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Isolation and characterization of three members of the multigenic family encoding ACC oxidase from *H. brasiliensis* during plant development

Kuswanhadi, Leclercq J., Sumarmadji, Rio M.A., Alemanno L., Montoro P.
UMR BEPC, CIRAD, TA 80 / 03, Avenue Agropolis, 34398 Montpellier Cedex 5, France.
Indonesian Rubber Research Institute, Sungei Putih & Sembawa Research Centres, Indonesia

Abstract

Applying Ethephon, an ethylene releaser, to increase rubber production in *H. brasiliensis* has been practised for a long time. The effect of ethylene on latex production has been amply described, notably for lengthening latex flow and its regeneration. Nevertheless, little is known about the expression of genes involved in ethylene biosynthesis and response to ethylene. In this paper, we isolated and characterized genes encoding ACC oxidase, a key enzyme in ethylene biosynthesis in the plant. We then studied the effect of ethylene stimulation on the expression of *ACO* genes in various tissues during plant development. Three members of the *ACO* multigenic family were isolated from a bark cDNA library with RACE technology: *Hb*ACO-H4, *HbACO-H5*, and *HbACO-O48*. Full length cDNA sequences encoded for peptides of 318, 315, and 318 amino acids respectively for these 3 members, which had 79 to 92% protein identity and 75 to 86% nucleotide homology between them. Two genomic sequences were isolated: *HbACO-H4*, which was 1504 bp long and consisted of 2 introns and 3 exons, while *Hb-ACO-H5* was 1456 bp long and consisted of 3 introns and 4 exons. These three genes were differentially expressed in different plant organs in response to ethylene stimulation.

1. Introduction

Improving productivity is a priority challenge for the future of rubber growing, which involves 50 million people. The risks of a drop in natural rubber production (33% of world production) and labour shortages are becoming substantial. Effective ways of reducing tapping time have been obtained whilst maintaining natural rubber yields through tapping systems using ethylene stimulation. Ethephon (ET), an ethylene generator, is applied to rubber tree bark on the tapping panel, to increase rubber production by stimulating latex regeneration and flow (D'Auzac, 1989). However, it is necessary to have a good command of the stimulation frequencies and ET concentrations to be used during tree exploitation, so as not to trigger cell dysfunctioning that might lead to the appearance of tapping panel dryness and production losses. In Indonesia, this syndrome is thought to affect up to 30% of the areas planted with rubber trees. Several studies have revealed the effect of exogenous ethylene on endogenous ethylene biosynthesis and it has been possible to characterize physiological responses depending on the rubber clones involved. Ethylene regulation of the expression of several genes involved in latex regeneration and flow, including the oxidative metabolism, has also been studied in depth.

The effect of Ethephon treatments on ethylene biosynthesis, latex production and tissue degeneration suggests major involvement of endogenous ethylene in the molecular mechanisms operating in laticifer tissues, but also within different bark tissues (Siwei et al.) (Etheridge et al., 2005) (Chen et al., 2005). Although the ethylene biosynthesis and perception pathways have been amply described, as has transduction of this signal, biochemical and molecular characterization of the genes involved in ethylene biosynthesis, and their regulation, remains tricky in the rubber tree due to problems with setting up a study design.

The existence of 1-aminocyclopropane-1-carboxylic acid (ACC) in the bark suggests that this biosynthesis occurs in a tissue of the bark other than the laticifer rings (Sumarmadji, 1999). We present here the preliminary characterization of the expression of genes encoding ACC oxidase (*ACO*) during plant development.

2. Results

Isolation of partial sequences of rubber tree cDNA corresponding to genes encoding ACOs. As the different members of the multigenic family encoding *ACOs* have spatio-temporal regulation, and given the interest shown in characterizing the expression of those genes in rubber trees, in callus and bark, the genes were amplified by PCR using degenerated primers from different DNA matrices, such has *Hevea* genomic DNA, single-stranded cDNA populations obtained from total RNA extracts of leaves, callus and bark of young rubber plants subjected, or not, to stimulation with Ethrel™ for 24 hours, or two phage libraries of cDNA from rubber tree meristems and bark. Thus, 13 PCR products of around 1000 bp each were obtained from matrices of different origins, single-stranded cDNA from callus, bark (stimulated or not with Ethrel) and leaves, and from the bark library, then cloned in pGEM-T Easy and sequenced.

In silico analyses of partial HbACO nucleic sequences. An initial comparison of sequences with the NCBI database, carried out with Blastn software, revealed that all the cloned products had a high degree of homology (>80%) with the *ACO* sequences of various species. The sequences were then aligned with Clustalw software and identical groups of sequences were established on all or part of their sequence. Three groups of sequences were found and three partial *ACO* clones (H4, H5, O48), representative of the whole, were selected. They displayed strong homology with an *ACO2* of *Populus euramericana* and an *ACO1* of *Vitis vinifera* respectively.

Isolation of a full-length cDNA encoding an ACO by screening a phage library of bark cDNA. A PB260 bark cDNA library constituted in the Triplex vectors was used. After screening the phages of the library by hybridization with probes from *ACO* partial clones, a positive clone was isolated; its plasmid was excised, sequenced and called *HbACO-H5*. The sequence of this full-length cDNA was 1115 bp, with an encoding sequence of 936 bp corresponding to 312 amino acids.

Isolation of two other full-length cDNAs by RACE-PCR type extension. With the single–stranded cDNAs obtained from different tissues and the partial genomic DNA sequence, amplifications were performed at 5’ and 3’ of the known partial sequences using the RACE-PCR kit (BD Bioscience ClonTech). The sscDNA obtained from total bark RNAs led to isolation of the corresponding fragments at the 5’ and 3’ ends of a cDNA called *HbACO-H4* and *HbACO-O48*. PCR amplification with high-fidelity Taq polymerase using primers positioned at the ends of those sequences and a matrix of bark sscDNA led to isolation of the two full-length cDNAs, *HbACO-H4* and *HbACO-O48* which were 1174 bp with a ORF of 954 bp (318 amino acids) and 1352 bp with a ORF of 954 bp (318 amino acids) respectively.

Isolation of genomic sequences by PCR amplification. With the primer pairs defined at the two ends of each full-length cDNA, PCR amplification was performed using genomic DNA from clone PB260 in order to isolate the corresponding genomic clones. After cloning, sequencing and sequence analysis, two full-length genomic clones were confirmed: *HbACO-*

H4 with a length of 1504 bp containing 2 introns and 3 exons, and *HbACO-H5* measuring 1456 bp and containing 3 introns and 4 exons.

Analysis of ACO gene expression by semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed using the gene encoding actin as an internal control (Table 1). Development of the PCR conditions showed that expression of this gene was constitutive and it was possible to determine the number of cycles required to display a variation in the expression of the *ACO* genes studied. When the number of amplifications was increased to 35 cycles, all the genes were found to be expressed. However, gene expression was not visible in calli and in latex at 28 cycles, meaning that they were weakly expressed in those tissues. On the other hand, expression in leaves increased between H5, H4 and O48. Although there was over-expression in the leaves of *in vitro* plantlets in response to ethylene for H4 and H5, O4.8 expression was independent from Ethrel stimulation. In older budded plants, H5 did not respond to stimulation. In the stems of *in vitro* plantlets, expression of H4 was strong and did not vary after stimulation. Accumulation of mRNAs was lower for O4.8 and even lower for H5 compared to H4, with expression of the H5 and O4.8 genes being reduced by Ethrel application. In the barks of budded plants, that tendency increased for H5 and O4.8 for which there was virtually no more expression after stimulation, whereas expression of H4, which was stable, increased substantially with Ethrel application. In mature untapped trees, H4 expression was greatest and it responded to stimulation. O4.8 also responded to Ethrel but to a lesser degree, reaching a peak after 8 hours of stimulation, and the weak basic expression of H5 was reduced by ethylene treatment. Lastly, in tapped trees, the activity of all the genes was reduced to the limit of detection for O4.8 and H5, and weak stimulation of H4 expression is worth noting. Expression of these genes was not detected in latex for the same level of amplification cycles.

Localizing expression of the HbACO-H5 and HbACO-O48 genes by in situ hybridization. In connection with a study on ethylene-regulation of the molecular mechanisms involved in natural rubber production, consideration was given to optimizing an *in situ* hybridization method for *Hevea brasiliensis*. Specific probes of the *ACO* genes isolated were used to study differential expression of those genes in different tissues of somatic plantlets and of shoot bark after ethylene stimulation for 8 h. For fine cell characterization of those genes, which were weakly expressed, label amplification was required using antibodies coupled to fluorochromes of the Alexa488 type. This method revealed expression of the *HbACO-H5* and *HbACO-O4.8* genes in all the tissues of shoot bark and stems, particularly in laticifer cells. In stems, stronger expression of those genes was found in the phloem, especially in the laticifer zone, than in the other tissues. In roots, expression of those genes was very weak or nonexistent. In leaves, autofluorescence was too strong to localize specific labelling. Alexa488 fluorochrome was not suitable in this case.

3. Discussion and conclusion

The genome of *Hevea brasiliensis* clone PB 260 carries at least three members of the multigenic family encoding ACOs. The genomic sequences and corresponding cDNAs were isolated. Their characterization at several stages of development revealed differential regulation of their expression, with *HbACO-H4* being the most strongly expressed gene in the bark of tapped trees and responding to Ethrel stimulation. These observations tallied well with the fact that Ethrel application triggers endogenous ethylene production *via* autocatalytic reactions. These results also suggested that the isoform HbACO-H4 might be responsible for endogenous ethylene biosynthesis in bark tissue. The very weak expression found in latex

tended to show that the seat of ethylene biosynthesis would appear to be in bark tissues in the vicinity of the laticifer cells. Although the *in situ* hybridization method is not quantitative, localizing the expression of *HbAco-H5* and *HbAco-048* in all the tissues meant that no conclusion could be drawn as to the specific role of *HbACO-H4* in the bark of tapped trees. However, generally speaking, the 3 genes were more strongly expressed in the different organs of 3-month-old *in vitro* plantlets. More in-depth studies currently being undertaken should determine how ethylene functions during rubber tree development in response to various types of abiotic stress.

References

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Tableau 1. Assessment of the degree of *HbACO* gene expression in response to ethylene stimulation (2.5% Ethrel) by semi–quantitative RT-PCR in different tissues/organs at different stages of development for rubber tree clone PB 260. (-) none (+) low (++) medium (+++) high.

	Expression of ACO members in various tissues and organs																
	Callus		*In vitro* plantlet (3 months old)				Budded plant (6 months old)				Untapped tree (5 years old)				Tapped tree (5 years old)		
			leaf		trunk	root	leaf		bark		bark				bark		latex
Time after stimulation with 2.5% Ethrel	0	24	0	24	24	24	0	24	0	24	0	4	8	24	0	24	0
H4	-	-	+	++	++	++	+	++	+	++	+	++	+++	++	+	++	-
H5	-	-	+	++	+	+	+	+	+	-	+	+	-	-	+	-	-
O-48	-	-	++	++	++	+	++	++	+	+	+	++	++	+	+	+	-