Tree and Forestry Science and Biotechnology ©2010 Global Science Books

Genetic Engineering for Tolerance to Fusarium Wilt Race 1 in *Musa sapientum* cv. Rastali (AAB) Using Biolistic Gun Transformation System

Sreeramanan Subramaniam[1,2*] • Maziah Mahmood[2] • Sariah Meon[3] • Xavier Rathinam[4]

[1] School of Biological Sciences, Universiti Sains Malaysia, Minden Heights, 11800 Penang, Malaysia
[2] Department of Biochemistry, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor DarulEhsan, Malaysia
[3] Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia
[4] Department of Biotechnology, AIMST University, Batu 3 ½, Jalan Bukit Air Nasi, Bedong, 08100, Kedah, Malaysia

Corresponding author: * **sreeramanan@usm.my, sreeramanan@gmail.com**

ABSTRACT

An effective method has been developed for the stable transformation and regeneration of silk banana cultivar 'Rastali' (*Musa* spp. AAB group) using biolistic gun method for tolerance to Fusarium wilt race 1 disease. Recent progress with advanced *in vitro* cultures of banana such as establishment of highly regenerable tiny single meristem buds opened the opportunity for the production of disease tolerant transgenic bananas. Chitinase and β-1,3-glucanase are important disease tolerance genes that were successfully transformed into banana together with *gfp* and *gus*A genes as reporter genes. Proliferating single buds were selected on geneticin G-418 to produce a number of putatively transformed bananas. Five different treatments using different chitinase and β-1,3-glucanase genes inserted singly or in combination were carried out. Molecular analyses such as polymerase chain reactions (PCR) and Southern blot were performed to confirm the integration and expression of the introduced genes into banana genome. The transgenic banana plantlets from each treatment were inoculated with 2×10^6 spores per ml conidial suspension of *Fusarium oxysporum* f.sp. (Race1) to evaluate the degree of tolerance to fungal infections. Evaluation of disease development in primarily and secondary infections showed that combination of the two transgenes gave substantially greater protection against the fungal than single-transgene introduction. Productive interactions between chitinase and β-1,3-glucanase transgenes *in planta* point to combinatorial expression of antifungal genes as an effective approach to enhanced tolerance to Fusarium wilt disease in banana through genetic engineering.

Keywords: biolistic gun, transgenic banana, chitinase, β-1,3-glucanase gene, Fusarium wilt tolerant

INTRODUCTION

Banana is the world's largest fruit crop with an annual production of 168 million tons in the year 2006, of which around a third is produced in each of the African, Asia-Pacific and Latin American and Caribbean regions (Anonymous 2008). In Malaysia, banana has been traditionally cultivated in smallholdings and also as an intercrop. Fungi are the cause of some of the most serious diseases of banana in Malaysia and thus are of the greatest economic concern to the commercial banana industry. Banana cultivar 'Rastali', a local dessert banana which belongs to the AAB group, is known to be susceptible to Fusarium wilt disease which causes heavy losses in banana plantation sectors in Peninsular Malaysia.

Conventional breeding of banana is hampered by a long generation time, polyploidy, the long cropping cycle and sterility of most edible cultivars (Vuylsteke *et al.* 1993). These difficulties could be overcome by genetic engineering techniques with the introduction of genes conferring resistance to fungal pathogens. The two most common genetic engineering systems in banana are *Agrobacterium*-mediated and particle bombardment transformation (May *et al.* 1995; Becker *et al.* 2000; Ganapathi *et al.* 2001; Wonget *et al*. 2005). Kido *et al.* (2005) reported plant transformation using particle bombardment on somatic embryogenesis of banana cultivar Maca (Silk banana, AAB). They had developed an efficient callus regeneration method for this important Brazillian banana cultivar which could offer an alternative for crop improvement especially for fungal disease resistance. In this study, biolistic gun-mediated transformation system was used in potential banana cultivar 'Rastali' (AAB) for introducing antifungal genes into single meristem bud explants.

Reliable optimized biological and physical conditions for biolistic transformation greatly influence the efficiencies of stable integration of foreign genes (Sreeramanan *et al.* 2005). Therefore, reporter gene markers that do not exist naturally in the host banana plant such as β-glucuronidase (GUS) and green fluorescent protein (GFP) are used for transient experiment as indication of the interation of a transgene (Sreeramanan *et al.* 2005; Wong *et al.* 2005). An efficient method for direct gene transfer of embryogenic cell suspension has been reported in cooking banana cultivar 'Bluggoe' and plantain (Sagi *et al.* 1995) and Becker *et al.* (2000) reported similar procedure on Cavendish banana cultivar 'Grand naine'. Particle bombardment technology (Sági *et al.* 1995) has enabled the production of several hundreds of transgenic lines, which express different genes encoding defensin-type AMPs and nsLTPs alone or in combinations, mainly in plantains (AAB, Three hand planty, Cemsa and Navolean) and Williams (AAA, Cavendish subgroup) at KULeuven.

Co-transformation is a process in which genes carried on separate plasmids are mixed prior to transfer by particle bombardment (Chen *et al.* 1998c). Co-transformation is a potential tool to create multiple and durable resistance in banana (Remy *et al.* 1998a). A large-scale molecular analysis has confirmed that a vast majority of these lines contain and express the introduced genes (Remy 2000). Simi-

Received: 26 August, 2008. Accepted: 12 August, 2010.

Research Paper

larly, transgenic tomato plants expressing only a chitinase or a β-1,3-glucanase transgene were susceptible to *Fusarium oxysporum*, but plants expressing both genes had significantly higher resistance than the plants expressing only chitinase or β-1,3-glucanase (Jongedijk *et al.* 1995). In addition, Jach *et al.* (1995) demonstrated that tobacco plants expressing a barley β-1,3-glucanase and a chitinase gene had a greatly enhanced resistance compared to plants expressing only one of these two enzymes. Bliffeld *et al.* (1999) introduced barley seed class 11 chitinase gene (*Pr3*) driven by maize *Ubi* promoter along with β-1,3-glucanase gene in cv. 'Bobwhite' of wheat using particle bombardment. Stable expression of transgenes was observed in successive three generations and transgenic plants showed increased resistance to infection with the powdery mildrew-causing fungus *Erysiphegraminis*. Recently, combined expression expression of both chitinase and β-1,3-glucanase genes in *indica* rice (*Oryza sativa* L.) was attempted to achieve higher levels of fungal resistance against *Rhizoctonia solani* using *Agrobacterium*-mediated transformation system (Sridevi *et al.* 2008). Although remarkable achievements have already been made in banana transformation by some scientists around the world, the identification and introduction of useful genes into banana to reduce losses caused by the fungal pathogen is still a major challenge.

In this study, five different treatments using different chitinases and β-1,3-glucanase genes inserted singly or in combination were carried out. Multiple genes encoding either chitinases (*RCC2* or *chi*) or β-1,3-glucanase (*Eg*) and both was bombarded together with *gfp* gene (pGEM.*Ubi1*-S*gfp*S65T) for early transient expression signal using optimised physical and biological conditions (Sreeramanan *et al.* 2005). In our previous study, we have demonstrated increased disease tolerance of transgenic banana plantlets to fungal pathogen *Fusarium oxysporum* f.sp. *cubense* (race 1; VCG 01217) using an optimized bioassay method (Sreeramanan *et al.* 2006b). Transgenic plants with a transgene locus resulting from genomic integration of a single, perfect copy of delivered DNA are the most desirable for banana improvement (Makarevitch *et al.* 2003). Therefore, molecular breeding of banana via transgenic approach is a necessity in view of its long life cycle, triploidy and sterility of most edible cultivars (Khanna *et al.,* 2004). Pillay and Tripathi (2007) reported that the chances of transfer of transgenes from improved material to wild species (the major public concern) are expected to be negligible in *Musa* in view of the sterility of many banana cultivars.

MATERIALS AND METHODS

Plant materials

Corm slices of *in vitro* Rastali banana (AAB) plantlets were cultured in MS (Murashige and Skoog 1962) medium supplemented with different concentrations of 6-Benzylaminopurine (BAP) to obtain multiple bud clumps (Sreeramanan *et al.* 2002). Single meristem buds (3 mm) were used as a target tissue for transformation studies. The cultures were incubated at 25 ± 2°C and a 16-h photoperiod with cool white fluorescent light of 150 µmol $m^{-2}s^{-1}$.

Plasmid constructs

The four plasmid constructs used throughout this study were as follows:

pBI333-EN4-RCC2

The plasmid pBI333-EN4-*RCC2* contains the *nptII* gene linked to the nopaline synthase gene (*promoter1*). Plasmid pBI333-EN4-*RCC2* was constructed to replace the *gus*A gene of pBI121 (Clontech, USA) with the cDNA (*RCC2*) of the rice chitinase gene.

Table 1 Five treatments with different combination of plasmids containing chitinase and β-1,3-glucanase were used together with *gfp* gene as a reporter gene.

Treatments	Combination of plasmids
1	pBI333-EN4-*RCC2* + pROkla-*Eg*
2	pMRC1301 + pROKla-*Eg*
3	pBI333-EN4-*RCC2*
4	pMRC 1301
5	pROKla-*Eg*

pMRC1301

The plasmid pMRC 1301 contains the *gusA* and *chitinase* genes, driven by the rice *actin 1* promoter.

pROKla-Eg

The plasmid pROKla-*Eg* contains the *nptII* gene linked to the nopaline synthase gene (*nos*) promoter and the soybean β-1,3-endoglucanase gene linked to the *CaMV35S* promoter in the T-DNA region.

pGEM.Ubi1-sgfps65T (GFP)

The plasmid contains a *gfp* gene driven by a maize *polyubiquitin* 1 (*Ubi*1) promoter.

Preparation of DNA and particle bombardment

The plasmid DNA was precipitated onto gold according to the instruction manual for the Biolistic PDS-1000/He device. Gold microcarriers were suspended in absolute ethanol (60 $mg.ml^{-1}$) and vortexed vigorously for 3 min to get rid of aggregated lumps. The suspensions were centrifuged for 1 min at 10,000 rpm and the supernatant was discarded. The pellet was resuspended in 1 ml of sterile distilled water, vortexed, centrifuged for 1 min and the supernatant discarded. The process was repeated once. The final pellet was resuspended in 1ml of sterile distilled water and 50 µl aliquoted each in microtubes while vortexing of the suspension continues. A 5 µl sample of DNA solution (1.5 $µg.µl^{-1}$), 50 µl of $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M) were added one by one to the 50 µl particle suspension. The mixture was vortexed for 3 min, centrifuged for 10 s at 10,000 rpm and the supernatant discarded. The pellet was washed with 250 µl of absolute ethanol. The final pellet was resuspended in 60 µl of absolute ethanol. A 6 µl sample of the aliquot was loaded onto the center of the macrocarrier, air dried and used for bombardment. Each bombardment was performed twice at 1100 psi rupture disk pressure, 4 mm rupture disk to macrocarrier distance, 11 mm macrocarrier to stopping screen distance, 9 cm stopping plate to target tissue distance and 28 mm Hg vacuum pressure (Sreeramanan *et al.* 2005).

Co-bombardment

There were five treatments used in this experiment (**Table 1**). Plasmid pGEM.*Ubi1*-s*gfp*s65T (GFP) was co-transformed in all treatments as a reporter marker to monitor early detections of successful gene insertions.

Selection and regeneration system

After bombardment, single buds were transferred to MS medium containing 10 $mg.l^{-1}$ of BAP and were allowed to recover for two weeks in the absence of antibiotic selection. After this recovery period, explants were transferred to the same medium containing 50 $mg.l^{-1}$ geneticin G-418 (Sigma) for two weeks. The survived explants were transferred back to the liquid medium containing 25 $mg.l^{-1}$ of geneticin G-418 for an additional two weeks. Liquid medium selection provides a better contact between the tissues and the antibiotic solution. The survived tissues were further selected in MS solid medium using geneticin G-418 at 25 $mg.l^{-1}$. The survived explants were maintained in hormone-free medium for plant regeneration. The regenerated plantlets from each putatively independent transformed cell line were maintained under *in vitro* conditions for further confirmation.

Table 2 Genes, primer, primer sequences and expected product length.

Genes	Primer	Sequence (5′-3′)	Product length (bp)
Gfp (pGEM.*Ubi1-SgfpS65*T)	Forward	ATGAGTAAAGGAGAAGAACTTTTC	726
	Reverse	TTTGTATAGTTCATCCATGCCA	
gusA (pMRC1301)	Forward	ATTAATGCGTGGTCGTGCAC	786
	Reverse	CGCCGATGCAGATATTCGTA	
Chitinase (pBI333-EN4-*RCC2*)	Forward	GGATCCAGCGGCTCGTCGGTTG	310
	Reverse	GTATAATTGCGGGACTCTAAT	
Chitinase (pMRC1301)	Forward	TACAACTTCAACTACGGGCCG	486
	Reverse	ACGACTCACTATAGGGCG	
β-1,3 glucanase (pROKla-*Eg*)	Forward	GATGTGATATCTCCACTGACGTAA	830
	Reverse	GTATAATTGCGGGACTCTAAT	
*npt*II	Forward	CCCCTCGGTATCCAATTAGAG	900
	Reverse	CGGGGGGTGGCCGAAGAACTCCAC	

Histochemical GUS staining

Bombarded tissues were assayed for GUS expression according to that of Jefferson *et al.* (1987).

Visualisation of GFP

A florescence microscope (Leica MZFL 111) equipped with GFP 2 filter set was used to monitor the GFP expression of transformed tissues.

2.8 Polymerase chain reactions (PCR)

Genomic DNA were extracted from eight putative transgenic lines using an improved and modified CTAB method adopted from Pasakinskiene and Paplauskience (1999). PCR was done using the DNA Thermal Cycler 480 machine (Perkin-Elmer). *RCC2* and *chi* (chitinases genes), Eg (β-1,3-glucanase) and *npt*II genes were amplified using standard protocols (Sambrook *et al.* 1989). The following primers were used to amplify the transgenes and part of the regulatory sequences (**Table 2**).

PCR amplifications were carried out in 100 µl reactions volume 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 5 U Taq DNA polymerase (MBI Fermentas). Amplification for *gfp* and *gus*A genes were performed using the following conditions: 1 cycle of 94°C for 5 min; 30 cycles of 94°C (30 sec), 60°C (1 min) and 72°C (2 min); and last cycle of 72°C for 7 min. Amplification of chitinase gene (*RCC2*) fragments was performed for 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, for denaturing, annealing and primer extension, respectively. The conditions for amplification of chitinase gene (*chi*) conducted were: 1 cycle of 95°C for 5 min; 35 cycles of 95°C (1 min), 58°C (1min) and 72°C (2 min); and 1 cycle of 72°C for 7 min. Amplification of β-1,3-glucanase gene (*Eg*) fragments was performed for 45 cycles at 94°C for 1 min, 40°C for 2 min and 72°C for 3 min, for denaturing, annealing and primer extension, followed by 72°C for 7 min. Amplification of *nptII* gene fragments was performed for 35 cycles at 94°C for 1 min, 62°C for 1 min and 72°C for 2 min, for denaturing, annealing and primer extension, respectively. All amplified PCR products were checked on 1.2% agarose gel.

Southern blot analysis

A nonradioactive method was used to confirm stable integration of chitinase (*RCC2* and *chi*) and β-1,3-glucanase (*Eg*) transgenes in the host banana genome. Genomic DNA (20 µg) from PCR positive transformants were subjected to digestion with *Hind*III in buffer H (Promega). For pMRC1301 plasmid, double digestions were carried out with *Nhe*I and *Kpn*I (*gusA* gene) or *Hpa*I and *Kpn*I (chitinase gene).

Extraction and determination of total acid soluble proteins

Young leaf samples from *in vitro* cultures (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 polyvinyl polypyrroline. The homogenate was centrifuged at 12,000 rpm for 20 min. The supernatant was used as crude extract for determination of total acid soluble protein, chitinase and β-1,3-glucanase activities. Protein was quantified by Bradford methods (1976), at wavelength of 595 nm. Total acid soluble protein was expressed in $mg.g^{-1}$ fresh weight by using bovine albumin as standard.

Chitinase activity assay

Methods of Tonon *et al.* (1998) were followed with a slight modification in enzyme concentration. Chitinase activity was determined based on the rate of *N*-acetylglucosamine production using chitin as the substrate. Amount of enzyme catalyzing the formation of 1 nm *N*-acetylglucosamine equivalent in one second under assay conditions equals 1 nkat (Anfoka and Buchenauer 1997).

β-1,3-glucanase activity assay

The β-1,3-glucanase activity assay methods of Tonon *et al.* (1998) were followed with a slight modification. β-1,3-glucanase activity determination was based on the rate of reducing sugar production using Laminarin (Sigma) as the substrate. Amount of enzyme catalyzing the formation of 1nm glucose equivalent in one second under assay conditions refers as 1 nkat (Anfoka and Buchenauer 1997).

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

Development of Fusarium bioassay method in transgenic banana for this study was referred by using an improved method adopted by in our previously published manuscript (Sreeramanan *et al.* 2006b). For plant inoculation bioassay protocol, untransformed banana cv. 'Rastali' (AAB) plantlets roots sections were cut at 3 cm from the root tips (**Fig. 1**). The plants dipped into spore con-

Fig. 1 The assembly of the single banana plantlet with perlite for *Fusarium* bioassay assay. The pipette tip indicates the initial point where inoculum (1×10^6 spores.ml^{-1}) was applied after the each single plantlet dipped for an hour in *Fusarium* spores suspension (1×10^6 spores.ml^{-1}). The bar in the bottom represents 2 cm.

centrations of 10^6 spores.ml^{-1} *Fusarium oxysporum* fsp. *cubense* (race 1; VCG 01217) for one hour and were then transplanted in a plastic container (8 cm × 8 cm) with sterile perlite. One ml of 10^6 spores concentration was again inoculated near the root or stem regions for double confirmation. The total spores used were 2 × 10^6 spores per ml. The plantlets were watered using basal MS liquid medium. The experiment was performed in three 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.

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

Evaluation of GFP and GUS expression as reporter genes using co-bombardment

Particle bombardment with an equimolar mixture of plasmids using single or double combinations were carried out (**Table 1**) together with GFP construct (pGEM.*Ubi1-sgfp*s65T), in which the antifungal proteins and the GFP plasmid were non-linked (all plasmids were containing *nptII* gene as selection marker). Expression of *gfp* gene could be observed transiently 48 hours after post-bombardment in all treatments (**Fig. 2, 4A**). Almost all bombarded single buds from each treatment showed *gfp* gene expression with some variations in percentage of fluorescent spots per bud (**Fig. 2**). However, no continuous expression could be detected after five months in regenerated plantlets. The maize *Ubi-1* promoter region has been shown to drive strong, constitutive transgene expression (Cornejo *et al.* 1993). GFP expression derived from the maize *Ubi-1* promoter was appear in mesophyll, epidermis, vascular cells of leaves, in cortex, endodermis and pith cells of young roots of maize (van der Geest *et al.* 1998).

The highest *gfp* gene expression could be detected in treatment 3 between week one and week two (**Fig. 2**). However, it decreased almost (50%) during week four. At week four, the highest expression could be detected in treatment 2 and the lowest in treatment 4. However, at week 5 and 6, GFP expression was the highest in treatment 1. Interestingly, GFP expressions had been observed on regenerated multiple bud clumps (Mbcs), shoots, leaves and roots (**Fig. 4**) under geneticin G-418 selection. These indicated that GFP regardless of targeted or not, could be expressed in banana tissue transiently in earlier stage and become stable at later stage of regeneration under optimise conditions (Tian *et al.* 1997). Becker *et al.* (2000) reported stable transformation of Cavendish banana (*Musa* spp., AAA group) cultivar 'Grand naine' via particle bombardment with a construct carrying *gfp* reporter gene under control of the *35S* promoter and the *nptII* gene under control of a novel promoter derived from banana bunchy top virus (BBTV).

Expression of the *gusA* gene could be observed transiently beginning 48 h post-bombardment in treatments 2 and 4 (**Fig. 3, 4B**). Highest GUS expressions observed during week 2 in both treatments [treatment 2 (65%) and treatment 4 (55%)]. It could due that the gene requires two days for it to express in a new environment from the injuries inflicted by scalpel and blasting conditions. Higher *gus*A expression observed in treatment 2 compared to treatment 4 throughout six weeks of observations (**Fig. 3**). Similarly, GUS expressions had been observed on regenerated multiple bud clumps (Mbcs), shoots, leaves and roots (**Fig. 5**) under geneticin G-418 selection. Becker *et al.* (2000) reported that selection of bombarded embryogenic cell suspension of banana with geneticin G-418 allowed transgenic embryo transformed but interfered with embryo germination development in the later stage.

Though both markers are useful, the *gfp* gene was more sensitive in following Rastali (AAB) banana transformation in transient assays. Previously, we obtained similar result in our experiment in *Agrobacterium*-mediated transformation results with *gfp* gene was more efficient compared to *gusA* gene (Sreeramanan *et al.* 2006a). The number of buds tes-

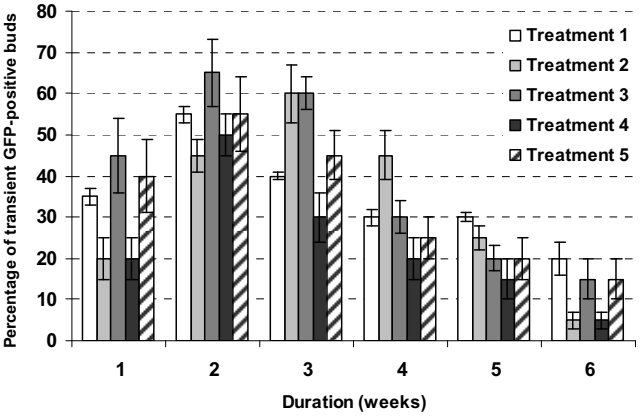


Fig. 2 Percentage of *gfp* gene expression (pGEM.*Ubi1-Sgfp*S65T) per bud obtained in different treatments. For each treatment, four replicates were used containing hundred single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

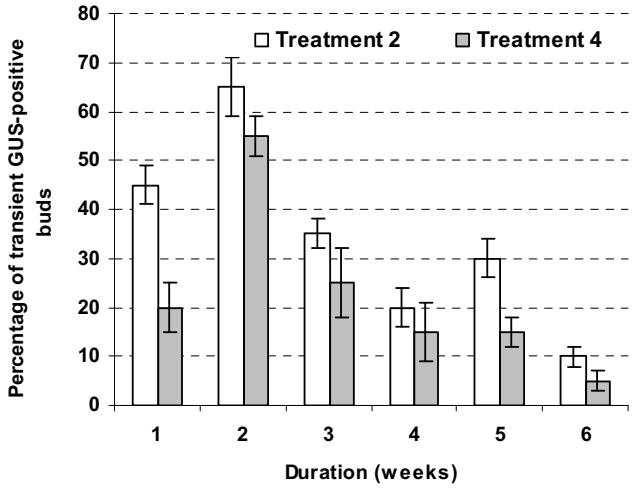


Fig. 3 Percentage of *gusA* gene expression (pMRC 1301) per bud obtained in different treatments. For each treatment, four replicates were used containing hundred single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

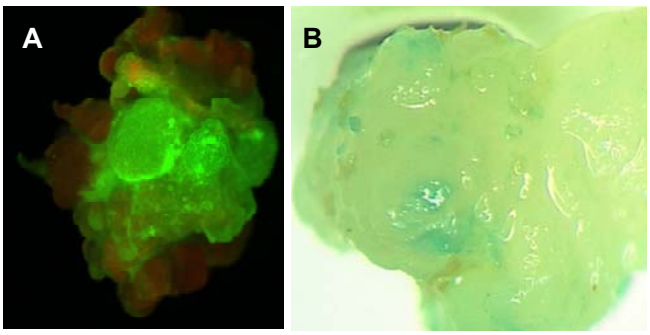


Fig. 4 Transient genes expression in single bud, two days after co-bombardment with antifungal genes. (**A**) Transient *gfp* (pGEM.*Ubi1-Sgfp*S65T) gene expression from Treatment 3; (**B**) Transient *gusA* (pMRC1301) gene expression from Treatment 2. Both pictures were taken under 80X magnification using a stereomicroscope (Leica MZFL 111).

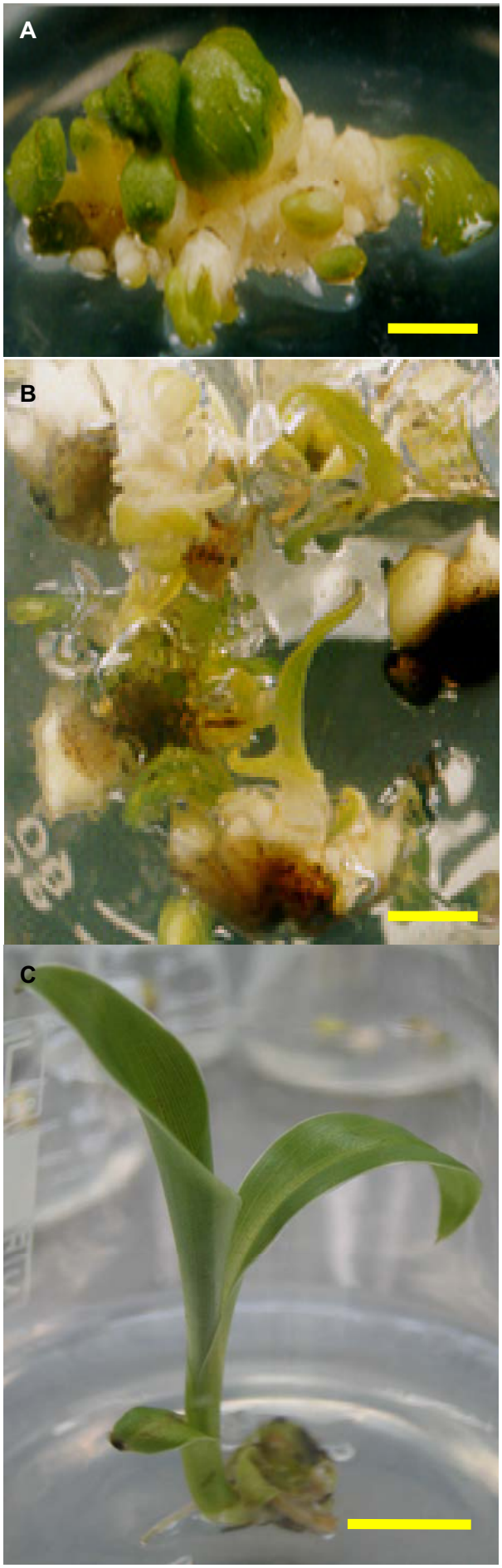


Fig. 5 Regeneration of transgenic banana plantlets, Rastali (AAB). (**A**) Three months in geneticin G-418 (50 mg.l^{-1}) selection media; (**B**) Multiple shoots proliferating in 1 mg.l^{-1} BAP; (**C**) Single regenerated banana plantlet. The bar in the bottom of each of the image represents 2 cm.

ting positive for the GFP reporter is higher than that for the GUS reporter gene. Furthermore, in buds that tested positive for either reporter (treatments 2 and 4), the number of spots with GFP expression typically is several times higher that seen with the GUS construct, indicating the greater sensitivity of detection of the *gfp* gene product (**Figs. 2, 3**).

An improved strategy for transformation in banana using single meristematic buds and plant regeneration

Selection was carried out on bombarded single buds on MS medium consisting of 10 mg.l^{-1} of BAP containing geneticin G-418 at 50 mg.l^{-1}, during a period of 4 to 8 weeks (**Fig. 5**). However, none of the negative control plates did ever give 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 mg.l^{-1} of BAP, whereas they turned to black and died after two months under selection condition even at the lower concentration tested. Percentage of transformation frequency was evaluated based on the number of buds regenerated from the total number of buds bombarded. However, regeneration frequency was highly dependent based on different treatments.

Particle bombardment with an equimolar mixture of two different plasmids pBI333-EN4-*RCC2* and pROKla-*Eg* (treatment 1) carrying two different antifungal protein gene (chitinase and β-1,3-glucanase) yielded in total of 180 shoots resistant in antibiotic selection during the first phase (50 mg.l^{-1} of geneticin G-418 in solid medium) and 140 shoots during the second phase. Only 5.25% of shoots survived after third selection. For treatment 2 (pMRC1301 and pROKla-*Eg*), a total of 153 shoots resistant were selected during the first phase (50 mg.l^{-1} of geneticin G-418 in solid medium) and 86 shoots during the second phase. Only 7.25% of shoots survived after third selection. However, some of regenerated plantlets turned black in both treatments. Transformation frequency is lower in treatment 1 (4%) compared to treatment 2 (6%). Similarly, the reduction of the regenerating potential has been described in bombarded banana cell suspension of Cavendish cultivar William (AAA) by Becker *et al.* (2000).

Transformation frequencies of the regenerated plantlets for these single plasmid transformants (treatments 3, 4 and 5) were in the same range as those for double co- transformants. So, the presence of another plasmid did not have an effect on the transformation frequencies of individual transgenes. Highest transformation frequency obtained from treatment 3 (7.50%) and the lowest from treatment 1 (4%). Factors that cannot be controlled completely but that probably contributed to this large variation include the competence for transformation of the single buds at the time of bombardment, the degree of aggregation of the coated gold particles and precise targeting of single buds.

Several studies have used GFP as a selection agent in order to improve transformation efficiency in plants (Vain *et al.* 1998; Elliot *et al.* 1999). In this study, GFP fluorescence together with geneticin G-418 was used to increase the accuracy and predictability of the banana selection process.

It has been reported that gene expression might decrease or be lost in the progeny of transgenic plants after several generative multiplication cycles or even with ageing of the plant (Cornejo *et al.* 1993). All independent lines from five treatments were analysed for GFP and GUS (treatments 2 and 4) expressions (**Figs. 6, 7**). Of the three banana plantlets tested from treatment 1, two (expression frequency of 67%) and one (expression frequency of 33%) developing bright green fluorescent in leaf and root tissues, respectively. Expression of GFP in regenerated was visually confirmed at the time of multiple bud clumps formation, shoot initiation, rooting and leaves (**Fig. 6**). Although GFP expression could be visually detected in all tissues, it was strongest in tissue containing only low amounts of chlorophyll such as cell cultures, meristematic regions, young

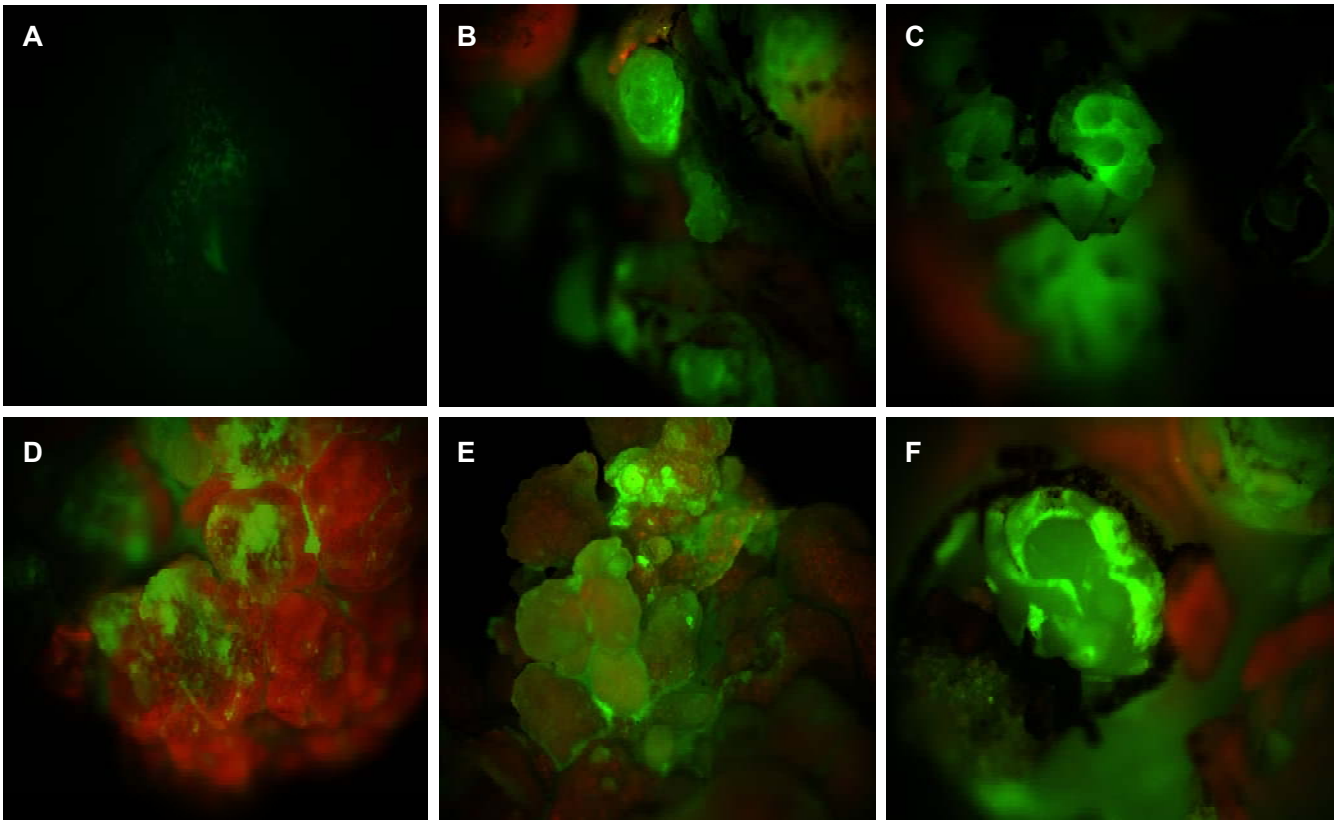


Fig. 6 Stable *gfp* gene expression in single buds and proliferating bud clumps from treatment 1 to 5. (**A**) Control explant; (**B**) Treatment 1; (**C**) Treatment 2; (**D**) Treatment 3; (**E**) Treatment 4 and (**F**) Treatment 5. Explants were viewed under a florescence microscope (Leica MZFL 111) equipped with GFP 2 filter. All pictures were taken under 60X magnification using a stereomicroscope.

plant tissues and reproductive structures of banana plantlets. From treatment 2, three (expression frequency of 75%) and one (expression frequency of 25%) expressed *gfp* gene in leaf and root tissues. However, GUS expression was slightly lower than GFP. Two plantlets (expression frequency of 50%) expressed *gusA* gene both in leaf and root tissues. For treatment 3, three (expressing frequency of 75%) and one (expression frequency of 25%) expressed *gfp* in leaf and root tissues. Both plantlets tested from treatment 4 (expression frequency of 50%) expressed *gfp* and *gusA* genes expression in leaf and root tissues, respectively. For treatment 5, one (expression frequency of 25%) and two (expression frequency of 50%) expressed GFP in leaf and root tissues. Zhu *et al*. (2004a) successfully transformed the papaya variety 'Kapoho Solo' with the *gfp* gene via microprojectile bombardment of embryogenic callus. A reduction in selection time (3-4 weeks as compared to the average 3 months experienced when using a geneticin [G-418] selection-based medium) was demonstrated, a 5- to 8-fold increase in the number of transformants (compared to antibiotic-based selection), and a 15- to 24-fold increase in transformation throughput.

No loss of GFP and GUS expressions was observed in transgenic *in vitro* leaf and root tissues at least three subcultures on selection medium. Thus, the *gfp* and *gusA* genes were not only transmitted but also stably expressed during vegetative multiplication *in vitro*. Regeneration of plants from transgenic tissue expressing GFP has been demonstrated in several different plants (Pang *et al.* 1996; Kohler *et al.* 1998), indicating that GFP has little impact on normal development embryonal cell. As the plantlets developed, GFP fluorescences were best visualized at the vascular tissue and root (**Fig. 6E, 6F**). Non-transgenic material at this stage fluoresced light red and was easily distinguished from the light to bright green of the transgenic plantlets. As the leaves developed and matured, the green fluorescence was less apparent and may have been masked by the increased red auto-fluorescence of chlorophyll. A similar observation was reported in sugarcane (Elliot *et al.* 1999).

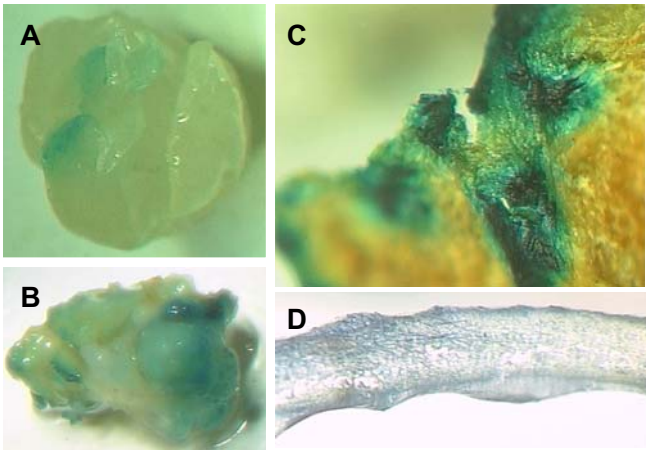


Fig. 7 Stable *gusA* gene expression on regenerating banana plantlets from treatment 2 and 4. (**A-B**) Regenerating single buds with GUS expression; GUS expression in leaf (**C**) and root (**D**). Only plasmid pMRC1301 contains *gusA* gene. All pictures were taken under 40X magnification using a stereomicroscope.

However, when the *in vitro* banana plant was at the end of its vegetative stage, it was difficult to differentiate between the leaves of the transgenic and non-transgenic as the result of the reddening of the GFP leaves by increased chlorophyll biosynthesis.

An extensive histochemical GUS analysis was carried out on different tissues of GUS expressing transformants from treatments 2 and 4 to determine if promoter regarded constitutive (rice *Act1*). When GUS expression was detected in leaf tissue of *in vitro* plantlets [Plant code: R1T2 (11) and R3T4 (7)], it was also found in all other tissues tested including the meristematic part and root hairs (**Fig. 7**). Staining of the meristem clearly demonstrated that plantlets were fully transformed and no chimaeric tissue was formed,

although a uniform blue staining was not always observed in all tissues (based on molecular analysis). Two types of GUS expression patterns in leaf tissue of plant code R1T2 (11) and R3T4 (7) can be distinguish. First, GUS expression was restricted to the leaf veins and midrib tissues (**Fig. 7C**). However, this expression pattern is not unusual to CaMV 35S promoter which is highly active in vascular tissue (Arokiaraj *et al.* 1998). Furthermore, it indicated that little or no GUS enzyme seemed to leak from GUS-expressing cells present in the veins into neighbouring cells, which rendered the histochemical assay useful for localization of GUS expression in banana leaf tissue. However, it has been reported that the variation in GUS expression could be explained by the existence of chimeras comprised of a mixture of transformed and untransformed cells, as been reported in transgenic chrysanthemum (Teixeira da Silva and Fukai 2003).

The second type of GUS expression pattern uniform as all cells of the leaf tissue including those of the lamina stained blue, while non-transformed controls were never positive. Without wounding, the X-Gluc solution did not fully penetrate and leaf disc did not stain completely blue, allowing misinterpretations of GUS expression. GUS expression was also present in longitudinal sections and root hairs of the main roots of transformants (**Fig. 6D**). Wong *et al.* (2005) reported the comparison of β-glucuronidase expression and anatomical localization in bombarded immature embryos of banana cultivar Mas (AA) via biolistic gun transformation. They reported that strong GUS staining in the deep layers of the cell structure were produced by higher acceleration pressure and shorter target distance, whereas weak GUS staining in the plant epidermis layer were observed in most lower acceleration pressure and higher target distance. The GUS and GFP expressions regarded constitutive in roots which is significantly important in developing transgenic banana tolerant to root attacking fungus such as *Fusarium oxysporum.* However, GUS expression in independent transformants could vary from confined to the leaf veins or root cylinder to a more uniform including all cells. Since *in vitro* plantlets were fully transformed, the observed differences between independent transformants might be the result of plasmid DNA integrations in different positions in the genome (position effects). These effects probably play a major role in particle bombardment transformation, since DNA integration happens in a complete random manner. Histochemical localization of GUS activity controlled by the rice *Act1* promoter equally active in all banana tissues like in cereals (maize: Zhong *et al.* 1996b; wheat: Nehra *et al.* 1994) or primarily in root meristems like in *Gladiolus* (Kamo and Blower 1999).

Verification of presence of the transgenes using polymerase chain reactions (PCR) analysis

PCR analysis was performed to confirm the presence of introduced gene (s) in putatively transformed plants. Genomic DNA extracted from *in vitro* banana plantlets derived from histochemical expressions of GFP or GUS (treatments 2 and 4) positive and geneticin G-418 resistant single buds.

Thirty-six DNA samples of resistant plantlets were picked from five treatments were subjected to PCR analyses. **Figs. 8-13** showed the results of PCR amplification with six different sets of primers of the *gfp*, *gusA*, chitinases (*RCC2* and *chi*), β-1,3-glucanase (*Eg*) and *nptII* genes. In all experiments, no bands were detected from the untransformed samples. Below is the summary of PCR results obtained from each treatment:

1. Treatment 1 (pBI333-EN4-RCC2 + pROKla-Eg)

Two [plant code: R2T1 (19) and R3T1 (7)] of eight samples assayed successfully amplified the expected band size of 726 bp for the *gfp* gene (**Fig. 8**: Lanes 2, 3), 310 bp for the *RCC2* chitinase gene (**Fig. 10**: Lanes 2, 3, 4, 6), 830 bp for the *Eg* gene (**Fig. 12**: Lanes 3, 4, 5, 6) and 900 bp for the *nptII* gene (**Fig. 13**: Lanes 2, 3).

2. Treatment 2 (pMRC1301 + pROKla-Eg)

Two [plant code: R1T2 (11) and R3T2 (24)] of five samples assayed successfully amplified the expected band size of 726 bp for the *gfp* gene (**Fig. 7**: Lanes 4, 5), 789 bp for the *gusA* gene (**Fig. 8**: Lanes 2, 3, 4, 5), 486 bp for the chitinase gene (*chi*) (**Fig. 10**: Lanes 3, 4, 5, 6, 7, 8, 9), 830 bp for the *Eg* gene (**Fig. 11**: Lanes 7, 8) and 900 bp for the *nptII* gene (**Fig. 12**: Lanes 4, 5).

3. Treatment 3 (pBI333-EN4-RCC2)

One [plant code: R2T3 (Y10)] of 11 samples assayed successfully amplified the expected band size of 726 bp for the *gfp* gene (**Fig. 10**: Lane 6). Meanwhile, plant R2T3 (Y10) and R2T3 (Y11) amplified 310 bp for the *RCC2* chitinase gene (**Fig. 10**: Lanes 7, 8, 11).

4. Treatment 4 (pMRC1301)

One [plant code: R3T4 (7)] of seven samples assayed successfully amplified the expected band size of 789 bp for the *gusA* gene (**Fig. 9**: Lanes 6, 7), 486 bp for the chitinase (*chi*) gene (**Fig. 11**: Lanes 10, 11).

5. Treatment 5 (pROKla-Eg)

One [plant code: R2T5 (42)] of five samples assayed successfully amplified the expected band size of 830 bp for *Eg* gene (**Fig. 12**: Lanes 9, 10, 11), and 900 bp for the *nptII* gene (**Fig. 13**: Lane 6).

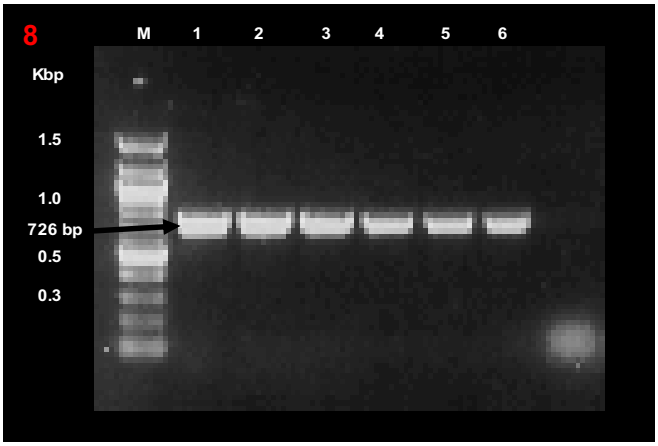
Genomic Southern blot hybridisation analysis

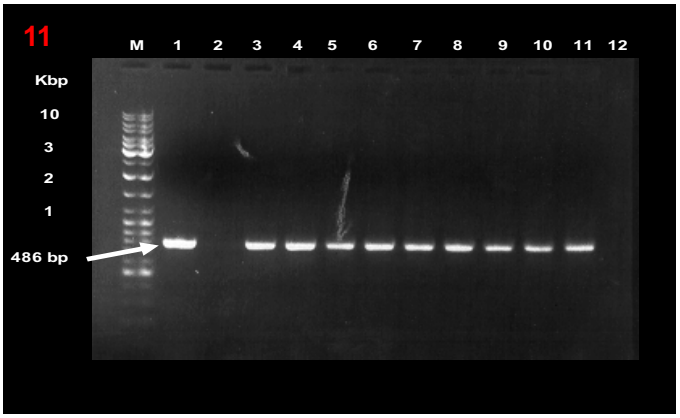
Southern blot analysis yields information on the number of integration sites per transgene and the copy number of the transgenes. For analysis of material produced by particle bombardment, there are two possible approaches. In one them, the restriction enzyme cuts only within the plant DNA and not within the vector used for transformation. The presence of multiple DNA bands indicates integration at multiple sites. The other alternative is to use a restriction enzyme that cuts only once within the plasmid, as used in this study except for pMRC1301. The genomic DNA was digested with *Hind*III, a unique cleavage site in the transgene used to estimate the number of insertion sites and hybridized with transgene specific probes. For Treatment 2 and 4 with the presence of pMRC1301, double digestions were performed using similar conditions with *Nhel* and *Kpnl* (*gusA* gene) and *Hpal* and *Kpnl* (chitinase gene).

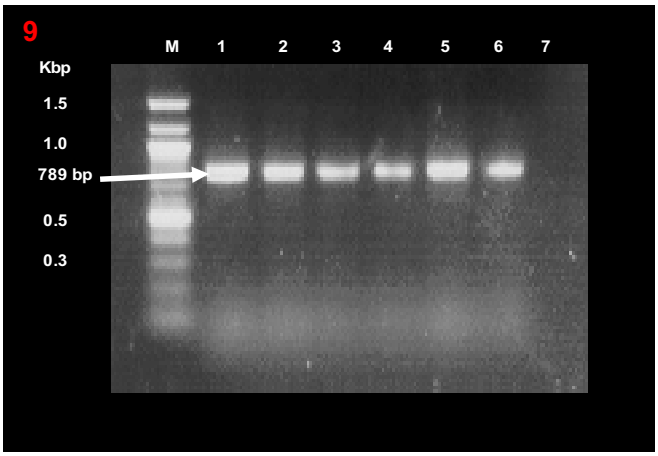
Southern blot analysis of transgenic plants revealed a set of unique and complex hybridization bands for each of the four transgenes {*gus*A (treatment 2 and 4), chitinases (*RCC2* and *chi*) and β-1,3-endoglucanase (*Eg*)}, indicating random integration of foreign DNA. Southern blots of PCR positive transgenic banana plantlets from five treatments are presented in Figure 14 to 17, with each representing a different transgene (**Fig. 14**, *gusA* gene; **Fig. 15**, *RCC2* gene; **Fig. 16**, *chi* gene and **Fig. 17**, *Eg* gene). Below is the summary of DNA analysis from Southern blots results characterised from each treatment.

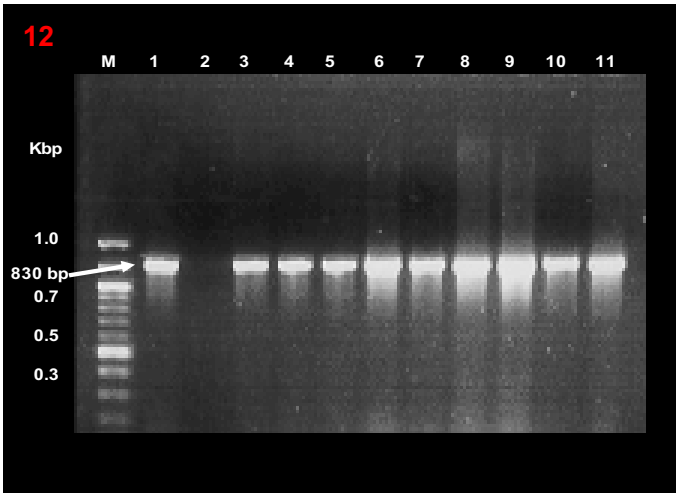
1. Treatment 1 (pBI333-EN4-RCC2 + pROKla-Eg)

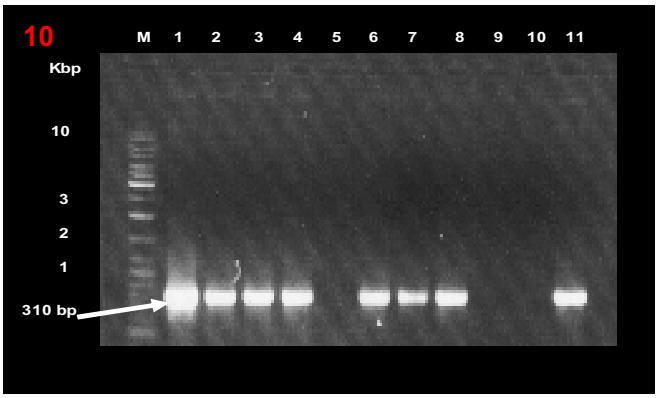
Two copies were found in each plantlet [plant code: R2T1 (19) and R3T1 (7)] for *RCC2* and *Eg* genes (**Fig. 15**: lanes 2, 3 4; **Fig. 17**: lanes 3, 4, 5, 6). Each of the sample from two plantlets successfully hybridized the expected band size of 1.4 kbp for the *RCC2* gene and 3.5 kbp for the *Eg* gene. The results obtained indicated co-transformation of both transgenes into the banana genome successful. No signals were detected in the negative control plantlets.











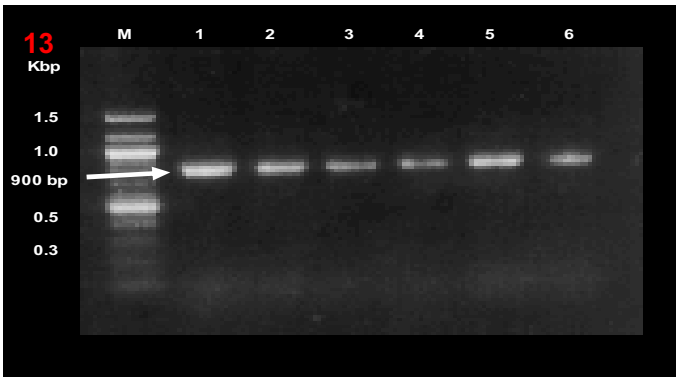


Fig. 8 PCR analysis of *gfp* gene in transgenic banana plantlets selected on geneticin G-418. M = Lambda DNA digested with *Hind*III was used as molecular weight marker; 1 = Transforming plasmid, pGEM.*Ubi1-Sgfp*S65T; 2-6 = Putative transformed plantlets showing the amplified 726 bp of *gfp* gene. **Fig. 9 PCR analysis of *gusA* gene in transgenic banana plantlets selected on geneticin G-418.** M = λ DNA digested with *Hind*III was used as the molecular weight marker; 1 = Transforming plasmid, pMRC1301; 2-6 = Putative transformed plantlets showing the amplified 789 bp *gusA* gene and 7 = non-transformed plantlet. **Fig. 10 PCR analysis chitinase gene (*RCC2*) in transgenic banana plantlets selected on geneticin G-418.** M = λ DNA digested with *Hind*III was used as the molecular weight marker; 1 = Transforming plasmid pBI333-EN4-*RCC2*; 2-4, 6-8, 11 = Putative transformed plantlets showing the amplified 310 bp *RCC2* gene; 5 = Untransformed plantlets and 9–10= chimeric plantlets. **Fig. 11 PCR analysis chitinase (*chi*) gene in transgenic banana plantlets selected on geneticin G-418.** M = λ DNA digested with *Hind*III was used as the molecular weight marker; 1 =Transforming plasmid, pMRC1301; 5 = Untransformed plantlets; 3-11 = Putative transformed plantlets showing the amplified 486 bp chitinase (*chi*) gene. **Fig. 12 PCR analysis of β-1,3-endoglucanse gene in transgenic banana plantlets selected on geneticin G-418.** M =λ DNA digested with *Hind*III was used as the molecular weight marker; 1 = Transforming plasmid, pROKla-*Eg*; 2 = untransformed plantlet; 3-11 = Putative transformed plantlets showing the amplified 830 bp of β-1,3-endoglucanse (*Eg*) gene. **Fig. 13 PCR analysis *npt11* gene in transgenic banana plantlets selected on geneticin G-418.** M =λ DNA digested with *Hind*III was used as the molecular weight marker; 1 = Transforming plasmid, pMRC 1301; 2-6 = Putative transformed plantlets showing the amplified 900 bp *npt*II gene.

2. Treatment 2 (pMRC1301 + pROKla-Eg)

Multiple copies (three to four copies each transgene) was found in one of the two plantlets from treatment 2 [plant code: R1T2 (11)] for *chi* gene (**Fig. 16**: lane 4). Except one copy of *chi1* transgene obtained with the expected band size of 3.3 kbp from plantlet treatment 2 [plant code: R3T2 (24); lanes 5, 6, 10, 11]. However one copy of β-1,3-endoglucanase and *gusA* transgenes obtained from those banana plantlets [β-1,3-endoglucanase (**Fig. 17**: lanes 7, 8, 9, 10)] and *gusA* (**Fig. 14**: lanes 2, 3, 4, 5) with the expected band size of 3.5 and 3.0 kbp. The results obtained indicated co-transformation of both transgenes into the banana genome successful in treatment 2 with strong bands especially for chitinase transgene. This probably indicates that multiple copies of the transgene had integrated into the same loci as containers in the genome.

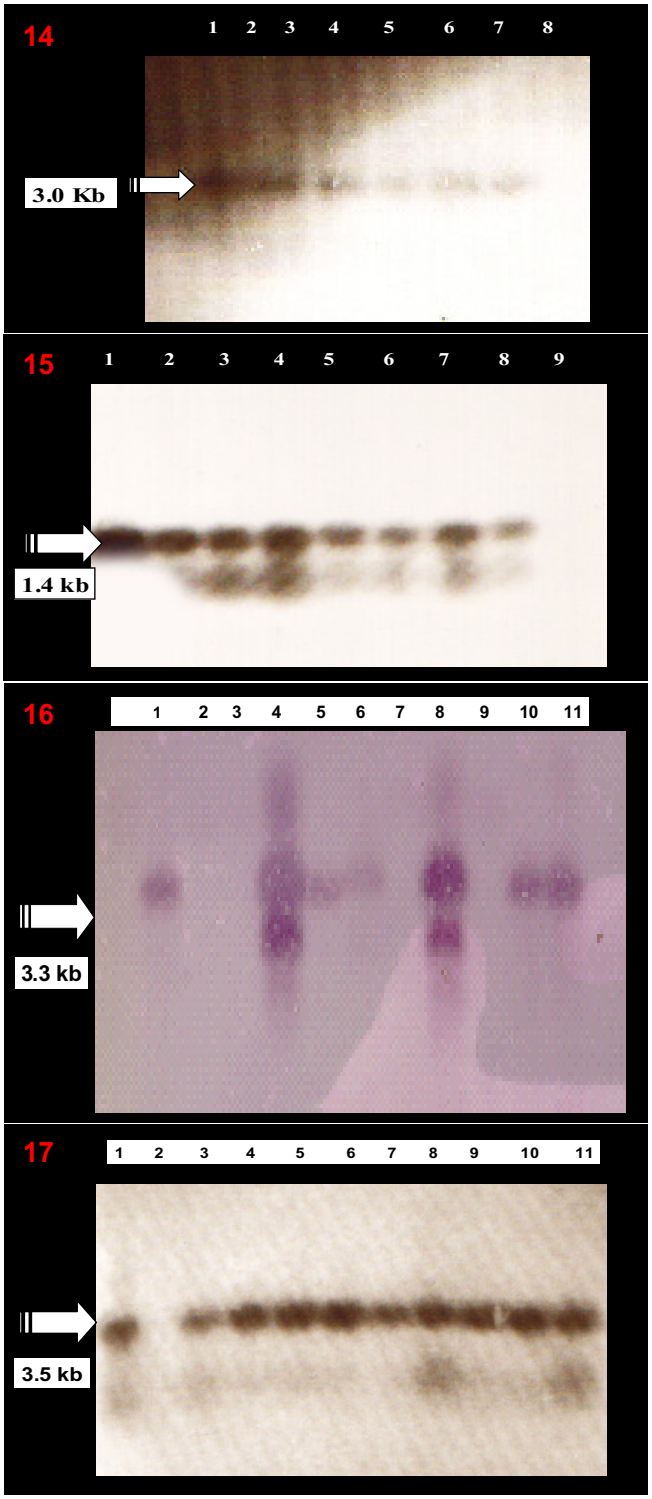


Fig. 14 Southern blot analysis of the *gusA* integration pattern in banana plantlets. Digested genomic DNA with *Nhe*I and *Kpn*I using PCR-amplified *gusA* gene (789 bp) as probe. 1 = Transforming plasmid, pMRC1301; 3-7 = genomic of putative transformed plantlets; 9 = untransformed control banana plantlets. **Fig. 15 Southern blot analysis of the chitinase integration pattern in banana plantlets.** Digested genomic DNA with *Hind*III using PCR-amplified *RCC2* gene (310 bp) as probe. 1 = Transforming plasmid, pBI333-EN4-*RCC2*; 2-8 = genomic of putative transformed plantlets; 9 = untransformed control banana plantlet. **Fig. 16 Southern blot analysis of the chitinase gene integration pattern in banana plantlets.** Digested genomic DNA with *Hpa*I *and Kpn*I using PCR-amplified chitinase gene (486 bp) as probe. 1 = Transforming plasmid, pMRC1301; 2, 3, 9 = untransformed control plantlet; 4-8, 10-11 = genomic of putative transformed banana plantlets. **Fig. 17 Southern blot analysis of the β-1,3-endoglucanse gene integration pattern in banana plantlets.** Digested genomic DNA with *Hind*III using PCR-amplified β-1,3-endoglucanse gene (830 bp) as probe. 1 = Transforming plasmid, pROKla-*Eg*; 2 = untransformed control plantlets; 3-11 = genomic of putative transformed banana plantlets.

3. Treatment 3 (pBI333-EN4-RCC2)

Two copies were found in each plantlet [plant code: B2Y (4) and B3Y 911)] for *RCC2* transgene (**Fig. 15**: lanes 5, 6, 7, 8). Each of the samples from two plantlets successfully hybridized the expected band size of 1.4 kbp for the *RCC2* gene. No signals were detected in the negative control (**Fig. 15**: lane 9).

4. Treatment 4 (pMRC1301)

One copy were found in plantlet [Plant code: R3T4 (7)] for *chi* transgene (**Fig. 16**: lane 8) and *gusA* transgenes (**Fig. 14**: lanes 7, 8). The DNA sample successfully hybridized the expected band size of 3.3 kbp for the *chi* gene and 3.0 bp for the *gusA* transgene.

5. Treatment 5 (pROKla-Eg)

One copy were found in plantlet [Plant code: R2T5 (42)] for β-1,3-endoglucanase (*Eg)* transgene (**Fig. 17**: lane 11). The DNA sample successfully hybridized the expected band size of 3.5 kbp for β-1,3-endoglucanase transgene. No signals were detected in the negative control (**Fig. 17**: lane 2).

Analysis of chitinase and β-1,3-glucanase protein production in transgenic banana plantlets

It has been observed differences in the amount of the chitinase enzyme produced in the T_0 transgenic plants of indica rice from the radiometric estimation of the chitinase protein (Kishimoto *et al.* 2002). The chitinase activity of R2T1 (19) and R3T1 (7) from treatment 1 and R1T2 (11) was four fold higher than untransformed plantlet (**Table 3**). The chitinase activity of R2T3 (11) (treatment 2) and R3T4 7 (treatment 3) was three to four folds higher than untransformed plantlet. The chitinase activity of R2T3 (10) (treatment 3) plantlet was only three fold higher than untransformed plantlet. Interestingly, the co-expression of chitinase and β-1,3-glucanase genes increased chitinase activity in transformants compared with single gene insertions. However, chitinase activity of R3T4 7 (treatment 4) was lower than the transformed plantlets by 1.5 units. The extra copy number of integrated *chi* fragment might be related to the weak expression of chitinase activity on R3T4 7 (treatment 4). Translation, mRNA expression and degradation might affect the integration and stabilty of rice chitinase protein in banana cell via biolistic gun transformation system.

The β-1,3-glucanase activity was assayed by measuring the rate of reduction of sugar production with laminarin as substrate. The β-1,3-glucanase activity in the whole plants of R2T1 (19) (treatment 1), R3T1 (7) (treatment 1), R1T2 (11) (treatment 2), R3T2 (24) (treatment 2) and R2T5 (42) (treatment 5) was measured (**Table 4**). The β-1,3-glucanase enzyme activity level was higher in transgenic plants than in the control banana plantlets.

The β-1,3 glucanase activity of R2T1 (19) and R3T1 7

Table 3 Chitinase enzyme activity in transgenic Rastali (AAB) banana plantlets transformed with chitinase gene (*RCC2* and *chi*).

Plant codes	Chitinase activity [a] (1 nkat µg protein)
R2T1 19[b]	54.2 ± 8.3
R3T1 7	48.2 ± 3.2
R1T2 11	46.4 ± 2.4
R3T2 24	41.4 ± 2.4
R2T3 Y10	38.3 ± 4.4
R2T3 Y11	36.4 ± 3.3
R3T4 7	11.4 ± 4.1
Control [c]	12.9 ± 1.8

[a] One unit of chitinase activity the amount of enzyme catalyzing the formation of 1nm.
N-acetlyglucosamine equivalent in one second under assay conditions refers as 1 nkat.
[b] Three replications were used for the assay to calculate the average activity.
[c] Control Untransformed banana Rastali (AAB).

Table 4 β-1,3-glucanase enzyme activity in transgenic banana Rastali (AAB) plantlets transformed with β-1,3-glucanase gene (*Eg*).

Plant codes	β-1,3 glucanase activity [a] (1 nkat ug protein)
R2T1 19 [b]	31.4 ± 6.2
R3T1 7	31.2 ± 3.9
R1T2 11	25.5 ± 2.7
R3T2 24	26.8 ± 2.6
R2T5 42	19.9 ± 2.2
Control [c]	10.1 ± 1.8

[a] One unit of β-1,3 glucanase activity the amount of enzyme catalyzing the formation of 1nm glucose equivalent in one second under assay conditions refers as 1 nkat.
[b] Three replications were used for the assay to calculate the average activity.
[c] Control Untransformed banana Rastali (AAB).

(treatment 1) was four-fold higher than untransformed plantlet (**Table 4**). The β-1,3-glucanase activity of R1T2 (11) and R3T2 (24) (treatment 2) was 2- to 3-fold higher than untransformed banana plantlets. The lowest β-1,3-glucanase activity detected in plant code R2T5 (42) of treatment 5 with only two fold higher than untransformed plantlet. The co-expression of two antifungal genes increased β-1,3-glucanase activity in transformants compared with single β-1,3-glucanase gene insertions from treatment 5. However, the β-1,3-glucanase activity from treatment 1 and 2 is lower than chitinase activity.

Transgenic banana plantlets obtained from biolistic gun mediated-transformation

Eight positive transgenic banana plantlets (confirmation based on PCR and genomic Southern blot analysis) obtained from particle bombardment using five different treatments. Below is the summary of final evaluation obtained for leaf disease symptoms after four weeks inoculation.

1. Treatment 1 [RCC2 (chitinase gene) + Eg (β-1,3-glucanase gene)]

Both plants [plant codes: R2T1 (19) and R3T1 (7)] appear healthy. However, plant code R3T1 (7) showed a slight yellow streaking in one leaf (lowest part) after 25 days of inoculation.

2. Treatment 2 [chi (chitinase gene) + Eg (β-1,3-glucanase gene)]

Both plants [plant codes: R1T2 (11) and R3T2 (24)] appear healthy. However, both plant showed a slight discolouration in one younger leaf (upper part) before transfer into polybags.

3. Treatment 3 [RCC2 (chitinase gene)]

Both plants [plant codes: R2T3 (Y10) and R2T3 (Y11)] showed slight streaking and yellowing of one older leaf (lower part) could be observed after 21 days of inoculation.

4. Treatment 4 [chi (chitinase gene)]

One yellowing of older leaf [plant code: R3T4 (7)] could be observed after 17 days of inoculation. Slight yellow discolouration of the third and fourth leaves (upper part) beginning to appear after 22 days of inoculation.

5. Treatment 5 [Eg (β-1,3-glucanase gene)]

This plant [plant code: R2T5 (42)] appears slight streaking and yellowing on 25% of the total leaves after 28 days of inoculation.

CONCLUSIONS

Fusarium wilt race 1 disease is a major constraint in banana cultivar, 'Rastali' (AAB) cultivation in South East Asia particularly. Since the cell wall of *Fusarium oxysporum* fsp. *cubense* has both chitin and β-1,3-glucan, combined expression of chitinase and β-1,3-glucanase genes is expected to increase Fusarium wilt race 1 tolerance to a higher level. Previously, this is thought to be a significant limiting factor in the generation of transgenic banana plants that has not been using with embryogenic callus or cell suspension cultures as a starting material for banana transformation work. Stable *gus*A and *gfp* genes expression were detectable in transformed single buds, shoots, multiple bud clumps, leaves and roots were successfully obtained. Integrative of the transgenes and stable of this system were assessed by PCR amplification of 726 bp of *gfp* gene, 789 bp of *gusA* gene, 310 bp of *RCC2* (chitinase gene), 486 bp of *chi* gene (chitinase), 830 bp of *Eg* gene (β-1,3-glucanase) and 900 bp of *npt*II gene. Genomic Southern blot hybridization analysis confirmed the incorporation of the *RCC2*, *chi* and *Eg* genes in host genome between one and five inserted transgene copies. Chitinase and β-1,3-glucanase enzyme activities of transgenic banana plantlets obtained was higher than untransformed plantlets. In addition, co-bombardment of chitinase and β-1,3-glucanase genes (treatment 1 and 2) gave a higher enzyme activity compared with single gene insertion (treatment 3,4 and 5). In conclusion, we produced transgenic banana cv. 'Rastali' (AAB) with increased tolerance to Fusarium wilt race 1 compared with the untransformed plants. Therefore, an efficient biolistic gun-mediated transformation protocol in this study and in other very similar, if not identical, studies by our group reported since at least 2002 in Colombia (Sreeramanan *et al.* 2005; Maziah *et al.* 2007a, 2007b; Sreeramanan *et al.* 2009) can facilitate further functional genomic study of this important banana cultivar. The continual need to increase food production necessitates the development and application of novel biotechnologies to enable the provision of improved banana crop cultivars in a timely and cost effective way (Kiggundu *et al.* 2003).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Masaaki Yokoshiwa from National Institute of Agrobiological Resources for generous gifts of a *RCC2* (rice chitinase), Dr. Ruslan Abdullah from Universiti Kebangsaan Malaysia (ex staff) for pMRC 1301 construct (rice chitinase), Dr. Yoji Takeuchi from Hokkaido University, Japan for pROKla-*Eg* (soybean β-1,3-glucanase) plasmid and Dr. Adrian Elliot for pGEM.*Ubi1*-s*gfp*S65T plasmid. This research supported financially by The Ministry of Science, Technology and Environmental of Malaysia through the PR project (01-02-04-0060-PR0010/041).

REFERENCES

Anfoka G, Buchenauer H (1997) Systemic acquired resistance in tomato against *Phytophthora infestans* by pre-inoculation with tobacco necrosis virus. *Physiology Molecular Plant Pathology* **50**, 85-101

Anonymous (2008) FAOSTAT database. Available online: http://www.fao.org/default.htm

Arokiaraj P, Yeang HY, Cheong KF, Hamzah S, Jones H, Coomber S, Charlwood BV (1998) CaMV 35S promoter directs β-glucuronidase expression expression in the laticeferous system of transgenic *Hevea brasiliensis* (rubber tree). *Plant Cell Reports* **17**, 621-625

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL (2000) Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv. Grand Naine via microprojectile bombardment. *Plant Cell Reports* **19**, 229-234

Bliffeld M, Mundy J, Potrykus I, Futterer J (1999) Genetic engineering of wheat for increased resistance to powdery mildew disease. *Theoretical and Applied Genetics* **98**, 1079-1086

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248-254

Chen WP, Gu X, Liang GH, Muthukrishan S, Chen PD, Liu PD, Gill BS (1998c) Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable

marker. *Theoretical and Applied Genetics* **97**, 1296-1306

Christou P (1992) Genetic transformation of crop plants using microprojectile bombardment. *Plant Journal* **2**, 275-281

Christou P, Ford T (1995) Parameters influencing stable transformation of rice immature embryos and recovery of transgenic plants using electric discharge particle acceleration. *Annals of Botany* **75**, 407-413

Cornejo M, Luth D, Blankenship KM, Anderson OD, Blechl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology* **23**, 567-581

Elliot AR, Campbell JA, Dugdale B, Brettell RIS, Grof CPL (1999) Green fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. *Plant Cell Reports* **18**, 707-714

Ganapathi TR, Higgs N, NS, Kurthi PJB, Arntzen CJ, May GD, Eck JMV (2001) *Agrobacterium*-mediated transformation of embryogenic cell suspension of the banana cultivar Rasthali (AAB). *Plant Cell Reports* **20**, 157-162

Grison R, Grezes-Besset B, Schneider M, Lucante N, Olson L, Leguay JJ, Toppan (1996) Field tolerance to fungal pathogens of *Brassca napus* constitutively expressing a chimeric chitinase gene. *Nature Biotechnology* **14**, 643-646

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Mass C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *The Plant Journal* **8**, 97-109

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* **5**, 387-405

Jongedijk E, Tigelaar H, van Roekel JSC, Bres-Vloemans SA, Dekker I, van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* **85**, 173-180

Kamo K, Blowers A (1999) Tissue specificity and expression level of *gus* under *rolD*, mannopine synthase and translation elongation factor 1 subunit α promoters in transgenic *Gladiolus* plants. *Plant Cell Reports* **18**, 809-815

Khanna H, Dong B, Kleidon J, Dale J (2004) Centrifugation-assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspension of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). *Molecular Breeding* **14**, 239-252

Kido LMH, Kido EA, Falco MC, Filho MdCS (2005) Somatic embryogenesis and the effect of particle bombardment on banana Maca regeneration. *Pesquisa Agropecuaria Brasileira* **40**, 1081-1086

Kiggundu A, Pillay M, Viljoen A, Gold C, Tushemereirwe W, Kunert K (2003) Enhancing banana weevil (*Cosmopolites sordidus*) resistance by plant genetic modification: A perspective. *African Journal of Biotechnology* **12**, 563-569

Kishimoto K, Nishizawa Y, Tabei Y, Hibi T, Nakajima M, Akutsu K (2002) Detailed analysisn of rice chitinase gene expression in transgenic cucumber plants showing different levels of disease resistance to gray mold (*Botrytis cinerea*). *Plant Science* **162**, 655-662

Kohler RH (1998) GFP for *in vivo* imaging of subcellular structures in plant cells. *Trends in Plant Science* **3**, 317-319

Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999) Transgene expression in rice engineered through particle bombardment: factors controlling stable expression and transgene silencing. *Planta* **208**, 88-97

Makarevitch I, Svitashev SK, Somers DA (2003) Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. *Plant Molecular Biology* **52**, 421-432

Marchant R, Davey MR, Lucas JA, Lamb CJ, Dixon RA, Power JB (1998) Expression of a chitinase transgene in rose (*Rosa hybrida* L.) reduces development of blackspot disease (*Diplocarpon rosae* Wolf). *Molecular Breeding* **4**, 187-194

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Biotechnology* **13**, 486-492

Maziah M, Sariah M, Sreeramanan S (2007) Transgenic banana rastali (AAB) with β-1,3-glucanase gene for tolerance to fusarium wilt race 1 disease via *Agrobacterium*-mediated transformation system. *Plant Pathology Journal* **6**, 271-282

Maziah M, Puad A, Sreeramanan S, Sariah M (2007) Production of transgenic banana cultivar, 'Rastali' (AAB) via *Agrobacterium*-mediated transformation with a rice chitinase gene. *Journal of Plant Sciences* **2**, 504-517

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 73-497

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha KK (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene contructs. *The Plant Journal* **5**, 285-297

Pang SZ, Jan FJ, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D (1996) Post-transcriptional transgene silencing and consequent topovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *The Plant Journal* **9**, 899-909

Pasakinskiene I, Paplauskiene V (1999) Floral meristems as a source of enhanced yield and quality of DNA in grasses. *Plant Cell Reports* **18**, 490-492

Pillay M, Tripathi L (2007) Genome mapping and molecular breeding in plants. In: Chittaranjan Kole (Eds) *Fruits and Nuts* (Vol 4 (15), Springer Berlin Heidelberg, pp 281-301

Remy S (2000) Genetic transformation of banana (*Musa* spp.) for disease resistance by expression of antimicrobial proteins, PhD thesis No. 420, Katholieke Universiteit Leuven, Belgium, 341 pp

Remy S, Francois I, Cammue BPA, Swennen R, Sagi L (1998a) Co-transformation as a potential tool to create multiple and durable resistance in banana (*Musa* spp.) *Acta Horticulturae* **461**, 361-365

Russell JA, Roy MK, Sanford JC (1992) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. *Plant Physiology* **98**, 1050-1056

Sagi L, Panis B, Remy S, Schoofs H, De Smet K, Swennen R, Gammue BPA (1995) Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. *Bio/Technology* **13**, 481-485

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edn), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY

Sreeramanan S, Maziah M, Abdullah MP, Sariah M, Xavier R, Nor'Aini M (2005) Physical and biological parameters affecting transient GUS and GFP expressions in banana via particle bombardment. *Asia Pacific Journal of Molecular Biology and Biotechnology* **13**, 35-57

Sreeramanan S, Marziah M, Mohd Puad Abdullah (2002) Optimization of multiple buds production in Pisang Rastali (*Musa sapientum* vv Rastali). In: *Proceedings of the 3rd National Banana Seminar*, Port Dickson, Negeri Sembilan, Malaysia, pp 31

Sreeramanan S, Maziah M, Sariah M, Abdullah MP, Xavier R (2006a) Transient expression of *gusA* and *gfp* gene in *Agrobacterium*-mediated banana transformation using single tiny meristematic bud. *Asian Journal of Plant Sciences* **5**, 468-480

Sreeramanan S, Maziah M, Sariah M, Abdullah MP, Xavier R (2006b) Bioassay method for testing Fusarium wilt disease tolerance in transgenic banana. *Scientia Horticulturae* **108**, 378-389

Sreeramanan S, Maziah M, Xavier R (2009) A protocol for *Agrobacterium*-mediated transformation of banana with a rice chitinase gene. *Emirates Journal of Food and Agriculture* **21 (2)**, 18-33

Sridevi G, Parasmeswari C, Sabapathi N, Raghupathy V, Veluthambi K (2008) Combined expression of chitinase and β-1,3-glucanase genes in *indica* rice (*Oryza sativa* L.) enhances resistance against *Rhizoctonia solani*. *Plant Science* **175**, 283-290

Stam M, Mol JNM, Kooter JM (1997b) The silence of genes in transgenic plants. *Annals of Botany* **79**, 3-12

Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). *Plant Cell Reports* **17**, 159-164

Teixeira da Silva JA, Fukai S (2003) Four gene introduction methods affect the shoot regeneration and localization of transgene expression in greenhouse stem explants and *in vitro*-grown chrysanthemum stem thin cell layers. *Journal of Biotechnology* **2 (5)**, 114-123

Tian L, Seguin A, Charest JP (1997) Expression of the green fluorescent protein in conifer tissues. *Plant Cell Reports* **16**, 267-271

Tonon C, Andreu A, Aued ME, Van Damme M, Huarte M, Daleo GR (1998) Defense reactions in two potato cultivars following infection with two races of *Phytophthora infestans*. *Potato Research* **41**, 319-325

van der Geest AHM, Petolino JF (1998) Expression of a modified green fluorescent protein gene in transgenic maize plants and progeny. *Plant Cell Reports* **17**, 760-764

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. *Theoretical and Applied Genetics* **96**, 164-169

Vuylsteke D, Swennen R, Ortiz R (1993) Development and early evaluation of Black sigatoka-resistant tetraploid hybrids of plantain (*Musa* spp., AAB group). *Euphytica* **65**, 33-42

Wong W-C, Othman RY, Khalid N (2008) Improvements in the efficiency of *Agrobacterium*-mediated transformation of embryogenic cell suspensions of banana cv. 'Mas' using a low-antibiotic liquid washing-assisted approach. *Transgenic Plant Journal* **2**, 75-85

Zhong H, Zhang S, Warkentin D, Sun B, Wu T, Wu R, Sticklen MB (1996b) Analysis of the functional activity of the 1.4 kb 5′-region of the rice actin 1 gene in stable transgenic plants of maize (*Zea mays* L.). *Plant Science* **116**, 73-84

Zhu YJ, Agbayani R, Moore PH (2004a) Green fluorescent protein as a visual selection marker for papaya (*Carica papaya* L.) transformation. *Plant Cell Reports* **22**, 660-667