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Transfer of β-1,3-glucanase Gene into Banana for Tolerance to Fusarium Wilt Disease Race 1 using *Agrobacterium*-mediated Transformation System

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ABSTRACT

Agrobacterium-mediated transformation of single buds *in vitro* grown banana cv. 'Rastali' (AAB) was done using the binary vector pROKla-*Eg*, harboring the soybean endo β-1,3-glucanase gene (*Eg*). Plasmid pROKla-*Eg* contained a neomycin phosphotransferase gene (*npt*II) as the selectable marker to identify the transformants. Treatment A contained kanamycin (kan) at 100 mgl^{-1} and treatment B contained geneticin G-418 at 50 mgl^{-1} in both MS medium supplemented 5 mgl^{-1} of BAP together with 2.7 g of gelrite agar. Single buds derived from multiple bud clumps, were the target explants for transformation. An assay was performed to identify the minimum concentration required for two antibiotics (carbenicillin and cefotaxime) that was most effective against *Agrobacterium* strains, LBA 4404, and the effect on tissue regeneration capacity. Even though the transformation frequency based on kan selection medium (treatment A) was higher, no transformants could be confirmed based on PCR and Southern blot analyses, compared to the use of geneticin (G-418) selection medium (treatment B). These results suggested that the use of G-418 as a selection agent is preferable to kan due to the lower concentration required to allow for the small numbers of putative transgenic cells in a large population of non–transformed ones to undergo multiplication and also reduced the occurrence of chimeras. The transgenic banana plantlets were inoculated with 2×10^6 spores ml^{-1} conidial suspension of *Fusarium oxysporum* f. sp. *cubense* (race 1) to evaluate the degree of tolerance and to investigate the effectiveness of the bioassay system as a potential tool for early screening. An assay of protein extract from the transgenic plantlets showed a significant increase in EG enzyme activity over the untransformed plantlets. The present *Agrobacterium*-mediated transformation protocol reported here is suitable for the use of tiny meristem tissues to obtain fungal disease tolerant or resistant banana through genetic engineering.

Keywords: β-1,3-glucanase gene, disease tolerance, Fusarium wilt, selection agents, transgenic banana
Abbreviations: AS, acetosyringone; **BAP**, 6-benzylaminopurine; **carb**, carbenicillin; **cef**, cefotaxime; ***Eg***, endoglucanase; **kan**, kanamycin; **MBC**, multiple bud clump; **MS**, Murashige and Skoog

INTRODUCTION

Genetic engineering offers a new method to improve disease resistance or tolerance via transforming a single gene or several genes of interest into target plants. *Agrobacterium*-mediated transformation transfers a defined segment of T-DNA from *Agrobacterium* spp., e.g. *Agrobacterium tumefaciens* and integrates it into the target plant genome precisely.

In the past, *Musa* species were considered outside the host range of *Agrobacterium*, but an increasing amount of research reports indicates that banana can be transferred by *Agrobacterium tumefaciens* (May *et al*. 1995; Ganapathi *et al*. 2001; Khanna *et al*. 2004; Sreeramanan *et al*. 2006; Wong *et al*. 2008). May *et al*. (1995) reported transformation of meristems and corm slices from 'Grand naine' (AAA). Ganapathi *et al*. (2001) successfully transformed 'Rastali' (AAB) by using embryogenic cell suspensions as a starting material. A total of 200 putative transformants were recovered, of which a set of 16 was tested by histochemical analysis for stable GUS expression and by Southern blot analysis. Therefore, the controversy over the susceptibility of banana plants for transformation using *A. tumefaciens* appears to be solved for a continuously increasing number of transgenic monocotyledonous plant species.

The rationale for selecting 'Rastali' in this study was based on the following criteria: (i) it is an excellent cultivar, having been one of the most important commercial cultivars used in Malaysia from the 1970s to the 1990s and due to severe Fusarium wilt race 1 outbreak in 1992, it was replaced by other, more tolerant cultivars such as Cavendish group banana cultivars; (ii) it shows a susceptibility to other types of fungal diseases such as Anthracnose disease, a condition that makes it very suitable for the evaluation of resistance/tolerance enhancement effects; (iii) a successful protocol was developed and optimized in our laboratory for the transformation of this important banana cultivar (Sreeramanan *et al*. 2006b).

This paper describes a series of experiments that were carried out by using *A. tumefaciens*, LBA 4404 which contained plasmid with the endo β-1,3-glucanase (E.C. 3.2.1.39) (*Eg*) gene (pROKla-*Eg*). A comparison between the use of kanamycin (kan) and geneticin (G-418) as selection agents were demonstrated in this study since both plasmids contained the neomycin phosphotransferase II (*npt*II) gene. The inhibition of single buds and the elimination of *A. tumefaciens* growth by carbenicillin (carb) and cefotaxime (cef) were demonstrated by using different concentrations of both antibiotics.

Generally, it is known that plants have defense systems which involve pathogenesis-related proteins such as EG (Nakamura *et al*. 1999; Jin *et al*. 2007), whose expression

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could be induced by fungal elicitors, ethylene, salicylic acid, wounding and other chemical inducers. EG enzyme catalyzes the hydrolysis of β-1,4 linkages of the *N*-acetyl-D-glucosamine polymer β-1,3-glucan, which is a component of mycelial cell walls. Thus, soybean EG appears to be a key enzyme involved in the generation of an elicitor signal to activate a disease resistance programme in the plant defense system. Yoshikawa *et al*. (1993) reported that transgenic tobacco (*Nicotiana tabacum*) plants expressing the soybean *Eg* gene exhibit high levels of resistance to fungal disease. In addition, Nakamura *et al*. (1999) produced transgenic kiwi fruit (*Actinidia chinensis*) engineered with *Eg* with increased resistance to *Botrytis cinerea* infections. Similarly, transgenic potato (*Solanum tuberosum*) expressing soybean *Eg* gene exhibited increased resistance to *Phytophthora infestans* (Borkowska *et al*. 1998). Recently, Jin *et al*. (2007) reported that a novel tissue-specific plantain (*Musa paradisica*) *Eg* gene was regulated in response to infection by *Fusarium oxysporum* f. sp. *cubense* (race 4). We have reported previously on the development of a bioassay method for testing Fusarium wilt disease tolerance in transgenic banana ('Rastali') by relating disease severity to the level of production of various plant compounds and enzymes (Sreeramanan *et al*. 2006c).

Currently, most genetic transformation protocols for banana use cell suspensions. However, establishing cell suspensions is a lengthy process and is cultivar dependent (Tripathi *et al*. 2008). In this study, we have developed an efficient *Agrobacterium*-mediated transformation method of for banana 'Rastali' with *Eg* using single buds (tiny meristem tissues) and demonstrated increased disease tolerance to the fungal pathogen *F. oxysporum* f. sp. *cubense* (race 1; VCG 01217). It has been proved that synergistic expression of plant defense genes is a preferred approach to protect crops against pathogen infection. To our knowledge, there is no previous report of banana being transformed with the *Eg* gene via *Agrobacterium*-mediated transformation system.

MATERIALS AND METHODS

Plant material and culture conditions

Banana 'Rastali' (AAB) was chosen as the plant transformation material. Corm slices (10 mm diameter) of 4 weeks old from *in vitro* banana plantlets obtained at 2nd generation from the suckers were cultured in MS medium (Murashige and Skoog 1962) supplemented with 10 mgl^{-1} of 6-benzylaminopurine (BAP; 99%, R&M, UK) to obtain multiple bud clumps (MBCs) after 6 weeks. The cultures were incubated at 25 ± 2°C in a 16-h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 $\mu mol\ m^{-2}\ s^{-1}$. Single buds (3 mm), excised from MBCs, were used for this study.

Bacterial strain, plasmid and culture conditions

Disarmed *A. tumefaciens* strain LBA 4404 (Hoekema *et al*. 1983) and the plant transformation plasmid harboring a binary vector pROKla-*Eg* (Yoshikawa *et al*. 1993) which originated in plasmid BIN 19 were used to introduce the soybean *Eg* gene into banana. The plasmid pROKla-*Eg* contains the *npt*II as a selective marker gene was linked to the nopaline synthase (*nos*) gene promoter and the soybean *Eg* cDNA linked to the CaMV 35S (35S) promoter in the T-DNA region (**Fig. 1**). The soybean *Eg* gene was a gift from Dr. Yoji Takeuchi, Japan.

LBA 4404 (pROKla-*Eg*) was streaked on LB medium (R&M, UK) supplemented with 50 mgl^{-1} kan and grown for 3 day at 28°C to form colonies. Each single colony with a diameter of 1 mm was picked out and cultured in 20 ml LB liquid containing the same antibiotic and the culture was allowed to shake at 28°C and 120 rpm for 20 h. After adjusting the optical density to 0.6 units at 600 nm (OD_{600}), the bacterium culture was used for transformation.

Effects of antibiotics on plant regeneration and bacterial suppression

For explant toxicity tests, single buds of 3 mm in size were transferred to MS soilid medium containing 5 mgl^{-1} of BAP and different concentrations of co-added carb and cef (0, 100, 200, 300, 400, 500 and 600 mgl^{-1}). After 30 min of co-cultivation of explants (100 single buds) in 20 ml *Agrobacterium* suspension culture, the percentage survival (easily observed through blackening or necrosis) of single buds and *A. tumefaciens* growth (number of single buds contamination with *Agrobacterium*) were determined within three days following the application of various concentrations of carb and cef.

Genetic transformation and plantlet regeneration

The protocol is described in shown in **Fig. 2** and described in detail next. Single buds (~3 mm) were pre-cultured on solid MS medium with 5 mgl^{-1} BAP for three days prior to *Agrobacterium* infection. The explants were mildly injured by dabbing with tips of scalpel (size 4) 4 times. About 100 single buds were immersed in *Agrobacterium* suspension for 30 min together with an optimized (Sreeramanan *et al.* 2006a) acetosyringone (AS) concentration at 100 μM. AS is known to activate the *vir* genes of the Ti plasmid and to initiate the transfer of T-DNA (reviewed by Karami 2008). The explants were then blotted dry on sterile filter paper (Whatmann #1) and co-cultivated for 3 days on BAP-free MS medium (without $CaCl_2$ during co-cultivation) designated as M1 medium containing 100 μM of AS, 60 mM D-(+)-glucose, 2 mM sodium phosphate and 40 mgl^{-1} of L-cysteine using Petri dishes (9 mm diameter; 10 single buds per Petri dish) at 22 ± 2°C in the dark. After a 3-day co-cultivation period, the buds were transferred onto the same medium but without AS but containing 300 mgl^{-1} carb or 200 mgl^{-1} cef, which was designated as M2 medium for a period of 5 days and then transferred back onto the BAP-free MS liquid medium with the same antibiotic concentration for another 5 days. Then, the buds were transferred onto MS solid medium containing 10 mgl^{-1} BAP with 100 mgl^{-1} kan or 50 mgL^{-1} G-418 for 2 weeks and transferred again into liquid medium with the same treatments for 5 days in 100 ml conical flasks. During this period, dead buds were removed before subculture back on MS solid medium containing 10 mgl^{-1} BAP and 25 mgl^{-1} G-418. The transformed buds regenerated into MBCs. Single buds were separated from MBCs and subcultured onto solid MS medium with 1 mgl^{-1} BAP until single plants regenerated. All plants regenerated from each putatively independent transformed bud line were maintained *in vitro*.

DNA extraction and PCR analysis

Genomic DNA was extracted from putative transformants using an improved and modified CTAB method adopted from Pasakinskiene and Paplauskience (1999). Approximately 0.5–1.5 g of tissue was preheated at 60°C in 20 ml of CTAB isolation buffer [3% w/v CTAB, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mm EDTA, 100 mM Tris-HCl pH 8.0, 1% (w/v) polyvinylpyrollidone (PVP-40, Sigma)]. The extracts were ground with 0.2 g of PVP-40 in a chilled mortar and pestle then preheated with buffer in 50 ml polypropylene centrifuge tubes which were inverted for thorough mixing. Tubes were then incubated in a water bath at 60°C for 1-2 h, swirling vigorously every 10-15 min. These were allowed to cool to room temperature and chloroform: isoamyl alcohol (24:1

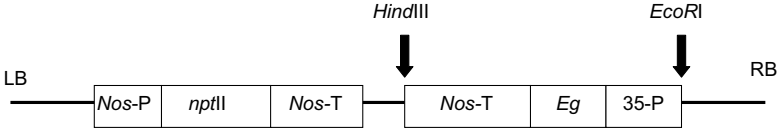

Fig. 1 Diagram of the binary vector T-DNA region of pROKla-*EG* used in the transformation experiment. RB, right border; LB, left border; *Nos*-P, nopaline synthase gene promoter; *npt*II, neomycin phosphotransferase gene; *Nos*-T, nopaline synthase gene terminator; 35-P, CaMV 35S promoter; EG, soybean β-1,3-glucanase cDNA.

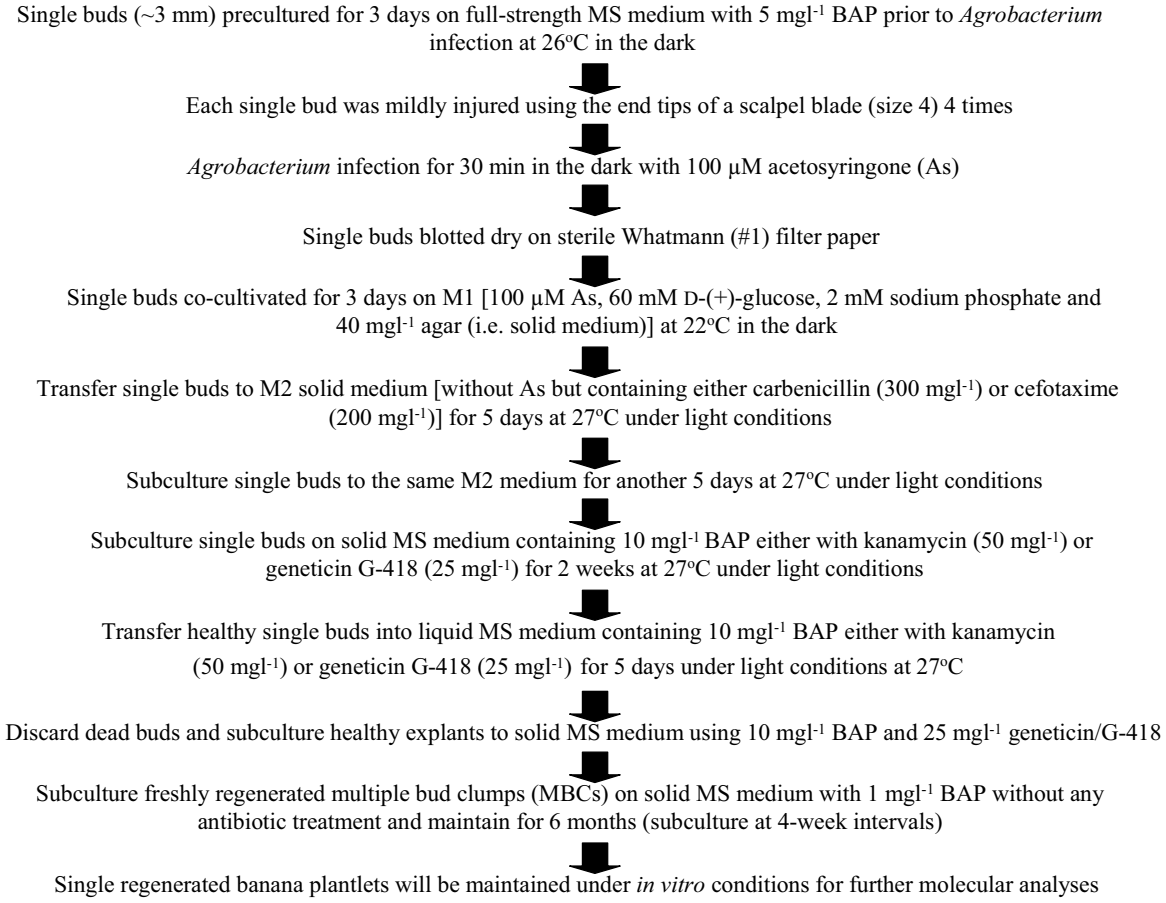

Fig. 2 Flow diagram showing various steps to selection of transgenic banana plants.

Table 1 Genes, primer, primer sequences and expected product length from PCR.

Genes	Primer	Sequence (5′-3′)	Product length (bp)
Eg	Forward	GATGTGATATCTCCACTGACGTAAG	830
	Reverse	GTATAATTGCGGGACTCTAAT	
*npt*II	Forward	CCCCTCGGTATCCAATTAGAG	900
	Reverse	CGGGGGGTGGCCGAAGAACTCCAC	

v/v) was added and mixed by inverting gently 15 to 20 times. Tubes were then centrifuged at 3000 rpm for 10 min at 28°C and the top aqueous phase was transferred with a wide-bore pipette to a clean 50 ml tube. Then, 2/3 volumes of cold isopropanol was added and gently mixed to precipitate the DNA and the tube was kept at 28°C for 30 min. This mixture was then centrifuged at 3000 rpm for 5 min and the supernatant decanted while the pellet was washed with 5 ml of wash buffer (76% ethanol, 10 mM ammonium acetate). The mixture was centrifuged again at 3000 rpm for 5 min and the pellet obtained was air-dried. The pellet was dissolved in 1000-15,000 µl of TE buffer (10 mM Tris-Hci, pH 7.5, 1 mM EDTA) and treated with 1 µl RNAse (10 mg.ml^{-1}, Sigma) per 100 µl DNA at 37°C for 30 min. 1/10 volume of 3 M sodium acetate and 2.5 vol of cold absolute ethanol was added and the tube kept at –20°C for 2 h. The tube was again centrifuged at 12,000 rpm for 5 min, the supernatant discarded and the pellet air-dried. The pellet was dissolved in an appropriate amount of TE buffer and the purity was assessed using a spectrophotometer at A_{260}/A_{280}. PCR was done on a DNA Thermal Cycler 480 (Perkin-Elmer). Both *Eg* and *npt*II genes were amplified by using a standard PCR protocol (Sambrook *et al*. 1989) using primers (Eg primer sequence obtained from Nakamura *et al.* (1999) and *npt*II primer sequence obtained from Ito *et al.* (1995)) specified in **Table 1**.

PCR amplifications were carried out in 100 µl reactions containing template DNA (500 ng genomic DNA or 60 ng plasmid DNA), 200 ng of each primer (forward and reverse), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 1X PCR buffer and 5U *Taq* DNA polymerase (MBI Fermentas). Amplification of *Eg* fragments was performed for 45 cycles at 94°C for 1 min, 40°C for 2 min and 72°C for 3 min (denaturation, annealing and primer extension), followed by 72°C for 7 min. Amplification of *npt*II gene fragments was performed for 35 cycles at 94°C for 1 min, 62°C for 1 min and 72°C for 2 min (denaturation, annealing and primer extension) followed by 72°C for 8 min. Amplified gene fragments were analyzed by electrophoresis in 1.2% Nusieve agarose gels visualized with ethidium bromide (0.5 µg.ml^{-1}).

Southern blot analyses

Genomic DNA (20 µg) from PCR positive transformants were subjected to digestion with *Hind*III in buffer H (Promega). Digestions were carried out for 2 h at 37°C in 20 µl final volumes. DNA fragments were separated by electrophoresis through a 1.0% agarose gel. A nonradioactive method was used to confirm stable integration of the *Eg* transgene in the host banana genome. DIG DNA Labeling and Detection Kit (Roche) were used in this study.

Fusarium oxysporum f. sp. *cubense* (race 1; VCG 01217) bioassay

Development of a *Fusarium* bioassay method in transgenic banana in this study was based on an improved method adopted in our previously published manuscript (Sreeramanan *et al*. 2006c). For plant inoculation bioassay protocol, untransformed banana 'Rastali' plantlet root sections were cut at 3 cm from the root tips. The plants were dipped into spore concentrations of 10^6 *Fusarium oxysporum* f. sp. *cubense* (race 1; VCG 01217) for 1 h and were then transplanted into a plastic container (8 cm × 8 cm) with sterile per-

lite. One ml (spore concentration = 10^6) was again inoculated near the root/stem regions for double confirmation. The total spores used were 2×10^6 spores ml^{-1}. The plantlets were watered using hormone- and sucrose-free MS liquid medium. The experiment was performed in three (3) replicates. Disease development and severity were monitored and measured over for a period of four weeks. Necrosis in new and old leaves tissue (yellowing) indicated Fusarium wilt symptoms occurred.

Extraction and determination of total acid soluble protein and β-1,3-glucanase enzyme activity

Tissue samples (0.25 g) were homogenised in 5 ml extraction buffer (0.1 M sodium buffer, 1 mM EDTA, 0.1% β-mercaptoethanol, pH 5.2), with 100 mg insoluble PVP-40. Protein was quantified by Bradford's (1976) method at λ = 595 nm. EG activity assay methods of Tonon *et al*. (1998) were applied in this experiment with a slight modification. EG enzyme activity determination was based on the rate of reducing sugar production using laminarin (Sigma) as the substrate. The reaction mixture consisted of 500 µl of 3.75 mg mL^{-1} laminarin in 0.3 M sodium acetate buffer, pH 5.0 and 1.0 ml of extract total acid soluble protein extract alone and substrate alone were used as control for each sample. The mixture was incubated at 37°C for 30 min and 1 ml alkaline copper reagent was added to stop the reaction. Reducing sugar was determined by using the Somogyi (1952) method. The mixture was heated at 100°C for 20 min in a water bath. When the mixture had reached room temperature, 1.0 ml of Nelson Somogyi reagent and 6.0 ml H_2O were added and mixed. The change of the colour was determined at λ = 510 nm. Three replications were applied. β-1,3-glucanase activity was expressed as nkat/mg protein with glucose as standard. The amount of enzyme catalyzing the formation of 1 nm glucose equivalent in 1 s under assay conditions is referred to as 1 nkat (Anfoka and Buchenauer 1997).

Statistical analysis

Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at 5% using SPSS 10.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Toxicity of carbenicillin and cefotaxime on banana single buds and *Agrobacterium-tumefaciens* growth

For effective *Agrobacterium*-mediated transformation, the antibiotic regime should control bacterial growth without inhibiting the regeneration of the plant tissues. Wong *et al.* (2008) reported that the lowest concentration of antibiotic preventing the appearance of turbidity was considered to be the minimal inhibitory concentration and the antibiotic considered as bacteriostatic in their work on the improvements in the efficiency of *Agrobacterium*-mediated transformation of embryogenic cell suspensions of banana. The effect of carb and cef on the percentage of single buds growth was evaluated after four weeks of culture on solid and liquid medium containing appropriate concentrations (**Figs. 3, 4**). No significantly differences were observed between the cultures when the percentage number of single buds growth was counted.

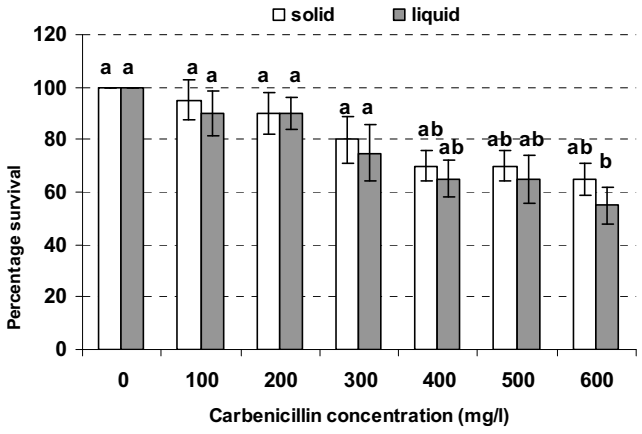

Fig. 3 Percentage of single buds growth obtained after four weeks in MS media using different carbenicillin concentration. Forty single buds 3 mm in size were plated onto 5 mgl^{-1} BAP-supplemented medium containing the antibiotic at various concentrations in both solid and liquid media. The data plotted were the means of four replicates. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

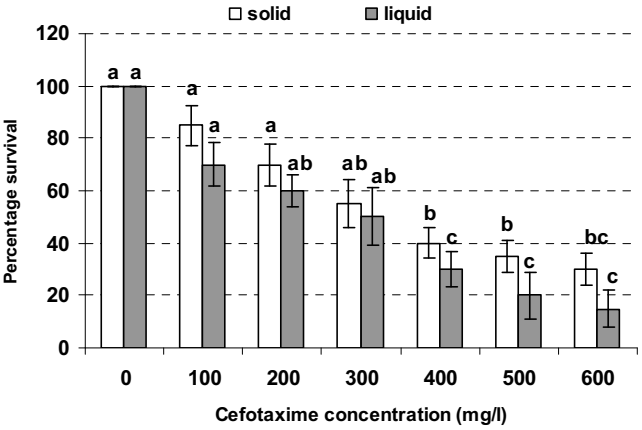

Fig. 4 Percentage of single bud growth obtained after four weeks in MS media using different cefotaxime concentrations. Forty single buds 3 mm in size were plated onto 5 mgl^{-1} BAP-supplemented medium containing the antibiotic at various concentrations in both solid and liquid media. The data plotted were the means of four replicates. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

However, by using 600 mgl^{-1} cef in solid medium reduced average growth from 100% (control) to 30%. The percentage of growth in liquid medium using the same concentration was 12% lower than on solid medium. In comparison to carb, using 600 mgl^{-1} reduced average growth to 62% in solid medium and 54% in liquid medium. The percentage growth was reduced to 8 and 12% in both solid and liquid media containing 150 mgl^{-1} carb, respectively. However in cef, the percentage growth dropped to 82% in solid medium and 63% in liquid medium using the same concentration, i.e. 600 mgl^{-1}.

A. tumefaciens strain LBA 4404 was consistently sensitive to the antibiotics tested. Using carb, the inhibition of cell growth started at 100 mgl^{-1} but complete inhibition occurred at 400 mgl^{-1} (**Fig. 5**). For cef, the inhibition of bacterial cell growth started at 100 mgl^{-1} and complete inhibition occurred at 200 mgl^{-1} (**Fig. 5**). These results demonstrate that *A. tumefaciens* LBA 4404 cells are more sensitive to cef than carb.

Previous studies showed that a higher concentration of *Agrobacterium* cells significantly increases the transformation efficiency of tobacco and *Arabidopsis thaliana* (Lin *et al*. 1994). Therefore, transformation with high concentrations of *A. tumefaciens* LBA 4404 is the preferred strain since it is easier to eliminate from the infected explants after co-cultivation period compared to other strains (Sreeramanan *et al.* 2006b).

Although cef is more effective in inhibiting *Agrobacterium* cell growth, carb is the antibiotic of choice in *Agrobacterium*–mediated transformation because cef has shown high toxicity in many different plant tissues (Antunez de Mayolo *et al.* 2003). In cacao, the addition of cef decreased somatic embryo production by 86% (Antunez de Mayolo *et al*. 2003). However, cef did not have a negative effect on

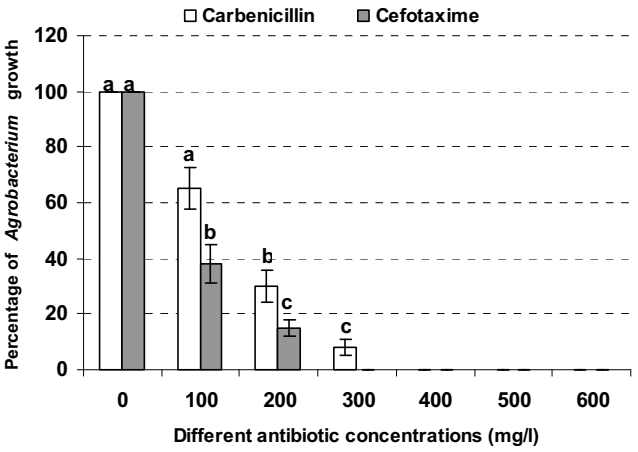

Fig. 5 Percentage of single buds with *Agrobacterium tumefaciens* LBA 4404 growth using different carbenicillin and cefotaxime concentrations. Forty single buds 3 mm in size were plated onto 5 mgl^{-1} BAP-supplemented medium containing both antibiotics at various concentrations in both solid and liquid media. The data plotted were the means of four replicates. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

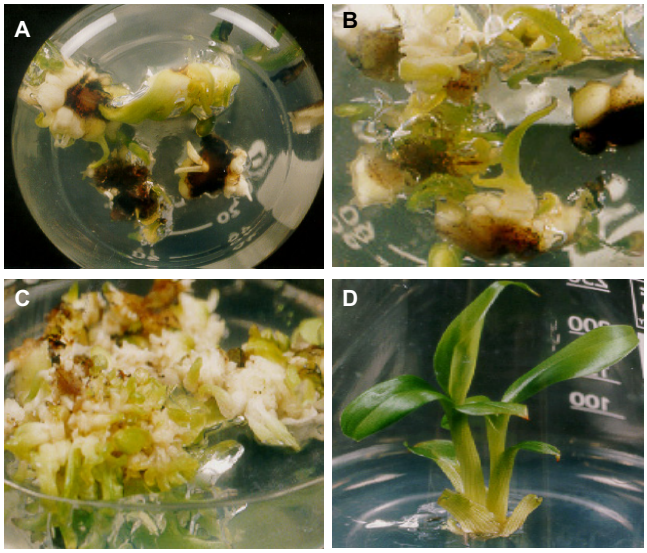

Fig. 6 Production of transgenic banana 'Rastali' (AAB). (**A**) Two-months old culture in geneticin G-418 selection media; (**B**) Some single buds develop into green multiple bud clumps in 5 mg L^{-1} BAP; (**C**) Multiple bud clump with shoot proliferation; (**D**) Putative transgenic plantlet confirmed from PCR and Southern blot analyses. Bar in **A-D** = 1.5 cm.

apple tissues (Maximova *et al*. 1998), adventitious buds and shoots induction in *Pinus radiata* (Holland *et al*. 1997) and chrysanthemum (*Dendranthema grandiflora* 'Lineker' and 'Shuhou-no-chikara' (Teixeira da Silva and Fukai 2002a). Other antibiotics such as augmentin, timentin and moxalactam were also used after co-cultivation to kill *Agrobacterium* as these did not reduce regeneration capacity (Park and Facchini 2000; Antunez de Mayolo *et al*. 2003).

Therefore, different carb and cef concentrations are required in solid and liquid medium of banana 'Rastali' explants in order to avoid toxicity or overgrowth of *A. tumefaciens* which could inhibit the explants' growth and cause contamination, in turn reducing transformation efficiency. Wong *et al.* (2008) demonstrated that the addition of 50 mgl^{-1} cefotaxime, 200 mgl^{-1} timentin and 1.0 mgl^{-1} PPM^{Tm} successfully eliminated *A. tumefaciens* LBA 4404 growth in their work on the transformation of embryogenic cell suspensions of diploid banana cultivar 'Mas' (AA). In addition, they suggested that the results of the antibiotic were significantly different at the somatic embryo stage, whereas there was no significant difference between the effects of cefotaxime and timentin either on plant regeneration or on the occurrence of normal characteristics.

An improved strategy for *Agrobacterium*-mediated transformation of banana single meristematic buds and plant regeneration

In this study, we have developed another efficient transformation method for banana 'Rastali' based on the results of various optimization transient expression parameters using both *gusA* and *gfp* genes as a reporter gene (Sreeramanan *et al.* 2006b). Previously, we reported particle bombardment-mediated co-transformation of chitinase and β-1,3-endoglucanase genes in Rastali (AAB) for the production of Fusarium wilt tolerance banana cultivar (Sreeramanan *et al.* 2006a). Single buds were infected and co-cultivated with induced *Agrobacterium* strain LBA 4404 (pROKLa-*Eg*) following the conditions described in the materials and methods. However, binary vector pROKLa-*Eg* does not consist of *gusA* or *gfp* genes as a visual marker gene. Therefore, this *A. tumefaciens* strain was used as a vector for transformation system with the two different treatments which were designed as treatment A (selection using kan) and treatment B (selection using geneticin/G-418). Similarly, the *npt*II gene was used as a selection marker and kanamycin at 100 mgl^{-1} was used as the selection agent for the production of transgenic East African Highland banana via similar transformation method by using intercalary meristematic tissues (Tripathi *et al.* 2008).

Co-cultivated single buds were selected on MS medium consisting of 10 mgl^{-1} of BAP containing kan at 100 mgl^{-1} (treatment A) or geneticin G-418 (treatment B) at 50 mgl^{-1}, during a period of 2 to 3 months, depending on the experiment. Selection medium containing geneticin G-418 produces fewer buds resistant to this antibiotic. However, none of the negative control plates ever gave rise to surviving cell aggregates under any of the selective conditions used in this experiment. In the absence of antibiotics, uninfected single buds proliferated normally on 10 mgl^{-1} BAP but turned black and died after two months under selection conditions even at lower concentrations.

The percentage transformation frequency was evaluated based on the number of explants regenerated. Transformation frequency from treatment A was higher than treatment B. More than 30 cell clusters arose per individual co-cultivated sample after selection, many of which initiated the formation of MBCs, while the rest of untransformed cells which could not survive selection, blackened and died (**Fig. 6**).

After the first level of selection, the remaining proliferating buds were subcultured in liquid medium containing 10 mgl^{-1} BAP using the same concentration of antibiotics that had previously been used in the first selection. After one week, the remaining healthy proliferating buds were then selected in the same concentration of selection in solid MS medium. MBC aggregates that formed during the selective process were regenerated in BAP and antibiotics-free medium. Single plantlets arising from geneticin G-418-resistant cell clumps in BAP-free medium formed shoots and root tips without any necrosis indicating that the tissue was fully transgenic without evidence of chimaeras. However, this is not true for explants regenerated *in vitro* from treatment A, in which kan was applied as the selection marker. Some transgenic plantlets produced albinos and semi-necrotic growth. This could be due to kan selection, which caused production of phenotypically abnormal plants, confirming similar observations in peas (*Pisum satium*) (Bean *et al*. 1997; Nadolska-Orcyzk and Orczyk 2000). Similary, the kan effect was reported in transgenic papaya by Yu *et al*. (2003). In plant cells, kan exerts its effect on mitochondria and chloroplasts by impairing protein synthesis, resulting in chlorosis (Weide *et al*. 1989). For several *Malus* cultivars

and rootstocks, kan at 50 mgl^{-1} is pyhtotoxic and causes shoot chlorosis and necrosis (Yepes and Aldwinckle 1994b).

For transforming papaya with *Agrobacterium*, 75-150 mgl^{-1} kan was used to kill non-transformed cells but with a low transformation efficiency and many abnormal transformed somatic embryos were noticed during the selection process (Yang *et al*. 1996). Similarly, Becker *et al.* (2000) reported that some antibiotics such as geneticin G-418 and kanamycin may interfere with embryo germination of Cavendish banana 'Grand naine' (AAA). Chimeric plantlets developed delay necrosis effect on a selection medium due to kan responses during proliferation of multiple bud clumps and regeneration into a single plantlets. The average frequency of regenerated plantlets rose to 10% when geneticin G-418 was used during the third phase of selection indicating that generation of 'escapes' was completely avoided by using *Agrobacterium* strain, LBA 4404.

Nevertheless, maintenance of cultures for long periods and with high antibiotic concentrations for more than a three-phase selection resulted in reduced numbers of surviving cell aggregates. He *et al*. (2008) reported that geneticin and cef were more effective than kan and carb to select transformants and eliminate *A. tumefaciens* growth in taro (*Colocasia esculenta* (L.) Schott) plants. Irrespective of the selective conditions used, the average frequency of plant regeneration ranged from 5 to 20% in putatively independent lines per co-cultivation in all samples. This strategy was designed to reduce the antibiotic stress on the transformed banana plantlets by accelerating plant regeneration and by shortening the incubation period of the transformed plants on the antibiotic selection medium prior screening system for detailed molecular analyses.

The use of highly regenerable single meristematic buds of banana 'Rastali' in combination with *Agrobacterium* as a vector for DNA transfer has been not described elsewhere previously. Tripathi *et al.* (2008) demonstrated that the use of intercalary meristematic tissues were chosen as the target for transformation because they have the potential to regenerate plants from many different cultivars, unlike somatic embryogenesis which is restricted to only a few cultivars. They also reported that transformation efficiency was greater with intercalary meristematic tissues than with apical meristems. However, using other types of banana meristem tissue such as corm slices in *Agrobacterium*-mediated transformation may have limited application because of the risk of generating chimaeric plants even though the transformation frequency obtained with particle bombardment could be markedly improved (May *et al*. 1995). Besides being more efficient, *Agrobacterium*-mediated transformation is technically simpler than particle bombardment, only requiring basic microbiology facilities and generally results in high levels of expression due to a simple integration pattern of well defined DNA sequences into transcriptionally active regions of the plant genome, which makes it the best option when both transformation systems are available (Hiei *et al*. 1997; Cheng *et al*. 1997).

Verification of presence of the soybean *Eg* and *npt*II genes by PCR analysis

Though *Agrobacterium*-mediated transformation is the most resorted method for the generation of transgenic plants with single integration of a precisely delimited DNA sequences (Smith and Hood 1995; Lawrence *et al*. 2001), the structure of the inserted T-DNA varies widely to include single or multiple copies, individual or tandem repeats, at a unique or several loci in the plant genome (Iglesias *et al*. 1997).

Total DNA isolated from the putative transformants was tested for the presence of the transgenes. The efficient and simplicity of the PCR analysis enable screening for transformed plants in a short period of time. Furthermore, techniques based on PCR are the best option for analyzing large amount amounts of transformants, since PCR is fast and demands low quantities of genomic DNA compared to other molecular methods. Perez-Hernandez *et al.* (2006) reported

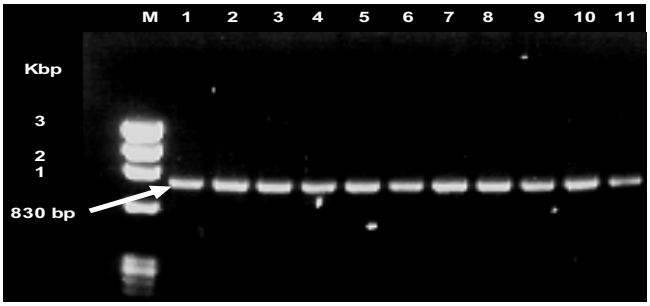

Fig. 7 PCR analysis of *Eg* gene in transgenic banana ('Rastali' (AAB)) plantlets selected on geneticin G-418. Lanes: M = λ DNA-digested with *Hind*III was used as a molecular weight marker; 1 = pROKla-*Eg*; 2-11 = putative transformed plantlets showing the amplified 830 bp *Eg* gene.

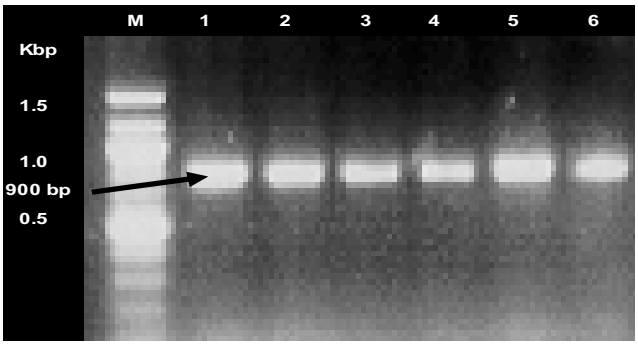

Fig. 8 PCR analysis *npt*II gene in transgenic banana ('Rastali' (AAB)) plantlets selected on geneticin G-418. Lanes: M = λ DNA digested with *Hind*III was used as molecular weight marker; 1 = pROKla-*Eg*; 2-6 = putative transformed plantlets showing the amplified 900 bp *npt*II gene.

an improved anchored PCR technique that amplified specific T-DNA border-containing genomic sequences in transgenic banana. PCR amplification confirmed that the *Eg* and *npt*II genes were present in the geneticin G-418 resistant regenerants (treatment B) compared to kan selection medium (treatment A). The PCR results of some transgenic plantlets are displayed in **Figs. 7** and **8**.

In all the experiments, no bands could be detected from DNA extracted from untransformed putative plantlets. Fourteen DNA samples picked from kan selection (treatment A) and six from geneticin G-418 selection (treatment B) were subjected to PCR analyses from transformation using *A. tumefaciens* LBA 4404 (pROKla-*Eg*). No bands (*Eg* and *npt*II genes) were detected from plants using kan selection (treatment A). These were probably escapes even though passing through a rigid selection system or the kan selection of plants and may have lost the *npt*II gene when the selective pressure was removed; alternatively they could be chimeric for expression of the *npt*II gene. Similar observations of high escape rates using kan have been observed in oil palm (*Elaeis guineensis*), rice (*Oryza sativa*) and orchid (*Dendrobium* Sonia-17) (Parveez *et al*. 1996; Kuvshinov *et al*. 1999; Janna *et al*. 2001).

Two out of the six DNA samples of putatively transformed plants that were analysed successfully amplified the expected band size of 830 bp of the *Eg* gene from geneticin G-418 selection medium (**Fig. 7**). Co-integration of *npt*II (900 bp) was detected in these two transformants (**Fig. 8**). Two transgenic plantlets were differentiated according to plant codes [B2E (2) and B3E (31); Symbol B means plants derived from geneticin G-418 selection system, second digit means experiment number, E means *Eg* gene and finally number in bracket means code for selected transgenic lines]. Similar co-integration of two genes – *npt*II and a gene encoding the antimicrobial protein *Ace-AmPI* – in seven transgenic scented geranium plants was confirmed by PCR (Bi *et al*. 1999).

In the present experiment, it was noted that a longer du-

ration of geneticin G-418 in the selection medium actually did not help to reduce the outcome of chimeric tissue but instead decreased the survival of regenerated plants. In addition, those putative plantlets produced pink colour pigmentation, especially in shoots and leaves. However, these pigmented plantlets returned back to normal green plantlet when the selection pressure was removed after six cycles of cultures and maintained in hormone-free MS medium.

Additionally, an extensive regeneration phase for MBCs formation and inefficient plant recovery on selection medium might further reduce the availability of competent cells for transformation. In oil palm (*Elaeis guineensis*), the presence of hygromycin or basta during five cycles of embryogenic callus clump formation was an essential factor or some of the regenerated plantlets were not actually transformed (Parveez *et al*. 1996). The fact that transgenic banana cells were selected during a longer period (four to six months) before regeneration might explain the low number (less than 1%) of transformants in geneticin G-418 selection medium. PCR analysis of transgenic papaya showed the presence of the *npt*II selectable marker gene and the linked transgene (the coat protein (*cp*) gene of the *Papaya ringspot virus* (PRV)) in 95 and 42% of putative transformants analysed, respectively (Fitch *et al*. 1992). As the latter transgene was located between the *npt*II and *gus*A transgenes, it appeared that complex rearrangements of transgenes occurred (Fitch *et al*. 1992).

Examination by PCR of 26 kan-resistant and thus, *npt*II-containing, regenerated kiwifruit shoots revealed that 17 (65%) also contained the linked soybean *Eg* gene (Nakamura *et al*. 1999). An 80% transformation frequency for linked transgenes (*gus*A and the *cp* gene of PRV), based on PCR analysis, occurred in 12 papaya transformants (Fitch *et al*. 1992).

Southern blot hybridization analysis

Southern blot analysis was carried out to evaluate further the transfer and insertion of the *Eg* gene in the genome of 'Rastali'. Although two lines of evidence, (i) the antibiotic resistant phenotype and (ii) the presence of the introduced genes in putative transformants as shown indeed by PCR demonstrated that the regenerated plants were transgenic, proof of stable integration of transgenes by Southern analysis was required.

Southern blot analysis also yields information on the number of integration sites per transgene, the copy number of the transgenes and whether transgenes are integrated in the same locus (linked co-integration) or not (non-linked co-integration) (Kohli *et al*. 1998). Results presented here comprise only the *Eg* hybridizations. No hybridisation was observed in the control plantlets. Hybridisation of high molecular weight DNA indicated stable integration of the transgenes into the banana genome.

Even two transgenic plantlets (treatment B) obtained from PCR-positive analysis, interestingly 100% were found to contain a single gene insertion in both leaves and roots tissue. *Hind*III digestion of transforming plasmid pROKLa-*Eg* (**Fig 9**; Lane 1), released an approximately 3.5 kb size fragment containing the CaMV 35S, *Eg* and Nos terminator (**Fig. 9**). No hybridization was observed from non-transformed plantlet (**Fig. 9**; Lane 2). Lanes 3 and 4 are the DNA samples from leaves and roots tissue of plantlet code B2E (2), lanes 5 (leaves), 6 and 7 (roots) of plantlet B3E (31). The presence of bands with sizes larger than the *Eg* fragment (3.5 kb) indicated the integration of the introduced plasmid. In contrast, multiple copy insertions (more than 2 copies) of the similar soybean *Eg* gene were obtained via particle bombardment system in our previous transformation experiment (Sreeramanan *et al*. 2006a).

Fig. 9 Southern blot analysis of the β-1,3-endoglucanse gene integration pattern in transgenic banana ('Rastali' (AAB)) plantlets. Digested genomic DNA with *Hind*III using PCR-amplified *Eg* gene (830 bp) as probe. Lanes: 1 = pROKla-*Eg*; 2 = untransformed control plantlets; 3-7 = putative transformed plantlets.

Patterns of integration, inheritance and expression of transgenes in a plant upon *Agrobacterium*-mediated transformation have a lower copy number of transgenes than a particle bombardment transformation system and a more predictable pattern of integration (Smith and Hood 1995; Klimaszewska *et al*. 2003). However, Kononov *et al*. (1997) reported that approximately 75% of transgenic tobacco plants from *Agrobacterium*-mediated transformation contained sequences from the binary vector 'backbone' sequences in the plant genome. Furthermore, there was no clear correlation between transgene expression and transgene copy number in tobacco (*Nicotiana tabacum*) (Hobbs *et al*. 1993). While single copies of transgenes may tend to be more stably expressed than multiple gene copies or scrambled inserts, there were additional factors that influenced transgene expression (Iglesias *et al*. 1997). The results from Southern blot analyses also rule out the possibility of *Agrobacterium* contamination of tissue resulting in false-positives.

Bioassay of transgenic banana plantlets challenged by the fungus pathogen, *Fusarium oxysporum* f. sp. *cubense* (race 1; VCG 01217)

Finally, in the laboratory fungal bioassay, two transgenic lines that exhibited increased tolerance to the fungal pathogen, *Fusarium oxysporium* f. sp. *cubense* were selected. Progress of disease development after four weeks was based on leaf symptoms of transgenic 'Rastali' plantlets (**Fig. 10**).

Next follows a summary of final evaluation obtained for leaf disease symptoms after four weeks inoculation (**Fig. 10**):

Plant code: B2E (2) containing the *Eg* gene. Yellowing of most of the older leaves and discolouration of younger leaves appear after 28 days of inoculation.

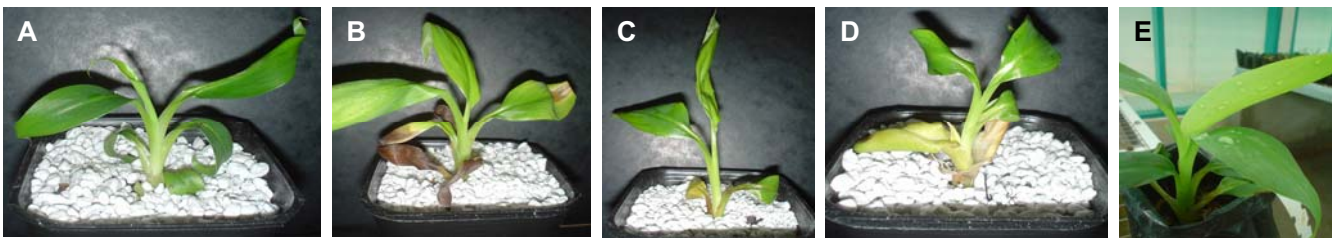

Fig. 10 Phenotypic observation on the effect of disease symptoms (secondary level) caused by *Fusarium oxysporum* f. sp. *cubense* (race 1) on leaves of transgenic banana ('Rastali' (AAB)) plantlets. (**A**) Control plantlet (no spores inoculated); (**B**) control plantlet with spores inoculated; (**C**) B2E (2); (**D**) B3E (31); (**E**) two-months old putative transgenic plants in the glasshouse.

Table 2 Total amount of EG enzyme activity in transgenic banana 'Rastali' (AAB) plantlets transformed during pre and post inoculation with *Fusarium oxysporum* f. sp. *cubense* race 1 (VCG 01217).

Plant code	EG activity (1 nkat μg^{-1} protein)	
	Before	After
B2E (2)	27.4 b	38.9 bc
B3E (19)	29.7 b	34.5 c
Untransformed banana plantlet	10.1 a	11.3 a

Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Plant code: B3E (31) containing the *Eg* gene. Slight discolouration appeared on one of the younger leaves at the end of 28 days. No yellowing of older leaves could be observed.

Similarly, leaves from transgenic tobacco plants highly expressing the soybean *Eg* gene were resistant to the fungus and almost no disease symptoms developed (Yoshikawa *et al*. 1993). They also indicated that EG-expressing transgenic tobacco leaves indeed induced the transcription of a plant defense gene in response to fungal attack more robustly than untransformed leaves.

Analysis of EG protein production in transgenic plantlets

The EG enzyme activity was assayed by measuring the rate of reduction of sugar production with laminarin as substrate. There were variations in the concentration of EG among the transgenic banana plantlets, including the untransformed plantlets before and after *F. oxysporum* spore inoculation demonstrating that all transgenic banana plantlets had significantly higher EG enzyme activity compared to the untransformed plantlets (**Table 2**).

The EG activity of B2E (2) and B3E (31) was 3-fold higher than the untransformed plantlet. The concentration of EG in transgenic plantlets ranged from 12.3 to 36.9 nkat mg^{-1} protein after infection with *Fusarium* spores. A correlation between constitutive EG levels and tolerance has been detected in all transgenic banana plantlets. EG activity and tolerance to the fungal pathogen was possibly strongly correlated since transgenic plants containing more than three times EG level than untransformed plants showed a high degree of disease resistance. Generally, it is known that transgenic plants which do not express the soybean EG at a higher level, untransformed plants or those transformed with only the vector contain low levels of EG activity are more uniformly susceptible to fungal attack (e.g. Ito *et al.* 1995; Borkowska *et al.* 1998; Nakamura *et al.* 1999). Previously, Yoshikawa *et al*. (1993) reported that a number of individual transgenic plants showed that the level of EG activity varied from one to four times the basal levels found in untransformed tobacco plants, probably due to different levels of expression of the integrated soybean *Eg* cDNA. In addition, transgenic potato plants expressing the soybean *Eg* gene exhibited an increase in the activity of EG in transgenic plants (Borkowska *et al.* 1998). EG is able to catalyse endo type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans. There is strong evidence that expression of the *Eg* transgene alone regulated by the strong CaMV 35S RNA promoter can reduce the susceptibility of plants to infection by certain fungi (*Phytohthora infestans*) (Borkowska *et al.* 1998). Transgenic tobacco plants expressing a soybean β-1,3-glucan-elicitor releasing EG or the tobacco class II β-1,3-glucanase show reduced symptoms when infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotiane* and *Peronospora tabacina* (Yoshikawa *et al*. 1993; Lusso *et al*. 1996). Dong *et al*. (2007) reported that expression of the alfalfa *Eg* gene in transgenic fescue conferred resistance to two major fungal diseases, *Magnaporthe grisea* and *Rhizoctonia solani*.

Similar pattern of EG levels and resistance has also been detected in leaves of resistant tomato lines (*Lycopersicon esculentum*) infected with *Alternaria solani* (Lawrence *et al*. 1996) and leaf rust resistance in wheat (Anguelova *et al*. 1999). Nakamura *et al*. (1999) reported that transgenic kiwifruit with soybean *Eg* cDNA showed up to 6-fold higher EG activity than control plants which resulted in disease lesion areas in transformants whose leaves were smaller than those of control plants. A similar phenomenon was reported by Ito *et al*. (1995) using transformed eggplants with soybean *Eg* cDNA. The transformed eggplants with higher EG activity showed a statistically significant degree of disease resistance to *Phytohthora capsici*. Yoshikawa *et al*. (1993) reported that transgenic tobacco with soybean *Eg* cDNA induced the transcription of a plant defence gene, phenylalanine ammonia lyase, in response to fungal attack to a greater extent than untransformed leaves.

Comparison with other transgenic banana studies

Three systems have so far been reported for genetic transformation of banana cultivars. Sagi *et al.* (1994) were the first group to report the successful transfer of the reporter gene, *gusA* using electroporation into protoplasts isolated from embryogenic cells of the cooking banana cv. 'Bluggoe'. In 1995, the same group reported stable transformation via particle bombardment system for the production of transgenic banana plants using embryogenic cell suspensions (Sagi *et al.* 1995). A similar approach was also used to produce transgenic Cavendish banana cv. 'Grand naine' in Australia (Becker *et al.* 2000). In general, most of the direct banana transformation systems using particle bombardment approach were carried out using embryogenic cell suspensions. In addition, resistance to the fungal pathogen in transgenic banana via particle bombardment transformation system has been reported by our group by using both antifungal genes, chitinase and β-1,3-glucanase in 2006 (Sreeramanan *et al.* 2006a). In addition to particle bombardment, the first *Agrobacterium*-mediated transformation in banana was reported by May *et al*. in 1995. They used the apical meristem dome and its underlying tissue wounded by bombardment with gold particles without coated DNA. In this study, they reported stable transformants, but chimerism was also noted. Pie *et al*. (2005) also reported a high frequency of banana transformation by a similar approach, a combination of particle bombardment and *Agrobacterium*-mediated transformation. They reported a transformation frequency of 0.95% by first bombarding with the naked gold particles and followed by co-cultivation with *A. tumefaciens* in conjunction with vacuum-infiltration.

An *Agrobacterium*-mediated transformation protocol was used to produce transgenic bananas cv. 'Rasthali' by co-cultivation of embryogenic cell suspensions (Ganapathi *et al*. 2001). Chakrabarti *et al.* (2003) reported that transgenic banana expressing a synthetic substitution analogue of magainin, a protein from skin secretions of *Xenopus laevis*, was resistant to two fungal pathogens, *Fusarium oxysporum* f. sp. *cubense* and *Mycosphaerella fijiensis*. The *Agrobacterium*-mediated transformation frequency also can be improved by the use of centrifugation and vacuum infiltration during the inoculation and co-cultivation phases (Khanna *et al*. 2004; Acereto-Escoffie *et al.* 2005). Khanna *et al.* (2004) demonstrated a three- to four-fold increases in transient gene expression and stable transformation of both 'Cavendish' and 'Lady finger' bananas by centrifugation of embryogenic cell suspensions co-cultivated with *A. tumefa-*

ciens strain AGL1 and LBA 4404 at 1000 rpm for 5 min at room temperatures. Apart from centrifugation, vacuum infiltration also benefited banana transformation by using an optimized parameter (4 min at 400 mm Hg) using meristematic tissues with *Agrobacterium* suspensions. Wong *et al.* (2008) reported improvements in the efficiency of *Agrobacterium*-mediated transformation of embryogenic cell suspensions of cv. 'Mas' using a low-antibiotic liquid washing-assisted approach. They transformed banana for early flower induction by using suppressor of over expression of CO 1 (SOC1) transgene. Tripathi *et al*. (2008) developed a rapid and efficient transformation protocol of a recalcitrant East Highland banana cultivar on direct shoot regeneration regeneration of *Agrobacterium* infected intercalary meristem tissues.

CONCLUSIONS

The present study demonstrated that the soybean *Eg* cDNA could be expressed in banana plants and that the transformed plants demonstrated EG activity up to 3-fold more than in untransformed plants. The transformed banana plantlets showed a higher degree of disease tolerance to *Fusarium oxysporum* f. sp. *cubense* (race 1; VCG 01217) and there was a high correlation between EG activity and disease tolerance. In conclusion, an alternative strategy using transgenic 'Rastali' (AAB) expressing the soybean *Eg* gene for increased tolerance to Fusarium wilt race 1 disease has been successfully produced. Thus, it is likely that our transgenic 'Rastali' lines may also exhibit tolerance or resistance to other types of common fungal diseases; however this has yet to be verified. Even though finally we only obtained two truly putative positive transgenic plantlets, we believe that they can be multiplied successfully using an optimised *in vitro* regeneration system. Previously, this was thought to be a significant limiting factor in the generation of transgenic banana plants that had not used embryogenic callus or cell suspension cultures as a starting material for banana transformation work. An efficient *Agrobacterium*-mediated transformation protocol such as that proposed in this study can facilitate further functional genomic studies of this important banana cultivar.

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