การแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์เอธิลีนในต้นยางพารา

Expression of Genes Involved in Ethylene Biosynthesis in Rubber Tree (*Hevea brasiliensis*)

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บทคัดย่อ

การเพิ่มผลผลิตของน้ำยางทำได้โดยการทาสารเคมีเร่งน้ำยางที่บริเวณเปลือกใกล้รอยกรีดของต้นยางพารา อีเทรล (Ethrel®) หรือ อีทีฟอน (ethephon) เป็นสารเคมีเร่งน้ำยางซึ่งสามารถปลดปล่อยเอธิลีนและทำให้เกิดการเปลี่ยนแปลงทั้งทางด้านสรีรวิทยาและกระบวนการเมแทบอลิซึมภายในเซลล์ท่อน้ำยาง อย่างไรก็ตามกลไกการทำงานที่ระดับอณูชีววิทยาของเอธิลีนภายในต้นยางพารายังไม่เป็นที่ทราบแน่ชัด ในงานวิจัยนี้จึงทำการศึกษาผลของเอธิลีนต่อการแสดงออกของยีนในกระบวนการสังเคราะห์เอธิลีน ได้แก่ *1-aminocyclopropane-1-carboxylic acid* (*ACC*) *synthase* (*ACS*) และ *ACC oxidases* (*ACO1*, *ACO2*, *ACO3*) จากผลการทดลองพบว่าเมื่อถูกกระตุ้นด้วยเอธิลีน ยีน *ACS* และ *ACO* ทุก isoform มีการแสดงออกเพิ่มขึ้นเฉพาะในเปลือกลำต้น แต่มีการแสดงออกน้อยในน้ำยางทั้งก่อนและหลังถูกกระตุ้นด้วยเอธิลีน แสดงให้เห็นว่ากระบวนการสังเคราะห์เอธิลีนในต้นยางพาราเกิดขึ้นที่บริเวณเปลือกลำต้นมากกว่าในท่อน้ำยาง โดยการกระตุ้นด้วยเอธิลีนจากภายนอกอาจสามารถเหนี่ยวนำให้เกิดการสังเคราะห์เอธิลีนซึ่งมีความสำคัญต่อกระบวนการพัฒนารวมทั้งผลผลิตน้ำยางของต้นยางพารา ทั้งนี้ควรจะมีการศึกษาการทำงานของเอนไซม์ ACS และ ACO เพื่อยืนยันสมมติฐานดังกล่าวต่อไป

คำสำคัญ : ผลผลิตน้ำยาง ยางพารา เอธิลีน *ACC oxidase*, *ACC synthase*, *Hevea brasiliensis*

ABSTRACT

Rubber tree latex yield can be improved through bark stimulation with ethylene. Ethrel® or ethephon, an ethylene releaser, induces marked changes in the physiology and metabolism of the latex cells. However, the molecular mechanisms of ethylene action on rubber tree are poorly understood. In this study, the expression of genes involved in ethylene biosynthesis, *1-aminocyclopropane-1-carboxylic acid* (*ACC*) *synthase* (*ACS*) and *ACC oxidases* (*ACO1, ACO2,* and *ACO3*), were studied in response to ethylene stimulation using quantitative real-time PCR. Our results showed that *ACS* and all 3 *ACO* isoforms were transiently up-regulated by ethylene in the bark tissues only. Their expressions were greatly higher in the inner bark tissue than in the latex, suggesting that ethylene biosynthesis tended to take place in the inner bark tissues only but not in the laticifers. The induction of these genes involved in endogenous ethylene production in bark of rubber tree by exogenous ethylene treatment

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confirmed the autocatalytic activity which may participate in the regulation of a variety of developmental processes and also latex production/regeneration. This hypothesis has to be verified through the study of the ACS and ACO enzymes activity.

Keywords : Latex yield, Rubber tree, Ethylene, *ACC oxidase*, *ACC synthase*, *Hevea brasiliensis*

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INTRODUCTION

Natural rubber produced by rubber tree (*Hevea brasiliensis*) has superior physical properties compared with synthetic rubber. It is an important raw material for rubber products which especially require the elastic character of this material. Therefore, several tapping systems and stimulants are used to improve production and maximize profits by adopting different tapping frequencies, cut lengths, stimulation frequencies and stimulant concentrations.

Nowadays, Ethrel® (ethephon: 2-chloroethylphosphonic acid) and ethylene gas are commonly used to increase rubber yield in numerous rubber plantations worldwide. Many studies have been performed at the latex biochemical and physiological level, showing that ethylene stimulation increases the production of latex through increase in the latex flow and regeneration processes (Coupé and Chrestin, 1989). But in-depth, molecular mechanisms of exogenous ethylene action on rubber tree are still poorly understood. Few data have been reported about the ethylene effect on genes expression in the latex and bark. Most of them depict genes that up-regulated in the latex after bark Ethrel® treatment (Pujade-Renaud *et al*., 1994; Kongsawadworakul *et al*., 1997; Sookmark *et al*., 2005; Hong *et al*., 2005). Few data have been published about the expression of genes involved in ethylene biosynthesis in the mature rubber tree (Kongsawadworakul *et al*., 2004; Kuswanhadi *et al*., 2007). Recently, the relative transcript abundance of genes involved in ethylene signaling was studied in the bark of 3-month-old epicormic shoots (Duan *et al*., 2010). In plant, ethylene is synthesized in two steps from adenosyl-S-methionine (SAM). ACC synthase (ACS) catalyses the first reaction step to produce 1-aminocyclopropane-1-carboxylic acid (ACC). Then ACC is converted into ethylene, cyanide and CO_2 through the action of ACC oxidase (ACO) (Yang and Hoffmann, 1984). Therefore, the relation of exogenous ethylene stimulation in an increase of endogenous ethylene inducing increase in latex yield needs to be further elucidated.

In this study, the relative expressions of *ACS* and three isoforms of *ACO* genes in bark and latex in response to Ethrel® stimulation were studied by quantitative real-time polymerase chain reaction (qRT-PCR). The increasing of endogenous ethylene production in rubber tree by exogenous ethylene treatment confirmed the autocatalytic activity which may participate in the regulation of a variety of developmental processes and also latex production and regeneration.

MATERIALS AND METHODS

Plant materials

The rubber tree samples were collected from the PB217 clone, in the Bongo/SAPH (Côte d'Ivoire) plantation. The samples were collected from mature 2-year-old half spiral-tapped trees of the PB217 clone, which had been selected for their medium homogenous growth and yield. The trees were kept unstimulated for about 4 months and left untapped for 10 days before treatments and samplings. Seven batches of 3 trees were set up: two as a control (unstimulated) and 5 others were treated on a 1 cm wide lightly scrapped 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.

Total RNA extraction from *Hevea* inner soft bark and latex

Latex collection and total RNA extraction were performed according to the method adapted from Pujade-Renaud *et al*. (1997). The bark total RNA was extracted according to the cesium chloride cushion method, adapted from Sambrook *et al*. (1989), as described by Kongsawadworakul *et al*. (2004). The quantity and purity of RNA were measured by UV spectrophotometry. The integrity of the RNA was checked by agarose gel electrophoresis.

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Preparation of DNA-free RNA

To ensure that the products obtained from RT-PCR are originated from the RNA template, the RNA sample used in the first strand cDNA synthesis reaction needs to be free from DNA contamination by using DNA-free™ kit (Ambion). Obtained RNA was incubated at 37°C for 30 min in the presence of 2 units DNase I, which digested single- and double-stranded DNA to oligodeoxyribonucleotides. The reaction was terminated by incubation with 0.1 volume of DNase I inactivation reagent at room temperature for 2 min. The mixture tube was centrifuged at 10,000 g for 1.5 min and the RNA supernatant was transferred to a fresh tube. The resulting RNA sample could be used as template in the first strand cDNA synthesis reaction.

First strand cDNA synthesis

First strand cDNA synthesis reaction was performed using Superscript™ III reverse transcriptase (Invitrogen). First, the reaction mixture containing 2 µg of total RNA, 2.5 mM oligo(dT)$_{20}$, and 0.5 mM dNTP was incubated at 65°C for 5 min, and immediately cooled on ice for 5 min. After the addition of 200 units SuperScript™ III reverse transcriptase and 40 units RNase™Out Recombinant Ribonuclease Inhibitor in 1X reverse transcriptase buffer, the reaction mixture was incubated at 50°C for 50 min. The enzyme was inactivated by heating the reaction at 70°C for 15 min.

Quantitative real-time PCR analysis

To amplify cDNA fragments, 20 µl reaction was prepared as follows: 2 µl of first strand cDNA (1:40 dilution), 0.2 mM dNTP, 0.4 µM each gene specific primer, 2 mM $MgCl_2$, 0.8 units Platinum *Taq* DNA polymerase (Invitrogen) and a 1:1000 dilution of SYBR Green I (Sigma) in 1X PCR buffer. PCR reactions were performed as follows: initial denaturation at 95°C for 15 sec, annealing and polymerization at 60-62°C, depending on primer pairs, for 1 min using an ABI-7500 real-time PCR machine. The relative expression of interested genes with the *40S ribosomal protein* or *actin* as internal control was calculated as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{stimulated} - Ct_{internal\ control}) - (Ct_{unstimulated} - Ct_{internal\ control})$.

RESULTS AND DISCUSSION

The analysis of *Hevea* ESTs and full-length cDNAs encoding *ACC synthase* (*ACS*) and *ACC oxidase* genes (*ACO*) reported in GenBank database showed that, in rubber tree, there were one and three isoforms of *ACS* and *ACO* genes, respectively. Among these three *ACO* isoforms, they shared 66-87% homology at the level of amino acid sequence. Therefore, the specific primer pairs of each *ACO* isoform were designed from their low homologous regions and used for the expression analysis by quantitative real-time PCR.

In bark, the expression of the *ACS* gene was transiently up-regulated by ethylene as soon as 4 hours after treatment and reached the maximum level at 24 hours (Figure 1A). As same as *ACS* gene, 3 isoforms of *ACO* genes (*ACO1*, *ACO2*, *ACO3*) were also transiently up-regulated by ethylene (Figure 1B-1D) and reached the maximum level at 16 hours after stimulation. However, the expression levels of each isoform were different by which *ACO1* was the highest expressed isoform.

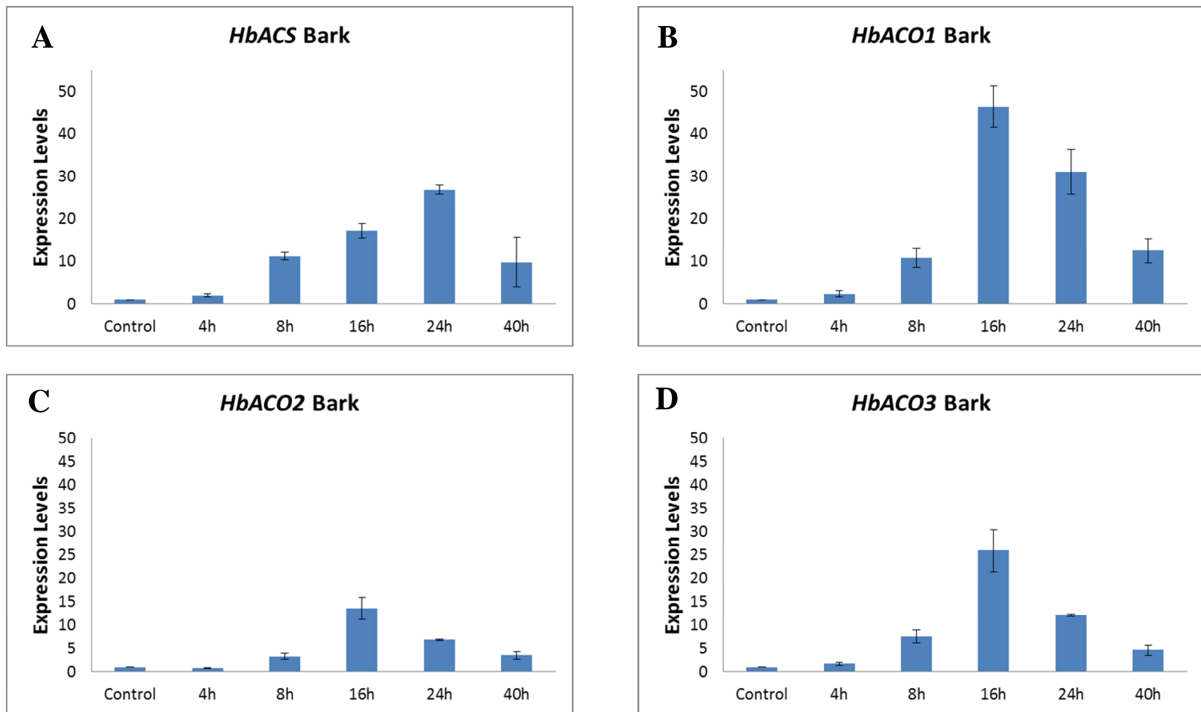

Figure 1. Expression of the *ACC synthase* (*ACS*) and three isoforms of *ACC oxidase* genes (*ACO1*, *ACO2*, *ACO3*) in the inner bark of tapped tree in response to bark treatment with EthrelÒ at 4, 8, 16, 24, and 40 hours before bark sampling. Untreated trees were used as control.

In latex, the transcripts of *ACS*, *ACO1*, *ACO2*, and *ACO3* were very slightly expressed and no effect of ethylene stimulation was detected (Figure 2A-2D).

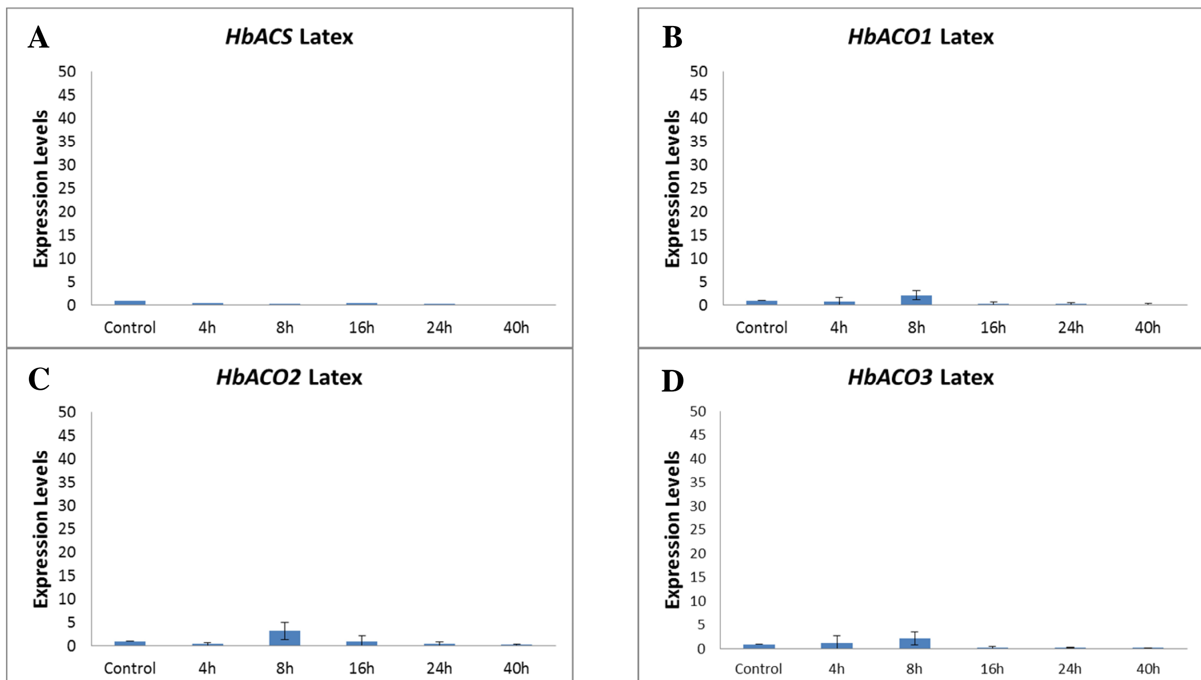

Figure 2. Expression of the *ACC synthase* (*ACS*) and three isoforms of *ACC oxidase* genes (*ACO1*, *ACO2*, *ACO3*) in latex of tapped tree in response to bark treatment with EthrelÒ at 4, 8, 16, 24, and 40 hours before latex sampling. Untreated trees were used as control.

In latex, there was very low or no detectable expression of the *ACS* and *ACO* genes both before and after ethylene stimulation. This confirmed the preliminary results reported no expression of *Hevea ACO2* in the latex (Kongsawadworakul *et al*., 2004). This further indicated that the fully differentiated latex cells may not express any of these genes of the *ACS* and *ACO* families, therefore they themselves may not be able to synthesize endogenous ethylene and thus be unable to emit any stress signal under the form of ethylene.

In the vicinity of the treated area, it is worth notice the very strong but transient induction by ethylene of the *ACS* and *ACO* genes expression, maximum at 24 and 16 hours after the treatment, respectively. This should lead, as previously reported by Kongsawadworakul *et al.* (2004), to a progressive longer term ethylene synthesis in the soft bark. The induction time of these genes in rubber tree bark (< 8 hours) is comparable to the ones reported (7 hours) when treating peach fruit with exogenous ethylene (Gallahan *et al*., 1993). It is most probable that, as in the tomato fruit maturation processes (Alexander and Grierson, 2002), exogenous ethylene promotes endogenous ethylene synthesis through an auto-activating mechanism, including *ACO* and *ACS* genes induction. This hypothesis should be verified through the study of the ACS and ACO enzymes activity and/or measurements of ethylene release by the bark samples.

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