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Ethylene-regulated genes in Hevea brasiliensis: effect of ethylene and wounding in young budded plants of three clones with contrasting metabolisms

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Ethephon, an ethylene generator, stimulates both latex flow and regeneration. Given ethylene triggers the expression of numerous genes in latex cells, a transcriptomics approach was taken in order to understand the molecular mechanisms underlying latex production controlled by this hormone. Two cDNA libraries were constructed using Suppression Subtractive Hybridization (SSH) technology from 4-year-old trees of clone PB 260 in their immature period without stimulation or stimulated with 2.5% ethephon stimulated. Among 1158 sequenced clones, 158 unique transcripts were identified. Putative functions were assigned by sequence analysis using BLASTX, which showed a large number of genes related to transcription and protein synthesis, unknown functions or defence proteins. A high density filter was completed with genes involved in latex metabolism such as rubber biosynthesis and ROS-scavenging protein. Macro-array analysis revealed a general differential expression between clones with a contrasting metabolism. A large proportion of genes was up-regulated for the active metabolic clone PB 260, and by contrast, a down-regulation was observed for lower metabolisms such as PB 217 or RRIM 600. Discrimination of the response to ethylene for these clones was significant for 35 genes, and 5 of them might discriminate between the responses of the 3 clones. The differential gene expression by Real Time PCR upon ethylene stimulation was confirmed for some of these candidate genes. These genes could be used as markers of expression under stress in a marker-assisted selection programme.

Key words: bark, ethephon, ethylene, gene expression, macroarray, real-time PCR, suppression subtractive hybridization, wounding.

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INTRODUCTION

Ethylene plays a pleiotropic role in plant growth and development processes, and one of its most economically important roles is in the regulation of natural rubber production [1]. Ethephon, an ethylene generator, is applied to rubber tree bark on the tapping panel to

increase rubber yield by stimulating latex flow and regeneration between two tappings [1]. Ethephon is a stimulator of many activities associated with latex cells. Among the changes, increases in RNA, invertase activity, sucrose content, latex stability, and a decrease in starch content have been known for many years [2-4]. More recently, ethephon application was shown to trigger the expression of numerous genes [5-7]. As ethylene triggers the expression of numerous genes in latex cells, a transcriptomics approach was taken in order to understand the molecular mechanisms underlying latex production.

RESULTS AND DISCUSSION

Analysis of expressed sequence tags from SSH libraries

Differential gene expression was detected by Suppression Subtractive Hybridization (SSH) in bark with ethephon treatment. The SSH method was used to isolate genes that were differentially regulated by ethylene in the bark of untapped trees. To obtain genes that are down- or up-regulated upon ethephon stimulation, two reciprocal libraries (namely ET^- and ET^+) were constructed by subtracting cDNA pools of 4-year-old trees of clone PB 260 in their immature period without stimulation or stimulated with 2.5%. A total of 1158 clones was sequenced, 309 and 849 for ET^- and ET^+ libraries respectively. Among these expressed sequence tags (EST), 158 unique transcripts were identified using a bioinformatics pipeline of analyses developed at CIRAD [8] (Table 1). Putative functions were assigned by sequence analysis using BLASTX that showed a large number of genes related to transcription and protein synthesis (48%), defence proteins (11%) and unknown functions (26%). The expression of defence and unknown genes might reflect the high specificity of latex cells. Hence, these data show that ethylene triggers the activation of the metabolism and the expression of genes related to specific latex cell functions not identified in other species.

Table 1. Classification of the 158 ethylene-regulated unique transcripts identified from the 2 SSH libraries.

Function	Down-regulated	Up-regulated	Total
Transcription & gene expression	1	6	7
Primary metabolism	9	67	76
Secondary metabolism	0	7	7
Cell structure	0	2	2
Development & cell differentiation	0	1	1
Signalling	1	3	4
Stress & defence	4	13	17
Transport & secretion	1	2	3
Unknown	8	33	41
Total unique transcripts	24	134	158

A global gene expression analysis carried out by the reverse-Northern technique (macroarray) revealed a significant up-regulation for 46% of genes from the ET^+ library and only 8% for the ET^- library (Table 2). The normalization and the elimination of identical sequences between the 2 SSH libraries were not sufficient to avoid redundancy and non-specificity of the genes in each library.

Table 2. Gene expression analysis by reverse-Northern carried out on clones identified in the ET^- and ET^+ libraries after 24h of ethylene treatment. Ethylene was applied as a gas at 1ppm on 3-month-old budded plants. Down and up-regulated genes are referred to respectively as genes having a level of expression under 0.5 and over twice the level of actin used as the internal control.

		Number of ethylene-regulated genes after 24h	
SSH Library	Total EST	Down-regulated (<0,5 X)	Up-regulated (>2X)
ET^-	24	2	1
ET^+	134	4	61

Analysis of gene expression by reverse-Northern

A global gene expression analysis was carried by reverse-Northern to compare the response of three clones with contrasting metabolisms (PB 217: low; RRIM 600: intermediate; PB 260: high) to stresses. A set of 189 EST clones consisting of both 158 ethylene-regulated and 31 latex-expressed genes were spotted on a high density filter. The latex-expressed genes were selected to be representative of its metabolism among genes involved in the reactive oxygen species-scavenging system, rubber biosynthesis, ethylene biosynthesis, nitrogen assimilation and plant defence. High density filters were hybridized with radio-labelled cDNA synthesized from the total RNA of bark from 3-month-old budded plants.

Differential gene expression in response to ethylene was recorded for the various clones studied. Global up-regulation of genes with ethylene stimulation was observed for clone PB 260, whilst in PB 217 or RRIM 600 genes were down-regulated (Table 3). Among 35 EST clones displaying a significant variation in gene expression upon ethylene treatment, 5 could discriminate between the 3 clones with contrasting metabolisms. Those EST clones had close identity with an aquaporin like protein (*PIP-like*), 2 genes of ethylene biosynthesis (*HbACS-F2*, *HbACO2*), a heat shock protein cognate protein 2 (*HSCP70*), and a ribosomal protein YL16.

Table 3. Analysis of gene expression by reverse-Northern upon ethylene and wounding treatments in 3 clones with contrasting metabolissm. Nd: non-determined.

		Number of genes up or down-regulated			
Clone	Ratio of gene expression related to actin	Control	Ethylene		
		0h	4h	8h	24h
PB 217	< 0,5	33	70	Nd	92
	2 < x < 5	20	8	Nd	8
	> 5	1	0	Nd	0
RRIM 600	< 0,5	29	41	Nd	Nd
	2 < x < 5	22	1	Nd	Nd
	> 5	2	0	Nd	Nd
PB 260	< 0,5	30	21	21	Nd
	2 < x < 5	16	26	79	Nd
	> 5	0	1	25	Nd

Analysis of gene expression by real-time PCR

Further validation of the differential gene expression was carried out for the *PIP-like* and *HSCP70* EST clones using the real-time PCR technique (Table 4). For the *PIP-like* gene, reverse-Northern analysis showed a down-regulation by the ethylene treatment for both the PB 217 and RRIM600 clones, and up-regulation for PB 260. An analysis based on real-time PCR showed that ethylene did not affect the level of transcripts for any clones. By contrast, the *PIP-like* gene was significantly induced by wounding for clones PB 217 and RRIM 600, with low and medium metabolisms respectively. Expression of the *HSCP70* gene analysed by reverse-Northern revealed down-regulation for PB 217, stability for RRIM 600 and a slight increase in transcript level for PB 260. Significant transient induction of the *HSCP70* gene was recorded 4h after ethylene treatment in clone PB 217 clone using real-time PCR (0.4 to 1.95). For the other clones, the expression of this gene remained stable. Similarly, the *HSCP70* gene is significantly induced 15 min after wounding for PB 217 and not for the other clones.

Table 4. Effects of clones, ethylene and wounding on the expression of genes corresponding to the EST clones PIP-like and HSCP70

Gene	Clone	Control 4h in box	Ethylene 4h in box	Control 24h in box	Ethylene 4h in box	Control 15 min	Wounding 15 min	Control 4h	Wounding 4h
PIP-like	PB217	0.07 ± 0.03^{ef}	0.14 ± 0.07^{def}	0.07 ± 0.03^{ef}	0.31 ± 0.14^{cd}	0.23 ± 0.15^{cdef}	0.02 ± 0.02^{f}	0.25 ± 0.12^{cde}	1.73 ± 0.35^{a}
	RRIM600	0.04 ± 0.01^{f}	0.03 ± 0.01^{f}	0.05 ± 0.01^{ef}	0.15 ± 0.12^{def}	0.13 ± 0.14^{def}	0.05 ± 0.03^{ef}	0.13 ± 0.08^{def}	0.42 ± 0.24^{c}
	PB260	0.06 ± 0.01^{ef}	0.07 ± 0.05^{ef}	0.04 ± 0.00^{ef}	0.08 ± 0.07^{ef}	0.08 ± 0.09^{ef}	0.06 ± 0.02^{ef}	0.21 ± 0.21^{cdef}	1.44 ± 0.24^{b}
HSCP70	PB217	0.4 ± 0.17^{cd}	1.95 ± 0.80^{a}	0.06 ± 0.10^{def}	0.19 ± 0.04^{cdef}	0.09 ± 0.07^{cd}	0.42 ± 0.20^{cd}	0.24 ± 0.05^{cd}	0.38 ± 0.19^{cd}
	RRIM600	0.21 ± 0.10^{cdef}	0.47 ± 0.58^{c}	0.02 ± 0.00^{f}	0.08 ± 0.05^{cdef}	0.02 ± 0.00^{d}	0.18 ± 0.12^{d}	0.37 ± 0.06^{cd}	1.03 ± 0.35^{b}
	PB260	0.16 ± 0.05^{cdef}	0.13 ± 0.09^{cdef}	0.02 ± 0.01^{f}	0.03 ± 0.01^{ef}	0.03 ± 0.03^{d}	0.37 ± 0.42^{cd}	0.41 ± 0.13^{cd}	0.68 ± 0.69^{bcd}

The discrepancy between the gene expression analyses performed by reverse-Northern and real-time PCR was probably related to the technique of hybridization used for Northern, which is not able to discriminate between genes with close sequence identity. Conversely, specific primers designed for each gene in the 3'UTR of EST sequences led to consistent data. For instance, the *HSCP70* gene was shown to be down-regulated by reverse-Northern and up-regulated by real-time PCR. Using this technique, ethylene treatment did not induce any change in gene expression for both tested genes except for *HSCP70* gene in PB 217. The wounding treatment triggered an accumulation of transcripts for the *PIP-like* gene for all clones and for the *HSCP70* gene in RRIM 600 only.

Given the consistency of data from real-time PCR compared to reverse-Northen, a high-throughput real-time PCR technique for gene expression is under development to identify genes likely to be used as markers of expression under stress. Ethylene, wounding and some other stresses will be tested in order to highlight specific patterns of expression for clones with contrasting metabolisms.

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