -20°C with 1/10 v of sodium acetate pH 5.2 and 2 v of absolute ethanol. The RNA pellet was collected by centrifugation at 14,000xg for 30 min at 4°C and washed with 1 ml cold 70% ethanol at 14,000xg for 15 min at 4°C. Subsequently, the pellet was allowed to air dry and resuspended in 100 µl of DEPC-H_2O.

The bark total RNA was extracted according to the cesium chloride cushion method adapted from Sambrook *et al*. (1989). Typically, 2-3 g of deep frozen fresh inner bark, ground into fine powder under liquid nitrogen (Kika A10 high speed grinder), were suspended in cold extraction buffer (4M Guanidine thiocyanate, 1% Sarcosine, 1% Polyvinylpyrrolidone, 20mM Ascorbic acid and 3% (v/v) β-Mercaptoethanol). The suspension was ground again on ice for 30 sec using an ultraturax. After vigorous shaking, the suspension was centrifuged at 35,000xg for 30 min at 4°C. The clear supernatant was transferred onto 5.7M cesium chloride cushion and centrifuged at 150,000x g for 20 h at 20°C. The RNA pellet was dissolved in DEPC-treated H_2O, then deproteinized two times by phenol/ chloroform/ isoamyl alcohol (25/24/1, v/v/v) extraction. Total RNA was precipitated overnight at -80°C with 1/10 v of sodium acetate and 2 v of absolute ethanol, then pelleted by centrifugation. After ethanol rinsing and air drying, the RNA pellet was resuspended in DEPC-treated H_2O.

3. Expression analysis

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

First-stranded cDNA synthesis for RT-PCR

The first-stranded cDNA synthesis reaction was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The reaction mixture containing 2 µg of total RNA, 250 ng of oligo$(dT)_{20}$, and 0.5 mM dNTPs were incubated at 65°C for 5 min and immediately cooled on ice for at least 1 min. After the addition of 200 units of SuperScript™ III RT, 40 units of RNaseOUT™, 10 mM DTT, 5 mM $MgCl_2$, and 1X RT buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), the reaction was incubated at 50°C for 50 min. Finally, the reaction was terminated by heating at 85°C for 5 min. To remove the RNA, the reaction was added with 1 unit of RNaseH (Invitrogen) and incubated at 37°C for 20 min.

Primer design

The specific primer for each candidate gene was designed from the EST sequences found in SSH libraries, using OligoExplorer 1.2 and OligoAnalyzer 1.2 program.

cDNA amplification

To amplify cDNA fragments, a 25-µl PCR reaction mixture was prepared as following: 2 µl of cDNA, 200 µM dNTP, 1 unit of *Taq* DNA polymerase (NEB), 0.4 µM of each gene specific primer in 1X PCR buffer

(20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$). The cycling conditions included initial denaturation at 94°C 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at suitable temperature depending on each primer pair for 30 sec and extension at 72°C for 30 sec. After the last cycle, additional extension at 72°C for 7 min was performed and the sample was kept at 4°C. To visualize the RT-PCR product, a 15-µl aliquot was electrophoresed on 1.2% agarose gel.

Real-time quantitative reverse transcriptase polymerase chain reaction (Real-time PCR)

The DNA contamination in the total RNA samples was eliminated by TURBO DNA-free™ Kit (Ambion®) according to the manufacturer's instruction. The cDNA was synthesized using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) as described in the manual.

The real-time quantification of RNA target was performed in the ABI 7500 System. The 25-µl PCR mixture composed of 2 µl of diluted cDNA, 0.3 µl of 1000 nM SYBR® Green, 1 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.25 µl of each 20 µM forward primer and reverse primer, 0.2 µl of Platinum *Taq* DNA Polymerase (Invitrogen) and then water was added to the final volume of 25 µl. The ABI 7500 Fast System SDS Software was programmed as follows: (I) denaturation at 95°C for 10 min (II) amplification for 40 cycles at 95°C for 15 sec and 58°C for 1 min. The data collection was carried out during the extension step (1 min at 58°C). The PCR reaction was followed by a melting curve analysis to verify specificity and identity of RT-PCR products, which can distinguish the specific PCR products from the non-specific PCR products resulting from a primer-dimer formation. The temperature of PCR products was elevated from 65°C to 95°C at a rate of 1°C/5 s, and the resulting data were analyzed by using the ABI 7500 Fast System SDS Software provided by the manufacturer.

To determine the different amount of cDNA which present in each sample, the obtained C_t for target gene was normalized by the C_t of reference gene. The normalized C_t for target gene was subtracted from the normalized C_t for reference gene to give the final difference in cycle numbers between the target gene and control gene. Expression ratio or the relative difference in gene expression was calculated as following formula.

$$\text{Relative fold change in gene expression or expression ratio} = 2^{-\Delta\Delta Ct}$$

$$\text{where : } \Delta\Delta C_t = \Delta C_t \text{ of treated} - \Delta C_t \text{ of untreated}$$

$$\text{and } \Delta C_t = (C_t \text{ of target gene} - C_t \text{ of reference gene})$$

4. Screening of a full-length cDNA from Ethrel®-treated bark cDNA library

The Ethrel®-treated bark cDNA library constructed in ZAP Express® vector (Stratagene) was used. The library contained double stranded cDNA of *Hevea* ligated to the ZAP Express® vector (Figure 1). The recombinant ZAP Express® vector was maintained by packaging into capsid head of lambda phage.

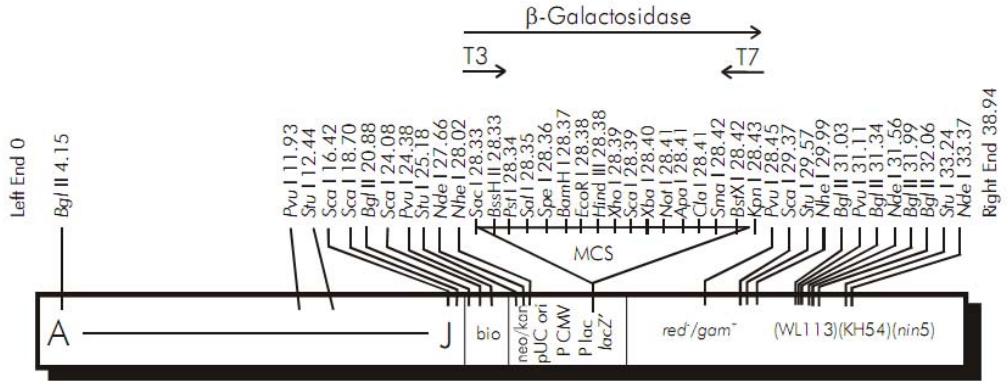


Figure 1. Map of the ZAP Express® vector (Stratagene).

Titration of the cDNA library

Phage solution was titered using *E. coli* XL1-Blue MRF´ as host cells. The *E. coli* cells were grown at 37°C overnight in LB broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$. The cells diluted in 10 mM $MgSO_4$ to an OD_{600} of 0.5 were incubated with 10, 100, 1,000 and 10,000 times diluted phage particles at 37°C for 15 min. The *E. coli*/phage mixtures were added to 3 ml of melted 0.7% NZY soft top agar maintained at 48°C. The warm melted top agar mixture was immediately poured on top of pre-warmed NZY agar plates. The plates were allowed to set for 20 min before incubation at 37°C for at least 6-8 h. The following formula was used to calculate the number of plaque forming units (pfu).

$$\text{pfu/ml} = \frac{\text{number of plaques x dilution factor x 1000 µl/ml}}{\text{µl of diluted phage plated}}$$

Plating

A single colony of freshly streak *E. coli* XL1-Blue MRF´ was grown at 37°C overnight in LB broth containing 0.2% maltose and 10 mM $MgSO_4$. Total of 200,000 pfu of the latex cDNA library were screened. Each 50,000 pfu of the latex cDNA library was mixed with 600 µl of *E. coli* cells diluted to an OD_{600} of 0.5 in 10 mM $MgSO_4$. The mixture was incubated at 37°C for 15 min to allow phage adsorption and introduction of its DNA into the cells. The mixture was mixed with 6 ml of melted (48ºC) NZY soft top agar and immediately poured onto pre-warmed NZY agar plates. After the top agar was solidified, the plates were incubated at 37°C for 6-8 h.

Plaque lifting

Plaque lifting was performed to transfer bacteriophage DNA from culture media to a nylon membrane. A plate containing bacteriophage was chilled at 4°C for at least 2 h to prevent any sticking of top agar to the membrane. The Hybond-N^+ membrane (Amersham) was placed onto the agar plate for 2-4 min to allow the transfer of phage particles to the membrane. Orientation of the membrane was noticed by cutting

the edge of membrane and mark on its plate. The membrane, with plaque side up, was subsequently placed on filter paper soaked with denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 5 min, and neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 8) for 5 min. Then membrane was submerged in rinsing buffer (2X SSC) for 30 sec and air-dried. The single-stranded DNA was fixed to the membrane using a UV cross-linker (GS GENE LINKER^{TM} UV chamber, Bio-Rad).

Hybridization

DIG labeling

Probe was prepared from plasmid extracted from ESTs clone from SSH library. DIG probe labeling was performed using PCR DIG Probe Synthesis Kit (Roche). The 50 µl of PCR labeling reaction contained 10 pg of plasmid, 5 µl of 10X PCR buffer with $MgCl_2$, 5 µl of PCR DIG Labeling Mix, 5 µl of each upstream and downstream primer, 0.75 µl of Enzyme Mix and 27.25 µl of PCR grade water. The cycling condition included initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 30 sec and elongation at 72ºC for 2 min. After the last cycle, additional elongation at 72°C for 7 min was performed and the probe sample was kept at -20°C.

Hybridization step

The membranes containing phage DNA were prehybridized at 65°C for at least 30 min in hybridization tube containing an appropriate volume of DIG Easy Hyb Buffer (10 ml/100 cm^2 membrane). Then DIG-labeled DNA probe (~25 ng/ml DIG Easy Hyb Buffer) was denatured by heating to 95°C for 5 min and rapidly cooled on ice. The denatured DIG-labeled probe was added to DIG Easy Hyb Buffer (3.5 ml/100 cm^2 membrane) then prewarmed at 65°C. Hybridization was carried out at 65°C overnight.

Washing step

After hybridization was completed, the membrane was washed 2 times under constant agitation in the 25 ml of low stringency washing buffer (2X SSC, 0.1% SDS) at room temperature for 5 min. Then membrane was washed 2 times with pre-warmed high stringency washing buffer (0.5X SSC, 0.1% SDS) at 65°C for 15 min.

Immunological detection and autoradiography step

To perform detection step on 100 cm^2 membrane, the membrane was rinsed briefly in DIG washing buffer (0.1 M Maleic acid, 0.15 M NaCl pH 7.5, 0.3% (v/v) Tween 20). Then the membrane was incubated for 30 min in 100 ml of Blocking Buffer (0.5% Blocking Reagent (Roche) in 0.1 M Maleic acid, 0.15 M NaCl pH 7.5), then incubated for 30 min in 20 ml antibody solution (75 mU/ml anti-Digoxigenin-AP in Blocking Buffer). The membrane was washed 2 times for 15 min in 100 ml of DIG washing buffer. Prior to the addition of CDP-Star, the membrane was equilibrated for 2-5 min in 20 ml of Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5). The membrane was placed on a development folder with DNA side facing up. The 1 ml of 25 mM CDP-Star (1:10,000 dilution) solution was applied to the membrane. The damp membrane was incubated for 5 min at room temperature in dark chamber before being exposed to X-ray film. Film was developed using developer and fixer reagent (Kodak) according to manufacturer's instruction.

Collection of positive plaques

After the film was developed, positive plaques were located by aligning the film to the original plate. Plaques corresponding to the positive signal were excised from the plate and put into 1 ml of SM buffer (0.1 M NaCl, 15 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) containing 20 µl of chloroform to prevent bacterial contamination. Secondary and/or tertiary screening was performed until a single plaque was obtained. The single plaque was excised from agar plate and transferred to a sterile tube containing 500 µl of SM buffer and 10 µl of chloroform. This phage solution was used for *in vivo* excision for the conversion of ZAP Express vector to pBK-CMV phagemid vector.

Conversion of ZAP Express vector to pBK-CMV phagemid vector

The ZAP Express vector was designed to allow simple and efficient *in vivo* excision and recircularization of any cloned insert within the lambda vector to form a circular phagemid. Excision of phagemid was performed for each positive clone selected. A mix of 200 µl of *E.coli* XL1-Blue MRF´ cells diluted to OD_{600} of 1.0 in 10 mM $MgSO_4$ and 250 µl of phage stock was prepared. The mixture was complemented with 1 µl of the ExAssist helper phage and incubated at 37°C for 15 min. Then, the mixture was transferred to the 3 ml of LB broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ and incubated overnight at 37°C with shaking. Afterwards, the mixture was heated at 65°C for 20 min and centrifuged at 1,000 g for 15 min. The supernatant was transferred into a new tube. This stock contained the non-infectious excised pBK-CMV phagemid and was stored at 4°C for 1-2 months.

To plate the excised phagemids, XLOLR cells were grown overnight in NZY broth, pelleted and resuspended to an OD_{600} of 1.0 in 10 mM $MgSO_4$. The 200 µl of this freshly prepared XLOLR cells suspension were mixed with 10 µl of the phage supernatant in a sterile microcentrifuge tube. After incubation at 37°C for 15 min, 300 µl of NZY broth were added and the mixture was incubated at 37°C for 45 min. Then, 200 µl of the cell mixture was spreaded on LB-kanamycin (50 µg/ml) agar plates and incubated overnight at 37°C to get the colonies containing the phagemid of interest. Single colony was then selected and cultured for plasmid extraction.

5. Plasmid DNA extraction

The cell culture was centrifuged at 12,000 g for 30 sec then the supernatant was discarded. The cell pellet was extracted for plasmid DNA by rapid alkaline lysis method or QIAprep® Spin Miniprep Kit (Qiagen).

Rapid alkaline lysis

The rapid alkaline lysis method was performed by using a modified method described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). After centrifugation, the cell pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and kept on ice. A 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and immediately mixed by gentle inversion. The microcentrifuge tube was stored on ice and 150 µl of ice-cold solution III (3 M potassium acetate, 5 M glacial acetic acid) was added. The mixture was gently inverted for 10 sec and kept on ice for 5 min before centrifugation at 12,000 g for 5 min. The supernatant was transferred to a new microcentrifuge

tube. The protein in the supernatant was removed by adding equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and centrifugation at 12,000 g for 5 min. The upper phase was transferred to a new microcentrifuge tube. The plasmid DNA was precipitated with 2 v of absolute ethanol for 2 min at room temperature and centrifugation at 12,000 g for 5 min. The DNA was rinsed once by adding 1 ml of cold 70% ethanol and centrifuged at 12,000 g for 5 min. The DNA was air-dried then resuspended in 20 µl of sterile deionized water containing RNase A (20 µg/ml) and stored at -20°C.

QIAprep® spin miniprep kit

This protocol was used to extract plasmid DNA for DNA sequencing. After the centrifugation at 13,000 rpm for 1 min, the cell pellet was resuspended by adding 250 µl of buffer P1 and mixed by vortex until the cell pellet was completely dissolved. The 250 µl of buffer P2 was added and the microcentrifuge tube was gently inverted several times or until the mixture became viscous and slightly clear. The 350 µl of buffer N3 was added to the mixture and the mixture was inverted immediately but gently several times then centrifuged at 13,000 rpm for 10 min. The supernatant was applied to the QIAprep® spin column, centrifuged at 13,000 rpm for 1 min, and then the flow-through was discarded. The QIAprep® spin column was washed by adding 750 µl of buffer PE and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at 13,000 rpm to remove residual ethanol in washing buffer. The plasmid DNA was eluted by adding 20 µl of sterile deionized water to the center of QIAprep® spin column. The column was let stand for 1 min and centrifuged at 13,000 rpm for 1 min. The eluted plasmid DNA was transferred to a new microcentrifuge tube and stored at -20°C.

6. Characterization of positive clones

The size of cDNA insert in positive clone was estimated by either PCR using T3 and T7 primers, which are complementary to the sequences flanking the cDNA cloning site, or by *EcoR*I and *Xho*I digestion, which releases the cDNA insert from the pBK-CMV phagemid vector (Figure 2). The PCR reaction was prepared as follows: 1 µl of plasmid DNA, 200 µM dNTP, 1 unit of *Taq* DNA polymerase (NEB), 0.4 µM of each forward and reverse primer in 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$). The cycling conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 45 sec and elongation at 72°C for 1 min. After the last cycle, additional elongation at 72°C for 7 min was performed.

The 10 µg of phagemid DNA extracted by rapid alkaline lysis method was digested with restriction enzyme *Eco*RI and *Xho*I. The reaction was performed in a 15 µl final volume. The final concentration of each component was 1X NEBuffer 2 (NEB), 10 units of *Eco*RI (20 units/µl, NEB) and 10 units of *Xho*I (20 units/µl, NEB). The reaction mixture was gently mixed, spun down and then incubated at 37°C for 1 h. The digested product was analyzed by agarose gel electrophoresis.

7. DNA sequencing and analysis

Plasmid DNA was extracted by the QIAprep® Spin Miniprep kit to obtain the purified DNA suitable for sequencing. The DNA was sequenced by 1st BASE DNA Sequencing Service (Malaysia). After the vector sequences were removed, the nucleotide sequences were searched for homology using BLASTN or BLASTX programs of NCBI. The ClustalW program of European Biotechnology Institute (EBI) and several programs of Expasy were also used to analyze the DNA sequences (http://www.expasy.ch/).

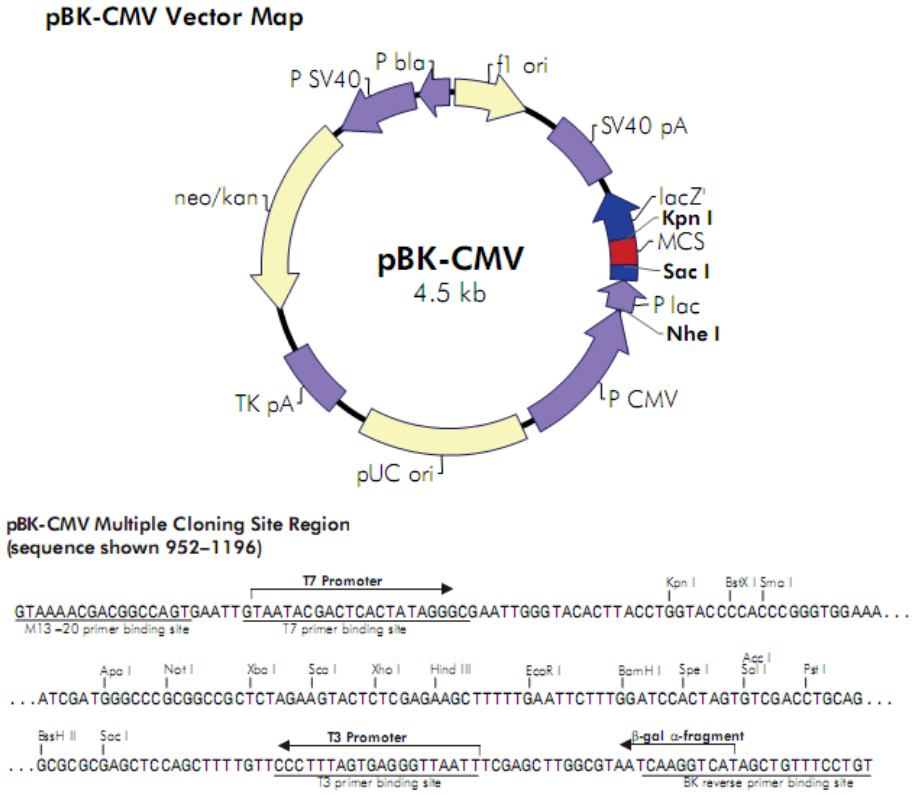


Figure 2. pBK-CMV vector map and multiple cloning site region.

Results and Discussion

Part I: Analysis and Classification of Sequenced ESTs from Latex SSH Libraries

In an attempt to identify some of the major genes that may be involved in the ethylene-induced increase in latex yield, the Suppression Subtractive Hybridization (SSH) technique was set up in collaboration between Mahidol University and Institut de Recherche pour le Développement (IRD) to compare the *Hevea* latex and bark transcriptomes from control and Ethrel® -stimulated (mix of the different time-treatments) trees (Kongsawadworakul and Chrestin, unpublished). In particular, various SSH cDNA libraries were constructed (control *vs* ethylene and ethylene *vs* control) with mRNA, of latex and inner soft bark (phloem), from control/ethylene-stimulated rubber trees, using PCR-select™ cDNA Subtraction Kit (Clontech, USA). Almost 5,000 cDNA clones picked up randomly from these four subtracted cDNA libraries were submitted to high throughput sequencing (ESTs) and analyzed using the bioinformatic software, so called "EST-DB pipeline", set up at IRD (France). The ESTs bioinformatic analysis could evidence marked differential gene expression between control and ethylene-stimulated rubber trees, at the level of the latex producing tissues (broadly speaking, i.e. in the latex cells and in the other inner bark tissues).

A total of 2,258 ESTs were sequenced from latex SSH cDNA libraries. About 1,130 ESTs were sequenced from each latex control (LC)- and latex Ethrel®-stimulated (LS)-SSH libraries (Table 3a). From almost the same amount of sequenced ESTs, these two SSH libraries differed in their amount of unique genes. The amount of unique genes were 898 ESTs for LC-SSH library and 257 ESTs for LS-SSH library (Table 3b). This higher number of unique genes in LC-SSH library suggested that many genes were down-regulated by ethylene. In addition, more number of ESTs in contig than in singleton from LS-SSH library implied that many genes in LS-SSH library were highly redundant, suggesting that some few major genes may be highly up-regulated in the latex cells in response to ethylene. All sequenced ESTs were classified into four groups, known function, unknown with information (stimuli responsive, membrane or organelle-targeted proteins, conserved motifs, etc.), unknown, and no hit, based on the similarity to genes in the public databases (Table 1).

Latex control SSH library (LC-SSH library)

For LC-SSH library, the known function gene was the largest group containing 552 ESTs (49%) (Figure 3). Unknown with information genes, unknown genes, and no hit showed 183 ESTs (16%), 211 ESTs (19%) and 184 ESTs (16%), respectively. However, the classification of unique gene in this library showed some differences (Figure 3). The genes in known function group of LC-SSH library were further classified based on gene function and divided into nine groups; cell organization, cellular communication and signaling, cellular metabolism/pathway regulation, energy production and conversion, gene expression/chromatin, protein fate/synthesis, rubber biosynthesis, stress/defense responses, and transport (Figure 4). Compared to LS-SSH library, the number of ESTs in the group of cell organization, cellular communication and signaling, cellular metabolism/pathway regulation, energy production and conversion, protein fate/synthesis, and stress/defense responses was higher in LC-SSH library. This result suggested that more genes in these groups from latex of rubber tree were down-regulated than up-regulated by ethylene (Figure 4).

Table 1. Classification of all ESTs (a) and unique genes (b) from latex SSH libraries.

(a) All ESTs

Library	Known function	Unknown with info	Unknown	No hit	Singleton	Contig	Total
LC	552	183	211	184	763	367	1130
LS	449	162	166	351	97	1031	1128
Total	1001	346	376	535	860	1398	2258

(b) Unique genes

Library	Known function	Unknown with info	Unknown	No hit	Singleton	Contig	Total
LC	405	152	171	170	763	135	898
LS	87	34	37	99	97	160	257
Total	492	186	208	269	860	295	1155

Latex Ethrel®-stimulated SSH library (LS-SSH library)

As in LC-SSH library, all ESTs in LS-SSH library were classified into four groups; known function (449 ESTs, 40%), unknown with information (162 ESTs, 14%), unknown (166 ESTs, 15%), and no hit (351 ESTs, 31%) (Figure 4). The known function group was also classified and divided into the same nine groups as in LC-SSH library but the energy production and conversion group was absent (Figure 4). The result suggested that this group might be down-regulated by ethylene. Moreover, the number of ESTs in the gene expression/chromatin and rubber biosynthesis groups was higher than in the LC-SSH library. This indicated that more genes in these groups were up-regulated than down-regulated by ethylene.

Part II: Analysis and Classification of Sequenced ESTs from Bark SSH Libraries

The 2,231 ESTs in total were sequenced from bark SSH cDNA libraries. About 1,120 ESTs were sequenced from each bark control (BC)- and bark Ethrel®-stimulated (BS)-SSH libraries (Table 2). Upon the almost equal amount of sequenced ESTs from the two libraries, the BC-SSH library contained higher number of singleton (784 ESTs) and a lower number of contig (327 ESTs) than the BS-SSH library (555 singletons and 565 contigs). This indicated that ethylene induced also a higher redundancy of gene expression in the bark.

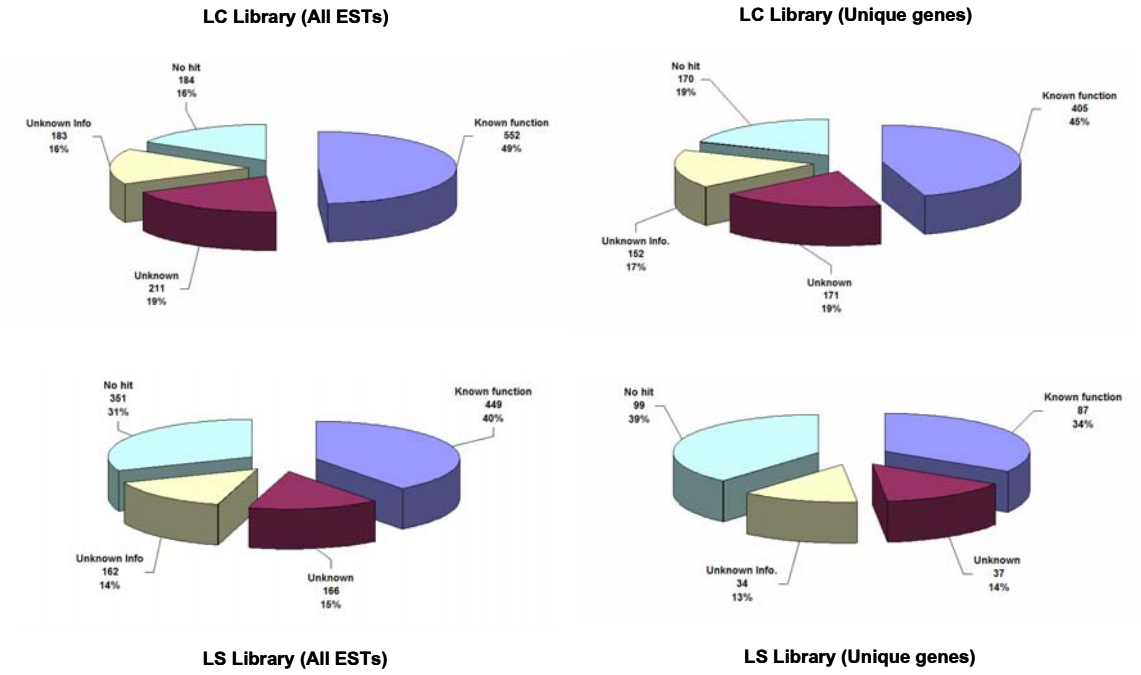


Figure 3. Classification of all ESTs and unique genes from LC- and LS-SSH libraries. All ESTs and unique genes were shown in these charts.

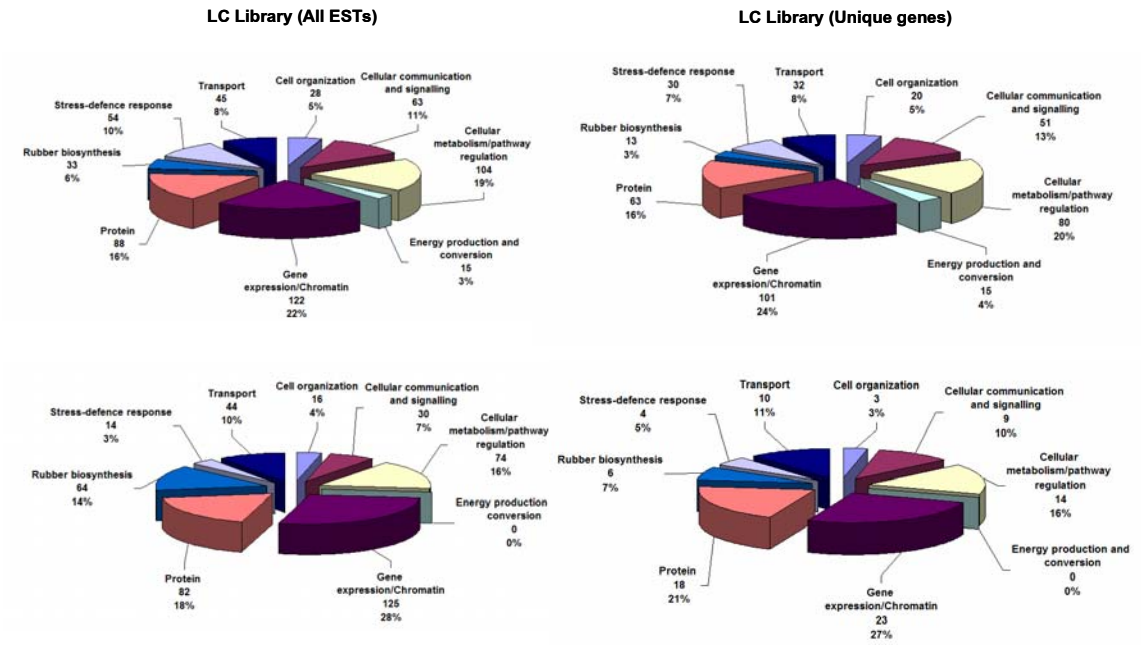


Figure 4. Classification of all ESTs and unique genes in known function group from LC- and LS-SSH libraries. All ESTs and unique genes were shown in these charts.

Bark control SSH library (BC-SSH library)

For BC-SSH library, the known function gene was the largest group containing 507 ESTs (46%) (Figure 5). Unknown with information genes, unknown genes, and no hit showed 235 ESTs (21%), 92 ESTs (8%) and 277 ESTs (25%), respectively. The genes in known function group of BC-SSH library were further classified based on gene function and divided into eight groups; cell metabolism, cell organization, energy production and conversion, gene expression/chromatin, protein fate/synthesis, signaling, stress/defense responses, and transport (Figure 6). Compared to BS-SSH library, the number of ESTs in the group of cell organization, protein fate/synthesis, stress/defense responses, and transport was higher in BC-SSH library. This result suggested that more genes in these groups from bark of rubber tree were down-regulated than up-regulated by ethylene (Figure 6).

Table 2. Classification of all ESTs (a) and unique genes (b) from bark SSH libraries.

(a) All ESTs

Library	Known function	Unknown with info	Unknown	No hit	Singleton	Contig	Total
BC	507	235	92	277	784	327	1111
BS	765	137	55	163	555	565	1120
Total	1272	372	147	440	1339	892	2231

(b) Unique genes

Library	Known function	Unknown with info	Unknown	No hit	Singleton	Contig	Total
BC	432	164	63	228	784	103	887
BS	452	104	43	140	555	184	739
Total	884	268	106	368	1339	287	1626

Bark Ethrel®-Stimulated SSH Library (BS-SSH Library)

As in BC-SSH library, all ESTs in BS-SSH library were classified into four groups; known function (765 ESTs, 68%), unknown with information (137 ESTs, 12%), unknown (55 ESTs, 5%) and no hit (163 ESTs, 15%) (Figure 5). The known function group was also classified and divided into the same eight groups as in BC-SSH library (Figure 6). The number of ESTs in the cell metabolism, energy production and conversion, gene expression/chromatin, and signaling groups was higher than in the BC-SSH library. This indicated that more genes in these groups were up-regulated than down-regulated by ethylene.

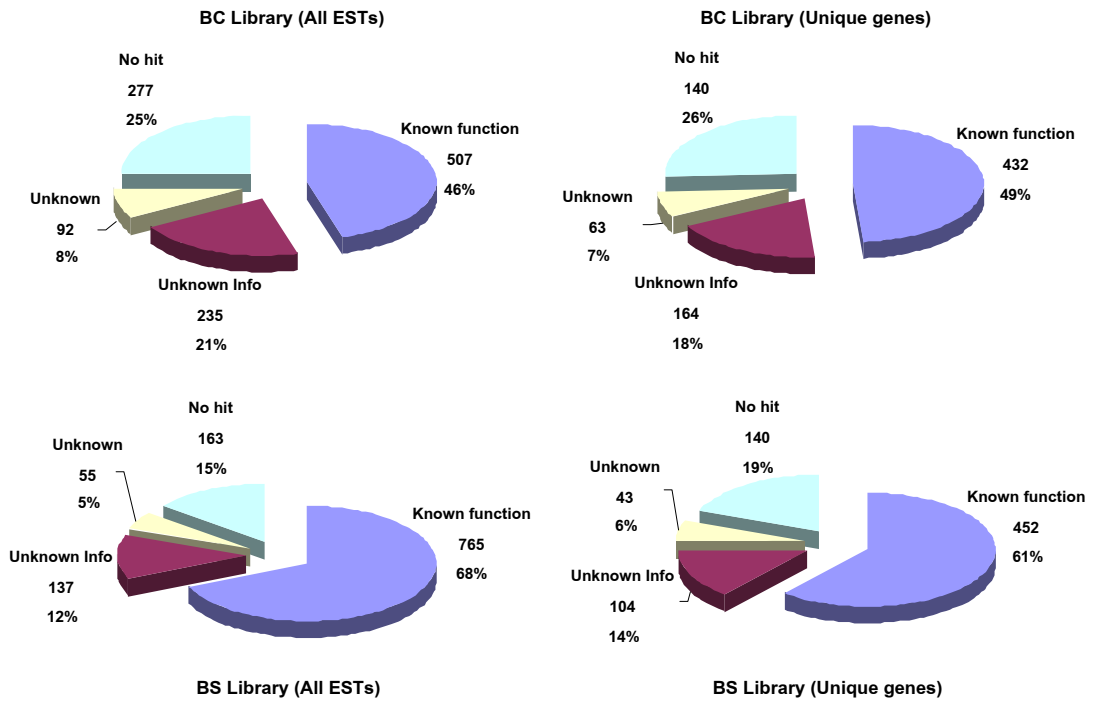


Figure 5. Classification of all ESTs and unique genes from BC- and BS-SSH libraries. All ESTs and unique genes were shown in these charts.

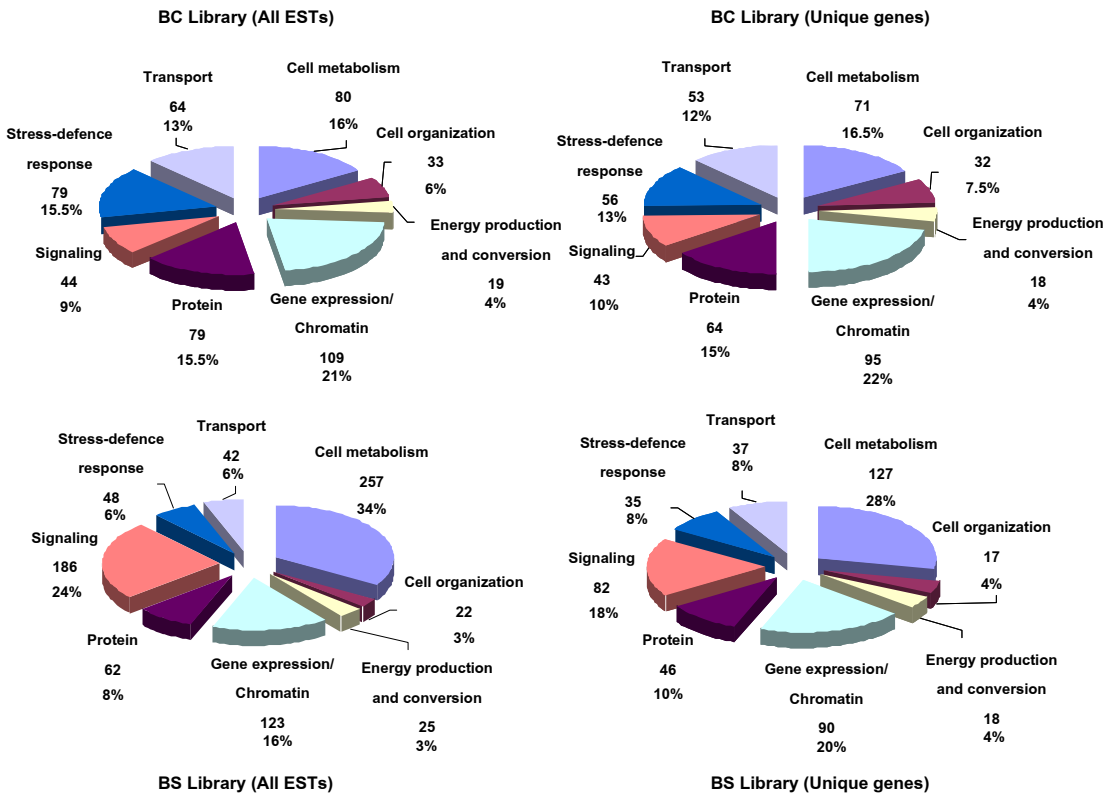


Figure 6. Classification of all ESTs and unique genes in known function group from BC- and BS-SSH libraries. All ESTs and unique genes were shown in these charts.

Part III: Macroarray Analysis

Gene expression profiles by macroarray

The expression profiles of genes in the LC- and LS-SSH libraries as well as BC- and BS-SSH libraries were then analyzed using the cDNA macroarray technique to compare the repressed or induced transcripts at different times after ethylene stimulation in each couple. In this experiment, the unique genes from ESTs of LC-SSH and LS-SSH libraries as well as BC-SSH and BS-SSH libraries were amplified by four PCR reactions, which were pooled and precipitated. After resuspension, 1 µl of PCR product was run on 2.5% agarose gel to estimate the insert size. An aliquot of 300 ng of each gene including *40S* and *ubiquitin* as positive control and *GFP* as negative control were spotted on macroarray nylon membranes as shown in Figure 7.

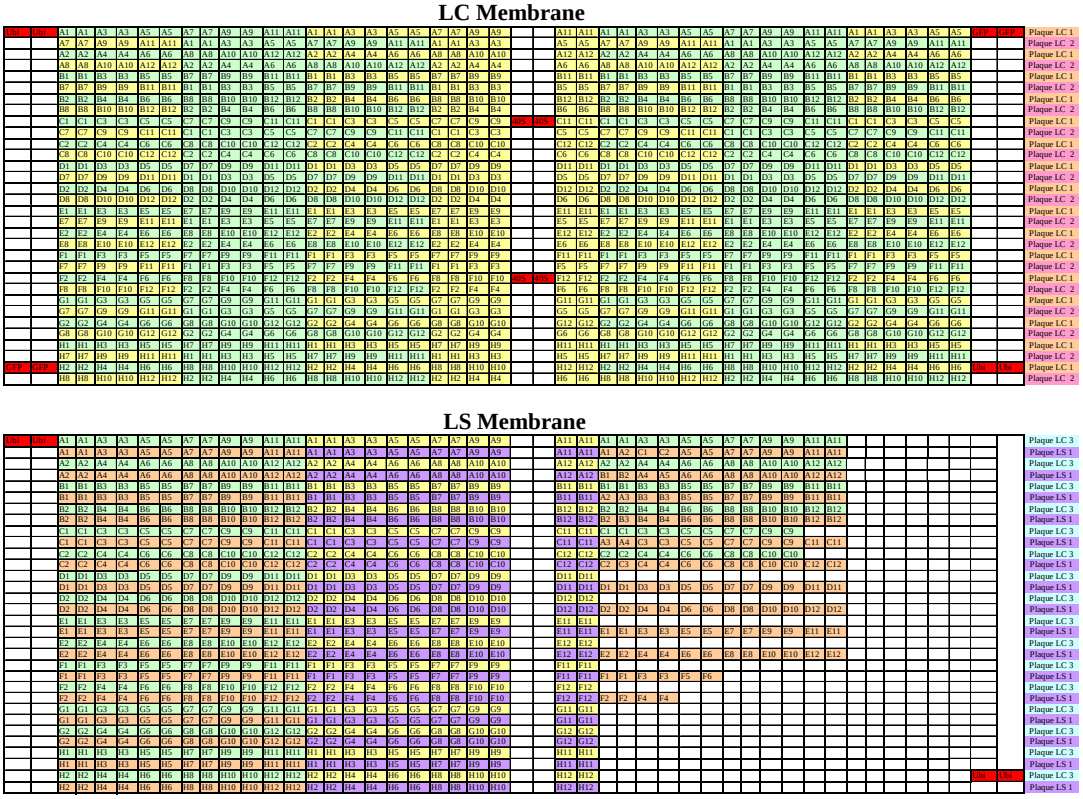


Figure 7. Membrane map for macroarray analysis. All unique and reference genes were spotted on the membrane corresponding to the position indicated in the map.

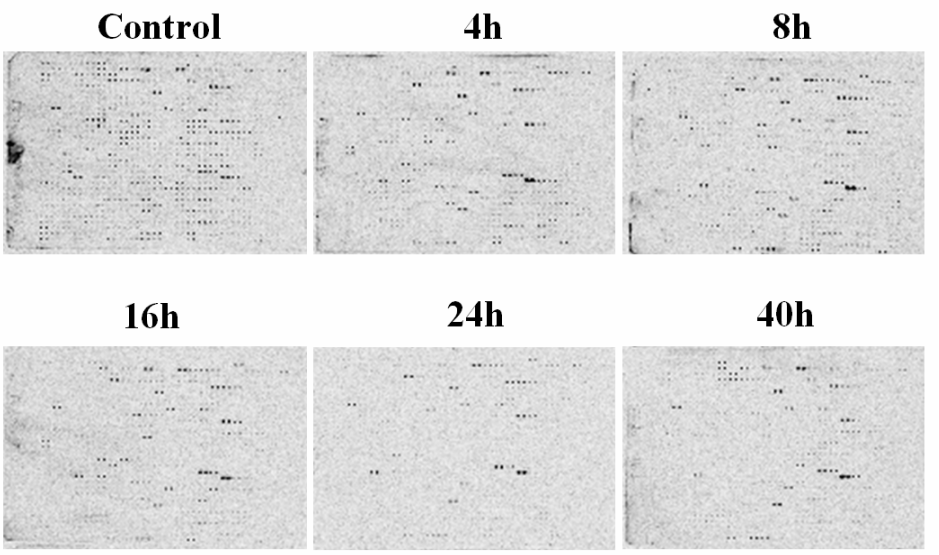
The 898 (including singletons and contigs) unique genes from LC-SSH library were spotted on latex control (LC) membrane and the only 257 unique genes from LS-SSH library were spotted on latex Ethrel®-stimulated (LS) membrane. The 887 unique gene ESTs and contigs from BC-SSH library and the 739 ones from BS-SSH library were spotted on bark control (BC) membrane and bark Ethrel®-stimulated (LS) membrane, respectively. The membranes carrying the unique genes were prepared and hybridized with ^{33}P-labeled probes synthesized from latex or bark RNA of control trees, and 4, 8, 16, 24 and 40 hours Ethrel®-stimulated trees. Then these membranes were exposed for three to four days to the phosphoimager screen (Amersham). The images were acquired by scanning the membranes with Typhoon Trio (Amersham) (Figure 8 and 9).

The result from LC membrane suggested that most genes in LC-SSH library were really down-regulated by ethylene, since lower signal intensity of most genes was found on the membranes hybridized with stimulated probes (Figure 8a). In the contrary, many genes in LS-SSH library were up-regulated by ethylene, since higher signal intensity of these genes was actually found when they were hybridized with stimulated probes (Figure 8b).

In the contrary, the result from BC membrane suggested that most genes in BC-SSH library were really up-regulated by ethylene, since higher signal intensity of most genes was found on the membranes hybridized with stimulated probes (Figure 9a) and *vice versa* (Figure 9b).

The signal intensity of each gene on membranes was analyzed using ArrayVision® software and all data were statistically analyzed by ANOVA test to determine the significant differences between means of two groups of control and stimulated samples. The statistical analysis was performed with the level of significance $P \leq 0.05$. All genes showing significant differences in expression level between control and stimulated condition were classified into six groups based on trend of expression as shown in Figure 10.

(a) LC membrane *vs* corresponding time treatment probes



(b) LS membrane *vs* corresponding time treatment probes

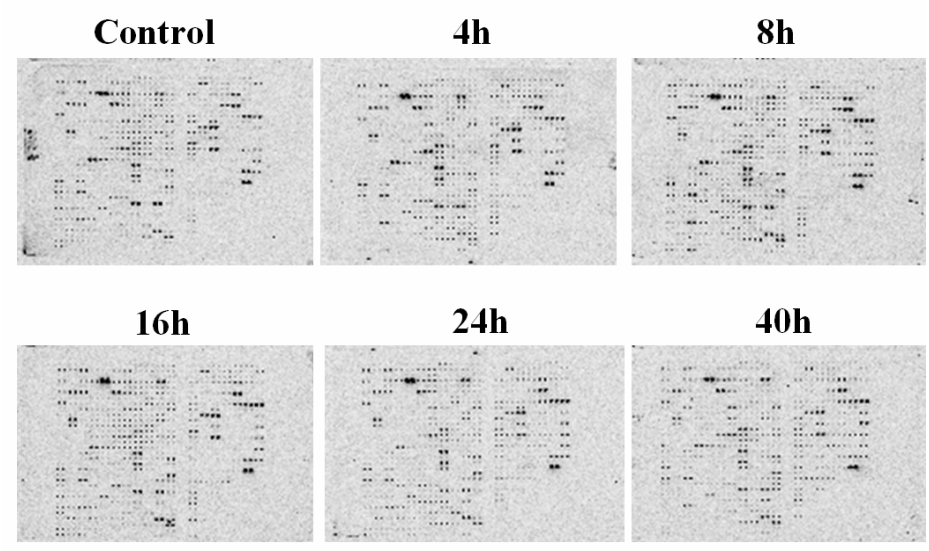
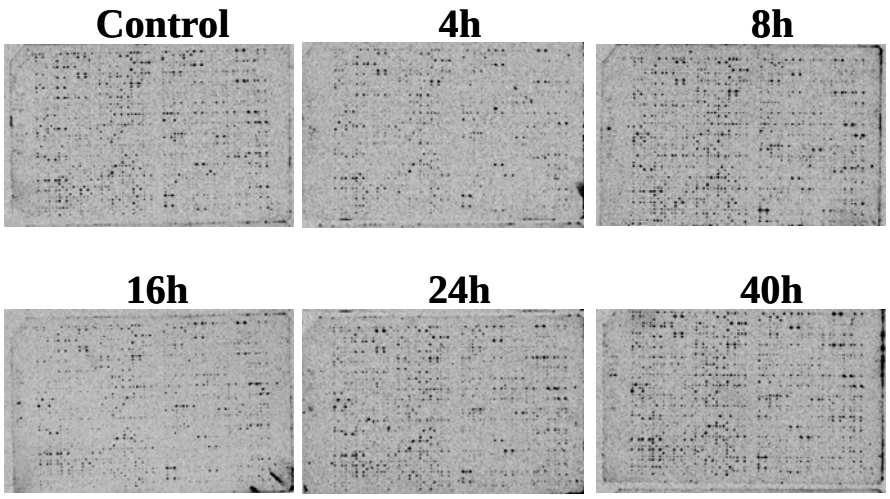


Figure 8. Macroarray analysis. LC membranes (a) and LS membranes (b) were hybridized with 1 control probe and 5 stimulated probes. Control: membrane hybridized with control (non-stimulated) latex cDNA probe; 4h, 8h, 16h, 24h, 40h: membrane hybridized with Ethrel®-stimulated latex cDNA probes from 4, 8, 16, 24, and 40 hours after Ethrel® treatment.

(a) BC membrane *vs* corresponding time treatment probes



(b) BS membrane *vs* corresponding time treatment probes

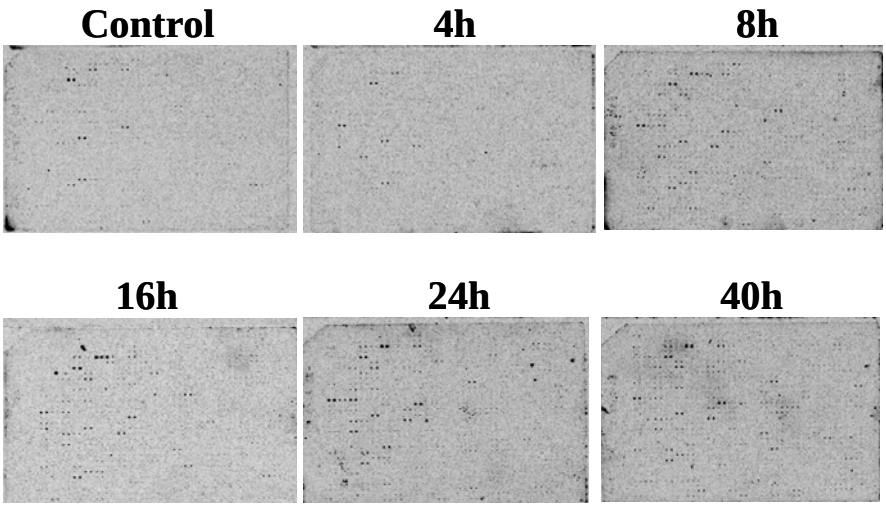


Figure 9. Macroarray analysis. BC membranes (a) and BS membranes (b) were hybridized with 1 control probe and 5 stimulated probes. Control: membrane hybridized with control (non-stimulated) bark cDNA probe; 4h, 8h, 16h, 24h, 40h: membrane hybridized with Ethrel®-stimulated bark cDNA probes from 4, 8, 16, 24, and 40 hours after Ethrel® treatment.

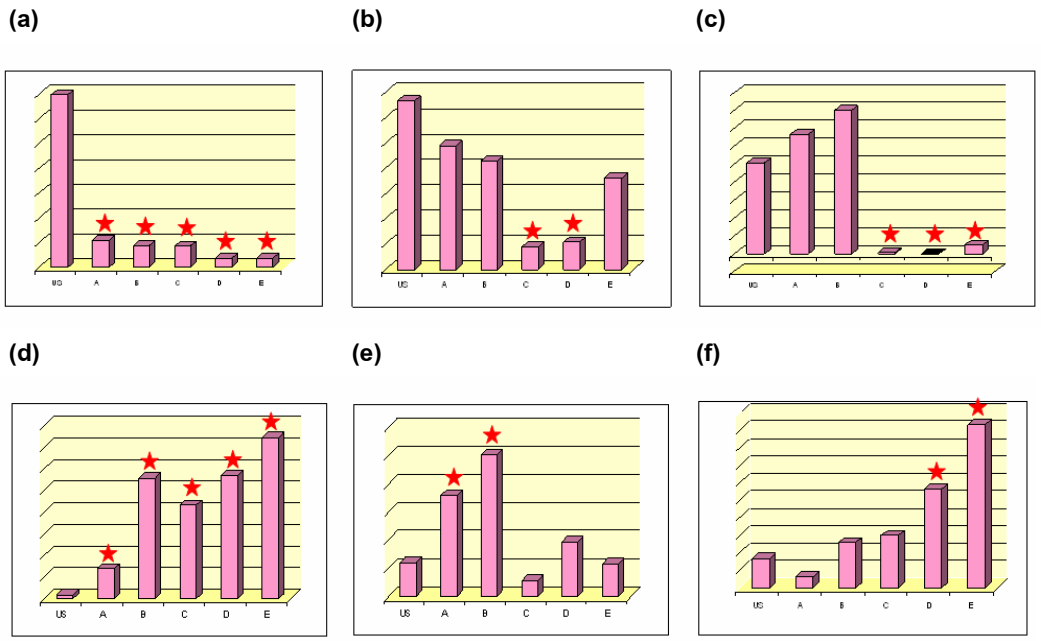


Figure 10. Gene classification based on trend of expression results from macroarray analysis. The graphs showed examples of gene expression in six different groups: (a) early down-regulated by ethylene; (b) transiently down-regulated by ethylene; (c) late down-regulated by ethylene; (d) early up-regulated by ethylene; (e) transiently up-regulated by ethylene; (f) late up-regulated by ethylene. US: unstimulated latex or bark cDNA probe; A, B, C, D and E: Ethrel®-stimulated latex or bark cDNA probes of 4, 8, 16, 24 and 40 hours after stimulation. ★: significantly different from unstimulated latex or bark cDNA probe.

Part IV: Expression Pattern of Candidate Genes

RNA extraction

The amount of RNA both from latex and bark was determined spectrophotometrically at OD 260 nm. The 260/280 OD ratio and integrity of RNA bands shown by gel electrophoresis were used as indicators for high quality of nucleic acid. The 260/280 OD ratio at 1.8-2.0 indicates low level of protein contamination in nucleic acid sample. By gel electrophoresis, intact RNA with prominent bands of 28S and 18S rRNA without smear indicates high quality of RNA without nucleic acid degradation. The RNA extraction methods used in this experiment was found suitable since the RNA products contain low protein contamination as suggested by 260/280 OD ratio (Table 3 and 4). Moreover, the gel electrophoresis also showed intact RNA with prominent 28S and 18S rRNA bands (Figure 11 and 12).

Table 3. Results of RNA extraction from latex RNA samples. OD 260/280 ratio indicates quality of nucleic acid. C1 and C2 are control (unstimulated) trees. 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

Latex RNA samples	OD260/OD280	Yield (µg/µl)	Yield (µg/ml latex)
C1	1.65	2.24	37.33
C2	1.72	2.06	17.17
4	1.63	1.30	21.67
8	1.70	1.96	32.67
16	1.67	2.00	33.33
24	1.70	1.46	24.33
40	1.73	1.38	23.00

Table 4. Results of RNA extraction from bark RNA samples. OD 260/280 ratio indicates quality of nucleic acid. C1 and C2 are control (unstimulated) trees. 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

Bark RNA samples	OD260/OD280	Yield (µg/µl)	Yield (µg/g tissue)
C1	1.70	2.80	112
C2	1.76	2.06	164.8
4	1.74	1.46	116.8
8	1.77	1.54	123.2
16	1.72	1.72	137.6
24	1.74	1.46	116.8
40	1.82	1.48	118.4

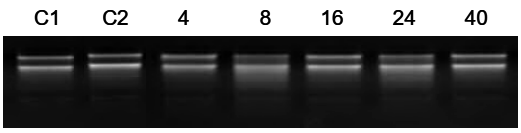


Figure 11. Gel electrophoresis of latex RNA. C1 and C2 are control (unstimulated) trees. 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

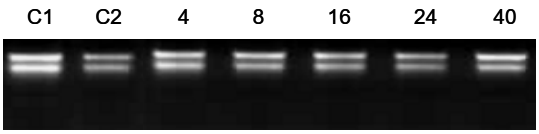


Figure 12. Gel electrophoresis of bark RNA. C1 and C2 are control (unstimulated) trees. 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

Identification of differentially expressed genes in response to ethylene from SSH

Latex SSH libraries: Among 2,258 sequenced ESTs from control and Ethrel®-stimulated latex SSH cDNA libraries, 43 ESTs identified as two isoforms of Abscisic acid Stress Ripening (*ASR*) gene, *ASR1* and *ASR2*, represented one of the highest percentage of the ESTs of a given class of gene. *ASR* genes are known to be regulated by stress and different hormones or phytochemicals, i.e. ABA, MeJA, but no data have ever been reported on their regulation by ethylene (Wang *et al*., 2002).

Bark SSH libraries: Among 2,231 sequenced ESTs from control and Ethrel®-stimulated inner soft bark SSH libraries, 55 ESTs, grouped in four contigs, identified as at least two isoforms of *lipoxygenase* gene, represented the highest percentage of the ESTs of a given class of gene. These genes were present only in the ethylene-treated bark SSH library indicating probable up-regulation of these genes in bark in response to ethylene. Moreover, a total of 53 ESTs identified as another isoform of Abscisic acid Stress Ripening (*ASR*) gene, *ASR3*, represented as high percentage in bark control SSH library only.

It is worth noticing that *cis-prenyltransferase*, a gene involved in polyisoprene synthesis, and *ACC oxidase*, a gene involved in ethylene biosynthesis, were identified to be down- regulated and up-regulated by ethylene, respectively.

Study on gene expression by RT-PCR

First-stranded cDNA was synthesized from latex and inner soft bark total RNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The expression of house- keeping 40S ribosomal RNA was monitored as control gene. *ASR* and *LOX* gene expression in response to ethylene treatment in the latex and in the inner bark of rubber tree was analysed.

Kinetic effects of bark Ethrel® stimulation on the *ASR* genes in latex and bark

The primers specific for *ASR1*, *ASR2* and *ASR3* were used to assess *ASR* gene expression in response to ethylene treatment in the latex and in the inner bark of rubber tree. The result showed that *ASR1* gene was expressed both in latex and inner bark, but higher in the latex (Figure 13). This confirms its abundant expression in the latex (Ko *et al*., 2003). However, this *ASR1* gene looked not to be regulated by ethylene. The *ASR2* gene is abundantly expressed in the latex, but not in the bark (Figure 13) whereas *ASR3* gene is abundantly expressed in the bark, but not in the latex. Thus, *ASR2* and *ASR3* genes appear to be specific for the latex cells and inner soft bark, respectively. Interestingly, Ethrel® treatment induced a marked down-regulation of both *ASR2* and *ASR3* genes. This correlated well with the result from macroarray showing also the down-regulation of *ASR3* in response to ethylene (Figure 14a). Even though it is presently not possible to specify whether down-regulation of latex *ASR2* and *ASR3* genes is directly or indirectly

induced by ethylene, this is the first report of the regulation of *ASR2* and *ASR3* genes after ethylene treatment.

Interestingly, Cakir *et al*. (2003) revealed that a grape ASR bound to the promoter of a hexose transporter gene. Recent data indicated that tomato *ASR1* localized to the nucleus where it bound to a specific DNA sequence (Kalifa *et al*., 2004a). The data were in close agreement with the proposed role of ASR as a transcription factor (Carrari *et al*., 2004; Kalifa *et al*., 2004b). In the rubber tree, Ethrel® stimulation induced an increase in sugar loading inside the laticifers, starting about 15 hours after the treatment then increasing with time (Lacrotte *et al*., 1984, 1985). Moreover, Dusotoit-Coucaud *et al*. (2007 and 2009) reported the up-regulation of several plasmalemma sugar transporters in the latex cells by ethylene. Based on all these data, ASR proteins in the latex and bark may play a role in the control of sugar uptake at the latex plasmalemma, through the negative control of some sugar transporter genes expression. It is proposed that, in the latex cells, through direct or indirect down-regulation of some *ASR* genes (among which at least *ASR2* and *ASR3*), ethylene might participate to the up-regulation of some sugar plasmalemma transporter genes in response to bark Ethrel® treatment.

Kinetic effects of bark Ethrel® stimulation on the *LOX* genes in latex and bark

Among the 2,231 ESTs sequenced from control and Ethrel®-stimulated bark SSH libraries, 55 ESTs, grouped in 4 contigs, represented the highest percentage of a gene family among the whole ESTs. They were present in the ethylene-treated bark SSH library only, indicating probable up-regulation of these genes in the liber in response to ethylene. All of them share high homology with the same published sequence of *LOX2* from *Populus deltoides*. This allowed location, through alignments, of four *Hevea LOX* contigs, to the same sequence, *LOX2 from P. deltoides* (Figure 15).

Two shorter contigs (172 and 173) harbour poly A tail, attesting they are 3'end. However, contig172 was 77 bp longer, at the 3'end, than contig173 demonstrating they are different isoforms of *LOX*, showing 94% identity. This allowed the design of specific primers to study their expression by semi-quantitative RT-PCR. Since the contigs76 and 88 do not overlap, it is presently impossible to certify whether they are two fragments of the same gene or different isoforms of the same *LOX* gene. Thus, two specific primers were also designed to study the expression of the corresponding genes. Figure 16 showed the time course of the four contigs expression in the latex and inner bark of rubber tree clone PB217. All four sequences were very slightly expressed in the inner bark of control trees and showed similar expression pattern, transiently but markedly up-regulated, in response to ethylene. *LOX* overexpression is maximum at 4-8 hours after Ethrel® treatment then decreases progressively from 16 hours and return to the basal level at 40 hours correlated with the macroarray result showing the transient up-regulation of *LOX* by ethylene (Figure 14b). There is no expression of the corresponding contigs in the latex, neither in the control nor in the stimulated trees, confirming that these *LOXs* which were found in the Ethrel®-treated bark SSH library only are really tissue (bark) specific. This led to propose that cell specialization (laticifer differentiation) might lead to modified regulation - or even loss - of such stress signal transduction pathway. Interestingly, bark Ethrel® treatment induced a marked up-regulation of all *LOX* contigs confirming the well known crosstalk between the ethylene

and jasmonate pathway, the one often inducing the other and *vice versa* (Devoto and Turner, 2005; Broekaert *et al*., 2006; Dreher and Callis, 2007).

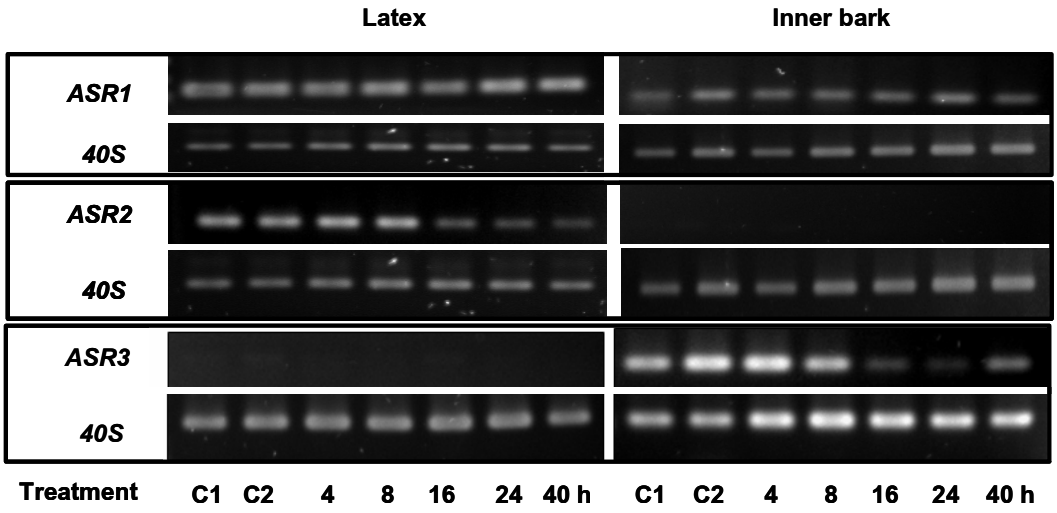


Figure 13. Kinetic effects of bark Ethrel® treatments on *ASR* gene expression in the latex and inner bark by semi-quantitative RT-PCR. *ASR1* and *ASR3* genes were amplified, using specific primers, for 30 cycles in both latex and bark templates. *ASR2* gene was amplified with specific primers for 25 and 35 cycles, with the latex and bark templates, respectively. The *40S ribosomal* gene was amplified for 25 cycles. C1 and C2 are control (unstimulated) trees. 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

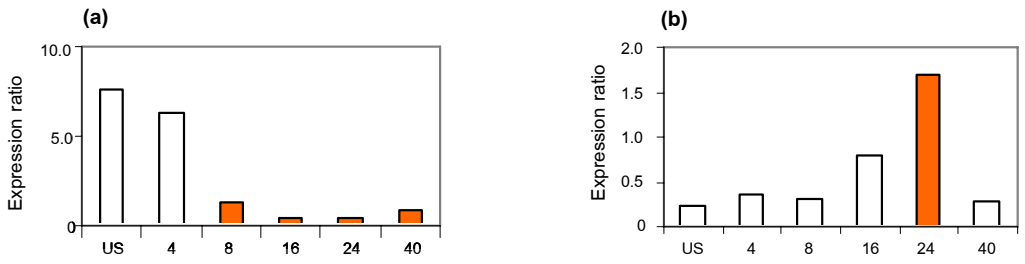


Figure 14. Macroarray results showing kinetic effects of bark Ethrel® treatments on *ASR3* (a) and *lipoxygenase* (b) genes expression in the inner bark. US: control (unstimulated) trees; 4, 8, 16, 24 and 40: trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

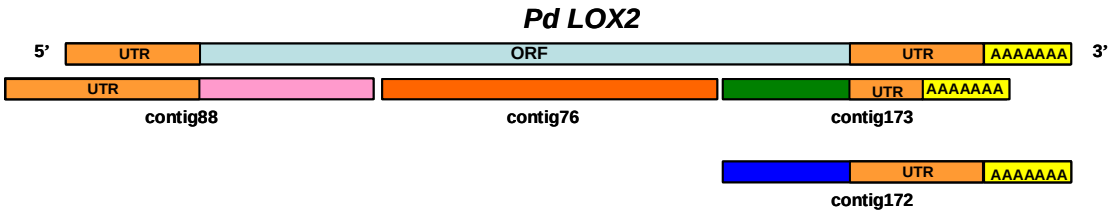


Figure 15. Alignment of four *Hevea* bark *LOX* gene contigs with the *LOX2* gene from *Populus deltoides*.

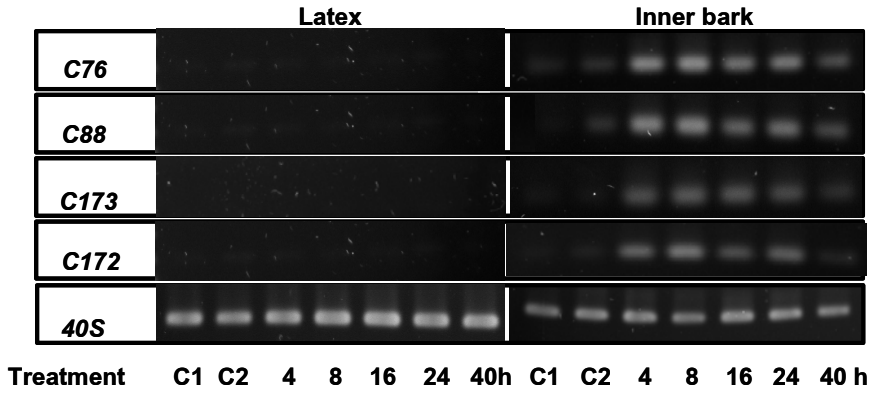


Figure 16. Kinetic effects of bark Ethrel® treatments on *LOX* gene expression in the latex and inner bark by semi-quantitative RT-PCR. The PCR amplification was performed for 30 cycles with specific primers of each contig. The *40S ribosomal* gene was used as control. C1 and C2 are control (unstimulated) trees. The 4, 8, 16, 24, 40 are trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

Lipoxygenase is the enzyme that catalyzes the first step of the jasmonate synthesis from linolenic acid (Vick and Zimmerman, 1984; 1987; Schaller *et al*., 2005). Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are genuine plant hormones which have regulatory function as signaling molecules in plant development, defense and adaptation to abiotic stress. They are produced in systemic response to wounding, pathogens, environmental stress, playing an important role to mediate development and defense response genes through the modulation of various gene expressions (Gundlach *et al*., 1992; Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Van der Fits and Memelink, 2000; Rossato *et al*. 2002; Cheong and Choi, 2003)

As far as the rubber tree is concerned, it has been established for long that the number of laticifers in a mantle as well as the number of functional laticifer mantle in the inner soft bark are very important factor related to rubber yield (Gomez, 1982; Hénon and Nicolas, 1989). Interestingly, application of exogenous JA, or its precursor, linolenic acid, to the bark of young rubber trees, has been reported to induce an increase in latex vessel numbers (Hao *et al*., 2000) and in secondary laticifers formation (Wu *et al*., 2002), respectively.

Given the importance of the jasmonate pathway in the regulation of cascade genes expression that may lead to increase rubber yield, full-length cDNA of *LOX* gene was screened from Ethrel®-treated bark cDNA library of rubber tree. After the first screening, 12 positive clones were selected and the secondary screening was performed. Six positive clones were obtained after the secondary screening and would be further characterized.

Study on gene expression by real-time PCR

Kinetic effects of bark Ethrel® stimulation on *cis-prenyltransferase* in latex and *1-aminocyclopropane-1-carboxylic oxidase* (*ACC oxidase*) in bark

The experiments through real-time PCR confirmed that *cis-prenyltransferase* and *ACC oxidase* genes were down-regulated and up-regulated by ethylene (Figure 17), respectively.

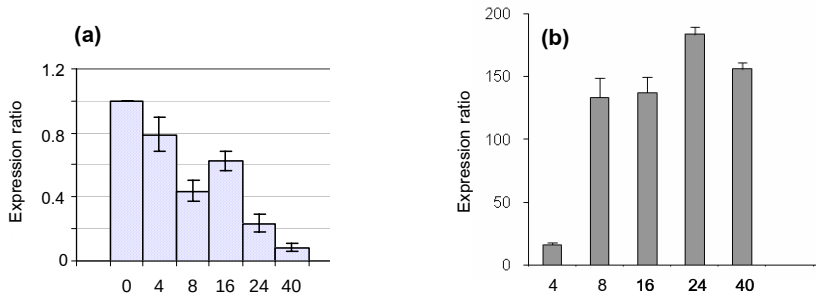


Figure 17. Kinetic effects of bark Ethrel® treatments on *cis-prenyltransferase* (a) and *ACC oxidase* (b) genes expression in the latex and inner bark, respectively, by real time qPCR technique. 0: control (unstimulated) trees. 4, 8, 16, 24, 40: trees stimulated with 5% Ethrel® for 4, 8, 16, 24 and 40 hours, respectively, before the first tapping.

The lower expression of *cis-prenyltransferase* gene which is involved in polyisoprene synthesis after ethylene stimulation may be due to the decrease in rubber biosynthesis efficiency (Chrestin, personal communication). In addition, the up-regulation of *ACC oxidase* gene involved in ethylene biosynthesis confirmed autocatalytic ethylene production.

Identification of differentially expressed genes in response to ethylene from macroarray

From the statistical analysis of macroarray results, 6 candidate genes showing highly up- or down-regulated by ethylene were selected for further characterization. The 6 candidate genes were protein with no similarity (singleton no. 823), unknown protein (singleton no. 353), *(1-4)-beta-mannan endohydrolase*, *b-keto acyl reductase*, phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (phosphorylating *GAPDH*), and non-phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (non-phosphorylating *GAPDH*) genes.

Study on gene expression by RT-PCR

Kinetic effects of bark Ethrel® stimulation on the *unknown gene (singleton no. 353) and no similarity gene (singleton no. 823)* in latex

The expression of *unknown gene* (singleton no. 353) and *no similarity* gene (singleton no. 823) were higher in stimulated tree than in control tree at 8 and 16 h after ethylene treatment, respectively (Figure 18 and 19).

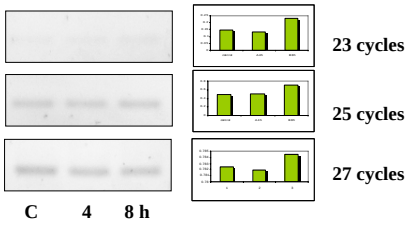


Figure 18. Semi-quantitative RT-PCR analysis of *unknown* gene (singleton no. 353). This gene was amplified with various PCR cycles. C: control sample; 4 and 8 h: stimulated samples collected at 4 and 8 h after ethylene treatment.

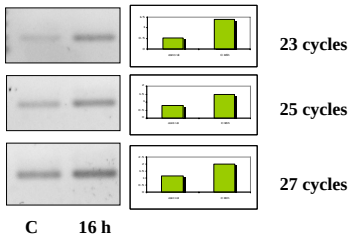


Figure 19. Semi-quantitative RT-PCR analysis of *no similarity* gene (singleton no. 823). This gene was amplified with various PCR cycles. C: control sample; 16 h: stimulated sample collected at 16 h after ethylene treatment.

The sequences of unknown and unknown with information groups were found in both LC- and LS-SSH libraries (Figure 3). These genes were reported in the public database but their molecular functions were unknown. However, if some unknown genes shows early up- or down regulated by ethylene, they may play an important role under the ethylene treatment condition. The data from RT-PCR suggested that the *unknown gene* (singleton no. 353) and *no similarity* gene (singleton no. 823) were up-regulated by ethylene. These genes may be *Hevea* specific candidate genes involved in ethylene response and maybe related to latex yield. Therefore, full-length cDNAs of these genes should be cloned for further characterization.

Kinetic effects of bark Ethrel® stimulation on the *(1-4)-beta-Mannan endohydrolase gene* in latex

To study the expression level of *(1-4)-beta-mannan endohydrolase*, control sample and samples collected at 16 and 40 h after ethylene treatment were used in RT-PCR. The result showed lower expression level of this gene in stimulated sample than in control starting at 16 h after ethylene treatment (Figure 20).

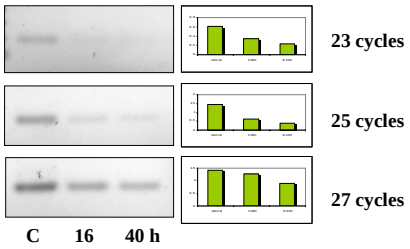


Figure 20. Semi-quantitative RT-PCR analysis of *(1-4)-beta-mannan endohydrolase* gene. This gene was amplified with various PCR cycles. C: control sample; 16 and 40 h: stimulated samples collected at 16 and 40 h after ethylene treatment.

The *(1-4)-beta mannan endohydrolase*, known as endo-beta-mannanase, was classified into the cellular metabolism/pathway regulation category in SSH functional analysis. This enzyme cleaves the β-1,4 links between the mannose residues in the mannan backbone (Mo and Bewley, 2003). In tomato, *endo-beta-mannanase* functions in the metabolism of cell wall polysaccharides during the endosperm mobilization in germinated tomato seeds (Bewley *et al.*, 1997) and also during the germination of coffee grains (Marraccini *et al.*, 2001). In *Cucumis*, a substantial activity of endo-beta-mannanase enzyme was found in the perisperm-endosperm (PE) envelope during the seed germination which may be necessary for the weakening of PE envelope to complete the seed germination (Ramakrishna and Amritphale, 2005). The endo-beta-mannanase enzyme is also active in ripe fruits of many plants such as tomato, watermelon, cantaloupe and peach (Bourgault *et al.*, 2001; Bourgault and Bewley, 2002; Brummell *et al.*, 2004). Based on these data, it can be suggested that the *Hevea (1-4)-beta mannan endohydrolase* gene may involve in the depolymerization of cell wall polysaccharides as in other plants.

The study of the expression profile of this gene using RT-PCR showed that the transcript level of *(1-4)-beta mannan endohydrolase* was significantly lower in stimulated tree than in control tree (Figure 20) suggesting that it was down-regulated by ethylene. This finding leads to the hypothesis that down-regulation of this gene after ethylene treatment results in the strengthening of the cell wall of the laticifers. However, the real function of *(1-4)-beta mannan endohydrolase* in rubber tree is necessary to be further characterized.

Kinetic effects of bark Ethrel® stimulation on the *b-keto Acyl reductase* gene in latex

The *b-keto acyl reductase* expression was studied on latex from tapped tree using RT-PCR. The result showed lower expression level in stimulated sample collected at 4 and 24 h after ethylene treatment than in control sample (Figure 21).

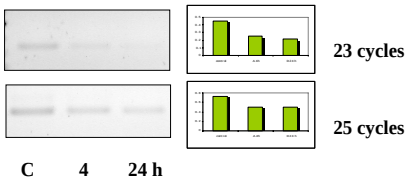


Figure 21. Semi-quantitative RT-PCR analysis of *b-keto acyl reductase* gene. This gene was amplified with various PCR cycles. C: control sample; 4 and 24 h: stimulated samples collected at 4 and 24 h after ethylene treatment.

It is known that the reduction of β-keto group in the biosynthesis of fatty acids was catalyzed by bacterial β-keto acyl reductase (FabG), but in mammalian, this function belongs to the β-keto acyl reductase (KR) domain which is involved in fatty acid synthase (FAS) (Smith, 1994; Rock and Cronan, 1996; White *et al.*, 2005). In plant, the reduction activity of KR in mutant lines resulted in the reduction of total fatty acid in seed. It also appeared that the slower rate of fatty acid synthesis triggered the switch of carbon from storage-lipid to storage-protein biosynthesis (O'Hara *et al.*, 2000). In rubber tree, total latex lipid of the clone RRIM 501 was 1.6% of the latex. Most of these lipids are associated with the rubber particles, none is present in the C-serum. Based on these data, it may be proposed *β-keto acyl reductase* gene function in the fatty acids biosynthesis in the latex cells.

In this study, the expression level of *β-keto acyl reductase* gene was extensively decreased in stimulated tree (Figure 21). According to the hyphothesis that ethylene stimulation may lead to a reduction of rubber biosynthesis (Chrestin, personal communication), thus, the lower expression of *β-keto acyl reductase* may lead to a decrease in fatty acid biosynthesis due to less requirement of lipids for rubber particles.

Study on gene expression by real time PCR

Kinetic effects of bark Ethrel® stimulation on the phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (phosphorylating *GAPDH*) and non-phosphorylating *glyceraldehyde-3-phosphate dehydrogenase* (non-phosphorylating *GAPDH*) genes in latex

In the 1st tapping of virgin tree, phosphorylating *GAPDH* transcript was decreased from 4 to 8 h after stimulation then increased upto 1.2-fold of the control tree. This result suggested that phosphorylating *GAPDH* was transiently down-regulated by ethylene in the 1st tapping of virgin tree. In the 1st tapping of tapped tree, phosphorylating *GAPDH* expression level was apparently lower in stimulated tree than that in control tree and the down-regulation could be observed until 40 h after stimulation (Figure 22). The result indicated that phosphorylating *GAPDH* gene was down-regulated by ethylene in the 1st tapping of tapped tree.

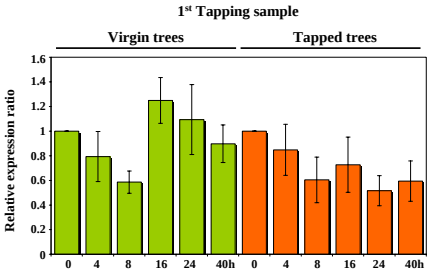


Figure 22. Real-time PCR analysis of phosphorylating *GAPDH* gene on ethylene-treated samples.

In the 1st tapping of virgin tree and tapped tree, the expression level of non-phosphorylating *GAPDH* was continuously decreased until 40 h after stimulation (Figure 23). These results suggested that non-phosphorylating *GAPDH* was truly down-regulated by ethylene.

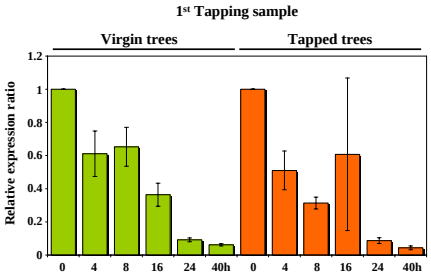


Figure 23. Real-time PCR analysis of non-phosphorylating *GAPDH* gene on ethylene-treated samples.

Hevea latex harbors at least two isoforms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One is the NAD-dependent, phosphorylating GAPDH which is a typical enzyme that catalyzes the oxidation of triose phosphates during glycolysis in all organisms. It phosphorylates 3-phosphoglyceraldehyde into 1,3-phosphoglyceric acid, hence involves in the production of NADH. Another enzyme is NADP-dependent, non-phosphorylating GAPDH that does not catalyze phosphorylation but catalyzes the simple oxidation of 3-phosphoglyceraldehyde into 3-phosphoglyceric acid (3-PGA) which involved in the production of reduction power (NADPH) in the cytosol (Jacob *et al.*, 1989; Bustosa *et al.*, 2008). The phosphorylating GAPDH is the key enzyme to the synthesis of energy by glycolysis since the diphosphoacid is at the origin of the two ATP synthesized by phosphoglycerate kinase (PGK) and pyruvate kinase (PK), while the non-phosphorylating GAPDH does not lead to 1,3-PGA but gives 3-PGA directly, thus avoiding the functioning of the phosphoglycerate kinase synthesizing ATP (Jacob *et al.*, 1989) (Figure 24). In rubber biosynthesis, there are the phosphoenolpyruvic crossroads whose functioning directs glycolysis either towards the production of oxaloacetic acid (OAA) and then malate and other acids in the Krebs cycle, or towards the production of pyruvate and then acetate which is a precursor of isoprene. According to the energy needed for latex production, NADPH and ATP are essential to *cis*-polyisoprene synthesis (Jacob *et al.*, 1989) (Figure 25).

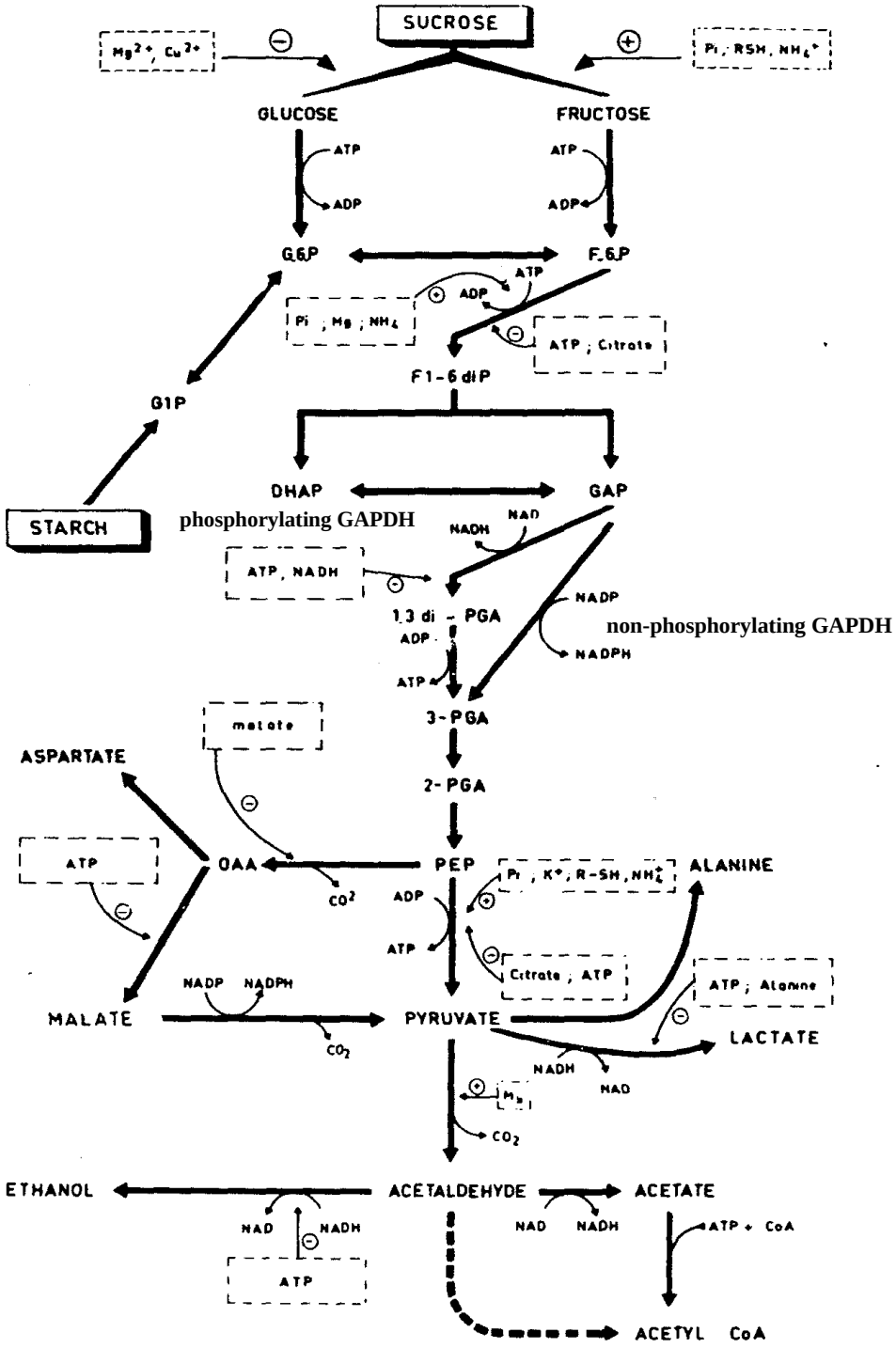


Figure 24. Glycolysis and some associated activities in *Hevea brasiliensis* latex. The effectors likely to affect the functioning of the reaction positively (+) or negatively (-) are shown in boxes (Jacob *et al.*, 1989).

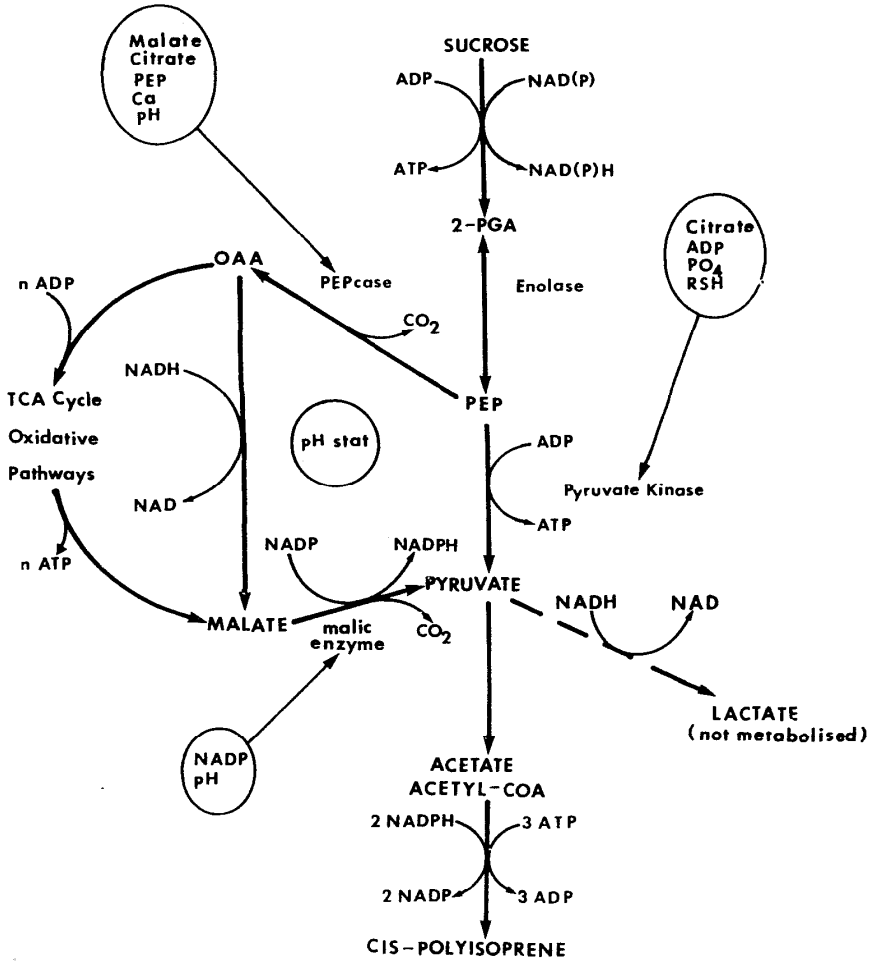


Figure 25. The phosphoenolpyruvic crossroads and its regulation in rubber tree latex (Jacob *et al.*, 1989).

In Arabidopsis, the cytosolic *glyceraldehyde-3-phosphate dehydrogenase* transcript level was increased when the plant was exposed to heat-shock, anaerobiosis, and increased sucrose supply conditions (Yang *et al.*, 1993). In filamentous fungi, *Drosophila* and *C. elegans*, *glyceraldehyde-3-phosphate dehydrogenase* is considered to be a constitutively expressed gene (Sun *et al.*, 1988; Huang *et al.*, 1989; Puyesky *et al.*, 1997).

In this study, the real-time PCR analysis showed that phosphorylating *GAPDH* was transiently down-regulated during 4 to 8 h after ethylene treatment then recovered to the basal level of expression from 16 to 40 h after treatment especially in the virgin tree (Figure 22). On the contrary, non-phosphorylating *GAPDH* was highly late down-regulated during 16 to 40 h after treatment both in virgin and tapped tree (Figure 23). The requirement of latex regeneration is increased after tapping especially after the ethylene treatment which benefits for more latex yield. According to latex regeneration, the energy (especially ATP) is required, thus the latex cells might try to produce more energy for whole cell components and *cis*-polyisoprene synthesis via the pathway which produces higher ATP. Based on this hypothesis, it implies that the latex cell favor the phosphorylating GAPDH than the non-phosphorylating GAPDH in order to obtain more ATP for latex production.

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Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Kongsawadworakul, P., Narangajavana, J., Chrestin, H. Effects of ethylene stimulation on differential genes expression in rubber tree (*in preparation*)

2. ผลงานอื่น ๆ

Kongsawadworakul, P., Viboonjun, U., Narangajavana, J., Chrestin, H. Studies on the physiological and molecular mechanisms involved in the onset of the physiological bark diseases and high rubber yield at the level of the *Hevea brasiliensis* latex producing tissues. The 1st Annual Seminar of Hevea Research Platform in Partnership (HRPP). Miracle Grand Hotel, Bangkok (2009).