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SHORT REPORTS

A simple and efficient agroinfiltration method in coffee leaves (*Coffea arabica* L.): assessment of factors affecting transgene expression

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Abstract

The establishment of a simple, rapid and efficient transient expression system is a necessary tool for the functional validation of candidate genes in coffee biotechnology. The effects of *Agrobacterium* strain, age of the donor plant, infiltration method, and infiltration medium on transgene expression in detached coffee leaves were evaluated. Regarding the effect of *Agrobacterium* strain, the expression of *uidA* was higher in GV3101-treated coffee disks than in LBA4404 and ATHV-treated samples. On the other hand, transient expression of *uidA* was significantly higher in leaf disks from young plants (6-weeks-old) (13.1 ± 1.4%) than in mature tissue (12-weeks-old) (1.6 ± 1.2%). Transient *uidA* expression was higher in detached coffee leaf disks from young plants infiltrated with one injection of 15 µL of *Agrobacterium* strain GV3101::1303 suspended in MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS with subsequent sanding of the abaxial epidermis. Using the optimized protocol, expression of the *uidA* gene was observed 6, 24 and 48 h and 5 weeks after bacterial injection. DNA was extracted from coffee disks with positive GUS expression and specific *mgfp5* and *uidA* fragments were amplified 5 weeks post-agroinfiltration. On the other hand, using the optimized protocol, a specific *cry10Aa* (500 bp) fragment was amplified in the agro-infiltrated coffee leaf disks 5 weeks post-agroinfiltration with the plasmid pB427-35S-cry10Aa. Moreover, the expression of the gene *cry10Aa* in two infiltrated coffee leaf disks was verified by RT-PCR and an expected 500 bp fragment was amplified.

Keywords Leaf agroinfection · Genetic transformation · In planta genetic transformation · *Agrobacterium tumefaciens* · *Bacillus thuringiensis* · *Cry10Aa*

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
AS	Acetosyringone
PCR	Polymerase chain reaction

Introduction

Coffee (*Coffea* spp.) is an economically important crop and one of the main export products of several countries in Latin America, Africa and Asia (Ivamoto et al. 2017). Recently, the genomes of *C. canephora* (Denoeud et al. 2014) and *C. arabica* (Van Deynze et al. 2017) as well as the transcriptomes of Arabica coffee (Ivamoto et al. 2017) and an Arabica coffee ancestor *Coffea eugenioides* (Yuyama et al. 2016) were reported. These advances open possibilities for the study of candidate genes related to agronomical traits and metabolic pathways and for assisted breeding applications through the introduction of new desirable traits by genetic engineering (Ribas et al. 2011).

Two genetic transformation strategies, stable transformation and transient transformation, have been used in plants for gene function analysis, protein production, protein–protein interaction and promoter activity evaluation (Cao et al. 2017; Zhao et al. 2017). Protocols for stable

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genetic transformation of *C. arabica* and *C. canephora* have been reported using *Agrobacterium tumefaciens* (Hatanaka et al. 1999; Leroy et al. 2000; Mishra et al. 2002; Ogita et al. 2002; Canche-Moo et al. 2006; Ribas et al. 2011), *Agrobacterium rhizogenes* (Alpizar et al. 2006; Kumar et al. 2006) electroporation (Fernandez-Da Silva and Menendez Yuffa 2003) and biolistics (van Boxtel et al. 1995; Rosillo et al. 2003; Ribas et al. 2005; Albuquerque et al. 2009).

Genetic engineering research on coffee has been done in the past 15 years to express herbicide resistance genes, insect resistance genes, genes controlling biochemical and physiological traits and reporter genes (Mishra and Slater 2012). Nevertheless, up to date, coffee plants expressing *cry* genes from *Bacillus thuringiensis*, which are effective against coffee berry borer (CBB), one of the major threats for its production, have not been produced. In this sense, Espinoza et al. (2010) indicated that *cry10Aa* and *cyt1Aa* genes might be effective against CBB. The results reported by these authors showed that bioassays against first-instar of *H. hampei* of *cry10Aa* and *cyt1Aa* gave estimated larval mortality of 20 and 50%, respectively.

Nevertheless, stable transformation of coffee under in vitro conditions is still a time consuming, inefficient and laborious process that requires the establishment of an efficient regeneration system (Ribas et al. 2011). Therefore, rapid, simple and efficient techniques for the functional study of candidate genes are particularly attractive (Rosillo et al. 2003). Transient transformation avoids the limitations of the stable transformation process such as transformation efficiency, selection, and regeneration of putative transgenic material (Zhao et al. 2017). A transient transformation system has been described for coffee by van Boxtel et al. (1995) and Rosillo et al. (2003). These authors evaluated factors affecting the transient expression of transgenes introduced into suspension cells using particle bombardment. To the best of our knowledge, an ex vitro protocol for transient expression using *A. tumefaciens*-mediated infiltration of coffee leaves has not been reported.

The delivery of genes into plant tissues for transient expression using *A. tumefaciens* has been reported in various species using vacuum or syringe-based infiltration methods (Shah et al. 2013; Cao et al. 2017; Zhao et al. 2017). Vacuum infiltration requires specialized equipment. Due to its speed, yield, and cost, this method has been used for the production of large quantities of recombinant proteins (Zhao et al. 2017). On the other hand, the syringe-based method requires only a needleless syringe. Due to its simplicity and speed, it has been widely used for promoter and gene function analyses, plant-pathogen interaction studies, abiotic stress-tolerance assays, and for the production of low quantities of recombinant proteins (Cao et al. 2017). In order to increase transformation efficiency, several factors need to be optimized (Matsuo et al. 2016; Cao et al. 2017). We studied the effects of the infiltration method, *Agrobacterium* strain, and age of the donor plant on Agrobacterium infiltration of coffee leaves to produce an efficient protocol for transient and stable expression of genes in coffee.

Materials and methods

Plant material

Seedlings of *C. arabica* L. variety Catuaí provided by CICAFE (Coffee Research Center, Barva de Heredia, Costa Rica) were grown in pots containing peat (Nutripeat, V-J Centroamericana S.A, Costa Rica) in a culture room at 100% relative humidity in darkness at 30 °C. The second fully expanded leaf below the apex was collected from 6 and 12-week-old plants and leaf disks were cut using a cork borer. Prior to agroinfiltration, leaf disks were rinsed in 95% (v/v) ethanol for 1 min and subsequently surface sterilized with a 1% (v/v) NaOCl solution for 1 min. After three washes with distilled water, the leaf disks were injected with bacterial suspensions as described below.

Bacterial strains and binary plasmids

For the optimization of the agroinfiltration method, three *Agrobacterium* strains with different chromosomal backgrounds and opines were used: *A. tumefaciens* GV3101 (50 mg/L rifampicin + 50 mg/L spectinomycin + 50 mg/L streptomycin + 50 mg/L kanamycin), LBA4404 (50 mg/L rifampicin + 50 mg/L ampicillin + 50 mg/L kanamycin) and ATHV (50 mg/L rifampicin + 50 mg/L kanamycin) containing the binary plasmid pCAMBIA 1303 (acquired from CAMBIA, Canberra, Australia). The T-DNA contained reporter genes *mgfp5* (which codes for green fluorescent protein) and *uidA* (which codes for beta-glucuronidase) and the selectable marker gene *hptII* coding for hygromycin phosphotransferase under the control of the CAMV 35S promoter (Fig. 1a). For the stable genetic transformation experiments, the *A. tumefaciens* GV3101 strain containing the binary plasmid pB427-35S-cry10Aa was used (acquired from DNA Cloning Service, Hamburg, Germany). The T-DNA cassette harbors the *cry10Aa* and *hptII* gene under the control of the CAMV 35S promoter (Fig. 7a). Plasmids were introduced into the agrobacteria by the freeze–thaw method. Their presence was verified by restriction analysis and by PCR (polymerase chain reaction) using specific primers for the *hptII* and *cry10Aa* gene.

Prior to transformation experiments, a single colony of each *Agrobacterium* strain was grown overnight at 26 °C in liquid LB medium supplemented with 20 µM acetosyringone (AS) (5-dimethoxy-4-hydroxyaceto-phenone) and the respective antibiotics on an orbital shaker at 250 rpm.

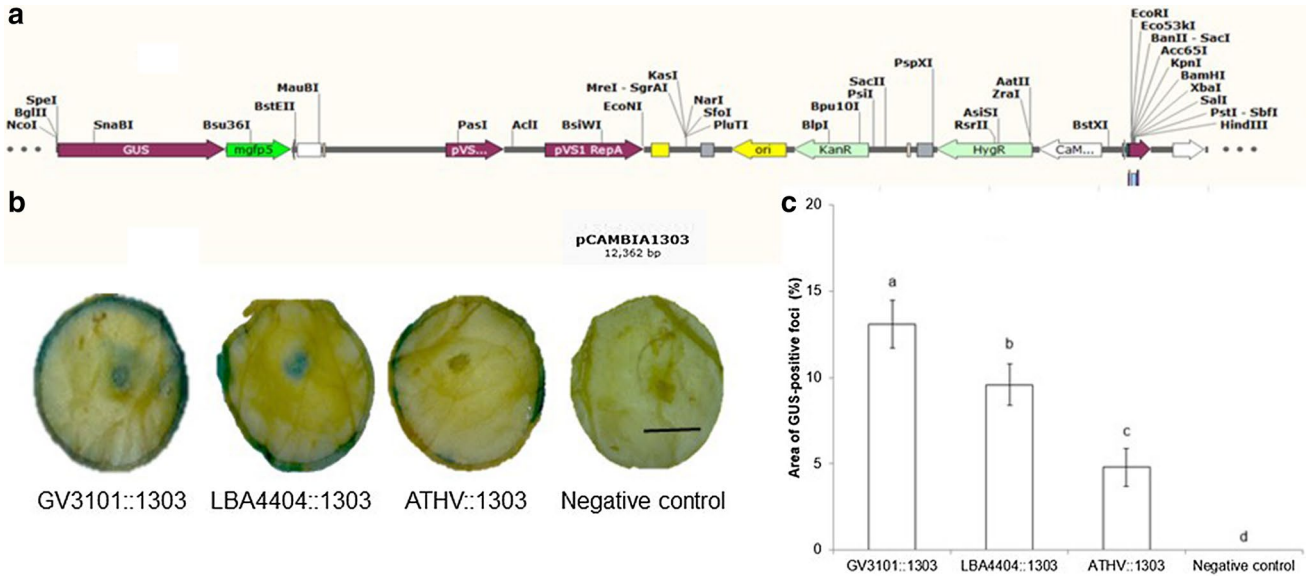

Fig. 1 Transient GUS expression in coffee leaf disks injected with three strains of *Agrobacterium tumefaciens*. **a** Linear map of pCAMBIA1303 plant expression vector **b** GUS staining observed 48 h after infiltration **c** Percentage of GUS-stained area detected using Image J measurements. Each value represents the mean ± SD of fifteen repetitions. Means with the same letter are not significantly different (Duncan test $p = 0.05$). Bar: 1 cm

The bacterial suspensions were centrifuged at 3500 rpm for 20 min. Pellets were resuspended in Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS to a final optical cell density (OD_{600}) of 0.6.

Optimization of agroinfiltration mediated transformation

To establish an agroinfiltration protocol for detached coffee leaf disks, four variables were evaluated: the strain of *Agrobacterium tumefaciens*, the age of the plant (6 or 12 weeks old) from which leaf disks were obtained, the type of wound made in the leaf disks and the infiltration medium in which the bacteria were resuspended.

In the first experiment, a 1 mL needleless syringe was used to inject surface sterilized coffee leaf disks (1 cm) with a suspension of the *Agrobacterium* strains GV3101, LBA4404 or ATHV (OD_{600}: 0.6) carrying the pCAMBIA 1303 plasmid. Injection was done as follows: a small wound was made in the abaxial epidermis of the leaf using the needle of the syringe. Subsequently, 15 µL of the bacteria solution was injected using a needleless syringe in the wound until the complete disk was covered. As the solution was injected, the disk becomes darker, which shows proper infiltration. In the second experiment, once the optimal *Agrobacterium* strain was chosen, coffee leaf disks from young (6-week-old) and adult (12-week-old) plants were injected with a solution of *A. tumefaciens* GV3101::pCAMBIA 1303 using a 1 mL needleless syringe. In the third experiment, once the best age of the donor plant was selected, a 1 mL needleless syringe was used to inject leaf disks from 6-week-old plants with *A. tumefaciens* GV3101::pCAMBIA 1303 using one of the following methods: (A) one injection of 15 µL of the bacterial suspension and subsequent sanding of the abaxial epidermis, (B) one injection of the bacterial suspension (15 µL) without sanding, (C) four equidistant injections of 15 µL of the bacterial suspension without sanding, (D) sonication for 1 min of the leaf disks together with the bacteria suspended in infiltration medium, and (E) sonication of the disks in infiltration medium for 1 min and then one injection of 15 µL of the bacterial suspension. Sanding of the abaxial epidermis was done with a 2000 grit sandpaper.

Finally, in the fourth experiment, coffee leaf disks from 6-week-old plants were infiltrated with one injection of *Agrobacterium* strain GV301::1303 suspended in MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS or 10 mM $MgCl_2$ supplemented with 1.9 g/L MES and 200 uM AS. In all experiments, the leaf disks were placed on wet tissue in Petri dishes with the adaxial side up and maintained at 25 °C in darkness for 48 h. Then, the explants were washed with a 1% (v/v) NaOCl solution for 1 min followed by three washes with distilled water of 10 min each one. All experiments were performed in triplicate using five leaf disks. Explants not exposed to *A. tumefaciens* were included as negative controls.

Transformation efficiency was evaluated 48 h after infiltration by observing GUS histochemical staining. The relative area of blue staining per explant was quantified using the program ImageJ (https://imagej.nih.gov/ij/index.html) (Rasband 1997–2016) as follows: [(area with blue foci/total area of the leaf disk) × 100]. Data were subjected to one-way analysis of variance (ANOVA) and means were compared using Duncan's (1955) multiple range tests ($p = 0.05$).

Histochemical GUS assays

GUS assays were performed by immersing tissues in staining buffer [50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, 0.1% Triton, 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide)] for 24 h at 37 °C in the dark (Jefferson 1987). Leaf disks were then washed with 95% ethanol (EtOH) and incubated at 65 °C for 12 h until the green color was removed. GUS-stained tissue was observed under 10-fold magnification using a stereomicroscope (Nikon SM2 8000) equipped with a digital camera (Moticam 5).

Transformation of the *cry10Aa* gene using the optimized method

Coffee leaf disks from 6-week-old plants were infiltrated with 15 µL of *Agrobacterium* strain GV301::1303 or GV301::pB427-35S-cry10Aa suspended in MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS. After agroinfiltration, the explants were placed on wet tissue in Petri dishes with the adaxial side up and maintained at 25 °C in darkness for 48 h. Then, the explants were washed with a 1% (v/v) NaOCl solution for 1 min followed by three washes with distilled water of 10 min each one. Following washing, the infiltrated disks were placed on wet tissue in Petri dishes with the adaxial side up and kept in plant tissue culture room with 16 h day light regime at 25 °C for 5 weeks. Explants not exposed to *A. tumefaciens* were included as negative controls.

DNA extraction and PCR analysis

Genomic DNA was extracted using the CTAB method outlined by Doyle and Doyle (1990) with minor modifications according to Bolívar-González et al. (2018). The concentration and purity (ratio 260/280) of genomic DNA was measured by spectrophotometric analysis and adjusted to 100 ng/µL. The integrity of the extracted DNA was verified by electrophoresis on 0.8% (*w*/*v*) agarose gels prepared in 1X TBE buffer.

PCR was used to test for the presence of the *mgfp5, uidA*, cry10Aa, and *virG* genes. The PCR reaction was performed in a mixture (25 µl) containing 1X Taq Buffer + $(NH_4)SO_4$ (Thermo Scientific), 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.3 µM of each primer, 0.5 U Taq Polymerase (Thermo Scientific) and 3 µL of genomic DNA. The primer sequences, as well as the amplification conditions, are shown in Supplementary Table 1. PCR products (10 µl) were separated on 1% (*w*/*v*) agarose gels stained with GelRed® (Phenix Research) in 1X TBE buffer at 50 V for 1 h. The gels were visualized in a Darkhood DH-20 transilluminator and photographed with the Gerix 1000 system (Biostep, Germany). The plasmid pCAMBIA 1303 and pB427-35S-cry10Aa were used as a positive control for the presence of the *uidA* and *cry10Aa* genes, respectively. Whereas DNA isolated from *A. tumefaciens* was used as positive control for *virG*.

RNA isolation and RT-PCR

Total RNA was isolated from two infiltrated and one non-infiltrated coffee leaf disks using the TRI Reagent (Sigma–Aldrich Chemie, Steinheim, Germany) following the manufactures' instructions. After RNA extraction, DNA was removed by DNase I treatment (MBI Fermentas, St. Leon-Rot). The total RNA was quantified using a spectrophotometer (NanoPhotometer TM, Germany) at wavelengths of 260 and 280 nm, and RNA integrity was verified by analyzing samples on a 1.2% (*w*/*v*) denaturing agarose gel. RNA (1 µg) was transcribed to DNAc using the RevertAid H Minus First Strand cDNA Synthesis Kit® (Thermo Scientific) according to the manufactures' instructions. For detecting possible DNA contaminations in RNA preparations, a negative control was included in which no reverse transcriptase was employed.

The transcript of the gene *cry10Aa* was analyzed by PCR in a mixture (20 µl) containing 1X Taq Buffer + $(NH_4)SO_4$ (Thermo Scientific), 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.3 µM of each primer [cry10Aa-f: (5′-aactacaagagaaccgattcataca-3′) and cry10Aa-r: (5′-acggtacgtgggaaagtaaca-3′)], 0.5 U Taq Polymerase (Thermo Scientific) and 2 µL of DNAc. Amplification conditions for were as follows: 94 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and a final step of 72 °C for 10 min. The RT-PCR products (10 µl) were evaluated on 1.2% (*w*/*v*) agarose gel stained with GelRed® (Phenix Research) in 1X TBE buffer at 50 V for 1 h, visualized in a Darkhood DH-20 transilluminator and photographed with the Gerix 1000 system (Biostep, Germany).

Results and discussion

Several factors, including plant genotype, type of explant, *Agrobacterium* strain, cell density in inoculation medium, and the conditions of inoculation and co-culture, influence the transfer of T-DNA from *Agrobacterium* to plant cells.

Each of these elements needs to be optimized for each transformation system (Amoah et al. 2001).

Effect of the *A. tumefaciens* strain on transient expression of *uidA*

To determine the most suitable strain for the transient expression of the *uidA* gene, fifteen coffee disks were injected using *A. tumefaciens* strain GV3101 (C58, nopaline), LBA4404 (Ach5, octopine) or ATHV harboring the plasmid pCAMBIA 1303. We observed histochemical staining 48 h post infection in 100% of the explants injected with either GV3101 or LBA4404. A positive reaction was seen in 86.7% of the explants injected with ATHV (Fig. 1b). Expression of *uidA* was 0.7 and 2.7 times higher in GV3101-treated coffee disks than in LBA4404 and ATHV-treated samples, respectively (Fig. 1c). The *A. tumefaciens* strain has been shown to affect the efficiency of transient expression in grapevine (Zottini et al. 2008), rose (Yasmin and Debener 2010), *Maesa lanceolata* (Faizal and Geelen 2012), and spinach (Cao et al. 2017). In coffee, Mishra et al. (2008) demonstrated that the *Agrobacterium* strain influenced the transient expression of *uidA* and *gfp* in embryogenic tissues. The virulence of *Agrobacterium* strains can be dependent on the target plant species (Faizal and Geelen 2012). This may be due to the ability of the bacteria to attach to plant cells or to differences in the T-DNA transfer mechanism (Wroblewski et al. 2005).

Effect of plant age on transient GUS expression

To determine the effect of leaf age on the transient expression of the *uidA* gene, fifteen coffee leaf disks from young (6-week-old) and adult (12-week-old) plants were injected with *A. tumefaciens* GV3101::pCAMBIA 1303. Transient expression of *uidA* was observed 48 h post infection in leaves of both stages injected with the bacterial suspension but not in the non-inoculated control (Fig. 2a). Transient expression of *uidA* was significantly higher in leaf disks from young plants than in mature tissue. Histochemical staining was observed in 100% of the leaf disks from 6-week-old plants and the relative area of blue-stained tissue per explant was 13.1 ± 1.4% (Fig. 2b). Similarly, leaf age affected transformation efficiency in cacao (*Theobroma cacao* L) (Fister et al. 2016) and strawberry (*Fragaria vesca*) (Cui et al. 2017). Transient expression of *yfp* and *gfp* genes decreased with increasing leaf age (Fister et al. 2016; Cui et al. 2017). Fister et al. (2016) attribute this result to both physiological and physical (toughness, rigidity) differences between leaves of different ages.

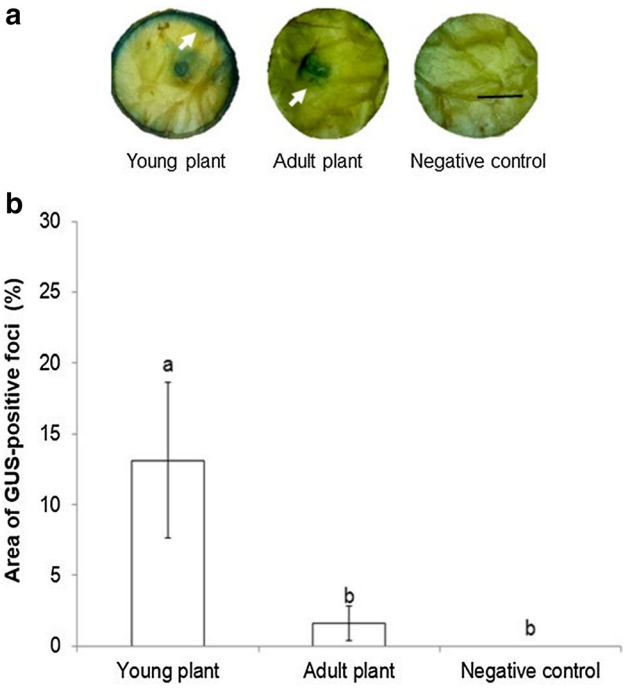

Fig. 2 Transient GUS expression in coffee leaf disks from young and adult plants injected with *A. tumefaciens* GV3101::pCAMBIA 1303. **a** GUS staining observed 48 h after infiltration. **b** Percentage of GUS-stained area detected using Image J measurements. Each value represents the mean ± SD of fifteen repetitions. Means with the same letter are not significantly different (Duncan test $p = 0.05$). Bar: 1 cm

Effect of infiltration method on transient expression of *uidA*

Fifteen coffee leaf disks from young (6-week-old) plants were injected with *A. tumefaciens* GV3101::pCAMBIA 1303 using different infiltration methods. Transient expression of *uidA* was observed 48 h post-infection in leaf disks injected with the bacterial suspension using the different methods, but not in the control (Fig. 3a). Agroinfiltration using one injection followed by sanding of the abaxial epidermis resulted in significantly higher transient *uidA* expression than the other injection methods (Fig. 3b). Histochemical staining was observed in 100% of the explants and the relative area of GUS-stained tissue was 84.8 ± 19.0% (Fig. 3b). Target genes have been effectively introduced into plants by agroinfiltration using syringe- and vacuum-based methods. Expression of target genes within tissue cells may differ between the two methods due to differences in the penetration and spread of the bacteria (Cao et al. 2017).

The use of surfactants, antioxidants, and abrasive substances such as Tween 20, Silwet L-77, ascorbate acid, and carborundum influences transformation efficiency (Zhao et al. 2017). Surfactants reduce surface tension, thereby allowing penetration of bacteria into plant tissues (Zhao et al. 2017). Here, we demonstrated that damaging the

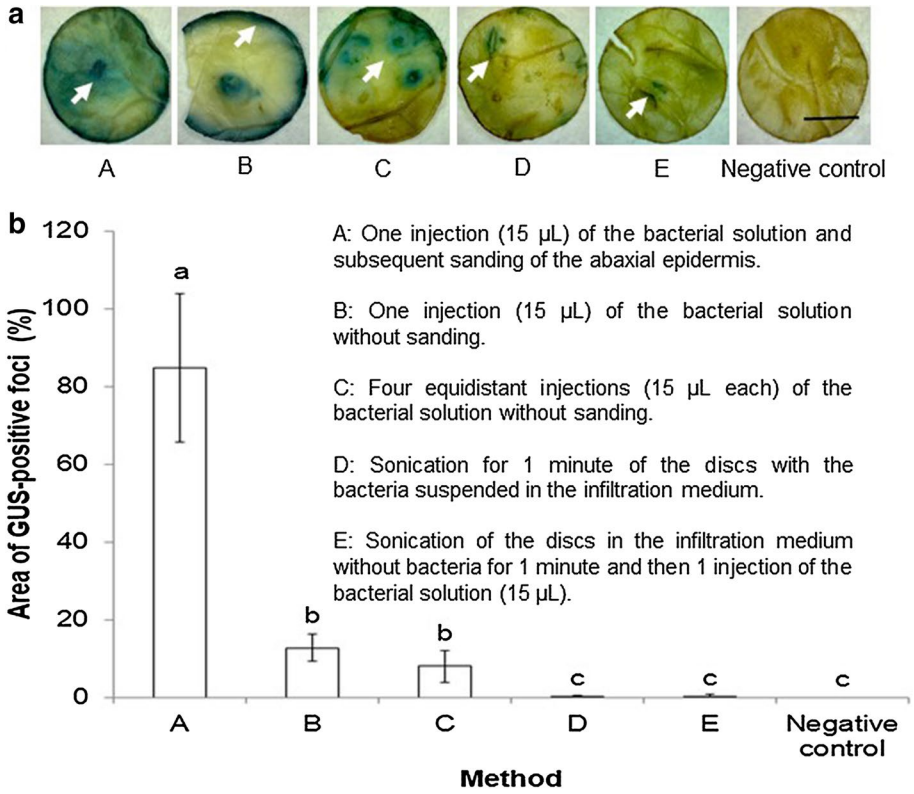

Fig. 3 Effect of *A. tumefaciens* GV3101::pCAMBIA 1303 infiltration method on transient GUS expression in coffee leaf disks. **a** GUS staining observed 48 h after infiltration. **b** Percentage of GUS-stained area detected using Image J measurements. Each value represents the mean ± SD of fifteen repetitions. Means with the same letter are not significantly different (Duncan test $p = 0.05$). Bar: 1 cm

coffee leaf epidermis using sandpaper increased transient expression of the *uidA* gene (Fig. 3a). Wounding the tissue probably triggered the *Agrobacterium* infection apparatus, resulting in an increased number of transformed cells. Andrieu et al. (2012) showed that wounding the rice leaf surface using a small apparatus carrying many 600 µm diameter needles favored the penetration of *Agrobacterium* and enhanced the expression of the *uidA* gene relative to unwounded infiltrated leaves.

Effect of infiltration medium on transient GUS expression

The infiltration medium is an important parameter to be considered for gene delivery in plant systems (Du et al. 2010; Kumar et al. 2017). In order to define the appropriate infiltration medium for the transient expression of the *uidA* gene, fifteen coffee leaf disks from young (6-week-old) plants were injected with *A. tumefaciens* GV3101::pCAMBIA 1303 using the best infiltration method described above and two different infiltration media (MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS or 10 mM $MgCl_2$ supplemented with 1.9 g/L MES and 200 uM AS). Transient expression of *uidA* was observed 48 h post-infection in coffee leaf disks injected with both media but not in the control (Fig. 4a). Transient expression of uidA was significantly higher in explants injected with bacteria suspended

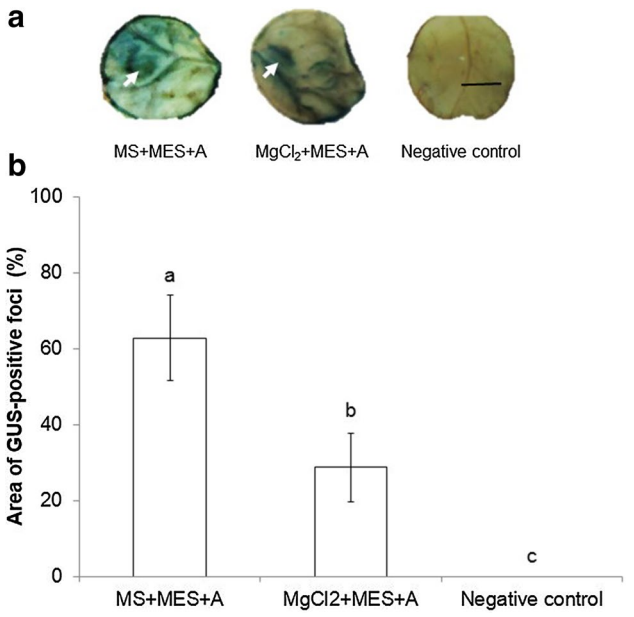

Fig. 4 Effect of infiltration medium on transient GUS expression in coffee leaf disks injected with *A. tumefaciens* GV3101::pCAMBIA 1303. **a** GUS staining observed 48 h after infiltration. **b** Percentage of GUS-stained area detected using Image J measurements. Each value represents the mean ± SD of fifteen repetitions. Means with the same letter are not significantly different (Duncan test $p = 0.05$). Bar: 1 cm

in MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS (Fig. 4b). GUS-positive foci were observed in 93% of the explants with an average stained leaf area of 62.8 ± 11.2%. Both MS and $MgCl_2$ media have been used successfully for genetic transformation by infiltration. The former has been used with *Nicotiana tabacum* (Yang et al. 2000), *Arabidopsis thaliana* (Dehestani et al. 2010), *Vigna unguiculata* (Adesoye et al. 2010) and *Pupulus sp* (Takata and Eriksson 2012). Infiltration media based on $MgCl_2$ have been used with *Nicotiana benthamiana, Nicotiana tabacum* (Koscianska et al. 2005), *Solanum melongena* and *Solanum lycopersicum* (Pratap et al. 2011).

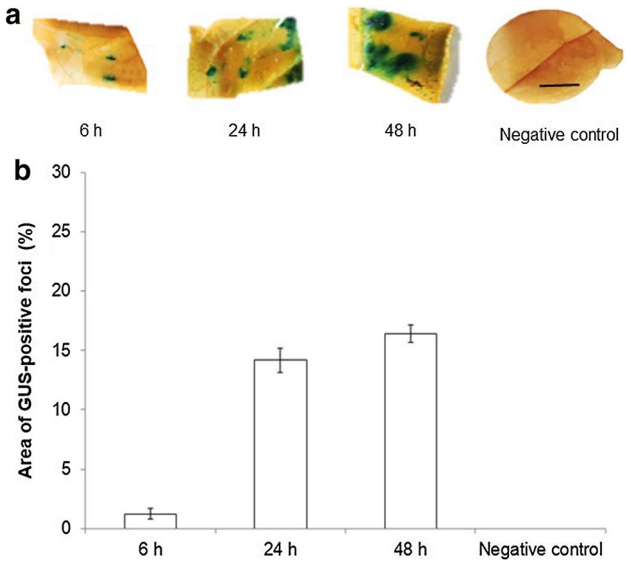

Fig. 5 Time course of GUS staining after infiltration of coffee leaf disks with *A. tumefaciens* GV3101::pCAMBIA 1303. **a** GUS staining observed 6, 24, and 48 h after infiltration. **b** Percentage of GUS-stained area detected using Image J measurements. Each value represents the mean ± SD of fifteen repetitions. Bar: 1 cm

Expression of the *uidA* gene

Expression of the *uidA* gene was evaluated over time using images of coffee leaves acquired 6, 24 and 48 h after infiltration with *Agrobacterium* using the optimized protocol and no phenolic damage due to sanding was observed (Fig. 5a). Transient GUS expression was first detected 6 h after bacterial injection. The relative area of GUS-stained tissue per explant was highest 48 h post-infiltration (Fig. 5b). The duration of co-cultivation of rose and cacao explants had a significant effect on the level of expression of the transgene over time (Yasmin and Debener 2010; Fister et al. 2016). In grapevine, rose and cacao, expression of the reporter gene increased over time until reaching a limit, after which it decreased (Zottini et al. 2008; Yasmin and Debener 2010; Fister et al. 2016).

After establishing a transient transformation assay, the expression of the *uidA* gene was monitored for 5 weeks after injection of coffee leaf disks with *A. tumefaciens* GV3101::pCAMBIA 1303. As shown in Fig. 6a, a positive reaction, indicated by blue staining of coffee leaves and no damage of the tissue due to phenolic exudation, was observed. As demonstrated in grapevine (Zottini et al. 2008) and *Maesa lanceolate* (Faizal and Geelen 2012), coffee leaves could potentially be infiltrated under in vitro conditions and cut from the plant after 5 weeks and cultured on shoot or embryogenic callus induction medium.

PCR was used to test for the presence of the *mgfp5, uidA*, and *virG* genes 5 weeks post-agroinfiltration. Specific *mgfp5* (790 bp) (Fig. 6b) and *uidA* (710 bp) (Fig. 6c) fragments were amplified in the agro-injected coffee leaf disks. No DNA fragments were amplified by PCR in wild-type leaf disks (non-injected control). Moreover, the *virG* gene was not detected in the agroinfiltrated coffee leaf disks, indicating the lack of contamination with *A. tumefaciens* (Fig. 6d).

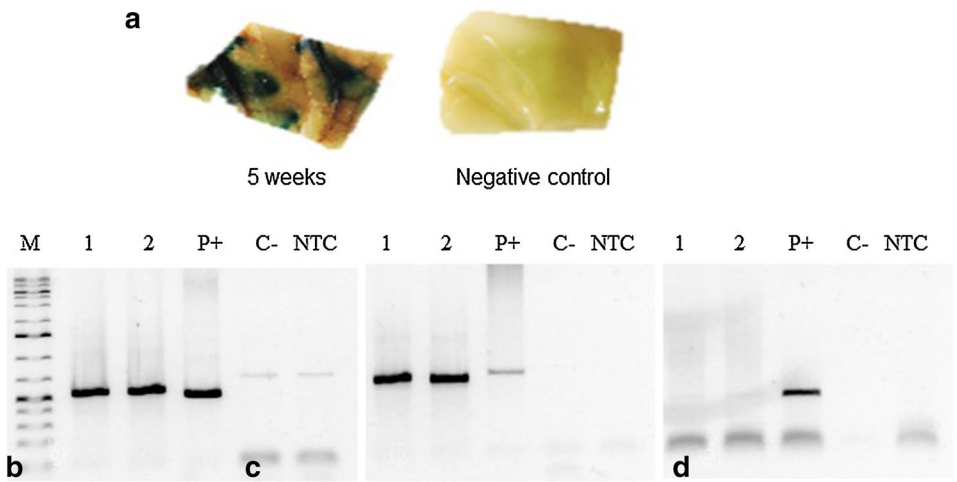

Fig. 6 **a** Expression of the *uidA* gene 5 weeks after infiltration. PCR analysis showing the presence of the genes **b** *mgfp5* (amplicon size 790 bp) and **c** *uidA* (amplicon size 710 bp) and the absence of the gene **d** *virG* in agroinfiltrated coffee leaf disks (amplicon size 390 bp). *M* molecular weight marker (1 Kb Plus, Fermentas), *NTC* negative control (PCR reaction mix without template), *P+* positive control (pCAMBIA 1303 DNA), *C-* wildtype leaf disks (non-injected control), 1 and 2: putative transgenic coffee leaf disks. Bar: 1 cm

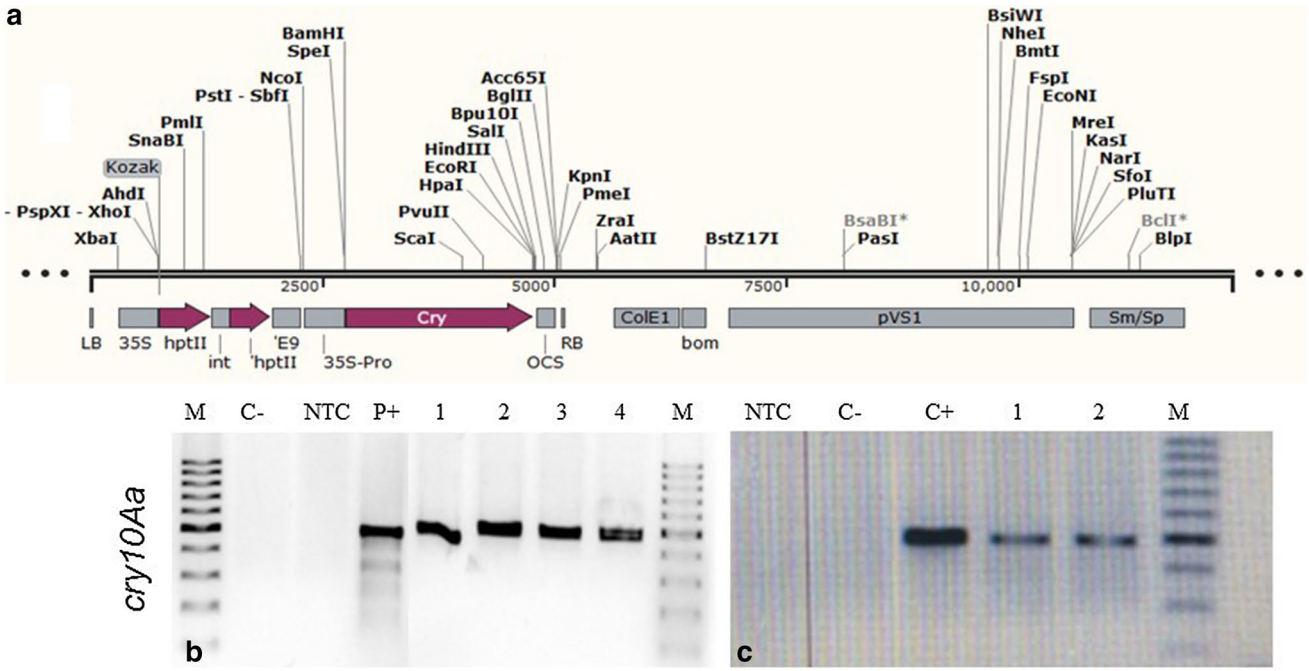

Fig. 7 **a** Linear map of *cry10Aa* gene cassette cloned in pB427-35S-cry10Aa plant expression vector. **b** PCR analysis showing the presence of the *cry10Aa* gene in agroinfiltrated coffee leaf disks (amplicon size 500 bp). *M* molecular weight marker (100 bp, Fermentas), *NTC* negative control (PCR reaction mix without template), *P+* positive control (pB427-35S-cry10Aa DNA), *C-* wildtype leaf disks (non-infiltrated control), 1, 2, 3, and 4: putative transgenic coffee leaf disks. **c** RT-PCR analysis demonstrating *cry10Aa* expression in agroinfiltrated coffee leaf disks (amplicon size 500 bp). *M* molecular weight marker (100 bp, Fermentas), NTC: negative control (RT-PCR reaction mix without template), *P+* positive control (pB427-35S-cry10Aa DNA), *C-* wildtype leaf disks (non-infiltrated control), 1, and 2: putative transgenic coffee leaf disks

Expression of the *cry10Aa* gene

Specific *cry10Aa* (500 bp) fragment was amplified in the agro-infiltrated coffee leaf disks 5 weeks post-agroinfiltration with the plasmid pB427-35S-cry10Aa (Fig. 7b). No DNA fragments were amplified by PCR in wild-type leaf disks (non-injected control). Moreover, the *virG* gene was not detected in the agroinfiltrated coffee leaf disks, indicating the lack of contamination with *A. tumefaciens* (data not shown).

The expression of the gene *cry10Aa* in two infiltrated coffee leaf disks was verified by RT-PCR and an expected 500 bp fragment was amplified (Fig. 7c). No signal was detected in the non-infiltrated leaf disk. In the controls where the reverse transcriptase was added to the reaction mixture, no amplicons were detected (data not shown). Production of insect-resistant coffee plants is one of the major objectives of the breeding programs. Previously, transgenic coffee plants expressing synthetic *cry* genes (*cry1Ac*) from *Bacillus thuringiensis*, which is effective against the coffee leaf miner, were regenerated (Leroy et al. 2000). In another experiment, an α-amylase inhibitor from *Phaseolus vulgaris* was tested against coffee berry borer and found to have an inhibitory effect on its growth and development (Barbosa et al. 2010). The effectiveness of *Bacillus thuringiensis* genes in controlling CBB has been well reported (Mendez-Lopez et al. 2003). Therefore, the development of coffee varieties resistant to coffee berry borer using transgenic technology will be a great benefit for the coffee industry.

In conclusion, the rapid, simple and economic transformation strategy reported in this study could be used to modify the expression of multiple genes in coffee. Potential uses include the development of control strategies for coffee berry borer (*Hypothenemus hampei* Ferrari), coffee rust (*Hemileia vastatrix*) or leaf miner (*Leucoptera coffeella*), as well as CRISPR/Cas9 mediated genome editing in coffee leaves.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest. All authors read and approved the final manuscript.

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