

SHORT COMMUNICATION

Transient expression of a reporter gene introduced by bioballistic bombardment into *Racosperma mangium* (Leguminosae family) tissues

M. Quoirin¹, F. Aragão², E. Rech² and D.E. De Oliveira³

ABSTRACT

We report on an assay of direct transfer of DNA into calli and seeds of *Racosperma* (*ex-Acacia*) *mangium*, using a bioballistic method. We observed transient expression of the GUS gene in the treated tissues.

INTRODUCTION

Racosperma (*ex-Acacia*) *mangium* is a legume species used in reforestation programs for marginal, low fertility soils. Its N-fixing ability contributes to satisfactory establishment and subsequent growth. *R. mangium* bears phyllodes, has good stem form, superior coppicing ability and great stability during storms. The timber is suitable for general construction, furniture, particle board, veneer and paper pulp. This species is important for reforestation in Northern Brasil, especially where soils are damaged by intensive cultivation or deforestation.

Gene transfer in this species is essential to introduce novel genetic characters such as disease and insect resistance. Gene transformation procedures are

also a means to study the sequences that regulate gene expression in tropical trees.

Leguminous trees are relatively recalcitrant to *in vitro* regeneration. Some success has been achieved with *Acacia koa* (Skolmen and Mapes, 1976), *A. auriculiformis* (Ranga Rao and Prasad, 1991), *A. albida* (Duhoux and Davies, 1985), *A. nilotica* (Dewan *et al.*, 1992) and *A. melanoxylon* (Meyer and van Staden, 1987).

Till now, no transformation experiment has been made with *Racosperma* or *Acacia* species. Transformation and regeneration of *Allocasuarina verticillata* and *Casuarina glauca*, other leguminous trees, have been done with *Agrobacterium rhizogenes* (Phelep *et al.*, 1991; Le *et al.*, 1996).

Microprojectile bombardment technology employs high velocity metal particles to deliver biologically active DNA into plant cells. This method has become widely used for transformation of species that are not susceptible to *Agrobacterium* infection or amenable to protoplast culture, as it is the only procedure capable of delivering DNA into cells in virtually any tissue of any organism. It has been very valuable for transient gene expression (Ludwig *et al.*, 1990) and stable transformation studies (Christou *et al.*, 1988), in a great variety of plant species.

¹ Departamento de Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), Caixa Postal 19031, 81531-970 Curitiba, PR, Brasil. Phone/Fax: 55 41 2662042. E-mail: quoirin@bio.ufpr.br. Send correspondence to M.Q.

² Centro Nacional de Recursos Genéticos (CENARGEN), EMBRAPA, Brasília, DF, Brasil.

³ Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil.

MATERIAL AND METHODS

Seed sterilization

Seeds of *R. mangium* were obtained from the Australian Tree Seed Center, CSIRO. The seeds were scarified for 1 min in boiling water and rinsed thoroughly. They were then disinfected for 10 min in sodium hypochlorite (2% active chlorine) and washed three times in sterile distilled water.

Seed treatment prior to bombardment

After disinfection, seeds were maintained in sterile water for 24 h. After that, the outer envelope was excised. The cotyledons and embryos were transversally cut into two halves, in order to expose the embryo meristem during bombardment.

Seed germination

The seeds were cultured on cotton in glass flasks containing 15 ml of liquid medium. The medium consisted of MS (Murashige and Skoog, 1962), salts and 15 g/l of sucrose. The pH was adjusted to 5.8 prior to autoclaving. Cultures were maintained at $27 \pm 3^\circ\text{C}$ and exposed to a 16-h photoperiod under mixed daylight and Sylvania GroLux fluorescent light ($45 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Callus production

Various kinds of explants were tested for callusing: hypocotyls from one-month old seedlings, adult leaves and roots from *in vitro* germinated plants. They were cut into 5-mm fragments and cultured in 100 x 20-mm petri plates (eight fragments per dish), containing 30 ml of solid medium. The medium consisted of MS salts and vitamins, 30 g/l of sucrose and 6 g/l of agar. 6-Benzyladenine (BA) and α -naphthaleneacetic acid (NAA) were added at 1.07, 2.68 and 10.74 μM for NAA and 0.44 to 22.19 μM for BA (Table I). The pH was adjusted to 5.8 after supplementing the medium with growth regulators. Six plates were used for every medium, giving a total of 48 explants per medium. The cultures were maintained under the same conditions as described for seed germination.

Plasmid construct

Plasmid pBI426 contains a GUS coding region under the control of 2CaMV35S promoters.

Bombardment of tissues

Calli obtained from hypocotyls, and seed halves, were placed on solid medium in 6-cm petri dishes. The seeds were placed with the cut surface upward. The medium consisted of MS salts and vitamins, 30 g/l of sucrose and 7 g/l of phytagel. The bombardments were made twice for each kind of explant.

Apparatus

A helium pressure apparatus was used, as previously described (Aragão *et al.*, 1996). For callus bombardment, two pressures of helium were tested: 600 and 1200 psi. The particle-target distance was 2 cm. In the case of seeds, the same pressures were used, and two distances were tested: 2 cm (level 1) and 4 cm (level 2).

Analysis of GUS expression

GUS expression was assayed in calli and seeds by staining with 5-bromo-3-chloro-2-indolyl- β -D-glucuronic acid (X Gluc) (Jefferson, 1987).

RESULTS AND DISCUSSION

Callus growth

After one month, calli had developed at both ends of the explants (Table I). In all cases, the percentage of explants forming calli was higher than 56%. For hypocotyl pieces cultured on 1.07 μM NAA and 6.66 μM BA, 100% callus formation was obtained and callogenesis was high. The first calli were compact and green. After two months on the same medium, friable and white calli appeared (Figure 1). Both kinds of callus were tested with bombardment. No organogenesis was observed in this type of callus, even after a subculture on the same medium.

Expression of gene *gusA* after bombardment

After 24 h, the expression of gene *gusA* was observed in both types of callus, being stronger in compact calli than in friable ones (Figure 2). The expression observed after four days was very low (number of blue points per callus) and observed in few calli (1/12 friable calli and 2/13 compact). The number of blue points in calli submitted to bombardment at 600

Table I - Callogenesis of *Racosperma mangium* explants treated with α -naphthalene-acetic acid (NAA) and benzyladenine (BA).

NAA concentration (μ M)	BA concentration (μ M)	Adult leaves		Hypocotyls		Roots	
		1	2	1	2	1	2
0	0	-	0	-	0	-	0
0	0.44	+	68.75	+	68.42	+	59.37
0	1.11	+	56.25	++	83.78	-	0
0	4.44	+	78.12	++	80.56	++	93.75
0	22.19	++	87.50	++	91.67	++	90.32
1.07	0.44	++	84.37	n.d.	-	n.d.	-
1.07	2.21	++	100.00	++	92.11	n.d.	-
1.07	6.66	n.d.	-	+++	100.00	+	84.37
2.68	4.44	++	96.88	+++	44.00	++	96.88
10.74	4.44	++	100.00	++	97.43	++	65.62

1. Callogenesis: -, no callogenesis; +, low; ++, medium; +++, high.

2. Percentage of explants forming calli.

n.d. = Not determined.

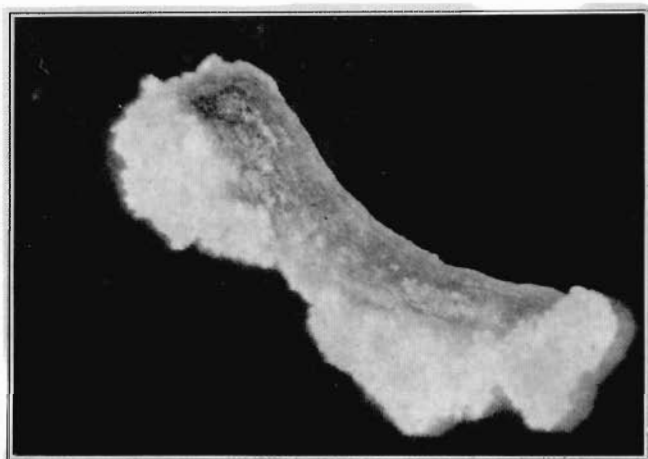


Figure 1 - Calli emerging from *Racosperma mangium* hypocotyl grown on nutrient medium containing 1.07 μ M NAA and 6.66 μ M BA.

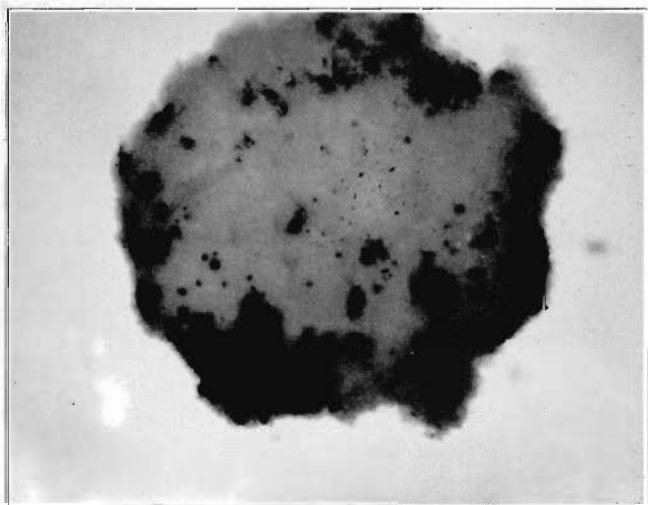


Figure 2 - β -glucuronidase-expressing callus developed from a hypocotyl 24 h after bombardment with pBI1426 containing a GUS gene.

psi vs. 1200 psi was approximately the same, indicating no difference of GUS expression in contrast to other experiments which indicate that transgenic expression

of the GUS gene increases with increasing pressure (Finer *et al.*, 1992). The blue coloration appeared 24 h after particle acceleration in 3/31 seeds at 2 cm and 2/30 at 4 cm (particle-target distance).

Callus formation was easily obtained from all three kinds of explants. Bombardment was effective for DNA transfer into calli and seeds of *Racosperma mangium*, as transient expression of the introduced reporter gene was observed. The compactness of the calli seems to be important to ensure particle introduction into the cells.

The expression of GUS was observed in cotyledon cells as well as in embryo cells of seeds, but not in meristematic cells. The small size of the embryo meristem results in a very low probability that it could be reached by a particle.

ACKNOWLEDGMENTS

M. Quoirin wishes to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a short-duration grant, and the Centro Nacional de Recursos Genéticos - EMBRAPA, for allowing us to make the bombardment assays in its laboratory of Biobalistics.

Financial support for this project was provided by the European Economic Community - DGXII (project TS3 CT94 0278).

RESUMO

Descreve-se um experimento de introdução de DNA em calos e sementes de *Racosperma* (ex-*Acacia*) *mangium*, utilizando um método biobalístico. A expressão do gene marcador GUS foi observada nos dois tipos de tecidos.

REFERENCES

- Aragão, F.J.L., Barros, L.M.G., Brasileiro, A.C.M., Ribeiro, S.G., Smith, F.D., Sanford, J.C., Faria, J.C. and Rech, E.L. (1996). Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. *Theor. Appl. Genet.* 93: 142-150.
- Christou, P., McCabe, D.E. and Swain, W.F. (1988). Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol.* 87: 671-674.
- Dewan, A., Nanda, K. and Gupta, S.C. (1992). *In vitro* micropropagation of *Acacia nilotica* subsp. *indica* Brenan via cotyledonary nodes. *Plant Cell Rep.* 12: 18-21.

- Duhoux, E. and Davies, D. (1985). Caulogénèse à partir de bourgeons cotylédonaire d'*Acacia albida* et influence du saccharose sur la rhizogénèse. *J. Plant Physiol.* 121: 175-180.
- Finer, J.J., Vain, P., Jones, M.W. and McMullen, M.D. (1992). Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Rep.* 11: 323-328.
- Jefferson, R.A. (1987). Assaying chimeric genes in plants: the Gus gene fusion system. *Plant Mol. Biol. Rep.* 5: 387-405.
- Le, Q.V., Bogusz, D., Gherbi, H., Lappartient, A., Duhoux, E. and Franche, C. (1996). *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. *Plant Sci.* 118: 57-69.
- Ludwig, S.R., Bowen, B., Beach, L. and Wessler, S.R. (1990). A regulatory gene as a novel visible marker for maize transformation. *Science* 247: 449-450.
- Meyer, H.J. and van Staden, J. (1987). Regeneration of *Acacia melanoxylon* plantlets *in vitro*. *S. Afr. Tidskr. Plantkunde* 53: 206-209.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Phelep, M., Petit, A., Martin, L., Duhoux, E. and Jacques, T. (1991). Transformation and regeneration of a nitrogen-fixing tree, *Allocasuarina verticillata* Lam. *Biotechnol.* 9: 461-466.
- Ranga Rao, G.V. and Prasad, M.N.V. (1991). Plantlet regeneration from the hypocotyl callus of *Acacia auriculiformis* - multipurpose tree legume. *J. Plant Physiol.* 137: 625-627.
- Skolmen, R.G. and Mapes, M.O. (1976). *Acacia koa* Gray plantlets from somatic callus tissue. *J. Hered.* 67: 114-115.

(Received November 27, 1996)