Optimization of coffee (*Coffea arabica*) transformation parameters using *uidA* and *hpt* genes: effect of osmotic pre-treatment, helium pressure and target distance

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Received 10-XI-2007. Corrected 15-VI-2009. Accepted 07-VII-2009.

Abstract: The aim of this work was to optimize the biolistic delivery parameters that affect the DNA delivery and stable expression of marker genes into coffee tissues (*Coffea arabica*. L. cvs. Caturra and Catuaí). The effect of osmotic preculture length, osmotic concentration of medium, Helium pressure and target distance on transient expression of the *uidA* gene in coffee leaves and somatic embryos were tested. The highest transient *uidA* expression was obtained when Caturra (18.3±2.8) and Catuaí (6.8±2.0) leaves and Catuaí embryos (80.0±7.4) were cultured for 5h on Yasuda medium complemented with 0.5M Mannitol +0.5M Sorbitol. The combination of 1100psi and a target distance of 9cm resulted in the highest number of blue spots per Caturra leaf segment (23.6±3.9), whereas for the Catuaí variety the combination of 1100psi and a target distance of six (10.2±1.9) and nine (8.2±1.9) cm gave the highest number of blue spots per leaf segment. The optimized protocol was tested with pCAMBIA 1 301 (*uidA* gene and the *hpt* gene), pCAMBIA 1 305.2 (*uidA* version GUSPlus ™ and the *hpt* gene) and pCAMBIA 1 301-BAR (*uidA* gene and the *bar* gene). The highest number of blue spots was obtained when Caturra (54.6±5.7) and Catuaí (28.9±4.3) leaves were bombarded with pCAMBIA 1 305.2. Selection of bombarded coffee tissues with 100mg/l hygromicyn caused the oxidation of tissues. Rev. Biol. Trop. 57 (Suppl. 1): 151-160. Epub 2009 November 30.

Key words: *Coffea arabica*, Caturra, Catuaí, biolistic delivery, osmotic treatment, *uidA,* GUSPlus™, *bar*, *hpt*, *nptII,* Costa Rica.

Coffee (*Coffea arabica* L.) is one of the most important cash crops for more than 50 countries in the world and it is highly valuable for beverage consumption. Nevertheless, this crop is susceptible to different diseases and pests, the coffee berry borer (*Hypothenemus hampei* Ferrari) (Coleoptera: Curculionidae: Scolytinae) being one of the major threats for its production (Carneiro 1999, Méndez-López *et al*. 2003). Actually, *H. hampei* control depends mostly on the application of synthetic insecticides, with the simultaneous damage to the environment (Méndez- López *et al*. 2003).

The genetic improvement of coffee to confer resistance to *H. hampei* is the most promising strategy to control the pest (Méndez-López *et al*. 2003). Nevertheless, introduction of resistance by conventional breeding is limited by the lack of natural resistance to *H. hampei* in both *C. arabica* and *C. canephora* (Fernandez-Da Silva & Menéndez-Yuffá 2003). As a result, genetic transformation represents an efficient alternative to incorporate insect resistance genes into commercial varieties (Fernandez-Da Silva & Menéndez-Yuffá 2003).

Genetic transformation of *C. arabica* and *C. canephora* has been reported using

Agrobacterium tumefaciens (Hatanaka *et al*. 1999, Leroy *et al*. 2000, Ogita *et al*. 2002, Canche-Moo *et al*. 2006, Ribas *et al*. 2006), *Agrobacterium rhizogenes* (Kumar *et al*. 2006, Alpizar *et al*. 2006), electroporation (Fernández-Da Silva & Menéndez-Yuffá 2003) and biolistic delivery method (Van Boxtel *et al*. 1995, Rosillo *et al*. 2003, Ribas *et al*. 2005). Direct gene transfer via biolistic delivery offers some advantages in relation to *Agrobacterium* and electroporation. Thus, with biolistic delivery diverse cell types can be targeted, there are no genotype limitations, the plant/bacteria relationship is eliminated and co-transformation is facilitated (Altpeter *et al*. 2005, Sharma *et al*. 2005).

In any plant transformation procedure, establishment of an efficient gene transfer method is imperative (Sharma *et al*. 2005). Moreover, genetic transformation sometimes results in low efficiency of stable transformed cells, in which case it is necessary to optimize transformation conditions (Tee & Maziah 2005). With the biolistic delivery method, the transformation efficiency is influenced by the composition and size of the microcarriers, precipitation and binding of DNA to the microcarriers, Helium pressure, bombardment distance, chamber vacuum pressure, temperature, humidity, light intensity and condition, the cell type, cell size, cell culture age, cell density and cell turgor pressure (Rasco-Gaunt *et al*. 1999, Kikkert *et al*. 2004). Therefore, in the present work various biolistic delivery parameters were evaluated with the aim of optimizing DNA delivery and stable expression of marker genes and identify the conditions which minimize damage to the coffee tissues (*Coffea arabica*. L. cvs. Caturra and Catuaí).

MATERIALS AND METHODS

Plant material and explant preparation: Leaf sections (0.5cm^2) of coffee vitroplants (*Coffea arabica* L. cvs Caturra and Catuaí) and somatic embryos of Catuaí variety were used as explants for particle bombardment. The zygotic embryos were excised from Caturra and Catuaí seeds and cultured in baby food jars, closed with polyethylene food wrap (Glad, Costa Rica), containing 20mL of MS medium (Murashige & Skoog 1962) supplemented with Morel vitamins (Morel 1965), 100mg/1 myo-inositol, 200mg/1 casein hydrolysate, 400mg/1 malt extract, 4.4μM BAP and 2g/1 Gelrite; pH was adjusted to 5.6 before autoclaving for 21min at 121°C and 1.07kg/cm^2. In vitro plantlets developed from these embryos were cultured with 20mL of the above medium under a 16h light photoperiod (30μmol/m^{2}s^1) at 26±2°C and transferred to fresh medium every 90 days. Somatic embryos were obtained through DSE from leaf sections of Catuaí vitroplants cultured on Yasuda et al. (1985) medium in the dark at 26±2°C.

Preparation of plasmid DNA: The plasmid pCAMBIA 1 301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) containing a uidA gene and the hpt gene, both under control of the constitutive CaMV35S promoter, was used for biolistic delivery optimization. The plasmid was isolated from Escherichia coli XL1 cells using the Wizard™ Plus Midipreps DNA purification systems (Promega). Ten μl of DNA (1μg/μl) was precipitated onto 50μL sterile gold particles (1μM, Bio-Rad Laboratories Inc, Hercules, CA, USA) according to the protocol described by Russell (1993).

Optimization of DNA delivery into coffee tissues: In order to determine the best osmotic preculture conditions, leaf explants (0.5cm^2) and somatic embryos were cultured for 5h and 20h prior to particle bombardment in the Yasuda *et al.* (1985) medium supplemented with 0.295M sucrose+4 g/1 Gelrite (MY_0) (Van Boxtel et al. 1995), 0.25M Mannitol+0.25M Sorbitol (MY_1) or 0.5M Mannitol+0.5M Sorbitol (MY_2) (Rosillo *et al.* 2003). Also, leaf explants and somatic embryos were cultured in the Yasuda *et al.* (1985) medium without osmotic agent. Particle bombardment was conducted with pCAMBIA 1301 using a PDS 1 000/ He Biolistic Particle Delivery System

(Bio-Rad) and conditions included a Helium pressure of 1 100psi, a 9cm target distance and a vacuum of 650mm of Hg.

Once the best osmotic preculture treatment was determined, leaf explants and somatic embryos were bombarded using Helium pressure of 650, 900, 1 100 or 1 550psi from a target distance of 6, 9 or 12cm. The optimized particle bombardment protocol was tested with the plasmid pCAMBIA 1305.2 (*uidA* version GUSPlus ™ and the *hpt* gene) and the pCAMBIA 1 301-BAR (*uidA* gene and the *bar* gene). All the genes are under control of the constitutive CaMV35S promoter. In all the experiments, 30 explants were bombarded once and cultured in the dark at 26±2°C. Non- bombarded explants were included as controls.

Assay for β-glucoronidase activity: Histochemical GUS assays were performed 48h after plasmid delivery following the protocol described by Van Boxtel *et al.* (1995). Briefly, bombarded explants were incubated in X-Gluc buffer (2mM X-Gluc, 100mM sodium phosphate buffer pH 8.0, 10mM EDTA, 1mM Potassium Ferricyanide, 1mM Potassium Ferrocyanide and 20% v/v Methanol) for 24h at 37°C in the dark. The explants were cleared using 95% (v/v) alcohol and the number of blue spots per explant and number of explants with uidA expression were counted using a binocular stereoscope.

Selection of putative transformants: Leaves and somatic embryos bombarded with pCAMBIA 1 301 and pCAMBIA 1 305.2 were cultured on semisolid Yasuda *et al.* (1985) medium supplemented with 100mg/1 hygromycin. During all culture period, the explants were maintained with 16h light photoperiod ($30\mu mol/m^2s^1$) at 26±2°C. Four weeks after the selective agent was supplied, the growth inhibition was determined taking into consideration the necrosis of the tissue.

The average and the standard error of the number of blue spots per explant and bombardment efficiency [(number of explants with GUS activity/total number of explants bombarded) x100] were determined. Data were analyzed using one-way ANOVA and the differences between means were contrasted using the Duncan test (Duncan 1955) at the level of 5%. The program STATISTICA (StatSoft, Tulsa, OK, USA) version 6.0 was used.

Abbreviations: CaMV-cauliflower mosaic virus; BAP-benzyladenine; DSE-Direct somatic embryogenesis; GUS- β-glucoronidase; GFP-green fluorescent protein; *hpt*-hygromycin phosphotransferase gene; MS-Murashige and Skoog; *nptII*- neomycin phosphotransferase gene; *uidA*- β-glucoronidase gene.

RESULTS

Transient *uidA* expression: The length of osmotic preculture, osmotic concentration of culture medium, Helium pressure and target distance had a significant influence on the number of blue spots per explant and percentage of explants with transient uidA expression.

The highest number of blue spots per explant and the highest percentage of explants with transient uidA expression were obtained when Caturra and Catuaí leaf explants and Catuaí somatic embryos were cultured on Yasuda *et al*. (1985) medium supplemented with 0.5M Mannitol+0.5M Sorbitol for 5h prior to particle bombardment (Table 1 and Fig. 1A).

The highest percentage of transient *uidA* expression on Catuaí somatic embryos was obtained using a Helium pressure of 1 100psi and a target distance of 6cm (83.3±6.9) and 9cm (70.0±8.5), whereas the lowest percentage of transient *uidA* expression on Catuaí somatic embryos was obtained using 1 550psi and 6cm (6.7±4.6) (Fig. 1B). On the other hand, the highest number of blue spots in Caturra leaf segments was obtained with 1 100psi and 9cm of target distance (23.6±3.9) and the lowest number of blue spots per explant was obtained using 900psi and 12cm of target distance (2.9±1.6) (Fig. 2). For the Catuaí variety, the combination of 1 100psi and target distance of 6 and 9cm gave the highest number of blue spots per explant (10.2±1.9 and 8.2±1.9). In contrast, the

TABLE 1

*Effect of osmotic preculture and osmotic concentration on transient uidA expression in coffee leaves (*Coffea arabica *L. cv. Caturra and Catuaí)*

Time	Osmotic pretreatment	Caturra		Catuaí	
		Average number of blue spots	Bombardment efficiency (%)	Average number of blue spots	Bombardment efficiency (%)
5 h	No osmoticum	12.6±2.3 b	70±9 b c	1.3±0.5 b c	27±8 c d
	0.295M Sucrose + 4g. L^{-1} Gelrite (MY_0)	2.8±0.9 d	43±9 d	3.8±1.5 b	50±9 a b
	0.25M Mannitol + 0.25M Sorbitol (MY_1)	8.9±2.4 b c	67±8 c	1.5±0.6 b c	27±8 c d
	0.5M Mannitol + 0.5M Sorbitol (MY_2)	18.3±2.8 a	90±6 a b	6.8±2.2 a	63±9 a
20 h	No osmoticum	5.7±1.3 c d	77±8 b c	0.8±0.5 c	17±7 d
	0.295M Sucrose + 4 g/l Gelrite (MY_0)	10.7±1.7 b c	90±6 a b	0.03±0.0 c	3±3 d
	0.25M Mannitol + 0.25M Sorbitol (MY_1)	8.7±1.0 b c	100±0 a	1.1±0.3 b c	40±9 b c
	0.5 MMannitol + 0.5M Sorbitol (MY_2)	9.6±1.4 b c	87±6 a b c	0.1±0.1 c	3±3 d

Data obtained 48h after particle bombardment by GUS assay. Bombardment efficiency: [(number of explants with GUS activity/total number of explants bombarded) x 100]. Different letters indicate values are significantly different by Duncan test at P>0.05.

combination of 650, 900 and 1 550psi with 6, 9 and 12cm respectively and the combination of 1 100psi with a target distance of 12cm resulted in the lowest number of blue spots per explant (Fig. 2). Moreover, the highest percentage of Caturra leaf explants with *uidA* expression was obtained using 1100psi and a target distance of 9cm. For the Catuaí variety, the combination of 1 100psi and a target distance of 6cm resulted in the highest percentage of explants with transient *uidA* expression (Fig. 2).

The bombardment of Caturra and Catuaí leaves with pCAMBIA 1 305.2 significantly influenced the transient *uidA* expression. The highest number of blue spots per explant was obtained when Caturra (54.6±5.7) and Catuaí (28.9±4.3) leaf explants were bombarded with the plasmid pCAMBIA 1 305.2. No significant differences in the transient *uidA* expression were obtained between pCAMBIA 1 301 and pCAMBIA 1 301-BAR (Fig. 3A and 3B).

Selection of putative stable transformants: Although transient uidA expression was obtained by bombarding leaves and somatic embryos with pCAMBIA 1 301 and pCAMBIA 1 305.2 (Fig. 4A and 4B), the explants turned brown after four weeks of culture on selection medium supplemented with 100mg/l of hygromycin (Fig. 4C and 4D). As a result, no transgenic plants with stable hpt and uidA gene expression were recovered.

DISCUSSION

The establishment of the best parameters in any plant tissue using particle bombardment is necessary for transient or stable gene expression (Tee & Maziah 2005). To the best of our knowledge this is the first report of optimization of biolistic delivery parameters for DNA delivery and gene expression into *C. arabica* cv. Caturra and Catuaí leaves and Catuaí somatic embryos.

The influence of the osmotic preculture and osmotic concentration of the medium was corroborated. It was shown that shorter periods of preculture in combination with higher concentration of osmotic agents resulted in a higher number of blue spots and percentage of explants with transient *uidA* expression. A similar result was obtained by Rosillo *et al*. (2003) who demonstrated that culture of *C.*

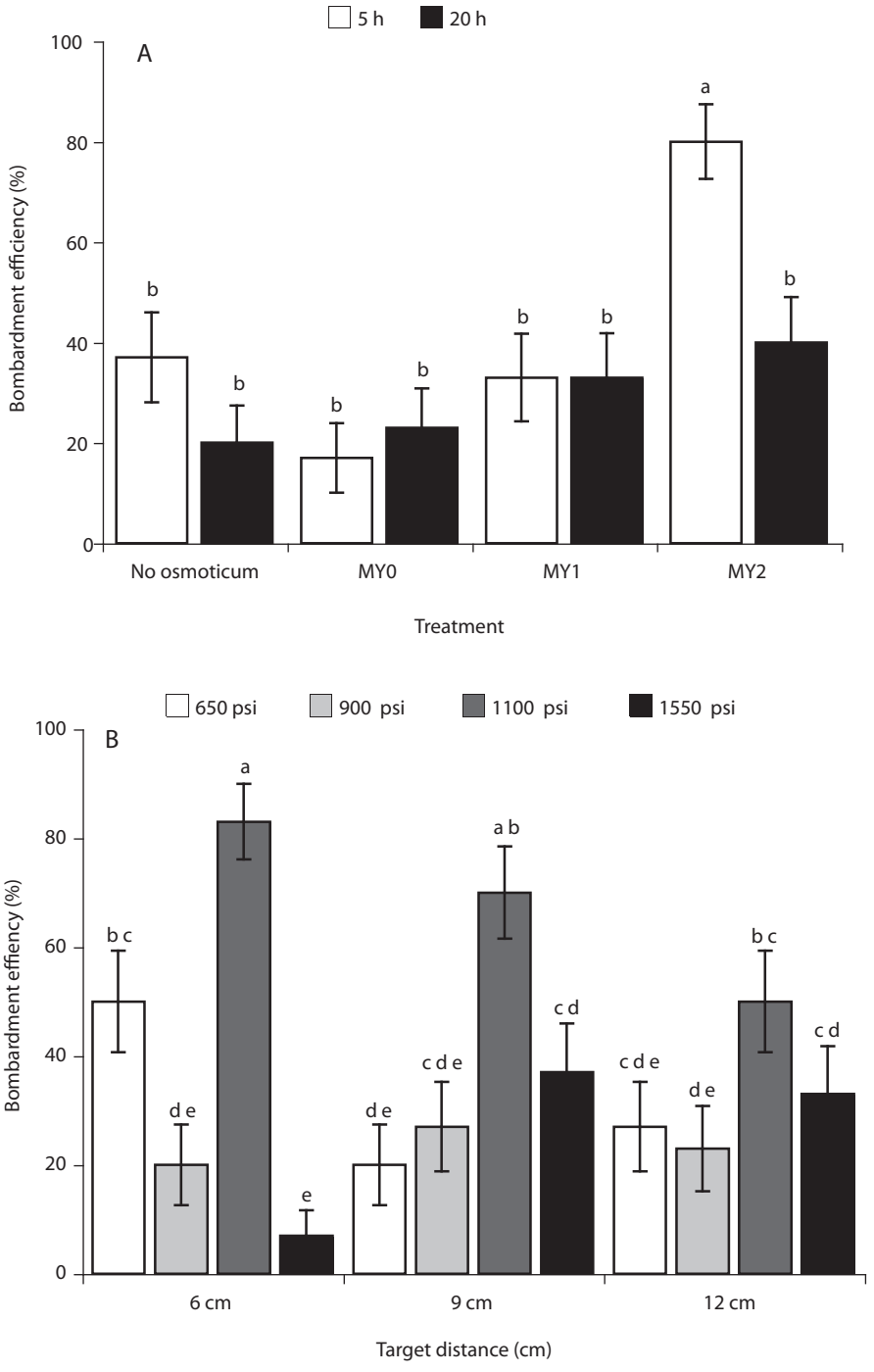

Fig. 1. A. Effect of osmotic preculture length and osmotic concentration and (B) Helium pressure and target distance on transient *uidA* expression in coffee somatic embryos (*Coffea arabica* L. cv. Catuaí). Treatments: MY0, 0.295M Sucrose + 4 g/l Gelrite; MY1, 0.25M Mannitol+Sorbitol; MY2, 0.5M Mannitol+Sorbitol. Data were recorded 48h after particle bombardment by GUS assay. Error bars correspond to SE. Different letters indicate values are significantly different by Duncan test at P>0.05.

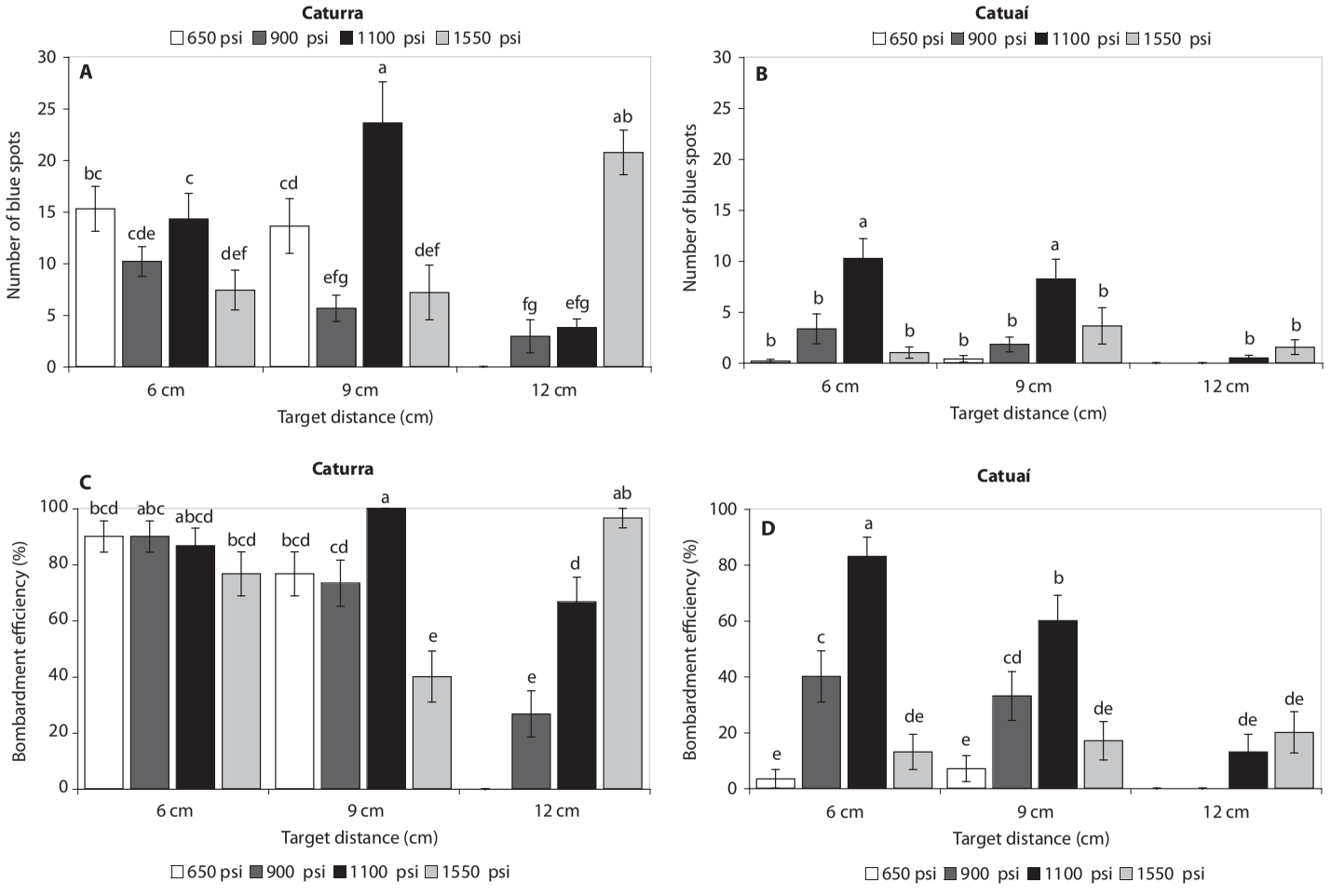

Fig. 2. Effect of Helium pressure and target distance on transient *uidA* expression in coffee leaf segments (*Coffea arabica* L. cv. Caturra and Catuaí). (A-B) Number of blue spots (mean±S.E); (C-D) bombardment efficiency. Data were recorded 48h after particle bombardment by GUS assay. Different letters indicate values are significantly different by Duncan test at P>0.05.

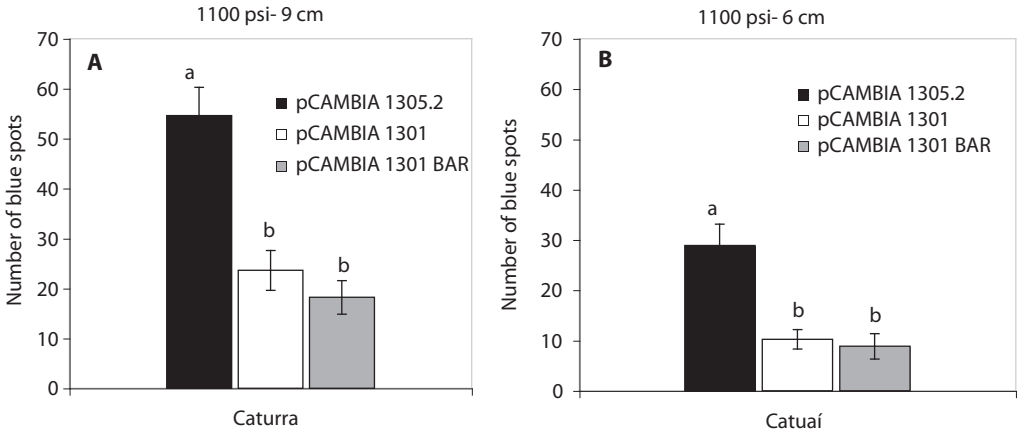

Fig. 3. Effect of plasmid on transient *uidA* expression in coffee (*Coffea arabica* L.) leaves. (A) Caturra and (B) Catuaí. Data were recorded 48h after particle bombardment by GUS assay. Error bars correspond to SE. Different letters indicate values are significantly different by Duncan test at P>0.05.

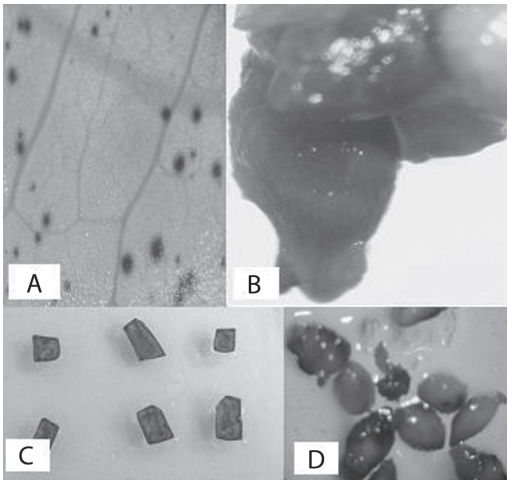

Fig. 4. Histochemical assay of *uidA* gene. (A) Coffee leaves bombarded with pCAMBIA 1301 or pCAMBIA 1305.2; (B) Catuaí somatic embryos bombarded with pCAMBIA 1301; (C) leaves and (D) somatic embryos cultured on selection medium supplemented with 100mg/l of Hygromycin.

arabica cv. Colombia suspension cultures for 4h prior to bombardment with a 0.5M Mannitol and Sorbitol mixture resulted in a higher number of blue spots. Moreover, the use of high concentrations of Mannitol, Sorbitol or Sucrose has improved transient *uidA* expression in maize (Vain *et al*. 1993), rice (Jain *et al*. 1996), wheat (Ingram *et al*. 1999, Rasco-Gaunt *et al*. 1999) and marigold (Vanegas *et al*. 2006). For *C. arabica* L. cvs. Caturra and Catuaí this is the first report of the use of 0.5M Mannitol and 0.5M Sorbitol as a high osmotic treatment to enhance transient *uidA* expression on leaves and somatic embryos. It is known that the type and concentration of osmotic agent may increase transient gene expression by reducing turgor pressure in cells. Therefore, the chance of cell survival increases by avoiding leakage following the shock wave created during bombardment (Rosillo *et al*. 2003). Moreover, a high concentration of osmotic agents may also induce changes in cell membranes, leading to increased cell tolerance to biolistic delivery impact (Ingram *et al*. 1999).

In the present work, significant differences in the expression of *uidA* in coffee leaves and somatic embryos were observed with respect to the Helium pressure and target distance (Fig 1B, Fig 2). It is known that Helium pressure and target distance influence the entry of DNA into the cell, the distribution of particles and tissue injury (Rasco-Gaunt *et al*. 1999, Rosillo *et al*. 2003, Rubio *et al*. 2004). It has been demonstrated that Helium pressure and target distance influence transient gene expression

in rice (Jain *et al*. 1996), wheat (Ingram *et al*. 1999, Rasco-Gaunt *et al*. 1999), pine (Fernando *et al*. 2000), carrot, sweet potato and ohelo (Deroles *et al*. 2002), coffee (Rosillo *et al*. 2003), sorghum (Tadesse *et al*. 2003), triticale (Rubio *et al*. 2004), *Dendrobium* Sonia 17 (Tee & Maziah 2005) and marigold (Vanegas *et al*. 2006). Moreover, optimization of Helium pressure and target distance for gene delivery into plastid genome has been reported in carrot (Kumar *et al*. 2004a) and cotton (Kumar *et al*. 2004b).

Although, the highest number of blue spots per Caturra and Catuaí leaves and Catuaí somatic embryos were obtained using a Helium pressure of 1 100psi, different target distances were required. These observations suggest different target tissues and genotypes require different bombardment conditions (Tadesse *et al*. 2003). Tee & Maziah (2005) reported bombardment of *Dendrobium* Sonia 17 type A calli using 1 100psi and 6cm but type B calli required 650psi and 6cm. Moreover, Tadesse *et al*. (2003) obtained higher transient *uidA* expression when sorghum immature embryos and shoot tips were bombarded using 1 100 or 1 300psi in combination with a target distance of 6cm.

The higher transient *uidA* expression obtained on coffee leaves bombarded with pCAMBIA 1 305.2 could be related to the properties of the new GUS*Plus*™ reporter gene isolated form *Staphylococcus* sp. (Broothaerts *et al*. 2005). In contrast to *uidA* gene isolated from *E. coli*, GUS*Plus*™ has a better catalytic activity for more rapid detection of GUS activity, greater stability at 60°C and in the presence of fixatives such as formaldehyde and glutaraldehyde. Moreover, GUS*Plus*™ represents an alternative to GFP since rice glycine-rich protein signal peptide for extracellular secretion provides rapid and non destructive *in vivo* GUS assays (Jefferson *et al*. 2003).

Van Boxtel *et al*. (1995) reported that somatic embryos are less appropriate for transient *uidA* expression than leaves. Nevertheless, high levels of transient *uidA* expression were observed on Catuaí somatic embryos using the optimized biolistic delivery protocol developed in the present work. Moreover, Van Boxtel *et al*. (1995) observed false GUS positive somatic embryos, probably due to the presence of endogenous bacteria, often encountered in culture tissue of tropical woody species such as coffee. In contrast, in the present work endogenous GUS activity caused by endogenous bacteria was not observed in any of the explants analyzed because of the elimination of endogenous GUS activity by using Methanol in X-Gluc solution (Kosugi *et al*. 1990).

Although, transient *uidA* expression was observed in leaves and somatic embryos bombarded with pCAMBIA 1 301 (*hpt* and *uidA*) or pCAMBIA 1 305.2 (*uidA* version GUS-*Plus* ™ and *hpt*), the use of 100mg/1 of Hygromycin led to oxidation of leaves and somatic embryos. Van Boxtel *et al*. (1994) indicated that transformed cells seemed to be hindered in their multiplication by abundant necrosis of surrounding tissue. In this regard, these authors reported that hygromycin caused severe necrosis of coffee leaves and suspension cultures. Nevertheless, if a lower concentration of hygromycin is used, as reported by Kumar *et al*. (2006) who used 20mg/1 of Hygromycin for the selection of *C. canephora* putative transformants, the method developed in the present work can be used for the genetic transformation of *Coffea*.

The conditions for transient gene expression in *C. arabica* L. cvs. Caturra and Catuaí have been established. These conditions consist of 5h preculture on 0.5M Mannitol and 0.5M Sorbitol, a Helium pressure of 1 100 psi and target distance of 6 or 9cm, depending on the explant used. Furthermore, the optimized protocol developed in the present study could be used for incorporation and stable expression of *cry* genes from *Bacillus thuringiensis* in order to confer resistance to *H. hampei.*

ACKNOWLEDGMENTS

The authors are grateful to Eric Guevara (CIGRAS, University of Costa Rica) and Paul Hanson (Escuela Biología, University of Costa

Rica) for the heplful disscusion, critical review and English correction of the manuscript. This research was supported by the Costa Rica-United States of America Foundation for Cooperation (CRUSA) and the University of Costa Rica.

RESUMEN

La presente investigación tuvo como objetivo optimizar los parámetros que afectan la incorporación y expresión de genes marcadores mediante biobalística en segmentos de hoja y embriones somáticos de café (*Coffea arabica*. L. cvs. Caturra y Catuaí). La mayor expresión transitoria del gen *uidA* en segmentos de hoja de Caturra (18.3±2.8) y Catuaí (6.8±2.0) y embriones somáticos de Catuaí (80.0±7.4) se obtuvo al cultivar los explantes por cinco horas previo al bombardeo en el medio Yasuda complementado con 0.5M mannitol+0.5M sorbitol. Asimismo, se obtuvo una mayor expresión transitoria del gen *uidA* al bombardear los segmentos de hoja de Caturra y Catuaí y embriones somáticos de Catuaí con una presión de helio de 1 100psi y una distancia de bombardeo de 6 o 9 cm.

Palabras clave: Caturra, Catuaí, biobalística, tratamiento osmótico, *uidA,* GUSPlus™, *bar*, *hpt*, *nptII*, Costa Rica.

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