

Molecular characterization of new members of the *Hevea brasiliensis* hevein multigene family and analysis of their promoter region in rice

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Abstract

The cloning of hevein genes from *Hevea brasiliensis* was undertaken with the objective to isolate useful promoters to drive transgene expression in genetically engineered rubber tree. Four different full length genes were cloned by library screening and a fifth, a partial gene, by adaptor-anchored PCR. Sequence alignment revealed that hevein genes, although highly conserved in their transcribed region, diverged in two groups, with major differences in their promoter region, suggesting a more rapid evolution of the upstream regulatory functions of the genes than the downstream functions of their protein products. The promoter regions from two hevein genes representative of each group were isolated and analyzed in rice. Although both were functional, only the longest promoter sequence (PHev2.1) conferred a high level of expression to the transgene in various tissues of this heterologous host. It was in addition up-regulated by mechanical wounding and fungal infection in leaves. A number of potential cis-regulatory elements were identified in silico and are discussed in view of the expression profiles observed in rice.

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1. Introduction

Hevea brasiliensis (Willd. Ex Adr. Juss), a tropical perennial Euphorbiaceae originating from the Amazon, is by far the main species exploited for the production of natural rubber in the world. In the bark, latex vessels formed by reticulated chains of anastomosed cells are dedicated to the synthesis of rubber particles (*cis*-1,4 polyisoprene chains surrounded by a single layer membrane). Upon tapping (excision of a thin layer of the trunk bark about 1 mm thick), the latex vessels are severed and the latex flows out until

latex coagulation processes occur to plug the wound. Reconstitution of the expelled latex is ensured by an active in situ regeneration metabolism [1].

Hevein is one of the major latex proteins that accumulates inside vacuolar elements known as luteoids where it represents about 50–70% of the soluble proteins [2]. It is a small chitin binding protein belonging to the lectin superfamily [3,4]. The mature hevein originates from a 204 amino acids precursor protein which is co- and post-translationally processed, giving rise to 2 distinct domains [5,6]: the 43 amino acids N-terminal chitin-binding domain (mature hevein), and a 144 amino acids C-terminal domain homologous to the wound-inducible proteins WIN1 and WIN2 from potato [7] and to type IV pathogenesis-related

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proteins (PR-IV) isolated from tobacco and tomato [8,9]. According to the classification established by Van Damme et al. [3], prohevein can thus be classified as a PR4 class I protein characterized by the presence of a chitin-binding N-terminal domain. A single full length cDNA sequence encoding the hevein precursor protein (GenBank M36986) was provided by Broekaert et al. [5] and used to demonstrate that rubber tree hevein genes are expressed at a very high level in the latex tissue compared to leaves. Furthermore it was demonstrated that the hevein genes are induced by wounding, ethylene, and ABA treatment [5]. In addition to sequence homologies with PR proteins, several evidences suggest that hevein plays a role in defense. First, hevein has the capacity to inhibit fungal growth as demonstrated using an in vitro assay [10]. Second, recombinant hevein when expressed in transgenic tomato plants, although poorly cleaved, displayed effective anti-fungal properties [11]. Third, Hevein has been proposed to participate in the latex coagulation processes, when released in the cytosol, by fixing the *N*-acetyl-D-glucosamine moiety of a receptor protein located at the surface of the rubber particles, thereby promoting the agglutination of rubber particles and the plugging of the tapping cut [12]. In summary, hevein is proposed to participate in the protection of the rubber tree trunk tissues, recurrently severed by the tapping process, by allowing the sealing of the tapping cut through latex coagulation processes and by preventing pathogenic infection.

We have focused on the genes encoding the hevein precursor as a source of potentially useful promoters for the control of transgene expression in rubber tree latex. In this paper we report the cloning of several genes encoding hevein precursor proteins, demonstrating that hevein originates from a multigene family. A detailed sequence comparison of various members of the hevein family is provided and discussed in terms of their evolution. In addition, the 5' upstream regions from two different members of the family were isolated and fused to the uidA (*gus*) reporter gene. As rubber tree genetic engineering was not routinely available at the time, they were introduced into heterologous host plants, namely rice and *Arabidopsis*, model systems for the monocotyledonous and dicotyledonous plants respectively, to test their functionality as promoters. This is justified since many aspects of abiotic and biotic mechanisms of stress perception, signal transduction and defence gene activation are conserved in monocots and dicots [13–15]. Rice offered the advantage of a well-established and highly efficient genetic transformation procedure, routinely conducted at CIRAD for functional genomics [16,17]. It proved to be a handy and reasonably quick system for preliminary investigation of the hevein gene promoters functionality. Although rice is neither a dicotyledonous nor a latex plant, interesting expression features were revealed which are presented in this paper. These results will later be compared to the expression profiles analyzed in *Arabidopsis*, a model system

for the dicotyledonous plants, and ultimately in the hevein genes natural environment, the rubber tree.

2. Material and methods

2.1. Cloning of *Hev1.1*, *Hev1.2*, *Hev2.1* by library screening and isolation of the 5' upstream region

Genomic DNA was extracted from young leaves of *H. brasiliensis* cultivar RRIM 600 using the method described by Dellaporta et al. [18]. A genomic library was constructed by ligating *Eco*RI-digested genomic DNA to the *Eco*RI site of the Lambda Zap II vector (Stratagene). Phages were packaged using the Stratagene "Gigapack III Gold Packaging extract" and plated in the host strain XL1-blue MRF, as described by the manufacturer. Two rounds of screening were performed using a 1 kb cDNA probe corresponding to the full length hevein cDNA [5]. Hybridization was performed overnight at 65 °C in 5×SSC, 10×Denhardt's reagent, 7% SDS, 20 mM sodium phosphate buffer pH 7.2 and 100 µg ml⁻¹ denatured salmon sperm DNA [19]. Final washes were carried out at 65 °C in 0.1×SSC and 0.5% SDS. From the selected phage colonies, the pBlue-Script SK(-) phagemids containing the cloned genomic inserts were excised and transferred into *E. coli* XL1 blue. The restriction mapping of the selected phagemids was performed using the enzymes *Kpn*I, *Eco*RI, *Sac*I, *Hind*III, *Pst*I and *Xho*I in TA buffer [19] at 37 °C. The restriction fragments were analyzed by Southern hybridization using two probes successively: a 91 bp fragment located at the 5' terminus of the hevein cDNA (GenBank accession M36986) including 82 bp of the coding sequence, and the last 339 bp of the cDNA 3'UTR. *Hind*III subclones were generated in pBlue-Script SK from four phagemids differing in their restriction maps (pBS-Hev1.1, pBS-Hev1.2, pBS-Hev2.1 and pBS-Hev2.2) and sequenced on both strands.

Two clones, pBS-Hev1.1 and pBS-Hev2.1, were chosen for PCR amplification of the hevein gene 5' upstream region. The forward primer SCH-S2 (5' AACAGCTATGACCATGATTAC 3') was designed from the pBlue-Script SK phagemid vector 12 bp upstream of the T3 promoter. The reverse primer SCH-R2 (5' TCAGATCTCCATTCTTCCCAATTCTTG 3'), tailed with a *Bgl*III artificial restriction site, matched the Hev2.1 hevein gene sequence at positions +48 to +27 from the transcription start site, 6 bp upstream of the ATG described by Broekaert et al. [5] as the translation initiation codon. Amplification was performed in a Perkin Elmer thermocycler, in a 50 µl final volume, using 2 ng of *Kpn*I-linearized phagemid as matrix, and DNA polymerase mix and buffer from the Expand High Fidelity PCR System (Boehringer) supplemented with MgCl₂ (3 mM). Amplification was performed over 35 cycles under the following conditions: denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and elongation at 72 °C for 2 min. The PCR fragments were ligated in pGEM-T easy vector (Promega).

The new constructs, named pGEMT-PHev1.1 and pGEMT-PHev2.1, were then restricted using the enzymes *Hind*III and *Bgl*II. The fragments corresponding to the promoter regions were isolated and ligated into the *Hind*III/*Bgl*II-restricted and dephosphorylated vector pCambia 1381Z (Cambia, Canberra, Australia) bearing the uidA (*gus*) reporter gene. The new constructs, named pCambia-PHev1.1 and pCambia-PHev2.1, were introduced into *Agrobacterium* cells (strain LBA 4404) by electroporation. The constructs were re-extracted from the *Agrobacterium* cells using a QIAGEN plasmid extraction system and checked both by restriction profile analysis, using the enzymes *Xho*I and *Eco*RV, and by sequencing.

2.2. Cloning of HevP (PHev2.3) by adaptor-anchored PCR

High-molecular weight DNA was isolated from young leaves of RRIM 600 (*H. brasiliensis*) using the method described by Dellaporta et al. [18]. Genomic mini-libraries were constructed using the Hevea genomic DNA by using the Universal GenomeWalker™ Kit (Clontech Laboratories, Inc, CA, USA). The mini-libraries were used as template DNA for the isolation of an hevein upstream sequence by PCR. The oligonucleotide primers were synthesised by Operon (Operon Technologies Inc, USA).

The primers used for the primary PCR reaction were the AP1 Genome Walker primer from Clontech (5' GTAA-TACGACTCACTATAGGGC 3') and the antisense primer GSP3 (5' CAGGTGAACAATATTCATCAGTGGAGC-CAC 3') located on the hevein cDNA at position [+205, +176] from the transcription start. DNA amplification was carried out in a 50 µl volume containing 5 µl of 10× Tth PCR reaction buffer (Clontech), 2.2 µl of 25 mM Mg(OAc)₂, 1 µl of 10 µM of each primer, 1 µl of 10 µM each dNTPs, 1 µl of Advantage Polymerase Mix (2.5 units) (Clontech) and 1 µl of the genomic mini libraries. The PCR reaction was performed in a DNA Thermal Cycler 480 (PE Biosystems), as follows: 7 cycles at 94 °C for 25 s and 72 °C for 3 min; 32 cycles at 94 °C for 25 s and 67 °C for 3 min; 67 °C for an additional 7 min after the final cycle.

A secondary PCR reaction was performed using a 1:50 dilution of the primary PCR product. The primers used were the AP2 Genome Walker primer from Clontech (5' ACTATAGGGCAGCGTGGT 3') and the antisense primer GSP2 (5' GTGGAGCCACACCACCCCACTGGCTA 3') designed from the hevein cDNA sequence, at position [+185, +159] from the transcription start. DNA amplification was carried out as previously described using 1 µl of the diluted (1:50) primary PCR product as template DNA. The PCR reaction was carried out as follows: 5 cycles at 94 °C for 25 s and 72 °C for 3 min; 20 cycles at 94 °C for 25 s and 67 °C for 3 min; 67 °C for an additional 7 min after the final cycle. The secondary amplified product (1.3 kb) was cloned into TOPO Cloning TA vector pCR®2.1TOPO® (Invitrogen, USA) and transferred into *E. coli* DH5α. The 1.3 kb insert was released by restriction using the enzyme *Eco*RI

and was then sequenced using the universal M13 Reverse and Forward primers.

2.3. Identification of the transcription initiation site by primer extension

Total RNA was isolated from latex of the RRIM 600 cultivar according to the method of Prescott and Martin [36]. Primer extension was carried out with latex total RNA using the SMART cDNA Synthesis by Primer Extension system (Clontech Laboratories, Inc, USA), using SMART III and CDS III/3' PCR primers in the first strand cDNA synthesis. The reverse transcriptase used was MMLV reverse transcriptase (Clontech Laboratories, Inc, USA). The ds cDNA synthesis by primer extension was carried out using the 5' PCR primer from Clontech (5' AAGCAGTGGTATCAACGCAGAGT 3') and GSP1 hevein specific primer (5' CTGGCTACAACATAGGT-TATTGGGGCAGAG 3') located near the translation start site of the cDNA, at position [+165, +136] and common to all the hevein sequences analyzed. The polymerase used was Advantage 2 Polymerase Mix (Clontech Laboratories, Inc, USA). The primer extension was commenced using GeneAmp 2400 with the following program: 72 °C for 10 min; 95 °C for 25 s; 3 cycles at 95 °C for 6 s and 68 °C for 8 min.

On the completion of the reaction, 5 µl sample of the product was analyzed on a 1.1% agarose gel alongside a 1 kb Plus ladder (Gibco BRL). The amplified product (0.2 kb) was purified and cloned into TOPO Cloning TA vector pCR®2.1TOPO® (Invitrogen, USA) and transferred into *E. coli* DH5α. Plasmid extractions were performed using Qiagen Plasmid Mini Kit (Qiagen Inc, USA) and the insert fragment was amplified using the universal M13 primers. The primer extension product was then sequenced using M13 primers.

2.4. Genetic transformation procedure

The Japonica rice (*Oryza sativa* L.) cultivar Nipponbare was transformed via *Agrobacterium tumefaciens* following the procedure described by Sallaud et al. [16]. T0 transgenic plants presenting a single T-DNA insertion, as determined by Southern blot analysis (see below), were transferred to the green house and grown for seed production (T1 population). The growth conditions were strictly controlled: 25 °C, 75% humidity and a photoperiod of 11 h 30 min per day under artificial light (400 µmol E m⁻² s⁻¹). Seeds were collected and dried at 37 °C for 3 days, then kept at room temperature. For the selection of hygromycin-resistant T1 lines (transformed plants of second generation), seeds were surface-sterilized and placed in a Petri dish on Whatman paper previously soaked with 50 µg ml⁻¹ hygromycin. After 5 to 7 days, germinated seeds were transferred to the green house.

2.5. Identification of transgenic rice plants with a single T-DNA insertion by Southern blot analysis

Genomic DNA was extracted from the hygromycin-resistant transgenic rice plants following a MATAB/CETAB procedure. Samples of 1.7 µg of genomic DNA restricted with *Bam*HI were separated on 1% agarose gel, transferred onto nylon membranes (Hybond N+, Amersham) under alkaline conditions and probed using a *Bgl*II/*Sph*I fragment corresponding to the *uidA* (*gus*) reporter gene from the plasmid pCambia1381Z. Hybridization was performed overnight at 65 °C in 50 mM Tris–HCl pH 8, 10 mM EDTA pH 8, 5×SSC, 1×Denhardt's reagent, 0.2% SDS, 10% dextran sulfate and 100 µg ml^{−1} denaturated salmon sperm DNA. Final washes were carried out at 65 °C in 0.1×SSC and 0.1% SDS.

2.6. Analysis of the β-glucuronidase activity

Histochemical and fluorimetric analyses of β-glucuronidase (GUS) activity were performed as described by Jefferson et al. [20]. For the fluorimetric analysis, explants were frozen in liquid nitrogen and stored at −80 °C. Total soluble proteins were extracted by grinding 200 mg of sample in 500 µl extraction buffer (50 mM Tris–HCl pH 8, 1 mM EDTA pH 8, 5% v/v glycerol, 1 mM DTT, 0.1% v/v Triton). After centrifugation at 20000×g for 15 min at 4 °C, the supernatant was collected and centrifuged again for 5 min in the same conditions. Proteins from the supernatant were quantified [21] using a Multiscan RC (Labsystems) spectrophotometer, coupled to the software Genesis version 2.00 (Labsystems). For the fluorimetric analysis, the fluorescence of 4-MU (4-methylumbelliferone) generated after the cleavage of the 4-MUG (4-methylumbelliferyl β-D-glucuronic acid) substrate by the GUS enzyme was measured using the fluorimeter “Fluoroscan II version 6.3 (Labsystems)” coupled to the software Genesis version 2.00, using wavelengths fixed at 365 nm and 460 nm for excitation and emission respectively.

2.7. *PHev1.1* and *PHev2.1* functionality assays in transgenic rice

Only transgenic rice lines carrying a single T-DNA insertion were analyzed. Each transgenic line was represented by 3 T1 progenies for each condition. T1 seeds were germinated in vitro in the presence of hygromycin then transferred to soil in a green house. Fluorimetric analyses were performed on 4–5 week old T1 seedlings.

2.7.1. Developmental gradient

Six different transgenic lines were analyzed, each line being represented by 3 T1 progenies. For each T1 plant, three different leaves (upper, intermediate and lower) were analyzed. Measurements were performed on 2-cm-long

leaf pieces cut at the tip (T), middle (M) and base (B) of the leaf blade.

2.7.2. Wounding

Mechanical wounding was performed by pricking the whole surface of intermediate leaves with needles. Four different transgenic lines were analyzed. For each line, 7 batches of 3 T1 plants were used. For one batch (T₀), the full intermediate leaves were collected before any wounding. Five other batches (T₁, T₃, T₆, T₁₀ and T₂₄) were submitted to mechanical wounding by pricking with needles the whole limb surface of the intermediate leaves, then the wounded leaves were collected respectively 1, 3, 6, 10 and 24 h after wounding. For the last batch (T₁₀S), the first and third leaves were wounded, then the intermediary unwounded leaf was collected 10 h after wounding in order to test for systemic induction in response to wounding. Fluorimetric analyses were performed on whole leaves to avoid artifacts linked with the existence of an expression gradient along the leaves.

2.7.3. Fungal infection

Six transgenic lines represented by 3 batches of 3 T1 progenies were analyzed. For each line, one batch was inoculated with spores (100 000 per ml, in 0.5% gelatin) from the fungus *Magnaporthe grisea* strain TH16 which generates rice blast disease symptoms of medium severity on the rice variety Nipponbare. One full leaf was collected for analysis 4 days after inoculation (I₄). The two other batches were sprayed with 0.5% gelatin only and one full leaf per plant was collected for analysis, either immediately (C₀) or 4 days after treatment (C₄).

2.8. In silico search of putative regulatory elements

The hevein gene promoter sequences and 5'UTR were scanned for the presence of cis-elements identical with or similar to the motifs registered in two different plant cis-acting regulatory elements databases, PlantCARE (<http://intra.psb.ugent.be:8080/PlantCARE>) and PLACE (<http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html>), using the database associated search tools.

Motif sampler (<http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html>), a matrix-based Gibbs sampling method [22], was used to detect overrepresented motifs in a set of sequences corresponding to the promoter and 5'UTR of the hevein genes *Hev1.1*, *Hev1.2*, *Hev2.1*, *Hev2.2*, *Hev2.3* and *AF327518*, using *Arabidopsis thaliana* genome as background model. Motif sampler analysis was reiterated one hundred times with each set of sequences. The parameters applied were 8 nt motif length, 0.5 probability of finding 1 motif instance, and 4 different motifs to find per analysis. Motifs appearing with a high frequency (10 to 45 times, out of 100 reiterations) were searched for homology (pattern matching) in the PLACE database using the PLACE Homology Search Program.

3. Results

3.1. The hevein multigene family

We have isolated 5 different genomic sequences encoding hevein precursors, demonstrating that hevein is encoded by a small multigene family in the rubber tree. Four clones (Hev1.1, Hev1.2, Hev2.1 and Hev2.2) isolated by library screening include the full transcribed region together with a 5' flanking sequence ranging from about 300 bp to 1.8 kb depending on the clone. Their complete nucleotide sequence is registered in GenBank under the accession number AY247787, AY247788, AY247789 and AY247790 respectively. A fifth clone (HevP, or PHev2.3, AF287016) including the leader peptide and 5' flanking region of another hevein gene (Hev2.3) was isolated by adaptor-anchored PCR. The transcription start site was identified by primer extension and is located 54 nt upstream the ATG codon previously described as the hevein mRNA translation start point (GenBank, M36986).

These five sequences were aligned together with a partial genomic sequence registered in GenBank (AF327518) by Deng and co-workers (Fig. 1). As deduced from the four full length sequences, the genes encoding hevein precursors appear highly conserved in their downstream region, with

93 to 100% identity depending on the domains analyzed, but highly divergent in their 5' upstream region, apart from a 40 bp conserved domain located immediately upstream of the transcription start site 100% conserved in all six sequences. Removing this 40 bp conserved domain allowed the classification of hevein genes into two groups. Members of group I (Hev1) versus group II (Hev2) share only 45–47% identity in their 5' upstream region. Whereas the members of group II (Hev2.1 and Hev2.2) appear almost identical over the whole gene sequence (99–100% identity), higher divergence is observed within group I, with 97% identity between the Hev1.1 and Hev1.2 upstream regions and down to 93% identity over the first exon. It is worth mentioning that all four genes are 100% conserved in the intron sequence, whatever their group of classification.

According to this classification based on sequence homology in the promoter region, HevP and AF327518 clearly belong to group II hevein genes, although they possess a 23 and 32 bp deletion respectively when compared to Hev2.1. When these deletions are excluded from the analysis, HevP and AF327518 share 96 and 99% identity respectively with the Hev2.1 promoter region.

3.2. Analysis of the promoters functionality in rice

Transgenic rice lines carrying the uidA (gus) gene under the control of the promoter sequences PHev1.1 and PHev2.1 were produced. Lines carrying the 35 S:gus construct were used as control.

Histochemical analysis of GUS activity in transgenic plants of first generation (T0) demonstrated that both constructs were functional in this heterologous system and expressed in various tissues (Fig. 2). In case of the longest promoter sequence (PHev2.1), GUS activity was observed in the pollen bags (Fig. 2a and b) and in 5–10% of the pollen grains (Fig. 2e). Patches of coloration were frequently observed on the lemma and palea, on the seed tegument, and occasionally in the scutellum (Fig. 2a and c). In roots, GUS expression was observed in the vascular tissues (Fig. 2d) in all plants tested, but in some of the roots only. In leaves, an intense blue coloration was observed in wounded tissues (cut edge or needle impacts) for all of the eight plants analyzed indicating that PHev2.1 is a functional promoter fragment containing wound-inducible elements active in rice (Fig. 2f and g). In case of PHev1.1, GUS activity was detected in some of the plants only (5 plants out of 9), in root vascular tissues (in 3 plants only, Fig. 2j) and in wounded leaves (Fig. 2k and l). In the flowers, the coloration was limited to the lemma and palea (Fig. 2h).

Fluorimetric analysis was performed in plants of second generation (T1), on leaves, in order to quantify the level of expression of the promoters and analyze their regulation in response to various effectors. It first revealed that the GUS activity was much higher in plants bearing PHev2.1 than in plants bearing PHev1.1, with an average value of 243

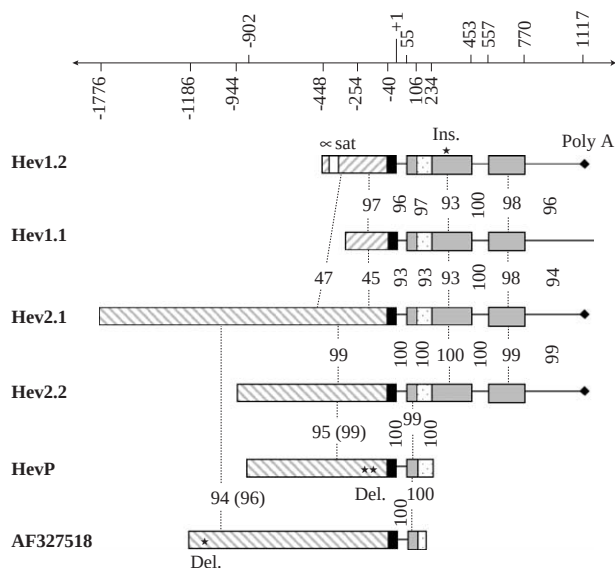


Fig. 1. Sequence alignment of the hevein multigene family. Percentages of nucleic acid identity between two sequences are indicated domain per domain. For the promoter regions, only sequence portions equivalent in size were compared, with the longest sequence shortened from the 5' end to the size of the shortest sequence. Grey boxes, exons; spotted boxes, mature hevein domain; dashed boxes, promoter region; black boxes, conserved domain in the promoter region (100% identity between all six sequences); white box (μ sat), 24 TA repeats microsatellite; + (Ins.), GAGTGG insertion; * (Del.), 23 nt deletion; ** (Del.), 32 nt deletion; $\diamond$, poly A signal. The percentages of identity in the promoter region are calculated without including the conserved 40 nt domain. Numbers between brackets are percentages of identity calculated after removing the sequence portions corresponding to deletions. The scale is numbered from the transcription start site (+1).

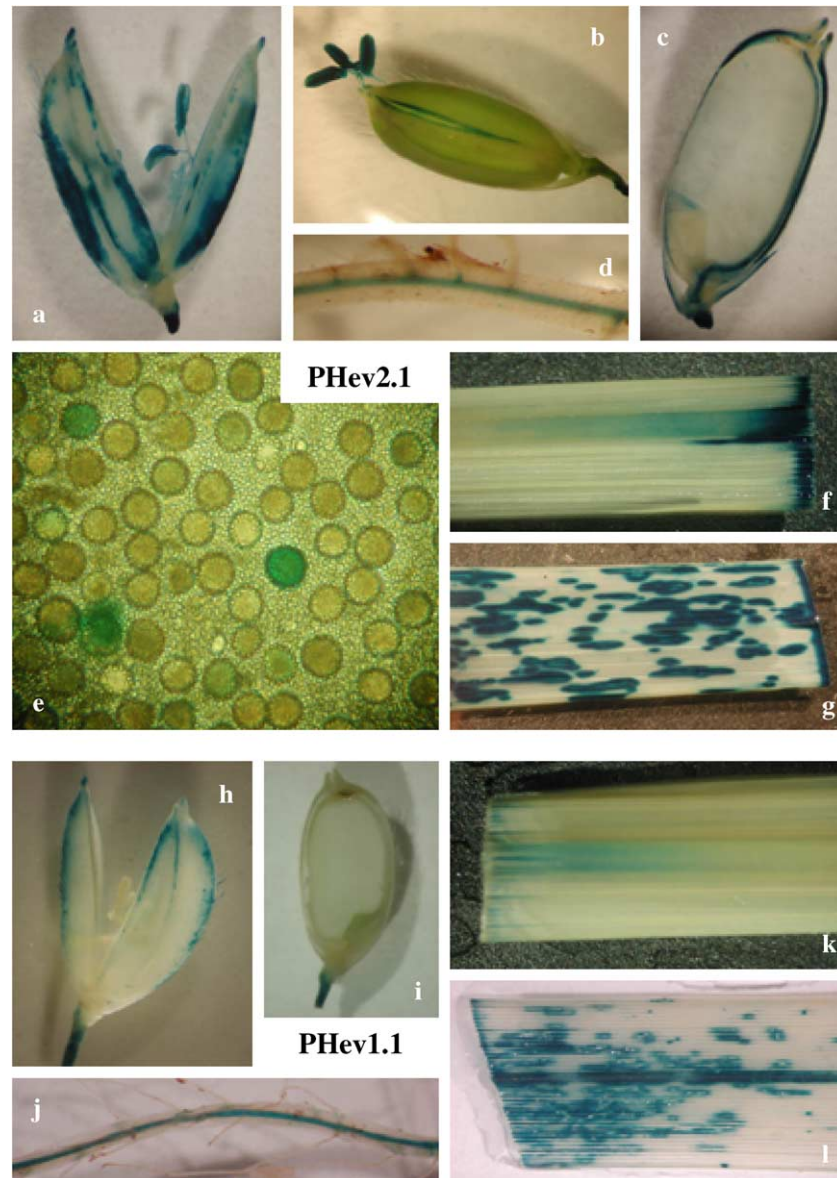


Fig. 2. Histochemical analysis of GUS activity in transgenic rice plants carrying a single copy of the PHev2.1-*gus* and PHev1.1-*gus* constructs. Upper bloc (a–g): PHev2.1-*gus* construct. Lower bloc (h–l): PHev1.1-*gus* construct. Immature flowers (a, h); longitudinal section of an immature seed (c, i); immature seed with pollen bags (b); roots (d, j); pollen grains (e); basal section of unwounded leaves (f, k); section of a leaf wounded with needles (g, l).

against 11 pmol MU/min/μg of proteins. Moreover, all the PHev2.1-carrying plants tested (8/8) expressed the *gus* gene, whereas for the PHev1.1-carrying plants, only two out of the nine tested displayed a measurable expression. Comparison of PHev2.1 with the CaMV 35 S promoter in T1 transgenic rice revealed that the strength of PHev2.1 was about half that of the 35 S promoter (243 against 470 pmol MU/min/μg of proteins in average) under normal (unstressed) conditions.

3.2.1. Developmental gradient

Gus gene expression under the control of promoter PHev2.1 followed a gradient along the leaf blade (Fig. 3A), with up to 1.3 fold higher GUS activity in the basal segment compared to the tip, as analyzed from six different trans-

genic lines. Such a gradient was statistically significant ($P < 0.01$) in the youngest (upper) and intermediate leaves, but not in the older (lower) leaves. No significant overall difference could be attributed to the leaf position, except the erosion of the inner-blade gradient when leaves get older, due to a slight decrease of expression in the basal part of the leaf over time. All subsequent fluorimetric analyses were performed on the whole intermediate leaves in order to avoid potential artifacts linked with the expression gradient along the leaf blade.

3.2.2. Mechanical wounding

The up-regulation of the hevein promoters by mechanical wounding, first observed after histochemical analysis, was quantified by fluorimetric analysis. Four transgenic lines

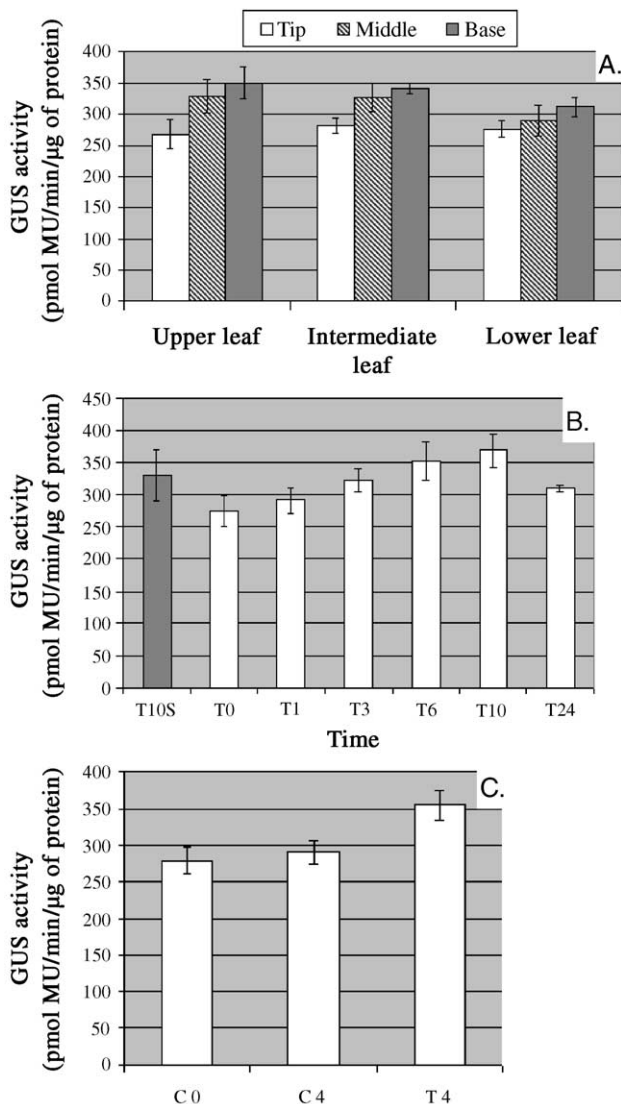


Fig. 3. Fluorimetric analysis of PHev2.1-*gus* expression in transgenic rice. Analyses were performed on transgenic lines carrying a single T-DNA insertion, on T1 progenies. For each motif analyzed, the GUS activity value presented is the average value from 4–6 lines, each line being represented by the average value from three T1 progenies. The statistical significance was evaluated by a Fisher test ($P < 0.01$). (A) PHev2.1-*gus* expression gradient along the leaf blade. Six different transgenic lines were analyzed. For each T1 plant, three leaves (upper, intermediate and lower) were analyzed, with measurements at three different points along each leaf blade: T, tip; M, middle; B, base. (B) PHev2.1-*gus* expression in response to wounding. Four different transgenic lines were analyzed. Wounding was performed by pricking leaves with needles. Wounded leaves were collected 1, 3, 6, 10 and 24 h after wounding (T1, T3, T6, T10 and T24 respectively). T0, unwounded plants. T10S, systemic response of unwounded intermediary leaves located between two wounded leaves and analyzed 10 h after wounding. (C) PHev2.1-*gus* expression in response to fungal infection. Six different transgenic lines were analyzed. Control plants were sprayed with 0.5% gelatine and analyzed at the time point 0 (C₀) or 4 days after treatment (C₄); T₄, plants inoculated with spores of *M. grisea* (TH16) in 0.5% gelatine and analyzed 4 days after inoculation.

bearing the PHev2.1 promoter region were analyzed in the T1 progeny (Fig. 3B). All four displayed a statistically significant ($P < 0.01$) transient increase of the *gus* gene

expression in response to wounding, with a maximum amplitude observed 10 h after wounding (the amplification factor was 1.4 to 1.6). No significant change was observed for the control plants bearing the CaMV 35 S promoter. The level of activity of the wound-stimulated PHev2.1 promoter is thus very close (80%) to the level attained by the 35 S promoter under similar conditions. The PHev2.1 promoter seems to respond systemically to wounding, although the amplification factor, measured from intact leaves framed by wounded leaves 10 h after wounding, was quite low, a factor of about 1.2 on average.

The variations observed after wounding were not an artefactual consequence of a circadian regulation. Indeed, only slight but not significant variations of PHev2.1 activity were measured in unwounded control plants at various time points of the day.

For the only two lines expressing the *gus* gene driven by the short promoter sequence PHev1.1, the expression level was very low and no increase in GUS activity in response to mechanical wounding could be detected by fluorimetric analysis. After histochemical staining, the accumulation of the very stable diXH-indigo crystals (final product of the GUS reaction) over time may compensate for the low expression and account for the apparent wound effect in the plants expressing PHev1.1-*gus* (Fig. 2I).

3.2.3. Pathogens

The inoculation of young T1 plantlets with the fungus *M. grisea* (strain TH16), agent of the rice blast disease, triggered a significant activation of the PHev2.1 promoter, as measured 4 days after inoculation (Fig. 3C). This was observed for all six transgenic lines tested, with amplification factors detected of between 1.2 and 1.4.

3.3. In silico search for potential regulatory elements

Two approaches were used to identify putative cis-acting regulatory elements from the hevein gene promoter sequences.

One approach (“pattern matching”) was to search each query sequence for motifs identical with or similar to previously reported cis-element registered in the PLACE and/or PlantCARE databases. Numerous putative cis-elements, often present in multiple copies, were identified in PHev1.1 and PHev2.1. Table 1 is a non exhaustive list of some of these elements, previously shown to be involved in cell- and tissue-specific expression or various regulatory processes, and which may contribute to the expression profiles observed in rice. The comparison of PHev1.1 and PHev2.1 over a sequence fragment comparable in length in the divergent region (position [−254, −41]) revealed a three fold higher number of putative elements in PHev1.1 compared to PHev2.1 (Fig. 4). Whether or not the shared elements were conserved in the same order along the sequence could not be ascertained, as the frequency of each element varied between the two promoters.

Table 1

Potential cis-acting regulatory elements identified in Phev1.1 and Phev2.1 by in silico “pattern matching” search against PLACE (P) and PlantCARE (PC) databases

Cis-element	Associated transcription factor	Number of copies		Expected function	Database (ID) P: PLACE; PC: PlantCARE	Reference
		PHev1.1	PHev2.1			
GTGA-motif		3	17	Expression in pollen	P (S000378)	[19]
AGAAA-motif		5	15		P (S000245)	[20]
GCN4		1	5	Expression in seeds	PC	[21]
AACA		0	1		P (S000353)	[21]
ACGT		0	1		P (S000278)	[21,22]
Prolamin-box		0	2		PC	[21]
Root-motif (ATATT)		2	17	Expression in roots	P (S000098)	[23]
GT-1	GT factor (Trihelix)	4	36	Cell-type-specific expression	P (S000198)	[24, 25]
WUN-motif		5	17	Wound-regulated expression	PC (BO~WUN-motif)	[26]
W-box	WRKY (Zn-finger)	1	8	Stress-regulated expression	P (S000310; 390; 442)	[27–29]
ERE		1	5	Ethylene-regulated expression	PC (DC~ERE)	[30]

The other approach (“pattern discovery”) used a statistical matrix-based method (Motif Sampler) to identify significantly overrepresented motifs from a set of upstream hevein gene sequences, using *Arabidopsis* genome as background model. Three major motifs were identified (Table 2). The almost palindromic sequence TGAGCT-TAGCTCA is homologous to as-1 elements, binding sites of bZIP trans-activating factors (ASF-1). As-1 elements are characterized by two typical TGAGCT inverted hexamer repeats separated by a spacer of variable length. The CACTTGGC motif contains an E-box (CANNTG consensus) which is the binding site of basic helix-loop-helix (bHLH) or MYC transcription factors. The third motif (AsCAGArA) contains an AGAAA sequence, required for pollen specific expression of the tomato late pollen gene lat52, and a GRWAAW sequence described as the consensus

binding site of GT-1 transcription factors. Degenerate homology was found also with GARE-4”, a Myb binding element required for giberellic acid induction in the promoter of the barley (H.v.) EPB-1 (cysteine proteinase) gene.

4. Discussion

The cloning by library screening of several full length genes encoding hevein precursors revealed that *H. brasiliensis* hevein originates from a multigene family, strikingly conserved over the whole transcribed region, including the intron and the UTRs. However, important divergences were observed in the promoter region, upstream a 40 bp proximal conserved domain identical in all hevein promoter sequences analyzed. Based on these divergences, we have classified the hevein precursor genes in two groups named Hev1 and Hev2. A closer look at putative cis-elements present in the divergent upstream region of Hev1.1 and Hev2.1, representative of each group of hevein gene, over a sequence comparable in length, revealed significant variations both in the number and nature of the elements identified. These observations are in accordance with recent demonstrations that the upstream regulatory regions of genes evolves more rapidly than downstream functions of their protein products. Maslov et al. [23] have shown that duplicated genes in *Saccharomyces cerevisiae* lose 3% of common transcription factors for every 1% divergence of their amino acid sequences. The degenerative complementation model proposes that after gene duplication, each daughter gene retains only a subset of promoter elements compared to the ancestral state [24,25]. At the end of this process, no new functions are gained but rather both genes are required to produce the full complementation functions of the ancestral gene. However, Papp et al. [26] demonstrated that this model could not be the sole mechanism as the number of shared regulatory motifs in duplicated yeast genes decrease with evolutionary time, whereas the total number of regulatory elements remained unchanged. This

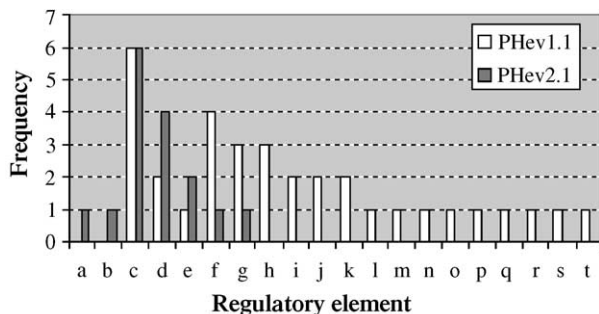


Fig. 4. Comparative analysis of cis-elements identified in silico in the divergent region of Phev1.1 and Phev2.1, over a sequence comparable in length. The query sequence for each gene was a 214 nt sequence located immediately upstream the promoter conserved domain (position [–254; –41]). Pattern matching was searched using the Signal Scan tool against the PLACE database. Elements identified: (a) CIACADIANLELHC; (b) SF1BOXSORPSIL21; (c) DOFCOREZM (equivalent: PYRIMIDINE-BOXOSRAMY1A; NTBBF1ARROLB; TAAGSTKST1); (d) GTGANTG10; (e) INRNTPSADB; (f) GT1CONSENSUS (equivalents: GATABOX; IBOXCORE); (g) CAATBOX1 (equivalent: CCAATBOX1); (h) POLLEN1LELAT52; (i) MYCCONSENSUSAT; (j) NTBBF1ARROLB; (k) ROOTMOTIFTAPOX1; (l) WBOXATNPRI (equivalent: ELRECOREPCRPI); (m) MARTBOX; (n) MYCATERD1; (o) POLASIG2; (p) POLASIG3; (q) RAV1AAT; (r) TATABOX4; (s) TATABOX5; (t) TRANSINITMONOCOTS.

Table 2

Motifs identified in silico using Motif Sampler (“pattern discovery”), in the upstream region of the hevein gene sequences

Motifs identified	Localization	Degenerate homology			Expected function	Reference
		Cis-element	Associated transcription factor	PLACE ID		
TGAGCTTAGCTCA	CD	As-1 elements	BZIP (ASF-1)	S000312; 073	Regulation by biotic and abiotic stresses	[31,32]
CACTTGGC	CD	E-box (CANNTG)	Myc	S000144; 407	Abscisic acid signaling	[33]
AsCAGArA	5'UTR	AGAAA-motif		S000245	Pollen-specific expression	[20]
		GrwAAw	GT-1	S000198	Cell-type specific expression	[25]
		GARE-4	Myb	S000297	Giberellic acid induction	[34]

Degenerate homologies were detected using the “Homology Search” function of the PLACE database. CD, promoter conserved domain. 5c'UTR, 5' untranslated region.

could not be quantified with any precision in our analysis due to the short size of the PHev1.1 promoter sequence. However, the variations we observe suggest that both genes have evolved with specific regulatory features.

Two promoter sequences (PHev1.1 and PHev2.1), representing each hevein group, were isolated in order to analyze their functions in the rubber tree as well as in various heterologous systems. In this paper we present the results of the analyses conducted in rice and we demonstrate that the hevein gene promoters are functional, and to some extent inducible, in monocotyledonous host plant. Promoters from dicotyledonous plants have been previously reported to be functional and similarly regulated in monocotyledonous plants, although with a lower strength, suggesting that the transduction of stress signals in dicots and monocots is conserved [14,15]. The hevein gene promoter constructs are currently being analyzed in the rubber tree as well as in *A. thaliana*, model plant for the dicots.

The 0.3 kb upstream sequence PHev1.1, representative of group I hevein genes, encompasses the full conserved domain that includes the CAAT and TATA boxes as well as potentially important regulatory elements (Table 2). This appeared sufficient to ensure minimal promoter activity in rice. However, PHev1.1 expression level was very low compared to the longest PHev2.1 sequence representative of group II hevein genes, and even undetectable in some plants. The probable variability linked to the T-DNA insertion site or to thin environmental/developmental regulations may explain, on such a weak expression basis, the lack of expression in some plants. Due to its short size, PHev1.1 promoter fragment is probably missing enhancers or regulatory elements necessary to ensure a high level of expression and responsiveness in this heterologous cellular environment.

PHev2.1 on the other hand had a strong and modulated expression level in all transgenic rice lines tested.

4.1. Tissue-specificity and developmental regulation

Tissue-specificity of PHev2.1 expression in rice was not highly restrictive and GUS activity was observed in various organs including leaf blades, root vascular tissues and in

pollen. Previous Northern blotting experiments suggested preferential gene expression of hevein in latex compared to leaves [5]. The reality of hevein gene expression in leaf parenchyma cells was even questioned, due to the presence of latex cells in most organs of the plants, including leaves. However, preliminary indications that the recombinant hevein gene promoters are functional in rubber tree callus (Arokiaaraj, unpublished data; Montoro, unpublished data), together with the expression profile observed in transgenic rice, suggest that the range of tissues and organs expressing the hevein promoters may be larger than expected and not restricted to latex cells only.

In rice, PHev2.1 steady-state expression measured in leaves was very high (half the expression level measured with the CaMV 35 S promoter) and increased after mechanical wounding. The expression gradient observed along the leaf blade suggests a developmental regulation of gene expression with a particularly strong expression observed in the expanding basal leaf cells.

A specific feature of PHev2.1 expression in rice, never observed in plants expressing PHev1.1, was its strong expression in pollen bags and in 5–10% of the pollen grains (Fig. 2a, b, e). The blue coloration in pollen grains cannot be accounted for in terms of diffusion of the diXH-indigo crystals, the product of the GUS reaction, from pollen bag to pollen grains, since staining after pollen expulsion out of the pollen bags similarly revealed the same percentage of stained grains. In addition, the proportion of blue grains observed did not correspond to predicted genetic (Mendelian) segregation ratios, suggesting that hevein promoter expression in pollen may rather depend on the pollen physiological state i.e. whether the pollen grains are maturing or senescing. Interestingly, potential regulatory elements linked with pollen expression were identified, highly repeated, in the hevein promoter sequences. “GTGA” is a conserved motif involved in pollen-specific regulation of the tobacco late pollen gene *g10* which is preferentially and maximally expressed in mature pollen, with bimodal peaks of expression just before and during pollen mitosis I and in mature anthers [27]. Another pollen-related motif AGAAA was also identified in multiple copies, with one repeat matching the motif identified using Motif Sampler in the 5'UTR. This motif is one of two co-dependent

regulatory elements responsible for pollen specific activation of the tomato late pollen gene *lat52* [28]. However, the second *lat52* co-dependent element (“TCCACCATA”) could not be identified in the hevein promoter sequences.

The hevein PHev2.1 sequence contains four motifs GCN4, AACA, ACGT and a prolamins box which are conserved in the upstream sequence of many seed storage proteins genes. It has been suggested that the GCN4 motif acts as an essential element determining endosperm-specific expression and that AACA, ACGT and the prolamins-box are involved in the quantitative regulation of the rice *GluB-1* gene. Low expression in the endosperm of transgenic rice seeds was occasionally observed with PHev2.1 but never with PHev1.1 which contains a single GCN4 motif but lacks the other three motifs.

The specific expression of both PHev1.1 and PHev2.1 in root vascular tissues is consistent with the presence in their sequence of frequently repeated ATATT “root motifs” initially found in the promoter of *rolD* genes which are preferentially expressed in roots compared to leaves, and more specifically in the root elongation zone and vascular tissue.

Concerning tissue-specificity, it is worth mentioning the presence in PHev2.1 of 36 GT1-elements, which potentially bind factors of the GT family. GT factors interact cooperatively with cognate binding factors of other cis-elements and play an important role in modulating cell-type-specific transcription, both positively and negatively.

4.2. Regulation by stress signals

We have demonstrated that the PHev2.1 hevein gene promoter is activated by wounding in transgenic rice consistent with the Northern blot expression profiles previously observed in rubber tree [5]. The systemic feature of the wound-response needs to be confirmed. The highly repeated WUN-motif, described as a wound-responsive element [29], may participate in the regulation of the hevein genes expression by wounding.

The demonstration of pathogen-induced up-regulation of the PHev2.1 promoter in transgenic rice, using spores of the rice pathogen *M. grisea*, brings additional argument supporting the thesis that hevein probably plays a role in Hevea's defence against pathogens [10,11]. The transduction pathways of wounding and biotic stress signals are often intricate and may involve common secondary messengers such as the plant hormones jasmonic acid, ethylene and salicylic acid. A number of potential regulatory elements associated with these transduction hormones have been identified in the hevein promoter sequences, such as the as-1-like motif identified by Motif Sampler (Table 2). As-1 elements are the binding sites of bZIP trans-activating factors (ASF-1) described as being essential for the response to wounding, auxin, methyl jasmonate and salicylic acid [30,31]. Several W boxes were also identified in the hevein promoter sequences. W boxes interact with zinc-fingered

WRKY proteins, an important family of transcription factors playing a role in the response to pathogen infection and other stresses in plants. Potential ethylene-responsive elements (ERE) were also identified, in agreement with previous Northern blot results demonstrating ethylene-induced up-regulation of the hevein mRNA production in rubber tree [5].

The expression features observed in rice now need to be confirmed in the rubber tree. An *Agrobacterium*-mediated transformation procedure initially developed from rubber tree anthers [32], and a newly developed procedure using integument-derived callus (Montoro P., personal communication), now allow efficient production of transgenic rubber tree lines. Therefore, the analysis of deleted constructs in the rubber tree can now be envisaged in order to evaluate the role of the various in silico-identified elements postulated to be involved in hevein gene regulation.

The regulatory features displayed by the recombinant hevein gene promoter PHev2.1 in rice are promising from the point of view of rubber tree genetic engineering objectives. Rubber tree transformation is envisaged as one possible way to improve the yield of natural rubber production through the over-expression of endogenous genes identified as being limiting factors in the metabolism of latex cells, such as in rubber synthesis, or by improving the defenses of the tree thereby indirectly contributing to increases in latex yield. It is also envisaged that the hevein gene promoter will be useful for the production of recombinant proteins of industrial or medical interest in the extractable latex of the rubber tree i.e. using the tree as a biotechnological factory [33–35]. In both cases, optimal expression of the transgene in the latex cells is of particular importance. Considering the strength of the PHev2.1 hevein gene promoter, together with its response to mechanical wounding, high levels of transgene gene expression is expected in the exploited bark tissues of the rubber tree, including in the latex vessels, as a result of the recurrent wound stress imposed on the tissues by the tapping process. In addition, the demonstration that transgene expression under the control of the PHev2.1 promoter was up-regulated by pathogen infection may open the door for applications in genetic engineering programs aimed at increasing the protection against pathogens in the rubber tree or potentially other plant systems.

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