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Transgenic coffee fruits from *Coffea arabica* genetically modified by bombardment

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Abstract The genetic modification of *Coffea arabica* fruits is an important tool for the investigation of physiological characteristics and functional validation of genes related to coffee bean quality traits. In this work, plants of *C. arabica* cultivar Catuaí Vermelho were successfully genetically modified by bombardment of embryogenic calli. Calli were obtained from 90% of the leaf explants cultivated in a callogenesis-inducing medium modified with 20 μM 2, 4-dichlorophenoxyacetic acid (2,4-D). The resulting calli were bombarded with the pBI426 vector containing a *uidA* and *nptII* gene fusion that was driven by the double CaMV35s promoter. Kanamycin-selected embryos were positive for β-glucuronidase (GUS) activity in histochemical assays and for target gene amplification by polymerase chain reaction. Integration of the *nptII* gene was confirmed by Southern blot and showed a low copy number (one to three) of insertions. Transformed plants showed normal development and settled fruits. GUS expression was assessed in the flower and fruit organs demonstrating the capacity of the double CaMV35s promoter to drive long-term stable expression of *uidA* in *C. arabica* fruit tissues. Moreover, we obtained a T_1 progeny presenting 3:1 Mendelian segregation of the *uidA* gene. This investigation is the first to report exogenous gene expression in coffee fruits and transgenic inheritance in *C. arabica* plants.

Keywords *Coffea arabica* · Genetic transformation · *uidA* gene · Coffee fruit expression · Bombardment

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Introduction

Coffee, a beverage appreciated worldwide, yielded more than USD$13 billion dollars in the international market in 2008 (ICO 2009). Approximately 130.7 million bags were produced in 2008–2009 generating millions of jobs in the tropical countries that produce coffee. Coffee species (*Coffea* spp.) are perennial woody shrubs. *Coffea arabica*, the most important commercialized species, is allotetraploid and autogamous, which makes it difficult to improve by conventional breeding (Etienne et al. 2002). The fruit of *C. arabica* requires 6 to 8 mo to mature covering the time between anthesis and full ripening.

Coffee biotechnology research has focused on isolating genes for improving fruit production, adaptability to the environment, and coffee-cup quality (Lin et al. 2005; Vieira et al. 2006). The importance of a better understanding of coffee fruit physiology was reviewed by De Castro and Marraccini (2006). They examined several important pathways involved in coffee berry development and metabolism, such as caffeine pathways, storage proteins (2S and 11S), α-D-galactosidase, biosynthetic pathway of chlorogenic acids (e.g., PAL), sucrose metabolism (e.g., sucrose synthase), and ethylene production involved in the pericarp maturation (e.g., synthesis and catalyzation of ACC 1-aminocyclopropane-1-carboxylic acid). Other workers have reported efforts toward genetically modified coffee plants with altered fruit traits.

Aiming to increase uniformity of bean maturation, coffee cDNAs coding for ACC enzymes were cloned in order to modulate ethylene production in the fruits by expression of antisense sequences (Pereira et al. 2005). To produce coffee beans that are intrinsically deficient in caffeine, Ogita et al. (2004) produced *C. canephora* plants containing caffeine content reduced by up to 70% through the use of RNA interference (RNAi) technology aimed at the gene encoding theobromine synthase (CaMXMT1). Reduced levels of caffeine were demonstrated in the leaves of transgenic plants, but it was not shown whether caffeine content was also reduced in the beans.

The improvement of commercial fruit trees by genetic transformation presents many difficulties. There are only a few crops successfully transformed with genes of agricultural importance. One example is the *Nicotiana alata* proteinase inhibitor (*Na-PI*) that was introduced into apple (*Malus domestica*) plants to confer resistance to insects (Maheswaran et al. 2007). To control fruit ripening, ACC oxidase (*ACO*) expression was modified in pear (*Pyrus communis*; Gao et al. 2007). However, in these works on perennial crop genetic modification, the transformed tissues analyzed are usually vegetative, i.e., leaves and shoots. One exception is the recent investigation by Torregrosa et al. (2008) in which transformed grapevine (*Vitis vinifera* L.) displayed altered levels of alcohol dehydrogenase (*Adh*) in grape berry tissues.

The large number of expressed sequence tags (EST) sequences currently available (Fernandez et al. 2004; Lin et al. 2005; Vieira et al. 2006; Salmona et al. 2008) and other sequence information that will become available from genome projects will permit the study of many coffee genes that may potentially impart agronomic and consumer-preferred traits. It will be of particular interest to investigate seed physiology for the elaboration of final characteristics of coffee beans to obtain better cup quality. However, the identification and characterization of genes activated or repressed during coffee fruit development requires validation *in vivo*.

The *uidA* reporter gene, coding for the β-glucuronidase (GUS), has been proven to be a useful marker for following gene transfer to coffee plants. Different coffee cells and tissues were evaluated by GUS activity in the optimization of direct transformation methods (Van Boxtel et al. 1995; Fernandez-Da Silva and Menéndez-Yuffá 2003; Rosillo et al. 2003). The first transformed coffee plants obtained expressed the *uidA* gene (Spiral et al. 1993; Hatanaka et al. 1999). The *C. canephora* and *C. arabica* plants transformed with the *cry*1Ac gene from *Bacillus thuringiensis* to confer resistance to the leaf miner (*Perileucoptera coffeella*), also contained the *uidA* gene (Leroy et al. 2000). Ribas et al. (2005) transformed *C. canephora* by particle bombardment with genes conferring both resistance to phosphinotricin and GUS activity.

In this paper, we report the recovery of transgenic *C. arabica* that highly expressed the *uidA* gene in the ovaries, anthers, and endosperm of their fruits. Our results indicated that the *uidA* gene is correctly expressed by the control of the dCaMV35s promoter in the flower and fruit tissues of *C. arabica*. We also describe a direct transformation methodology that has potential to produce sufficient numbers of low copy transformants for both the improvement of *C. arabica* varieties and the functional validation of candidate genes.

Materials and Methods

Induction of embryogenic calli. The second pair of leaves from plagiotropic branches of *C. arabica* cv. Catuaí Vermelho was collected from plants that were grown in a greenhouse. These leaves were then surface-disinfected by a 3 min soak in 70% ethanol, followed by a soak in 2% sodium hypochlorite for 20 min and a rinse with distilled water repeated three times. Leaves were segmented (0.5× 0.5-cm pieces) and cultivated in petri dishes for 6 mo in the dark at 25±2°C on high frequency embryogenic induction medium, here called C20, based on medium C as described by Van Boxtel and Berthouly (1996) and modified by Teixeira et al. (2004). C20 consisted of half-strength Murashige and Skoog (Murashige and Skoog 1962) salts, 20 mg/l sucrose, 100 mg/1 casein hydrolysate, 400 mg/1 malt extract, 10 mg/1 thiamine, 1 mg/1 nicotinic acid, 1 mg/1 pyridoxine, 1 mg/1 glycine, 100 mg/1 myoinositol, 20 μM 2,4-D, 4.9 μM indole-3-butyric acid, and 9.8 μM 2-isopentenyladenine, solidified with 2.4 g/l Phytagel (Sigma Chemical Co.; St. Louis, MO). The pH was adjusted to 5.6 before autoclaving. During the incubation period in C20, explants were subcultured onto fresh medium only after the first month and then left in the same plates until the sixth month of culture.

Transformation procedure. After 6 mo of calli induction, yellow friable masses that had formed around the leaf explants were transferred to bombardment plates. Each bombardment plate was composed of a membrane containing approximately 50 mg of embryogenic callus. Embryogenic mass was spread on a nitrocellulose membrane (BA85 with 0.45 μm pore size; Schleicher and Schuell; Dassel, Germany) that was placed over modified C20 medium containing 10 μM 2,4-D (C10) and incubated at 25±2°C in the dark for 6 d prior to microparticle bombardment. Membranes were then transferred to osmotic-conditioning medium (C10 containing 0.5 M mannitol and 8 g/l Phytagel) and incubated at 25±2°C in the dark for 24 h before bombardment.

The transformation experiment was composed of 12 bombarded plates. The vector used for bombardment was

pBI 426 (Datla et al. 1991), which contains the *npt*II gene conferring resistance to kanamycin and the *uidA* gene coding for the enzyme β-glucuronidase, which is driven by the double CaMV35s (dCaMV35s) promoter with an enhancer. Microprojectile sterilization and DNA precipitation were conducted according to Aragão et al. (1996). In a horizontal flux chamber, DNA was surface-deposited over 1.2 μm tungsten microparticles (M10, Sylvania Inc.; New York, NY) previously prepared in suspension (60 mg/l in 50% glycerol). For each shot, a 50-μl aliquot of microparticles was mixed with 5 μl of vector (1 μg/ml), 50 μl of 2.5 M $CaCl_2$, and 20 μl of 100 mM spermidine (Sigma), in this order. The mixture was gently inverted during 10 min and then centrifuged at 15,000×*g* for 10 s. The supernatant was gently removed by pipetting, and the pellet was washed twice with absolute ethanol. Covered microparticles were finally suspended in 24 μl absolute ethanol and passed three times (1 s each time) in a sonication bath. Aliquots (3.2 μl) were pipeted over carrier membranes (Kapton, 2-mil; DuPont, Belle, WV) and placed in a high-pressure helium-driven particle acceleration device adapted from the system described by Sanford et al. (1991). Briefly, the bombardment chamber was maintained at approximately 50% relative humidity and 27 in. of Hg vacuum when 1,200 psi of helium pressure was applied to the shock-wave generation membrane. Bombardment physical parameters (Aragão et al. 1996) were 8-mm distance between the shock membrane and the DNA carrier membrane, 13-mm flying distance of the carrier membrane to the stopping screen, and 80-mm flying distance of the DNA-coated microparticles to the explants. The bombardment plates were covered with a metallic screen (0.85-mm overture) to avoid spreading of the embryogenic callus during the shot. Immediately following bombardment, the membranes carrying bombarded calli were transferred to C10 medium without selection pressure and maintained at 25±2°C in the dark for 2 wk. Afterwards, the calli were subcultured on C10 medium that contained increasing concentrations of the selective agent kanamycin as follows: first week with 200 mg/l, second week with 300 mg/l, and then monthly with 400 mg/l until the appearance of resistant calli or embryos. Selected calli were cultured physically apart from each other to maintain traceability of the different transformation events. Regenerated somatic embryos were cultivated until they reached the torpedo stage on R medium (Van Boxtel and Berthouly 1996) that contained 400 mg/l kanamycin. Regenerated embryos were then cultivated in baby food jars without the selection agent on woody plant medium (WPM; Lloyd and McCown 1981) containing 3 g/l activated charcoal and 2 g/l Phytagel at 25±2°C under low-light conditions (7.5 μmol/m^2/s) with a photoperiod of 16 h. Derived plantlets were potted in vermiculite and soil (1:1 *v*/*v*) and covered with plastic to be acclimated in a greenhouse with 40% to 50% shade. Covers were gradually opened to be completely removed after 2 wk of acclimatization and permit the direct irrigation of the pots.

In the second year of greenhouse growing, fruits were collected from the selected event, identified as T4, to perform GUS activity tests and *in vitro* germination of the transformed zygotic embryos aiming to further propagate the T1 progeny by tissue culture. Red cherry fruits were disinfected before aseptic extraction of the zygotic embryos. Embryo cotyledonary samples were GUS-stained while axes were cultivated in WPM until acclimatization.

Histological analysis. Embryogenic calli, kanamycin-resistant somatic embryos, and plantlets were tested before acclimatization for the histochemical detection of GUS activity (Jefferson et al. 1987). Samples were incubated overnight in the staining buffer modified by Lacorte (1998) and were observed under a stereomicroscope. The same GUS staining assay was used to analyze cut samples of flowers and fruits by microscopy. Four stages of the development relative to the days after anthesis (DAA; De Castro and Marraccini 2006) were collected corresponding to: (1) pin heads (15 DAA); (2) green immature cherry (115 DAA); and (3) mature cherry (230–240 DAA). Fruit materials from transformed plants were stained, dehydrated in a crescent ethanol battery, and embedded in paraffin. Longitudinal serial, semithin sections were treated with xylol and observed using a Zeiss axiophoto microscope. Cotyledonary samples from zygotic embryos (T_1 generation) of the T4 event were analyzed in X-gluc buffer as described previously.

Molecular analysis. The *uidA* gene was amplified by polymerase chain reaction (PCR) using the primers described by Moore et al. (1992). Genomic DNA from leaves of kanamycin-resistant *in vitro* plantlets were extracted by the CTAB method (Doyle and Doyle 1987) and analyzed as follows: 20 ng of template were mixed with the buffer containing 1.2 mM $MgCl_2$, 0.128 mM dNTP, 1.5 U *Taq* polymerase (Qiagen; Hilden, Germany), and 0.2 mM primers (forward: 5′-TTGGGCAGGCCAGCGTATCGT-3′ and reverse: 5′-ATCACGCAGTTCAACGCTGAC-3′). This mix was submitted to thermocycling (95°C /5 min; 36 cycles: 95°C/1 min, 55°C/1 min, and 73°C/1 min; 73°C/5 min) and the amplification products were analyzed by electrophoresis on a 1% (*v*/*v*) agarose gel.

The *nptII* gene was detected by Southern blot analysis (Sambrook et al. 1989) from four different transformation events. Genomic DNA was extracted using the DNAeasy Plant Kit (Qiagen) following manufacturer's instructions. Ten-microgram DNA samples were digested with B*gl*II, S*ca*I, or S*sp*I restriction enzymes and subjected to electrophoresis on a 1% (*v*/*v*) agarose gel. The DNA fragments were transferred to a Hybond N^+ membrane (Amersham™ Biosciences; Freiburg, Germany) by capillary blotting. The

DNA-containing membrane was exposed to ultraviolet radiation to permanently attach the transferred DNA to the membrane. The *nptII* probe was obtained by B*gl*II linearization of the pBI426 vector (Fig. 1) and isolation of the corresponding 811 bp fragment on 1% (*v*/*v*) agarose gel. The purified probe fragment was oligolabeled (Ready-To-Go kit, AGE Healthcare Biosciences Ltd.; Freiburg, Germany) and then used for overnight hybridization with the membrane at 65°C. After excess probe washing, the membrane was maintained for 2 d at −80°C for visualization by autoradiography (Kodak X-ray film, Eastman Kodak Co., Rochester, NY).

Results

Calli bombardment and regeneration of transformed embryos. Intense cellular proliferation was observed at the leaf explant's edge leading to primary calli formation in all explants (data not shown). Friable yellow masses from secondary embryogenic calli were obtained from 90% of the *C. arabica* leaf explants after 6 mo cultivation (Fig. 1*a*). Two leaf explants rendered sufficient material (50 mg of friable calli) for one bombardment shot. To avoid antibiotic toxicity on freshly bombarded calli, a new selection protocol was used wherein kanamycin doses were slowly increased during 4 wk before reaching the highest dose (400 mg/l). After 2 mo on the selective medium supplemented with 400 mg/l kanamycin, most of the bombarded calli showed necrosis. In the third month of selection, we observed the appearance of both yellow friable calli and differentiating globular (Fig. 1*b*) and torpedo embryos (Fig. 1*c*). After a total of 4 mo under selective pressure with monthly subculture, 65 potential transformation events were obtained, most of them callus, but we also obtained some torpedo embryos. During a regeneration period on R medium for 2 mo in the dark, we transferred the fully developed embryos as soon as they reached the cotyledonary stage to light conditions on WPM containing activated charcoal (Fig. 1*d*). After approximately 6 months of subcultivation in WPM, we regenerated 178 somatic embryos with well-developed, green-colored cotyledons. Two months later, development of rootlets and leaves was observed (Fig. 1*e*). A total of 30 putative transformed plantlets were acclimated in soil in the greenhouse (Fig. 1*f*).

Analysis of the GUS stable expression and integration of the uidA gene. GUS histochemical assays were performed on randomly selected bombarded materials growing on medium supplemented with 400 mg/l kanamycin. Regenerated somatic embryos (Fig. 2*a*, *b*) sampled from such kanamycin-resistant material showed blue-colored stained cells indicating the presence of β-glucuronidase activity. Blue staining was also observed in the root tips and leaves (Fig. 2*c*, *d*) of all the embryos germinated in WPM.

Eleven samples from 12 events (T1–T12) assayed by PCR amplified 420-bp fragments (expected size) of the *uidA* gene (Fig. 3). Southern blots on DNA from four events (T3, T4, T5, and T8) showed integration of the *nptII* gene at different loci. Hybridization profiles obtained with the *nptII* probe confirmed integration and an estimated two to three copies of the *nptII* gene for the events T3, T5, and T8 (Fig. 4, lines 5, 6, 7). One event (T4) showed single-copy integration as revealed by the hybridization patterns of the DNA digestion with B*gl*II, S*ca*I, and S*sp*I (Fig. 4, lines 2–4).

Analysis of the genetically modified fruits. Transformed *C. arabica* plants from different events showed normal development and life cycle comparable to the nontransformed controls growing in the same greenhouse conditions. Forty-two flowers and 12 fruits (immature pin heads and green cherries) that developed after flowering in the second year of four transformed coffee plants, which were cloned from the single-copy event T4, were pooled for transgene

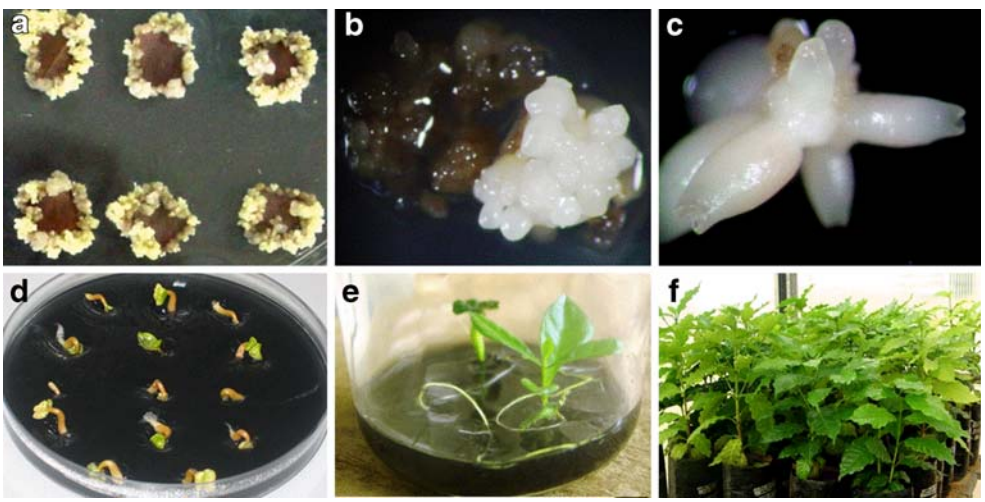

Figure 1. Explants for transformation and regeneration of transformed somatic embryos: (*a*) yellow masses of embryogenic calli produced from *C. arabica* cv. Catuaí Vermelho leaf explants; (*b*) appearance of kanamycin-resistant calli from bombarded explants; (*c*) torpedo-stage embryos grown on the R medium; (*d*) cotyledonary stage, selected embryos cultivated in WPM; (*e*) rooted plantlets before acclimatizing in greenhouse; (*f*) transformed plants.

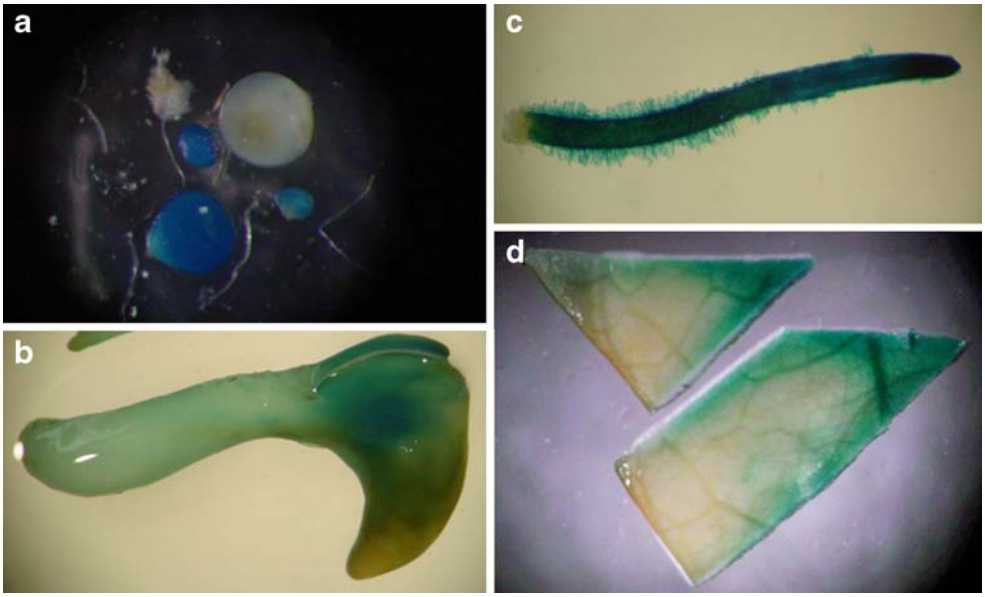

Figure 2. Stable GUS expression evidenced by histochemical analysis (*blue* coloration) of *C. arabica* kanamycin-resistant samples: (*a*) globular embryos; (*b*) cotyledon embryos; (*c*) roots; and (*d*) leaves.

expression analysis. Flower buds showed various stained tissues including the floral verticil, receptacle, stigma, and anther (Fig. 5*a*) as well as pedicel and ovary (Fig. 5*b*). Histological cuttings showed that the ovary cells accumulated greater blue staining than the surrounding tissues (Fig. 5*c*). Similarly, coffee fruits collected 15, 115, and 230 DDA presented GUS staining mostly throughout the cut edge and the endosperm layers (Fig. 5*d–f*). Cotyledon samples from zygotic embryos (T_1 generation) of the T4 event plants (T_0 generation) showed blue staining (Fig. 5*g*) in 19 (73%) samples out of the 26 total samples. This result corresponds to the segregation pattern of a single dominant gene (3:1) in a classical Mendelian fashion.

Discussion

The transformation procedures presented here show that the bombardment of embryogenic calli can be effectively used for introducing genes into *C. arabica*. We obtained an average of 5.4 putative transformation events per 50 mg of bombarded explants from which at least one event/plate was positive for PCR amplification of the *uidA* gene. The osmotic treatment by mannitol performed before the bombardment could have favored the bombardment as reported for the transient expression in coffee cells (Rosillo et al. 2003). All samples from the 65 selected materials were GUS positive. Also, the progressive selection strategy, beginning with a 2-wk interval without kanamycin after bombardment, seemed to positively influence the regeneration of the transformed *C. arabica* cells. About 178 embryos regenerated after more than 2 mo of selection in 400 mg/l kanamycin. Since we observed a good transformation rate, it would be interesting to perform additional repetitions and new experiments to test variations in the selection strategy.

We analyzed 30 GUS-positive and kanamycin-resistant *C. arabica* plants from different transformation events after 2 years of maintenance in greenhouse. The *nptII* hybridization band patterns from the transformed *C. arabica* plants showed low-copy insertions, ranging from one to three copies. Transformed plants showed normal development and life cycle over this period comparable to the non-transformed control growing in the same conditions. Genetically-modified coffee fruits presented normal seed development, as reviewed by Eira et al. (2006), and generated zygotic embryos (T1) presenting Mendelian segregation pattern of the *uidA* gene expression.

We found that the dCaMV35s promoter drove high expression of the *uidA* gene in coffee beans. This marker system is still useful to determine the optimum conditions for gene transfer to fruit trees (Padilla et al. 2006; Maghuly et al. 2008) and to study gene expression in fruit tissues (Honda and Moriguchi 2006). It is important to notice that fruit-specific expression could be misinterpreted if indirectly assessed from different organs as observed by Torregrosa et al. (2008) in the responses of leaves and fruits of the

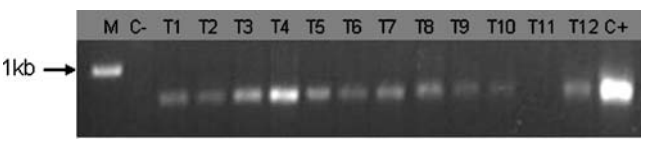

Figure 3. Detection of the *uidA* gene by PCR in 12 *C. arabica* transformants. PCR products were analyzed by electrophoresis on a 1% (*v*/*v*) agarose gel as follows: (*M*) molecular marker, 1-kb DNA ladder; (*C*-) negative control, DNA extracted from *C. arabica* cv. Mundo Novo; (*T1* to *T12*) DNA extracted from GUS-positive plants acclimatized in greenhouse; and (*C*+) positive control, plasmid DNA from pBI426.

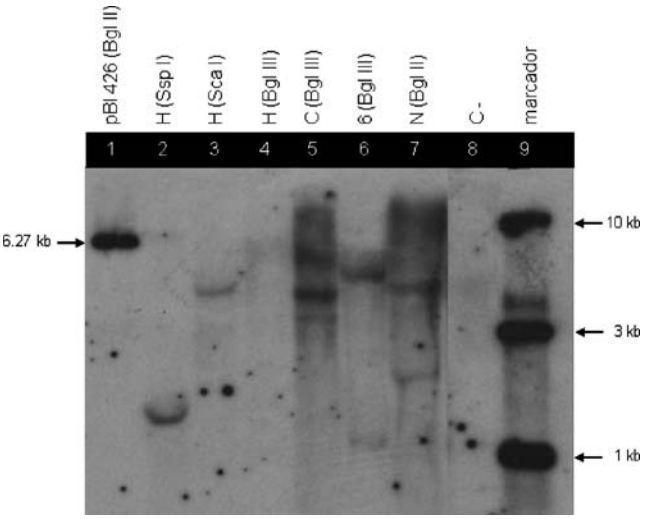

Figure 4. Southern blot analysis of *C. arabica* plants transformed with plasmid vector pBI426. DNA samples (10 μg) from the T3 (*5*), T8 (*6*), and T5 (*7*) events were digested with *Bgl*II while DNA from the T4 event was digested with *Sca*I (*2*), *Ssp*I (*3*), and *Bgl*II (*4*). The *Ssp*I enzyme cuts at least twice into the vector producing a fragment near 1,524 bp. DNA-restricted fragments were hybridized with an oligolabeled *nptII* probe (811 bp) obtained by digestion of pBI426 vector with *Sst*I. The *Bgl*II-linearized vector (350 pg) was used as positive control (*1*) while the *Bgl*II-digested DNA from a non-transformed *C. arabica* plant was the negative control (*8*). A high molecular mass ladder (*9*) from Promega® was used throughout.

same transformed grapevine individuals. Coffee fruits presented deepest blue staining in endosperm tissues in the present study (Fig. 5*d–f*) probably due to their metabolically high activity, which provides a source of carbohydrate and other reserves for the growing seedling (Dentan 1985; Bewley and Black 1994). Flower tissues were responsive to the GUS staining, but this material also presented some degree of oxidation, which partially hampered visualization of the GUS expression. Nevertheless, the presence of GUS in inner organs, such as the flower ovary, was confirmed in histological sections (Fig. 5*c*).

There is a demand for improving the genetic modification of *C. arabica* varieties because Arabica varieties are the most important genotypes for the global coffee market. While the genetic transformation of *C. arabica* tissues has already been achieved (Van Boxtel et al. 1995; Fernandez-Da Silva and Menéndez-Yuffá 2003; Rosillo et al. 2003), only a few regenerated and genetically modified plants were reported (Leroy et al. 2000), and no data is available about their offspring. The viability of the bombardment method to transform coffee was demonstrated previously in *C. canephora* (Ribas et al. 2005). Here, we describe the genetic modification by bombardment of *C. arabica* fruits that is similar to precursor work for transformation experiments with the α-amylase inhibitor (Valencia et al. 2000), aiming to develop resistance of coffee plants to the coffee berry borer parasite (unpublished data). This methodology may be transferred

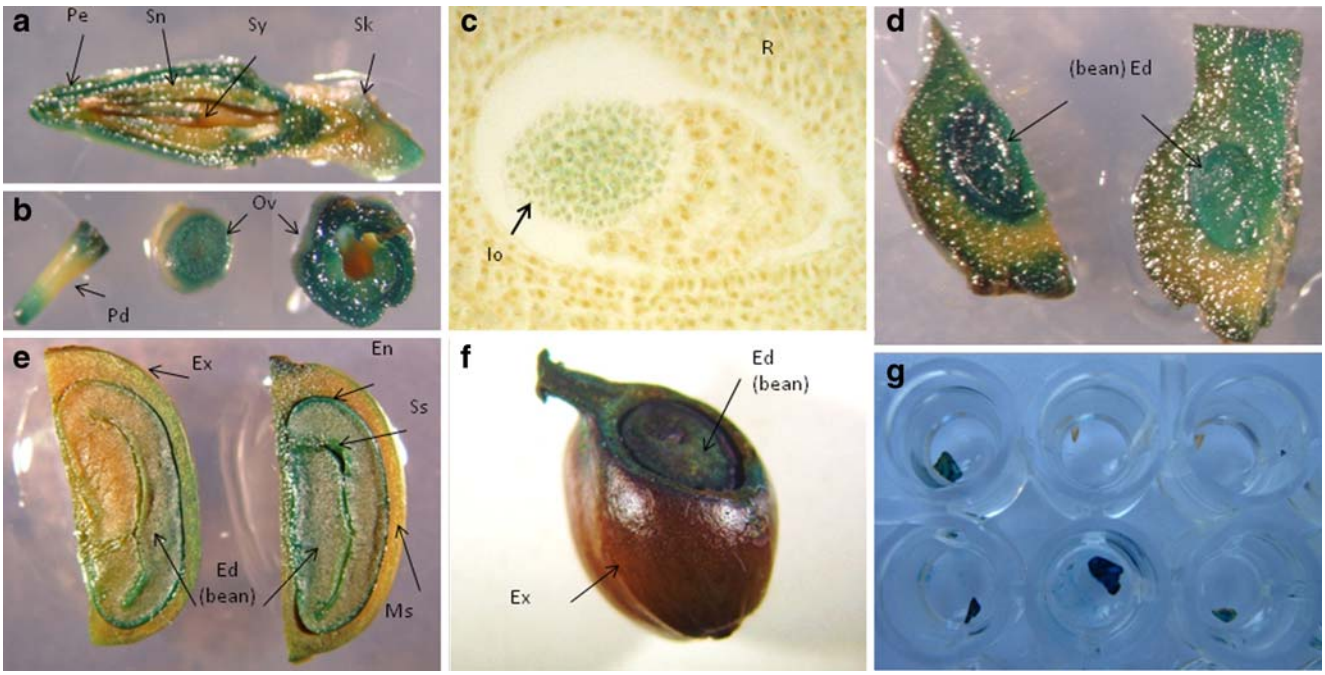

Figure 5. Cut samples from transformed *C. arabica* plants after GUS staining assays: (*a*) flower bud divided in two; (*b*) pedicel and initial seed development from ovary at the pin head stage; (*c*) chopped pin heads; (*d*) sliced bean of green immature cherry; (*e*) whole mature cherry; (*f*) ovary section under light microscopy (×200), pin head longitudinal serial semithin sections stained with xylol; and (*g*) cotyledon samples from zygotic embryos (T_1 generation) from the transformation event T4. *Ed* endosperm, *Ex* exocarp, *En* endocarp, *Ms* mesocarp, *Ss* silver skin, *Ov* ovary, *Sk* stalk, *Sy* style, *Sn* stamen, *Pe* petals, *Pd* pedicel, *Io* immature ovule, *R* receptacle.

from *C. arabica* Catuaí Vermelho to other coffee varieties considering that bombardment is not genotype-dependent as is *Agrobacterium*-mediated transformation. To our knowledge, the results presented in this paper constitute the first report of long-term, stable GUS expression and heritability of such a transgene in coffee fruits. While an initial report, the protocol described here constitutes a useful tool for the introduction of agronomic traits and for the functional validation of candidate genes in *C. arabica* fruits.

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