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Particle Bombardment-mediated Co-transformation of Chitinase and β-1, 3 Glucanase Genes in Banana

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Abstract: An efficient transformation system for Pisang Rastali (AAB) was developed using an optimised particle bombardment device, improved antibiotic selections and co-transformations procedure and a simple protocol for regeneration of transformants. 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 glucanase the important disease tolerant genes were successfully transformed into banana together with *gfp* and *gusA* genes as reporter gene. Five different treatments using different chitinase and glucanase genes inserted singly or in combination were carried out and resulted in earlier detection of transient expression of *gfp* and *gusA* genes. Proliferating single buds were selected on geneticin G-418 (solid-liquid-solid medium) to produce a number of putatively transformed bananas. Molecular analyses such as Polymerase Chain Reactions (PCR) and Southern blot was performed to confirm the integration and expression of the introduced genes in genome. Protein assay of extract from the transgenic plantlets showed an increased in chitinase and β-1,3-glucanase enzymes activity over the untransformed plantlets. The present of particle bombardment-mediated transformation system reported here is suitable for using tiny single meristem buds to obtain fungal disease tolerant or resistant banana through genetic engineering.

Key words: Banana, Particle bombardment, GUS, GFP, Chitinase, β-1,-3 glucanase

INTRODUCTION

Banana is the world's largest fruit crop with an annual production of 58.6 million tons (Anonymous, 2001). In Malaysia, the banana has been traditionally cultivated in smallholdings and also as an intercrop. Pisang Rastali is 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 long generation time, triploidy, 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 of genetic engineering system in banana is via *Agrobacterium*-mediated and particle bombardment transformation (May *et al*., 1995; Becker *et al*., 2000; Ganapathi *et al*., 2001). In this study, particle bombardment was used for Pisang Rastali (AAB) transformation using tiny single meristem buds.

Co-transformation is a process in which genes carried on separate plasmids are mixed prior to transfer by particle bombardment (Chen *et al*., 1998). Co-transformation is a potential tool to create multiple and durable resistance in banana (Remy *et al*., 1998a). 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). Similarly, 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*.

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(1999) introduced barley seed class 11 chitinase gene (*Pr3*) driven by maize *ubi* promoter along with β-1, 3-glucanase gene in Bobwhite cultivar 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*.

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.Ubi-S*gfp*S65T) for early transient expression signal using an optimised physical and biological condition (Sreeramanan *et al*., 2005).

MATERIALS AND METHODS

Plant materials: Corm slices of *in vitro* 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). 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} (supplied by Philips TLD fluorescent light tubes). Single meristem buds (3 mm) were used as a target tissue in this transformation study.

Plasmid constructs: The three plasmid constructs used throughout this study were as follows:

pBI333-EN4-*RCC2*: The plasmid pBI333-EN4-*RCC2* contains the *npt11* gene linked to the nopaline synthase gene (*nos*). Plasmid pBI333-EN4-*RCC2* was constructed to replace the *gusA* gene of pBI121 (Clontech, USA) with the cDNA (*RCC2*) of the rice chitinase gene.

pMRC1301: The plasmid pMRC 1301 contains the *gusA* and *Chi*, *chitinase* gene, driven by the rice *actin 1* promoter. It carriers the *npt11* gene that confers kanamycin resistance.

pROKla-*Eg*: The plasmid pROKla-*Eg* contains the *npt11* gene linked to the nopaline synthase gene (*nos*) promoter and the soybean β-1,3-endoglucanase cDNA linked to the CaMV 35S (35S) promoter in the T-DNA region.

pGEM.*Ubi1*-s*gfp*s65T (GFP): The plasmid contains a *gfp* gene driven by a maize *polyubiquitin* 1 (*Ubi* 1) promoter.

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*

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. 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 mmHg vacuum pressure.

Co-bombardment: There were five treatments used in this experiment (Table 1). Plasmid pGEM.*Ubi*1-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 for two weeks. The survived explants were transferred back to the liquid medium containing 25 mg L^{-1} of geneticin G-418 for 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.

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.

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

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

Genes	Primer	Sequence	Product length
gfp (pGEM.*Ubi*1-S*gfp*S65T)	Forward	5'-ATGAGTAAAGGAGAAGAACTTTTC-3'	726 bp
	Reverse	5'-TTTGTATAGTTCATCCATGCCA-3'	
gusA(pMRC1301)	Forward	5'-CGCCGATGCAGATATTCGTA-3'	789 bp
	Reverse	5'-ATTAATGCGTGGTCGTGCAC-3'	
Chitinase (pBI333-EN4-*RCC2*)	Forward	5'-TGGATCCAGCGGCTCGTCGGTTG-3'	310 bp
	Reverse	5'-GTATAATTGCGGGACTCTAAT-3'	
Chitinase (pMRC1301)	Forward	5'-TACAACTTCAACTACGGGCCG-3'	486 bp
	Reverse	5'-ACGACTCACTATAGGGCG-3'	
β-1,3 glucanase (pROKla-*Eg*)	Forward	5'-GATGTGATATCTCCACTGACGTAAG-3'	830 bp
	Reverse	5'-GTATAATTGCGGGACTCTAAT-3'	
npt11	Forward	5'-CCCCTCGGTATCCAATTAGAG-3'	900 bp
	Reverse	5'-CGGGGGGTGGCCGAAGAACTCCAC-3'	

was done using the DNA Thermal Cycler 480 machine (Perkin-Elmer). RCC2 and *Chi* (Chitinases gene), *Eg* (β-1, 3 glucanase) and *npt11* 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 amplications 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 *gusA* 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 1 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 extention, 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 (1 min) 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 extention, followed by 72°C for 7 min. Amplification of *npt11* 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 extention, respectively. All amplified PCR products were checked on 1.2% agarose gel.

Southern blot analyses: 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*111 in buffer H (Promega). For pMRC1301 plasmid, double digestions with *Nhe*I and *Kpn*I (*gusA* gene) or *Hpa*I and *Kpn*I (chitinase gene).

Extraction and determination of total acid soluble proteins: Tissue samples (0.25 g) were homogenised in 5ml extraction buffer (0.1 M sodium buffer, 1 mM EDTA, 0.1% mercaptoethanol, pH 5.2), with 100 mg insoluble polyvinyl polypyrroline. Protein was quantified by Bradford methods (1976), at wavelength 595 nm. Total acid soluble protein was expressed in mg/g 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-acetlyglucosamine equivalent in one second under assay conditions refers as 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 1 nm glucose equivalent in one second under assay conditions refers 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

Evaluation of GFP and GUS constructs as reporter gene using co-bombardment: Co-transformation studies have important applications in the area of resistance breeding which are required for the development of a durable and

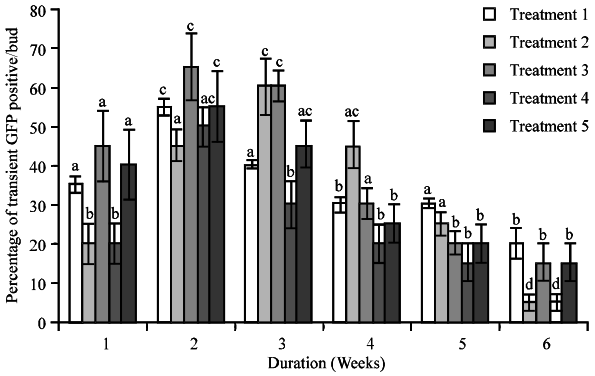

Fig. 1: Percentage of *gfp* gene expression (pGEM.*Ubi*1-S*gfp*S65T) per bud obtained in different treatments. For each treatment, four replicates were used containing hundred single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

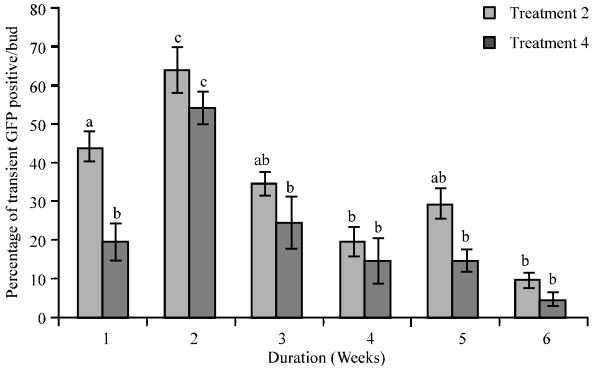

Fig. 2: Percentage of *gusA* gene expression (pMRC 1301) per bud obtained in different treatments. For each treatment, four replicates were used containing hundred single buds per replicate. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

broad-spectrum disease resistance (Punja, 2001). Since *Fusarium oxysporum* f.sp. *cubense* attack banana roots, respectively, this will probably require several antifungal proteins and perhaps different constitutive and tissue-specific promoters, which might be realized by co-transformation.

Particle bombardment with an equimolar mixture of plasmids using singly or double combinations were

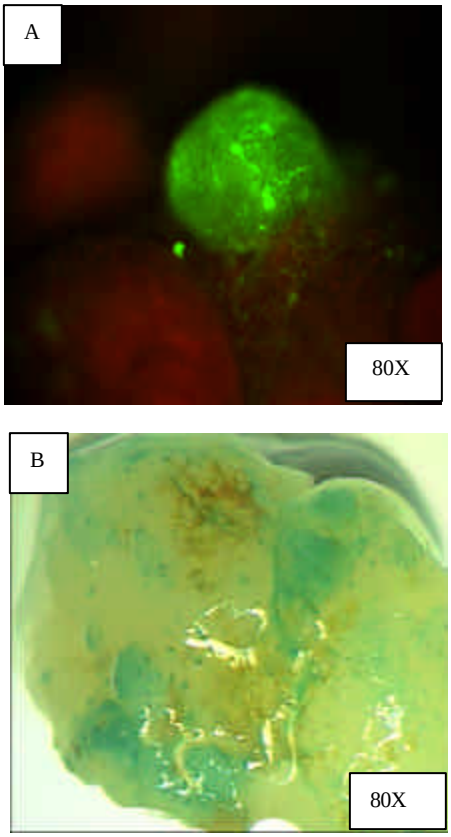

Fig. 3: Transient genes expression in single bud, two days after co-bombardment with antifungal genes. (A) Transient *gfp* (pGEM.*Ubi*-S*gfp*S65T) gene expression from Treatment 3; (B) Transient *gusA* (pMRC1301) gene expression from Treatment 2. All pictures were taken under 80X magnification using a stereomicroscope

carried out (Table 1) together with GFP construct (pGEM.*Ubi*1-s*gfp*s65T), in which the antifungal proteins and the GFP plasmid were non-linked (all plasmids used contain *npt11* gene). Expression of *gfp* gene could be observed transiently beginning 48 h post-bombardment in all treatments (Fig. 1 and 3A).

The highest *gfp* gene expression could be detected in treatment 3 between week one and week two (Fig. 1). 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. 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

Table 3: Comparison of transformation frequency based on regeneration of putative plantlets obtained from five different treatments

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
a. Total buds bombarded	400	400	400	400	400
b. Explants resistant in antibiotic selection using solid medium [a] (1st subculture)	180	153	163	131	147
c. Explants resistant in antibiotic selection using liquid medium [b] (2nd subculture)	74	86	75	78	64
d. Explants resistant in antibiotic selection using solid medium [b] (3rd subculture)	21	29	32	18	27
e. Plants regenerated in hormone free medium [c]	16	24	30	15	27
f. Transformation frequency (%)[d]	4.00	6.00	7.50	3.75	6.75

[a]Contain geneticin G-418 (50 mg L^{-1}) in 10 mg L^{-1} of BAP, [b]Contain geneticin G-418 (25 mg L^{-1}) in 5 mg L^{-1} of BAP, [c]MS medium without BAP
[d]Transformation frequency (%) = Number of explants regenerated in hormone free medium (e)/total explants bombarded (a) × 100%

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 *npt11* gene under control of a novel promoter derived from Banana Bunchy Top Virus (BBTV).

Expression of *gusA* gene could be observed transiently beginning 48 h post-bombardment in treatment 2 and 4 (Fig. 2 and 3B). 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 *gusA* expression observed in Treatment 2 compared to treatment 4 throughout six weeks of observations (Fig. 2). Similarly, GUS expressions had been observed on regenerated Multiple Bud Clumps (MBCs), shoots, leaves and roots (Fig. 5) under geneticin G-418 selection However, no continuous GUS expression could be detected after four months in regenerated plantlets.

Though both markers are useful, the *gfp* gene was more sensitive in following Pisang Rastali (AAB) transformation in transient assays. The number of buds testing positive for the GFP reporter is higher than that for the GUS reporter gene. Furthermore, in buds that tested positive for either reporter (treatment 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 (Fig. 1 and 2).

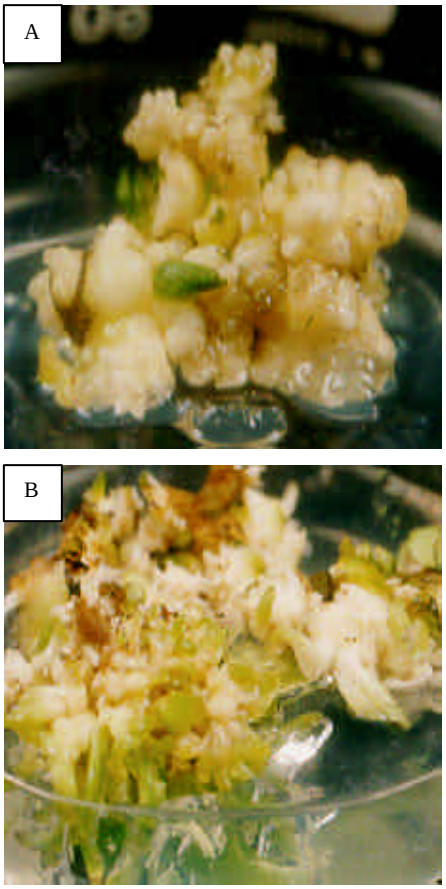

Fig. 4: Regeneration of transgenic Pisang Rastali (AAB) (A) Three months in geneticin G-418 (50 mg L^{-1}) selection media ; (B) Multiple shoots proliferating in 1 mg L^{-1} of BAP concentration. The bar in the bottom of each of the image represents 2 cm

Selection of stable transformation: 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. 4). 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 (Table 3).

Particle bombardment with an equimolar mixture of two different plasmids pBI333-EN4-*RCC2* and pROKIa-*Eg*

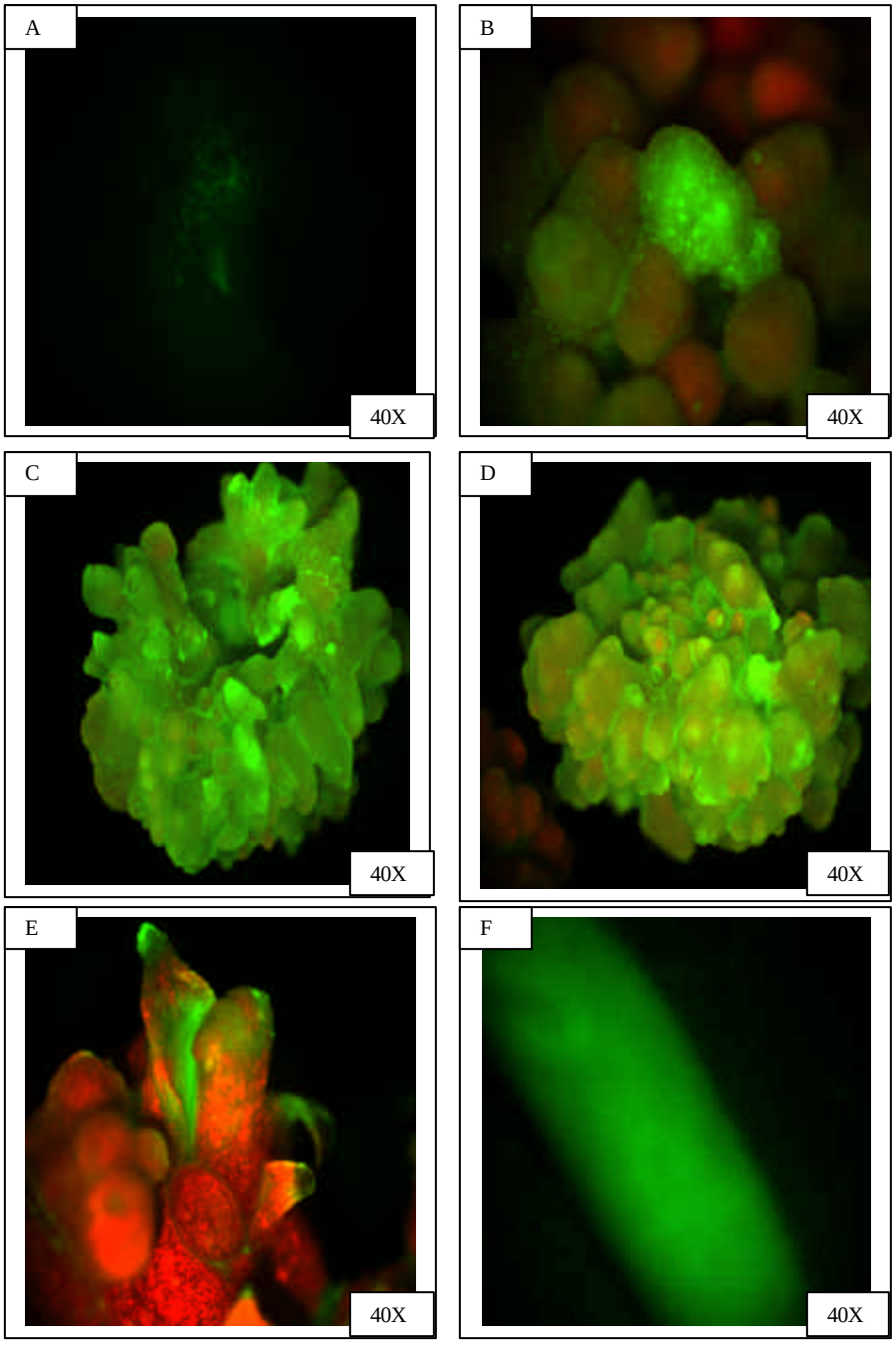

Fig. 5: Stable *gfp* gene expression in regenerating plantlets. (A) Control explant; (B) Single buds ; (C) and (D) multiple bud clumps; (E) Stable *gfp* gene expression in transformed shoot and (F) Stable *gfp* gene expression in transformed root. Explants were viewed under a florescence microscope (Leica MZFL 111) equipped with GFP 2 filter. All pictures were taken under 40X magnification using a stereomicroscope

(treatment 1) carrying two different antifungal protein gene (chitinase and β-1,3-glucanase) yielded in total of 180 explants resistant in antibiotic selection during the first phase (50 mg L^{-1} of geneticin G-418 in solid

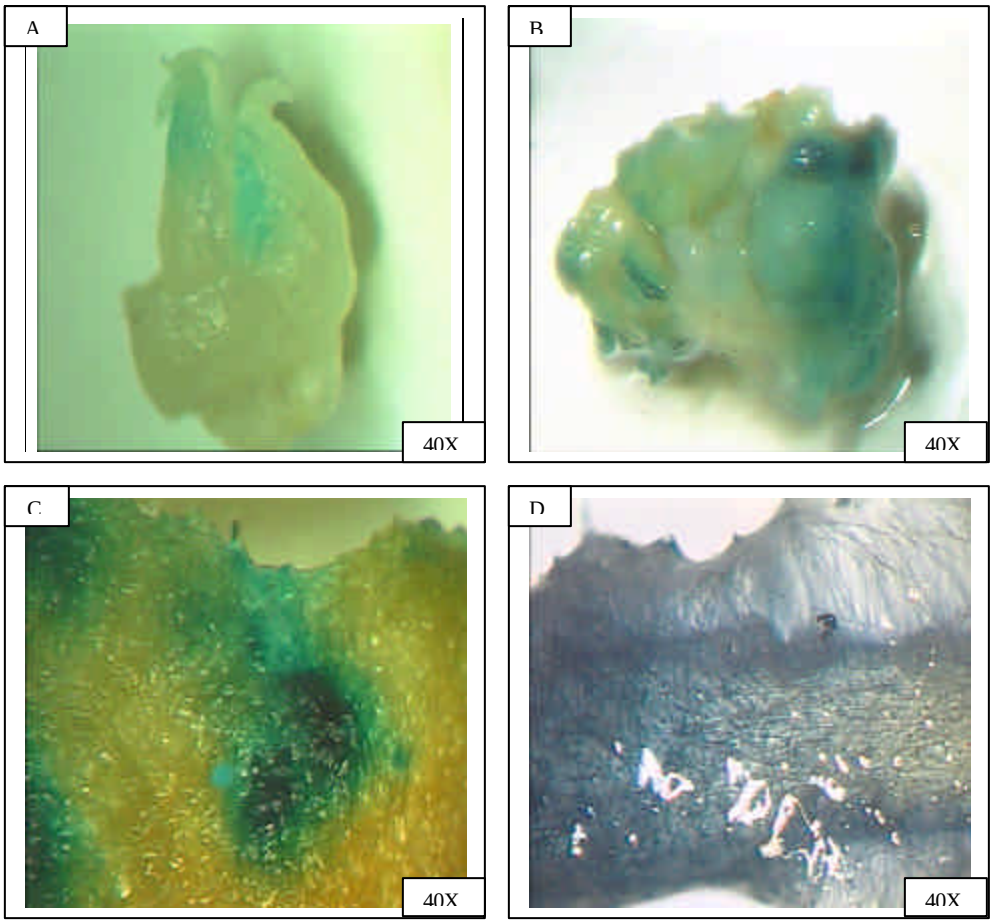

Fig. 6: Stable *gusA* gene expression on regenerating plantlets from treatment 2 and 4. (A) Regenerated single bud; (B) Single buds regenerated into clump buds; (C) Stable *gusA* gene expression in transformed leaf; (D) Stable *gusA* gene expression in transformed root. All pictures were taken under 40X magnification using a stereomicroscope

medium) and 140 explants during the second phase (Table 3). Only 5.25% (21/400) explants survived after third selection. For treatment 2 (pMRC1301 and pROKIa-*Eg*), a total of 153 explants resistant were selected during the first phase (50 mg L^{-1} of geneticin G-418 in solid medium) and 86 explants during the second phase. Only 7.25% (29/400) explants survived after third selection (Table 3). However, some of regenerated plantlets turned black in both treatments. Transformation frequency is lower in treatment 1 (4%) compared to treatment 2 (6%).

Transformation frequencies of the regenerated plantlets for these single plasmid transformants (treatment 3, 4 and 5) were in the same range as those for double co-transformants (Table 3). 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.

Interestingly, GFP expressions had been observed on regenerated multiple bud clumps (Mbcs), shoots, leaves and roots (Fig. 4) under geneticin G-418 selection. Several studies have used GFP as a selection agent in order to improve transformation efficiency in plants (Elliot *et al*., 1999; Vain *et al*., 1998). 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 (treatment 2 and 4) expressions (Fig. 5 and 6). Of the three 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. 5). 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 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.

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. 5E and F). 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). 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 chrophyll biosynthesis.

An extensive histochemical GUS analysis was carried out on different tissues of GUS expressing transformants from treatment 2 and 4 to determine if promoter regarded constitutive (rice *Act 1*).

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. 6). 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. 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. 6C). 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. 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. 5D). The GUS and GFP expressions regarded constitutive in roots which is significantly important in developing transgenic banana tolerance 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. The *gusA* gene driven by the 35S promoter was expressed in all parts of transgenic rice, but various degrees of GUS expression were measured (fluorometrically) in different organs with highest expression in roots (Peng *et al*., 1992). 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).

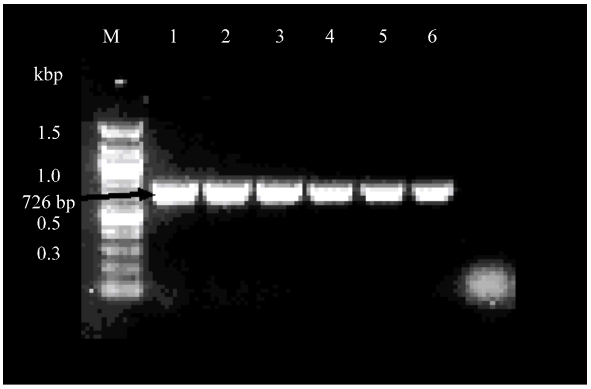

Fig. 7: PCR analysis of *gfp* gene in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hind111* was used as molecular weight marker; lane 1 = Transforming plasmid, pGEM.*Ubi*1-S*gfp*S65T; lane 2-6 = Putative transformed plantlets showing the amplified 726 bp *gfp* gene

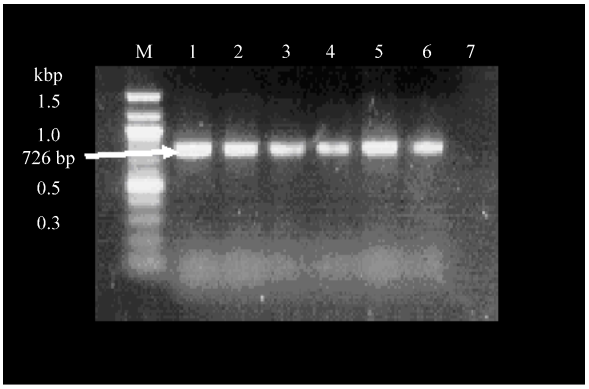

Fig. 8: PCR analysis of *gusA* gene in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hind111* was used as molecular weight marker; lane = Transforming plasmid, pMRC1301; lane 2-6 = Putative transformed plantlets showing the amplified 789 bp *gusA* gene and lane 7 = non transformed plantlet

PCR analysis of transformants: 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 (treatment 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. Figure 7-12 showed the results of PCR

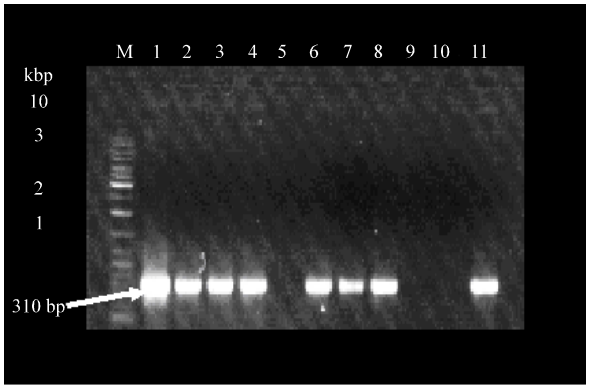

Fig. 9: PCR analysis chitinase gene (*RCC2*) in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hind11*1 was used as molecular weight marker; lane 1 = Transforming plasmid pBI333-EN4-*RCC2*; lane 2-4, 6-8, 11 = Putative transformed plantlet showing the amplified 310 bp *RCC2* gene; lane 5 = Untransformed plantlets and lane 9-10 = chimeric plantlet

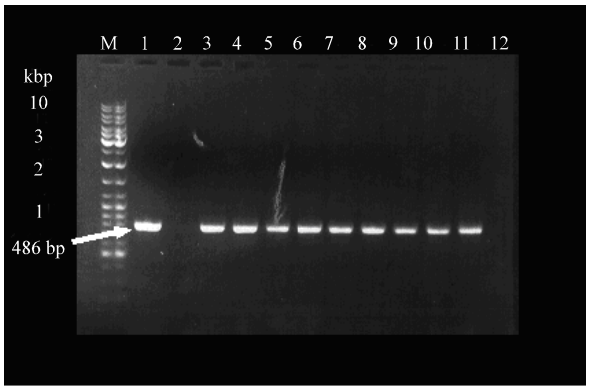

Fig. 10: PCR analysis chitinase (*Chi*) gene in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hind111* was used as molecular weight marker; lane 1 = Transforming plasmid, pMRC1301; lane 5 = Untransformed plantlets; lane 3-11 = Putative transformed plantlets showing the amplified 486 bp chitinase *(Chi)* gene

amplification with six different sets of primers of the *gfp*, *gusA*, chitinases (*RCC2* and *Chi*), β-1,3-glucanase (*Eg*) and *npt11* genes. In all experiments, no bands were detected from the untransformed samples. Below is the summary of PCR results obtained from each treatment.

Treatment 1 (pBI333-EN4-*RCC2* + pROKIa-*Eg*): Two [Plant code: R2T1 (19) and R3T1 (7)] of eight samples

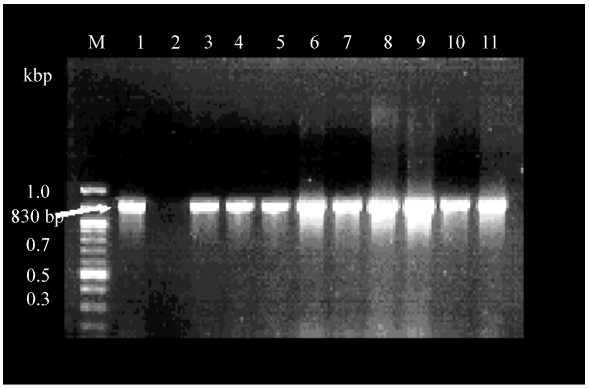

Fig. 11: PCR analysis of β-1,3-endoglucanse gene in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hindl11* was used as molecular weight marker; lane1 = Transforming plasmid, pROKla-*Eg*; lane 2 = untransformed plantlet; lane 3-11 = Putative transformed plantlets showing the amplified 830 bp of β-1,3-endoglucanse (*Eg*) gene

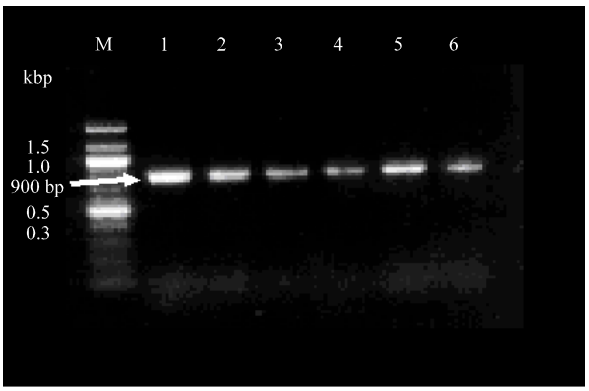

Fig.12: PCR analysis *npt11* gene in transgenic banana plantlets selected on geneticin G-418. Lane M = Lambda DNA digested with *Hindl11* was used as molecular weight marker; lane 1 = Transforming plasmid, pMRC 1301; lane 2-6 = Putative transformed plantlets showing the amplified 900 bp *npt11* gene

assayed successfully amplified the expected band size of 726 bp for the *gfp* gene (Fig. 7: Lane 2 and 3), 310 bp for the chitinase gene (Fig. 9: Lane 2, 3, 4 and 6), 830 bp for the β-1,3-glucanase gene (Fig. 11: Lane 3, 4, 5 and 6) and 900 bp for the *npt11* gene (Fig. 12: Lane 2 and 3).

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: Lane 4 and 5), 789 bp for the *gusA* gene (Fig. 8: Lane 2, 3, 4 and 5), 486 bp for the chitinase gene (*Chi*) (Fig. 10: Lane 3, 4, 5, 6, 7, 8 and 9), 830 bp for the β-1,3-glucanase gene (Fig. 11: Lane 7 and 8) and 900 bp for the *npt11* gene (Fig. 12: Lane 4 and 5).

Treatment 3 (pBI333-EN4-*RCC2*): One [Plant code: R2T3 (Y10)] of eleven samples assayed successfully amplified the expected band size of 726 bp for the *gfp* gene (Fig. 9: Lane 6). Meanwhile, plant R2T3 (Y10) and R2T3 (Y11) amplified 310 bp for the chitinase gene (Fig. 9: Lane 7, 8 and 11).

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. 8: Lane 6 and 7), 486 bp for the chitinase (*Chi*) gene (Fig. 10: Lane 10 and 11).

Treatment 5 (pROKla-*Eg*): One [Plant code: R2T5 (42)] of five samples assayed successfully amplified the expected band size of 830 bp for the β-1, 3-glucanase gene (Fig. 11: Lane 9, 10 and 11) and 900 bp for the *npt11* gene (Fig. 12: Lane 6).

Genomic Southern blot hybridisation analysis: The genomic DNA was digested with *Hindl11,* 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 {*gusA* (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 plantlets from five treatments with each representing a different transgene (Fig. 13, *gusA* gene; Fig. 14, *RCC2* gene; Fig. 15, *Chi* gene and Fig. 16, *Eg* gene). Below is the summary of DNA analysis from Southern blots results characterised from each treatment.

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. 14: lane 2, 3 and 4; Fig. 16: lane 3, 4, 5 and 6). Each of the sample from two plantlets successfully hybridized the expected band size of 1.4 bp for the *RCC2* gene and 3.5 bp for the β-1, 3-endoglucanase gene. The results obtained indicated

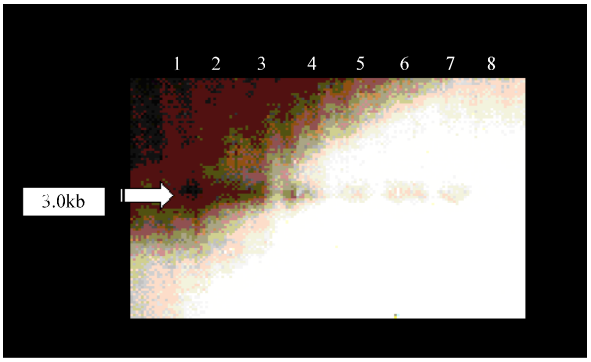

Fig. 13: Southern blot analysis of the *gusA* integration pattern in banana plantlets. Digested genomic DNA with *Nhel* and *Kpnl* using PCR amplified *gusA* gene (789 bp) as probe. Lane 1 = Transforming plasmid, pMRC1301; lane 3-7 = genomic of putative transformed plantlets and lane 9 = untransformed control plantlets

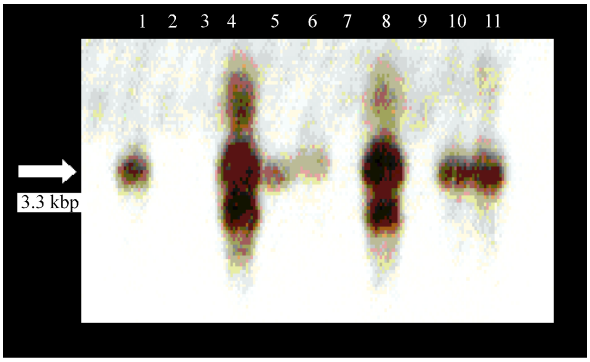

Fig. 15: Southern blot analysis of the chitinase gene integration pattern in banana plantlets. Digested genomic DNA with *Hpal and Kpnl* using PCR amplified chitinase gene (486 bp) as probe. Lane 1 = Transforming plasmid, pMRC1301; lane 2, 3 and 9 = untransformed control plantlet; lane 4-8, 10-11 = genomic of putative transformed plantlet

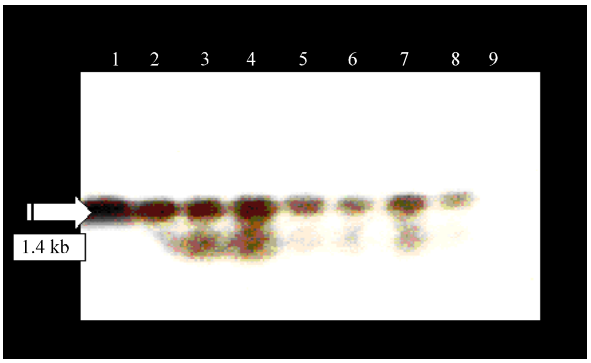

Fig. 14: Southern blot analysis of the chitinase integration pattern in banana plantlets. Digested genomic DNA with *Hind111* using PCR amplified *RCC2* gene (310 bp) as probe. Lane 1 = Transforming plasmid, pBI333-EN4-*RCC2*; lane 2-8 = genomic of putative transformed plantlets and lane 9 = untransformed control plantlet

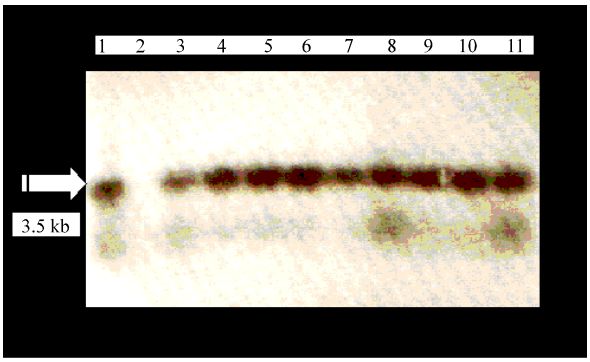

Fig. 16: Southern blot analysis of the β-1,3-endoglucanse gene integration pattern in banana plantlets. Digested genomic DNA with *Hind111* using PCR amplified β-1,3-endoglucanse gene (830 bp) as probe. Lane 1 = Transforming plasmid, pROKla-*Eg*; lane 2 = untransformed control plantlets; lane 3-11 = genomic of putative transformed plantlet

co-transformation of both transgenes into the banana genome successful. No signals were detected in the negative control.

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. 15: lane 4). Except one copy of *Chi* transgene obtained with the expected band size of 3.3 bp from plantlet treatment 2 [Plant code: R3T2 (24); lane 5, 6, 10 and 11]. However one copy of β-1,3-endoglucanase and *gusA* transgenes obtained from plantlets with this treatment (Fig. 16: lane 7, 8, 9 and 10)) and *gusA* (Fig. 13: lane 2, 3, 4 and 5) with the expected band size of 3.5 and 3.0 bp. 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 contamers in the genome.

Treatment 3 (pBI333-EN4-*RCC2*) Two copies were found in each plantlet [Plant code: B2Y (4) and B3Y 911)] for *RCC2* transgene (Fig. 14: lane 5, 6, 7 and 8). Each of

Table 4: Chitinase enzyme activity in transgenic Pisang Rastali (AAB) plantlets transformed with chitinase gene (*RCC2* and *Chi*)

Plant code	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 1 nm 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 Pisang Rastali (AAB)

Table 5: β-1,3 glucanase enzyme activity in transgenic Pisang Rastali (AAB) plantlets transformed with β-1,3 glucanase gene (*Eg*)

Plant code	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 1 nm 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 Pisang Rastali (AAB)

the samples from two plantlets successfully hybridized the expected band size of 1.4 bp for the *RCC2* gene. No signals were detected in the negative control (Fig. 14: lane 9).

Treatment 4 (pMRC1301): One copy were found in plantlet [Plant code: R3T4 (7)] for *Chi* transgene (Fig. 15: lane 8) and *gusA* transgenes (Fig. 13: lane 7 and 8). The DNA sample successfully hybridized the expected band size of 3.3 bp for the *Chi* gene and 3.0 bp for the *gusA* transgene.

Treatment 5 (pROKIa-*Eg*): One copy were found in plantlet [Plant code: R2T5 (42)] for β-1,3-endoglucanase transgene (Fig. 16: lane 11). The DNA sample successfully hybridized the expected band size of 3.5 bp for β-1,3-endoglucanase transgene. No signals were detected in the negative control (Fig. 16: lane 2).

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 4). 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 particle bombardment 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 5). The β-1,3 glucanase enzyme activity level was higher in transgenic plants than in the control plants.

The β-1,3 glucanase activity of R2T1 (19) and R3T1 7 (treatment 1) was four fold higher than untransformed plantlet (Table 5). The β-1,3 glucanase activity of R1T2 (11) and R3T2 (24) (treatment 2) was 2 to 3 fold higher than untransformed plantlet. 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.

CONCLUSIONS

Co-transformation of chitinase and β-1,3-glucanase genes was successfully carried out. Stable *gusA* 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 *npt11* gene. Genomic Southern blot hybridization confirmed the incorporation of the *RCC2*, *Chi* and *Eg* genes in host genome between one and five inserted copies. Chitinase and β-1,3-glucanase enzyme activities of transgenic plantlets obtained from particle bombardment 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). The above transformation

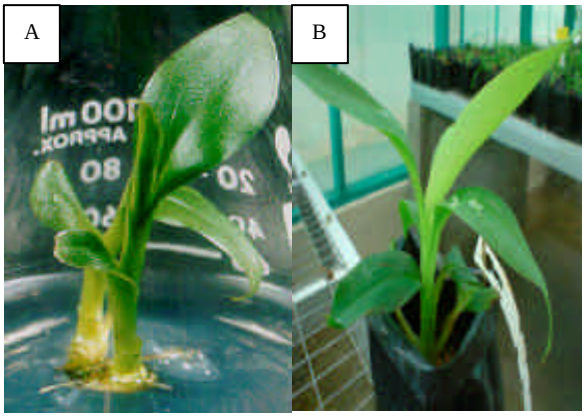

Fig. 17: Production of transgenic Pisang Rastali (AAB) (A) Putative transgenic plantlets confirmed from PCR and Southern blot analyses; (B) Transgenic banana in the glasshouse

technology has a great potential for molecular improvement of banana especially to tolerance Fusarium wilt disease. Experimental are now in progress to assay for the tolerance of transgenic banana against *Fusarium oxysporum* (race 1) exposure in potted plants in the glasshouse (Fig. 17).

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