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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression

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Abstract We describe a protocol for *Agrobacterium*-mediated genetic transformation of *Theobroma cacao* L. using cotyledonary explants from primary somatic embryos (SEs) and *A. tumefaciens* strain AGL1. Transgenic plants carrying the visible marker, gene green fluorescent protein (*EGFP*), the selectable marker gene neomycin phosphotransferase II (*NPTII*), the class I chitinase gene from cacao (*Chi*), and tobacco nuclear matrix attachment regions (MARs) in different combinations were successfully produced via regeneration of secondary SEs. The presence of the *Chi* gene or MARs did not influence the number of transgenic plants produced compared to the marker genes alone. However, the inclusion of MARs contributed to increased mean GFP expression in the population of transgenics. Additionally, the presence of MARs reduced the occurrence of gene silencing and stabilized high levels of GFP expression in lines of transgenic plants multiplied via reiterative somatic embryogenesis. Ninety-four transgenic plants were acclimated in a greenhouse and grown to maturity. Detailed growth analysis indicated that there were no differences in various growth parameters between transgenic and non-transgenic SE-derived plants. Seeds produced from two genetic crosses with one of the transgenic lines were analyzed for *EGFP* expression—a near-perfect 1:1 segregation was observed, indicating that this line resulted from the insertion of a single locus of T-DNA.

Keywords *Theobroma cacao* · *Agrobacterium tumefaciens* · Genetic transformation · Matrix attachment region · Somatic embryogenesis

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Introduction

Theobroma cacao, a tropical perennial tree species, was originally prized by the ancient meso-Americans as an ingredient in ritual drinks (Hurst et al. 2002). The processed form of cacao seeds, cocoa, is now the basis of a multi-billion dollar chocolate industry (Wood and Lass 1987). Recently, the international agricultural research and development communities have recognized the benefits of cacao farms as ecologically beneficial agro-ecosystems and thus have begun to promote the sustainable production of cocoa as a means of environmentally friendly poverty amelioration (http://www.treecrops.org).

Cacao production suffers from a number of serious plant pests and diseases, which annually reduce the potential yields by as much as 40% (McGregor 1981; Fulton 1989). While traditional approaches to reduce pest and pathogen pressure are used in cacao, the high costs, difficulty of chemical application, and remoteness of most of the plantings severely limit their use. Strong genetic resistance in the cacao genome against most of these pests and pathogens has not been identified in primary germplasm. Partial resistance or tolerance has been identified and deployed in some breeding programs over the past century (Eskes and Lanaud 1997). However, to date, cacao breeding programs, which suffer from a long life cycle, funding constraints, and other limitations, have made limited progress with respect to the incorporation of durable, horizontal resistance to cacao pests and diseases into widely used cacao germplasm.

Modern biotechnology offers an array of approaches to enhance traditional plant breeding programs. Chief among these are plant tissue culture, molecular marker-assisted selection (MAS), and genetic transformation (Eskes and Lanaud 1997; Wilkinson 2000). Tissue culture can be a rapid system for clonal propagation of elite germplasm, while MAS can potentially speed up and reduce the costs of breeding programs. However, the plant breeder will still be limited by the genes and sources of resistance that are in the genomes of a species and its close relatives. Genetic transformation, on the other hand,

opens an avenue for incorporating novel sources of resistance, or other added value traits, into the genome, which can subsequently be utilized in breeding programs along with endogenous traits (Hansen and Wright 1999; Persley 2000). Additionally, genetic transformation provides a very powerful research tool, an invaluable approach for the analysis of gene structure and function that has revolutionized the field of plant molecular biology (Hansen and Wright 1999). While this approach has been utilized for many plant species and has resulted in a new sector of the agricultural economy, consumer resistance has slowed its application and development in many areas.

To date, only one publication on cacao transformation has been published, and only transformed callus cells were produced from this study (Sain et al. 1994). With an eye towards the future, we report here the development of a genetic transformation system for cacao. Using somatic embryogenesis as a regeneration system, coupled with *Agrobacterium tumefaciens* co-cultivation, transgenic *Theobroma cacao* plants were produced and grown to maturity. Physiological and morphological observations of many of the resulting transgenic cacao plants indicated no detectable pleiotropic phenotypic changes. The inclusion of tobacco matrix attachment regions (MARs) (Allen et al. 1996; Spiker and Thompson 1996) in the T-DNA did contribute to increased green fluorescent protein (GFP) fluorescence in the primary transgenics and stable and uniform GFP expression among the tertiary somatic embryos produced from the primary transgenics. Segregation and expression of the transgenes in the subsequent T_1 generation indicated stable integration and expression in one of the transgenic lines evaluated.

Materials and methods

Vector construction

Standard gene cloning methods (Ausubel et al. 2001) were used to construct three binary *Agrobacterium tumefaciens* transformation vectors: the primary vector (pGH00.0126), the MARs-containing vector (pGH00.0131), and the chitinase-containing vector (pGAM00.0511) (Fig. 1). All vectors contained the backbone, left, and right T-DNA borders of the pBin19 transformation vector (Bevan 1984). The T-DNA region of the primary vector included reporter gene *EGFP* (Clontech, Palo Alto, Calif.) driven by the E12-Ω promoter (Mitsuhara et al. 1996) and the marker gene neomycin phosphotransferase II (*NPTII*, De Block et al. 1984), also driven by the E12-Ω promoter. E12-Ω is a strong constitutive promoter containing the 5′-upstream sequence of the cauliflower mosaic virus (CaMV) 35S promoter (–419 to –90) (twofold); P35S, the 5′-upstream sequence of the CaMV 35S promoter (–90 to –1); and Ω, the 5′-untranslated sequence of the tobacco mosaic virus (TMV, Mitsuhara et al. 1996). Tobacco MARs (Allen et al. 1996; Spiker and Thompson 1996) flanking the T-DNA were incorporated into the primary vector to create the MARs vector. For construction of the chitinase vector, we digested the primary vector with *Cla*I next to the right T-DNA border and inserted a transgene cassette containing a genomic fragment of the Class I chitinase gene (*Chi*) isolated from *Theobroma cacao* (accession no. U30324) (Snyder 1994; Snyder-Leiby and Furtek 1995). The *Chi* cassette was constructed with a 1.241-kb polymerase chain reaction (PCR) fragment (Ausubel et al. 2001) amplified from position 444 to 1,666 of the genomic chitinase clone. This fragment contains 33 bp of 5′ untranslated region, three exons encoding the entire chitinase protein, two introns and 30 bp of 3′ untranslated region. To facilitate cloning, restriction fragment recognition sites were added to the ends. The following linkers were used in the PCR reaction:

- 5′ primer: *Sma*I: 5′-ggcccggg (RE site).....cattgacaaaatcacagatatt-3′ (chitinase sequence)
- 3′ primer: *Not*I 5′-gggcggccgc (RE site).....gtttaccaaagtcattc-3′ (chitinase sequence).

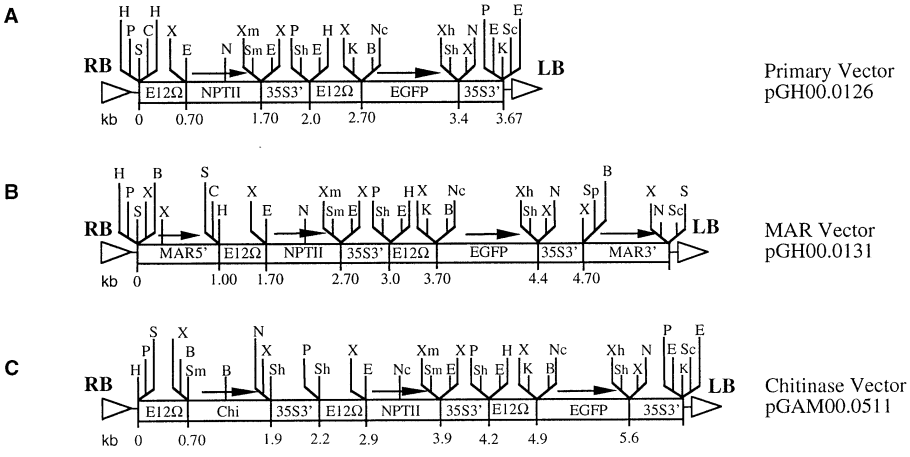

Fig. 1A–C Structures of binary transformation vectors for *Agrobacterium tumefaciens*. All vectors have backbone and left (*LB*) and right (*RB*) T-DNA borders from pBin19. **A** The primary vector (pGH00.0126) contains a 3.7-kb T-DNA, including an *NPTII* gene for plant resistance to aminoglycoside antibiotics and *EGFP* gene encoding for green fluorescent protein. **B** The MARs vector (pGH00.0131) contains 5′ and 3′ tobacco matrix attachment regions (*MARs*) flanking the *NPTII* and *EGFP* genes. **C** The chitinase vector (pGAM00.0511) includes *NPTII* and *EGFP* gene cassettes with the addition of a cacao chitinase gene (*Chi*) inserted next to the RB. The E12-Ω strong constitutive promoter (a CaMV 35S derivative) drives the transgenes in all three vectors. Restriction enzyme recognition sites are indicated at the *top* of the maps and are abbreviated as follows: *B Bam*HI, *C Cla*I, *E Eco*RI, *K Kpn*I, *N Not*I; *Nc Nco*I, *P Pst*I, *S Sal*I, *Sc Sac*I, *Sh Sph*I, *Sm Sma*I, *Sp Spe*I, *X Xba*I, *Xh Xho*I, *Xm Xma*I. Distance from the *left-most* site to each restriction enzyme site is indicated *below* the maps in kilobase pairs (kb)

The fragment was digested with *Sma*I and *Not*I enzymes and ligated to the intermediate cloning plasmid pE2113-*EGFP* (cut with *Sma*I and *Not*I to remove the GFP coding sequence). The resulting plasmid, pChi0329, contained the E12-Ω promoter upstream of the chitinase fragment followed by the 35S terminator. The *Chi* cassette was excised with *Hin*dIII, filled in with Klenow and ligated to *Cla*I digested and filled in primary vector, resulting in the chitinase vector. All binary transformation vectors were introduced into the disarmed *A. tumefaciens* strain AGL1 (Lazo et al. 1991) by electroporation (Lin 1994).

Tissue culture and genetic transformation

Primary somatic embryos were produced from the greenhouse-grown PSU Scavina 6-1 cacao genotype as previously described (Li et al. 1998). Healthy mature primary embryos with developed cotyledons were selected. The cotyledons had glossy surfaces without visible trichomes and an opaque white, pink, or ivory color. The cotyledons were excised and cut into approximately 4-mm^2 pieces for secondary embryogenesis as recently described (Maximova et al. 2002). Prior to culture, the pieces were inoculated with *A. tumefaciens* AGL1 (Lazo et al. 1991) containing one of the three binary vectors. *A. tumefaciens* cultivation, inoculation, and co-cultivation with cocoa tissue were performed as previously described for apple (Maximova et al. 1998) with minor modifications, as follows. *A. tumefaciens* was grown in 20 ml of 523 bacterial medium (Dandekar et al. 1989) at 25 C and agitated at 200 rpm on an orbital shaker until the cultures reached an optical density (O.D.) of 1, measured at 420 nm. Bacterial cells were collected by centrifugation (800 *g*) at 25°C. The pellet was resuspended in induction medium (Maximova et al. 1998) and the O.D. was adjusted to 0.5 at 420 nm. The induction medium included DKW basal medium, 30 g/l sucrose, 1 g/l glucose, 0.0001 *M* acetosyringone, and 0.0013 *M* proline. Virulence was induced for 5 h at 25°C and 100 rpm agitation. After induction, between 100 and 200 cacao cotyledon explants were added to 20 ml of *A. tumefaciens* solution in 50-ml conical tubes. The explants in the *A. tumefaciens* solution were sonicated for 30 s in a Branson water bath (Model 1510, VWR, Pittsburgh, Pa.) followed by 10 min of infection at 25°C and 50 rpm agitation. After infection, the *A. tumefaciens* solution was vacuum-aspirated, and the explants were transferred to modified solid SCG medium (Maximova et al. 2002) overlaid with one sheet of Whatman filter paper no. 5 and incubated for 48 h of co-cultivation at 25°C in the dark. Following co-cultivation, the explants were transferred to fresh solid SCG medium containing 200 mg/l moxalactam (Sigma-Aldrich, St. Louis, Mo.) for *A. tumefaciens* counter-selection and 50 mg/l geneticin (Sigma-Aldrich) for *NPTII* selection. The infected explants were cultured on geneticin selection for 2 weeks in the dark at 25°C, then transferred and maintained on ED medium (Li et al. 1998) with 200 mg/l moxalactam following the secondary embryogenesis protocol as previously reported (Maximova et al. 2002). Ten different sets of transformation experiments involving three different transformation vectors with 100 to 400 explants infected per experiment were initiated over a period of 12 months. Cultures were observed biweekly, and transgenic embryos were counted. The Fisher Protected LSD test at the $P<0.05$ level of significance was used for mean separations (StatView5, SAS Institute, Cary, N.C.).

The regenerated transgenic embryos were grown to maturity and their cotyledons excised and cultured for tertiary and quaternary embryo production/multiplication as described by Maximova et al. (2002). Only cotyledons with high GFP florescence (measured as described below) were selected for tertiary and quaternary embryogenesis. Mature tertiary somatic embryos (SEs) from 12 transgenic lines (six lines transformed with the primary vector, three lines transformed with the MARs vector, and three lines transformed with the chitinase vector) and three non-transgenic lines were transferred to in vitro PEC medium for conversion to plants (Traore 2000). Between 10 and 54 tertiary embryos—subsequently transferred to conversion medium—were produced per line.

Plant acclimation

Three- or four-centimeter-tall, well-developed SE-derived plants with two to six leaves and roots were transferred from in vitro culture directly to a climate-controlled greenhouse exceeding NIH PBL2 biosafety conditions. The plants were transplanted to deep pots [6.5 (diameter) × 25 cm] with silica sand for adequate drainage. The acclimation and growth conditions were: 14/10-h (light/dark) photoperiod; 28/25°C (light/dark). For the first 7–10 days, the plants were placed under low light (<100 μmol m^{-2} s^{-1} PAR) on a mist bench with approximately 100% humidity; after that, the relative humidity was decreased to 75%. Natural light was supplemented with 430-W high-pressure sodium lamps as needed to maintain a minimum of 250 μmol m^{-2} s^{-1} PAR, while automatically retractable shading limited light levels to a maximum of 1,000 μmol m^{-2} s^{-1} PAR. Irrigation with 1/10-strength Hoagland's nutrient solution (160 ppm N) was applied daily at multiple times to maintain adequate pot moisture.

Four sets of 10–43 individual transgenic plants from different lines—containing the primary and MARs vectors—and control non-transgenic plants were transferred to the greenhouse. The percentage of acclimated plants was calculated, and the variation (mean separation) between transformation vectors was analyzed with the Fisher Protected LSD test at $P<0.05$ level of significance (StatView5, SAS Institute).

Growth measurements

A completely randomized design with two treatments (ten transgenic and ten control SE-derived plants) was set up to monitor growth parameters. The transgenic plants evaluated were randomly selected from the first established single transgenic line (T980603) containing the *EGFP* and *NPTII* genes. Height, stem diameter, and numbers of leaves were recorded at 5 months after acclimation. To non-destructively estimate whole-plant leaf area, we employed the following approach: leaves of various sizes from both treatments were randomly selected (70 per treatment) and cutouts of their outlines made. The lengths of the cutouts were recorded and their areas measured with a LI-3000A portable area meter (Li-Cor, Lincoln, Neb.). Two regression equations—one for transgenic and one for non-transgenic plants—were determined relating leaf length to leaf area. Leaf areas were calculated for individual plant leaves using the regression equations after measuring all leaf lengths from each plant. Total leaf area (in square centimeters) for each plant was then determined by summing the calculated individual leaf areas. Means of the two treatments were calculated and compared using a non-paired, two-tailed *t*-test (Microsoft Excel, Microsoft, Seattle, Wash.).

Pollination method and gene segregation analysis

Controlled hand-pollinations were performed following standard procedures as described by Wood and Lass (1987). Freshly open flowers from the Pound 7 genotype were fertilized with pollen from 18-month-old transgenic PSU Scavina 6-1. Additionally, flowers from transgenic PSU Scavina 6-1 were fertilized using pollen from the Catongo genotype. A total of six cacao pods, three per cross, were set and formed seeds. All pods were harvested at 5– 6 months after pollination, and *EGFP* expression/fluorescence in the seed cotyledons was scored under the fluorescent microscope as described below. Chi-square analysis was used to establish the segregation distribution.

GFP visualization

Bright field and fluorescent images of SEs were taken with a Nikon SMZ-4 dissecting microscope equipped with an epi-fluorescence attachment, a 100-W mercury light source, and a 3 CCD video camera system (Optronics Engineering, Goleta, Calif.). The microscope filters used were a 520- to 560-nm emission filter (transmitting only green light) and a 450- to 490-nm excitation filter. The whole plant images were taken with a 3 CCD video camera and 35-mm Nikon lens equipped with a 520- to 560-nm emission filter. The video camera was connected to a Macintosh computer with NIH Image 1.6 image processing and analysis software (Maximova et al. 1998). The GFP fluorescence intensity of different transgenic and non-transgenic cacao tissues was measured under the microscope as previously described in Maximova et al. (1998). The fluorescence intensity of cotyledons from etiolated tertiary transgenic SEs from three independent lines per vector (10–12 SEs per line) was measured and compared to that of control non-transgenic SEs. The cotyledons measured were randomly selected from mature SEs and had the same characteristics as explants selected for *A. tumefaciens* transformation (see above). The measurements were taken at a magnification of 30× and an exposure time of 0.5 s. Additionally, young leaves and flowers from 1-year-old transgenic plants (line no. T980603) grown in the greenhouse were observed. Five samples per tissue type per tree were collected from five different transgenic trees and five control trees. Measurements of fluorescence intensity were taken at a magnification 30× and an exposure time of 1 s. Mean intensities (integrated pixel values, IPV) were measured using NIH Image 1.6 image processing and analysis software (Maximova et al. 1998) and were compared using the Fisher Protected LSD test at $P<0.05$ level of significance (StatView5, SAS Institute).

Genomic PCR analysis

Three sets of PCR primers were used for the analysis. The first amplifies a 700-bp *EGFP* fragment (5′-CAG ATC TTT ACT TTG ACA GCT CGT CCA TGC CGT-3′ and 5′-GGG ATC CAT GGT GAG CAA GGG CGA GGA GCT GTT-3′) (Maximova et al. 1998); the second produces a 500-bp fragment from the cacao actin gene (5′-TGG TGT CAT GGT TGG NATG-3′ and 5′-GGCACAGTGTGAGANACACC-3′); the third set amplifies a 411-bp fragment from the backbone sequence of the pBin19 vector located approximately 2,000 bp upstream of the right T-DNA border in the tetA region (5′-CTG GCG GCC GTC TAT GG-3′ and 5′-CAG CCC CTC AAA TGT CAA-3′). Genomic DNA leaf extracts from fully developed transgenic plants from independent lines transformed with the primary vector and with the MARs vector and non-transgenic Scavina 6 plants were prepared using the REDExtract-N-Amp plant PCR kit (Sigma, St. Louis, Mo.). The single-step DNA extraction included incubation of 4-mm leaf disks with 100 µl proprietary extraction solution from the PCR kit at 95°C for 10 min, followed by the addition of an equal volume of proprietary dilution solution. Dilutions (1:5) of the extracts were made with water. Four-microliter aliquots of the diluted leaf extracts were added to PCR mix. Each PCR reaction also contained 10 µl REDExtract-N-Amp PCR jumpstart reaction mix and both forward and reverse primers at a final concentration 0.25 µ*M* in a final volume of 20 µl. Reactions were prepared at 25°C. Control PCR reactions were also performed with DNA from the primary vector and backbone vector pBin19 containing only the left and right borders without the rest of the T-DNA region (pBin19A), and with the PCR reaction mix without DNA. Plasmid DNA was prepared via dilution series with DNA from salmon sperm (SS) (Sigma-Aldrich no D-1626). A total of 0.15 pg of the respective plasmid DNA was added to each control PCR reaction. This represents an equal molar amount of plasmid DNA compared to the GFP DNA contained in 5 ng total cacao genomic DNA present in the leaf extract, assuming single copy/insertion of the GFP gene. The DNAs added to the control reactions were as follows: SS DNA only; primary vector DNA and SS DNA; primary vector DNA, SS DNA, and Scavina 6 genomic DNA; primary vector DNA, SS DNA and genomic DNA from different transgenic plants. For the plasmid reactions, DNA was isolated using the QIAGEN plasmid midi-purification kit (QIAGEN, Valencia, Calif.). Reactions were prepared at 25°C as a REDExtract-N-Amp PCR reaction mix, and primers were added as described above. PCR conditions for all reactions were: 94C for 2 min; then 35 cycles of 94°C for 45 s, 54°C for 45 s, 72°C for 1 min. The final cycle was followed by incubation at 72°C for 7 min. Eight microliters of each PCR reaction were loaded onto a 2% high-resolution agarose gel (Sigma-Aldrich, no. A-4718) for electrophoresis.

Results and discussion

Transgenic embryo formation and transformation efficiency

Recently, we described a highly efficient system for the regeneration of secondary SEs from primary SE explants originally initiated from floral tissue explants (Maximova et al. 2000, 2002). This system produces more embryos per explant then the primary SE system and, in addition, the embryos have a higher morphological conformity and percentage of conversion to plantlets. We coupled this secondary SE system with *A. tumefaciens* infection and co-cultivation in order to develop a genetic transformation system for *T. cacao* L. Another important factor that contributed to the success of this protocol was our recent finding that the antibiotic moxalactam was very effective for *A. tumefaciens* counter-selection and, surprisingly, increased the regeneration frequency of secondary embryos (Antúnez de Mayolo 2003).

Three binary vectors were constructed based on the Bin19 T-DNA system (Fig. 1; Bevan 1984)). Each plasmid contained the *NPTII* and GFP genes under the control of the CaMV 35S promoter derivative E12-Ω (Mitsuhara et al. 1996). The primary vector contained these two genes alone. The MARs vector contained matrix attachment regions flanking the GFP and *NPTII* genes. The MAR sequences were previously shown to stabilize levels of gene expression in other transgenic plant systems (Allen et al. 1996). The chitinase vector contained a cacao *Chi* gene (Snyder 1994), placed under the control of the same E12-Ω promoter in addition to the GFP and *NPTII* genes. The *Chi* gene was included in a construct as a potential gene for resistance to fungal pathogens.

The binary T-DNA vectors were electroporated into *A. tumefaciens* AGL1, and bacteria were co-cultivated with primary SE cotyledon explants as described above. Green fluorescence in the infected explants due to GFP expression was first detected 48 h post *A. tumefaciens* infection (Fig. 2A, D). The fluorescence was mainly associated with the cut edges of the explants, as has been observed earlier in other species (Maximova et al. 1998). During the following 10 days, the fluorescent areas enlarged and their fluorescence intensities increased. Occasionally the fluorescence covered entire explants (Fig. 2B, E). The large number of fluorescent areas and the high fluorescence intensity shortly after transformation demonstrated

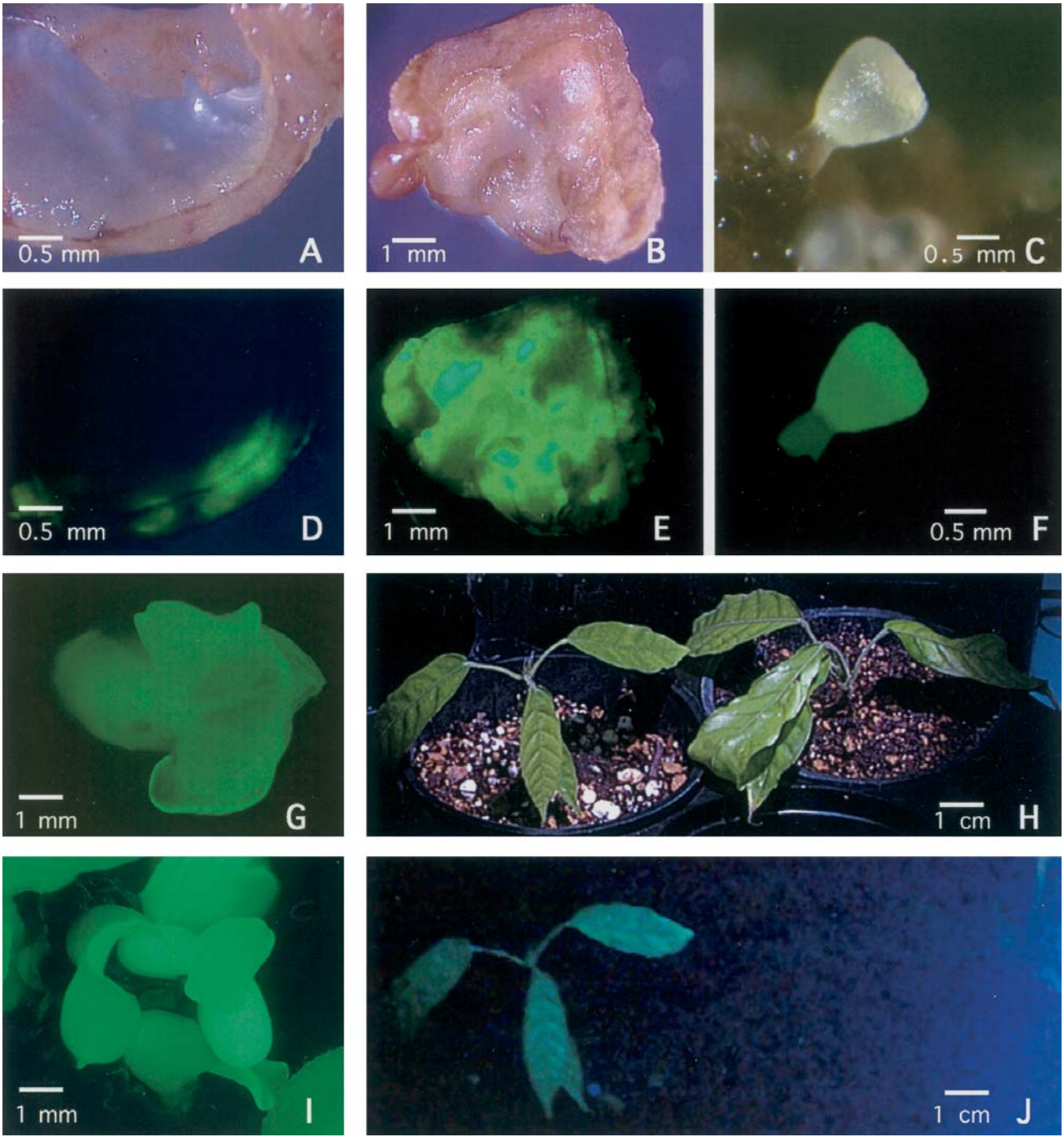

Fig. 2A–J Green fluorescent protein expression in cacao tissues and plants. **A–C**, **H** Light images, **D–G**, **I**, **J** fluorescent images. **A**, **D** The cut edges of a somatic embryo (SE) cotyledon explant 48 h after *A. tumefaciens* infection. **B**, **E** SE cotyledon explant 10 days after *A. tumefaciens* infection. **C**, **F** Transgenic SE in early-heart stage. **G** Mature transgenic SE. **H**, **J** Transgenic and non-transgenic SE-derived plants. **I** Tertiary transgenic SEs produced from a cotyledon of the embryo on panel **G**

the high frequency of *A. tumefaciens* infection and DNA transfer to the plant cells. However, by 60 days after infection, the fluorescence was reduced to a few small areas, indicating either high transient expression shortly after transformation without gene incorporation or high levels of gene silencing in the cacao genome.

The formation of individual transgenic secondary SEs (TSSE) was observed between 44 days and 180 days after infection (Fig. 2C, F). A total of 31 secondary transgenic embryos were regenerated from the ten experiments over a period of 1 year (July, 2000–August, 2001) (Table 1). From these embryos, 14 were transformed with the primary vector, ten with the MARs

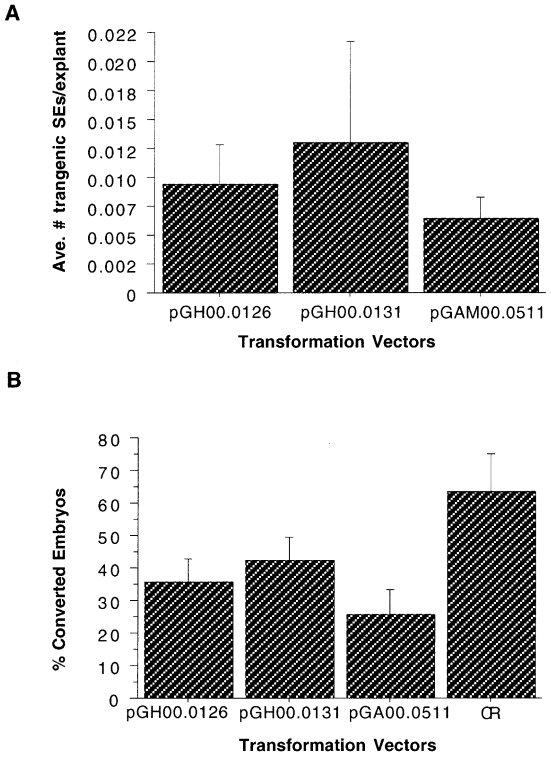

Fig. 3A, B Mean number of transgenic embryos produced and plant conversion rates from ten transformation experiments. Vector designations are as follows (see Materials and methods for details): *p126* the primary vector, *p131* the MARs-containing vector, *p511* the chitinase-containing vector. The vectors used for transformation were described in Fig. 1. Mean separation was performed with the Fisher Protected LSD test at $P<0.05$ level of significance. **A** Mean numbers ± standard error (SE) of transgenic embryos produced per infected explant, **B** percentages ± SE of mature transgenic and non-transgenic somatic embryos converted to plants

vector, and seven with the chitinase vector. The mean numbers of transgenic embryos per infected explant produced from the individual transformation experiments were low, ranging from 0 to 0.043 TSSE (Table 1). From the seven transformations performed with the primary vector, only one did not generate TSSEs. Similarly only one of the five transformations with pGAM00.0511 did not produce TSSEs. Two of the five transformations carried out with the MARs vector produced TSSEs (Table 1). The mean efficiencies (no. of transgenic embryos per infected explant) were calculated for the different vectors, and mean separation indicated no significant differences (P=0.61, 0.69, 0.40, respectively) (Fig. 3A). Compared to the high frequency of transient expression, stable expression frequencies were much lower on a per explant basis. The relatively low production of transgenic embryos was consistent among all vectors. However, despite the low efficiency of this protocol, we are able for the first time to consistently and reproducibly generate transgenic cacao plants. Future optimizations of the protocol for increasing the T-DNA incorporation efficiency should include evaluation of different vectors, selectable marker and reporter genes, selection conditions, and *A. tumefaciens* strains.

The occurrence of gene silencing is a possible explanation for the low frequency of transgenic embryo regeneration. In other plant species tobacco nuclear MARs have been shown to enhance and stabilize transgene expression after transformation with *A. tumefaciens* and to reduce the frequency of gene silencing (Allen et al. 2000). We reasoned that if the dramatic difference observed between transient and stable expression in cacao was due to gene silencing, the incorporation of MARs could enhance transgenic embryo recovery. To

Table 1 Transgenic secondary (TSSE) and tertiary embryos (TTSE) produced from ten *Agrobacterium tumefaciens*-mediated transformation experiments with three different binary vectors[a]

Experiment ID	Transformation vector	Total no. explants infected	TSSE	Mean no. TSSE per explant	Number of transgenic lines	TTSE
1	Primary	161	4	0.025	4	35
2	Primary	250	1	0.004	0	0
3	Primary	113	0	0.000	0	0
4	Primary	200	3	0.015	1	25
5	Primary	642	2	0.003	1	14
6	Primary	705	2	0.003	1	52
7	Primary	127	2	0.016	2	40
1	MARs	155	0	0.000	0	0
2	MARs	185	8	0.043	3	41
3	MARs	91	2	0.022	1	26
7	MARs	129	0	0.000	0	0
9	MARs	109	0	0.000	0	0
4	Chitinase	201	2	0.010	2	10
5	Chitinase	116	0	0.000	0	0
8	Chitinase	235	2	0.009	1	54
9	Chitinase	113	1	0.009	0	0
10	Chitinase	400	2	0.005	1	33

[a] Somatic embryo cotyledon explants were transformed in ten different experiments with three different transformation vectors over a period of 12 months. Cultures were observed biweekly, and transgenic embryos were counted. The regenerated individual secondary transgenic embryos were grown in vitro to the mature embryo stage after which their cotyledons were excised and cultured for tertiary embryo production/multiplication following the protocol for secondary embryogenesis (Maximova et al. 2002)

test this hypothesis, we incorporated tobacco MAR sequences within the T-DNA of the resulting MARs plasmid. However, after five transformation experiments with this vector, no significant difference in the number of transgenic embryos recovered was observed (Fig. 3A). It is possible that any expected positive effect of the MARs was reduced or eliminated due to the slow growing nature of the cotyledon cells involved in embryo regeneration (Allen et al. 2000). Compared to some of the faster plant regeneration systems—for example, like tobacco (Fisher and Guiltinan 1995), which requires only 20 days to produce regenerants—cacao cotyledon tissue regenerates slowly and takes 60–200 days to produce the first SEs. Alternative possible explanations are that the low stable transformation frequency is due to *trans* silencing mediated by sequence homology somewhere else in the cacao genome, against which the presence of MARs is ineffective, or that the MARs effect was decreased by the *cis* interactions among the repeated sequences of the E12-Ω promoter (Allen et al. 2000). Considering the possibility of promoter homology-dependant silencing, we should note that the incorporation of *Chi* as the third transgene driven by the E12-Ω promoter without the presence of MARs did not result in a lower transgenic embryo production. However, in view of the general low regeneration frequency of the system, additional experiments, including larger number of samples and transgenic lines, are needed to substantiate this observation.

In order to clonally propagate independent transgenic somatic embryo lines, we initiated tertiary somatic embryogenesis. Cotyledon explants from mature T_0 transgenic embryos were cultured for multiplication and the establishment of transgenic lines by the re-initiation of somatic embryogenesis on embryo induction medium. A total of 16 transgenic lines were established, each of which produced between 10 and 52 individual tertiary transgenic SEs. Nine of these lines originated from T_0 transformations containing T-DNA from the primary vector, four from the MARs vector, and three from the chitinase vector (Table 1). The successful multiplication of the single T_0 regenerants was an important step of the protocol for the establishment of transgenic lines for further transgene expression and growth analysis. Considering the relatively low transgenic embryo regeneration (T_0) frequency and the 50–60% embryo-to-plant conversion rates (Fig. 2B) in this system, the high rates of tertiary embryo production is an important attribute of this system for obtaining sufficient transgenic plants for subsequent analysis.

Transgenic plant conversion, growth, and gene segregation analysis

Conversion of in vitro somatic embryos into functional autotrophic plants (conversion) is an essential and sometimes difficult process for some species. Furthermore, it is possible that this process may be sensitive to DNA transformation and/or transgene expression. Between 10 and 54 transgenic embryos from different transgenic cacao lines were converted to plants in vitro, and the effect of different T-DNAs on the conversion rates were evaluated (Fig. 3B). The mean percentage of transgenic SEs containing T-DNA from the primary and chitinase vectors that converted to plants (35.8% and 25.7%, respectively) was significantly lower than the percentage of control non-transgenic secondary embryos converted (63.5%, P=0.031, P=0.016, respectively). No significant difference between control SEs and SEs transformed with the MARs vector (41.9%, P=0.135) was observed. However, at the same significance level (5%), no differences were measured between the two other vectors and the MARs vector (P=0.628, P=0.279, respectively). Hence, the slightly higher conversion rates of plants transformed with the MARs vector could be due to the inclusion of the MARs sequences or, alternatively, the difference could be a result of positional effects of the insertions recovered in this study.

Growth and maturation of transgenic plants

To test the possible effects of transgene insertion on the growth and physiology of the transgenic cacao plants, we carefully compared control and transgenic plants for morphological characters. Ten plants from transgenic line T980603 containing the *EGFP* and *NPTII* genes and ten control SE-derived plants were grown to maturity in a greenhouse. All stages of development of these plants exhibited GFP expression as observed by fluorescence imaging (data not shown). At 5 months after acclimation, plant height, stem diameter, and the number and surface area of all leaves were recorded. Both sets of plants grew at similar rates (Table 2). No significant differences in their height, stem diameter, and leaf area were observed,

Table 2 Comparison of the growth parameters of transgenic and control somatic embryo (SE)-derived plants after 5 months in the greenhouse[a]

Treatment	Plant height (cm)	Stem diameter (cm)	Numbers of leaves	Leaf area (cm^2)
Transgenic	89.0±4.3	1.23±0.04	54.0±2.9	5,340±480
Control	95.2±4.9	1.25±0.06	59.2±3.7	6,400±540
P value	0.35	0.79	0.29	0.16

[a] The growth to maturity of ten transgenic and ten control SE-derived plants was monitored. The transgenic plants evaluated were from a single line containing the *EGFP* and *NPTII* genes. Data were recorded at 5 months after acclimation. Each value is a mean ± standard deviation of ten repetitions. *P* values greater than 0.05 are non-significant as determined by a non-paired, two-tailed *t*-test (Microsoft Excel)

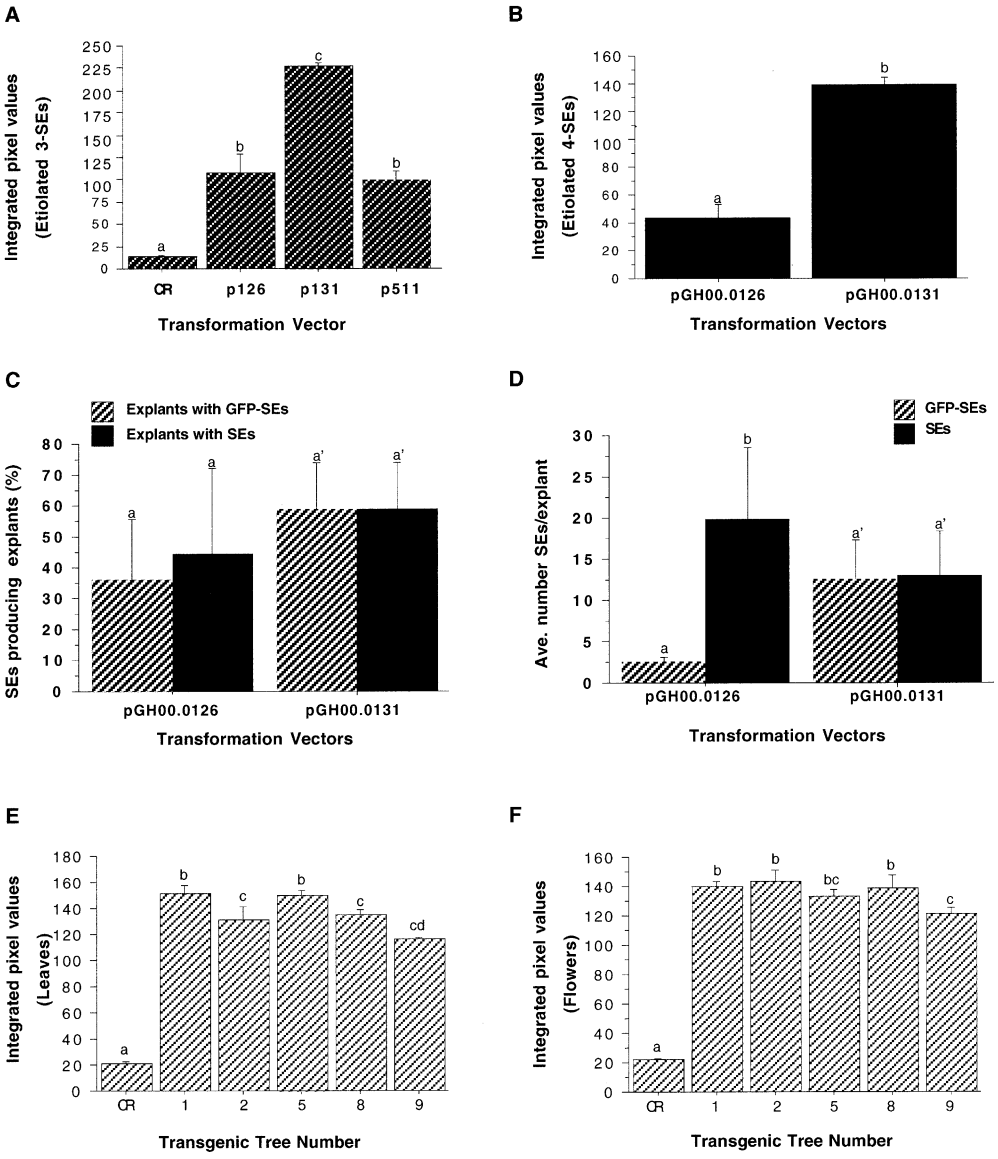

Fig. 4A–F Evaluation of green fluorescent protein fluorescence of control and transgenic tissues from multiple somatic embryo lines and mature cacao plants. Vector designations are as follows (see Materials and Methods for details): *p126* the primary vector, *p131* the MARs-containing vector, *p511* the chitinase-containing vector. Fluorescence intensity was expressed as integrated pixel values (IPVs). **A** IPVs of tertiary control and transgenic etiolated SEs regenerated in the dark. **B** IPVs of transgenic, quaternary etiolated SEs regenerated in the dark. **C** Percentages ± SE of tertiary, transgenic cotyledons producing at least one quaternary somatic embryo and producing at least one fluorescent somatic embryo. **D** Average numbers ± SE of quaternary somatic embryos per producing cotyledon and average number ± SE of fluorescent quaternary somatic embryos per producing cotyledon. **E** Mean IPV ± SE of control and transgenic leaves. **F** Mean IPV ± SE of control and transgenic flowers. The mature plants measured for **E** and **F** were from a single line (T980603) containing the *EGFP* and *NPTII* genes without MARs. Mean separations were performed with the Fisher Protected LSD test at $P<0.05$ level of significance. Means marked with *different letters* are significantly different from each other

indicating that the transformation process and expression of the transgenes in this line did not result in any visible phenotypic alterations. Additionally, 94 newly converted and acclimated plants from ten independent transgenic lines were observed in the greenhouse for a period of 3–4 months and these also did not demonstrate any visible phenotypic alterations (data not shown).

Transmission of GFP expressi on through sexual reproduction

After both set of plants had developed flowers, two crosses were performed: transgenic T980603 (female) × Catongo (male) and Pound 7 (female) × transgenic T980603 (male). Six cacao pods were produced with a total of 282 T_1 seeds (44–50 seeds per pod). All maternal tissue in the cacao pods produced from the first cross

exhibited high levels of GFP expression, including the pod surface, exocarp, placental tissues, and seed coats. Segregation of GFP expression was clearly observed after removal of the seed coats, and very high levels of GFP accumulation occurred in the cotyledons and embryos of the transgenic seeds. From all the crosses, 143 seeds scored positive and 139 negative for GFP expression in the cotyledons. The goodness-of-fit Chi-square for a 1:1 distribution demonstrated that the GFP gene in the T_1 generation of line T980603 segregated as a single locus insertion (χ^2=0.422, 3 *df*). All seeds were germinated and grown in the greenhouse. Leaf and root samples from all of the seedlings were observed for *EGFP* expression at 6 months after germination. All of the originally GFP-positive seeds produced plantlets expressing GFP, indicating stability of the *EGFP* transgene expression through meiosis, fertilization and into the T_1 progeny.

Stability of GFP expression level

In order to assess the stability and variation in GFP expression in different transgenic lines, and to evaluate the influence of the MARs in further detail, we measured GFP expression in vivo using semi-quantitative fluorescence microscopy. The fluorescent intensity (represented as IPVs) of etiolated SE cotyledons due to expression of the GFP gene was measured in transgenic SEs and compared to background fluorescence in non-transgenic SEs. The values recorded for highly fluorescent cotyledons of transgenic SEs were up to 17.7-fold higher than the background fluorescence levels found in non-transgenic SEs (P<0.0001) (Fig. 4A). The variation among the fluorescence intensities of SE lines was assessed in three different ways: among lines transformed with different vectors, among lines transformed with the same vector, and variation among multiple SEs regenerated from individual lines.

We observed that despite the high green fluorescence/GFP expression of all transgenics (T_0) selected for multiplication via tertiary embryogenesis, the new embryos that were produced were not always fluorescing. Complete silencing (no green fluorescence) was observed in two lines from a total of eight lines established containing the primary vector and in one line from a total of four lines established with the chitinase vector. In contrast, all four lines containing the MARs vector were highly fluorescent (Fig. 4A). The overall expression levels (±standard error) (IVPs) recorded were: 103.06±20.45, 226.49±4.42, and 98.89±81.29 for the primary, MARs and, chitinase vectors respectively. The fluorescence intensity of non-transgenic SEs was 14.47±0.03 IPVs. The overall mean intensities of the lines transformed with the primary and chitinase vectors were significantly lower than that of the MARs vector (P<0.0001). Additionally, the lines transformed with the primary and chitinase vectors were not significantly different from each other (P=0.7508). All of the transgenic SEs lines were significantly different from the controls (P<0.003). On the basis of the overall intensity data and the data recorded for the mean intensities of individual lines (Fig. 4A, Table 3), it appears that the presence of the MARs may have contributed to higher mean expression levels. Similar observations of a correlation between increased mean transgene expression levels of populations of transformants and MARs-containing transgene constructions have been reported for other plant species (Allen et al. 2000).

Additional comparisons among the mean intensities of three individual lines per transformation vector supported this observation (Table 3). The largest differences were recorded among the lines containing the primary and chitinase vectors. In contrast, no differences were recorded among the lines with the MARs vector.

To establish the variation within the individual transgenic lines, we compared the standard errors and

Table 3 Mean values of green fluorescent protein fluorescence intensity ± standard error and their variances[a]

Line no.	Transformation vector	Mean intensity	Standard error	Variance
1	Primary	42.34	13.91	1354.86
2	Primary	14.44	0.02	0.03
3	Primary	209.38	12.21	1340.57
1	Chitinase	80.14	13.25	5442.12
2	Chitinase	190.32	7.83	736.30
3	Chitinase	62.04	19.40	5273.28
1	MARs	223.44	6.47	419.04
2	MARs	225.18	8.49	576.75
3	MARs	237.26	7.41	219.53
1	CR[b]	14.53	0.13	0.02
2	CR	14.43	0.07	0.01
3	CR	14.44	0.07	0.01

[a] Fluorescence intensity of cotyledons from etiolated tertiary transgenic SEs from three independent lines per vector (10–12 SEs per line) was measured and compared to that of control non-transgenic SEs. Fluorescent images were taken with a Nikon SMZ-4 dissecting microscope equipped with an epi-fluorescence attachment, a 100-W mercury light source and a 3 CCD video camera system (Optronics Engineering). The microscope filters used were a 520–560 nm emission filter and a 450–490 nm excitation filter. Mean intensities (integrated pixel values) were recorded using NIH Image 1.6 image processing and analysis software (Maximova et al. 1998)

[b] Control regeneration

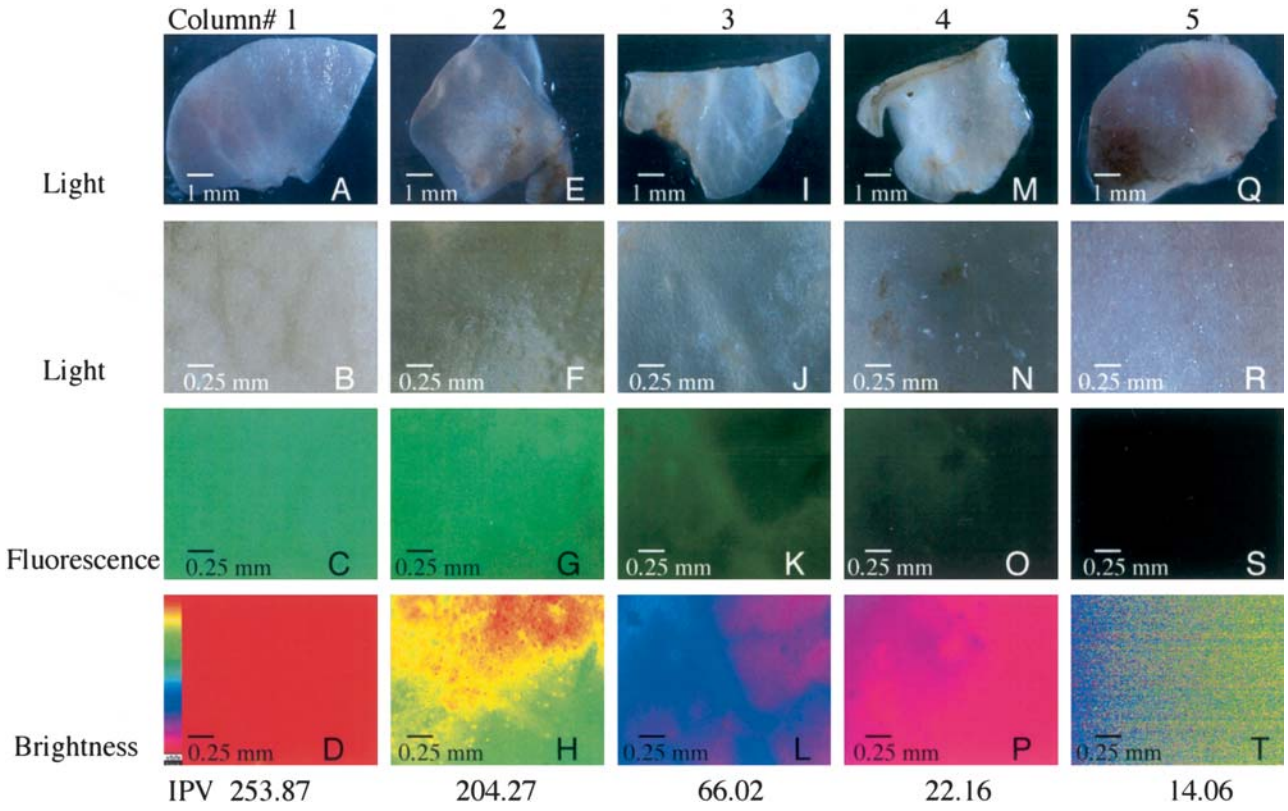

Fig. 5A–T Microscope images of the variation in green fluorescent protein (GFP) fluorescence intensity among different tertiary SEs from a single transgenic line containing the primary vector pGH00.0216. *Rows 1*, *2* Light images, *row 3* fluorescent images, *row 4* color-coded brightness of GFP fluorescence—the brightness scale is from *red* to *purple*, with *red* representing the brightest fluorescence and *purple* representing the lowest fluorescence. Each *column* contains images of one of the five explants selected from different somatic embryos. *A–D* Transgenic explant no. 1, *E–H* transgenic explant no. 2, *I–L* transgenic explant no. 3, *M–P* transgenic explant no. 4, *Q–T* control non-transgenic explant. *Rows 3, 4, 5* are magnified images of the explants in *row 1*. The measurements of GFP brightness were taken at high magnification to ensure a uniform and flat surface. *IPV* Integrated pixel value

variances of the lines (Table 3). These data demonstrated that the standard errors and variances of the transgenic lines containing MARs are significantly lower, suggesting that the presence of the MARs sequences contributed to stable, high expression levels within the individual lines of tertiary embryos. To our knowledge, this is the first report of a positive MARs influence on gene silencing during somatic embryogenesis and regeneration of clones from a single transgenic plant. The differences in GFP fluorescence within a single primary vector line are demonstrated in Fig. 5. The brightness of the fluorescence varied from very high to very low.

To further evaluate the influence of MARs on somatic embryogenesis, highly fluorescent cotyledons from tertiary SEs containing the primary vector and MARs vector were cultured and regenerated. Explants were selected from three different lines per vector. At 60 days after culture initiation, the fluorescence intensities (IPVs) of quaternary randomly selected embryos were measured (30 SEs × three lines × two vectors) (Fig. 4B). The results from the separation of the mean IPVs for the two vectors were similar to those previously obtained for tertiary SEs. The mean fluorescence of the embryos containing MARs was significantly higher ($P<0.0001$) than those without MARs (Fig. 4B). A comparison of the percentage of explants producing one or more SEs to the percentage of explants producing one or more fluorescent SEs (GFP-SEs) demonstrated that all of the SE-producing explants containing the MARs vector were also producing GFP-SEs (Fig. 4C). In contrast, 25% of the cotyledons of one of the primary vector lines produced only silenced SEs. No statistical differences between the percentages of SE and GFP-SE cotyledons were recorded within the individual vectors ($P=0.818$, 0) (Fig. 4C). Further analysis of the average numbers of GFP-SEs per producing explant established that the majority of SEs regenerated from the primary vector lines were silenced and that close to 100% of the SEs regenerated from the MARs vector lines were highly fluorescent (Fig. 4D). These results provide additional evidence of the preventive effect of MARs on transgene silencing during cacao somatic embryogenesis.

Variation in GFP levels was also investigated in leaves and flowers removed from mature trees, which were clonal replicates of the same line (T980603) (Fig. 4E, F). Minor variation was observed among the values recorded for individual trees for both leaves and flowers (Fig. 4E, F), with differences of between 1.15- and 1.3-fold for the

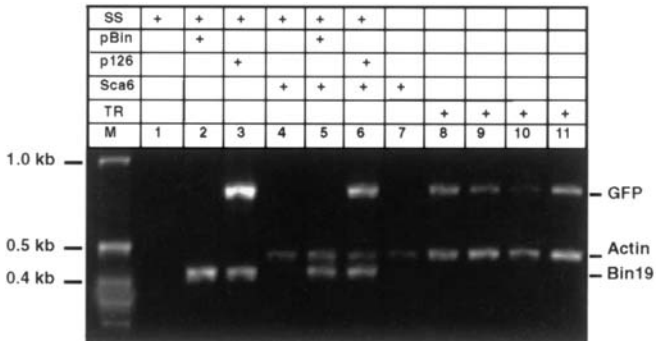

Fig. 6 Genomic PCR analysis of non-transgenic and transgenic lines containing the *EGFP* gene. PCR amplification was performed with three primer sets: pBin19 backbone primers amplifying a 400-bp fragment, cacao actin gene primers producing a 500-bp fragment, and *EGFP* gene primers amplifying a 700-bp fragment. The control plasmid DNAs used were Bin19A DNA (*pBinA*), containing only left and right borders without the rest of the T-DNA region, and the primary vector pGH00.0126 DNA (*p126*), including the *EGFP* gene. The plasmid DNA was diluted to 0.15 pg in salmon sperm DNA (*SS*). Leaf extract from non-transgenic Savina 6 (*Sca6*) and transgenic plants (*TR*) from four independent lines were prepared using the REDExtract-N-Amp plant PCR kit (Sigma) and used for cacao genomic DNA analysis

leaves and 1.15- and 1.57-fold for the flowers. The lower intensity values for leaves and flowers compared to etiolated SEs could be attributed to the partial absorption of the fluorescence by the pigments in these tissues.

Molecular analysis of transgene insertion into the genome

The incorporation of the *EGFP* gene was verified by genomic PCR (Fig. 6). As a result of using specific PCR primers, our strategy allows the detection of T-DNA insertion, while also demonstrating the lack of non-T-DNA region sequences of the binary plasmid. This allows us to rule out the possibility of *A. tumefaciens* contamination contributing to GFP expression or to the amplification of T-DNA region sequences. As a positive control for the amplification of the non-T-DNA region, (binary PCR test), we used a cacao actin gene sequence. The three primer sets were multiplexed to allow amplification in one tube and the display of each line in a single electrophoretic lane. This strategy enables the molecular verification of transgene insertion into genomic DNA using very small amounts of tissue, thus facilitating the early analysis of the SE lines. Furthermore, this protocol requires one simple and easy DNA extraction step without purification (see Materials and methods).

A series of dilution and mixing controls were performed to demonstrate the specificity and sensitivity of the procedure. Plasmid DNAs containing the binary plasmids used in this study were diluted to genome equivalent amounts (15 pg of plasmid equals approximately molar-equivalent to 5 ng of cacao genomic DNA) using ssDNA as carrier (5 ng per reaction). No amplification was observed with all three of the primer sets and the ssDNA used to dilute the plasmid DNAs (Fig. 6, lane 1). The PCR of vector pBin19A (no GFP gene) resulted only in the amplification of one 411-bp fragment corresponding to the pBin19 backbone fragment (Fig. 6, lane 2). As expected, the primary vector produced two fragments: a 411-bp pBin19 backbone fragment and a 700-bp *EGFP* fragment (Fig. 6, lane 3). The mixture of equal amounts non-transgenic Scavina 6 DNA and ssDNA produced only the expected 500-bp cacao actin fragment (Fig. 6, lane 4). The combination of pBin19A with non-transgenic Scavina 6 resulted in amplification of the 411-bp pBin19 backbone fragment and the 500-bp cacao actin fragment (Fig. 6, lane 5). When pBin19A was substituted with the primary vector (containing the GFP gene), an additional 700-bp *EGFP* fragment was observed (Fig. 6, lane 6). No *EGFP* gene or pBin19 backbone fragment amplification was observed using the non-transgenic SE DNA template in the reaction (Fig. 6, lane 7). This reaction produced only the expected 500-bp cacao actin fragment. All of the transgenic lines evaluated produced a 500-bp cacao actin fragment and the 700-bp *EGFP* fragment. Representative of these data, reaction products from two lines with the primary vector and two with the MARs vector are presented in Fig. 6 (lanes 8–11). These results demonstrate that the *EGFP* gene was stably incorporated into the genomes of these plants and that the amplification was not due to *A. tumefaciens* contamination. Additionally, we confirmed that the PCR method described in this study provides a fast and reliable means for screening for transgenic plants with minimal DNA extraction step.

The study presented here demonstrates that using co-cultivation of SEs with *A. tumefaciens* led to the recovery of stably transformed cacao plants in ten consecutive experiments. The number of T_0 plants regenerated per explant was relatively low, but the consistency of the regeneration, the high efficiency of tertiary embryogenesis, and the reasonable conversion rates provide a working protocol for cacao transformation. Because of the low frequency of transgenic embryos recovered, the use of *EGFP* was of major importance for the non-lethal identification of transgenic embryos at early stages of development. The use of the GFP marker gene enabled the optimization of many variables in this protocol quickly. Further improvement in transformation efficiency would be necessary to allow the elimination of the GFP gene for generation of transgenic plants. Alternatively, other methods for gene removal following transformation may be utilized for cacao (Hare and Chua 2002). The transgenes incorporated into these plants did not interfere with the regeneration or growth of the plants, and they were stably integrated and expressed into the next generation of progeny of one line. The incorporation of MARs appears to be important for stabilizing transgene expression during somatic embryogenesis and transgenic line establishment. Future experiments incorporating genes of interest into the MAR vectors would further establish the reliability of the system. This system can be used as a basis for analyzing gene structure and function

in cacao, and as a model system for testing the effectiveness of transgenes for enhancement of desired traits in cacao, such as fungal or viral disease resistance.

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