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Genetically modified coffee plants expressing the *Bacillus thuringiensis* *cry*1Ac gene for resistance to leaf miner

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Abstract A synthetic version of the *cry*1Ac gene of *Bacillus thuringiensis* has been used for the transformation of coffee species (*Coffea canephora* and *C. arabica*) to confer resistance to an important pest, the coffee leaf miner (*Perileucoptera coffeella* and other *Leucoptera* spp). Somatic embryos were co-cultivated with the LBA4404 strain of *Agrobacterium tumefaciens* containing the *cry*1Ac gene. More than 100 transformed plants from independent transformation events were obtained for each coffee genotype. The integration and expression of the *cry*1Ac gene was studied, and effective resistance of transgenic plants against leaf miner was verified in bioassays with the insects. These plants could represent a good opportunity to analyse the impact of genetic engineering of perennial crops for sustainable resistance to an obligate endocarpic pest using a *B. thuringiensis* insecticidal protein.

Key words *cry*1Ac gene · *Coffea* spp. · Genetic transformation · *Perileucoptera coffeella* · *Leucoptera* spp.

Introduction

Coffee is an extremely important agricultural crop with more than 6.5 million tons of green beans produced every year on about 11 million hectares. In terms of economic importance on the international markets, it is second only to oil and contributes more than 9000 M US $. One of the major pests threatening this production is the leaf miner *Perileucoptera* spp., which has an economically important impact in East Africa and Brazil (Guerreiro et al. 1990). This insect pest is characterised by a strict endocarpic larval stage which makes useless any sprayable formulations of chemical or biological pesticides (Crowe 1964). Systemic chemical pesticides are usually harmful, and the implementation of an environmentally friendly way of controlling this pest would be a preferable option. Since insecticidal proteins do not work, in contrast to chemicals, as contact or external poisons but instead as internal poisons, the insecticidal protein must be eaten by the pest, and the only strategy available would therefore be the transgenic approach. As use of *Bacillus thuringiensis* genes to transform plants for protection to this insect pest is currently considered to be the most reliable strategy (Estruch et al. 1997; Schuler et al. 1998), investigations were initiated to determine the susceptibility of *Perileucoptera* sp. to *B. thuringiensis* insecticidal proteins and identify the candidate genes for transformation of coffee (Guerreiro et al. 1998). To date, six different endotoxins of *B. thuringiensis* have been tested against coffee leaf miner (Guerreiro et al. 1998), and the toxin expressed by the *cry*1Ac gene, widely used to confer resistance to lepidopterae (Dandekar et al. 1998), has been demonstrated to be the most effective.

In addition to pest control, conventional breeding of woody species is a low-efficiency and time-consuming process due to their long life cycles. Genetic engineering could alleviate these problems by incorporating known genes into elite genetic backgrounds.

The first genetic transformation of coffee cells reported (Barton et al. 1991) was by protoplast electroporation. Genetic transformation with *Agrobacterium sp.* has also been reported (Feng et al. 1992; Freire et al. 1994). The regeneration of transgenic coffee trees

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was first obtained by the transformation of somatic embryos *via Agrobacterium rhizogenes* (Spiral and Pétiard 1993; Spiral et al. 1993; Sugiyama et al. 1995), and more recently *via Agrobacterium tumefaciens* (Leroy et al. 1997). Although somatic embryogenesis is still a tedious process for coffee species (Yasuda et al. 1985; Berthouly and Michaux-Ferrière 1996), regeneration is easy obtained. Thus somatic embryos are of great interest as primary explants for genetic transformation (Fisk and Dandekar 1993).

In this article we report the first transformation of coffee using *Agrobacterium tumefaciens* for expression of an agronomic trait. In the investigation reported here, a synthetic *cry*1Ac gene was introduced into three coffee genotypes from the two cultivated species, *C. arabica* and *C. canephora*. The data revealed the correct integration and expression of the *cry*1Ac gene within the coffee genome. Furthermore there was a good correlation between *cry*1Ac gene expression and insect bioassays performed on transformed plants.

Materials and methods

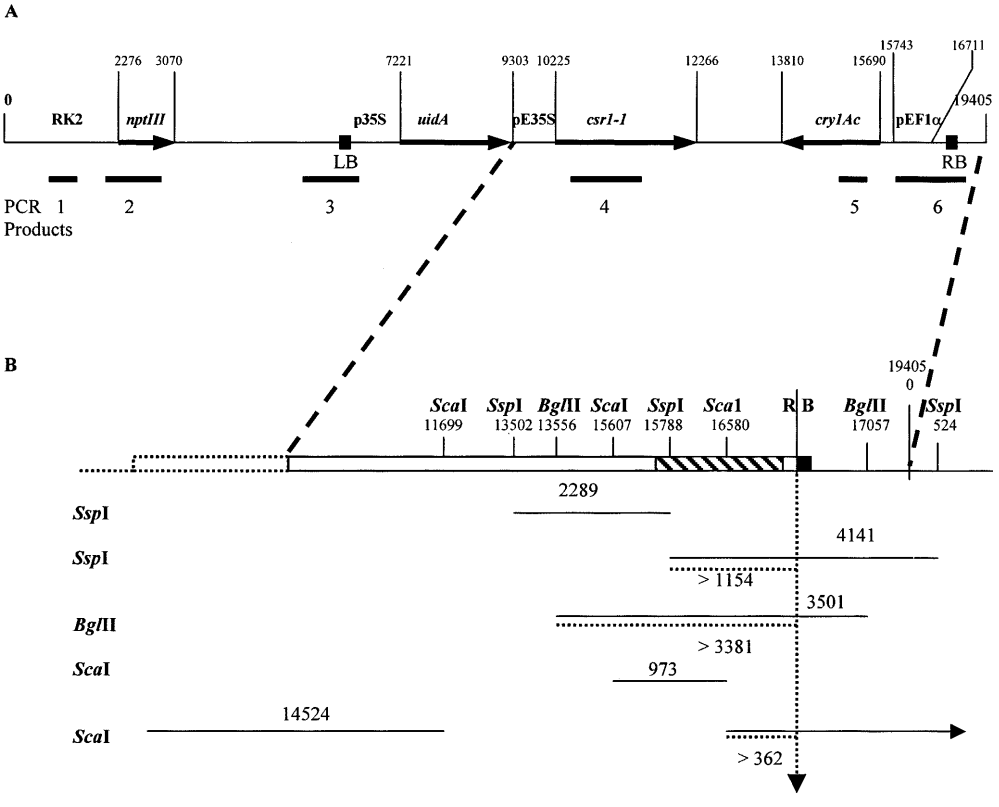
Plant material

Three genotypes were used for these experiments, one from *Coffea canephora* and two from *Coffea arabica*. The *C. canephora* genotype (126) was a selected clone of good agronomic value; a Catimor (8661–4) and an F_1 hybrid (Et29 X Ca5) were used as *C. arabica* genotypes.

Transformation vector.

The *Agrobacterium tumefaciens* disarmed strain LBA4404 was used for transformation with the pBin19 plasmid. The *nptII* gene, located within the T-DNA, was eliminated, and three genes were introduced (Fig. 1A): (1) the *uidA* bacterial gene isolated from *E. coli* coding for β-glucuronidase (GUS), with an additional intron for specific expression in plants (Vancanneyt et al. 1990); the gene is controlled by the cauliflower mosaic virus (CaMV) promoter 35S and the 35S terminator; (2) the *csr1–1* gene isolated from *Arabidopsis thaliana* (Brasileiro et al. 1992) conferring resistance to the herbicide chlorsulfuron; this herbicide was used for selection of transformed cells and is also controlled by the CaMV promoter 35S with a duplicated enhancer sequence (Kay et al. 1987) and by the *csr1–1* terminator; (3) the *cry*1Ac gene from *B. thuringiensis* in a modified form synthesised at the University of Ottawa (Sardana et al. 1996). After preliminary studies on coffee cells demonstrating its efficiency in transient expression (Van Boxtel et al. 1995), the EF1α promoter from *Arabidopsis thaliana*

Fig. 1A Linear plasmid map indicating the localisation of the different genes (*nptIII*, *uidA*, *csr1–1*, *cry*1Ac), the p35S, pE35S and pEF1α promoters, RK2 origin and T-DNA borders (*LB* left border, and *RB* right border). The *arrows* indicate the translation orientation of the genes. The *bold lines below* the linear map represent the PCR amplification fragments: *1* RK2 (63–572), *2* NPTIII (2200–3014), *3* BG (5384–6367), *4* CHLOR (10299–11080), *5* BtSynt (15007–15678), *6* BD (16000–16962). **B** The probe used is pEF1α (*dashed box*). *Uppermost, in bold* the restriction enzymes, with their positions indicated immediately *below*. *Below bar* to the *right* of each enzyme, *above the regular lines*, the size of the fragment between two known restriction sites in the plasmid. Sizes *under the dashed lines* indicate the minimum length of fragments expected after the hybridisation of the probe against T-DNA integrated into the plant genome



(Curie et al. 1991) was chosen, together with a promoter enhancer sequence Ω' derived from tobacco mosaic virus (Gallie and Kado 1989) and the nopaline synthase terminator.

Transformation protocol

Leaf explants were cultured on semi-solid medium, with 5 μM benzylaminopurine (BAP) and 0.09 M sucrose for the *C. canephora* and Catimor genotypes, and successively on the two media defined for the F_1 hybrid genotype (Berthouly and Michaux-Ferrière 1996). Afterwards, they were subcultured every 5 weeks for 3–5 months until somatic embryos appeared at the edge of the explants. Somatic embryos were harvested at the torpedo stage and then wounded with a scalpel. They were soaked in a 0.9% NaCl solution for 2 h with bacteria at a DO_{600nm} of 0.3–0.5. The embryos were then co-cultivated in the dark on semi-solid MS medium (Murashige and Skoog 1962) without hormones for 3 days. They were then rinsed in liquid MS medium supplemented with cefotaxim (1 g/l) for 3–5 h and subsequently were cultivated on semi-solid medium with 5 μM BAP, 0.09 M sucrose, and supplemented with cefotaxim (400 mg/l) under low-light conditions (16 h per day).

After a period of 21–28 days, they were transferred to a selective MS medium supplemented with 400 mg/l of cefotaxim and 80 μg/l of chlorsulfuron. Due to a decrease in herbicide concentration with time, the embryos were transferred to new selective medium every 4 weeks. After regeneration from calli, the transformed embryos were sub-cultured on the semi-solid, germination MS medium with Morel vitamins, 1 μM BAP and 0.03 M sucrose. The rooting medium was identical but without BAP.

GUS histochemical assay

Calli, shoots, leaves or roots were incubated overnight at 37 °C in the classical medium modified by the use of phosphate buffer (0.2 M) at pH 7. Methanol (20% v/v) was used to eliminate any eventual endogenous expression of the non-integrated GUS gene.

Molecular analyses

Plants that presented a positive reaction to the GUS histochemical test were used for total DNA extraction. Extractions were made from plantlets cultivated *in vitro* and also from greenhouse-cultivated plants.

Seven pairs of primers were chosen in order to analyse the quality of the DNA and the structure of the integrated T-DNA. These primers defined various sequence domains of the plasmid (Fig. 1A). Another pair of primers were used to test the presence of residual bacteria by amplification of the *virD2* gene from the virulence plasmid. Amplifications were performed on 25 ng of genomic DNA in a final volume of 50 µl containing 1 × PCR (polymerase chain reaction) buffer, primers and *Taq* DNA polymerase, as recommended by the manufacturer (Stratagene). Forty cycles of amplification were performed (94 °C, 1 min; Thyb, 1 min; 72 °C, 2 min), where Thyb was specific for each pair of primers. A predenaturation step of 3 min and a final elongation step of 7 min were used. The amplification products were observed by electrophoresis on 1% agarose gels stained by ethidium bromide.

Total DNA (5 µg) was digested with *Ssp*I, *Bgl*II and *Sca*I (10 U/µg). Standard methodology (Sambrook et al. 1989) was used to separate DNA on a 0.7% agarose gel in tris-borate-EDTA buffer; it was then transferred to a Hybond N+ membrane (Appligene) and hybridised with α-[^{32}P]-labelled DNA probes using the random priming labelling method (Feinberg and Volgestein 1983). The pEF probe, corresponding to the EF1α promoter, was used for this analysis (Fig. 1B).

Western blot hybridisation and bioassays

Proteins were extracted from about 1 g of fresh leaves, and the detection of the insecticidal protein was performed by Western blotting (Rogers et al. 1991) using a rabbit polyclonal antiserum, raised against the Cry1Ac protein previously purified in our laboratories (dilution of 1/1200 v/v). A secondary goat anti-rabbit antiserum alkaline phosphatase conjugate (Sigma), was then used for final detection, at a dilution of 1/1000 (v/v).

Bioassays were performed using a leaf miner species from Tanzania (*Leucoptera caffeina*). Plants to be tested were put in cages for 24 h to let the adult insects lay eggs on the leaves. Fifteen days later, an overall score was attributed to the plants using the following scale: 0, death of larvae after hatching, no galleries observed; 1, galleries shorter than 3 cm, death of the larvae usually before 1 week; 2, galleries larger than 3 cm before the death of the larvae; 3, living larvae on the leaves, possible development of pupae; 4, complete development of the larvae with formation of pupae.

Results and discussion

Transformation and regeneration

Approximately ten experiments were completed involving between 1000 and 10 000 somatic embryos each. After 3–6 months on a selective medium with 80 μg/l of chlorsulfuron, calli or embryos appeared on the edge of the necrotic embryos. Fig. 2A illustrates a transformed callus from *C. canephora* with growing embryos that has formed on a necrotic embryo after 6 months of culture. Such calli occurred on approximately 1% of the embryos. For the Catimor genotype, small groups of embryos appeared on 0–5% of the primary embryos, with considerable variability. On the F_1 *C. arabica* genotype, less than 0.1% of the explants produced calli. While the transformation process has been improved with further experimentation, it remained difficult to obtain transformed calli or embryos for each experiment; most of the results were obtained from two transformation assays for the *C. canephora* and Catimor genotypes.

For the *C. canephora* and the F_1 *C. arabica* genotypes, isolated calli were then cultivated on the same selective medium. After a few weeks of culture somatic embryos were observed. The rate of calli giving rise to embryos was lower than 30% for *C. canephora*, and lower than 10% for the F_1 *C. arabica* genotype. When the embryos were sub-cultured on the germination medium (3–6 weeks) more than 80% of them germinated correctly. Plantlets were then sub-cultured on the rooting medium for 1–3 months before transfer to the greenhouse. For the Catimor genotype, groups of between 1 and 10 transformed embryos grew directly on primary explants. More than 50% of these embryos were converted into plantlets.

The histochemical GUS assay was performed on calli and plantlets. A transformed stained callus is shown in Fig. 2B. Approximately 50% of the calli or embryos on selective medium exhibited a blue staining, indicating *uidA* gene expression. However, a high rate

Fig. 2A–F Calli and transgenic plants obtained with *C. arabica* and *C. canephora* and evaluation of susceptibility to the coffee leaf miner. **A** Transformed callus (*1*) and small somatic embryos (*2*) growing from a necrotic embryo (*3*) on selective medium, 6 months after the transformation process (magnification: ×37.5). **B** Callus obtained from a primary explant and tested by the GUS histochemical assay. All its cells are transformed, since it is totally stained (magnification: ×37.5). **C** Transformed plantlets from *C. canephora* (126). From *left to right*: just after transfer to the greenhouse and 1, 3, 6 and 12 months later. **D** Transformed plants from Catimor compared with a control obtained by *in vitro* culture without transformation (on the *right*), 6 months after transfer to the greenhouse. **E** Untransformed control plant of *C. canephora* obtained by in vitro culture, and susceptible to the coffee leaf miner during the bioassays. Most of the leaves have many galleries with larvae. **F** Transformed plants from *C. canephora* during bioassays. The Cry1Ac protein was detected by Western blotting in both plants, and they were exposed to the insects at the same time. *Plant 1* shows leaves susceptible to the pest, *plant 2* is resistant to the pest

of calli or embryos recovered from selective medium failed to show positive GUS activity. Increasing the chlorsulfuron concentration did not alleviate this problem and drastically decreased the rate of transformation events. This high error rate led us to use the GUS test as a secondary screening of transformed calli or plantlets.

According to the protocol described, more than 300 transformation events were obtained with Catimor, more than 200 with *C. canephora* and only 10 with the F_1 *C. arabica* genotype. Between 20 and 60 plantlets were generated from each of the 120 calli obtained from the *C. canephora* genotype (Fig. 2C). For Catimor, 1 to 10 plantlets were obtained from each group of embryos from the 100 transformation events (Fig. 2D). Only 10 regeneration events were obtained for the F_1 *C. arabica* hybrid due to a lack of regeneration from transformed calli.

Molecular characterisation of transformed plants

Six PCR amplifications were performed on each of 51 plants from independent events obtained with *C. canephora.* Three PCRs were performed with the RK2, NPTIII and BG primers (Fig. 1A) in order to evaluate the size of the plasmid DNA segment outside the left border of the T-DNA eventually integrated into the

plant genome. No DNA sequences from the plasmid replication origin (about 6000 bp from the left border) were amplified using the RK2 primers. Results from other PCRs, (data not shown) can be summarised as follows: out of the 51 plants analysed, 1 (2%) integrated a plasmid fragment which includes the *nptIII* gene, located between 3000 and 6000 bp outside the left border, 22 (43%) integrated a plasmid fragment between 700 and 3000 bp outside the left border and 28 (55%) may have integrated a plasmid fragment of between 0 and 700 bp in length. The two PCR amplifications with primers CHLOR and BtSynt, which were designed to amplify the T-DNA genes *csr1–1* and *cry*1Ac, were positive except for 2 plants. Nevertheless, a correct hybridisation was observed by Southern blotting for these 2 plants. This result could be explained by internal recombinations inside the T-DNA. Finally, no amplification was observed with the BD primers for the right border, indicating that no integration of the plasmid occurred beyond 356 bp downstream of the T-DNA right border. The control plasmid and also untransformed plants behaved as expected for all amplifications.

Southern blotting was used to evaluate the number of T-DNA copies integrated into the plant genome. Restriction enzymes (*Ssp*I, *Bgl*II and *Sca*I) and probe (*cry*1Ac promoter pEF1α) were chosen in order to detect fragments of the T-DNA of known size and flanking fragments of the T-DNA the sizes of which were unknown (Fig. 1B). It was postulated that the number of copies of the *cry*1Ac gene, located on the right side of the T-DNA, is in fact representative of the copy number of the whole T-DNA. Among the 51 events studied, 35 plants (69%) presented one T-DNA copy, 8 (16%) two copies, 2 (4%) three copies as shown in Fig. 3, but 1 (2%) plant also showed four copies and 1 (2%) five copies (data not shown). These results are consistent with observations made in various dicotyledonous plants including tobacco, petunia, tomato and sunflower (Zambryski 1988).

In most cases, no integration of the plasmid occured beyond 116 bp (*Bgl*II site) downstream of the T-DNA right border. In 5 cases this *Bgl*II plasmid restriction site was integrated, but none of them indicated an integration of more than 356 bp downstream of the right border (PCR assays with BD primers). This is in agreement with other observations (Tinland 1996) that show an asymmetry between the recombination of the left- and the right-hand sides of the T-DNA, suggesting that the integration involves different processes. Possible internal recombination within the T-DNA was not studied, although it was suspected in 1 case (data not shown).

Protein expression and bioassays

Expression of the *cry*1Ac gene was tested by Western analyses on proteins extracted from 23 transformed plantlets of the 51 independent events analysed in this investigation. In 18 of them, the polyclonal antibody (Table 1) detected protein. As shown in Fig. 4, an expected signal of 66 kDa molecular weight was observed with purified Cry1Ac protein and with leaf extract from transformed plants. However, in the other 5 transformed plants, although the *cry*1Ac gene was detected by PCR and Southern blotting, the protein was not detected by Western analyses (Fig. 4, lane 2).

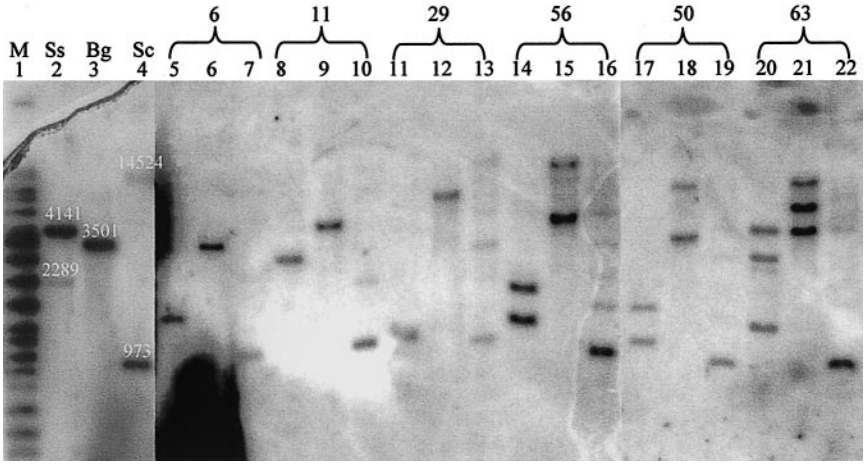


Fig. 3 Southern blot analysis of putatively transformed plants. DNA was prepared as described in the experimental protocol section and was hybridised with the pEF probe, corresponding to the pEF1α promoter of the *cry*1Ac gene. *Lane 1* Size DNA marker (*M*). *Lanes 2–4* Digestion of plasmid by *Ssp*I (*Ss*), *Bgl*II (*Bg*) and *Sca*I (*Sc*); the size (bp) of each digested fragment is indicated on each lane by *white figures*. *Lanes 5–22* Transformed plants, with each plant digested by *Ssp*I (first lanes), *Bgl*II (second lanes) and *Sca*I (third lanes). Plant codes are indicated *above the curly brackets*. Plants *6*, *11* and *29* present one integrated copy. For plant 6, the *Bgl*II fragment has a size of 3500 bp, signifying that the integration of a plasmid fragment downstream of the right border of the T-DNA occurred beyond the *Bgl*II site (Fig. 1B). For plants 56 and 50, the two fragments of different size observed with the *Ssp*I and *Bgl*II digests indicate the integration of two copies. Plant 63 presents a pattern that characterises an integration of three copies of T-DNA

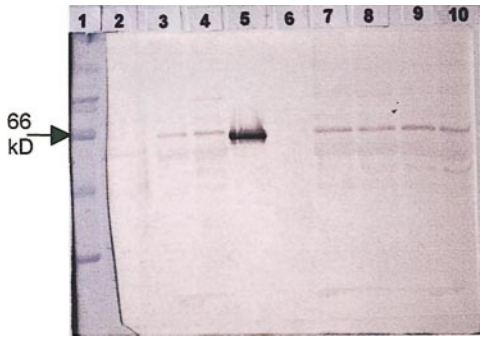


Fig. 4 Western blot analysis of total proteins from fresh leaves separated on 10% SDS-PAGE and probed with rabbit anti-Cry antibody; 14–24 μg of total protein was used as template. *Lane 1* Standard protein molecular-weight marker, *lane 5* pure Cry1Ac protein (0.5 μg), *lane 6* negative control, with an untransformed plant, *lane 2* transformed plant where no Cry1Ac protein was detected by the test, *lanes 3, 4*, and *7–10* transformed plants containing Cry1Ac protein

This may be due either to a level of protein below the detection threshold of the antibody, which is about 0.1% of total protein in this assay, or to a lack of expression of the protein. It could explained be also by a recombination event within the T-DNA which has altered the structure of the *cry*1Ac gene. Bioassays were performed on these 23 plants. The resistance/susceptibility of the plants was estimated with an overall score based on the symptoms observed 2 weeks after the release of adult insects and by the number of pupae counted 1 week later (Table 1). The control plants, as expected, showed a high susceptibility (Fig. 2E), with an overall score higher than 3.5 and more than 20 pupae produced for most of them. When plants were ranked according to their overall score in the bioassays (Table 1), two distinct observations could be made. Firstly (Fig. 2F-2), the Cry1Ac protein was detected in all plants with a score below or equal to 2 (14 events), and the height of plants was diversified, from 5 cm (plant 21) to 28 cm (plant 24). Thus, a good correlation does exist between the presence of the insecticidal protein and the resistance, as expressed by the lack of pupae on the plants. The expression of the resistance appears to be constitutive, as expected with the promoter pEF1α.

Table 1 Results of Western analyses and bioassays on plants from 23 independent transformation events. Overall score and number of pupae are an average value of 1–3 plants observed for each event

Plant	Presence of Cry1Ac protein[a]	Overall score in bioassays	Number of pupae per plant	Height of plant (cm)
45	+	0.0	0	12
13	+	0.2	0	18
21	+	0.5	0	5
63	+	0.5	0	10
18	+	0.5	0	10
65	+	0.6	0	8
62	+	0.9	0	10
6	+	1.0	0	10
44	+	1.0	0	12
16	+	1.0	0	27
24	+	1.0	0	28
48	+	1.5	0	10
31	+	1.7	0	25
75	+	2.0	0	16
59	−	2.1	2	25
23	+	2.5	2	22
7	+	2.5	20	25
9	−	2.5	3	25
46	+	3.0	23	24
22	−	3.0	1	20
76	−	3.1	20	30
8	+	3.4	24	26
10	−	3.8	13	29

[a] +, Protein detected; −, protein not detected

Secondly, for the other transformation events, whether the Cry1Ac protein was detected or not, the score of the bioassays was not correlated with the level of resistance expressed by the average number of pupae per plant after 15 days. Three plants with a score of 2.1 (plant 59), 2.5 (plant 9) and 3.0 (plant 22) and where the Bt protein was not detected had a low number of pupae (1 to 3). On the contrary, 3 plants where the protein was detected were highly susceptible (Fig. 2F-1) with 20–24 pupae per plant and scores of 2.5 (plant 7), 3.0 (plant 46) and 3.4 (plant 8). We can hypothesise that below a given threshold, for the presence of the Cry1Ac protein, the plant is more or less susceptible, and that this threshold is at the limit of our immunological detection. However, all plants of this group were higher than 20 cm. Our results do not allow us to draw any definitive conclusion, but they do point out that we have to question, for future observations, whether age and susceptibility could be related in some transformation events.

Moreover, bioassays performed on 6 transformed plants from both the Canephora 126 and Catimor genotypes showed results where half of the plants were highly resistant and higher than 15 cm in height compared to control plants showing more than 50 pupae per plant. Molecular and Western analyses are in progress for these transformed plants.

Here we report for the first time the regeneration of transgenic coffee plants containing a *B. thuringiensis* gene which has been integrated into the coffee genome. As previously foreseen (Guerreiro et al. 1998), the *cry*1Ac gene conferred resistance to leaf miner, as demonstrated by the bioassays. A correlation between the detection of the insecticidal protein in leaf extracts from transformed plants and resistance to leaf miner was observed in most cases. However, the observations suggested that, in some cases, the level of resistance and development stage could be related. This aspect will be checked carefully when the plants are transferred in 1999 to French Guyana for a field trial. Suitable insect management will be implemented in parallel with monitoring the expression of the *cry*1Ac gene

throughout the 5 year experiment. Up to now, all the results dealing with the evaluation of the efficiency of a *B. thuringiensis* (B. t.) gene in agricultural conditions have been obtained on annual crops. Thus, the observations that we intend to carry out on coffee will be very valuable for the future development of a B. t. strategy on perennial crops. Besides the stability of gene expression, it must be borne in mind that a targeted insect can develop resistance to toxins from *B. thuringiensis* (Rousch 1997; Gould 1998; Tabashnik et al. 1998). Therefore, the use of genes with different resistance mechanisms is ideally required to maintain a B. t. strategy. The *cry*1B gene could be used in order to obtain a cumulative effect with *cry*1Ac (Guerreiro et al. 1998), since the gut receptors are different for the two proteins. Furthermore, integrated pest management, including trapping, using parasitoides, nematodes or entomophagous fungi and adapted agricultural practices (optimising of herbicides and other pesticide uses), also have to be considered. This is particularly relevant for a perennial crop like coffee.

The transformation of coffee for resistance to the coffee leaf miner is a first step in the process of creating insect-resistant transgenic coffee plants. Although economically important, the coffee leaf miner is restricted to Brazil and East Africa and the practicality of this work will benefit only part of the coffee producers. Another obligate endocarpic pest, the coffee berry borer, *Hypothenemus hampei*, is present worldwide and considered to be the most devastating and economically important insect pest of coffee. The next step could therefore be the development of coffee plants resistant to the coffee berry borer.

The same approach has been applied to other coffee genotypes, and more than 100 plants from two *C. arabica* accessions, corresponding to independent transformation events, are already available. It is intended that genetically modified plants from both species, *C. canephora* and *C. arabica*, will soon be produced. The results presented in this work open the way to new opportunities to improve the coffee species, not only for other agronomic traits but also for those of technological interest. With respect to these perspectives, the recent cloning of a coffee seed specific promoter (Marraccini et al. 1999) is an important contribution towards further developments.

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