Emir. J. Food Agric. 2009. 21 (2): 18-33
http://cfa.uaeu.ac.ae/ejfa.shtml

A protocol for *Agrobacterium*-mediated transformation of banana 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 (hptII) as the selectable marker and gusA gene as a reporter marker to identify the transformants.. Single buds derived from multiple bud clumps (Mbcs), were the target explants for transformation. Transformation frequency based on hygromycin selection (25 mg L-1) was higher, although no positive transformant was confirmed based on PCR and Southern blot analyses.. Stable gusA gene expression was detectable in transformed single buds, multiple bud clumps, shoots, leaves and roots derived from hygromycin selection at 50 mg L-1). An assay was performed to identify the minimum concentration of two antibiotics most effective against *Agrobacterium* EHA 101. Protein assay showed an increase in chitinase enzyme activity of transformed plantlets. The *Agrobacterium*-mediated transformation protocol reported here is suitable for future selection of banana meristem tissues resistant to fungal disease.

Key words: Single buds, Hygromycin, Chitinase, Transgenic banana.

طريقة التحول الوراثي باستخدام بكتيريا التدرن التاجي في الموز بواسطة جين الرز الكايتينيز

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الملخص: تم مضاعفة جين الرز الكايتينيز (RCC2) في سلالة بكتيريا التدرن التاجي (EHA 101)، وإدخاله في برعم الموز الأحادي صنف راستالي (AAB) مخبريا. تم استخدام البلازميد (pBI333-EN4-RCC2) المحتوي على جين هيجرومايسين فوسفوترانسفيرز (hygromycin phosphotransferase gene) كواسم اختياري وجين (gusA) كواسم مراسل لتحديد عملية التحول. البرعم الأحادي المأخوذ من مجموعة كتل متعددة هو الجزء النباتي المستهدفة في عملية التحول. تكرار عملية التحول بناءا على اختيار هيجرومايسين (25 مجم/لتر) كانت أعلى على الرغم من عدم ثبوت إي تحول ايجابي بناءا على تفاعل البلمرة المتسلسل (PCR) وتحليل التهجين الجنوبي Southern Blot Analysis. تم الكشف عن التعبير الثابت للجين (gusA) في عملية التحول للبرعم الأحادي وكتل البراعم المتعددة والسيقان والأوراق والجذور الماخوذة من اختيار هيجرومايسين عند 50 مجم/لتر. تم إجراء التحليل للكشف عن اقل تركيز للمضادين الحيويين الأكثر فعالية ضد بكتيريا التدرن التاجي (EHA 101). بين تحليل البروتين زيادة في نشاط إنزيم الكايتينيز للنباتات المتحولة. طريقة التحول الوراثي باستخدام بكتيريا التدرن التاجي التي تم ذكرها مناسبة لاختيار خلايا مريستمية من الموز مقاومة للإمراض الفطرية مستقبلا.

الكلمات المفتاحية: البرعم أحادي، هيجرومايسين، كاينتينز، موز معدل وراثيا.

Introduction

Banana is considered outside the host range of *Agrobacterium*, but an increasing number of research reports indicate 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 in banana.

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), orchids (Belarmino and Mii, 2000 ; Yu et al., 2003 and 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 slice explants from cultivar Grand naine (AAA). Antioxidants used such as L-cysteine during pre-culture, infection, and pre- and post- co-cultivation were reported to favor stable transgenic plant 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 led to an increase in the survival rate of *Agrobacterium tumefaciens*-infected cells and a corresponding raise in stable transformation efficiency in soybean. Frame et al. (2002) reported that L-cysteine in co-cultivation medium minimized cell death caused by the hypersensitive response of maize scutellum cells to *Agrobacterium* infection.

Generally, it is known that plants have defense 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. Several studies have been made on transgenic plants integrated with chitinase genes. Transgenic tobacco and canola which have been engineered with bean endochitinase gene were shown to exhibit resistance to *Rhizoctonia solani* (Broglie et al., 1991). Transgenic rice integrated with rice endochitinase driven by the 35S promoter showed enhanced resistance to sheath blight (Lin et al., 1995) whereas transgenic tobacco harboring rice endochitinase gene also possessed increased resistance against powdery mildew, *Erysiphe cichoracearum* (Nishizawa et al., 1993).

This paper describes a series of experiments that were carried out by using *Agrobacterium tumefaciens* supervirulent strain, EHA 101, containing plasmid with chitinase gene (pBI333-EN4-*RCC2*). Comparisons using hygromycin in two different low and high concentrations were demonstrated in this study as the plasmid contained *hptII* gene. The inhibition of single buds of banana and an elimination of *Agrobacterium tumefaciens* growth by carbenicillin and cefotaxime were also demonstrated.

Materials and Methods

Plant material and culture conditions

Banana (Musa cultivar, Rastali; AAB) was chosen as the plant transformation material. In vitro corm slices of were cultured in MS medium (Murashige and Skoog, 1962) supplemented with 10 mgl^{-1} of 6-Benzylaminopurine (BAP) to obtain multiple bud clumps. The cultures were incubated at 25-27°C under 16h photoperiod using cool white fluorescent bulbs (Philips fluorescent light tubes) of 150 $\mu molm^{-2}s^{-1}$. Single buds (approximately 3 mm) excised from

multiple bud clumps were used for this study.

Agrobacterium tumefaciens strain, EHA 101 and binary vector (pBI333-EN4-*RCC2*) plasmid

Disarmed *A. tumefaciens* strain EHA 101 and the binary vector (pBI333-EN4-*RCC2*) contains the *hptII* gene linked to the nopaline synthase (*nos*) promoter and also *gusA* genes of pBI121. A binary vector, pBI333-EN4-*RCC2* was constructed with the cDNA of the *RCC2* gene (Nishizawa et al. 1993). Plasmid pBI333-EN4-*RCC2* was introduced into *A. tumefaciens*, EHA 101 strains by heat shock method.

Agrobacterium cultures were plated on Luria-Bertani (LB) medium supplemented with 50mg L^{-1} kanamycin and grown for 3 d at 28°C to form colonies. Each single colony with a diameter of 1mm was picked out and cultured in 20 ml LB liquid containing the same antibiotic. The culture was agitated at 120 rpm for 20 h at 28°C. After adjusting the optical density to 0.6 units at 600nm (OD_{600nm}), the *Agrobacterium* cultures were used for transformation experiment.

Effects of carbenicillin and cefotaxime antibiotics on plant regeneration and suppression of growth of *Ag. tumefaciens* strain, EHA 101 (pBI333-EN4-*RCC2*)

For explant toxicity tests, single buds were transferred to MS medium containing 5mgL^{-1} of BAP and different concentrations of carbenicillin and cefotaxime (0, 100, 200, 300, 400, 500 and 600mgL^{-1}). After 30 min of co-cultivating the explants in *Agrobacterium* suspension culture, the percentage of single bud survival was determined. Growth of *A. tumefaciens* strain EHA 101 was determined in the 3-day treatment period.

Transformation and regeneration of banana plantlets

Single buds that were mildly injured using a scalpel were precultured for three days prior to *Agrobacterium* infection. About 100 single buds were immersed in *Agrobacterium* suspension for 30 min with an optimized acetosyringone concentration of 100µM. 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 co-cultivated for three days in the dark at 22°C on hormone-free MS medium. The medium designated as M1, contained 100µM acetosyringone, 60mM glucose, 2mM sodium phosphate and 40mgL^{-1} of L-cysteine.

After the 3-d co-cultivation period, the buds were transferred to fresh medium of the same composition but without acetosyringone, together with 300 mg L^{-1} of carbenicillin or 200mgL^{-1} cefotaxime (designated as M2 medium) for 5 days and transferred back to hormone free MS liquid medium with the same antibiotic concentrations for another 5 days.

The buds were next transferred to MS solid medium containing 10mg L^{-1} BAP and 25mgL^{-1} or 50mgL^{-1} hygromycin. After two weeks they were transferred again to fresh liquid medium in conical flasks and kept for 5 days. During this period, dead buds were removed before being sub-cultured back to 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 sub-cultured onto solid MS medium with 1mgl^{-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.

Histochemical GUS Staining

The method of Jefferson (1987) was used for a GUS histochemical assay. Explants were immersed in X-Gluc solution (2 mM X-Gluc, 100 mM NaH_2PO_4 (pH 7.0), 0.5 mM potassium ferricyanide and 50 mM ferrocyanide at 30°C. After staining, the materials were treated with 70% ethanol for 3 d to remove chlorophyll before observation.

PCR verification of the rice chitinase (*RCC2*) and *hptII* genes

Total genomic DNA was isolated from the control putative and transformed plantlets regenerated on selective media using a modified CTAB method adopted from Pasakinskiene and Paplauskience (1999). PCR was done using the DNA Thermal Cycler 480 machine (Perkin-Elmer).

PCR amplifications were carried out in 100µl reaction volumes 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 $MgCl_2$, 1 X PCR buffer, and 5 U Taq DNA polymerase (MBI Fermentas). Amplification of chitinase *RCC*2 gene 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. Amplification of *hptII* 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. The amplification was analyzed by electrophoresis in 1.2% agarose gel laced with ethidium bromide.

Southern blot analysis

The DIG DNA Labelling nd Detection System (Roche) was used to confirm stable integration of chitinase (*RCC2*) transgene in the host banana genome. Genomic DNA (20µg) from PCR positive transformants were subjected to digestion with *Hind*III in buffer H (Promega).

Extraction and determination of total acid soluble protein and chitinase activity assay

Tissue samples (0.25g) were homogenised in 5ml extraction buffer (0.1M 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 minutes. 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 the Bradford method (1976), at wavelength 595 nm.

The chitinase activity assay methods of Tonon et al. (1998) were applied in this experiment with a slight modification in enzyme concentration. Chitinase activity was determined based on the rate of N-acetylglucosamine production using chitin as the substrate. The amount of enzyme catalyzing the formation of 1 nm N-acetlyglucosamine equivalent in one second under assay condition is referred to as 1 nkat (Anfoka and Buchenauer, 1997).

Statistical Analysis

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

Results and Discussion

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

For effective *Agrobacterium*-mediated transformation, the antibiotic regime should control bacterial growth without inhibiting the regeneration of the plant cells. The effect of carbenicillin and cefotaxime on the percentage of single

bud growth was evaluated after four weeks of culture on solid and liquid media containing appropriate antibiotic concentrations (Figure 1, 2). Average bud growth in solid medium containing 600 mg L^{-1} cefotaxime was reduced from 100% (control) to 30%. The percentage of growth in liquid medium using same concentration was 12% lower. In comparison to carbenicillin, using 600 mg L^{-1} reduced average growth by 62% in solid medium and 54% in liquid medium. The percentage of growth in 150 mg L^{-1} carbenicillin was reduced by 8% and 12% in solid and liquid medium, respectively. However in cefotaxime, percentage of growth dropped tremendously to 82% in solid medium and 63% in liquid medium using the same concentration of 600 mgL^{-1}.

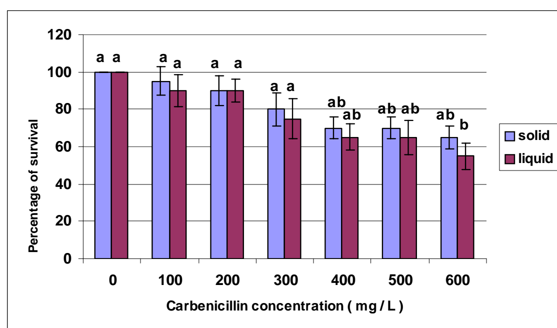

Figure 1. Percentage of single bud growth obtained after four weeks in MS media using different carbenicillin concentration. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different (p<0.05).

In order to examine the bactericidal effects of carbenicillin and cefotaxime, *Agrobacterium* strains EHA 101 were inoculated on 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 was obtained using 400 mgL^{-1} (Figure 3). For cefotaxime, the inhibition of cell growth started at 100 mg l^{-1} and complete inhibition of cell growth occurred at 300 mgl^{-1}. The results demonstrated that, *Agrobacterium* cells were more sensitive to cefotaxime than carbenicillin.

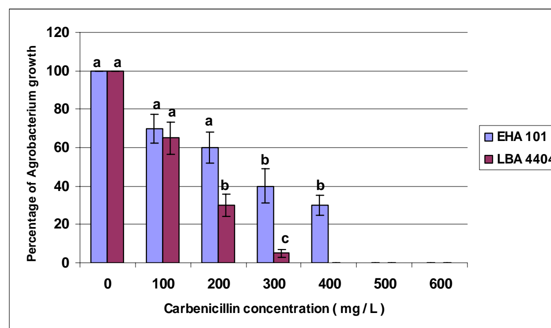

Figure 2. Percentage of single buds growth obtained after four weeks in MS media using different carbenicillin concentration. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different (p<0.05).

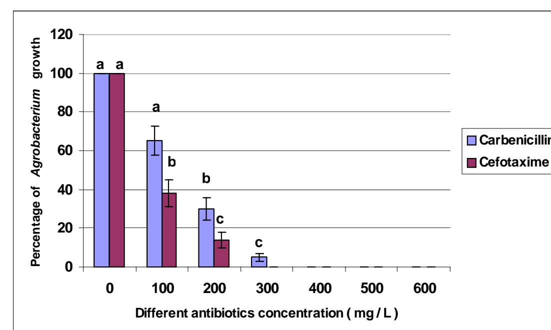

Figure 3. Percentage of single buds with *Agrobacterium tumefaciens*, EHA 101 growth using with different carbenicillin and cefotaxime concentrations.

Although cefotaxime is more effective in inhibiting *Agrobacterium* cell growth, 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 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).

Other antibiotics such as augmentin, timentin and moxalactam have also been used after co-cultivation to kill the *Agrobacterium tumefaciens* strains as they did not reduce regeneration capacity (Park and Facchini, 2000; Antunez de Mayolo et al., 2003). Therefore, different concentrations of carbenicillin and cefotaxime were required in solid and liquid medium of banana cultivar, Rastali (AAB) explants in order to inhibit growth of *Agrobacterium tumefaciens* which could affect explant growth and cause contamination, reducing transformation efficiency.

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

Single buds of banana cultivar, Rastali (AAB) were infected and co-cultivated with induced *Agrobacterium* strain harboring different binary vectors, following the condition described in materials and methods. Co–cultivated single buds were selected on MS medium with 10mgL^{-1} BAP and hygromycin at 25mg L^{-1} (treatment A) or 50mg L^{-1} (treatment B), for 2-3 months. Selection medium containing hygromycin at 50mg L^{-1} produced a lower number of resistant buds. However, none of the negative control plates gave rise to surviving cell aggregates under any of the selective conditions used. Uninfected single buds proliferated normally on 10mg L^{-1} BAP, turning black and dead after two months on selective medium, even at the lower concentration.

Percentage of transformation frequency was evaluated based on the number of explants regenerated. Transformation frequency of *Agrobacterium,* from treatment A was higher than treatment B. More than 30 cell clusters arose per individual co–cultivated sample after selection, many of which initiated the formation of multiple bud clumps, while the rest did not stand selection, blackened and died (Figure 4).

After the first level of selection, the remaining proliferated buds were subcultured into liquid medium containing 10mg L^{-1} of BAP using the same concentration of antibiotics (indicate conc here). After one week, the remaining healthy proliferating buds were selected in the original concentration (indicate conc here) of antibiotics in solid MS medium. Multiple bud clump aggregates formed during the selective process were regenerated in 10mgL^{-1} BAP and antibiotic-free medium. Single plantlets arising from hygromycin resistant cell clumps in hormone-free medium formed shoots and root tips without any necrosis.

However, this has not been true for explants regenerated *in vitro* using kanamycin as a selectable marker in transgenic plant experiments. Some transgenic plantlets produced albinos and semi–necrotic growth in kanamycin selection which caused production of phenotypically abnormal pea plants (Bean et al., 1997; Nadolska-Orcyzk and Orczyk, 2000). Similar kanamycin effects were reported in transgenic papaya by Yu et al. (2003). In plant cells, kanamycin exerts its effect on mitochondria and chloroplasts by impairing protein synthesis, resulting in chlorosis (Weide et al., 1989). For several *Malus* cultivars and rootstocks, kanamycin at 50mg L^{-1} is phytotoxic and causes shoot chlorosis and necrosis (Yepes and Aldwinckle, 1994).

The single buds treated with L-cysteine which is known as an antinecrotic compound that minimises the *Agrobacterium*-induced hypersensitive response. Enriquez-obregon et al. (1999) showed that the co-culture of sugarcane explants with *A. tumefaciens* induced a rapid necrosis of the tissue.

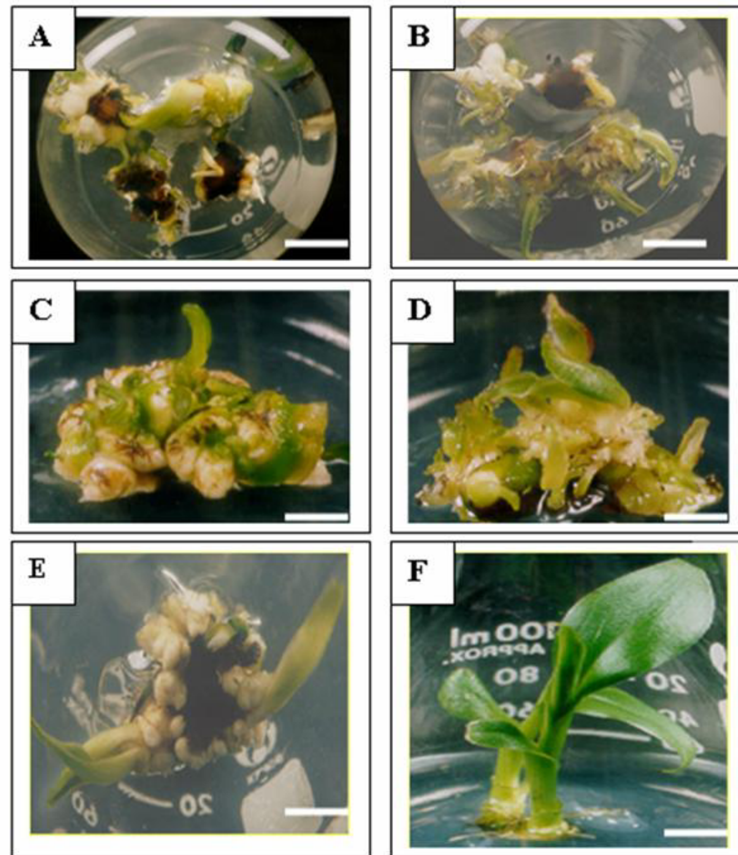

Figure 4. Production of transgenic banana cultivar, Rastali. (A) Regeneration of plantlets after two months in hygromycin selection treatment B; (B) Shoot proliferation from antibiotic resistant meristematic clumps in 5mg L^{-1} BAP-containing MS medium; (C) Some single buds develop into green multiple bud clumps in 5mg L^{-1} of BAP; (D) Shoots developed from green multiple bud clumps; (E) Multiple shoots proliferating; (F) Putative transgenic banana plantlet confirmed from PCR and Southern blot analysis. The bar in the bottom of each image represents 1.5 cm.

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 were reported to decrease 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 co-cultivation. 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 (Sanders et al., 1999). Hence, a lack of 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. Irrespective 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 *A. 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.

Puttive expression of GUS gene in regenerated buds was visually confirmed from treatment B at the time of single bud regeneration, shoot initiation, rooting and leaf formation of the transformants (Figure 5). Histochemical GUS staining of the meristem clearly demonstrated that banana plantlets were fully transformed and no chimaeric tissue was formed, although a uniform blue staining was not always observed in all tissues. No loss of GUS expression was observed for at least three subcultures on the selection medium from the putative banana plantlets from treatment B only. Thus, the *gusA* gene was not only transmitted but also stably expressed during vegetative multiplication *in vitro*. However, no stable GUS expression could be observed from the regenerated multiple bud clumps and other type tissues from treatment A. Previously, it has been reported that reporter gene such as GUS 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).

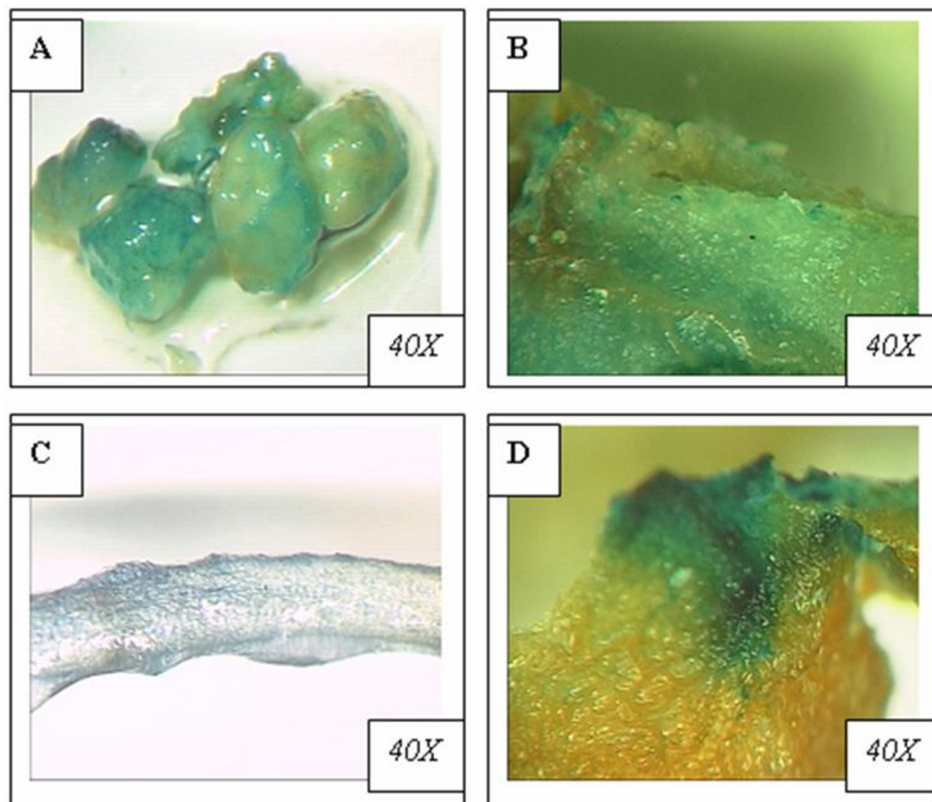

Figure 5. Stable gusA gene expression in different types of tissue. (A) Single buds; (B) Regenerated shoots; (C) Single root and (D) Newly -formed leaf.

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 have limited application because of the risk of generating chimaeric plants even though the transformation frequency obtained with particle bombardment could be markedly improved (May et al., 1995). Besides being more efficient, *Agrobacterium*-mediated transformation is technically simpler than particle bombardment, only requiring basic microbiology facilities and generally results in high levels of expression due to a simple integration pattern of well defined DNA sequences into transcriptionally active regions of the plant genome, which make it the best option when both transformation systems are available (Hiei et al., 1997; Cheng et al., 1997).

Verification of presence of the rice chitinase (*RCC2*) and *hptII* genes using polymerase chain reactions (PCR) analysis

Though *Agrobacterium* mediated transformation is the most common method for the generation of transgenic plants with single integration of a precisely delimited DNA sequences (Smith and Hood, 1995; Lawrence et al., 2001), the structure of the inserted T-DNA varies widely to include single or multiple copies, individual or tandem repeats, at a unique or several loci in the plant genome (Iglesias et al., 1997). Total DNA isolated from the putative transformants was tested for the presence of the transgenes. The efficient and simplicity of the PCR analysis, enabled screening for transformed plants in a shorter period. PCR amplification confirmed that the chitinase (*RCC2*) and *hptII* genes were present in a high proportion at 50mg L^{-1} hygromycin (treatment B) compared to lower hygromycin (25mg L^{-1}) selection medium (treatment A). The PCR results of some transgenic banana plantlets are shown in Figures 6 and 7.

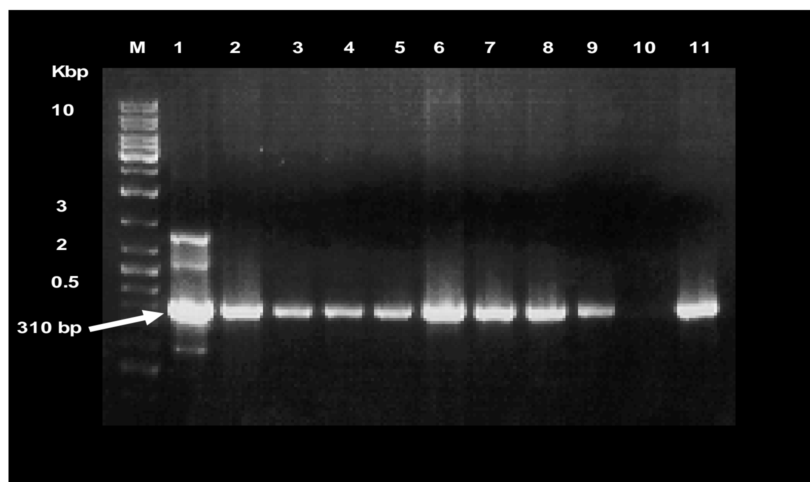

Figure 6. PCR analysis of chitinase gene in transgenic banana plantlets selected on 50mg L^{-1} hygromycin. Lane M = Lambda DNA digested with *Hind*III was used as molecular weight marker; Lane 1 =Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2-9, 11 = Putative transformed plantlets showing the amplified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

In all the experiments, no bands could be detected from DNA extracted from the control. Eleven DNA samples selected from treatment A and five from treatment B were subjected for PCR analyses. No bands (*RCC2* and *hptII* genes) were detected from treatment A. Three out of the five DNA samples assayed successfully amplified the expected band size of 310 bp of chitinase gene (*RCC2*) from treatment A (Figure 6). Co-integration of the *hptII gene* (900 bp) as expected was detected in these putative transformants (Figure 7). In a similar study, putative transgenic *Agrostis palustris* plants showed a band of expected size in PCR for the linked *hph* and *gusA* transgenes with transformation frequencies of 100% (Xiao and Hu 1997). (Three transgenic plantlets obtained in this study containing chitinase gene were differentiated according to plant code [B2Y (4); B2Y (16) and B3Y (11)].

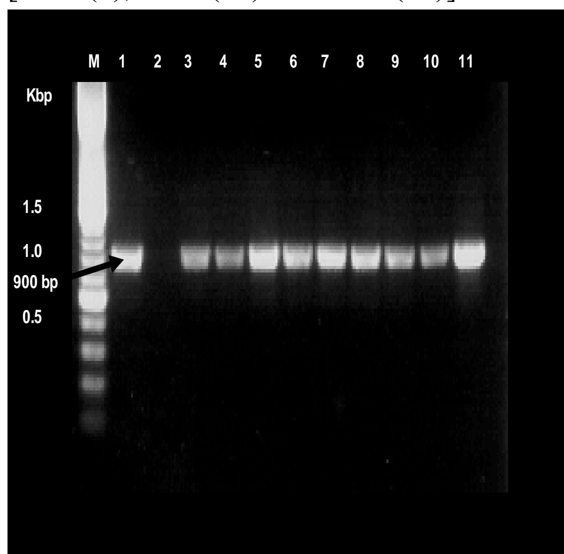

Figure 7. PCR analysis of *hptII* gene in transgenic banana plantlets selected on 50 mg L^{-1} hygromycin. Lane M = Lambda DNA digested with *Hind*III was used as molecular weight marker; Lane 1 =Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2 =Untransformed plantlets; Lane 3-11 = Transformed plantlets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

A longer duration of hygromycin selection did not reduce the incidence of chimeric tissue but instead decreased the survival of regenerated. In addition, putatively transformed plantlets produced pink colour pigmentation especially in shoots and leaves. However, these pigmented plantlets returned to normal green colour after removal from selective medium after six cycles of culture in hormone-free MS medium. Parveez et al., 1996 noted that the presence of hygromycin or basta during five cycles of oil palm embroyogenic callus clumps was essential in the production of transgenic plants. The fact that transgenic banana cells were selected over a longer period (four to six months) in this experiment may explain the low number of non-transformants in higher hygromycin selection medium.

Southern blot hybridization analysis

Hybridisation of chitinase gene (*RCC2*) to *Hind*III 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 *A. tumefacien*, EHA 101 (pBI333-EN4-*RCC2*) contained chitinase genes in leaves and roots (Figure 5).

*Hind*III digestion of transforming plasmid, p*RCC2*, released an approximately 1.3 kb size fragment containing the p35S, RCC2 and nos terminator [Figure 8 (lane 1)]. Lane 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.

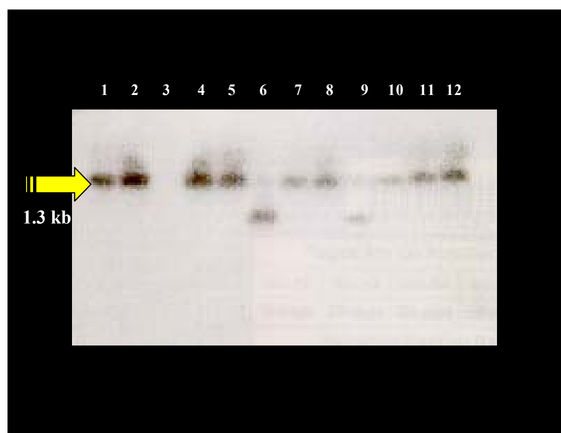

Figure 8. Southern blot analysis of the chitinase integration pattern in banana plantlets. Digested genomic DNA with *Hind*III using PCR amplified *RCC2* gene (310 bp) as probe. Lane1=Transforming plasmid, pBI333-EN4-*RCC2*; Lane 2, 4-12 = genomic of putative transformed plantlets and Lane 3 and 9 =untransformed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

Even though three transgenic plantlets tested were from different independent transformation events (treatment B), surprisingly two plantlets showed a similar hybridisation patterns with except of leaf 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).

Analysis of chitinase protein production in transgenic banana 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 1).

The chitinase activity of B2Y (4) and B2Y (16) was 3 to 4 fold higher than untransformed plantlet (Table 1). 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, being only 2-fold higher than untransformed plantlets. 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 production in banana tissues.

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

Genes	Primer	Sequence	Product length
Chitinase	Forward	5’T-GGATCCAGCGGCTCGTCGGTTG-3’	310 bp
	Reverse	5’-GTATAATTGCGGGACTCTAAT-3’	
*hpt*II	Forward	5’-CCCCTCGGTATCCAATTAGAG-3’	900 bp
	Reverse	5’-CGGGGGGTGGCCGAAGAACTCCAC-3’	

Table 2. Chitinase enzyme activity in transgenic banana Rastali (AAB) plantlets transformed with chitinase gene (RCC2).

Plant code	Chitinase activity (1 nkat / µg protein)
B2Y (4)	39.2 $\pm$ 8.3
B2Y (16)	43.2 $\pm$ 3.2
B3Y (11)	24.4 $\pm$ 2.4
Control [c]	12.9 $\pm$ 1.8

Conclusion

Single buds are more sensitive at higher cefotaxime concentrations compared to carbenicillin. The design of an adequate artificial environment to favour the interaction of *A. tumefaciens* with the banana single buds is critical to the success of genetic transformation experiments. An oxidative burst, phenolization and subsequent cell death have been described as frequent phenomena during the interaction of *A. tumefaciens* with monocot plant cells. Stable *gusA* gene expression was detectable in transformed single buds, multiple bud clumps, shoots, leaves and roots derived from treatment B (Figure 5) Genomic DNA samples from transgenic banana cultivar, Rastali (AAB) were obtained only from selection media A which contained a higher hygromycin concentration (50 mg l^{-1}). They also tested positive for the presence of the chitinase (*RCC2* gene) and *hptII* coding sequences by PCR analysis. Integration of transgene and stable genetic transformation of *A. tumefaciens* (EHA 101) using chitinase gene were assessed by PCR amplification of 310 bp of *RCC2* gene and 900 bp of the *hptII* 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. Even though at the end, we have only obtained three truly putative positive transgenic plantlets, but we do believe that these plantlets can be multiplied at larger number successfully using an optimised *in vitro* regeneration system. 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. Therefore, an efficient *Agrobacterium* mediated transformation protocol such as that proposed in this study can facilitate further functional genomic study of this important banana cultivar.

Acknowledgements

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

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