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Improvements in the Efficiency of *Agrobacterium*-Mediated Transformation of Embryogenic Cell Suspensions of Banana cv. 'Mas' using a Low-Antibiotic Liquid Washing-Assisted Approach

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ABSTRACT

Agrobacterium-mediated transformation is a convenient gene transfer process for many plants, including banana. However, the use of various antibiotics using this method, either to eliminate *Agrobacterium* or for screening of putative transformed plants significantly affects plant growth as well as transformation efficiency. In this study, we have improved currently reported methods for transformation of banana embryogenic cell suspension by using a hormone-free Murashige and Skoog (MS) liquid washing-assisted medium supplemented with an optimum concentration of cefotaxime at 50 mg l^{-1} for bacterial elimination followed by 200 µg l^{-1} hygromycin for screening of putative transformed plants. This strategy was designed to reduce the antibiotic stress on the transformed plants by accelerating plant regeneration and by shortening the incubation period of the transformed plants on the antibiotic selection medium. The highest number of transformation events was achieved after 30 minutes incubation with *Agrobacterium tumefaciens* strain LBA4404 carrying pCAMBIA/SOC1 encoding a MADS-box transcription factor followed by 4 days of co-cultivation in darkness at 30 ± 1°C. The integration of the *Elaeis guineensis* Jacq. (oil palm) *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) transgene was confirmed by both Southern Blot and real-time RT-PCR analysis.

Keywords: antibiotic stress, cefotaxime, hygromycin, liquid medium, *Musa*, transgenic banana
Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **6-BA**, N^6-Benzyladenine; **CaMV**, *Cauliflower mosaic virus*; **hph**, hygromycin phosphotransferase gene; **MIC**, minimal inhibitory concentration; **MS**, Murashige and Skoog; **NOS**, nopaline synthase; **PPM™**, Plant Preservative Mixture; **SCV**, settled cell volumes; **SE**, somatic embryo; **SOC 1**, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* gene

INTRODUCTION

Over the last decade, the cultivation of banana has been significantly threatened by pests and diseases such as *Fusarium* wilt, leaf spot diseases, *Banana bunchy top virus*, *Banana streak virus*, nematodes and weevils. Research worldwide is now focused on the improvement of banana varieties for disease resistance (Johanson and Jeger 1993; Vuylsteke *et al*. 1993b; Carlier *et al*. 2002; Tripathi *et al*. 2007). However the use of conventional breeding approaches for developing new resistant varieties remains difficult because of the sterility (i.e. seedless fruit) and polyploid nature of the edible banana varieties. It is also known that a conventional breeding program, when possible, is very time-consuming and the plantlets produced are usually not genetically uniform. Therefore, the development of *in vitro* micropropagation methods for banana by somatic embryogenesis has become an alternative method to produce a rapid multiplication single cell regeneration system for large scale production (Côte *et al*. 1996; Wong *et al*. 2006; Xu *et al*. 2008). This approach has also become a useful tool for genetic transformation and somatic hybridization, which could potentially overcome the factors limiting conventional approaches to banana improvement.

For banana, May *et al*. (1995) reported the development stable transformants by co-cultivating wounded banana meristems with *Agrobacterium tumefaciens*. However, their protocol could not transform all cell types and the putative transformed plants regenerated via organogenesis were often chimeras. Hence, in the late 1990s, several groups proposed the use of embryogenic cell suspensions, a single cell culture, as the target plant material for plant transformation. The concept of using embryogenic cell suspensions for banana transformation has been reported by Hernandez *et al*. (1999), Ganapathi *et al*. (2001), Piñeda *et al*. (2002), Khanna *et al*. (2004) and Sunil Kumar *et al*. (2005) for *Agrobacterium*-mediated transformation of embryogenic cell suspension of Banana cv. 'Rasthali' (AAB), 'Dominico Hartón' (AAB), 'Grand Nain' (AAA) and 'Lady Finger' (AAB), respectively. It was also reported that the regenerants derived from somatic embryogenesis appeared to produce lower rates of somaclonal variation compared to organogenesis (May *et al*. 1995; Ganapathi *et al*. 2001). While gene transfer methods have been well resolved, the efficiencies of transformation have been shown to be greatly affected by many factors, including environmental factors such as recalcitrance and poor regeneration of the transformed plants (Hernandez *et al*. 1999; Lessard *et al*. 2002; Zambre *et al*. 2003; Wong *et al*. 2005). Thus, the objective of this paper was to optimize factors during the co-cultivation and post-cultivation phase of *Agrobacterium*-mediated transformation of *Musa acuminata* cv. 'Mas', which would influence the efficiency of its transformation. The plasmid used in this study contained the *Elaeis guineensis* Jacq. (oil palm) *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) transgene and was from a project investigating early flowering in monocots (K. Harikrishna pers. comm.).

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Original Research Paper

MATERIALS AND METHODS

Explant source and culture conditions

Cell suspensions of *Musa acuminata* cv. 'Mas' (AA) were established from embryogenic callus derived from immature male flower clusters cultured for six months according to the method developed by Côte *et al*. (1996). The embryogenic cell suspension cultures were subcultured at 2 week-intervals in M2 medium (Côte *et al*. 1996) containing Murashige and Skoog (MS) (1962) macronutrients and micronutrients supplemented with 0.40 mg l^{-1} thiamine, 0.50 mg l^{-1} nicotinic acid, 0.50 mg l^{-1} pyridoxine, 2.0 mg l^{-1} glycine, 100.0 mg l^{-1} myo-inositol, 10.0 mg l^{-1} ascorbic acid, 1.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 250.0 µg l^{-1} *trans*-zeatin (Sigma) and 20.0 g l^{-1} sucrose. The pH was adjusted to 5.7 prior to autoclaving. Throughout the experiment, approximately 2.0 ml of settled cell volumes (SCV; the volume of settled embryogenic cell aggregates) of the cell aggregates were inoculated into 50.0 ml M2 media in a 250.0 ml Erlenmeyer flask. The cultures were maintained on an orbital shaker at 70 rpm, at 25 ± 1°C, 16 hours light: 8 hours dark photoperiod with a light intensity of 31.4 µmol$m^{-2}s^{-1}$.

Somatic embryo (SE) development and plantlet regeneration

For embryo development, suspension cells in M2 medium were sieved through a 450-µm mesh, the filtrate was left to settle down in a 50.0 ml Falcon tube followed by the adjustment of the SCV to approximately 1:5 (SCV: liquid M2 medium). Cell aggregates were then re-suspended and 200.0 µl aliquots were dispensed into 20 ml of hormone-free liquid-based MS medium (MSOL). Cultures were placed on an orbital shaker at 80 rpm, at 25 ± 1°C in the dark until the formation of somatic embryos were observed. The cultures were subcultured once every 14 days. The immature embryos were then transferred to differentiation medium containing half-strength MS macronutrients and micronutrients supplemented with 1.0 mg l^{-1} thiamine, 1.0 mg l^{-1} nicotinic acid, 10.0 mg l^{-1} pyridoxine, 100 mg l^{-1} *myo*-inositol, 30.0 g l^{-1} sucrose, 2.6 g l^{-1} phytagel, pH 5.8. For continued plantlet regeneration, the embryos that differentiated were transferred to full strength MS basal medium supplemented with 7.0 mg l^{-1} N^6-Benzyladenine (6-BA) and placed at 25 ± 1°C, 16 hours light: 8 hours dark photoperiod with a light intensity of 31.4 µmol $m^{-2}s^{-1}$.

Agrobacterium and plasmid

Agrobacterium tumefaciens strain LBA4404 harboring the plasmid pCAMBIA/SOC1 (**Fig. 1**) was used for the transformation experiment. This plasmid contains the *SUPPRESSOR OF OVEREXPRESSION OF CO 1* gene (*SOC1*) which encodes a MADS-box transcription factor associated with early flowering originally from oil palm (*Elaeis guineensis* Jacq.) and the hygromycin phosphotransferase gene (*hph*) which confers resistance to hygromycin for transgenic plant selection. Both the *SOC1* and *hph* genes were under the control of a *CaMV35S* promoter and a *NOS* terminator. The bacteria stock cultures were maintained and recovered as described in Sambrook *et al*. (1982).

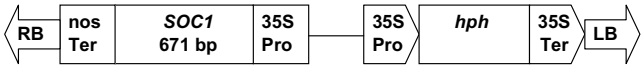

Fig. 1 Linear map of plasmid pCAMBIA/SOC1.

Determination of bacterial growth curve and cell density

The *A. tumefaciens* culture was prepared by streaking the frozen cells onto LB agar plate containing selection antibiotics (100 mg l^{-1} streptomycin or 100 mg l^{-1} kanamycin; Amresco, Ohio). After an incubation period of 16 hours, single bacterial colonies were picked from the fresh plate and grown in LB broth supplemented with selection antibiotics and incubated in the dark at 30°C on a rotary shaker (200 rpm). The optical density (OD_{550}) reading was measured at 1-h intervals until the log phase was achieved. The cell density of the exponential bacterial growth stage was determined by using a spread-plate method. A correlation between bacterial cell density and optical density was obtained from a standard graph which was performed using a serial dilution plating method.

Identification of antibiotics for elimination of *A. tumefaciens*

This experiment was carried out by using a broth tube dilution method to determine the minimal inhibitory concentration (MIC) of the antibiotic to be used for eliminating *A. tumefaciens*. The three tested antibiotics were cefotaxime, timentin and Plant Preservative Mixture (PPM™, PhytoTechnology Lab., USA). Ten different dilutions of each antibiotic (50, 100, 150, 200, 250, 300, 350, 400, 450, 500 mg l^{-1}) were made in LB broth medium. The lowest concentration of the antibiotic preventing bacteria growth was considered to be the MIC. At this dilution the antibiotic was considered bacteriostatic. Subsequently, the minimal bactericidal concentration was determined by subculturing the contents of the tubes onto antibiotic-free LB agar plates and incubation at 30°C in the dark. After 48 h, the bacterial growth was examined.

Determination of MIC of hygromycin for selection of transgenic plants

To identify the MIC of hygromycin for selecting transgenic plants, immature embryos were placed on optimal differentiation and regeneration media supplemented with various concentrations of hygromycin (0, 200, 400, 600, 800, 1000 µg l^{-1}). Three replications were used for each treatment.

An improved strategy for *Agrobacterium*-mediated transformation

An overnight bacteria culture from a single *Agrobacterium* colony was grown in the dark (250 rpm) at 30°C for 8-9 hours to an OD_{550} of 0.5. Suspension culture cells of cv. 'Mas' in M2 medium were passed through a 450-µm mesh and the settled cell volume: liquid M2 medium ratio adjusted to approximately 1:5. Cell aggregates were then resuspended and dispensed as 500.0 µl aliquots into each 10 ml liquid culture medium containing 1.0 ml *Agrobacterium* culture. The infection period (15, 30, 45 and 60 minutes) and co-cultivation period (1, 2, 3, 4 and 5 days) were evaluated through 3 replications in two experiments. After co-cultivation in darkness at 80 rpm for 30°C, the suspension cells were transferred to 20 ml fresh liquid medium supplemented with the optimum concentration of cefotaxime. Forty eight hours later, the suspension cells were transferred again to fresh liquid medium without cefotaxime. Bacterial testing was carried out at this stage to ensure the efficacy of elimination of the *Agrobacterium*. The transformed suspension cells were allowed to grow in liquid medium with a 10 days subculture interval. The OD_{550} of *Agrobacterium* culture was not monitored at this stage. After 4-6 weeks, immature SEs were transferred to solidified medium for plant regeneration. Hygromycin at the optimum concentration was incorporated into all subsequent medium.

Polymerase chain reaction (PCR)

Genomic DNA was isolated from control and transformed plants regenerated on selective media, respectively, according to a rapid CTAB DNA isolation protocol (Stewart and Via 1993). This method had been used with success in isolation of banana DNA for PCR and subsequent Southern blotting (May *et al*. 1995). In this study, the putative transgenic cell line was validated by the integration of the *SOC1* transgene only. The forward and reverse primer sequences used for the PCR amplification of the *SOC1* gene were 5′-GGACTAGTGGAAGATGGTGAGGACTAGTGGAAGA TGGTGA-3′ and 5′-GGACTAGTAATTGCCTGTCA-3′, respectively. The reaction mixture (25 µl) contained 2.5 U *Taq* DNA polymerase (Promega, Madison, WIS), 5 pmol of each forward and reverse primers, 0.75 µl 25 mM $MgCl_2$, 0.25 µl 10 mM dNTP, 2.5 µl 10X buffer, 100-300 ng template DNA and nuclease-free

water (Promega, Madison, WIS). The PCR programme used was one cycle of 94°C for 60 s; 94°C for 60 s; 55°C for 60 s; 68°C for 60 s followed by 29 cycles of 94°C for 60 s; 55°C for 60 s; 68°C for 60 s. Finally, the reaction was completed with an additional extension of 10 min at 68°C before cooling to 10°C. The PCR analysis was performed in an Eppendorf PCR thermocycler (Model 5330). PCR products were then determined by gel electrophoresis (1.0% agarose) at 80 V for 45 min to assess the PCR product.

Southern blot analysis

Genomic DNA of the putative transformed plants was extracted from leaves according to the method of Stewart and Via (1993). Undigested genomic DNA samples (25 µg) were electrophoresed through a 1% (w/v) agarose gel (Sagi *et al*. 1995) and the separated DNA transferred to Hybond-N nylon membrane (Amersham Biosciences, USA) according to Sambrook *et al*. (1989). Additionally, genomic DNA (25 µg) was digested with *Bam*HI (NEB, USA) which cuts once in the flanking site of the *SOC1* transgene prior to membrane transfer (Sambrook *et al.* 1989). Estimation of transgene copy number were made by including on the Southern blots, a double digested genomic DNA using the combination of *Eco*RI, *Bam*HI and either *Cla*I or *Sac*II (New England Biolabs Inc., USA) which cuts once in the *SOC1* sequence to release a 255 or 209 bp fragment of the partial *SOC1* transgene, respectively. The Southern hybridization process was carried out using a DIG DNA labelling and detection kit (Roche, Germany). The membrane was then washed as recommended by the manufacturer and the hybridization signal was detected by autoradiography (Gel-Pro Analyzer, MicroLAMBDA, USA).

Real-Time PCR analysis

The integration of *SOC1* sequences were analysed by Real-Time PCR (Rotor Gene, Corbett Research) using DNA extracted from putative transformed and control plants. The forward and reverse primers used to amplify the specific internal sequences of the *SOC1* gene of 150 bp were 5′-AGTGGCATCAACAACAATGAA-3′ and 5′-TGGCAGAGGCTTTGCTCTA-3′, respectively. The Taqman dual-labeled hybridization probe was designed as 5′FAM-ATCGAATCCCTTGAAGTTTCCAAAC-3′Tamra. The reaction components consisting 1X QuantiTect Probe PCR master mix, 0.4 µM forward primer, 0.4 µM reverse primer, 0.1 µM probe, RNase-free water and 250 ng template DNA. The real-time cycler conditions using TaqMan probes was one cycle of 95°C for 15 min (initial activation step); 94°C for 15 s (denaturation); 60°C for 60 s (combined annealing and extension steps) followed by 39 cycles of 94°C for 15 s and 60°C for 60 s. The fluorescence data was acquired during the combined annealing and extension step.

RESULTS AND DISCUSSION

Determination of bacteria growth curve and cell density

There are several methods for counting bacterial cell numbers e.g. direct microscopic count, turbidimetric measurements, filtration, viable or plate counts etc. (Madigan *et al*. 2000). In this study, viable counts were preferred for determination of the living bacterial cells for plant transformation which was *A. tumefaciens* strain LBA4404 carrying the vector pCAMBIA/SOC1. The growth curve (data not shown) reflected three parts of the growth phases: The lag phase (OD_{550nm} = 0–0.05), the exponential phase (OD_{550nm} = 0.05–1.00) and stationary phase (OD_{550nm} = 1.00–1.05). Bacterial cells in exponential growth are usually in a vigorous state due to the abundance of nutrients and permissive environmental conditions. Thus, the cells in the exponential phase are optimal for *Agrobacterium*-mediated transformation. The number of viable cells in the exponential phase was measured at OD_{550nm} = 0.2, 0.4, 0.5, 0.6 and 0.8 according to the standard graph (data not shown). The selected cell density at OD_{550nm} = 0.5 (7.0×10^7 single forming colony per one ml of sample) was used for plant transformation.

The determination of cell density for this study was important as previous studies have shown that a higher cell density of *A.tumefaciens* strain LBA4404 significantly enhances the transformation efficiency of tobacco and Arabidopsis (Lin *et al.* 1994). However, elimination of bacterial cells after co-cultivation may require incorporating high concentration of antibiotic into the culture medium. This may interfere with the growth and development of the transformed plant cells either by overgrowth of the bacterial cells or toxicity of antibiotic used (Lin *et al.* 1994; Lin *et al.* 1995; Nauerby *et al*. 1997; Cheng *et al*. 1998). Hence, the bacteria growth curve was generated to determine the optimal incubation period and bacterial cell density during the various growth phases.

Identification of antibiotic for suppression of *A. tumefaciens*

The broth tube dilution method is the standard method for determining levels of bacterial resistance to an antibiotic. The *A. tumefaciens* carrying pCAMBIA/SOC1 was incubated for the prescribed time to achieve an OD_{550nm} = 0.5 and then inoculated into different dilutions of antibiotics in LB broth. Cefotaxime, timentin and Plant Preservative Mixture (PPM™, P820, Phytotechlab) were tested for their bacteriostatic and bactericidal effects as well as their effects on plant regeneration of banana plantlets following co-cultivation with *A. tumefaciens*. The lowest concentration of antibiotic preventing the appearance of turbidity was considered to be the MIC and the antibiotic considered as bacteriostatic. The contents of the tubes without any sign of bacterial growth were subcultured onto antibiotic-free LB agar plates and examined for colony growth. No growth of bacteria indicates that the antibiotic was bactericidal at that particular concentration. However the growth of bacteria shows that the antibiotic was bacteriostatic but not bactericidal at that dilution. The results showed that minimum bactericidal concentration of cefotaxime, timentin and PPM™ were 50.0 mg l^{-1}, 200.0 mg l^{-1} and 1.0 ml l^{-1} respectively.

Effects of antibiotics on plant regeneration

For the plant transformation experiments, *Agrobacterium* has to be eliminated after co-cultivation so as not to interfere with the growth and development of transformed plant tissues. Therefore, the effects of a selective media containing *Agrobacterium*-eliminating antibiotics on plant regeneration were studied in detail. In this study, the potential of 50.0 mg l^{-1} cefotaxime, 200.0 mg l^{-1} timentin and 1.0 mg l^{-1} PPM™ for eliminating *Agrobacterium* growth when incorporated into hormone-free liquid-based MS medium (MSOL) was tested individually. In our earlier study of a banana regeneration system, we suggested that liquid MSOL medium was the best SE development medium (Wong *et al.* 2006) and most mature SE developed from this improved liquid protocol showed differentiation and regeneration within 4 months. Thus, liquid MSOL medium was used as the optimal medium in the co-cultivation stage with cell suspensions. It was then followed by transferal of the developed SE to differentiation and regeneration media without any *Agrobacterium*-eliminating antibiotics. The regenerated shoots and any abnormal characteristic were compared at each treatment (**Fig. 2A-D**). The data presented in **Table 1** indicates that 50.0 mg l^{-1} cefotaxime in MSOL potentially the most suitable *Agrobacterium*-eliminating antibiotic for use in banana cell suspension transformation. This is because of the low percentage of abnormalities (23.1%) observed in the regenerated plantlets compared to that seen in 200.0 mg l^{-1} timentin (47.1%). No plants developed when PPM™ was incorporated into the liquid MSOL medium. Statistical analysis applied to the data using one-way analysis of variance (ANOVA) showed that the P value of the germination rate was much less than 0.05 (**Table 2**). This suggests that the results of the antibiotic treatments were significantly different at the SEs germination stage, whereas

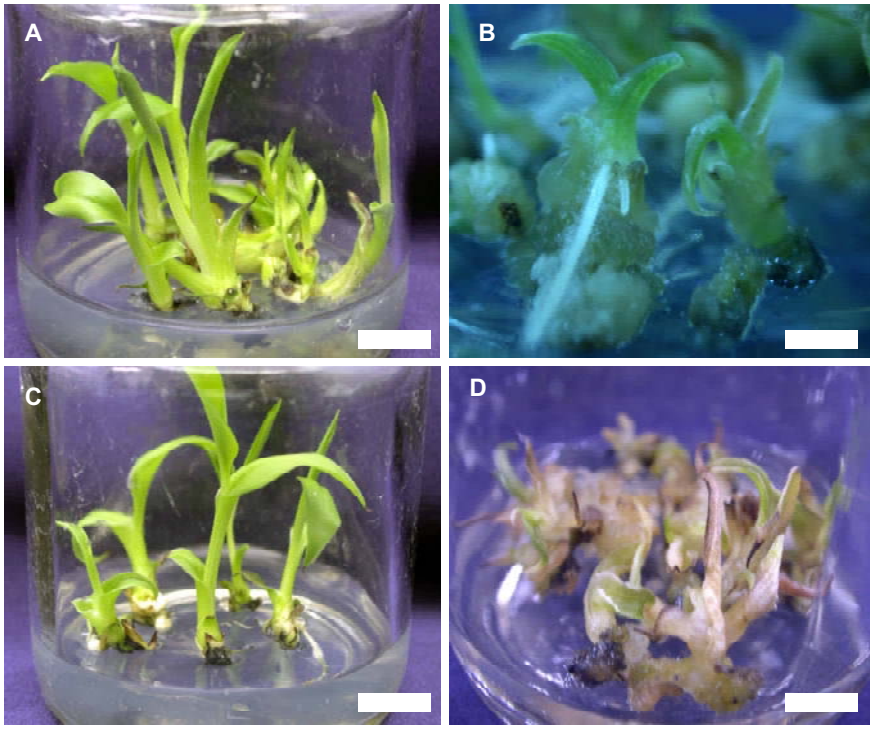

Fig. 2A-D The effects of *Agrobacterium*-eliminating antibiotic on plant regeneration. (**A**) Plantlets regenerated from somatic embryos developed on MSOL with 50.0 mg l^{-1} cefotaxime (bar: 1.5 cm); (**B**) Abnormal plantlets were observed in plantlets regenerated from somatic embryos developed on MSOL with 50.0 mg l^{-1} cefotaxime (bar: 1.0 cm); (**C**) Plantlets regenerated from somatic embryos developed on MSOL with 200.0 mg l^{-1} timentin (bar: 1.5 cm); (**D**) Abnormal plantlets were observed in plantlets regenerated from somatic embryos developed on MSOL with 200.0 mg l^{-1} timentin (bar: 1.0 cm).

Table 1 The effects of *Agrobacterium*-suppressing antibiotic on SE germination and regeneration.

Antibiotic in MSOL	Concentration	№ of somatic embryos	№ of germinated somatic embryos	№ of regenerated plantlets	№ of regenerated shoots with abnormal characteristics
Cefotaxime	50.0 mg l^{-1}	50	31	13	3
Timentin	200.0 mg l^{-1}	50	24	17	8
PPM	1.0 ml l^{-1}	50	0	0	0

Table 2 One-way analysis of variance (ANOVA) of antibiotic effects on rate of SE germination, regeneration and abnormal characteristics

Treatment	Variance of germination rate (%)			Variance of regeneration rate (%)			Variance of abnormal characteristics (%)		
	Mean	StDev	P	Mean	StDev	P	Mean	StDev	P
Cefotaxime	62.00	15.62	0.001	20.65	20.98	0.079	26.45	45.82	0.227
Timentin	47.33	11.02		39.38	20.81		45.98	19.91	
PPM™	0.00	0.00		0.00	0.00		0.00	0.00	

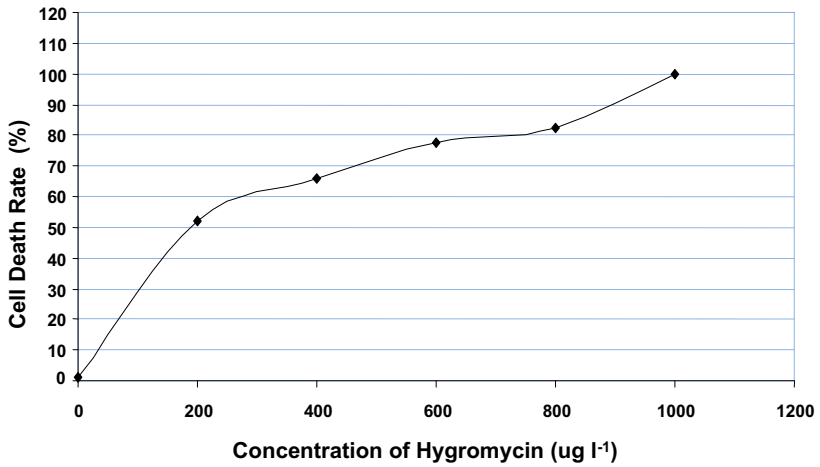

Fig. 3 Determination of minimal inhibitory concentration of hygromycin on differentiation media.

there was no significant difference between the effects of Cefotaxime and Timentin either on plant regeneration or on the occurrence of abnormal characteristics.

Determination of MIC of hygromycin for plant selection

Hygromycin was used as the plant selection antibiotic as the vector pCAMBIA/SOC1 contains the *hph* gene that confers resistance to hygromycin B. The MIC of hygromycin was determined for non-transformed somatic embryos of banana. The experiment was conducted to determine the stringency of the antibiotic selection level (75-100% cell death) or non-stringent selection level (50-74%cell death) to be used for screening transformed plants. This was determined by analyzing the percentage of cell death in non-transformed tissues at different concentrations of hygromycin. The results of MIC experiments on the differentiation stages showed that serial concentrations of hygromycin, 200, 400, 600, 800 and 1000 µg l^{-1} induced 52.3%, 65.7%, 77.7%, 82.3%, and 100% cell death, respectively (**Fig. 3**). Based on the MIC results, non-stringent selection level at 200 µg l^{-1} hygromycin was suitable as the primary selection concentration in the differentiation stage. This was necessary to allow the putative transformed plants to undergo multiplication before further analyses. Although non-stringent selec-

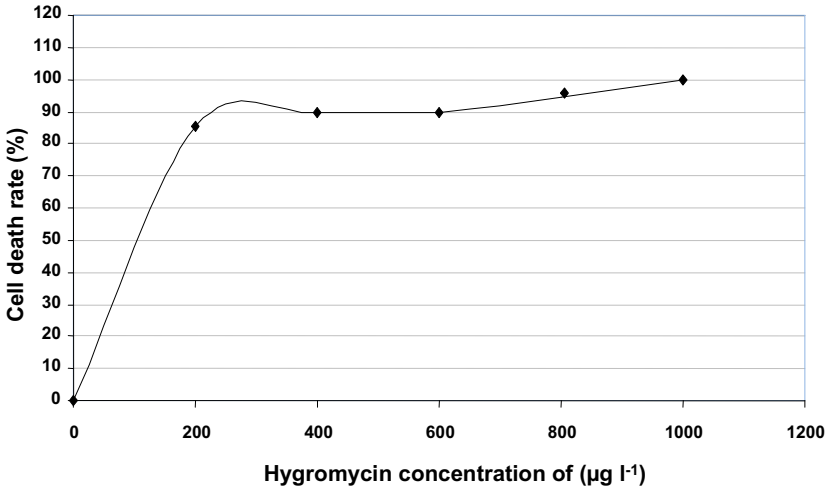

Fig. 4 Determination of minimal inhibitory concentration of hygromycin on regeneration media.

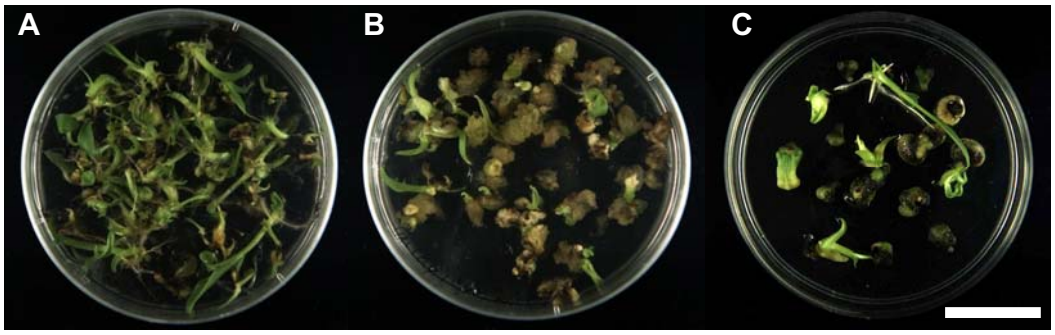

Fig. 5A-C The effects of hygromycin on plants differentiation and regeneration. (**A**) Control plants; (**B**) The effects of 200 µg l^{-1} of hygromycin on differentiated somatic embryos; (**C**) The effects of 200 µg l^{-1} of hygromycin on regenerated banana plantlets (bar: 3.0 cm).

tion might increase the regeneration of non-transformed plants, this could be overcome through frequent transfer to fresh medium containing less stringent selective agent with stringent selection re-introduced in subsequent culture medium (Manoharan and Dahleen 2002; Lee *et al*. 2006). Consequently, the transformed plants with low copy numbers will also have a greater chance of survival.

At the regeneration stage, 200, 400, 600, 800, and 1000 µg l^{-1} of hygromycin induced 85.7%, 90.0%, 90.0% and 94.4% cell death respectively (**Fig. 4**). These results showed that most non-transformed banana plantlets at the regeneration stage are highly sensitive to hygromycin (**Fig. 5**), where 200 µg l^{-1} of hygromycin is considered as a low antibiotic concentration compared to the standard concentrations used for putative transformed cell selection (Blochlinger and Diggelmann 1984) and also as recommended by the Sigma Handbook 2006-2007 (Sigma, USA) i.e. 150-400 µg ml^{-1} for higher eukaryotes. It was suggested that the incorporation of hygromycin in the regeneration medium could increase the selection pressure to non-transformed banana plants. Ebinuma *et al*. (2001) reported that identification and separation of transgenic and non-transgenic plants were the critical steps during antibiotic selection because the dying cells would inhibit the nutrient uptake of transgenic plants and also excrete undefined toxic compounds which could impede the growth of transgenic plants. Therefore, in the transformation experiment, 200 µg l^{-1} hygromycin was used in the selection medium to allow for stringent selection of untransformed plants at the regeneration stage. The rapid transfer of cultures to fresh medium was complementary to allow for complete selection of putative transformed plants.

Co-cultivation of embryogenic cell suspension with *A. tumefaciens*

The efficiency of *Agrobacterium*-mediated transformation depends on several factors, such as plant material (May *et al*. 1995; Ganapathi *et al*. 2001), bacterial cell density (Pi-

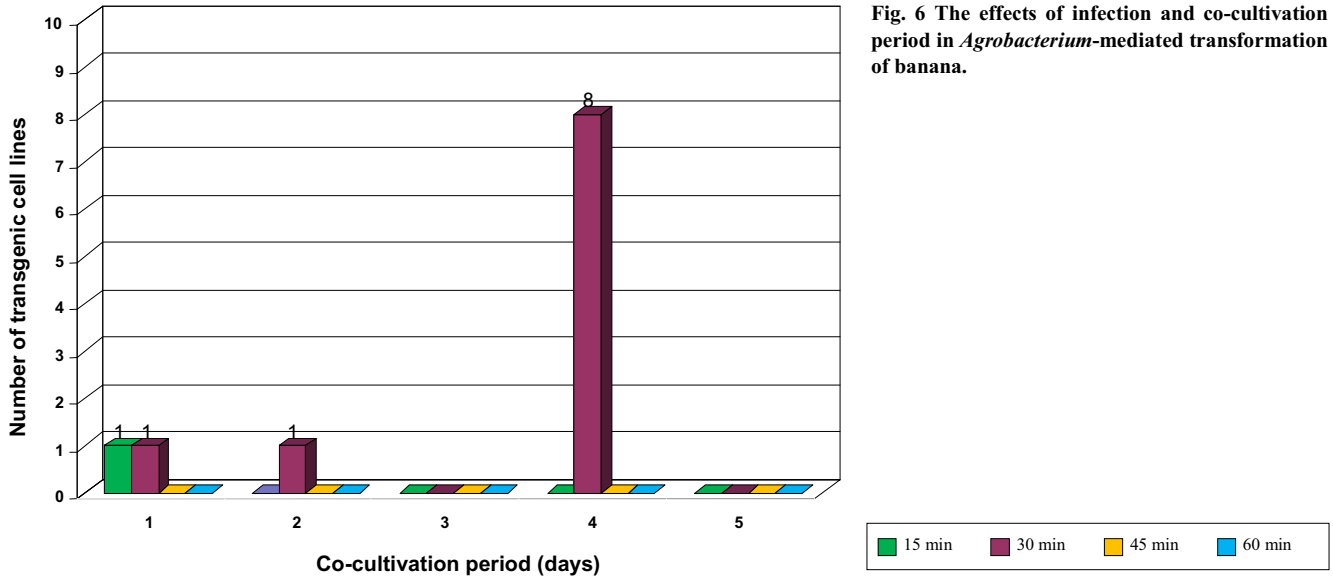

Fig. 6 The effects of infection and co-cultivation period in *Agrobacterium*-mediated transformation of banana.

Table 3(A) Quantitation data of Real-Time PCR for sample number 151(4), 301(1), 302(1), 304(1) and 304(2). This report generated by Rotor-Gene Real-Time Analysis Software (C) Corbett Research 2000.

No.	Colour	Name	Type	Given Conc. (copies/ul)	Calculated Conc. (copies/ul)	% Variation	Ct	Ct Std. Dev.
1		1	Standard	61,200,000.	70,490,432.	15.18%	17.23	0.01
2		2	Standard	61,200,000.	69,991,583.	14.37%	17.24	0.01
3		3	Standard	61,200,000.	69,496,264.	13.56%	17.25	0.01
4		4	Standard	6,120,000.	5,351,906.	12.55%	20.86	0.23
5		5	Standard	6,120,000.	6,391,715.	4.44%	20.61	0.23
6		6	Standard	6,120,000.	4,643,242.	24.13%	21.06	0.23
7		7	Standard	612,000.	461,748.	24.55%	24.31	0.27
8		8	Standard	612,000.	663,296.	8.38%	23.8	0.27
9		9	Standard	612,000.	499,267.	18.42%	24.2	0.27
10		10	Standard	61,200.	65,962.	7.78%	27.05	0.04
11		11	Standard	61,200.	65,495.	7.02%	27.06	0.04
12		12	Standard	61,200.	62,763.	2.55%	27.12	0.04
13		13	Standard	6,120.	6,560.	7.18%	30.3	0.03
14		14	Standard	6,120.	6,653.	8.72%	30.28	0.03
15		15	Standard	6,120.	6,421.	4.92%	30.33	0.03
16		151(4)	Sample		65,657,841.		17.33	
17		151(4)	Sample		62,031,423.		17.41	
18		151(4)	Sample		43,490,448.		17.91	
19		301(1)	Sample		5,745,821.		20.76	
20		301(1)	Sample		4,545,360.		21.09	
21		301(1)	Sample		7,367,236.		20.41	
22		302(1)	Sample		12,198,149.		19.7	
23		302(1)	Sample		15,529,665.		19.36	
24		302(1)	Sample		12,819,894.		19.63	
25		304(1)	Sample		425,081,842.		14.7	
26		304(1)	Sample		304,444,241.		15.17	
27		304(1)	Sample		462,897,506.		14.58	
28		304(2)	Sample		9,116,650.		20.11	
29		304(2)	Sample		15,419,764.		19.37	
30		304(2)	Sample		11,122,369.		19.83	

ñeda *et al*. 2002), infection and co-cultivation period (Mondal *et al*. 2001; Piñeda *et al*. 2002), the vector system (Gelvin 2000; Mondal *et al*. 2001; Tang 2003), selective agent (Lessard *et al.* 2002) and culture conditions (Schnurr and Guerra 2000; Zambre *et al*. 2003). In this study, banana embryogenic cell suspension was used as the plant material for transformation. The embryogenic cell suspension maintained in high 2,4-D medium (M2 medium) will divide rapidly to produce a large number of competent cells and encourages loosening of cell walls which will facilitate the transfer of the T-DNA strand into the plant genome (Lee *et al*. 2006; Wong *et al*. 2006). For explant infection, an OD_{550} of 0.5 that produced a concentration of *Agrobacterium* of 10^7/ml was used to infect the embryogenic cell suspension with co-cultivation period between 1 to 5 days. The results indicated that a high efficiency of transformation was

Table 3(B) Quantitation data of Real-Time PCR for sample number 304(3), 304(4), 304(7), 304(11), 304(12) and 304(13). This report generated by Rotor-Gene Real-Time Analysis Software (C) Corbett Research 2000.

No.	Colour	Name	Type	Given Conc. (copies/ul)	Calculated Conc. (copies/ul)	% Variation	Ct	Ct Std. Dev.
1		1	Standard	61,200,000.	82,204,221.	34.32%	17	0.39
2		2	Standard	61,200,000.	48,073,737.	21.45%	17.77	0.39
3		3	Standard	61,200,000.	67,635,055.	10.51%	17.28	0.39
4		4	Standard	6,120,000.	7,276,413.	18.90%	20.48	0.29
5		5	Standard	6,120,000.	6,508,855.	6.35%	20.64	0.29
6		6	Standard	6,120,000.	4,925,755.	19.51%	21.04	0.29
7		7	Standard	612,000.	484,042.	20.91%	24.37	0.37
8		8	Standard	612,000.	436,010.	28.76%	24.52	0.37
9		9	Standard	612,000.	710,071.	16.02%	23.82	0.37
10		10	Standard	61,200.	46,907.	23.35%	27.72	0.38
11		11	Standard	61,200.	54,298.	11.28%	27.51	0.38
12		12	Standard	61,200.	78,005.	27.46%	26.99	0.38
13		13	Standard	6,120.	8,750.	42.98%	30.13	0.36
14		14	Standard	6,120.	5,336.	12.81%	30.84	0.36
15		15	Standard	6,120.	7,301.	19.29%	30.39	0.36
16		304(3)	Sample		8,600,752.		20.24	
17		304(3)	Sample		11,129,890.		19.87	
18		304(3)	Sample		8,481,738.		20.26	
19		304(4)	Sample		3,941,371.		21.36	
20		304(4)	Sample		2,880,624.		21.81	
21		304(4)	Sample		3,575,084.		21.5	
22		304(7)	Sample		4,375,567.		21.21	
23		304(7)	Sample		4,499,223.		21.17	
24		304(7)	Sample		4,530,679.		21.16	
25		304(11)	Sample		13,621,939.		19.58	
26		304(11)	Sample		14,104,832.		19.53	
27		304(11)	Sample		12,616,973.		19.69	
28		304(12)	Sample		10,899,673.		19.9	
29		304(12)	Sample		7,910,918.		20.36	
30		304(12)	Sample		10,526,512.		19.95	
31		304(13)	Sample		7,966,227.		20.35	
32		304(13)	Sample		12,973,536.		19.65	
33		304(13)	Sample		10,095,544.		20.01	

achieved after 30 min infection with *Agrobacterium* followed by 4 days co-cultivation in darkness at 30 ± 1°C (**Fig. 6**). It was also observed that infection time of more than 30 min and a co-cultivation period of longer than 4 days will immediately kill the suspension cells. This may be due to sensitivity of the suspension cells to prolonged exposure to

a high temperature (30 ± 1°C) as the viable cell suspensions were constantly maintained in 25 ± 1°C (Jalil *et al*. 2003; Wong *et al*. 2006). Moreover, the cell death of the suspension culture could be attributed to the presence of high inoculums of bacterial cells within a longer co-cultivation period, which would repress the viability and competence of plant cells for infection by *A. tumefaciens* (Gelvin 2000; Khanna *et al*. 2004).

Verification of *SOC1* transgene in putatively transformed *in vitro* banana plants

With the development of a new transformation strategy, a total of 11 banana transgenic cell lines were generated (**Fig. 6**). Based on the *in vitro* transgenic plants produced, there were no observable differences in the morphology of one month *in vitro* non-transformed (control plants) and transformed plants (**Fig. 7**). The integration of the *SOC1* gene into the regenerated plantlets was confirmed at the early developmental stage by PCR amplification of a fragment size of 671 bp (**Fig. 8**) and by Southern blot. Both Southern blot analysis of *Bam*HI digested (**Fig. 9A**) and undigested DNA samples (**Fig. 9B**) also strongly indicate the integration of the *SOC1* transgene in the putative transformed plants. Southern blot analysis of 11 transformation events confirmed the integration of the *SOC1* transgene into the plant genome. The putative transgenic plant total genomic DNA digested with *Bam*H1 revealed specific single cutting sites on the target T-strand which generated the faint hybridization signals at above 10 kb (**Fig. 9A**), whereas, Southern blotting of 5 undigested transgenic plant genomic DNA produced clear hybridization signals (**Fig. 9B**). However, we failed to determine the copy number in the transgenic plants using restriction enzyme (RE) *cum* Southern blot technique due to the limited amount of high quality DNA. Additionally, dedicated two single cutting RE of *SOC1* sequence, *Cla*I (cuts position: 253/255 bp) and *Sac*II (cuts position: 209/207 bp) were also selected for RE digestion. However the hybridization signal could not be unambiguously detected in this study.

Real-Time PCR was also used to study the integration of the *SOC1* transgene in putative transformed plants (**Fig. 10A, 10B**). This is a convenient technique compared to Southern hybridization for the assessment of transgene integration levels if the sample available is limited (Bubner and Baldwin 2004; Bubner *et al*. 2004). The TaqMan dual-labeled hybridization probe was designed as 5′FAM- ATCG AATCCCTTGAAGTTTCCAAAC-3′Tamra. The donor fluorophore and quencher are fixed to the opposite ends of the probe oligonucleotide which anneals to the target sequences of the *SOC1* transgene. During PCR, the exonuclease activity of *Taq* polymerase degrades the probe oligo, freeing the fluorophore from the quencher and the fluorescence can be detected.

In this experiment, the Real-Time quantitative results also indicated the presence of *SOC1* transgene in the genomic DNA extracted from eleven transgenic cell lines (**Fig. 6** and **Table 3**). These results further confirmed the integration of *SOC1* transgene into the putative transformed plants which complements the Southern blot analysis. For this particular transgene absolute quantification assay i.e., concentration of the transgene molecules per 1 µl sample could be estimated using a 10-fold titration curve of plasmid pCAMBIA/SOC1 as the standard in Real-Time PCR analysis (**Table 3A, 3B**). The highest numbers of transgenic plants were produced using 30 minute infection times followed by 4 days co-cultivation to allow for the stable integration of the T-DNA into the banana plant genome. The concentration of these eight transgenic samples were sample number 304(1), $3.97 \pm 0.83 \times 10^8$ molecules/µl; 304(2), $1.19 \pm 0.32 \times 10^7$ molecules/µl; 304(3); $9.40 \pm 1.50 \times 10^6$ molecules/µl; 304(4), $3.47 \pm 0.54 \times 10^6$ molecules/µl; 304(7), $4.47 \pm 0.082 \times 10^6$ molecules/µl; 304(11), $1.34 \pm 0.076 \times 10^7$ molecules/µl; 304(12), $9.78 \pm 1.63 \times 10^6$ molecules/µl and 304(13), $1.03 \pm 0.25 \times 10^7$ molecules/µl. The above parameters were considered as the most optimum parameter for *Agrobacterium*-mediated transformation in this study. On the other hand, lower transformation efficiency was observed when 15 minutes *Agrobacterium* infection time was used followed by 1 day co-cultivation [sample number 151(4) with concentration of $5.71 \pm 1.19 \times 10^7$ molecules/µl]. Additionally, the parameters of 1 and 2 days co-cultivation following 30 minutes infection time also gave lower numbers of transgenic plants. These were designated as samples 301(1) and 302(1), which had the concentrations of $5.89 \pm 1.42 \times 10^6$ and $1.35 \pm 0.18 \times 10^7$ molecules/µl, respectively.

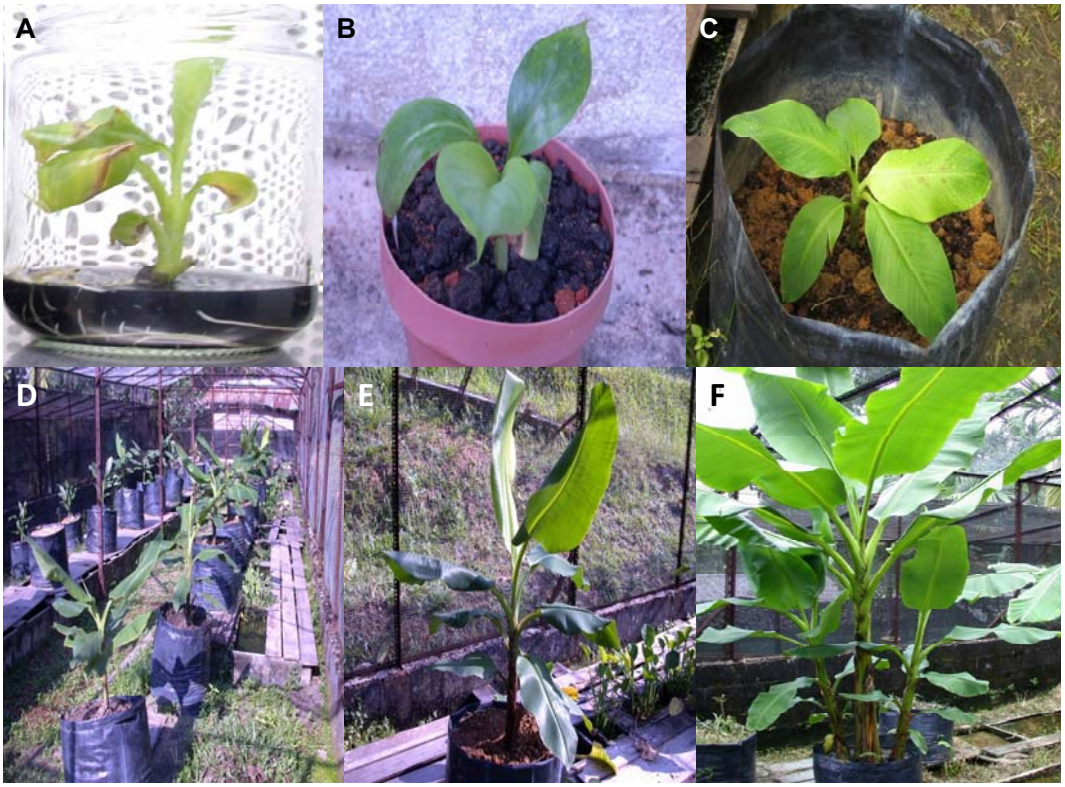

Fig. 7 Phenotypic observation on the effect of *SOC1* transgene in the putative transgenic plants. (**A**) *In vitro* transgenic plant; (**B**) Acclimatization of transgenic plant; (**C**) Transgenic plant was transferred to a large soil-filled polybag with no contact with the bare ground; (**D**) Three-months old putative transgenic plants were planted in a gated nursery; (**E**) Seven-months old putative transgenic plants; (**F**) Seven-months old wild-type banana cv. 'Mas' plant (control) with it's daughter plants in a large polybag.

Fig. 8 PCR amplification of the 671 bp fragment of the *SOC1* gene. M: 100 bp DNA marker. B: blank. Lanes 1-4, 7, 11-13 show the presence of the *SOC1* gene in independently transformed plants derived from a 30 min infection period and co-cultivated for 4 days. Lane 5-6, 8-9 and 10: non-transformed plants.

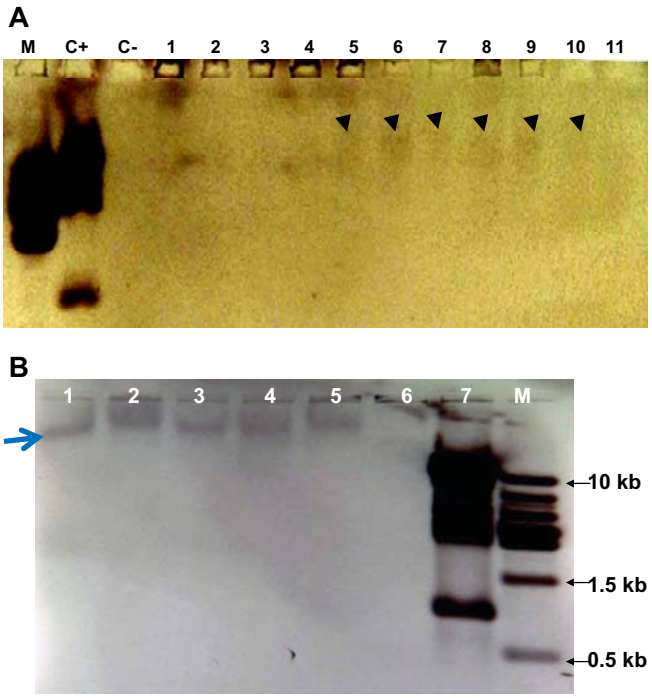

Fig. 9A-B Southern blotting of RE-digested and undigested genomic DNA. (**A**) Southern blot analysis of RE digested genomic DNA isolated from putative transformed plants. The arrows show the positive signal for the *SOC1* transgene. M: 100 bp ladder (Fermentas, US); C+: *Bam*H1 digested plasmid DNA (positive control); C-: non-transformed plant (negative control); Lane1-11: *Bam*H1 digested genomic DNA; (**B**) Southern blot analysis of undigested genomic DNA. The blue coloured arrow indicates the positive signal of *SOC1* transgene. Lane 1-5: Undigested genomic DNA extracted from transgenic nursery grown transformed plants; Lane 6: Non-transformed plant (negative control); Lane 7: *Bam*H1 digested plasmid DNA (positive control); M: 1kb DNA marker.

Agrobacterium-mediated transformation using a low-antibiotic liquid washing-assisted approach

Several reports have shown that the major drawback of *Agrobacterium*-mediated transformation was not the gene delivery method itself but the recalcitrance of the plants especially for monocotyledons, which are not the natural host of *Agrobacterium*. Another significant limitation of this transformation method appeared to be its genotype-dependence in monocotyledonous crops where the success in one cultivar might not be repeated on another cultivar (Murray *et al*. 2004). Additionally, an extensive regeneration phase for cell suspensions and inefficient plant recovery on selective medium might further reduce the availability of embryogenesis-competent cells for transformation (Strosse *et al*. 2006).

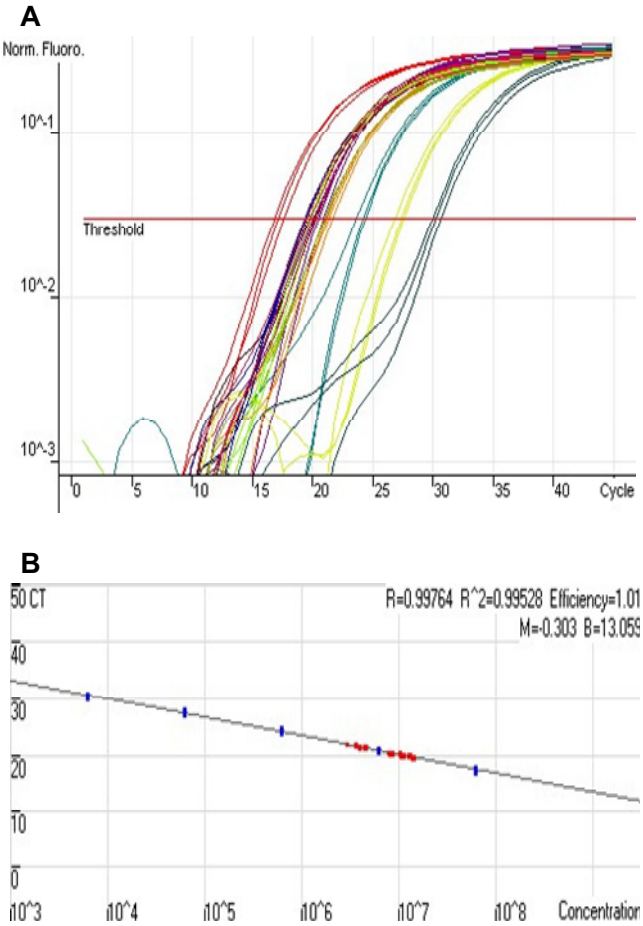

Fig. 10A-B Verification of *SOC1* transgene using Real-Time PCR (Rotor Gene, Corbett Research). (**A**) Quantitation data for Cycling A.FAM/Sybr; (**B**) Standard dilution curve of target gene (pCAMBIA/SOC1, blue spot) and the presence of target gene (*SOC1*, red spot) in genomic DNA extracted from putative transformed plants. (Data was analysed using Rotor-Gene Real-Time analysis software© Corbett Research 2000).

In this experiment, higher transformation efficiency in 'Mas' variety was obtained from 30 minutes infection with *Agrobacterium* strain LBA4404 carrying a pCAMBIA/SOC1 plasmid, followed by 4 days co-cultivation in MSOL

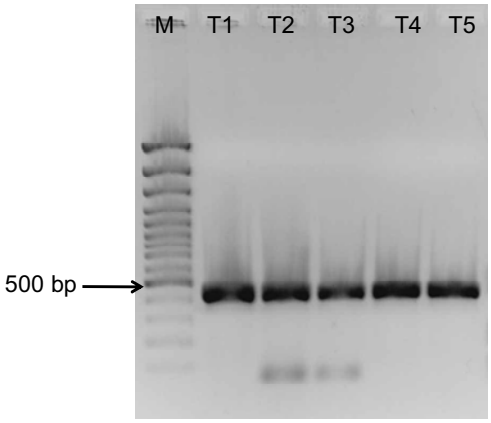

Fig. 11 RT-PCR analysis in leaf samples from transgenic plants (T1, T2, T3, T4 and T5) over-expressing *SOC1* transgene. The initial RT-PCR analysis revealed that *Elaeis guineensis SOC1* transgene by a new set of primers which specify the partial cDNA sequence of the *SOC1* transgene which was designed with a product length of about 440 bp including the *E. guineensis SOC1* C-terminal domain sequence. M: GeneRuler™ 100 bp DNA ladder.

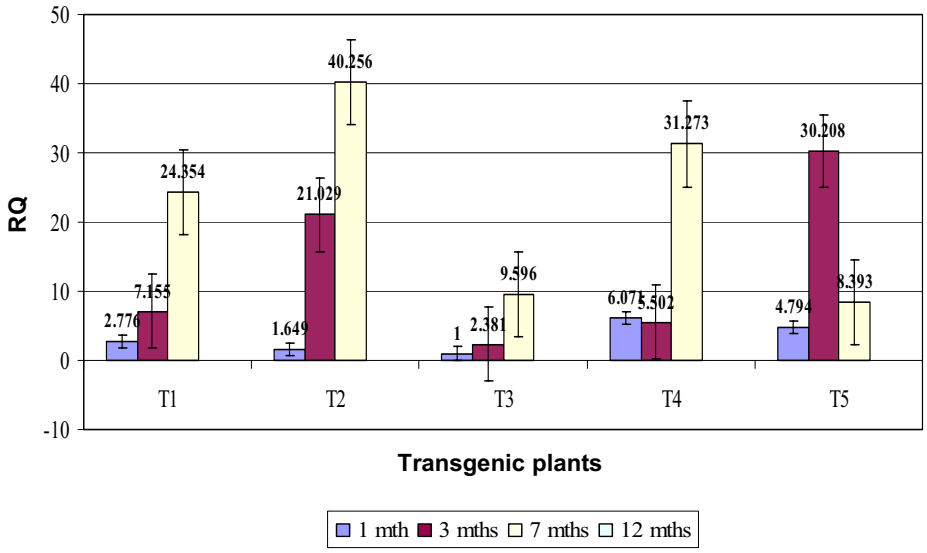

Fig. 12 Expression profile of *SOC1* transgene (440 bp) in transgenic banana plants during different development time. Relative expression levels of *SOC1* of 5 transgenic plants were calculated by using comparative $\Delta\Delta C_T$ method according to ABI 7500 System Sequence Detection software v1.2 (Applied Biosystem, US). RQ**:** Fold difference in gene expression.

medium. Custom suspension subculturing was done after 4 days of co-cultivation, in which co-cultivated cells were transferred to fresh MSOL substantiated with 50 mg l^{-1} cefotaxime to eliminate the *Agrobacterium* (2 days incubation in similar shaking conditions). Uniform dispersion of cefotaxime could completely eliminate the *Agrobacterium* because the plant cells were equally exposed to the cefotaxime during this washing process. Subsequently, the cell cultures were transferred to antibiotic-free MSOL media until the formation of immature embryos. This strategy significantly helped to avoid bacterial growth while promoting the growth of putative transformed cells. On the other hand, as there is no requirement of excess or continuous addition of *Agrobacterium*-eliminating antibiotic in the following hygromycin selective medium this may further reduce *in vitro* antibiotic selection pressure.

The transformation efficiency in this study was not directly comparable to published results (Hernandez *et al*. 1999; Pérez 2000; Ganapathi *et al*. 2001; Piñeda *et al*. 2002; Khanna *et al*. 2004) as the former studies did not investigate transformation using similar parameters. However, different factors were found to increase banana *Agrobacterium*-mediated transformation frequency significantly, including plant material cell-type and regeneration of genotype-dependent embryogenic cell suspension (May *et al.* 1995; Ganapathi *et al*. 2001); appropriate inoculation or cell density of *Agrobacterium* (Piñeda *et al*. 2002); the presence of acetosyringone or other surfactants e.g. Pluronic F68 (Hernandez *et* al. 1999; Piñeda *et al*. 2002; Khanna *et al*. 2004); physical conditions and media used for the transformation procedure (Khanna *et al*. 2004); *Agrobacterium* strain and plasmid construct (Khanna *et al*. 2004); the effects of heat shock and programmed cell death in banana (Khanna *et al*. 2004). In this present study, we demonstrated that a low-antibiotic liquid washing-assisted protocol by modifying Ganapathi's (2001) co-cultivation and post-cultivation steps potentiated the generation of transgenic banana plants.

In conclusion, an alternative strategy using a low-antibiotic liquid washing-assisted protocol during the antibiotic selection process that potentially reduces the environmental stress factor by reducing exposure time to the antibiotic. This is thought to be a significant limiting factor in the generation of transgenic banana plants. The functionality and expression profiles of the *SOC1* transgene driven by the *CaMV35S* promoter has been analysed using RT-PCR (**Fig. 11**) and Real-Time RT-PCR (**Fig. 12**, data were inconclusive). In order to characterize the temporal expression pattern of the *SOC1* single MADS-box transgene and its effects on multiple *Musa* endogenous MADS-box genes, RT-PCR was first conducted to confirm that there was transcript expression before subsequent Real-Time relative quantification. Total RNA from the transgenic banana plants were collected from the acclimatization stage and plants at three and seven months after transferring to the contained transgenic nursery (**Fig. 7D, 7E**).

Relative expression levels of the *SOC1* transgene in the five transgenic plants were calculated from the results obtained by real-time RT-PCR using the comparative $\Delta\Delta C_T$ method. The expression level was initially low, the transcript levels increased gradually in 3 month-old transgenic plants to be the 2.381-30.208 fold of difference. In 7 month-old transgenic clones, the levels reached between 8.393-40.256 fold of differences relative to calibrator. In general, expression of *SOC1* appeared to increase over the growth period from acclimatization stage (1 month) to matured plant development stage (7 months) (**Fig. 12**). However, the Real-Time relative quantitative analysis also showed that the expression patterns differed considerately between individual transgenic plants at the different plant development stages, even though the transgene was the same and was introduced under similar transformation conditions. This may be a consequence of the random insertion of the transgene and its positional effect in the plant genome which may influence the developmental patterns of the transgene or a reflection of copy numbers.

The induction of flowering in plants is an important process in its reproductive strategy, in particular, for monocarpic plants which flower only once in their lifecycle (Komeda 2004). Such information can lead to a mechanistic understanding of plant growth which is of fundamental importance in plant biology and opens up possibilities of crop improvement via molecular approaches. An efficient transformation protocol such as that proposed in this study can facilitate further functional genomic study of this process in banana.

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