Plant Cell, Tissue and Organ Culture (2006) 84: 373–377
DOI 10.1007/s11240-005-9036-4

Research note

Genetic transformation of C*offea canephora* by vacuum infiltration

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Received 9 June 2005; accepted in revised form 19 September 2005

Key words: *Agrobacterium* transformation, coffee, somatic embryogenesis, vacuum infiltration

Abstract

Agrobacterium-mediated plant transformation protocol was evaluated as a fast method to obtain genetically modified *Coffea canephora* plantlets. Leaf explants were used as source material for *Agrobacterium tumefaciens*-mediated transformation involving a vacuum infiltration protocol, followed by a step of somatic embryogenesis induction and a final selection of the transformed plants. *A. tumefaciens* strain C58CI containing the binary vector pER10W-35SRed was used. PCR amplification of DsRFP gene and visual detection of the red fluorescent protein demonstrated 33% transformed embryos. The protocol presented here produces reliable transgenic coffee embryos in two months.

Abbreviations: EDTA – ethylenediaminetetraacetic acid; MS – Murashige and Skoog; PCR – polymerase chain reaction; SDS – sodium dodecyl sulfate

The genetic modifications of coffee cultivars have been carried out since 1991 by electroporating DNA into protoplasts of *Coffea arabica* (Acuna and de Pena, 1991). However, this method yielded low and unreliable amounts of transformed plants which took over a year to develop. Later, *Agrobacterium* sp. was used to generate transformed roots (Carneiro, 1997). The first regeneration of transgenic coffee plants by this method was by the transformation of somatic embryos using *A. rhizogenes* (Spiral et al., 1993), and more recently transformation has been carried out with *A. tumefaciens* (Leroy et al., 1997, 2000; Hatanaka et al., 1999; Ogita et al., 2004).

Although the latter studies have taken advantage of somatic embryogenesis for the transformation this event has been done at a relatively late stage leading to a long waiting period for the recovery of potentially chimeric transgenic plants. Furthermore, the use of indirect somatic embryogenesis relies on the generation of embryogenic calluses for the formation of embryos, which can take several months to develop. Here we take advantage of a previously reported simple protocol to generate somatic embryos directly from coffee leaf explants (Quiroz-Figueroa et al., 2002), combined with vacuum infiltration of *A. tumefasciens* to induce transformation of the wounded tissue (Acereto-Escoffié et al., 2005). This provides a fast and reliable method of producing transgenic coffee plants.

Plantlets of *Coffea canephora* cv. Robusta were grown in Magenta® boxes containing 40 ml of MS medium, supplemented with thiamine (11.86 μM), myo-inositol (0.56 μM), L-cysteine (0.16 μM), sucrose (87.64 mM), α-naphthalene acetic acid

(0.54 μM), kinetin (2.32 μM) and Gelrite® (0.25% w/v); pH was adjusted to 5.8 before autoclaving (20 min, 110 °C). The plantlets were cultured under photoperiod (16 h/8 h light/dark) at 25 ± 2 °C and were transferred to fresh medium every 90 days. For somatic embryo induction 200 leaf segments, were excised with a 1 cm diameter corkborer and cultured in the BA-based Yasuda medium (Yasuda et al., 1985) modified as follows: 0.748 mM $CaCl_2$, 5.15 mM NH_4NO_3, 4.7 mM KNO_3, 0.624 mM KH_2PO_4, 0.5 mM Na_2MoO_4, 50 mM H_3BO_3, 0.375 mM $MgSO_4$, 40 $MnSO4 \cdot 7H_2O$, 0.2 mM $CuSO_4$, 15 mM $ZnSO_4$, 75.53 mM $FeSO_4$, 74.95 mM Na_2EDTA, 4.86 mM Piridoxin, 8.12 mM nicotínic acid, 29.6 mM thiamine–HCl, 550 mM myo-inositol, 87.64 mM sucrose and 5 mM 6-benzil amino purine; pH was adjusted to 5.8 and Gelrite® (0.25% w/v) was used as gelling agent. Cultures were incubated under photoperiod (16 h/8 h light/dark) with a light intensity 50 μmol m^{-2} s^{-1} at 25 ± 2 °C.

Agrobacterium tumefaciens strain C58Cl was used for the transformation of coffee explants. Bacterial selection for this strain was carried out with 100 mg l^{-1} of rimfampicin and 100 mg l^{-1} of espectomicin. The binary vector was created by cloning the *Sma*I and *Sac*I fragment of pCd-35SRed (Gallie et al., 1989), that contains the DsRFP reporter gene under the constitutive 35S promoter into pER10W that contains neomycin phosphotransferase gene for selection as published earlier (Zuo et al., 2002), digested with *Spe*I. The ligation reaction was carried out after fill-in of the 5′ overhangs with DNA polymerse I (Klenow fragment from Invitrogen Life Technologies) to generate pER10W-35SRed.

Bacterial culture. An aliquot of *A. tumefaciens* strain C58Cl was inoculated in 5 ml of YEP medium (10 g l^{-1} peptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl) containing the appropriate antibiotics and allowed to grow at 27 °C with 110 rpm agitation for 2 days. Subsequently, this bacterial suspension was added to 100 ml of YEP supplemented with the required antibiotics. The culture was allowed to grow at 27 °C with 110 rpm agitation until it reached 1 OD. Afterwards, the culture was centrifuged at 1940 × *g* for 30 min. *A. tumefaciens* was induced by resuspension of the bacterial pellets in 100 ml of YEP containing 55 mM dextrose, allowing growth for 4 h with agitation at 110 rpm at 27 °C followed by induction with 100 μM acetosyringone for 16 h at the same growing conditions to an OD of 0.8. The coffee explants were transferred to the induced bacterial culture and submitted to vacuum infiltration at 400 mm of Hg for 4 min as described earlier (Acereto-Escoffié et al., 2005). The explants were then transferred to MS medium without antibiotics (Murashige and Skoog, 1962), and shaken in the dark at 27 °C and 110 rpm for 24 h. Afterwards the explants were rinsed with 200 mg l^{-1} timentin and claforan to eliminate excess bacteria. Controls were treated in a similar way in absence of *A. tumefaciens*.

The *A. tumefaciens* treated explants were washed in each of the following medium for 10 min: sterile water, timentin and timentin–claforan (200 mg l^{-1} of each). Afterwards, the explants were transferred to liquid MS-medium containing 200 mg l^{-1} timentin and 100 mg l^{-1} kanamycin, used as a selection agent. The antibiotic washings were repeated 48 and 98 h later. Subcultures were carried out every 15 days. Each explant yields between 10 and 50 somatic embryos. Two torpedo stage somatic embryos were chosen after 2 months from each explant.

For DNA isolation, samples were macerated with 200 μl extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), and centrifuged at 17,949 × *g* for 3 min. The extracts were washed with 100 μl of phenol: chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with ethanol and resuspended in water.

For PCR analysis, the *DsRFP* gene primers used were: forward (5′-ATGAGTTGTTCCAA GAATGT-3′) and reverse (5′-TACCGCTCACC CACGTTTGA-3′) which amplify a 675 bp fragment. The Vir E2 selected primers for detection of *A. tumefaciens* contamination were (5′-TGC CCA CCA AGG CGG AAT T-3′) and (5′-CTT TGC CGA CCC ATC GA-3′). The final composition of the reaction mixture was: 1× PCR buffer, 0.2 mM dNTPs, 0.2 mM $MgCl_2$, 0.5 μM forward and reverse primers, 200 ng sample DNA and 0.04 U μl^{-1} *Taq* polymerase. The reaction conditions were: initial denaturalization 96 °C for 1 min, 30 amplification cycles (denaturalization 94 °C for 1 min, primer annealing 55 °C for 1 min, elongation 72 °C for 1 min) and a final extension step of 10 min at 72 °C before lowering the temperature to 4 °C. The amplified fragments were separated in 1%(w/v) agarose gels.

DNA (50 μg) from a selected transformed plant and from a non-transformed plant (Control) was digested with *Eco*RI or *Xho*I restriction enzymes, separated on a 0.7% agarose gel and blotted onto charged nylon membranes (Hybond N^+, Amersham Pharmacia Biotech). For the probe labelling and hybridization we used the Gene Images Alkphos direct labelling and detection system (Amersham Biosciences) kit with the sequence of DsRFP as a template. The hybridization was carried on at 65 °C and all steps were carried according with the manufacturer's instructions.

Red fluorescent protein was detected by microscopy in a Axioplan microscope with an excitation wavelength of 546/12 nm and 580 nm filters. Fluorescent stereoscopy was carried out in a Leica MZFLIII with an excitation wavelength of 546/10 nm and filters D600/40.

In Table 1 we show the number of embryos selected from the explants and tested for transformation, as well as the number of red fluorescent embryos obtained after the transformation. A total of 200 transformed and 200 non-transformed embryos were tested from 4 independent sets of experiments. Thirty three percent of the transformed embryos showed a strong red fluorescence at 580 nm with an excitation at 540 nm (Figure 1a).

Transformed embryos were selected under a fluorescent stereoscope at this stage and subsequently grown in selective media to obtain the transformed plants. We employed this simple visual test for initial selection. Once the somatic embryos have been selected, they were placed in a selective media containing kanamycin.

To test the presence of the inserted DNA fragment in genomic DNA extracts and to assure the lack of bacterial contamination, PCR assays were carried using primers for amplification of *DsRFP* and *Vir* E2 genes, respectively (Figure 1b). To prove that transformation had occurred and that fluorescence was not due to bacterial expression of the *DsRFP*, a Southern blot was carried out from the DNA obtained from a 5-month-old transformed coffee plants and an equivalent non-transformed plants. The *DsRFP* DNA hybridized with the genomic DNA of the transformed plant. Southern blot analysis combined with PCR of the *VIR* genes are useful tools to ensure the genetic integration of the transgenes because the fluorescence expression in plant tissues can result of leftover *A. tumefaciens* expressing DsRF.

Figure 2 depicts different stages of the transformation protocol in relation to the somatic embryogenesis induction. The leaves are cut and incubated in Yasuda media for 1 week prior to vacuum infiltration with *A. tumefaciens*. After the infection, daily check of the explants and proper wash must be carried out to prevent the bacteria from degrading the explants tissue. The number of somatic embryos produced was lower than in explants that were not treated with bacteria (data not shown); this is probably due to the constant cleaning or to the toxicity of the compounds used to remove the bacteria. This is a crucial step for reducing the time for the transformation of coffee plants. However, over 90% of the torpedo stage embryos germinated into new plants. Recent transformation of this cultivar has been carried out using embryogenic calluses (Hatanaka et al., 1999), which take several months to establish. However, maintenance of embryogenic calluses can only be done for a limited period of time before its regeneration ability is lost. This protocol allowed the generation of transformed somatic embryos at torpedo stage that where able to express *DsRFP* in 2 months.

Table 1. Transformation efficiency of coffee explants

Treatment	Number of embryos	Number of embryos that show fluorescence (%)	Number of embryos that show a positive DsRFP PCR (%)	Mortality percentage in selective media[a] (%)	Mortality percentage for fluorescent embryos in selective media[b] (%)
Control	200	0	0	100	n/a
Transformed	200	33	32	67	0

Two hundred transformed and 200 non-transformed embryos were selected from different explants and observed under fluorescence microscopy. Hundred embryos from either non-transformed explants (Control) or transformed explants were used for DNA extraction and PCR. The remaining embryos where grown in media supplemented with 100 mg ml^{-1} kanamycin as a selective agent. Mortality is given in percentage either from the total 100 plants[a] or from the embryos that showed fluorescence[b] that survived after 4 months.

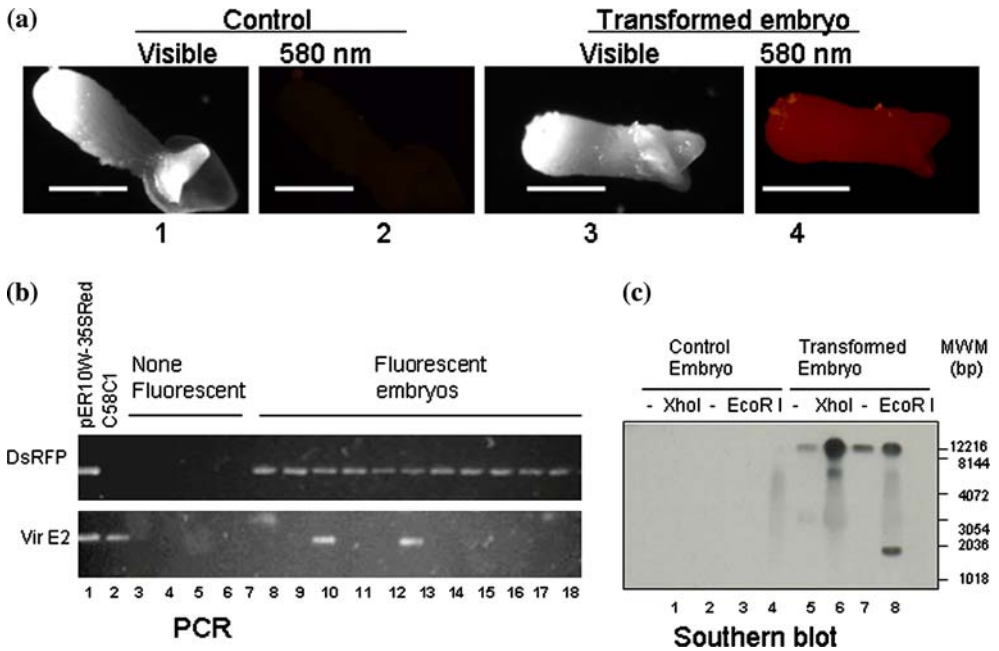

Figure 1. Analyses of transformed coffee somatic embryos. (*a*) micrographs in visible and fluorescent selected with a 580 nm filter of control (1,2) and transformed (3,4) coffee embryos; bars = 1 mm. Expression of DsRFP observed in a transformed embryo (4). (*b*) PCR analyses for DsRFP and Vir E2 of non-fluorescent and fluorescent embryos showing DsRFP amplifications in the control plasmid (lane 1) and in all fluorescent embryos (lanes 7–18), but not in non-fluorescent embryos (lanes 3–6). Amplification of *Vir* E2 gene shows *A. tumefaciens* contaminations in lanes 9 and 12. (*c*) Southern blot analysis of non-transformed plants (lanes 1–4) and transformed plant (lanes 5–8).

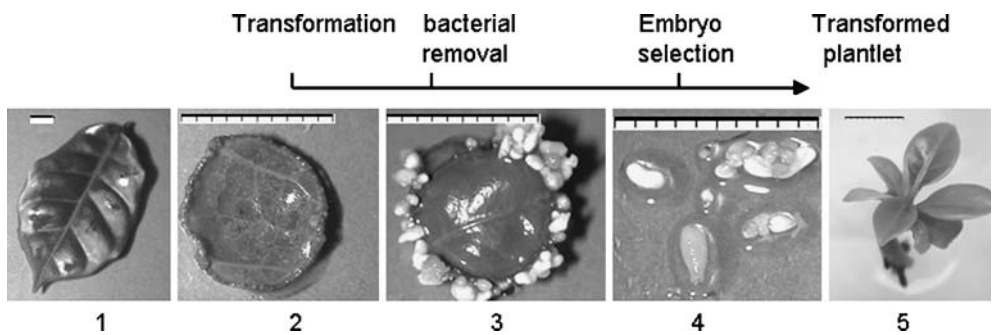

Figure 2. Development of somatic embryos from coffee leaf explants. The diagram shows the points of *A. tumefaciens*-mediated transformation, relative to the different steps of embryo development. The transformed plantlet shown in box five took 4 months to develop after transformation; bars = 1 cm.

Acknowledgements

We wish to thank Francisco Quiroz and Mirian Monforte for their technical help and Luis Joel Figeroa from Accesolab for the contribution of reagents for this study. Dr Castano is partially funded by CONACYT Grant 39731-z.

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