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Isolation of Three Members of the Multigene Family Encoding ACC Oxidases in Hevea brasiliensis *and Investigation of their Responses to Ethylene Stimulation and Wounding*

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Ethylene is a major stimulating factor for natural rubber production in Hevea brasiliensis *and is often applied in the form of Ethephon, an ethylene releaser. In a positive feedback type mechanism, the application of ethylene leads to the enhanced expression of genes involved in ethylene biosynthesis such as ACC oxidases, which are also responsive to wounding. We studied the regulation of ethylene biosynthesis in rubber trees through the characterisation of the ACC oxidase multigene family and the responses of individual genes to ethylene and wounding. Three full-length cDNAs,* HbACO1*,* HbACO2 *and* HbACO3*, were isolated. The* HbACOs *were 1115, 1174 and 1074 bp long, respectively, with open reading frames encoding polypeptides of 312, 318 and 318 amino acids, respectively. The genomic sequences of* HbACO1 *and* HbACO2 *were also characterised, revealing divergent gene structures:* HbACO1 *(1456 bp) possesses three introns and four exons, while* HbACO2 *(1418 bp) consists of two introns and three exons. All of the* HbACO *genes were expressed at all stages of development studied, from in vitro callus to the exploited plant, but with different expression profiles. Nevertheless, no expression was detected in latex. In the bark of the shoot of the first flush of budded plants,* HbACO1 *was expressed at a higher level than the other genes, and was down-regulated by ethylene and wounding in both leaf and bark tissues. In contrast,* HbACO2 *and* HbACO3 *were transiently induced in response to ethylene and wounding. Treatment with 1-MCP, an ethylene action inhibitor, abolished the ethylene induction of* HbACO2 *gene, showing that this gene was under positive feedback regulation. The expression of* HbACO3 *gene was also induced by ethylene treatment and wounding, again with an antagonistic effect of 1-MCP. In conclusion,* HbACO1 *appears to be responsible for basal levels of ethylene production while* HbACO2 *and* HbACO3 *are up-regulated in response to external factors.*

Keywords: *Hevea brasiliensis*; ACC oxidase; 1-methylcyclopropene; ethephon; ethylene; gene expression; latex cell; RT-PCR; wounding

Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; ACO: 1-aminocyclopropane-1-carboxylic acid oxidase; AOA: amino oxyacetic acid; 1-MCP: 1-methylcyclopropene.

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Ethylene is a simple hydrocarbon gas which acts as a plant growth regulator. Ethylene plays a pleiotropic role in plant growth and development processes, such as fruit ripening, leaf abscission, stress and pathogen responses[1]. One of its most economically important roles is in the regulation of natural rubber production[2].

Hevea brasiliensis is the main source of natural rubber and accounts for about 33% of total rubber production (synthetic and natural). Rubber biosynthesis takes place in latex cells. These cells are differentiated from the cambium in soft bark. After anastomoses of latex cells, they form an articulated network, called laticifer mantels, which are independent but located in the phloem vascular system[3]. Ethephon, an ethylene generator, is applied to the rubber tree bark on the tapping panel, to increase rubber yield by stimulating latex flow and regeneration between two tappings[2].

Ethephon is a stimulator of many activities associated with latex cells. Among the changes induced by ethephon are increases in RNA, invertase activity, sucrose content, latex stability and a decrease in starch content have been known for many years[4,5]. More recently, ethephon application was shown to trigger the expression of numerous genes[6,8]. Ethephon application makes it possible to reduce bark consumption and tapping frequencies. However, it is necessary to have a good command of stimulation frequencies and of the ethephon concentrations to be used during tree exploitation, so as not to trigger a physiological disorder that might lead to the appearance of tapping panel dryness and production losses.

Although ethylene biosynthesis, perception, transduction and responses have been amply described in model plant species, the biochemical and molecular characterisation of the genes involved in ethylene biosynthesis[9,10] and their regulation remains difficult for rubber trees due to problems with setting up an experimental system. Ethephon application provokes endogenous ethylene production[11]. The existence of 1-aminocyclopropane-1-carboxylic acid (ACC) in the bark suggests that such biosynthesis occurs in tissues of the bark, but not only in latex cells[12].

The ethylene biosynthesis pathway is well characterised in many plant species. 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), which converts *S*-adenosyl-methionin to ACC, is the first committed and generally rate-limiting step in ethylene biosynthesis[13–15]. The level of ACS activity closely parallels the level of ethylene production in most plant tissues[13,16,17]. ACC is converted to ethylene by the enzyme ACC oxidase, the production of which is also highly regulated. These two enzymes are encoded by a multigene family and their expression regulated differentially by various developmental, environmental and hormonal signals[18–20].

The characterisation of genes encoding ACC oxidase began in 1987, when Holdsworth published the first ACO cDNA isolated from tomato fruit. Transcript levels of this gene were found to increase in response to exogenous ethylene and wounding[21]. Recently, many genes encoding ACC oxidase were isolated from monocotyledon and dicotyledon species[22–25]. ACC oxidase is encoded by a small gene family, which is highly conserved throughout the protein coding region but shows a degree of sequence divergence within the 3' untranslated regions[26–27]. It has been reported that there are five *ACO* genes in tomato[26,28], and three in sunflower[29], melon[30] and tobacco[31–32]. The expression of genes encoding ACC oxidases in higher plants is regulated developmentally and environmentally. They are expressed either in specific organs or tissues such as in the hypocotyl, leaf, root, petiole, flower,

petal, pistil, stamen and fruit or in response to various biotic and abiotic stimuli such as wounding, low O_2, high CO_2, flooding, plant growth substances, chilling or pathogens[33]. Although individual members of the *ACO* gene family are able to respond to numerous different developmental and environmental signals[26,29,34], a single developmental cue or environmental factor may also induce the co-ordinated expression of several *ACO* genes[26,35,36].

Isolate genes encoding ACC oxidase and their spatio-temporal expression in *Hevea brasiliensis* were studied. The isolation of three members of the multigene family encoding *ACO* and their expression in response to ethylene stimulation and wounding during plant development was reported. Spatial analysis of expression was also performed using mRNA *in situ* hybridisation on the bark tissues of plantlets.

MATERIALS AND METHODS

Plant Material

RNAs were extracted from several plant tissues of clone PB 260 at various stages of development. Embryogenic callus and *in vitro* plantlets were produced according to the procedure developed by Carron[37]. Young budded plants were grown for three months in the greenhouse up to development of the first growth unit. Untapped and tapped trees were 5 years old and grown at the Sembawa Centre (Rubber Research Institute, Palembang, Indonesia).

Ethephon, ethylene, 1-methyl cyclopropane and wounding treatments

Plant material was treated either with 2.5% ethephon, 1 p.p.m. ethylene or by wounding (scarification of bark with a blade, or squashing leaves with forks). As treatment with 2.5% ethephon releases about 1 p.p.m. of ethylene after 24 h, that quantity was applied in an airtight culture container. The effects of ethephon, ethylene and wounding were studied at various times after treatments: 0, 1 h, 4 h, 8 h, 24 h, 48 h, 96 h and 168 hours for plants treated with ethephon and ethylene, and 0, 15 min, 1 h, 4 h, 8 h and 24 hours for wounding. To demonstrate the specific effect of ethylene, an inhibitor of its receptor, 1-MCP (1-methyl cyclopropane), was applied at 1 or 5 p.p.m. for 16 hours before treating with 1 p.p.m. of ethylene for 8 hours. Plants were placed in an airtight Plexiglass box (1.2 m × 0.5 m × 0.5 m) one day prior to treatment with 1-MCP or ethylene. Ethylene concentration was measured at the beginning and at the end of treatments. After 24 h of treatment, the ethylene concentration was adjusted to 1 p.p.m. each day. Ethylene content was measured by gas chromatography (Type HP 5280 with FID detector).

DNA Extraction

One gram of leaf tissue was ground to a fine powder in a mortar containing liquid nitrogen. The powder was transferred to a tube containing 5 mL of extraction buffer composed of 2% MATAB, 100 mM Tris-HCl pH8, 1.4 M NaCl, 20 mM EDTA pH8, 1% PEG 6000, and 0.5% of sodium sulphide. After incubation at 74ºC for 30 min, 5 mL chloroform isoamyl alcohol were added and mixed before centrifugation at 6000 g at 20ºC for 15 minutes. The supernatant was transferred to a new tube containing 0.7 volume of isopropanol and mixed. The DNA pellet was collected and transferred to a new tube containing 300 µl Tris-EDTA pH 8.

Southern-blot Hybridisation

Ten micrograms of genomic DNA were digested with restriction enzymes and

fractionated by electrophoresis in 0.8% (w/v) agarose gels. The nucleic acids were transferred to Hybond N+ nylon membrane (Amersham). To identify all genomic fragments related to *HbACO*, the conserved coding region of *HbACO* cDNAs was selected as a probe. Hybridisation was performed under conditions specified in Sambrook[38,39]. The membranes were washed 3 times with 1x SSC and 0.1% SDS. Autoradiograms were exposed at –80ºC for 7 days.

RNA Extraction

For each replicate, one gram of plant tissue was ground to a fine powder in a mortar containing liquid nitrogen. The powder was transferred to a tube containing 30 mL of extraction buffer consisting of 4 M guanidium isothiocyanate, 1% sarcosine, 1% PVP and 1% ß-mercapto-ethanol. After mixing, the tubes were kept on ice and then centrifuged at 10 000 r.p.m. at 4ºC for 30 minutes. The supernatant was transferred to a new tube containing 8 mL of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 25 000 r.p.m. at 20ºC for 20 hours. The supernatant and cesium cushion were discarded and the RNA pellet was washed with 70% ethanol. After 30 min of air drying, the pellet was resuspended in 200 µL of sterile water. RNAs were conserved at –80ºC.

Isolation of Partial *HbACO* Sequences

Several pairs of degenerated primers were designed based on the conserved amino acid region of about 100 ACC oxidase from various plant species. PCR amplifications were performed on reverse transcripts of total RNA isolated from various tissues at different stages of development before or after treatment with 1 p.p.m. ethylene or 2.5% ethephon. PCR products were cloned in pGEM-T easy vector and then sequenced. Sequences were compared to NCBI databases by using BLASTn and BLASTx (*http://www.ncbi.nlm.nih.gov/BLAST/*). Three groups of sequences were identified (H4, H5, O48). One representative fragment was selected for each group. The following degenerated pairs of primers were those that amplified the representative clones H5, H4 and O48: ACO-D1-F/ACO-D3b, ACO-D1-F/ACO-AS and ACO-D1-F/ACO-D3-R (*Table 1*). Specific primers were then designed for each representative *Hevea* sequence: ACO-H4-S/ ACO-H4-R, ACO-H5-S/ ACO-H5-R and ACO-O48-S/ ACO-O48-R in order to synthesise probes for screening of a phagemid cDNA library (*Table 1*).

Screening of Phagemid cDNA Library

A cDNA library from bark tissues was screened using radiolabelled *ACO* partial fragments (Lamda-TriplEx, Clontech, USA). Phagemid clones (lysis plaques) were transferred to Hybond N^+ nylon membrane and hybridised with the ^{32}P labelled probes (Megaprime™ DNA Labelling systems, Amersham Bioscience). Hybridisation was carried out according to the procedure established in Sambrook[38,39]. Five hybridised clones were selected by a PCR test using previous specific primers and diluted for a second round of screening. Of the hybridised clones, ten were sequenced and one was identified as a full-length cDNA encoding ACC oxidase: that clone was called *HbACO-H5*.

RACE-PCR

For the remaining two partial sequences (H4 and O48), cDNA end extension was carried out using the SMART™ rapid amplification of cDNA ends (RACE) kit (Clontech, USA). The cDNA was synthesised by reverse transcription from 1 µg total RNA

TABLE 1. DEGENERATED PRIMER SEQUENCES FOR THE CLONING OF PARTIAL ACC OXIDASE cDNA, AND SPECIFIC PRIMER SEQUENCES FOR AMPLIFYING THE FRAGMENT USED FOR PROBE IN cDNA LIBRARY SCREENING, FOR CLONING OF H4 AND O48 ACC OXIDASE CLONES, FOR AMPLIFYING BOTH cDNA AND GENOMIC FULL LENGTH SEQUENCES, RESPECTIVELY

Name of primers	Primer sequence
ACO-S	GATGCNTGYSARAAYTGNGGNTT
ACO-AS	TTCATNGCYTCRAANCKNGGCTC
ACO-D1-F1	TGYGARAAYTGGGGNTTYTTYG
ACO-D1-F2	RAYGYNTGYGARAAYTGGGGNTT
ACO-D2a-F	GGNCTNCGNGCNCAYWSNGAYGC
ACO-D2b-F	GCNCAYWSNGAYGCNGGNGG
ACO-D3b-R	TCRAANCGNGGYTCYTTNGC
ACO-H4-S	TCATTGCTTCGAACCGGGGC
ACO-H4-R	AAAGCACCTTCTTCCTCCGCCACC
ACO-H5-S	TTCATGGCTTCGAAACGGGG
ACO-H5-R	GGGCGAGAATCTTGGGTTGG
ACO-O48-S	GGAAGGTGATGAAGGAGTTTG
ACO-O48-R	TGCATCACTTCCAGGGTTGT
H4-R1-69	CAACTGTGTCCATGAACTCTGGCTCT
H4-R2-98	TTTCCTGTAGTGACCCTTTGTCATCC
H4-R3-181	TCCCAATCCATATCTTTGATCTCAGT
H4-R4-330	CAAGATTCTCACACAACAGGTCTAAG
H4-S1-283	TGGAGAAACTGGCCGAGGAGCTCTTA
H4-S2-724	AAGAAGCAGAGGAGAAGAAGCAAGTC
H4-S3-799	AATTCCAGCCCAAGGAGCCCCGGTTC
O48-R1-63	ACAGGTCCAGAAGCTCCTCTGCAAGC
O48-R2-78	CAAGATTTTCACATAACAGGTCCAGA
O48-R3-557	CAAAGAACCACGCTTTGAAGCACTAG
O48-S1-483	CAGAAGAAGCTGGTGTACCCAAAATT
O48-S2-121	GGGGTCCAACCTTTGGGACCAAGGTG
H4-ext-5’	ACGCGGGGGTCTCAACAAGCACAA
H4-ext-3’-R1	TTAGAAGTAAAACACATATT
H5-ext-5’	CAGAGACAAGTTTAATTTAG
H5-ext-3’	TTAGACCAAACTTTCGGCAT
O48-ext-5’	ACGCGGGGAACAAACACAAGGCA
O48-ext-3’	TTGATAATATTAAACAACAC

from both 5’ and 3’ ends respectively with 5’-CDS primer A and 3 -CDS primer A provided by the manufacturer. Then, cDNA ends were amplified with Universal Primer A and internal primers designed in the specific *HbACO* sequences (*Table 1*):

- for 3’ ends: H4-S1-283-3’RACE (H4 clone) and O48-S2-121-3’RACE (O48 clone)
- for 5’ ends: H4-R2-98-5’RACE (H4 clone) and O48-R1-65-5’RACE.

PCR reactions were performed with the following programme: 5 amplification cycles (94ºC for 30 s, 72ºC for 3 min), 5 amplification cycles (94ºC for 30 s, 70ºC for 30 s, 72ºC for 2 min), and 40 amplification cycles (94ºC for 30 s, 68ºC for 30 s, 72ºC for 2 min). PCR

products were cloned into pGEM-T-Easy vector (Invitrogen) and then sequenced. Full-length cDNA sequences of *HbACOs* were constructed in silico and specific primers for each *ACO* gene were designed at the ends of cDNA sequences (*Table 1*). PCR amplification of full-length cDNA sequences was carried out with high-performance Taq enzyme (BD Clontech Bioscience) with three replicates. The PCR programme was: 94ºC for 3 min, then 30 amplification cycles (94ºC for 30s, 55ºC for 1 min, 72ºC for 2 min). PCR products were cloned into pGEM-T-Easy vector and sequenced from both ends.

Semi-quantitative RT-PCR

cDNAs were synthesised from 2 µg of total RNA with M-MLV reverse transcriptase using oligo(dT)18 primer (Fermentas). Specific primers for each *HbACO* genes were designed according to their full-length sequences for an expected length of PCR product of about 400-600 bp (*Table 2*). The specificity of primers was checked by crossing PCR reactions for each primer pair and the three sources of *HbACO* genes. *HbActin* was used as an internal control because of its relatively stable constitutive expression irrespective of the treatment. Actin primers were designed for an expected PCR product of 260 bp. For all reactions, the PCR programme was as follows: 94ºC for 3 min, 19–29 cycles of (94ºC for 30s, 55ºC for 1 min and 72ºC for 1 min), 72ºC for 7 min. The number of PCR cycles was adjusted so as to obtain visible bands on the gel in non-saturating amplification conditions. The number of PCR cycles was 28 for the amplification of H4 and H5 sequences in both bark and leaf and 26 for the Actin internal control. For O48 sequence, 19 and 21 cycles were used respectively for leaf and bark cDNA samples with 23 cycles for the actin internal control. Band intensity was analysed by ImageQuant software (Molecular Dynamics).

RESULTS

Isolation of Full-length *HbACO* Sequences

Three full-length cDNAs encoding *HbACO* were isolated from various DNA templates (bark phagemid cDNA library, genomic DNA and cDNA from leaves, callus and bark of young plants subjected or not to ethephon treatment for 24 h). First, three partial cDNA sequences were isolated by PCR amplification of cDNA from non-stimulated bark (clone H5), RNA from ethephon-stimulated bark (clone H4) and from genomic DNA (clone O48). One full-length cDNA was directly obtained by screening a bark cDNA library with the H5 radiolabelled partial sequence as a probe (*HbACO1*, EMBL accession AM743170). The sequenced cDNA was 1115 bp long with an open reading frame of 936 bp corresponding to a polypeptide of 312 amino acids. For the other two partial sequences (H4 and O48), extension of 5' and 3' ends was carried out by Rapid Amplification of cDNA Ends (RACE) using single stranded cDNA obtained from total bark RNA. Two full-length cDNAs were isolated after amplification using primers designed at the ends of extended fragments from the same single stranded cDNA source. These cDNAs were named *HbACO2* (EMBL accession AM743171) and *HbACO3* (EMBL accession AM743172) and were respectively 1174 bp and 1074 bp long, each with an open reading frame of 954 bp encoding 318 amino acids.

Structure of *HbACO* Genes

Southern hybridisation showed that the *HbACO* multigene family consisted of more than 3 members and potentially up to seven members if restriction enzymes did not cut into the *HbACO* genomic sequences (*Figure 1*). Using a probe located in the conserved coding sequence, and restriction by the *Eco*RI,

TABLE 2. PRIMER SEQUENCES USED FOR GENE EXPRESSION ANALYSES BY SEMI-QUANTITATIVE RT-PCR AND SIZE OF EXPECTED PCR PRODUCT

Name of primers	Primer sequences	Expected PCR product (bp)
H4-S5'ext	ACGCGGGGGTCTCAACAAGCACAA	510
H4-R330-5'RACE	CAAGATTCTCACACAACAGGTCTAAG	
H5-S692	GGCGACCAACTTGAGGTAAT	379
H5-R1071	TCAATCAGTAACTTCATCAT	
O48-S483-3'RACE	CAGAAGAAGCTGGTGTACCCAAAATT	524
O48-R1007	TTGATAATATTAAACAACAC	
Actin-S	TCCATAATGAAGTGTGATGT	260
Actin-R	GGACCTGACTCGTCATACTC	

*Eco*RV and *Sac*I enzymes, the variation in pattern of the seven bands in terms of size and intensity revealed polymorphisms between the three clones studied (RRIM 600, PB 260 and PB 217).

To analyse gene structure, two full-length genomic clones were isolated by PCR amplification from genomic DNA of rubber tree clone PB 260 using primers designed at the ends of each full-length cDNA. The *HbACO1* gene was 1456 bp long and contained 3 introns and 4 exons (EMBL accession AM743173; *Figure 2*). The *HbACO2* gene was 1418 bp long and consisted of 2 introns and 3 exons (EMBL accession AM743174; *Figure 2*). The intron/exon junction used the general GT/AG splicing site.

Features of the Deduced Protein Sequences

The alignment of deduced *HbACO* polypeptide sequences showed that they shared 80 to 90% of identical amino acids between them, and 80 to 100% with various other plant ACC oxidases (*Table 3*). Phylogenetic analysis of the *HbACO* proteins compared to other ACOs available in the SWISSPROT database showed that *HbACOs* could be classified into two different groups (*Figure 3*). *HbACO1* belonged to a group consisting of *ACO* from kiwi fruit, pea, apple and melon, whilst *HbACO2* and *HbACO3* belonged to another group consisting of *ACOs* from apricot, tomato and petunia.

Alignment of the amino acid sequences of *HbACOs* revealed conserved and functionally essential residues (*Figure 4*). The HD and HR motifs are involved in the interaction with Fe^{2+} and the RS motif with co-substrate. Some potential motifs of Leucine Zipper are also present in the sequences.

Expression of *HbACO* Genes during Plant Development and Response to Ethephon Stimulation

The three *HbACO* genes were found to be differentially expressed in various plant organs in response to ethephon (*Figure 5*). Semi-quantitative RT-PCR analyses were performed using the actin gene (*HbACT1*) as an internal control. Expression of Actin was relatively stable for each tissue and stage of development. For all *HbACO* genes, transcripts were not detected in callus and latex. Conversely, *HbACO* genes were expressed in leaf stems and roots of 3-month-old in vitro plantlets, in bark and leaves of

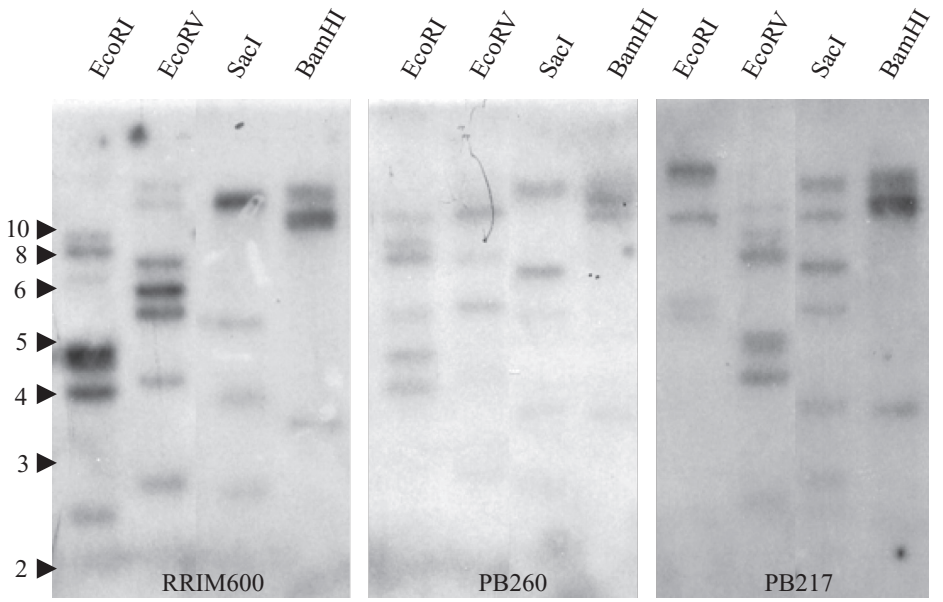

Figure 1. Southern-blot analysis of HbACO. *Genomic DNA of rubber tree clones RRIM 600, PB 260 and PB 217 was digested with* EcoRI, EcorV, SacI *and* BamHI *and fractionated by gel electrophoresis. Fragments were hybridized with a [32P]-labelled probe obtained after digestion of the full length cDNA* HbACO1.

CmACO1	105	103	227	107	334	125	291
CpACO1	105	128	227	151	334	434	291
LeACO1	105	278	227	106	334	89	282
LeACO2	105	334	227	825	334	398	285
LeACO3	105	225	227	106	334	346	285
MdACO1	105	236	227	177	334	218	279
MdACO2	105	205	227	171	334	219	327
MdACO3	105	236	227	177	334	217	279
PpACO1	105	118	227	117	334	78	294
MhACO2	105	87	224	90	337	69	288
HbACO1	**105**	124	**227**	98	**334**	119	**270**

CmACO2	102	166	221	206	580
MhACO1	102	96	224	91	592
HbACO2	**102**	92	**227**	143	**625**

Figure 2. Comparison of the Hevea brasiliensis HbACO1 *and* HbACO2 *genomic structures with* CmACO1 (X95551), CmACO2 (X95552), CpACO1 (AF379855), LeACO1 (X58273), LeACO2 (Y00478), LeACO3 (Z54199), MdACO1 (X98627), MdACO2 (AF015787), MdACO3 (AF030859), MhACO1 (AF030411), MhACO2 (U86045); PpACO1 (AF532976). *Numbers below box represent number of base pairs. Exon* ▭ *and Intron* ▭ .

TABLE 3. THE HOMOLOGY OF THE HbACOS WITH *ACO* FROM THE OTHER SPECIES

Gene	Homology with (aa)	Score (%)	Exp Value	Gaps
HbACO-H4	Hevea brasiliensis (AAP41850)	100	1e-169	0 % (0/318)
	Populus euramericana (BAA94601-1)	89	2e-152	0 % (1/319)
	Polargonum hortorum (AAC48977)	87	6e-149	0 % (1/316)
	Prunus armeniaca (AAC33524.1)	86	2e-148	0 % (0/316)
	Diospyros kaki (BAB89351.1)	86	2e-147	0 % (1/315)
	Prunus mume (BAA90550.1)	85	6e-147	0 % (0/316)
HbACO-H5	Gossypium hirsutum (AAZ83342.1)	87	2e-142	1 % (6/315)
	Carica papaya (AAC988808.1)	85	7e-142	0 % (0/311)
	Prunus persica (AAF36484.1)	84	1e-139	1 % (6/317)
	Malus domestica (AAA18566.1)	83	4e-139	2 % (7/318)
	Betula pendula (CAA71738.1)	81	8e-138	1 % (6/318)
	Actinidia deliciosa (BAA21541.1)	85	1e-137	0 % (1/306)
HbACO-O48	Hevea brasiliensis (AAP41850)	94	1e-158	0 % (0/318)
	Populus euramericana (BAA94601-1)	89	2e-149	0 % (1/319)
	Polargonum hortorum (AAC48977)	87	3e-145	0 % (1/316)
	Prunus armeniaca (AAC33524.1)	85	6e-144	0 % (0/316)
	Fagus sylvatica (CAD21844.1)	85	7e-143	0 % (0/316)
	Prunus mume (BAA90550.1)	84	1e-142	0 % (0/316)

3-month-old budded plants and in bark of trees. For *HbACO1*, a general reduction in expression was observed in response to ethephon and in relation to age, transcripts reaching an undetectable level in 5-year-old trees. For *HbACO2*, transcript accumulation was higher in ethephon-stimulated tissues than under control conditions. For *HbACO3*, expression was stimulated by ethephon application, particularly in leaves of budded plants and untapped trees but not in bark. The undetected level of transcripts in budded plants and tapped trees after 24 h of stimulation could be explained by the transient peak of expression of this between 4 h and 8 h as it is observed in untapped trees.

Expression of *HbACO* Genes in Response to Ethephon, Ethylene or Wounding

In order to achieve fine characterisation of the regulation of *HbACO* genes, analyses were carried out on the bark of 3-month-old shoot of budded plants. *HbACO* genes were differentially expressed in response to ethephon, ethylene or wounding treatments (*Figure 6*). The gene expression in budded plants treated either with 2.5% ethephon or 1 p.p.m. ethylene were analysed in kinetics from 1 to 168 hours. The expression of *HbACO1* was down-regulated in bark and leaf tissues, whereas *HbACO2* and *HbACO3* were up-regulated by ethephon and ethylene treatments. Gene expression patterns were similar in response to ethephon and ethylene, although ethylene action was faster.

For *HbACO1*, the level of transcripts decreased 1 h after ethephon or ethylene treatments to reach a minimum after 24 h. Then it increased slightly to its initial level before ethephon application, whereas it was still down-regulated when the ethylene treatment was maintained in the culture container. In the same way, *HbACO1* was dramatically down-regulated by wounding within 15 min of treatment.

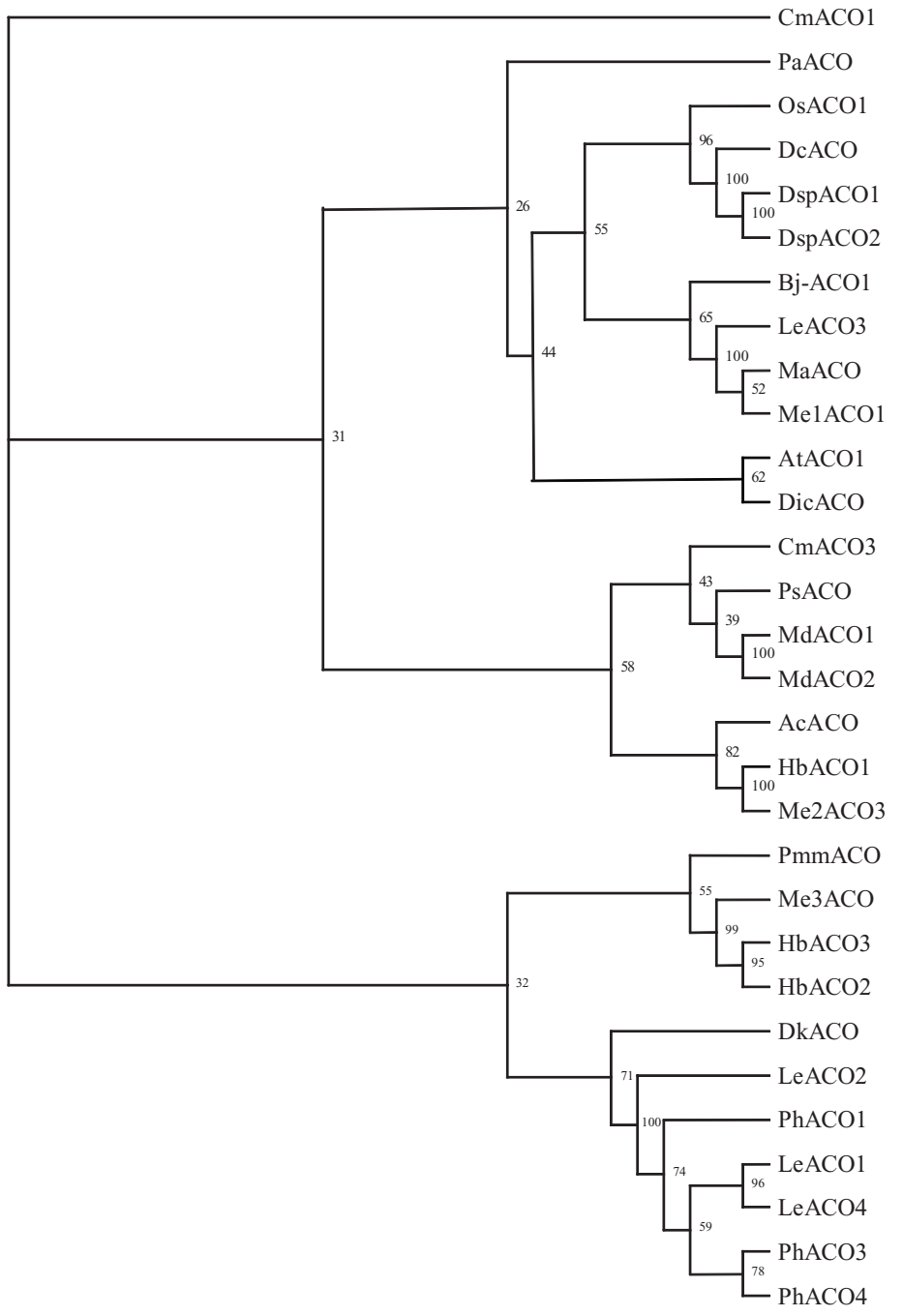

Figure 3. Phylogenetic analysis of the HbACO*-deduced proteins compared to other ACO available in the SWISSPROT database: AcACO (P31237), AtACO1 (Q06588), BjACO1 (Q09052), CmACO1 (Q04644), CmACO3 (P54847), DicACO (Q9ZQZ1), DcACO (P31528), DkACO (Q85932), DspACO1 (P31238), DspACO2 (Q39705), HbACO1(AM743170), HbACO2(AM743171), HbACO3(AM743172), LeACO1(P05116), LeACO2 (P07920), LeACO3 (P10967), LeACO4 (P24157), MaACO (Q9FR99), MdACO1 (Q00895), MdACO2 (O48882), Me1ACO2 (AAX84675), Me2ACO3 (ABK59094), Me3ACO(ABK58140), OsACO1 (Q40634), PaACO (P19464), PhACO1 (Q08506), PhACO3 (Q08507), PhACO4 (Q08508), PmmACO (Q9MB94), PsACO (P31239).*

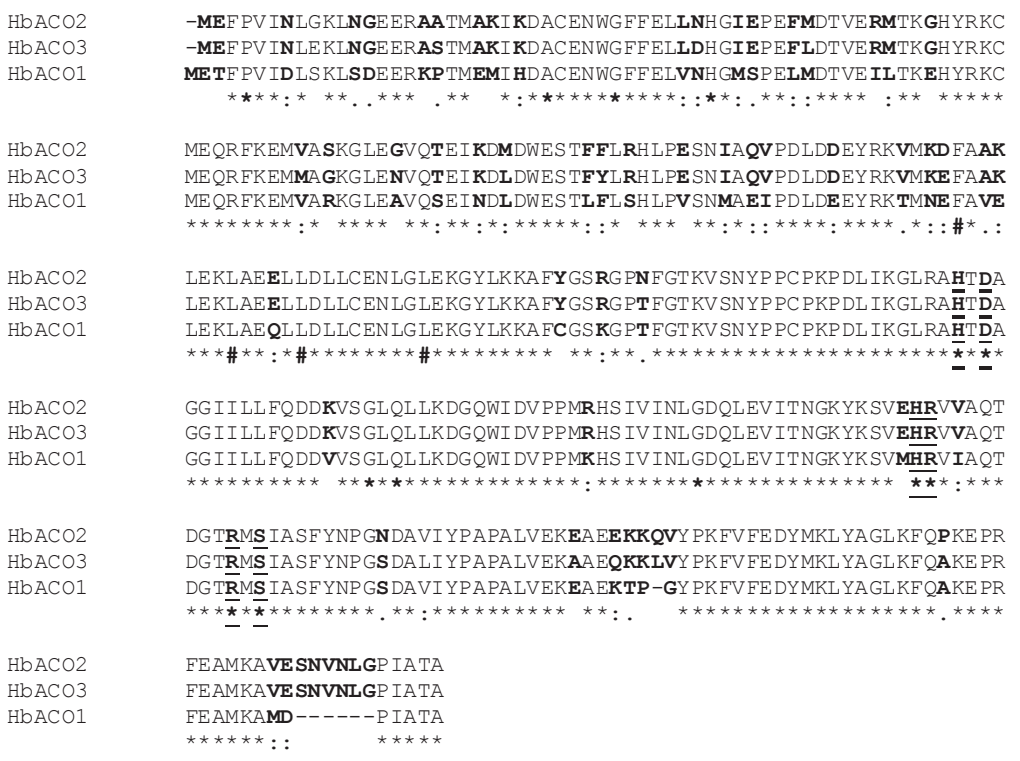

Figure 4. Alignment of the amino acid sequences of clone PB 260 HbACOs*. The HD and HR motifs involved in the interaction with* Fe^{2+} *are in bold and underlined. The RS motif for relation with co-substrate is also in bold and underlined. The potential motifs of leucine zipper are marked by #. The different residues among the sequences were marked in bold.*

For *HbACO2* and *HbACO3*, gene expression peaked between 8 to 24 h after ethephon application whereas the ethylene effect only took 4 h. The accumulation of *HbACO3* transcripts appeared much greater than that of *HbACO2* in view of the low number of PCR cycles required for the amplification of its transcripts. After wounding, *HbACO2* and *HbACO3* transcripts were not accumulated in bark, and only slightly in leaves.

Pre-treatment with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, before ethylene stimulation inhibited the ethylene induction of *HbACO* genes (*Figure7*). Expression of *HbACO2* declined in line with the 1-MCP concentration. However, the induction of *HbACO3* transcript accumulation in response to ethylene was slightly decreased when 1-MCP treatment was applied. This is likely to mean that 1-MCP acts differently on the expression of *HbACO3* compared with *HbACO2*.

DISCUSSION

Latex flow and regeneration between two tappings are activated by applying ethephon to the bark of tapped rubber trees. Ethylene

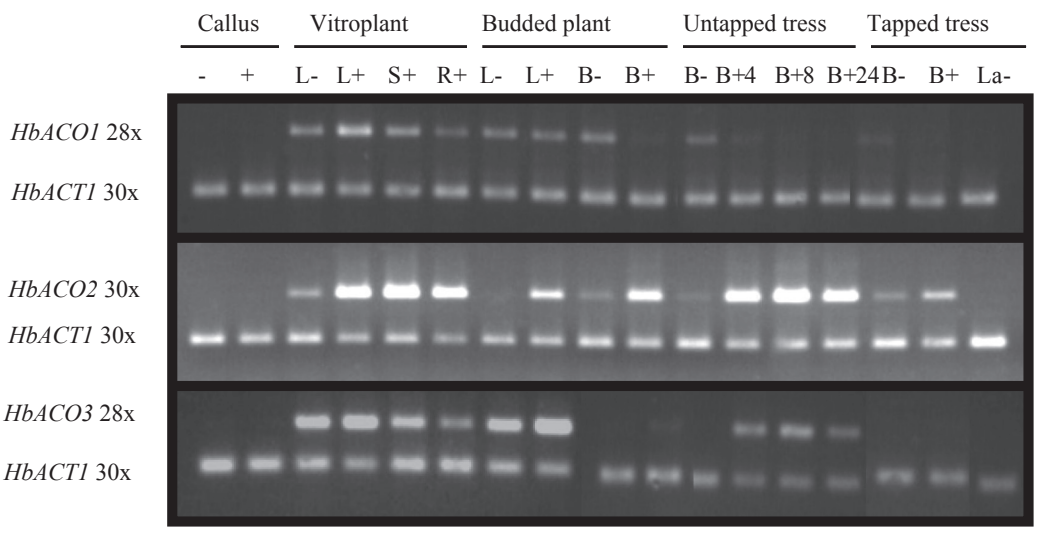

Figure 5. Analysis of the expression of HbACO *genes by semi-quantitative RT-PCR in various plant tissues and stages of development in response to ethephon stimulation. Leaf (L), Bark (B), Root (R), Stem (S), Latex (La), with (+) and without (-) stimulation, stimulated for 4, 8 and 24 h.*

might therefore coordinate several metabolic pathways conducive to latex production. In order to elucidate the molecular mechanisms underlying latex production and response to exploitation stress (tapping and ethephon stimulation), it is essential to characterise the genes involved in ethylene biosynthesis and the genes regulated by ethylene. The identification of the genes encoding ACC oxidase is the first step in the process of understanding these mechanisms.

The *ACO* multigene family comprises at least 3 members in *H. brasiliensis*. Prior to this work, a single gene had previously been isolated in *Hevea* clone RRIM 600 from a bark cDNA library (Accession NCBI AY207387.1)[40]. The isolation of the full-length sequences of 3 members of the *ACO* family and their characterisation in clone PB 260 represents an important contribution to our knowledge of *ACO* gene diversity in *H. brasiliensis*. The cDNA of *HbACO2* isolated from clone PB 260 encoded an identical protein to that of the cDNA isolated previously from clone RRIM 600, but was highly divergent 5' and 3' UTR regions. The largest *ACO* multigene family characterised to date, namely that of tomato, contains 5 members[28]. The 5th member isolated recently is induced during anaerobia and has a leucine zipper domain that enabled its identification by yeast two-hybrid screening. Three *ACO* genes have been identified in tobacco, and just one in *A. thaliana*. In *H. brasiliensis*, it may be possible to add up to 4 other genes to the three members already isolated. Indeed, Southern blot analysis indicated a group of at least 7 genes. The banding profiles also revealed fragment length polymorphism between the *Hevea* clones studied. Of the 2 members isolated, a structural analysis of the genome sequences of *HbACO1* and *HbACO2* showed that different genes were indeed involved. The genomic structure of *ACO* generally consists of three introns, but a loss

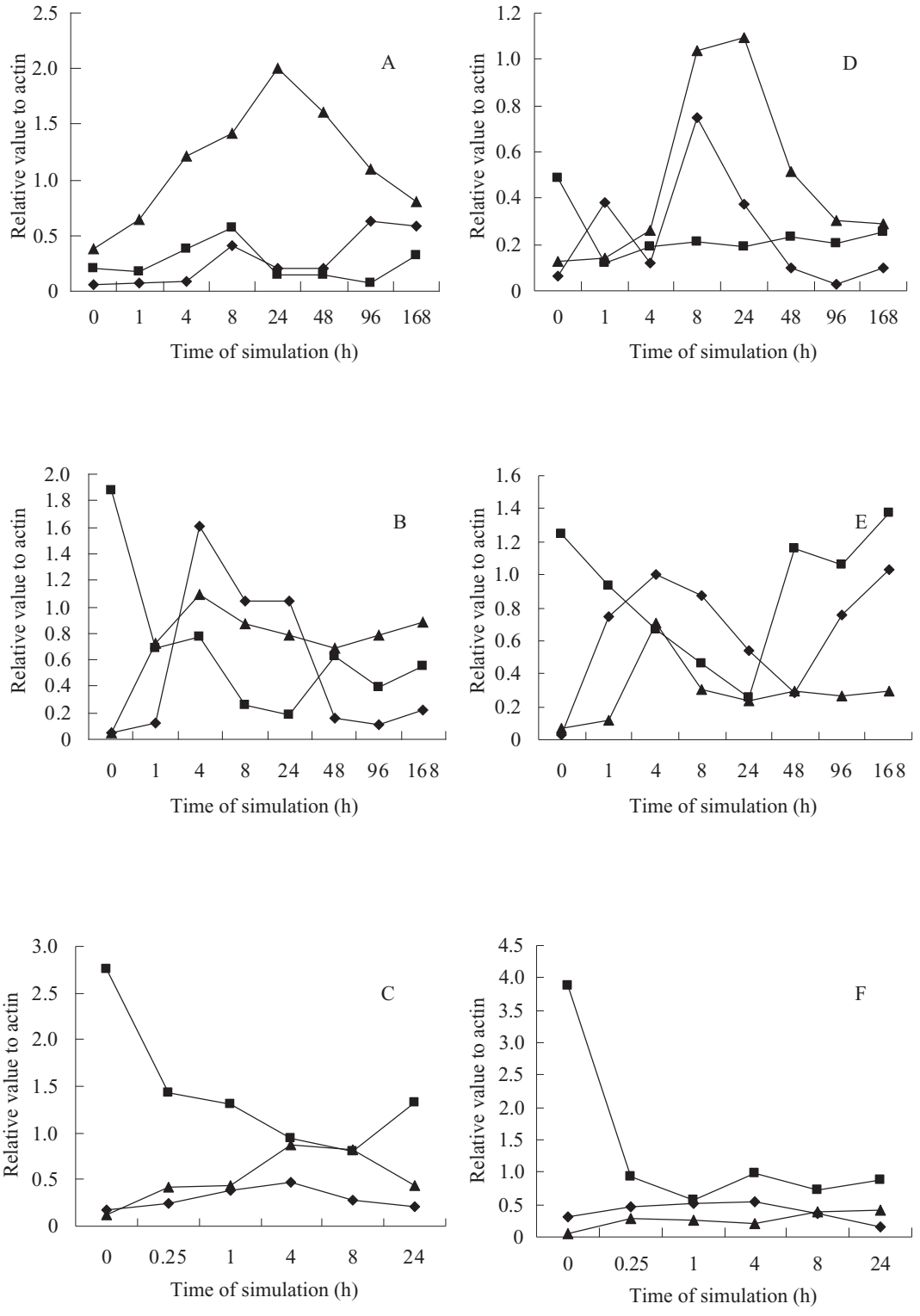

Figure 6. Analysis of the expression of HbACO *genes by semi-quantitative RT-PCR in leaf (A, B, C) and bark (D, E, F) of 3-month old budded plants treated with 2.5% ethephon (A, D), 1 p.p.m. ethylene (B, E) or wounding (C, F).*

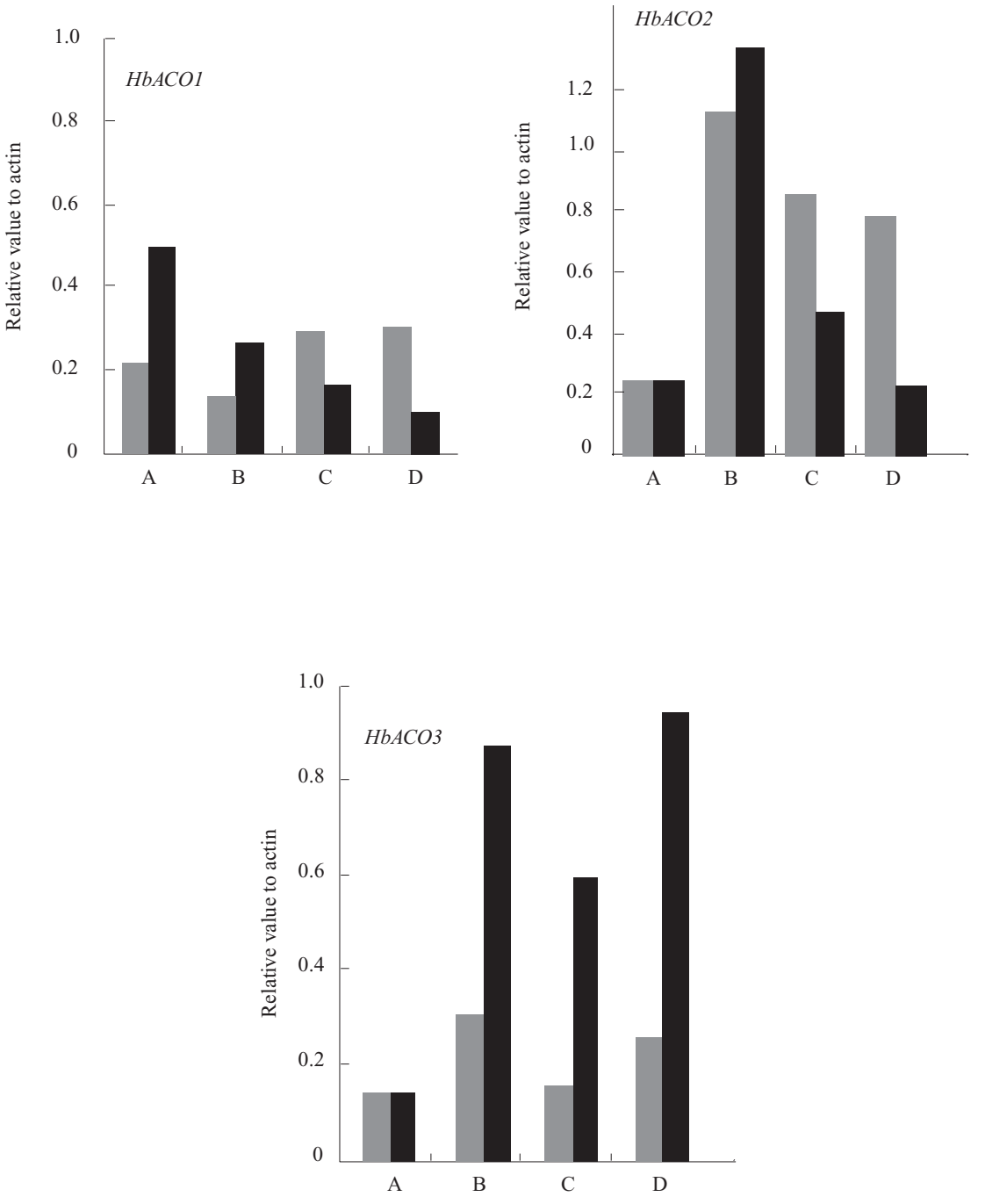

Figure 7. Effect of a pre-treatment with 1-methyl cyclopropane (1-MCP) before ethylene stimulation on the expression of HbACO *genes in bark (■) and leaf (■) tissues of 3-month old budded plants.*

of one intron, the second or the third intron, is observed for many species[27]. Given *HbACO2* contains only 2 introns, and according to the sequence, we propose that *HbACO2* might lose its third intron. Based on a phylogenetic analysis, *HbACO* genes were divided into two groups: the first group consisted of *HbACO2* and *HbACO3* sharing homology with *LeACO* and *PhACO*, while *HbACO1* belonged to another group with *MdACO*. An analysis of protein structure also revealed 2 functional sub-groups.

The potentially large number of *ACO* genes and their strong regulation in rubber trees might possibly reflect the particularly important and original role of ethylene in this species. Indeed, the large number of *HbACO* genes might be explained in part by the need for complex spatio-temporal modulation of expression, as a means of obtaining a highly coordinated regulatory system for ethylene biosynthesis and related functions. Ethylene induces numerous physiological changes[41], such as an increase in adenyl nucleotide content, which is one of the earliest changes to be observed[42]. Such changes are linked to changes in transcript abundance[6,7,43,44]. Over and above the regulation of latex production, ethylene production plays a central role in defence mechanisms, by activating PR-proteins and other anti-microbial proteins[45]. The differential regulation of the three studied genes by ethylene and wounding is noteworthy, *HbACO1* being repressed by both whereas *HbACO2* and *HbACO3* were induced. Blockage of ethylene action by 1-MCP confirmed the effect of ethylene on *HbACO1* in bark, but not in leaves, suggesting that there are two regulatory mechanisms which govern the expression of this gene. On the other hand, a unique influence of ethylene was demonstrated for *HbACO2*, since pre-treatment with 1-MCP totally blocked the induction of its expression. The *HbACO3* gene did not appear to be strictly dependent on ethylene and a more complex regulation phenomenon would seem to be involved.

The three *ACO* gene family members isolated were not observed to be expressed in callus; however, since ethylene production is known to occur during different stages of *in vitro* micropropagation[46], it can be assumed that another member of the *ACO* gene family must be responsible for the biosynthesis of the molecule in this case. Ethylene accumulation is conducive to callus browning and inhibits the development of embryogenic structures[46]. The inhibition of ethylene synthesis by application of AOA (an ACC synthase inhibitor) enables the formation of embryogenic cells[46]. However, it was only the application of $AgNO_3$, Ag^+ being an inhibitor of ethylene receptors that enabled the development of embryogenic structures into somatic embryos[46]. Conversely, application of ACC, an ethylene precursor, favours tissue senescence[46].

Characterisation of *ACO* gene expression at several different stages of plant development revealed that they are differentially regulated, with *HbACO2* being the gene most expressed in exploited tree bark and responding itself to ethephon stimulation. These findings agreed well with the fact that ethephon application causes endogenous ethylene production *via* auto-catalytic reactions[11]. Our results suggest that the isoform *HbACO2* could be the major source of endogenous ethylene biosynthesis in bark tissue of mature trees. A lack of transcript accumulation in latex suggested that the site of ethylene biosynthesis might be in bark tissues surrounding the laticifer cells. In general, the 3 genes were more expressed in the different organs of 3-month-old *in vitro* plantlets. The strong response of *HbACO2* to stimulation in bark and leaves tended to decline as tree exploitation progressed. The *HbACO3* gene, like *HbACO2*, responds positively to ethephon. From the budded plant stage, *HbACO3* is weakly expressed in bark

and ethylene stimulation makes it possible to achieve a minimal expression level. The *HbACO1* gene, on the other hand, is inhibited by ethylene stimulation. Its expression tends to become undetectable in trees with age, notably in those which are subjected to tapping stress. This observation is corroborated by the fact that tapping induces endogenous ethylene production[11].

Characterisation of the expression of these genes in young budded plants confirmed the repression of *HbACO1* and over-expression of *HbACO2* and *HbACO3* by ethylene applied either in ethephon form or in gas form. The specific role of ethylene was demonstrated using 1-MCP. As expected given its gaseous nature, ethylene was found to act more rapidly than ethephon. The 3 genes studied were regulated in the same way by wounding, but with an even faster response, since a reduction in the quantity of *HbACO1* transcripts and increase for the other two genes was found just 15 minutes after treatment.

The *HbACO* genes appear to be regulated at the transcriptional level, probably *via* multiple *cis*-acting promoter elements present in their 5' gene flanking regions. Blume and Grierson[48] studied the expression of the *ACO1* gene in *Lycopersicum esculentum* and *Nicotiana plumbaginifolia* by promoter-GUS fusions. They thus showed that expression of the reporter gene was induced by wounding, ethylene and infection by pathogens. In another article, the same team analysed the promoter and intron regions of the two *LeACO* genes[49]. They found inserts of class 1 and class 2 mobile elements that caused intron rearrangements. All the analysed promoters of the *ACO* genes except *PpACO1* had at least one copy of an ethylene *cis* response element (ERE, consensus AT/ATTCAA)[50]. This clearly demonstrates the auto-catalytic nature of ethylene synthesis[51]. In Prunus persea, the *ACO1* promoter displays numerous other potential sites for the attachment of transcription factors[50]. The motifs of interest include a potential MADS box transcription factor binding site[52] and the following *cis* elements: DRE/LTRE[53,54], Saur[55], ACGT[56], 2S[57], AGC (consensus AGCCGCC)[58] and DPBF[59].

In terms of post-translational regulation, an analysis of the protein sequences deduced from the *HbACO* genes revealed that all of the motifs determining enzyme functionality were present. To our knowledge, no information is as yet available on the influence of developmental and environmental factors on the regulation of protein stability or activity. However, the impact of the presence or absence of cytoplasmic bicarbonate on enzyme activity requires investigation[60]. The crystal structures of both apo- and metal-complexed ACC oxidase derived from *Petunia hybrida* have recently been reported[60]. The common Fe^{2+}-binding motif (His-X-Asp-X(54)-His) and the putative co-substrate hydrogen-binding residues (Arg-X-Ser) are well conserved among members in the non-heme iron enzyme family. The ascorbate is located in the cofactor Fe^{2+}-binding pocket, which consists of His_{177}, Asp_{179}, and His_{234}; the ascorbate interacts with the side chains of Arg_{244} and Ser_{246}, composing hydrogen-bonding network in the hydrophobic wide cleft of the active site near the C-terminus. These residues are conserved in *HbACO* at the positions of H_{176}, D_{178}, H_{233}, R_{243} and S_{245}. Mutagenesis studies have led to proposed roles for residues in *ACO* catalysis, including the iron binding ligands, various lysyl residues possibly involved in ascorbate binding, and arginine (Arg_{244}, using the tomato pTOM13 ACCO numbering system), serine (Ser_{246}), and threonine (Thr_{157}) residues in binding the ACC carboxylate or bicarbonate[61,62]. In *Hevea*, residues Arg_{243}, Ser_{245} and Tyr_{161}.may constitute this RXS motif. Recently, it was highlighted another conserved motif Lys_{296}-Glu_{301} located in C-terminus from a study based on the analysis

of ACC oxidases from 24 different species[63]. By performing the site-directed mutagenesis studies substituting the Lys_{296} and Arg_{299} residues of *MdACO1* by Gly, Lys, Arg and Glu residues, respectively, it was shown that the mutation to Gly resulted in the loss of a positive charge and the substitution by Glu caused an electrostatic repulsion, whereas mutations of Lys_{296} by Arg and Arg_{299} by Lys did not make any change in net charge except the size of the side chains. In *Hevea*, these residues are also conserved at the positions of Lys_{296}, Arg_{299} and Glu_{301}. Two conserved regions corresponding to the self-cleavage sites involved in the degradation of ACO proteins were found. As regards the post-translational regulation of ACC oxidase, it has been noted that the enzyme undergoes rapid inactivation in the absence of bicarbonate. ACC oxidase also undergoes metal catalyzed auto-cleavage. N-terminal sequencing allowed the identification of two cleavage sites in ACC oxidase: one is between Leu_{186} and Phe_{187}, and the other is between Val_{214} and Val_{215}[60]. These residues are conserved in *Hevea* ACO and *MdACO1*, but the second cleavage site was replaced by Ile_{215} in the position of Val_{215}, so that its function needs to be determined for both *Hevea* and *Malus*. Correspondences between the levels of expression, protein amounts and ACC oxidase activity will need to be studied further to gain a clearer understanding of the different levels of regulation in the experimental system used here.

Young budded plants are a model for studying the regulation of genes involved in ethylene biosynthetic pathway in rubber trees. The use of controlled growing conditions make it possible to envisage using a larger number of inhibitors in order to more closely define the mechanisms which operate. For future studies, the availability of a genetic transformation system for rubber trees offers prospects for the functional validation of the genes studied[64,65].

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