Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 2 (5): 504-517, 2007
ISSN 1816-4951

Production of Transgenic Banana Cultivar, Rastali (AAB) via *Agrobacterium*-mediated Transformation with a Rice Chitinase Gene

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Abstract: A rice chitinase gene (*RCC2*) multiplied in *Agrobacterium* strain (EHA 101), was simultaneously introduced into single buds of *in vitro* grown banana cultivar, Rastali (AAB). Plasmid pBI333-EN4-*RCC2* contained a hygromycin phosphotransferase gene (*hpt*11) as the selectable marker to identify the transformants. Treatment A contained hygromycin at 25 mg L^{-1} and treatment B contained hygromycin at 50 mg L^{-1} in both MS medium supplemented 5 mg L^{-1} of BAP together with 2.7 g of gelrite agar. Single buds derived from multiple bud clumps (Mbcs), were the target explants for transformation. An assay was performed to identify the minimum concentration required for two antibiotics (carbenicillin and cefotaxime) that is most effective against *Agrobacterium* strain, EHA 101 and the effect on tissue regeneration capacity. Even though the transformation frequency based on hygromycin selection medium (treatment A) is higher, but there is no transformant could be confirmed based on PCR and Southern blot analyses, as compared using 50 mg L^{-1} hygromycin selection medium. Assay of protein extract from the transgenic plantlets showed an increased in chitinase enzyme activity over the untransformed plantlets. The present of *Agrobacterium*-mediated transformation reported here is suitable for using tiny meristem tissues to obtain fungal disease tolerant or resistant banana through genetic engineering.

Key words: Single buds, hygromycin, chitinase, transgenic banana

INTRODUCTION

Banana were considered outside the host range of *Agrobacterium*, but an increasing amount of research reports indicates that banana can be transferred by *Agrobacterium tumefaciens* (May *et al*., 1995; Ganapathi *et al*., 2001; Sreeramanan *et al*., 2006). These reports include studies on transient expression of transferred genes, stable transformation, regeneration of transformed plants and heritability of transgenes.

Application of *Agrobacterium*-mediated transformation method in monocotyledons plants, with some minor modifications, resulted in the successful production of transgenic rice cultivars (Aldemita and Hodges, 1996; Dong *et al*., 1996; Rashid *et al*., 1996). In addition to these results obtained in rice, efficient *Agobacterium*-mediated transformation of maize (Ishida *et al*., 1996; Frame *et al*., 2002), Anthurium (Chen and Kuehnle, 1996), sugarcane (Arencibia *et al*., 1998), barley (Wu *et al*., 1999),

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orchids (Belarmino and Mii, 2000; Yu *et al*., 2003; Liau *et al*., 2003), sorghum (Zhao *et al*., 2000) and wheat (Wu *et al*., 2003) has been demonstrated. May *et al*. (1995) reported transformation of banana using meristems and corm slices explants from cultivar Grand naine (AAA).

Antioxidants used such as L-Cysteine during pre-culture, infection and during and after co-cultivation were reported to favor stable transgenic plants recovery in *Agrobacterium*-mediated transformation experiments (Enriquez-Obregon *et al*., 1999). Olhoft and Somers (2001) concluded that the increase in host-*Agrobacterium* compatibility mediated by the presence of antioxidants during transformation moderated the detrimental effect of hypersensitive response, which in turn lead to an increase in the survival rate of *Agrobacterium tumefaciens*-infected cells and a corresponding rise in stable transformation efficiency in soybean. Frame *et al*. (2002) reported that L-Cysteine in co-cultivation medium function to minimize cell death caused by the hypersensitive response of maize scutellum cells to *Agrobacterium* infection.

Generally, it is known that plants have defence systems which involve pathogenesis-related proteins such as chitinase (Nishizawa *et al*., 1999). Chitinase catalyzes the hydrolysis of β-1, 4 linkages of the N-acetyl-D-glucosamine polymer, chitin. The transgenic tobacco and canola which have been engineered with bean endochitinase gene were shown to exhibit resistance to *Rhizoctonia solani* (Broglie *et al*., 1991). The transgenic rice integrated with rice endochitinase driven by the 35S promoter also shown enhanced resistance to sheath blight (Lin *et al*., 1995) and the transgenic tobacco harboring rice endochitinase gene (Nishizawa *et al*., 1993) also possessed increased resistance against powdery mildew (*Erysiphe cichoracearum*).

This study describes a series of experiments that carried out using EHA 101 contained plasmid with chitinase gene (pBI333-EN4-*RCC2*). Comparisons between using hygromycin in two different concentrations were demonstrated in this study since both plasmid contained *hpt*11 gene. The inhibition of explants and bacterial growth by carbenicillin and cefotaxime were also presented here.

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). Single buds were used as a target tissue for transformation studies.

Agrobacterium Cultures

Disarmed *Agrobacterium tumefaciens* strain, EHA 101 (Hood *et al*., 1986) and the binary vector (pBI333-EN4-*RCC2*) contains the *hpt 11* gene linked to the nopaline synthase gene (*nos*). A binary vector, pBI333-EN4-*RCC2* was constructed to replace the *gusA* gene of pBI121 (Clontech, USA) with the cDNA (*RCC2*) of the rice chitinase gene (Nishizawa *et al*., 1993) (Fig. 1). Plasmid pBI333-EN4-*RCC2* was integrated into *Agrobacterium tumefaciens*, EHA 101 by heat shock method.

Agrobacterium cultures were streaked out on LB medium supplemented with 50 mg L^{-1} of hygromycin and grown at 28°C for colonies to form. Single colony was transferred to 20 mL LB liquid

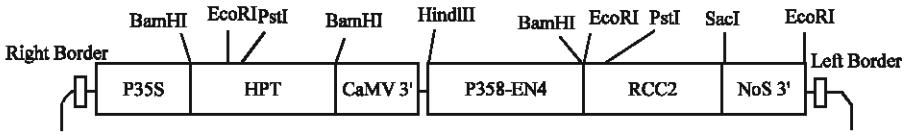

Fig. 1: Diagram of T-DNA region in pBI333-EN4-*RCC2*

contained the same antibiotic and the culture was allowed to shake at 28°C and 120 rpm for 20 h. After adjusting the Optical Density to 0.7 units at 600 nm (OD_{600}), the bacterium culture was used for transformation.

Explant and Bacterial Toxic Effects of Carbenicillin and Cefotaxime

For explant toxicity tests, single buds of 3 mm in size were transferred to MS medium contain 5 mg L^{-1} of BAP and different concentrations of carbenicillin and cefotaxime (0, 100, 200, 300, 400, 500 and 600 mg L^{-1}). In order to examine the bactericidal effects of carbenicillin and cefotaxime, *Agrobacterium* strains EHA 101 (pBI333-EN4-*RCC2*) was used. After 30 min co-cultivation of explants in bacterial cultures, the percentage of single buds with *Agrobacterium* strains were determined after three days using various carbenicillin and cefotaxime concentrations.

Transformation Protocols of Single Meristematic Buds

Single buds (3 mm) were precultured for three days prior to *Agrobacterium* infection. The explants injured mildly using scalpel. About 100 single buds immersed in *Agrobacterium* suspension for 30 min together with an optimized acetosyringone concentration at 100 μM. Acetosyringone is known to activate the virulence genes of the Ti plasmid and to initiate the transfer of the T-DNA. The explants were then blotted dry on sterile filter paper and cocultivated for three days on hormone free MS medium (without $CaCl_2$ during co cultivation) designated as M1 medium containing 100 μM of acetosyringone, 60 mM D-(+)-glucose, 2 mM sodium phosphate and 40 mg L^{-1} of L-Cysteine using Petri dish at 22°C temperature in the dark condition. After co-cultivation period for three days, the buds were transferred on same medium but without acetosyringone concentration together with 300 mg L^{-1} of carbenicillin or 200 mg L^{-1} cefotaxime designated as M2 medium for 5 days and transferred back on hormone free MS liquid medium with the same antibiotic concentrations for another 5 days. Then, the buds were transferred to MS solid medium containing 10 mg L^{-1} of BAP with 25 or 50 mg L^{-1} of hygromycin for 2 weeks and transferred again on liquid medium with the same treatments for 5 days in 100 mL conical flasks. During this period, death buds were removed before subcultured back on M2 medium. The transformed buds regenerated into clumps which are known as multiple bud clumps (Mbcs). Single buds were separated from multiple bud clumps and subcultured onto solid MS medium with 1 mg L^{-1} of BAP until the single plants were regenerated. All plants regenerated from each putatively independent transformed bud line were maintained under *in vitro* conditions.

Polymerase Chain Reactions (PCR)

Genomic DNA was extracted from putative transformants 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). The following primers were used to amplify the transgenes and part of the regulatory sequences (Table 1).

PCR amplications were carried out in 100 μl reactions volume containing template DNA (500 ng genomic DNA or 60ng 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

Table 1: Genes, primer, primer sequences and expected product length

Genes	Primer	Sequence	Product length (bp)
Chitinase	Forward	5'T-GGATCCAGCGGCTCGTCGGTTG-3'	310
	Reverse	5'-GTATAATTGCGGGACTCTAAT-3'	
hpt 11	Forward	5'-CCCCTCGGTATCCAATTAGAG-3'	900
	Reverse	5'-CGGGGGGTGGCCGAAGAACTCCAC-3'	

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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. Amplication of *hpt11* 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 of PCR products were checked on 1.2% agarose gel.

Southern Blot Analyses

A nonradioactive method was used to confirm stable integration of chitinase (*RCC2*) transgene in the host banana genome. Genomic DNA (20 μg) from PCR positive transformants were subjected to digestion with *Hind*111 in buffer H (Promega). DIG DNA Labeling and Detection Kit (Roche) were used in this study.

Extraction and Determination of Total Acid Soluble Proteins

Tissue samples (0.25 g) were homogenised in 5 mL extraction buffer (0.1 M sodium buffer, 1 mM EDTA, 0.1% mercaptoethanol, pH 5.2), with 100 mg insoluble polyvinyl polypyrroline. The homogenate was spun 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 (1976) methods, at wavelength 595 nm. Three replications were applied to all treatments and extraction buffer was used as a blank reference. 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-acetlyglucosamine 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 the level 5% using SPSS 10.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Toxicity of Carbenicillin and Cefotaxime on Single Buds and *Agrobacterium* Growth

For effective *Agrobacterium*-mediated transformation, the antibiotic regime should control bacterial growth without inhibiting the regeneration of the plant cells. The effect of carbenicillin and cefotaxime on the percentage of single buds growth, were evaluated after four weeks of culturing on the solid and liquid medium containing an appropriate concentrations (Fig. 2 and 3). No significantly differences were observed between the cultures when the percentage number of single buds growth was counted.

However, using 600 mg L^{-1} cefotaxime in solid medium reduced average growth from 100% (control) to 30%. The percentage of growth in liquid medium using same concentration is lower 12% than solid medium. In comparison to carbenicillin, using 600 mg L^{-1} reduced average growth from 62% in solid medium and 54% in liquid medium. The percentage of growth is reduced to 8 and 12% in solid and liquid of 150 mg L^{-1} carbenicillin. However in cefotaxime, percentage of growth dropped tremendously to 82% in solid medium and 63% in liquid medium using the same concentration of 600 mg L^{-1}.

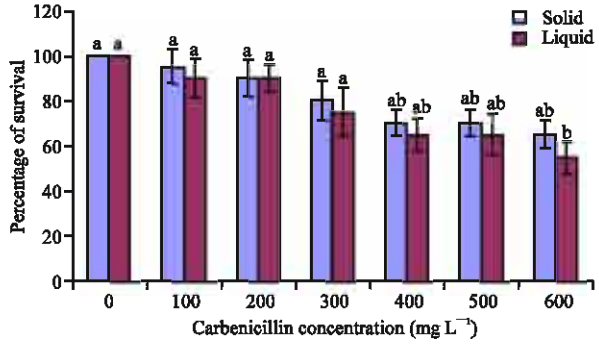

Fig. 2: Percentage of single buds growth obtained after 4 weeks in MS media using different carbenicillin concentration. The data plotted were the means of four replicates. Different letters() indicate values are significantly different ($p<0.05$)

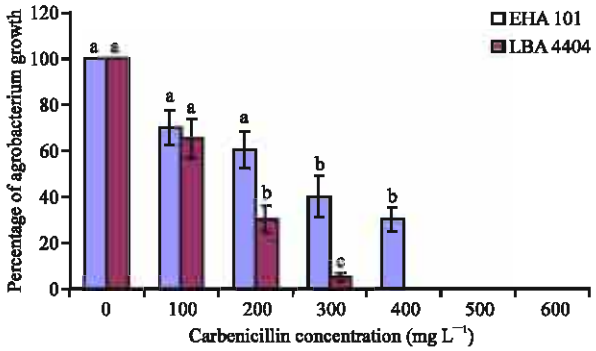

Fig. 3: Percentage of single buds growth obtained after 4 weeks in MS media using different cefotaxime concentration. The data plotted were the means of four replicates. Different letter(s) indicate values are significantly different ($p<0.05$)

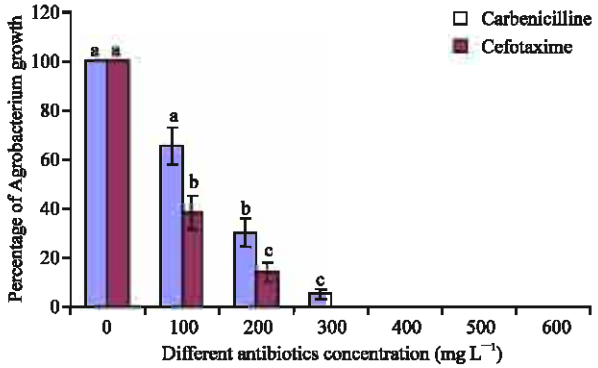

Fig. 4: Percentage of single buds with *Agrobacterium tumefaciens*, EHA 101 growth using with different carbenicillin and cefotaxime concentrations. The data plotted were the means of four replicates. Different letter(s) indicate values are significantly different ($p<0.05$)

In order to examine the bactericidal effects of carbenicillin and cefotaxime, *Agrobacterium* strains EHA 101 were inoculated with different concentrations of antibiotics. Using carbenicillin, the inhibition

of cell growth started at a concentration of 100 mg L^{-1} for both strains, but complete inhibition obtained using 400 mg L^{-1} for EHA 101 (Fig. 4). For cefotaxime, the inhibition of cell growth started at 100 mg L^{-1} and complete inhibition of cell growth at 300 mg L^{-1} for *Agrobacterium* strains.

The results demonstrated that, *Agrobacterium* cells were more sensitive to cefotaxime than carbenicillin.

Although cefotaxime is more effective in inhibiting *Agrobacterium* cell growths, carbenicillin is the antibiotic of choice in *Agrobacterium*-mediated transformation because cefotaxime has shown high toxicity in many different plant tissues (Lin *et al*., 1994; Antunez de Mayolo *et al*., 2003). In cacao, the addition of the cefotaxime decreased somatic embryo production by 86% (Antunez de Mayolo *et al*., 2003). However, it has been reported that cefotaxime did not show negative effect in apple tissues (Maximova *et al*., 1998), adventitious buds and shoots induction in *Pinus radiate* (Holland *et al*., 1997) and chrysanthemum (Teixeira da Silva and Fukai, 2002). The other antibiotic, augmentin, timentin and moxalactam were also used after co-cultivation to kill the *Agrobacterium* as it was not reduce regeneration capacity (Park and Facchini, 2000; Antunez de Mayolo *et al*., 2003).

Therefore, different concentration of carbenicillin and cefotaxime required in solid and liquid medium of banana cultivar, Rastali (AAB) explants in order to avoid toxicity or overgrowth of *Agrobacterium tumefaciens* which could inhibit the explants growth and caused contamination which could reduces transformation efficiency.

Transformation of Single Meristematic Buds and Plant Regeneration

Single buds of banana cultivar, Rastali (AAB) were infected and co-cultivated with induced *Agrobacterium* strains harboring different binary vectors, following the condition described in materials and methods. Co-cultivated single buds were selected on MS medium consisting of 10 mg L^{-1} of BAP containing hygromycin at 25 mg L^{-1} (treatment A) or hygromycin at 50 mg L^{-1} (treatment B), during a period of 2 to 3 months, depending on the experiments. Selection medium containing hygromycin at 50 mg L^{-1} produces lower number of buds resistant to this antibiotic for both *Agrobacterium* strains, EHA 101. 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 explants regenerated. Transformation frequency of *Agrobacterium*, from treatment A is higher than treatment B. More than 30 cell clusters arose per individual co-cultivated sample after selection with many of which initiated the formation of multiple bud clumps, while the rest of untransformed cells which did not stand selection, blackened and died (Fig. 5).

After first level of selections, the remaining proliferation buds were subcultured into liquid medium containing 10 mg L^{-1} of BAP using the same concentration of antibiotics that had been used previously in first selections. After one week, the remaining healthy proliferating buds then selected in same concentration of selections in solid MS medium. Multiple bud clumps aggregates formed during the selective process were regenerated in BAP and antibiotics free medium. Single plantlets arising from hygromycin resistant cell clumps in hormone free (without BAP) formed shoots and root tips without any necrosis been observed. The observation patterns revealed that the tissue were fully transgenic without evidence of chimaeras. However, this is not true for explants regenerated *in vitro* using kanamycin as a selection marker in some of the transgenic plants experiment. Some of transgenic plantlets produced albinos and semi-necrosis growth in kanamycin selection which caused production of phenotypically abnormal plants confirmed observations in peas (Bean *et al*., 1997; Nadolska-Orcyzk and Orczyk, 2000). Similarly, kanamycin effect has been reported in transgenic papaya by

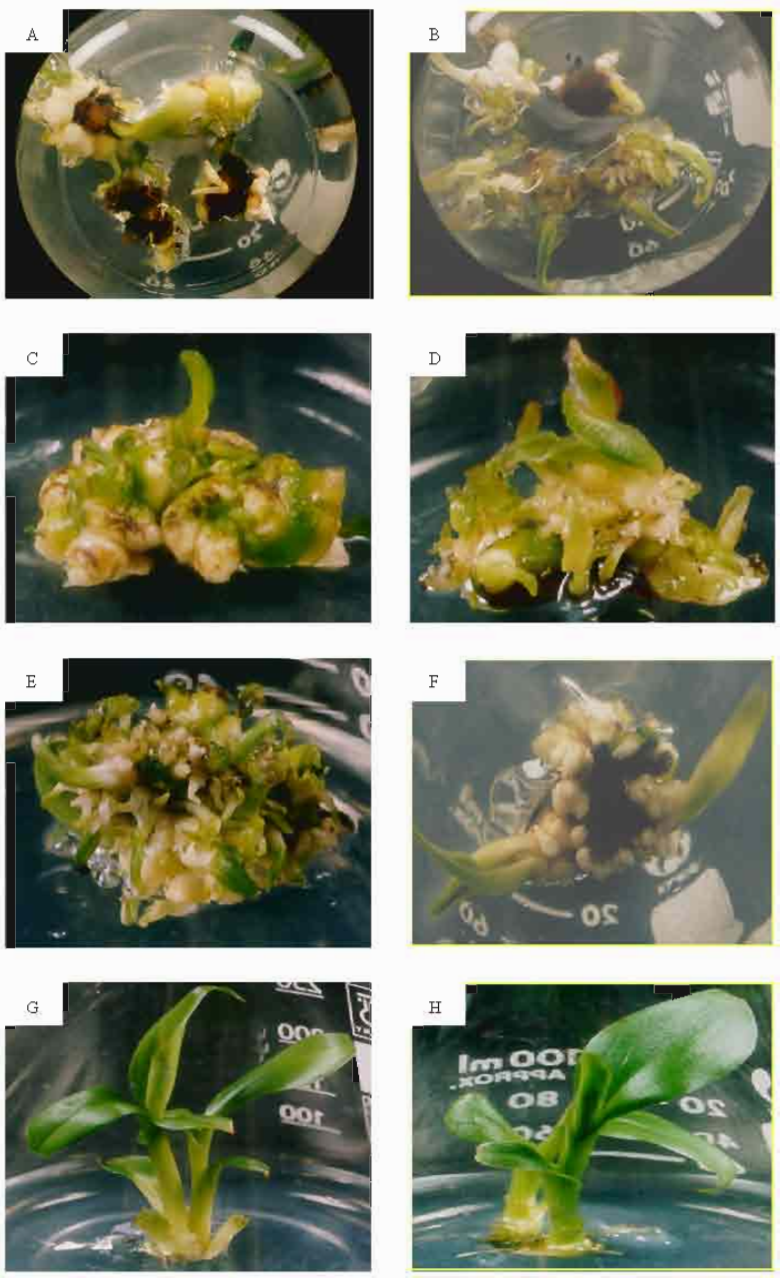

Fig. 5: Production of transgenic banana cultivar, Rastali. (A) Two months in geneticin G-418 selection media; (B) Shoot proliferation from antibiotic resistant meristematic clumps in 5 mg L^{-1} of BAP concentration; (C) Some single bud develop into green multiple bud clumps in 5 mg L^{-1} of BAP concentration; (D) Shoots developed from green multiple bud clumps; (E) Multiple shoots proliferating; (F) Two single plantlet resistant to antibiotic selection; (G) and (H) Putative transgenic plantlet confirmed from PCR and Southern blot analyses

Yu *et al*. (2003). In plant cells, kanamycin exerts its effect on mitochondria and chloroplasts by impairing protein synthesis, resulting chlorosis (Weide *et al*., 1989). For several *Malus* cultivars and rootstocks, kanamycin at 50 mg L^{-1} is pyhtotoxic and causes shoot chlorosis and necrosis (Yepes and Aldwinckle, 1994 b).

The single buds treated with L-Cysteine which is known as antinecrotic compounds in order to minimise the *Agrobacterium*-induced hypersensitive response. Enriquez-obregon *et al*. (1999) showed that the co-culture of sugarcane explants with *Agrobacterium tumefaciens* induces a rapid necrosis of the tissue. Similarly, Olhoft and Somers *et al*. (2001) demonstrated increased *Agrobacterium*-mediated transformation efficiencies in soybean cotyledonary-node cells using 400 mg L^{-1} of L-Cysteine. In addition, L-Cysteine and ascorbic acid has been reported decreased tissue necrosis of Japonica rice meristem cultures used for *Agrobacterium*-mediated transformation (Enriquez-Obregon *et al*., 1999).

The influence of calcium deprivation on the cell wall is considered during the interaction of *Agrobacterium tumefaciens* with explants by using $CaCl_2$ free medium during cocultivation period. Ca^{2+} acts directly as an ionic cross-linkage of the carboxyl groups of linear macromolecules in the cell wall and directly as an inducer of changes in the cell wall composition through its influence on gene expression (Sander *et al*., 1999). Hence, lack calcium reduced cell wall matrix by modifying cell wall structure and made banana single buds more susceptible to *Agrobacterium* cell attachment. This was consistent with observations made on *Arabidopsis thaliana* tissues (Sangwan *et al*., 1992), *Pisum sativum* (de Katen and Jacobsen, 1995) and *Hevea brasiliensis* (Montoro *et al*., 2003).

Nevertheless, maintenance of cultures for long periods and with high antibiotics concentration for more than three phase selection resulted in reduced numbers of surviving cell aggregates. Irrespectively of the selective conditions used, average frequencies of plant regeneration ranged from 5 to 20% putatively independent lines per co-cultivation in all samples. This study demonstrated that the use of *Agrobacterium tumefaciens* as a transformation system for banana cultivar, Rastali (AAB) could be highly efficient when a rapid screening system for the identification of lines of interest developed before performing more detailed molecular analyses. Techniques based on the Polymerase Chain Reactions (PCR) are the best option for analyzing large amount amounts of transformants, since they are fast and demand low quantities of genomic DNA.

The use of highly regenerable single meristematic buds of banana cultivar, Rastali (AAB) in combination with *Agrobacterium* as a vector for DNA transfer has been not described elsewhere previously. However using other banana meristem tissue such as corm slices in *Agrobacterium*-mediated transformation may be limited application because of the risk of generating chimaeric plants even though the transformation frequency obtained with particle bombardment could be markedly improved (May *et al*., 1995). Besides being more efficient, *Agrobacterium*-mediated transformation is technically simpler than particle bombardment, only requiring basic microbiology facilities and generally results in high levels of expression due to a simple integration pattern of well defined DNA sequences into transcriptionally active regions of the plant genome, which make it the best option when both transformation systems are available (Hiei *et al*., 1997; Cheng *et al*., 1997).

Analysis of T-DNA Integration via PCR Amplification

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

Total DNA isolated from the putative transformants was tested for the presence of the transgenes. The efficient and simplicity of the PCR analysis, enable screening for transformed plants in a shorter period. PCR amplification confirmed that the chitinase (*RCC2*) gene and *hpt11* gene were

present in a high proportion of the 50 mg L^{-1} hygromycin (medium B) compared to lower hygromycin selection medium (medium A). The PCR results of some transgenic plantlets are displayed in Fig. 6 and 7.

In all the experiments, no bands could be detected from DNA extracted from untransformed control. Eleven DNA samples were picked from hygromycin selection (medium A) and five from selection medium B were subjected for PCR analyses. In all experiments, no bands (*RCC2* and *hpt11* genes) were detected from plants using hygromycin selection (medium A).

Three out of the five DNA samples assayed successfully amplified the expected band size of 310 bp of chitinase gene (*RCC2*) from medium A (Fig. 6). Co-integration of the *hpt 11 gene* (900 bp) as expected, detect in these putative transformants (Fig. 7). Similarly to this result, all putative transgenic *Agrostis palustris* plants tested showed a band of expected size in PCR for the linked *hph* and *gusA* transgenes with transformation frequencies of 100% (Xiao *et al*., 1997). Three transgenic plantlets contained chitinase gene were differentiate according to plant code [B2Y (4); B2Y (16) and B3Y (11)].

Longer duration (more than eight cycles) of hygromycin in selection medium did not help to reduce the outcomes of chimeric tissue instead it decreases the survival of regenerating plants, appeared to effect fertility of the regenerated plants and surprisingly produced pink colour pigmentation in shoots and leaves. However, these pigmented plantlets return back to normal green plantlet when the selection removed after six cycles and maintained in hormone free MS medium.

The presence of hygromycin or basta during five cycles of oil palm embroyogenic callus clumps remained essential as otherwise some of the regenerated plants were not transgenic (Parveez *et al*., 1996). The fact that transgenic banana cells were selected during a longer period (four to six months) before regeneration is started probably explains the low number of transformants in higher hygromycin selection medium.

Southern Blot Hybridization Analysis

Southern blot analyses were carried out to evaluate further the transfer and insertion of the chitinase gene (*RCC2*) in the genome of the transformed banana cultivar, Rastali (AAB). Although two lines of evidence, (I) the antibiotic resistant phenotype and (ii) the presence of the introduced genes in putative transformants as shown indeed by PCR demonstrated that the regenerated plants were transgenic, proof of stable integration of transgenes by Southern analysis was required.

Hybridisation of chitinase gene (*RCC2*) to *Hind111* digested genomic DNA from the transgenic plantlets was shown in Figure 8. The results confirmed that all three transgenic plantlets [Plant code: B2Y (4); B2Y (16) and B3Y (11)] derived from PCR positive results using *Agrobacterium tumefacien*, EHA 101 (pBI333-EN4-*RCC2*) contained chitinase genes in leaves and roots. *Hind111* digestion of transforming plasmid, p*RCC2*, released an approximately 1.3 kb size fragment containing the p35S, RCC2 and nos terminator (Fig. 8 (lane 1)). Lane 1, 2, 4-5, 7-8 and 10-12 revealed bands with sizes larger than the *RCC2* gene fragment (1.3 kb) were indicative of integration of the introduced plasmid into the host plant genome. The presence of bands with molecular weights different from the original transforming plasmid indicates that it is a possibility transgene rearrangement or multiple independent insertions had occurred and the observed bands represent plasmid-chromosome junction fragments (Janna *et al*., 2001).

Even though 3 transgenic plantlets tested were from different independent transformation events (treatment B), surprising 2 plantlets showed similar hybridisations patterns with except of root tissue from plant B3Y (11), which revealed an addition band in its hybridization pattern. DNA fragments smaller than expected size could be due to rearrangement of the transgene in the genome (Kohli *et al*., 1999).

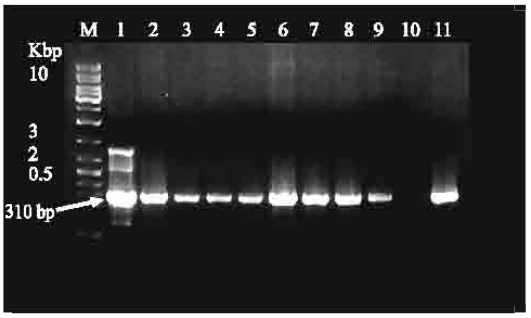

Fig. 6: PCR analysis chitinase gene in transgenic banana plantlets selected on hygromycin 50 mg L^{-1}. Lane M = Lambda DNA digested with *Hind*111 was used as molecular weight marker; Lane 1 = Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2-9, 11 = Putative transformed plantlets showing the amplified 310 bp *RCC2* gene and Lane 10 = Untransformed plantlet

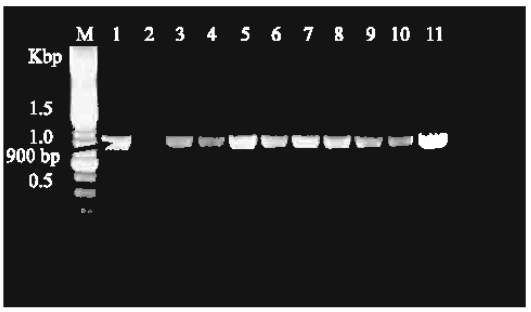

Fig. 7: PCR analysis *hpt11* gene in transgenic banana plantlets selected on hygromycin 50 mg L^{-1}. Lane M = Lambda DNA digested with *Hind*111 was used as molecular weight marker; Lane 1 = Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2 = Untransformed plantlets; Lane 3-11 = Putative transformed plantlets showing the amplified 900 bp *hpt11* gene

Fig. 8: Southern blot analysis of the chitinase integration pattern in banana plantlets. Digested genomic DNA with *Hind 111* using PCR amplified *RCC2* gene (310 bp) as probe. Lane1=Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2, 4-12 = genomic of putative transformed plantlets and Lane 3 and 9 = untransformed control plantlets

Table 2: Chitinase enzyme activity in transgenic Pisang Rastali (AAB) plantlets transformed with chitinase gene (*RCC2*)

Plant code	Chitinase activity [a] (1 nkat μg^{-1} protein)
B2Y (4) [b]	39.2±8.3
B2Y (16)	43.2±3.2
B3Y (11)	24.4±2.4
Control [c]	12.9±1.8

[a] One unit of chitinase activity: the amount of enzyme catalyzing the formation of 1 nm N-acetylglucosamine 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 cultivar, Rastali (AAB)

Analysis of Chitinase and β-1,3 Glucanase Protein Production in Transgenic Plantlets: The accumulation of chitinase activity in transformed banana cultivar, Rastali (AAB) plantlets was examined by enzyme assay. The chitinase activity in the whole plants of B2Y (4), B2Y (16) and B3Y (11) was measured (Table 2).

The chitinase activity of B2Y (4) and B2Y (16) was 3 to 4 fold higher than untransformed plantlet. Neuhaus *et al*. (1991) reported a similar observation in chitinase activity upon introducing the similar chitinase (*chi I*) gene via *Agrobacterium*, LBA 4404 in transgenic tobacco. However, chitinase activity of B3Y (11) is slightly lower than B2Y (4) and B2Y (16) plantlets with only 2 fold higher than untransformed plantlet. The extra copy number of integrated *RCC2* fragment might be related to the weak expression of chitinase activity on B3Y (11). Translation, mRNA expression and degradation might affect the differences in accumulation of rice chitinase protein in banana cell.

CONCLUSION

Single bud is more sensitive at higher cefotaxime concentrations compared to carbenicillin. DNA samples from transgenic banana cultivar, Rastali (AAB) obtained only from selection media contained higher hygromycin concentration (50 mg L^{-1}) and were tested positive for the presence of the chitinase (*RCC2* gene) and *hpt11* coding sequences by PCR analyses. The results from PCR analyses suggested that the selective system protocol (solid media-liquid media-solid media) using higher hygromycin concentration as selection agent in banana is effective in reducing chimeras when compared to lower concentration (25 mg L^{-1}). Integration of transgene and stable genetic transformation of *Agrobacterium tumefaciens* (EHA 101) using chitinase gene were assess by PCR amplification of 310bp of *RCC2* gene (chitinase gene) and 900 bp of the *hpt11* gene. Genomic Southern blot hybridization confirmed the incorporation of the RCC2 gene in host genome between one and two inserted copies. The accumulations of chitinase activities in transformed banana cultivar, Rastali (AAB) plantlets were higher than untransformed plantlets. The design of an adequate artificial environment to favor the interaction of *Agrobacterium tumefaciens* with the banana single buds is critical to the success of genetic transformation experiments. Oxidative burst, phenolization and subsequent cell death have been described as frequent phenomena during the interaction of *Agrobacterium tumefaciens* with monocot plant cells.

ACKNOWLEDGMENT

The generous gifts of a *RCC2* (rice chitinase) plasmid by Dr Yokoshiwa from National Institute of Agrobiological Resourses, Japan is gratefully acknowledged. This research was supported by the Ministry of Science, Technology and Environmental funded this research through IRPA PR Research Grant (01-02-04-0000-PR0010/04)

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