

Expression of the *HEV2.1* gene promoter in transgenic *Hevea brasiliensis*

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Abstract Hevein is involved in the coagulation of rubber particles and is known to be highly expressed in latex cells from *Hevea brasiliensis*. As all *HEVEIN* genes are highly conserved in their transcribed region, a promoter functional analysis was carried out in transgenic rubber tree in order to evaluate the expression of *HEV2.1*, one member of the *HEVEIN* multigenic family. Three out of the 14 established independent transgenic lines carrying the *HEV2.1::GUS* construct were selected and set to regenerate plantlets. In situ hybridization of transgenic and wild-type plant tissues was respectively carried out with *gusA* and *HEVEIN* antisense mRNA probes. This showed co-localized-expression of the *HEV2.1* gene with the overall *HEVEIN* gene expression. Laticifer-specific expression was observed in roots and stems, but not in leaves where *HEVEIN* genes were expressed in all cell types. Since *HEV2.1::GUS* callus lines responded significantly to light stimulation, up-regulation of the expression of *HEV2.1* genes by light was suggested in leaves.

Keywords *Hevea brasiliensis* · Hevein · Latex cell · Genetic transformation · In situ hybridization

Abbreviations

ABA	Absciscic acid
BAP	Benzylaminopurine
3,4-D	3,4-Dichlorophenoxyacetic acid
DEV	Medium for embryo development
DM	Decontamination medium
EXP	Medium for embryo expression
GUS	β -Glucuronidase
HEV	Hevein
MU	4-Methylumbelliferone

Introduction

Hevein was identified in 1960 as a 4.7 kDa polypeptide (Archer 1960), located in small vacuole-derived organelles, called lutoids, from *Hevea brasiliensis* latex cells (Archer et al. 1969). This specific expression in latex cells was confirmed by immunolocalization (H. Chrestin, pers. comm.). The first cDNA clone to be isolated (*HEV 1*) is 1018 base pairs long and includes an open reading frame encoding a 204 amino acids polypeptide (Broekaert et al. 1990). This amino acids sequence consists of a putative signal sequence of 17 amino acids and a polypeptide of 187 amino acids corresponding to a 20 kDa pro-protein. This prohevein undergoes co- and post-translational processing to give a 43 amino acid N-terminal chitin-binding domain, the 5

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kDa hevein (Lee et al. 1991), and a 144 amino acid C-terminal domain showing homology to type IV pathogenesis-related proteins (Stanford et al. 1989).

Hevein is a major component of latex (50–70% of soluble proteins) with antifungal properties (Van Parijs et al. 1991). It was shown to be involved in latex coagulation, playing a bridging role between rubber particles *via* a surface receptor protein carrying a *N*-acetylglucosamine group (Gidrol et al. 1994). The International Union of Immunological Societies recognizes ten allergens, including prohevein (20 kDa), hevein (4.7 kDa) and prohevein C terminus, which were classified as Hev b 6.01, Hev b 6.02 and Hev b 6.03 respectively (Yeang et al. 2002), Hev b 6.01 being one of the most important allergens for glove-sensitized latex allergies in people (de Silva et al. 2004).

Hevein is an acidic protein belonging to the lectin super-family and is encoded by a multigene family consisting of at least five members (Pujade-Renaud et al. 2005). These genes are highly conserved in their transcribed region but highly divergent in their regulatory sequences upstream the position –40 from the transcription start. Functional analysis of the *HEV2.1* gene promoter in fusion with the *gusA* reporter gene in transgenic rice revealed GUS activity in leaf blades, root vascular tissues and in pollen, and additional up-regulation by wounding and pathogen infection in leaves (Pujade-Renaud et al. 2005). These results were in agreement with previously described wound-induced overexpression of the hevein genes in rubber tree (Broekaert et al. 1990) and the proposed role of hevein in plant defence (d'Auzac et al. 1995).

As an efficient *Agrobacterium tumefaciens*-mediated transformation procedure was recently established for *H. brasiliensis* (Blanc et al. 2006), the fusion of the promoter region of the *HEV2.1* gene to the *gusA* reporter gene sequence was analysed in the rubber tree in order both to gain insight into the molecular mechanisms involved in the regulation of the expression of hevein genes and to control transgene expression for applications in genetic engineering programmes. As the hevein protein is assumed to be specifically expressed in latex cells, the localization of the expression of the *gusA* and *HEV2.1* genes was studied by mRNA in situ hybridization. Finally, the effect of the light on the induction of *HEV2.1* gene was characterized in embryogenic calli.

Materials and methods

Vector construction

The construct pCAMBIA-(35S-*nptII*)-(PHEV2.1-*gusA*) used for functional analysis of the hevein gene promoter in the rubber tree is derived from the construct pCambia-PHEV2.1 described by Pujade-Renaud et al. (2005). The plasmid pCAMBIA-PHEV2.1 bears the *HEV2.1* hevein gene promoter fused to the *gusA* reporter gene (Jefferson et al. 1987), in a pCAMBIA1381Z-derived vector (CAMBIA, Canberra, Australia). This construct carries a “35S promoter-*hptII* gene-35S terminator” cassette allowing hygromycin selection in plants. The *hptII* gene was removed by Xho I digestion and replaced by the *nptII* gene (Xho I-Xho I fragment from the pCAMBIA2301 vector) in order to allow kanamycin selection. The final construct was introduced into *Agrobacterium tumefaciens* EHA 105 cells by electroporation.

Genetic transformation procedure

A callus line called VP10 from the *Hevea brasiliensis* clone PB 260 was established in accordance with the culture conditions described by Carron et al. (1995). Friable calli were transformed according to the procedure reported by Blanc et al. (2006). First, friable callus was precultured on CaCl₂-free MM medium for 21 days on plates (100 × 20 mm) (Montoro et al. 2003). *Agrobacteria* were grown in liquid LB medium (Sigma, USA) supplemented with 50 mg l⁻¹ kanamycin and 100 µM acetosyringone at 28°C until an OD₆₀₀ = 0.6 was obtained. The suspension was centrifuged at 1,000g for 10 min and then the pellet was resuspended in liquid MM medium deprived of Fe-EDTA, CaCl₂ and growth regulators and supplemented with 100 µM acetosyringone. The precultured calli were inoculated with 50 µl of the *Agrobacterium* suspension adjusted to 10⁷ cells ml⁻¹. After a 5-day co-culture period at 20°C, calli were placed on decontamination medium (DM), which was a modified MM medium supplemented with 500 mg l⁻¹ ticarcillin (Sigma, USA). Co-cultured tissues were subcultured every 3 weeks as small tissue aggregates of 1–2 mm on plates. After two subcultures on DM, ticarcillin was reduced to 250 mg l⁻¹ and paromomycin added at increasing concentrations (50–100 mg l⁻¹) from the third to the

sixth subculture. Resistant tissues were then assayed for GUS (β -glucuronidase) activity, and the GUS-positive tissues were used for the establishment and molecular characterization of transgenic callus lines.

Southern blot analysis of transgenic callus lines

Genomic DNA was extracted from control and transformed callus lines, as described by Dellaporta et al. (1983). The 35S::GUS lines used as control have been previously established by Blanc et al. (2006). Restriction of 8 μ g DNA per sample was performed using 5 units/ μ g of *EcoRI* enzyme in an appropriate buffer, at 37°C for 5 h. Restricted DNA fragments were separated by electrophoresis in 0.8% agarose gel in TAE buffer, then transferred onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia, Buckinghamshire, England) according to Sambrook's protocol (Sambrook et al. 1989). Two hybridizations were performed overnight using as probes the 674 bp and 394 bp radiolabelled fragments respectively for *gusA* and *nptII* genes, amplified from the plasmid pCAMBIA2301 using the following primers:

Forward *gusA*: 5'-GTGGGTCAATAATCAGGAA G-3'

Reverse *gusA*: 5'-CCAATCCAGTCCATTAATG C-3'

Forward *nptII*: 5'-CCGGCTACCTGCCCATTG C-3'

Reverse *nptII*: 5'-GCGATAGAAGGCGATGCG-3'

Plant regeneration

One gram of callus from each transgenic line and the non-transformed line VP 10 were transferred for 4 weeks to 250 ml bottles containing the expression medium (EXP) corresponding to a modified MM in which 3,4-D (Sigma-Aldrich), a synthetic auxin (Carron et al. 1995), and BAP concentrations were reduced to 0.44 μ M, AgNO₃ eliminated, and sucrose replaced by 60 g l⁻¹ of maltose and 20 g l⁻¹ of sucrose. The EXP medium was also supplemented with 250 mg l⁻¹ of ticarcillin. Two subsequent subcultures were conducted in a temporary immersion culture system (Rita[®], CIRAD, France) for one month each (Etienne et al. 1997). The RITA[®] system contained 200 ml of liquid development medium (DEV; Blanc et al. 2006). At the end of the second month, somatic embryos were

counted. Embryos with a normal morphology were subcultured in glass tubes (200 × 25 m) containing 20 ml of solidified DEV medium for the germination step. After 15 days in the dark, they were transferred to a culture chamber with a 12/12 h photoperiod (60 μ M m⁻² s⁻¹ produced by Sylvania, Grolux, Germany). All cultures were carried out at 27°C. One month later, plantlets were acclimatized in the greenhouse.

Treatments applied to transgenic callus lines

Callus lines are grown under dark condition. In order to study the effect of light, HEV2.1::GUS transgenic callus lines were exposed for 16 h to 60 μ M m⁻² s⁻¹ of light intensity (Sylvania, Grolux, F36W/GRO-T8, Germany).

Fluorimetric GUS assay

Callus and plant tissues were deep-frozen in liquid nitrogen, then ground. Fifty milligrams of tissue powder were homogenized in 1 ml of an extraction buffer containing 10 mM EDTA, 3.4 mM *N*-laurylsarcosine, 0.1% Triton X-100 in phosphate buffer 50 mM pH 7. The suspension was centrifuged at 15,000 rpm at 4°C for 15 min (Gallagher 1992). The protein concentration was determined by the Bradford's method (Bio-Rad Protein assay kit). Two micrograms of protein were adjusted to 2 mM 4-methyl umbelliferyl β -D-glucuronide (MUG). Reactions were incubated for 30 min at 37°C, and fluorescence emission at 460 nm was measured at 10 min intervals following excitation at 355 nm. Protein and GUS analyses have been carried out with the Fluoroskan Ascent's devices and software version 2.4 (Thermo, Electron Corporation, Waltham, USA). For callus tissues, each value was the mean ($\pm$ standard deviation) of three independent samples per treatment. For plant tissues, only one plant was analysed per treatment.

Histological analysis

Roots, stems and leaves from in vitro somatic plantlets were fixed for 24 h in phosphate buffer (0.2 M, pH 7.2) containing 2% paraformaldehyde, 1% glutaraldehyde and 1% w/v caffeine. They were then dehydrated in an alcohol series: 70% ethanol, 60 min; 95% ethanol twice 30 min; 100% ethanol/100% butanol (v/v),

30 min; 100% ethanol/100% butanol (30/70), 30 min; 100% butanol, 30 min; and 100% butanol, overnight. The samples were subsequently transferred for seven days to Technovit 7100 resin (Kulzer, Wehrheim, Germany) and finally embedded in the same material. A double PAS (periodic acid-Schiff)-naphthol blue-black stain was used. Soluble and insoluble proteins were stained blue specifically with naphthol blue-black (Fisher 1968) and polysaccharides were stained red with PAS (Gabe 1968). Slides of 3 µm thick were observed with a DMRXA microscope (Leica, Wetzlar, Germany) under white light. Pictures were taken with a Coolpix 4500 camera (Nikon, Tokyo, Japan). Digital images were edited using Adobe Photoshop 5.0 (Mountain View, CA).

mRNA in-situ hybridization

Parts of *ribosome*, *gusA* and *HEV* cDNAs were PCR amplified with designed specific primers:

Forward *ribosome*: 5' CCAAGTCAGACGAACG ATTTGCACG 3'

Reverse *ribosome*: 5' CCGACCCTGATCTTCTG TGAAGGG 3'

Forward *gusA*: 5' CCTGTAGAAACCCCAACC CGT 3'

Reverse *gusA*: 5' TCTTCGGCGTG GTGTAGAGC 3'

Forward *HEV2.1*: 5' TTGCTGAGCAATGTGGT CG 3'

Reverse *HEV2.1*: 5' CAAGCACCTTTCCACAGG AG 3'

The fragments obtained were used for partial ligation (Lig'n scribe Kit, Ambion Inc., Austin, TX) for 11 min at 20°C, in order to ligate the T7 adapter on one side only of the fragment of interest. Fragments were then amplified a second time by PCR with specific primers to obtain the desired probe: forward or reverse. After purification (Qiaquick PCR Purification Kit, Qiagen Inc., Valencia, CA) the fragments were used to generate sense and antisense digoxigenin-labelled transcripts by in vitro transcription (T7 MAXIScript Kit, Ambion Inc., Austin, TX). Final purification of the probes was carried out using the Rneasy mini kit (Qiagen Inc., Valencia, CA). The final length of the different sequences used as probes were: ribosomal probes (150 bp), *gusA* probes (535 bp), *HEV2.1* probes (350 bp).

Roots, stems and leaves of transformed and control in vitro somatic plantlets were fixed with 4%

paraformaldehyde in phosphate buffer (0.2 M, pH 7.5) for 10 h. The samples were washed in phosphate buffer containing 0.1 M glycine and then dehydrated through a series of ethanol–butanol solutions: 50% ethanol, 30 min; 70% ethanol, 30 min and 1 h; 95% ethanol, twice 30 min; 100% butanol, twice 1 h; 100% butanol, three days. They were then embedded in paraffin (ParaplastPlus®, Structure Probes, West Chester, PA), cut into 8 µm sections and mounted on silanised slides (Dako, Carpinteria, CA). Sections were subjected to de-waxing with Safesolv (Labonord, Templemars, France) and rehydration through an ethanol series: 100% ethanol, three times 10 min; 70% ethanol, 2 min; 50% ethanol, 2 min and two 2 min baths in sterile water. The slides were then subjected to protein digestion with proteinase K (3 µg ml⁻¹) for 30 min at 37°C. They were then washed with PBS plus 0.2% glycine, 2 min, and twice 2 min in PBS only, and finally re-hydrated through an ethanol series: 50% ethanol, 1 min; 70% ethanol, 1 min; 100% ethanol, twice 1 min.

Hybridization was performed overnight at 45°C in a humidity chamber, with a denatured (5 min at 65°C) hybridization mixture: 50% deionized formamide, 10% 10× SSC, 10% Dextran sulfate, 2% Denhardt reagent, 0.1 mg ml⁻¹ tRNA, 200 ng of the probe of interest, adjusted with DEPC water. NTE solution consists of 0.5 M NaCl, 1 mM EDTA in Tris–HCl pH 7.5. The slides were subjected to washing (2× SSC twice 5 min, 0.2× SSC twice 30 min at 50°C, 1× NTE 5 min at 37°C); RNase treatment (NTE, A RNase 20 µg ml⁻¹ 30 min at 37°C); washing (1× NTE twice 5 min at 37°C, 0.2× SSC twice 30 min at 55°C); blocking (1× Blocking Reagent (Roche, Basel, Switzerland) 30 min at 37°C); incubation with antibody (1:500 dilution of anti-DIG (Roche, Basel, Switzerland) 1 h at 37°C); and washing (PBS three times 10 min at room temperature), colour development (100 mM Tris–HCl pH 9.5, NaCl 100 mM, Levamisole (Sigma-Aldrich Co., Saint Louis, MO) 5 mM, twice 10 min at room temperature). Hybridization signals were detected with NBT and BCIP (Roche, Basel, Switzerland) in colour development buffer in the dark at 37°C until the signal appeared (antisense ribosomal probe: 20 min; antisense *GUS* probe: 24 h; antisense *HEV* probe: 24 h). Colour development was stopped by incubation in water and sections were mounted in the Mowiol 4–88 solution (CABIOCHEM, ref.

475904, La Jolla, CA, USA). Slides were observed with a DMRXA microscope (Leica, Wetzlar, Germany) under white light. Pictures were taken with a Coolpix 4500 camera (Nikon, Tokyo, Japan). Digital images were edited using Adobe Photoshop 5.0 (Mountain View, CA).

Results

Production and characterization of the transgenic lines

Fourteen independent transgenic callus lines harbouring the HEV2.1::GUS construct were established within 6 months after *Agrobacterium*-mediated transformation of *Hevea brasiliensis* friable callus line VP10 of clone PB 260. Southern-blot analysis was performed on genomic DNA from transgenic callus lines hybridised with *gusA* and *nptII* probes. This analysis confirmed the presence of T-DNA insertions in all the resistant lines and DNA band patterns showed that HEV2.1::GUS lines were issued from independent transformation events (Fig. 1a, b). Wild-type plants and 3 transgenic 35S::GUS callus lines containing one T-DNA insertion (Blanc et al. 2006) were respectively used as negative and positive controls. With regards to HEV2.1::GUS, eight of them carried 1 copy of the T-DNA, while 2 lines carried 2 copies, 3 lines carried 3 copies and 1 line carried 4 copies (Table 1). After this molecular characterisation, production of transgenic plants was carried out by somatic embryogenesis. Fluorimetric GUS activity was checked in plants before further histological analyses. Four callus lines selected both for their growth and regeneration abilities. GUS activity was recorded in roots, stems and leaves of 1 plantlet for each PIT6A4, PIT5A14, PIT5A17 callus lines (data not shown).

In planta localization of HEV2.1 gene expression

HEV2.1 gene expression was localized by in situ hybridization in wild-type plants using a HEV cDNA-derived probe and in transgenic plants using a *gusA*-derived probe (Fig. 2).

Histological analysis of wild-type vitroplants clearly revealed that primary latex cells are

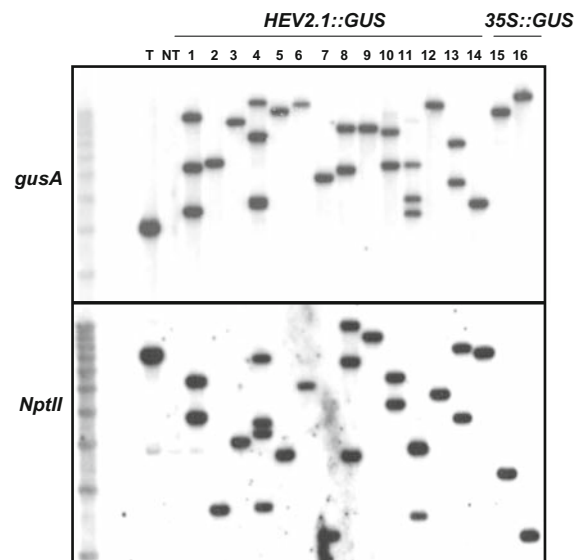


Fig. 1 Southern blot analysis of DNA radiolabelled with (a) *gusA* probe, (b) *nptII* probe. T 35S::GUS transformed control (E4T6A44), NT non transformed tissue (VP10), and transgenic callus lines with constructs HEV2.1::GUS: 1 PIT6A1, 2 PIT6A5, 3 PIT6A3, 4 PIT6A4, 5 PIT5A10, 6 PIT3A7, 7 PIT3A8, 8 PIT5A16, 9 PIT5A11, 10 PIT5A13, 11 PIT5A14, 12 PIT5A17, 13 PIT5A15, 14 PIT5A18, and constructs 35S::GUS: 15 PIT2A19, 16 PIT2A20

differentiated in the phloem of roots, stems and leaves (Fig. 2 a1, b1, c1). In some case these cells anastomose with each other (Fig. 2a2, b2, c2). For each in situ hybridization assay, several controls were prepared. The “hybridized sections without probes” and “sections treated with RNase” controls never showed any signal (data not shown). The hybridization controls with ribosomal probes revealed good RNA preservation with very strong signals after a few minutes of detection for both wild-type and transformed plants (Fig. 2d2, e2, f2). The negative controls with the sense ribosomal probe, homologous to a rRNA fragment, did not show any signal (Fig. 2d1, e1, f1). The *gusA* transcripts were detected in all the cells of the 35S::GUS transformed plants, which were used as positive control (Fig. 2g2, h2, i2). Conversely, *gusA* transcripts were detected specifically in the latex cells of roots and stems from HEV2.1::GUS transformed plants (Fig. 2j2, j3, k2, k3), while it was observed in all cells of leaves (Fig. 2l2, l3). Generally speaking, the labelling obtained by hybridization with the *gusA* probe was more precise (cytoplasmic labelling without

Table 1 Molecular characterisation of transgenic callus lines

Constructs	Lines	Nb of T-DNA copies
None	VP10	0
None	VP10	0
None	VP10	0
None	VP10	0
None	VP10	0
35S::GUS	E4T6A44	1
35S::GUS	P1T2A19	1
35S::GUS	P1T2A20	1
HEV2.1::GUS	P1T6A5	1
HEV2.1::GUS	P1T6A3	1
HEV2.1::GUS	P1T5A10	1
HEV2.1::GUS	P1T3A7	1
HEV2.1::GUS	P1T3A8	1
HEV2.1::GUS	P1T5A11	1
HEV2.1::GUS	P1T5A17	1
HEV2.1::GUS	P1T5A18	1
HEV2.1::GUS	P1T5A13	2
HEV2.1::GUS	P1T5A15	2
HEV2.1::GUS	P1T6A1	3
HEV2.1::GUS	P1T5A16	3
HEV2.1::GUS	P1T5A14	3
HEV2.1::GUS	P1T6A4	4

Number of T-DNA copies was identified by Southern-blot hybridisation of 8 µg of genomic DNA from each line using as probes the 674 bp and 394 bp radiolabelled fragments respectively for *gusA* and *nrpII* genes

diffusion) than with the probe corresponding to the native *HEV2.1* gene (Fig. 2m1, m2, n1, n2).

Regulation of the *HEV2.1* gene promoter by light in rubber tree callus

Given *HEV2.1* transcripts were accumulated in all leaf cell types, the regulation of the *HEV2.1* gene expression by light was tested. Fluorimetric GUS activity was compared on four callus lines cultivated either in standard darkness conditions or after 16 h under light exposure (Table 2). The mean of GUS activity increased significantly from 118 to 402 µM MU/min/mg proteins after light induction. An analysis line by line showed that 2 out of 4 were significantly induced by light. This is consistent with the fact that the promoter of the *Hevea brasiliensis* *HEV2.1* gene directs latex cell-specific expression in

Fig. 2 Histology and in situ hybridization of roots (**a, d, g, j**), stems (**b, e, h, k, m**) and leaves (**c, f, i, l, n**) of control somatic plants (**a, b, c, m, n**), GUS transformed somatic plants (**d, e, f, g, h, i**) and HEV2.1::GUS transformed somatic plants (**j, k, l**). (**a1**) Control plant root cross-section. C: Cortex; Ep: Epidermis; La: Latex cells; VT: Vascular Tissues. (**a2**) Higher magnification of latex cells. La: Latex cells. (**b1**) Control plant stem cross-section. C: Cortex; Ep: Epidermis; La: Latex cells; Ph: Phloem; P: Pith; Sc: Sclerenchyma; VT: Vascular Tissues; X: Xylem. (**b2**) Higher magnification of latex cells. La: Latex cells. (**c1**) Control plant leaf cross-section. La: Latex cells; L Ep: Lower Epidermis; PM: Palisade Mesophyll; SM: Spongy Mesophyll; U Ep: Upper Epidermis; VT: Vascular Tissues. (**c2**) Higher magnification of latex cells. La: Latex cells. (**d1**) (**e1**) (**f1**) No signal with sense ribosomal probe. (**d2**) (**e2**) (**f2**) High specific signal with antisense ribosomal probe. (**g1**) (**h1**) (**i1**) No signal with sense *gusA* probe. (**g2**) (**h2**) (**i2**) Specific signal with antisense *gusA* probe. (**j1**) (**k1**) (**l1**) No signal with sense *gusA* probe. (**j2**) (**k2**) Specific signal with antisense *gusA* probe in latex cells. (**j3**) (**k3**) Higher magnification showing the signal restricted to the latex cells. La: Latex cells. (**l2**) Uniform signal with antisense *gusA* probe in any type of leaf cells. (**l3**) Higher magnification of **l2**. (**m1**) (**n1**) Signal with antisense hevein probe in latex cells. La: Latex cells. (**m2**) (**n2**) Higher magnification of **m1** and **n1**. Bars = 50 µm in (**a2**), (**b2**), (**c2**), (**j3**), (**k3**), (**l3**), (**m2**) and (**n2**), 100 µm in (**c1**), (**f1**), (**f2**), (**g1**), (**g2**), (**i1**), (**i2**), (**j1**), (**j2**), (**k1**), (**k2**), (**l1**), (**m1**) and (**n1**), 200 µm in (**b1**), (**d1**), (**d2**), (**e1**), (**e2**), (**h1**), (**h2**) and (**l2**) and 400 µm in (**a1**)

non-photosynthetic tissues and global expression in chlorophyll leaf tissues.

Discussion

All members of the multigene family encoding Hevein share high sequence homology over their whole transcribed region (Pujade-Renaud et al. 2005). Consequently, functional characterization of the promoters of those genes was an alternative for characterizing the specific expression of each member of that family.

Analysis by in situ hybridization of mRNAs showed that the global expression of *HEV* genes and of the *HEV2.1* gene in particular, was restricted to latex cells in roots and stems of PB 260 plantlets. Co-localization of the global expression of all *HEV* genes, using a non-specific probe designed in the conserved translated region of *HEV* genes, and of the *gusA* gene under the control of the *HEV2.1* gene promoter, showed that *HEV* genes were expressed mostly in latex cells from root and stem, and surprisingly in all cells of leaf tissues. Although the promoter sequences of *HEV* genes were different

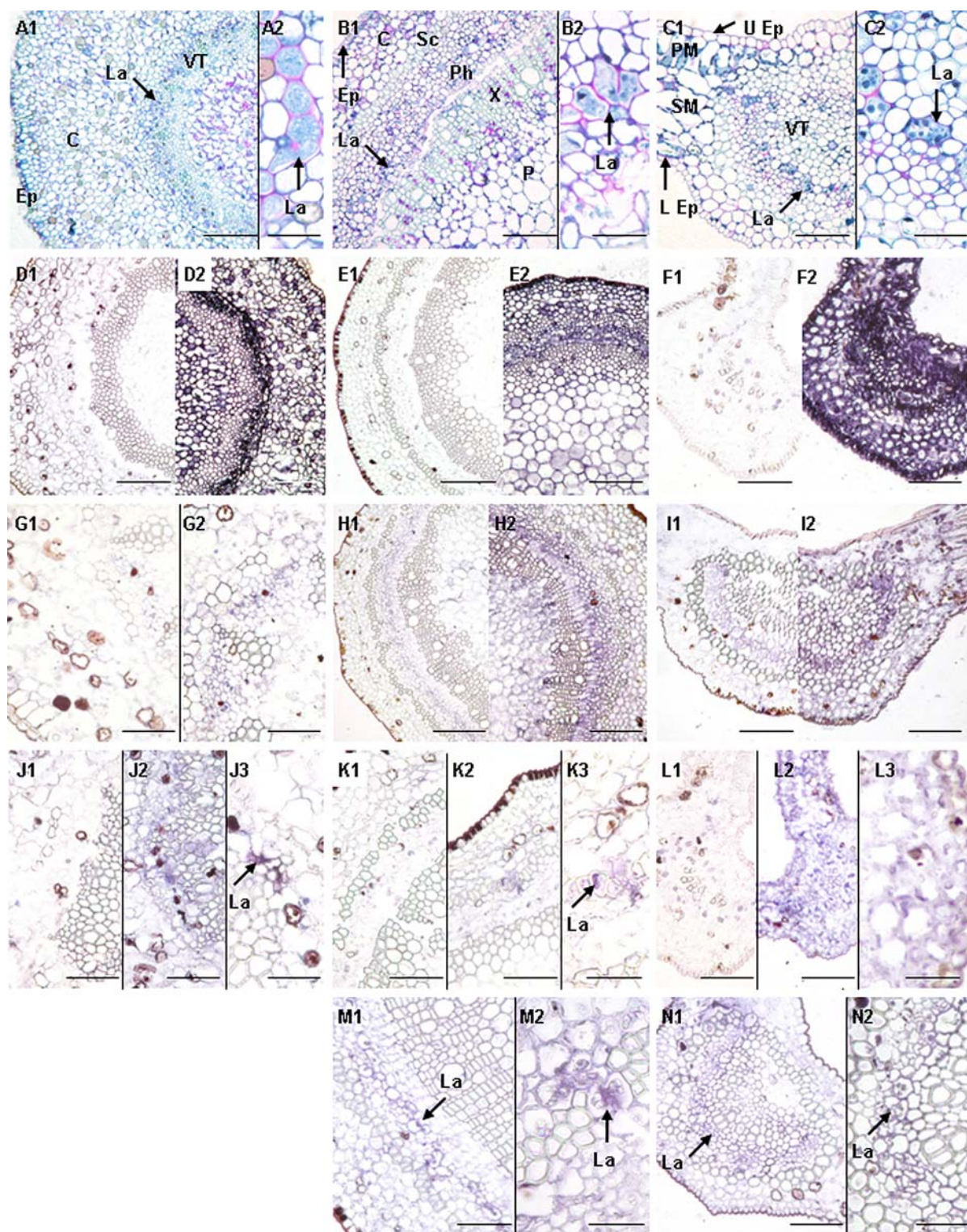


Table 2 Effects of light induction on the fluorimetric GUS activity of HEV2.1::GUS transgenic callus lines

Lines	T-DNA copy (Nb)	GUS activity GUS ($\mu\text{M MU/min/mg protein}$)*	
		Control	Light
PIT5A17	1	233 $\pm$ 12 ^a	183 $\pm$ 210 ^a
PIT5A14	3	60 $\pm$ 94 ^a	131 $\pm$ 22 ^a
PIT6A1	3	62 $\pm$ 32 ^b	681 ^a
PIT6A4	4	117 $\pm$ 127 ^b	613 $\pm$ 310 ^a
Means		118 $\pm$ 81 ^b	402 $\pm$ 285 ^a

Callus lines PIT6A1, PIT6A4, PIT5A14 and PIT5A17 were cultivated for 16 h under light condition before sampling. (*)^{a–b} Fisher test (LSD). All values with the same letter are not significantly different ($P < 0.05$). Each value is the mean of three replications

from each other, the expression of the *HEV2.1* gene has got identical pattern of expression than the other *HEV* genes.

A more precise characterisation under the influence of different effectors might reveal the response specificity of a given promoter. This approach would mean isolating and characterizing each *HEV* gene promoter. An analysis of the expression of promoter-*gusA* fusions might be subject to artefacts and might not reflect precisely the *in vivo* regulation of the genes studied. Elements of sequences upstream of the transcription initiation site (Callis et al. 1987), or in the 5'UTR or even in the intron, which may play a role in native gene regulation (Fu et al. 1995a, b), may be absent from the isolated promoter sequence. Although the sequence of the *HEV2.1* gene is 1835 bp, some regulation elements may be missing.

Analyses of histochemical GUS activity in the transgenic calli and plants of clone PB 260 revealed that the *HEV2.1* gene was expressed in embryogenic calli, roots, stems and leaves. These results confirmed the fluorimetric GUS activity in transgenic plants (data not shown) and the analyses of the overall hevein gene expression by Northern hybridization carried out during the process of somatic embryogenesis and development of young clone PB 260 rubber plants (Montoro et al. 1997). In fact, Northern hybridization gave a global image of the expression of *HEV* genes, as the probe used corresponded to the encoding part of the gene, which is highly conserved in all the members of this multigene family (Pujade-Renaud et al. 2005). Interestingly, Broekaert reported in 1990 that basic

expression of the *HEV1* gene observed in leaves, stems and latex, increased from 2 to 5 times in 24 h after wounding or spraying with ethephon solutions at 0.1% or ABA at 50 μM , but no expression was detected in the roots of 4-month-old clone RRIM 600 seedlings (Broekaert et al. 1990). Expression of *HEV* genes in roots therefore seems to be dependent either upon a genotype effect or the physiological state or developmental stage of the roots.

For the first time, to our knowledge, we demonstrate that the *HEV* genes are expressed in all cell types of leaf tissues. These results are consistent both with data on callus response to light and with *in silico* analysis of the sequence of the *HEV2.1* gene promoter comprising 7 I-boxes et 36 GT1 consensus boxes potentially involved in regulation by light (Pujade-Renaud et al. 2005). Thereby the regulation of the *HEV2.1* gene expression in latex cells is also likely prone to trans-regulation involving light induction.

Using genetic modification is no trivial matter for the rubber tree. The development of an efficient procedure has required sustained efforts since 2000 (Montoro et al. 2000; Rattana et al. 2001; Montoro et al. 2003; Blanc et al. 2006). This functional analysis demonstrates that the *HEV2.1* promoter has the ability to direct a strong gene expression in latex cells of root, stem and leaf. This promoter is also inducible by light, and consequently drives expression in all cells of leaves. By taking this characterization further, it might be possible to specify the role of hevein in latex and leaves, which is already known to be involved in the coagulation of rubber particles and defence against fungi (Gidrol et al. 1994; Van Parijs et al. 1991). For instance, the role of external stresses (wounding, ethephon or ethylene) might be informative to have a better understanding of its regulation under exploitation of rubber trees. This promoter will be also useful for applications in genetic engineering programmes of rubber tree, in particular to control the expression of gene of interest in latex cells.

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