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# Transient GUS and GFP Expression in Spanish Red Cedar (*Cedrela odorata* L.) Somatic Embryos. Optimization of Bombardment Conditions and Evaluation of Selective Agent Lethal Dose

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*Cedrela odorata* is a tropical tree widely appreciated for its wood. Commercial plantations are frequently hampered by the attack of the meliacea borer, *Hypsipyla grandella*, and the lack of resistant varieties. *C. odorata* traditional breeding would consume very long periods of time, thus direct transfer of entomotoxic coding genes to generate resistant varieties is a promising alternative. There are two prerequisites for gene manipulation of this species: 1) to set the conditions for transgene delivery and 2) to have a way to select regenerating transformed plants. In this paper, we report the optimal biolistics conditions for transient expression of *uid*A and *gfp* reporter genes in *C. odorata* somatic embryos and the selective doses for kanamycin, spectinomycin, phosphinotrycin and hygromycin to screen transformed cells.

*Keywords*: Biolistics; Genetic Transformation; Tree Genetic Modification; Tropical Wood

## Introduction

The establishment of commercial plantations of some tropical hardwood species native to the Americas, such as Spanish red cedar (*Cedrela odorata*), mahogany (*Swietenia macrophylla*), and ipé (*Tabebuia spp.*), among others, faces serious limitations because of the lack of domesticated varieties able to sustain cultivated plantations (Merkle & Nairn, 2005). *C. odorata* (Meliaceae) is the second most valued wood tropical product, thus it has a huge economical importance as a crop, however, its high susceptibility to the attack of the borer *Hypsipyla grandella* (Lepidoptera: Pyralidae) has hindered the efforts to grow this species as a managed crop (Pérez-Salicrup & Esquivel, 2008). Among all the existing strategies for *C. odorata* improvement, breeding through modern genetic manipulation remains to date a pending issue. Direct transfer of a desirable gene into the trees could generate novel traits without any significant modification of their genetic background, one of those traits could be pest resistance (Campbell et al., 2003). Gene manipulation of tree species has already been employed as a tool for basic studies, for manipulation of lignin/cellulose content, for improving wood quality or kraft pulping, for modification of flowering time and tree architecture, to confer abiotic stress tolerance, and for developing pest-resistant tree varieties (Giri et al., 2004).

In order to move forward to the genetic manipulation of C. odorata, our group has recently developed an efficient regeneration system of juvenile material via somatic embryogenesis using immature zygotic embryos as initial explants (Peña-Ramírez et al., 2011). However, to establish a reliable gene transfer protocol, it is also necessary to find an efficient gene transfer procedure and a methodology to select transformed cells. Nowadays, gene transfer mediated by biolistic bombardment is a widely used tool, though the efficiency of this procedure varies depending on a number of physical and biological factors, such as the amount of DNA loaded onto the projectiles, the projectile's speed, their size and density (Sanford et al., 1993), the helium pressure used (Able et al., 2001; Casas et al., 1993; Ikea et al., 2003; Tadesse et al., 2003), the target explant characteristics, and the osmotic pressure in the medium (Klein & Jones, 1999). Therefore, the optimum efficiency of heterologous DNA transfer into plant cells can only be achieved by a fine balance between the factors involved in bombardment efficiency and factors related to target-tissue damage (Tadesse et al., 2003; Zuker et al., 1995). Optimizing these conditions is frequently a tedious and time consuming issue, mainly when a stable expression of the genes used for detection and selection is required. These limitations can be circumvented by assessing transient expression of reporter genes, allowing almost immediate detection of transformed cells (Hunold et al., 1994). Transient expression assays have proven useful to find the optimal conditions for transformation (Able et al., 2001; Heim & Tsein, 1996; Jeoung et al., 2003). The genes that are most frequently employed as reporters for transient gene expression are: 1) the bacterial gene *uid*A (GUS) coding for β-glucuronidase, which catalyses the hydrolysis of X-Gluc (Jefferson et al., 1987), and 2) the *gfp* gene coding for the green fluorescent protein (GFP) cloned from jellyfish *Aequorea victoria* (Elliott et al., 1999), which allows a simple detection by visualizing its expression in

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intact tissues, without adding exogenous substrates. In addition to an optimized DNA-delivery system, to achieve a successful plant transformation requires an efficient selection of the transformed cells, to allow only the regeneration of transgenic plants, preventing the proliferation of non-transformed sectors (Pérez-Barranco et al., 2009). The main goals in the present work were to optimize the conditions to transform *C. odorata* somatic embryos by biobalistics, using transient expression of *uid*A and *gfp* as reporter genes, and to determine the inhibitory doses of four selective agents to be used for T0 plant selection. Both achievements will lead to the establishment of an efficient *C. odorata* transformation protocol.

## Materials and Methods

### Plant Material

Embryogenic calluses were initiated and maintained according to the methodology previously reported by our group (Peña-Ramírez et al., 2011). For biolistic experiments, calli were subcultured one week prior to transformation. Each shoot was performed over approximately 0.1 g of embryogenic calli type II-A located inside of a 3 cm-diameter circle at the center of petri dishes containing full-strength MS (Murashige and Skoog 1962) medium, 80 mM sucrose, 13.4 μM dicamba and 2.5% (w/v) GelRite® (PhytoTechnology Laboratories, Lenexa, KS), pH 5.7. For lethal-dose experiments, 0.2 g of embryogenic clusters were spread in each Petri dish containing the same basal medium supplemented with the appropriate antibiotics (see below).

### Plasmid Preparation and Biolistics

The plasmids pCAMBIA1201 and pCAMBIA1302 (www.cambia.org) carrying either *uid*A or *gfp* gene driven by the constitutive CaMV 35S promoter, previously cloned in *E. coli* DH5αF', were extracted using QIAGEN (Germantown, MD) Maxi Prep kits. The concentration of the plasmid DNA was calculated using a spectrophotometer (SmartSpec, BioRad, Hercules, CA). Embryo bombardment was carried out using a PDS-1000/He Biolistic Particle Delivery System (BioRad). The plasmid DNA was coated onto gold particles as described by the manufacturer (BioRad). Six microlitres of the DNA-microcarrier suspension (see **Table 1**) were dispensed onto each macrocarrier membrane and allowed to dry. The standard bombardment procedure was performed using manufacturer's instructions. In the first treatment, variables were set as follows: 6 cm of shooting distance, 1 μm particle diameter, 6 μg of DNA, 0.1 M spermidine, and vector pCAMBIA1302. To assess the subsequent parameters, further variables were set according to the optimal condition previously determined. Each treatment was repeated 3 times, each consisting of 5 shoots.

### Evaluation of GUS and GFP Transient Expression

Transient expression was analyzed 36 hr after bombardment. GUS assays were carried out using the protocol reported by Jefferson et al. (1987) with minor modifications. GUS expression was tested by immersing explants in 5-*bromo*-4-*chloro*-3-*indolyl*-β-d-*glucuronic acid* (X-Gluc) buffer overnight, at 37˚C in the dark, with a subsequent wash for 24 h in absolute ethanol. The number of blue spots (foci) on explants was observed under a stereomicroscope using a white light source. For GFP analysis, calli were observed under an Olympus microscope equipped with a GPP-2 filter and ultraviolet illumination source. For both systems, the number of foci per petri dish was quantified by analyzing digital photographs with the assistance of the software Quantity One® (BioRad).

### Determination of Antibiotic Lethal Dose

Wild type embryogenic calli were cultured on MS medium prepared as previously described and supplemented with different concentrations of Kanamycin (KAN) (0.0, 20.0, 50.0, 100.0, 150.0, 200.0, 350.0, 500.0, or 750.0 μM); hygromycin (HYG) (0.0, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 35.0, or 50.0 μM); Spectinomycin (SPE) (0.0, 20.0, 50.0, 100.0, 150.0, 200.0, 500.0, 750.0, or 1000.0 μM); or phosphinotricin (PPT) (0.0, 0.5, 1.0, 2.0, 3.5, 5.0, 10.0, 15.0, 20.0, 35.0, or 50 μM) (PhytoTechnology Laboratories, Lenexa, KS). Antibiotics were filter-sterilized and added to the autoclaved medium at 45˚C. 10 calli, 20 mg each, were used for each treatment and the procedure was repeated 3 times. After three weeks, plant material was transferred to an antibiotic-free medium. Viability was assessed 3 weeks later as the ability of embryos to proliferate (measured as weight gain) on an antibiotic-free medium.

### Statistical Analysis

Each parameter was evaluated by using an experimental sample of five Petri dishes and repeated twice. The data were analyzed using standard ANOVA procedures. The differences between the means were determined by Fisher's least significant difference (LSD) tested with the assistance of Statistica® software package (StatSoft, Tulsa, OK).

## Results and Discussion

### Biolistic Optimization

To find the optimal conditions for microparticle delivery, 16 treatments were analyzed, which involved different membrane rupture pressures, shooting distances, microcarrier diameters, plasmid DNA amounts, spermidine concentrations, and two pCAMBIA vectors. Treatment efficiency was evaluated by the number of transient expression foci produced. As can be seen in **Table 1**, the results show significant differences between the different pressures assayed. 1200 psi produced the best results, followed by 1600 and 900 psi. These acceleration pressures might be considered relatively high for this kind of explants, according to Rasco-Gaunt et al. (1999) who found that bombardment pressures over 900 psi cause a drastic drop in transient expression. However, other authors have reported similar results for transformation of embryogenic calli (Abdollahi et al., 2009; Tee & Maziah, 2005).

Shooting distance also had a significant effect. 9 cm resulted the best, followed by 7.5 and 6 cm; longer distances caused an abrupt decrease in the number of foci. Tee and Maziah (2005) and Abdollahi et al., (2009) have found that short distances combined with high shooting pressures might cause an increase in cell damage or injury, resulting in low regeneration rates. We observed this to happen in the case of *C. odorata* regeneration index, which was significantly affected in treatments 5, 6, and 7, where high shooting pressures were combined with short distances. In those cases the regeneration rate dropped by nearly

 

**Table 1.**
Optimization of biolistic parameters.

| Treatment | Membrane rupture pressure (psi) | Shoot distance (cm) | Microcarrier diameter (μm) | DNA amount (μg) | Spermidine addition | pCambia vector | Number of foci/100 mg of embryogenic calli |
|---|---|---|---|---|---|---|---|
| 1 | 400 | 6 | 1 | 6 | + | 1302 | $21.0 \pm 1.1^{c}$ |
| 2 | 600 | 6 | 1 | 6 | + | 1302 | $28.6 \pm 1.9^{d}$ |
| 3 | 900 | 6 | 1 | 6 | + | 1302 | $49.4 \pm 1.5^{e}$ |
| 4 | 1100 | 6 | 1 | 6 | + | 1302 | $49.2 \pm 1.0^{fg}$ |
| 5 | 1550 | 6 | 1 | 6 | + | 1302 | $45.6 \pm 1.9^{ef}$ |
| 6 | 1100 | 3 | 1 | 6 | + | 1302 | $15.0 \pm 2.0^{b}$ |
| 7 | 1100 | 4.5 | 1 | 6 | + | 1302 | $19.6 \pm 1.5^{c}$ |
| 8 | 1100 | 7.5 | 1 | 6 | + | 1302 | $56.0 \pm 1.4^{h}$ |
| 9 | 1100 | 9 | 1 | 6 | + | 1302 | $60.6 \pm 2.6^{i}$ |
| 10 | 1100 | 12 | 1 | 6 | + | 1302 | $12.4 \pm 2.4^{ab}$ |
| 11 | 1100 | 9 | 0.6 | 6 | + | 1302 | $32.2 \pm 1.1^{d}$ |
| 12 | 1100 | 9 | 1.6 | 6 | + | 1302 | $10.4 \pm 1.1^{ab}$ |
| 13 | 1100 | 9 | 1 | 2 | + | 1302 | $32.2 \pm 0.9^{c}$ |
| 14 | 1100 | 9 | 1 | 10 | + | 1302 | $67.2 \pm 0.7^{j}$ |
| 15 | 1100 | 9 | 1 | 10 | - | 1302 | $8.8 \pm 1.2^{ab}$ |
| 16 | 1100 | 9 | 1 | 10 | + | 1201 | $57.6 \pm 1.2^{h}$ |

The average value of the number of foci per treatment is presented ± Mean Standard Error. Cursive letters next to values correspond to significant difference levels by LSD test at $p \leq 0.05$; $n = 5 \times 3$.

50% (data not shown). With regard to particle size, the 1 μm microprojectile resulted in the highest numbers of foci. Optimal results were obtained when 10 μg of plasmid DNA were precipitated onto 2 mg of gold particles. In contrast, spermidine depletion strongly decreased the number of foci, demonstrating its importance for the adequate adsorption of the plasmid onto the gold particle. These results coincide with the findings reported by Rasco-Gaunt et al., (1999), who reported a drop of *uid*A transient expression in samples without spermidine. Moreover, the differences observed between pCAMBIA plasmids 1302 and 1201could be an effect of the image analysis, because fluorescent foci can be quantified more efficiently than blue ones. The lower contrast between expressed *uid*A foci and their background, as well as the difficulty to get spots arrangements in a single plane, making it hard to get good images for foci quantification (Schöpke et al., 1997) and therefore the analysis might be less sensitive. Nonetheless it can be concluded that both *uid*A and *gfp* can be successfully used as reporter genes for transformation in *C. odorata* embryogenic calli (**Figures 1(a)-(c)**).

## Selective Dose Determination

To determine a selective concentration of antibiotics useful to inhibit the proliferation of untransformed plant cells, four selective agents were evaluated cultivating *C. odorata* type II-A embryogenic calli in the presence of different concentrations of KAN, HYG, SPE and PPT. As shown in **Figure 1(d)**, callus viability was affected by all the tested antibiotics following similar patterns. PPT was the most toxic agent, requiring a concentration of 5 μM to kill 100% of calli, followed by 20 μM HYG, 500 μM SPE, and 1 mM KAN. Similar concentrations have been reported to inhibit embryo regeneration of chestnut (Rothrock et al., 2007), grape (Geier et al., 2008), oil palm (Parveez et al., 1997), *Eucalyptus* (Sartoretto et al., 2002) and poplar (Okumura et al., 2006). The obtained lethal doses could be considered as common for several plant species, however it was very important to establish them for *C. odorata* because sometimes even slight variations in the physiological conditions of a particular tissue or in its the genetic background may change its susceptibility to the toxicity of a given selective agent (Duke, 1996). Finally, it was observed a non linear susceptibility curve for all the tested antibiotics, which quickly drops to around 20% to 25% of survival, followed by a weaker slope to finally reach 0% of somatic embryo viability. A biphasic behavior has also been reported for other embryogenic cultures (Catlin, 1990; Parveez et al., 1997) and is in agreement with classic data for cell culture systems with high mitotic activity (Drewinko et al., 1974), thus it supports our previous observations that suggest an unsynchronized and highly proliferative nature of *C. odorata* embryogenic callus.

# Conclusion

As far as we know, this work is the first report of an approach to establish a *C. odorata* gene manipulation protocol. The optimized set of biolistic parameters and the determination of lethal dosages for four antibiotics commonly used for selective screening of transformed plants, provide the necessary foundations for future efforts to generate improved varieties of *C. odorata* through modern genetic manipulation, including those resistant to *H. grandella*. Most of the previous work related with genetic modification of Meliacea has been constricted the genus *Azadirachta* (Morimoto et al., 2006) via *Agrobacterium*-mediated gene delivery. This work provides experimental data that could be used not only for *C. odorata*

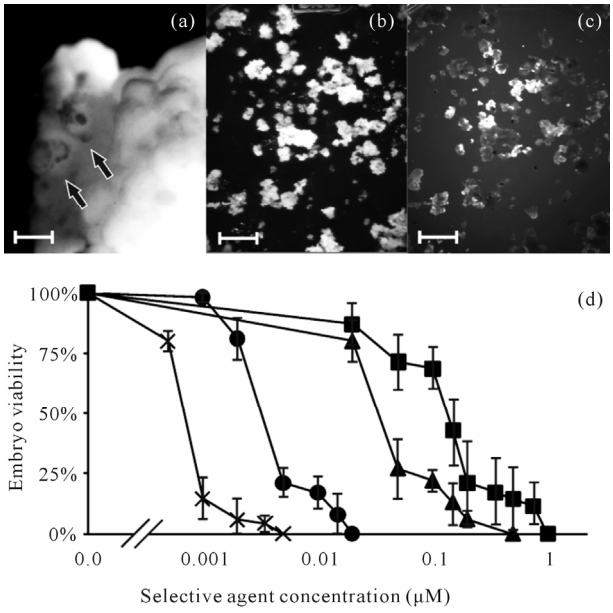


**Figure 1.**
Transient expression in embryogenic calli and lethality curves. Bombarded embryogenic calli expressing transient foci of GUS (a) or GFP under white (b) or UV light (c). Arrows in (a) point to foci clusters in a representative bombarded callus. The white bar in a) corresponds to a length of 50 μm whereas in (b) and (c) it is equivalent to 1 cm. d) shows the lethality curves of PPT (×), HIG (●), SPE (▲), and KAN (■) measured as viability of *C. odorata* embryogenic callus cultured under several concentrations of selective agents. Bars at each point correspond to the Mean Standard Error.

manipulation, but also lay the foundations to generate transformed Meliacea trees of other genus via biolistic approach.

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