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CHARACTERIZATION OF THE *HEV2.1* PROMOTER IN TRANSGENIC *HEVEA BRASILIENSIS* CALLI AND PLANTS

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INTRODUCTION

Hevein is a major latex component with antifungal properties (Van Parijs et al. 1991). 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 and highly divergent in their regulatory sequences from the fortieth base upstream of the transcription start. As an efficient *Agrobacterium tumefaciens*-mediated transformation procedure was recently established for *H. brasiliensis* (Blanc et al. 2006), the promoter region of the *HEV2.1* gene was analysed in the rubber tree in order both to gain insight into the molecular mechanisms involved in the expression of genes encoding hevein and to direct transgene expression for applications in genetic engineering programmes.

MATERIAL AND METHODS

Vector construction

The construct pCAMBIA-(35S-*nptII*)-(P*HEV2.1-gusA*) used for functional analysis of the hevein gene promoter in the rubber tree is derived from the construct pCambia-P*HEV2.1* described by (Pujade-Renaud et al. 2005). The plasmid pCAMBIA-P*HEV*2.1 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.

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 (Table 1). After acclimatization in the greenhouse, no difference in terms of growth and morphology between wild types and transformed plants was detected.

Southern blot analysis performed on the transgenic callus lines with *gusA* and *nptII* probes confirmed the presence of T-DNA insertions in all the resistant lines and DNA band patterns showed that HEV2.1::GUS lines had independent transformation events: 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. Growth and regeneration characteristics of transgenic callus lines.

Constructs	Lines	Nb of T-DNA copies	Nb plants.Rita^{-1}
HEV2.1::GUS	P1T6A5	1	0
HEV2.1::GUS	P1T6A3	1	0
HEV2.1::GUS	P1T5A10	1	0
HEV2.1::GUS	P1T3A7	1	0.8
HEV2.1::GUS	P1T3A8	1	2
HEV2.1::GUS	P1T5A11	1	1
HEV2.1::GUS	P1T5A17	1	2
HEV2.1::GUS	P1T5A18	1	0
HEV2.1::GUS	P1T5A13	2	0
HEV2.1::GUS	P1T5A15	2	0.3
HEV2.1::GUS	P1T6A1	3	4
HEV2.1::GUS	P1T5A16	3	0
HEV2.1::GUS	P1T5A14	3	11
HEV2.1::GUS	P1T6A4	4	4

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

GUS activity was quantified by fluorimetry on a sample of a few transgenic callus lines available in sufficient quantities for carrying out the various abiotic stress treatments (Table 2). The basic GUS activity level recorded in the lines varied from 60 to 233 µM MU/min/mg protein in HEV2.1::GUS. A significant effect was found for light, Ethrel or ethylene depending on the lines. Knowing that callus are cultured in dark conditions, that was reflected in the mean of the HEV2.1::GUS callus lines through a significant effect of light on GUS activity.

Table 2. Effects of various types of stress on the fluorimetric GUS activity of H2.1::GUS transgenic callus lines. Applied treatments were as follows: light = 8/16 hours photoperiod; Ethrel = 2%; Ethylene = 10 ppm. Fisher test (LSD). All values with the same letter are not significantly different (P <0.05). Each value is the mean of 3 replications. Nd: not determined

Lines	GUS activity GUS (µM MU/min/mg protein)*			
	Control	Light	Ethrel	Ethylene
P1T6A1	62±32[b]	681[a]	441±212[a]	234±225[b]
P1T6A4	117±127[b]	613±310[a]	647±112[a]	208±147[b]
P1T5A14	60±94[b]	131±22[ab]	92±127[ab]	70±120[b]
P1T5A17	233±12[ab]	183 ±210[abc]	232±37[ab]	367±155[a]
Means	118±81[c]	402±285[a]	353±243[ab]	220±122[abc]

Influence of ethylene stimulation in plantlets

Ethylene regulation of the *HEV2.1* gene promoter was evaluated through a preliminary study on young transgenic plantlets 1 month after germination (Table 3). Three plants per line were treated for each of the three regenerating lines used: P1T6A4, P1T5A14, P1T5A17. Of those three lines, two (P1T6A4 and P1T5A14) displayed stronger GUS activity in roots with 171 and 330 MU/min/mg protein respectively. For line P1T5A17, the average GUS activity level was well above the other two lines for all organs, with very strong expression in leaves, at 885 MU/min/mg proteins. Although the response to ethylene was positive on average, it varied depending on the organs and type of treatment (ethylene or Ethrel).

Table 3. Effects of ethylene stimulation on the fluorimetric GUS activity of various HEV2.1::GUS transgenic *in vitro* plantlets. Applied treatments were as follow: Ethrel = plantlets were spayed with 2% Ethrel solution diluted in glycerin; Ethylene = C_2H_4 at 10 ppm. (*) [a-b] Fisher test (LSD). All values with the same letter are not significantly different (P <0.05). Only one measurement was performed on plantlet samples. Nd: not determined

Lines	Organs	GUS activity (µM/MU/min/mg protein) *		
		Control	Ethylene	Ethrel
P1T6A4	Root	171	250	325
	Stem	58	36	105
	Leaf	72	75	84
P1T5A14	Root	330	539	387
	Stem	234	373	646
	Leaf	337	577	429
P1T5A17	Root	574	523	874
	Stem	683	943	Nd
	Leaf	885	815	1270
Means	Root	358±203[a]	437±162[a]	529±301[a]
	Stem	325±322[a]	451±458[a]	376±383[a]
	Leaf	431±415[a]	489±378[a]	594±610[a]

CONCLUSIONS

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 (data not shown). 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). This promoter will be also useful for applications in genetic engineering programmes of rubber tree, in particular to control gene expression in latex cells.

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