Comparison of GUS activity in self-rooting and budded primary transformant plants in Hevea brasiliensis.

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Successful both somatic embryogenesis and Agrobacterium tumefaciens-mediated genetic transformation systems have been developed in PB260 clone. Several transgenic callus lines and plants expressing two reporter genes (gusA and/or GFP) and CuZnSOD gene driven by the CAMV 35S promoter are respectively maintained by cryopreservation and in greenhouse. Functional genomic analysis requires homogenous population of plants for further characterization. However, the primary transformant plants are considered unstable leading to a variation in transgene expression. With regards to this epigenetic control, the variability of the beta-D-glucuronidase activity has been assessed by fluorimetric assays in primary transformant plants both self-rooting and budded state. Our preliminary results show a relative homogeneity of GUS activity in the population of self-rooting primary transformant plants obtained directly by somatic embryogenesis. After green budding, a decrease in GUS activity is recorded leading to a higher variability. The effects of budding, rootstock, rootstock/scion interaction, quality and position of the scion are discussed.

Keyword: beta-D-glucuronidase activity, budding, *Hevea brasiliensis*, genetic transformation, somatic embryogenesis

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Hevea brasiliensis is the main source of natural rubber which is biosynthesized in latex cells. Ethephon application can enhance the biosynthesis activity required for latex regeneration after each tapping, optimizing the yield potential of rubber tree. However, a good management of both tapping frequencies and ethephon applications is required for avoiding excessive metabolic activation which can lead to Tapping Panel Dryness (TPD). Oxidative stress was previously suggested to be not involved in TPD since *Catalase* and *MnSOD* genes were not regulated upon TPD[1]. More recently *CuZnSOD* gene has been shown to be down-regulated in TPD tree compared to its healthy counterpart [2]. This physiological disorder is a consequence of an oxidative stress in the latex cells, leading to membrane damages, flocculation of rubber particles and plugging of the latex vessels [3-4].

With the successful plant regeneration from *H. brasiliensis* PB260 somatic embryos [5] an A*grobacterium tumefaciens*-mediated genetic transformation has been developed [6] and has lead to genetically transformed PB260 plants expressing two reporter genes (*gusA* and/or *GFP)* and a gene of interest (*CuZnSOD)* driven by the *CAMV 35S* promoter.

For functional genomic analysis, the need of a homogenous population of plant is required. Because of the apparent heterogeneity of primary transformant plants, we plan to propagate these plants either by budding or microcutting. The variability of beta-D-

glucuronidase activity has been assessed by fluorimetric assays in the transgenic plants before and after budding.

EXPERIMENTAL

Genetic transformation

Agrobacterium tumefaciens–mediated genetic transformation procedure has been done[6], using the GFP reporter gene [7].

Genomic DNA extraction from calli and Southern blot

Rubber tree genomic DNA was isolated [8] and Southern blot were performed [9].

Production of transgenic plantlets

First somatic embryogenesis process was induced on the expression medium on semi-solid medium, and then induced calli were placed into the RITA (air pulsed temporary immersion container) on the development medium. Well-shaped embryos were then selected and placed on a conversion medium for the germination of the plantlets. The acclimatization was done on the confined greenhouse of type safety 2 (*Figure 1*).

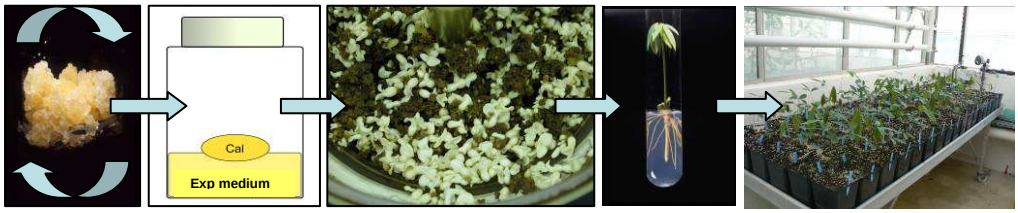

Figure 1: Production of transgenic plantlets from transgenic callus lines.

Plant materials

Some primary transformant plants of the first-generation expressing two reporter genes (*gusA* and/or *GFP)* were propagated by green budding on illegitimate GT1 seedlings coming from CIRAD in French Guyana.

Protein extraction and dosage

After gridding in liquid nitrogen, 100 mg of leaves stage D were mixed with 1 mL of extraction buffer (3.4 mM N-laurylsarcosin, 50 mM phosphate buffer and 0.1% Triton X100, 10mM Na_2EDTA). Cells debris were separated by centrifugation at 15000 rpm at 4°C for 15 minutes. The supernatant is centrifuged again in the same conditions. The protein content is measured in duplicate by Bradford method with a spectrophotometer using a standard curve done with known quantity of bovin serum albumin.

Beta-D-glucuronidase activity

Activity of beta-D-glucuronidase is quantified thanks to its substrate MUG (4-methyllumbelliferyl-beta-D-glucuronide) which is cleaved into a compound MU (4-méthyllumbelliferon) fluorescing at 460 nm after excitation at 355 nm (*Figure 2*).

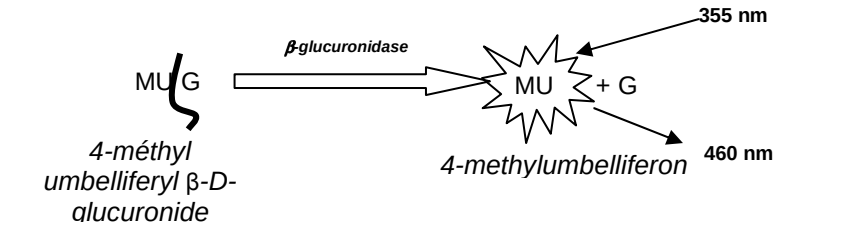

Figure 2: Beta-glucuronidase activity is measured thanks to its substrate MUG. Once it is cleaved, the fluorescent compound MU is released.

The reaction is performed in duplicate at 37°C in 200 µL of extraction buffer, 2 µg of total soluble proteins and 2 mM of MUG. The MU fluorescence is measured by fluorimetry (fluoroscan Ascent version 2.4). The values obtained are converted in MU molarity thanks to a standard curve of known quantity of MU. Activity of beta-D-glucuronidase is expressed in nmol of MU formed per minute and per µg of soluble proteins.

RESULTS

Genetic transformation and molecular analysis of the transgenic lines

An *Agrobacterium tumefaciens*-mediated genetic transformation has been developed [6] based on the successful plant regeneration procedure using *H. brasiliensis* PB 260 somatic embryos [5]. The construct used for generating control plantlet genetically modified, contained two reporter genes *gusA* and *GFP* placed under the control of *CaMV35S* promoter (*Figure 3*).

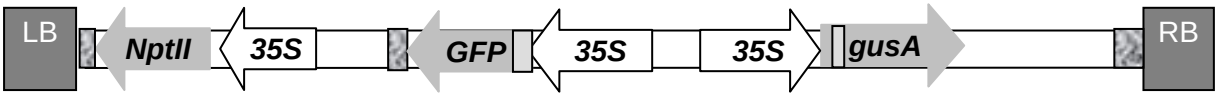

Figure 3: Construct used for generating control plantlets with two reporter genes.

Genomic DNA from independent transgenic callus lines were subjected to Southern-blot hybridisation with at least two probes corresponding to the *gusA*, *NptII* or *GFP* genes. As shown in table 1, a majority of the callus lines contains only one copy of the T-DNA.

Table 1: Summary of Southern blot analysis

	Number of T-DNA insertion		
Constructs	One copy	Two copies	Truncated/multiple
35S::GUS; 35S::GFP	28	2	1

From those callus lines, 6 lines containing one insertion of the T-DNA were selected: TS3T4A24 13.2 and TS3T4A24 13.1 are sister lines, TS3T4Ab 4.4, TS3T4A24 31.2, TS3T7Ac 1.1 and TS3T4A22 6.1.

Characterization of the transgenic plants expressing the two reporter genes

Production of transgenic plants was initiated from a few numbers of selected lines. As shown in table 2, the transgenic callus lines have got the same embryogenic potential as the non-transformed line (wild type) but there is an important decrease in the number of

well-shaped embryos per gram of callus as well as plantlet acclimatized per gram of callus in the transgenic lines compared to the wild type.

Table 2:Comparison of the regeneration data obtained for the 6 selected transgenic lines

N°Lines	Nb embryos/g of callus		Total SE cultivated	Nb plantlet acclimatized / g of callus	Nb plantlet acclimatized
	Total	Well-shaped			
Wild type Line CI05519	275 ± 46 a	59 ± 13 a		13,3 ± 7,5	
TS3T4 Ab 4.4	256 ± 51 a	15 ± 3 bc	213	5,5 ± 1,6	76
TS3T4 A24.13.2	226 ± 52 a	19 ± 8 b	445	8,4 ± 2,8	160
TS3T4 A24.31.2	133 ± 43 b	6 ± 2 c	141	1,1 ± 0,7	20
TS3T7 Ac1.1	45 ± 29 c	7 ± 6 c	137	6,7 ± 5,2	47
TS3T4 A22.6.1	253 ± 92 a	22 ± 11 b	219	5,0 ± 2,7	60
TS3T4 A24.13.1	322 ± 130 a	26 ± 9 b	157	8,3 ± 4,7	58

Quantification of the beta-D-glucuronidase activity in self-rooting primary transformant plantlets.

For each transgenic line, 10 plantlets were submitted to beta-D-glucuronidase activity. The table 3 shows that the activity is different between the lines with three levels statistically different and one line does not show any activity. For the two sister lines, TS3T4 A24-13-1 and TS3T4 A24-13-2, the activity is statistically different.

Table 3: Beta-D-glucuronidase activity (nmol MU.min^{-1}.µg^{-1}) in ten first-generation plantlets for six transgenic lines (Newman - Keuls test P<0.05).

N°line	Mean m	Std error se	dispersion (Se/m)x100
TS3T4A22-6-1	0,196^{a}	0,047	24,2
TS3T4 A24-13-2	0,095^{b}	0,025	26,8
TS3T4 A24-13-1	0,051^{c}	0,014	26,8
TS3T7Ac 1-1	0,051^{c}	0,022	44,1
TS3T4 Ab 4-4	0,041^{c}	0,008	18,2
TS3T4 A24.31.2	0,0000	0,000	

The dispersion analysis shows also heterogeneity between plantlets from the same line (from 24 to 44%).Variance analysis shows that 80% of the total variation of the beta-D-glucuronidase activity can be explained by the variation between independent transgenic lines that is to say by the insertion point of the T-DNA in the genome.

Quantification of the beta-D-glucuronidase activity in budded transgenic plantlets

A population of green budded plant has been done with all buds coming from four plants of the transgenic line TS3T4A24-13-2 budded on illegitimate GT1 seedlings. Table 4 shows a decrease of beta-D-glucuronidase activity in a population of budded plants compared to the one of the first-generation plantlets. Moreover, the heterogeneity is higher in the population of budded plantlets (51.5 %).

Table 4: Beta-D-glucuronidase activity in a population of self-rooting and budded plantlets from the same transgenic line TS3T4 A24-13-2 *(Newman - Keuls test P<0.05)*

TS3T4 A24-13-2		
	self-rooting	budded
Mean	0,095[a]	0,053[b]
Std	0,025	0,028
Dispersion	26,8	51,5

When the four sub-populations of budded plantlet are considered separately, two levels of beta-D-glucuronidase activity statistically different are observed (*Figure 4*). Moreover, the variability in a sub-population of budded plantlet is higher than in the first-generation population.

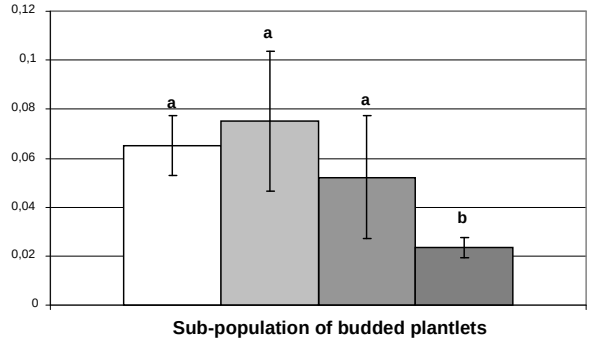

Figure 4: Beta-D-glucuronidase activity in the four sub-populations of budded plantlets for one transgenic line (Newman - Keuls test P<0.05)

DISCUSSION

The present work summarises the results concerning the analysis of the beta-D-glucuronidase activity in primary transformant plants and their budded descendants in rubber tree.

In the first-generation plants, the beta-D-glucuronidase activity allows the discrimination between the independent transgenic lines. As the two sister lines display a level of activity statistically different, it is difficult to dissociate the effect on the T-DNA position in the genome and the physiological state effect.

In budded plantlets, the beta-D-glucuronidase activity decreases and the variability is higher in the budded population than in the first-generation plants. For functional genomic analysis, the need of a homogenous population of plant is required but budding seems to introduce some variability probably due to some parameters such as rootstock, rootstock/scion interaction, quality and position of the scion. This work will be continued with more budded population coming from other transgenic lines and microcutting will also be tested.

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