Transformation of coffee (*Coffea Arabica* L. cv. Catimor) with the *cry1ac* gene by biolistic, without the use of markers

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(With 3 figures)

Received November 3, 2008 – Accepted June 1, 2009 – Distributed May 31, 2010

Abstract

The transformation of coffee plantlets with the *cry1ac* gene of *Bacillus thuringiensis* was achieved by biolistic using either the whole pUBC plasmid or only the ubi-*cry1ac*-nos genetic cassette. The *cry1ac* gene was inserted into coffee plants in order to confer resistance to the leaf miner *Leucoptera coffeella*, an insect responsible for considerable losses in coffee crops. Bearing in mind that the genetic cassettes used for this study lack reporter genes and/or selection marker genes, the parameters for the transformation procedure by biolistic were previously standardised with a plasmid carrying the *gus* reporter gene. The presence of the *cry1ac* gene in young plantlet tissues was determined by PCR, Southern blot and reverse transcription-PCR. Our results show that the obtainment of viable coffee plantlets, transformed by bombardment with the *cry1ac* gene and without selection markers nor reporter genes, is feasible.

Keywords: coffee, biolistic, genetic cassette, *cry1ac* gene, transformation without markers.

Transformação do café (*Coffea arabica* L. cv. Catimor) com o gene *cry1ac* por bombardeamento, sem usar marcadores

Resumo

A transformação das plântulas de café com o gene *cry1ac* de *Bacillus thuringiensis* foi realizada por biobalística, utilizando todo o pUBC plasmídeo ou só o cassete genético UBI-*cry1ac*-nos. O gene *cry1ac* foi inserido no cafeeiro a fim de conferir resistência à folha mineiro *Leucoptera coffeella*, um inseto responsável por perdas consideráveis nas culturas de café. Tendo em conta que ao plasmídeo e ao cassete genético utilizados para este estudo faltam genes repórteres e/ou de seleção, os parâmetros para o processo de transformação por biobalística foram previamente padronizados com um plasmídeo transportando o gene repórter *gus*. A presença do gene *cry1ac* em tecidos de jovens plântulas foi determinada por PCR, Southern blot e transcrição reversa-PCR. Nossos resultados mostram que a obtenção de plântulas de café, transformado por bombardeamento com o gene *cry1ac* sem genes de seleção genética nem repórteres é viável.

Palavras-chave: café, biobalística, cassette genético, gene *cry1ac*, transformação sem marcadores.

1. Introduction

Coffee stands among the first five crop products sold worldwide and the number of people that live from this crop is estimated at 125 million, including some 25 million producers and processors (O´Brien and Kinnaird, 2003; ICO, 2007). In 2006/07 world production reached over 125 million bags, an increase of 14% compared to crop year 2005/06, when output was 110 million bags (ICO, 2007). Insects have been blamed as the most diverse and numerous plagues affecting this crop, and the lepidopteran *Leucoptera coffeella* (leaf-miner) and the coleopteran *Hypothenemus hampei* (borer) stand out as devastators (Kranz et al., 1978). Production losses caused by the miner are put at 30%, due to the widespread defoliations brought about by the burrows on leafs, opened by the larvae while feeding. The latter are controlled by systemic insecticides such as endosulfane or cypermethrine, with the inherent risks of contamination and the usually high costs of chemical controls (Mondragón et al., 2004). A useful alternative appears to be the genetic transformation of coffee plants with the *cry1ac* gene from *Bacillus thur-*

ingiensis, which codifies for a crystalline protein that is specifically toxic to lepidopteran larvae (Guerreiro et al., 1998). Using *Agrobacterium tumefaciens* or *A. rhizogenes* several genes have been inserted into coffee plants, such as *bar* (for resistance to the Finale™ herbicide), *gus* (reporter gene for β-glucuronidase), *nptII* (resistance to kanamycin), *hpt* (resistance to hygromycin), *csr1* (resistance to the herbicide chlorosulphuran), *rolB* (a gen from *A. rhizogenes*) and *cry1ac* (resistance to lepidopterans). Hatanaka et al. (1999), Leroy et al. (2000), Perthuis et al. (2005), Ribas et al. (2006), Alpizar et al. (2006) and Kumar et al. (2006) have all reported regeneration of such transformed tissues. The genes *gus*, *bar* and *nptII* have also been incorporated by electroporation; notable are the studies of van Boxtel et al. (1995) who described the transitory expression of the *gus* gene, Barton et al. (1991) who transformed coffee-plant embryos with the *nptII* gene, and Fernández-Da Silva and Menéndez-Yuffá (2003) who reported the regeneration of transformed tissues with positive results for the GUS and PCR reactions for *gus* and *bar* genes, while da Cunha et al. (2004) achieved the transformation of *C. arabica* using biolistics with the *nptII* and *gus* genes. The insertion of the *cry1ac* gene into coffee plants has been accomplished by transformation with *A. tumefaciens* (Leroy et al. 2000); however, the biolistic procedure has the advantage of simple construction vectors besides easier transformation protocols as complex plant-bacteria interactions are eliminated (Gray and Finer, 1993, cited by Ribas, et al. 2006).

Transformations by microparticle bombardment are usually carried out using whole plasmids, which carry sequences that may negatively affect the transgen stability and its expression. Plant transformations have been described in recent years using genetic blocks assembled by the gene of interest flanked by the promotor and ending sequences, which in turn eliminate, or at least minimise, those negative effects; also this procedure reduces the number of foreign sequences introduced into the plant genome (such as reporter genes, selection markers and genes for resistance to bacterial antibiotics), all of which could represent an advantage regarding biosafety (Fu et al., 2000; Breitler et al., 2002; Vianna et al., 2004). In view of this, the present study was aimed at the transformation of arabica coffee by means of biolistic using a whole plasmid or a genetic block with the *cry1ac* gene, in order to introduce resistance to the leaf-miner. It is noteworthy that the conditions of the procedure as followed (including kind of explant, helium pressure, distance between the microparticle carrier and explant, shot time and post-bombardment recovery medium) were determined in a previous work (De Guglielmo et al., 2007, in preparation), which in turn facilitated the transformation with the *cry1ac* gene, as the genetic construction used for the present study lacked both reporter genes and selection markers.

2. Materials and Methods

2.1. Plant material

Torpedo-shape somatic embryos of *C. arabica* cv. Catimor were used. This material was obtained by the induction methods for somatic embryogenesis of coffee as described by Menéndez-Yuffá et al. (1994). Conditions in the growth room were: illumination 12 $\mu mol.m^{-2}.s$ at a mean temperature of 24 °C.

2.2. Genetic constructions

We used the pUBC plasmid (approximately 7,000 bp), kindly donated by Dr. I. Altosaar (University of Ottawa, Canada), which is based on the pGEM 4Z vector (Promega) containing a synthetic version of the *cry1ac* gene under control of the ubiquitine promoter of corn and the nopaline synthetase terminator (Sardana et al., 1996). This genetic block (approximately 4,200 bp), was introduced in the *Hind*III site of the base vector which also contains the resistance gene to ampicillin, an antibiotic used as a selection agent for transformed bacteria. Plasmid DNA was isolated using the Wizard® Plus Midipreps DNA Purification System Kit (Promega), and following its instructions, from *E. coli* bacterial strain DH5α transformed with the pUBC plasmid as described by Chung et al. (1989). This DNA was digested with the *Hind*III enzyme (Invitrogen), following the manufacturer`s recommendations, and subjected to electrophoresis in 0.8% agarose gel (Promega), containing TAE 1X buffer (4.84 g Tris base, 1.14 mL glacial acetic acid and 2 mL EDTA 0.5 M at pH 8, final volume 1 L) at 80 V. The gel was then stained with ethidium bromide and the resulting bands were analysed using the Gel Doc 2000 Gel Documentation System (BIORAD). The ubi-*cry1ac*-nos genetic block was also used, liberated from the whole plasmid by digestion with the *Hin*dIII enzyme (Invitrogen). The band concerned (approximately 4,200 bp) was separated from the agarose gel and the DNA from this block was purified by centrifuging on glass wool, followed by precipitation with absolute ethanol.

2.3. Biolistic-mediated transformation

Bombardments of somatic torpedo embryos of *C. arabica* cv. Catimor with the whole pUBC plasmid or with the ubi-*cry1ac*-nos genetic block were carried out using a low-pressure helium pistol manufactured by the Instituto Venezolano de Investigaciones Científicas (IVIC), based on Vain et al. (1963). Relevant procedures were as in Klein et al. (1987, 1988) with the following standard conditions: helium pressure 70 psi, shot time 0.1 seconds, distance between the microparticle carrier and explants 14 cm, vacuum pressure 21 $mm.Hg^{-1}$; recovery medium was the solid medium for germination without hormones described by Hermoso-Gallardo and Menéndez-Yuffá (2000).

Of the experiments performed, 11 replicates correspond to whole-plasmid shots and three to those of the genetic cassette; these numbers ensured enough material to assess detection of the transgen in the transformed tissues, bearing in mind that variable and generally low efficiencies of transformation by biolistic have been reported (McCabe and Christou, 1993).

2.4. Evaluation of transformation

2.4.1. PCR analysis

Plant DNA was extracted according to Doyle and Doyle (1990) with some modifications to reduce the amount of foliar tissues used, as well as DNA extraction time and length of PCR tests (Fernández-Da Silva and Menéndez-Yuffá, 2003). This material was used for PCR tests with specific primers for the *cry1ac* gene, under conditions as described by Sardana et al. (1996). The primer pair Ac-f (ATGGACAACAACCCAAACATC) and Ac-r (TCATGTCGTTGAATTGAATAC) were used to amplify a sequence of 416 bp. The fragments were visualised by electrophoresis on 2% agarose gels, TAE 1X buffer and ethidium bromide staining (Chandler and Vaucheret, 2001; Jana et al., 2004).

2.4.2. Southern blot hybridization

This procedure was done with the DIG DNA Labelling and Detection Kit (Roche), according to its indications to attain a non-radioactive label of the probe. Roughly 30 µg of plant DNA from each sample were treated with *Hind*III enzyme. The ubi-*cry1ac*-nos genetic block was used as probe; it was obtained by enzymatic digestion of pUBC plasmid DNA with *Hind*III, followed by labelling with digoxigenin.

2.4.3. Reverse transcription-PCR (RT-PCR)

This was carried out using the Super Script II reverse transcriptase (Invitrogen). Total RNA of the bombarded plantlets was used, extracted with the Plant RNA Purification Reagent (Invitrogren), and the synthesis of cDNA was performed with random primers (Invitrogen). PCR was then carried out with specific primers for the *cry1ac* gene, as detailed above for PCR analysis.

2.5. Statistical analyses

Non-parametric Kruskal-Wallis tests were applied to compare results of bombardments with the whole plasmid or with the genetic block containing the *cry1ac* gene, regarding numbers of bombarded embryos, surviving embryos, regenerated plantlets, analysed plantlets and transgene detection by means of the various molecular techniques employed (see above).

3. Results

3.1. Bombardments with the pUBC plasmid or the genetic block

Twelve torpedo embryos were used for each bombardment, corresponding to the area covered by the shots under our experimental conditions (Table 1). The embryos usually showed a dark brown colouration after bombardment, probable due to mechanical damages. Out of the 12 embryos bombarded in each assay, four or five (roughly 42%) died within two weeks of the treatment and showed necrosis, further darkening and tissue softening. Two months after the bombardments some of the surviving embryos showed the emergence of secondary somatic embryos, which died off shortly afterwards. At two and a half months the development of cotyledons was noted in an average of six surviving embryos from each of the replicates. One cotyledon was removed from each for PCR analysis with the specific primers for the *cry1ac* gene.

3.2. PCR

The primers used revealed an amplification product of 416 bp. For the transformation with the pUBC plasmid 51 plantlets were analysed, of which 7 (14%) were positive for the PCR test (Figure 1). Of these seven, one died shortly afterwards. Of the embryos bombarded with the *cry1ac*-sequence carrier block, all the regenerated plantlets (19/19) were examined by PCR, but only one (5%) gave positive results.

3.3. Southern blot hybridisation

The probe used for this assay was prepared with the genetic block containing the *cry1ac* sequence. Positive control was represented by DNA from the pUBC plasmid and negative control by DNA from untransformed coffee plants. Controls and PCR-positive plantlets DNA were digested with the *Hind*III enzyme, which frees the

Table 1. Results of the bombardments of torpedo somatic embryos of coffee with the plasmid or the genetic cassette.

Inserted DNA	pUBC plasmid	ubi-*cry1ac*-nos cassette
Nº of bombardments	11 (12 embryos each)	3 (12 embryos each)
Nº of bombarded embryos	132	36
Nº of surviving embryos	82 (61.9%)	22 (61%)
Nº of regenerated plantlets	60/82 (73.2%)	19/22 (86.4%)
Nº of examined plantlets	51/60 (85%) *	19/19 (100%)
Nº of positive PCR-*cry1ac*	7/51 (13.7%) **	1/19 (5.3%)
Nº of positive Southern blot	6/6 ***	1/1
Nº of positive RT-PCR	1/5	None

*Nine plantlets could not be analyzed due to poor growth. **One plantlet died after removal of cotyledons. ***One plantlet was discarded due to contamination.

cry1ac fragment together with the promoter and the terminator. Results are shown in Figure 2. The positive control showed a strong band whereas no hybridisation took place between the probe and the negative control, nor with the plantlets which were negative for PCR tests with *cry1ac* primers (results not shown). The seven surviving plantlets positive for the later test, of which six belonged to the whole-plasmid bombardment and one to the single genetic cassette, hybridised with the probe. Of these plantlets (including the latter) three revealed a single band presenting a similar migration observed in the positive control, corresponding to the *cry1ac* gene fragment of 4,200 bp (Figure 2a). One of them, resulting from the whole-plasmid assay, showed a mixed hybridisation pattern expressed as one band similar to the positive control (4,200 bp) and another heavier (7,500 bp approximately) but less intense one (Figure 2b, lane 2). In the three remaining samples from the whole-plasmid bombardments a complex pattern of bands was noticed, represented by two heavier bands (7,500 and 10,000 bp approximately) than that of the positive control (Figure 2b, lane 4) (Spiral, 1993).

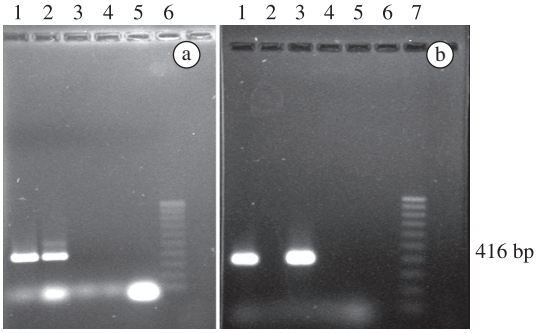

Figure 1. PCR results with specific primers for the *cry1ac* gene.1a and 1b: positive controls (pUBC DNA); the expected amplification product of 416 bp is evident. 2 to 4a and b: DNA from the bombarded tissues (lanes 2a and 3b show the positive samples). 5a and b: negative control (DNA from non-transformed coffee). 6a and 7b: Molecular weight markers (100 bp, Invitrogen).

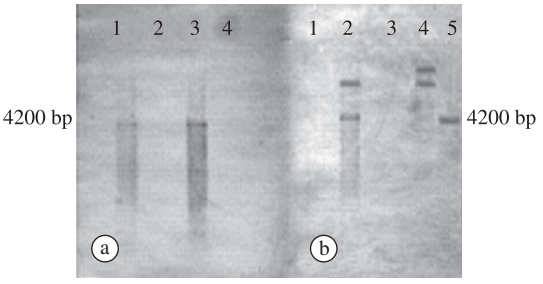

Figure 2. Southern blot hybridisation with the probe based on the ubi-*cry1ac*-nos block, of DNA from coffee plantlets positive to PCR with *cry1ac* primers. 3a and 5b: positive controls (pUBC DNA). 4a and 1b: negative controls (DNA from non-transformed coffee). 1a, 2b and 4b: DNA from 3/7 coffee plantlets positive to PCR. 2a and 3b: DNA from plantlets which gave negative results for *cry1ac* primers PCR. All DNA were digested with *Hind*III enzyme.

3.4. Reverse transcription-PCR

This test was performed on six surviving plantlets positive by PCR and by Southern blot, as of the remaining seven, one showed transgenic rearrangements by hybridisation when tested by Southern blot but had to be discarded due to contamination. Positive control was the pUBC plasmid DNA while negative controls corresponded to non-transformed coffee cDNA and the reaction mixture. As shown in Figure 3 (lane 5) only a single sample, from the bombardments with the whole plasmid and which revealed a single band of the expected size by Southern blot, has the expected band of 416 bp corresponding to the *cry1ac* gene as revealed by PCR. This band also shows in the positive control but it is absent from the negative controls.

3.5. Statistical analyses

Based on the non-parametric test used, no significant differences ($p > 0.05$) were obtained between the results of survivorship, regeneration and PCR detection in the bombardments with the whole plasmid or with the genetic cassette.

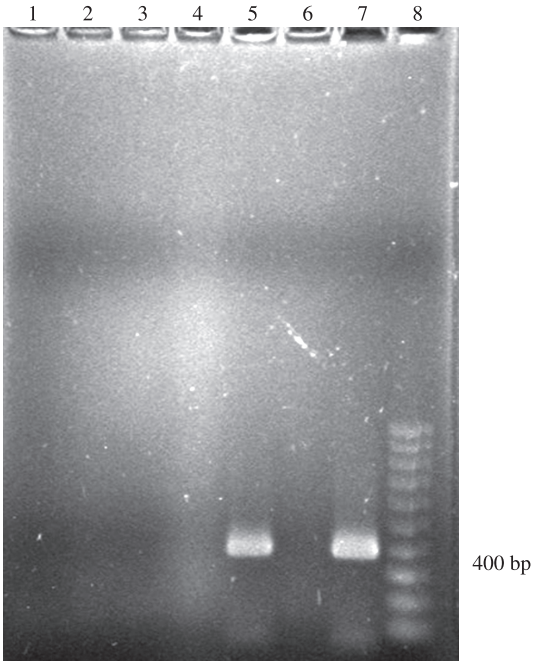

Figure 3. Reverse Transcription-PCR with *cry1ac* primers and cDNA. 1 to 5: studied samples. 6: negative control (non-transformed coffee). 7: positive control (pUBC DNA). 8: Molecular weight markers (100 bp). Overall, only a single studied sample (lane 5) showed a positive test-result. Electrophoresis of the reaction mixture (a negative control) was performed on another gel (not shown), with a negative result.

Discussion

The present work achieved the transformation of *C. arabica* plants with the *cry1ac* gene to confer resistance to the leaf-miner, by means of biolistic bombardments using whole plasmids or genetic blocks. Table 1 summarises the results of such bombardments. The use of simple genetic blocks or cassettes built with promoter-sequence of interest-terminator has not been previously reported in the literature concerning transformation of coffee plants. This technique for genetic modifications has been used by Fu et al. (2000) and Breitler et al. (2002) to transform rice by means of biolistic, without a significant loss of transformation efficiency nor affecting the responses of bombarded explants regarding survival or regeneration, in comparison with the use of whole plasmid DNA. Those authors also pointed out that the rice plants transformed by genetic cassettes exhibited simple integration patterns and low frequencies of transgene rearrangements (less than those observed with the use of whole plasmid). This favours the stable expression of the transgene and improves biosafety as less foreign rearranged sequences become inserted in the bombarded tissues. This also reduces or eliminates the possible risk of horizontal or vertical transfer of genes for the resistance to antibiotics or herbicides, used as selection marker genes in the transformation procedure.

Recently, Vianna et al. (2004) transformed bean plants by biolistic using a genetic cassette containing the *bar* gene, and their findings agree with those of Fu et al. (2000) and Breitler et al. (2002). The use of the whole plasmid could cause plasmid-plasmid recombination events brought about by sequences rich in A-T (i.e. highly recombinogenic) on the backbone of the transformation vectors, which in turn might be involved in plasmid multimerization events (Fu et al., 2000). Our own findings did not reveal any statistically significant differences between responses of tissues bombarded about survivorship, regeneration and detection by PCR, either with the whole plasmid or the genetic block, in agreement with other results published elsewhere. Anyhow, the advantage of using limited genetic blocks appears to support transgenic stability expression and inheritance over a long range.

Our hybridisation results tested by Southern blot evidenced that seven plantlets of coffee from the bombarded and regenerated embryos selected by PCR incorporated the *cry1ac* gene. The test also showed that of seven plantlets, three had a single band agreeing with the genetic block, indicating that no rearrangements had occurred. Additionally, four plantlets showed extra bands or did not have the one corresponding to the block, as was shown in Figure 2 (lines 2b and 4b); one possible cause could be the transgene rearrangements.

The six surviving plantlets selected by PCR and which were also positive by Southern blot hybridisation, were examined by RT-PCR. As was shown in Figure 3, only a single sample had the expected 416 bp band on the PCR test with the *cry1ac* primers. This suggests that five of the six plantlets positive for PCR and Southern blot hybridisation (including the one resulting from the bombardment limited to the genetic cassette) do not express the gene *cry1ac*. Only the plantlet positive for RT-PCR would possibly show any resistance to the leaf-miner by its expression of the cry1ac protein. Anyhow, we do not disregard further assessments of the plantlets which gave negative results for RT-PCR tests (these negative results could be associated with molecular technique or DNA detection system sensibility). Their tissues ought to be propagated, while examining their proteins and performing bioassays to expose plantlets to the insect larvae.

Several hypothesis could explain the lack of expression at mRNA level; one of them could be the sensibility of the test, and as reported by Matzke and Matzke (1995), Hiei et al. (1994) and Kumpatla et al. (1997), this can be due to genetic rearrangements, gene silencing by insertion in transcriptionally inactive or highly repetitive regions, or by methylation.

The transformation obtained in this study was performed without the use of markers of selection or report genes. This is a very important feature for biosafety in a plant with a product intended for human consumption. However, the marker genes are important to establish the procedure of transformation, for this reason, the parameters for transformation by bombardment were previously established using the plasmid pCAMBIA3201 (De Guglielmo et al, in preparation) which contains the reporter gene *gus*. The best conditions were selected according to the survivorship and the expression of *gus*. It is remarkable, that even though *gus* is considered transient, the expression of this gene was observed seventeen months after bombardment of torpedo shape embryos. Bombardments with pCAMBIA3201 were made in parallel with those of the gene *cry1ac*, they were done as a control for the procedure, and to have a faster and easier procedure to follow the procedure of transformation, this control showed *gus* expression in all the experiments of bombardments (results not presented).

According to the results, it is advisable to carry out new experiments in the future, to evaluate pre and post bombardment treatments, and also conditions for the regeneration of transformed products. It would also be interesting to improve the efficiency of secondary somatic embryogenesis in the bombarded material, to propagate the transformed material and also to diminish the risk of chimerism.

To conclude, this work proved that the transformation of coffee plants by means of microprojectile bombardment is feasible, applying for the first time a method that produces coffee plantlets without selection markers or reporter genes. Also, an improvement was added to the procedure of bombardment, using the isolated genetic block or cassette, this technique could eventually produce plants without the vector plasmid sequences, including those for antibiotic resistance used for its selection. We obtained plantlets transformed with the *cry1ac*

gene which showed expression at the mRNA level, with the further advantage that they contained neither reporter genes nor selection markers that could be expressed in the plants, a rather important aspect in view of the recent tendencies concerning biosafety in plant transformation research.

Acknowledgements — Thanks to FONACIT and to CDCH for financial support of this research project. We also thank Dr. Eva de García, Head of the Lab. of Biotecnología Vegetal, IBE-UCV; and Dr. Maira Oropeza for useful tips on the Southern blot methodology.

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