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Enhanced Tolerance Against a Fungal Pathogen, *Fusarium oxysporum* f.sp. *cubense* (Race 1) in Transgenic Silk Banana*

[1, 2]S. Sreeramanan, [1]M. Maziah, [1]N.M. Rosli, [3]M. Sariah and [2]R. Xavier
[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor DarulEhsan, Malaysia
[2]Department of Biotechnology, Asian Institute of Medicine, Science and Technology, Amanjaya, 08000, Sungai Petani, Kedah
[3]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia

Abstract: An effective method has been developed for the stable transformation and regeneration of silk banana (*Musa* spp. AAB group) cv 'Rastali' using microprojectile bombardment. 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 using microprojectile bombardment system together with *gfp* and *gusA* genes as reporter gene. Proliferating single buds were selected on geneticin G-418 to produce a number of putatively transformed bananas. Five different treatments using different chitinase and glucanase genes inserted singly or in combination were carried out. 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. The transgenic banana plantlets from each treatment inoculated with conidial suspension of fungal to evaluate the degree of tolerance and to investigate the effectiveness of the bioassay system as a potential tool for early screening. Different chemical compound such as hydrogen peroxide (H_2O_2) and relevant enzyme activities such as Phenylalanine Ammonia Lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (PER) and polyphenol oxidase (PPO), were determined for each treatment including control plantlets. Evaluation of disease development in primarily and secondary infections showed that combination of the two transgenes gave substantially greater protection against the fungal than single-transgene introduction. Productive interactions between chitinase and glucanase transgenes *in planta* point to combinatorial expression of antifungal genes as an effective approach to enhanced tolerance to Fusarium wilt disease.

Key words: Transgenic banana, chitinase, glucanase, fusarium wilt tolerant

Introduction

Fusarium wilt disease causes severe damage in commercial plantations and represents a major constraint to banana production in Malaysia. Pisang Rastali (AAB) is a local dessert banana, which belongs to the AAB group and also known as Pisang Keling and Pisang Tali. Large-scale cultivation of this cultivar is difficult, as it is highly susceptible to *Fusarium oxysporum* f.sp. *cubense* race 1. Genetic engineering offers an alternative route to increase resistance to Fusarium wilt disease. Microprojectile bombardment was used for Pisang Rastali (AAB) transformation using tiny single

Corresponding Author: Dr. S. Sreeramanan, Department of Biotechnology, Asian Institute of Medicine, Science and Technology (AIMST), 2, Persian Cempaka, Amanjaya, 08000, Sungai Petani, Kedah, Malaysia

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meristem buds. Therefore, genetic engineering with the introduction of genes conferring resistance to fungal pathogens such as chitinase and β-1,3-glucanase is a useful complementary tool to overcome this problem.

Co-transformation is a potential tool to create multiple and durable resistance in banana (Remy *et al*., 1998). 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*. (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.

In this study, five different treatments using different chitinases and β-1,3-glucanase genes inserted singly or in combination were carried out. Multiple genes encoding either chitinases (*RCC2* or *Chi*) or β-1, 3-glucanase (*Eg*) and both was bombarded together with *gfp* gene (pGEM.Ubi-S*gfp*S65T) for early transient expression signal using an optimised physical and biological condition. For the analysis of transgene integration patterns, a polymerase chain reactions (PCR) and Southern blot hybridization techniques have been elaborated and evaluated. Transgenic plants with a transgene locus resulting from genomic integration of a single, perfect copy of delivered DNA (Makarevitch *et al*., 2003) are most desirable for banana improvement. Finally, to assess practical applications of the developed transformation protocol for Fusarium wilt disease control, the chitinase and β-1,3-glucanase genes, was transferred into Pisang Rastali (AAB) and the transgenic banana produced were tested using an optimised fungal bioassay conditions.

Materials and Methods

Plant Material

Corm slices of *in vitro* banana cultivar, Rastali (AAB) plantlets were cultured in MS (Murashige and Skoog, 1962) medium supplemented with 10 mg L^{-1} of 6-Benzylaminopurine (BAP) to obtain multiple bud clumps. The cultures were incubated at 25±2°C and 16 h photoperiod with cool white fluorescent light of 150 μmol m^{-2} s^{-1} (supplied by Philips TLD fluorescent light tubes). Single buds (3 mm), excised from multiple bud clumps, were used for this study.

Minimal Inhibitory Concentration of Selective Agents

The effectiveness of kanamycin, geneticin G-418, neomycin, paromomycin, basta and hygromycin as selection agents to inhibit the growth of single buds derived from multiple bud clumps were evaluated. The concentration of selection agents tested was 0, 25, 50, 100, 150, 200, 250 and 300 mg L^{-1} in both solid and liquid medium. For control and each treatment, four replicates were used and were carried out twice to ensure reproducibility. In each plate (solid medium) and flasks (liquid medium), 40 single buds of approximately 3 mm in size and controls were cultured without selection agents and incubated in 24 h photo period for 4 weeks. Data were recorded based on percentage growth of the single buds. 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).

Physical and Biological Parameters of Microprojectile Bombardment

Optimisations of the physical and biological parameters were carried out based on GUS and GFP transient gene expressions. Optimisation of the physical parameters was carried out under the following conditions, rupture disc pressure; distance from stopping plate to target tissue; vacuum

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

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

Genes	Primer	Sequence	Product length
gfp	Forward	5'-ATGAGTAAAGGAGAAGAACTTTTC- 3'	726 bp
(pGEM.Ubi1-SgfpS65T)	Reverse	5'-TTTGTATAGTTCATCCATGCCA- 3'	
gusA	Forward	5'-CGCCGATGCAGATATTCGTA- 3'	789 bp
(pMRC1301)	Reverse	5'-ATTAATGCGTGGTCGTGCAC-3'	
Chitinase, *RCC2*	Forward	5'-TGGATCCAGCGGCTCGTCGGTTG-3'	310 bp
(pBI333-EN4-RCC2)	Reverse	5'-GTATAATTGCGGGACTCTAAT-3'	
Chitinase, *Chi*	Forward	5'-TACAACTTCAACTACGGGCCG-3'	486 bp
(pMRC1301)	Reverse	5'-ACGACTCACTATAGGGCG-3'	
β-1,3 glucanase, *Eg*	Forward	5'-GATGTGATATCTCCACTGACGTAAG-3'	830 bp
(pROKla-Eg)	Reverse	5'-GTATAATTGCGGGACTCTAAT-3'	
npt 11	Forward	5'-CCCCTCGGTATCCAATTAGAG-3'	900 bp
	Reverse	5'-CGGGGGGTGGCCGAAGAACTCCAC-3'	

pressure; gold microcarrier size and number of bombardments per Petri dish. The biological parameters included the explant types, preculture treatment prior bombardment, DNA concentrations and various explants sizes. There are five treatments used in this study (Table 1). We introduced multiple genes encoding chitinases (*RCC2* and *Chi*) and β-1,3-glucanase (*Eg*) together with *gfp* gene (pGEM.Ubi-S*gfp*S65T) for early transient gene expression using an optimised physical and biological parameters as mentioned above.

Antibiotic Selection of Transformants

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 and Visualisation of GFP

Bombarded tissues were assayed for GUS expression according to Jefferson *et al*. (1987). A florescence microscope (Leica MZFL 111) equipped with GFP 2 filter set used to monitoring GFP expression of transformed tissues.

PCR and Southern Blot Hybridization Analysis

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). *RCC2* and *Chi* (Chitinases gene), Eg (β-1,3 glucanase) and *npt 11* 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 extension, 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 were carried out using a non-radioactive method was used to confirm stable integration of chitinase (*RCC2* and *Chi*) and β-1,3 glucanase (*Eg*) transgenes in the host banana genome. DIG DNA Labeling and Detection Kit (Roche) were used in this study. DIG-labeled DNA probes are generated according to the method of random primed labeling (Holtke *et al*., 1995) which is based on the hybridization of random oligonucleotides to the DNA template.

Development of Fusarium Bioassay Method

Induction of sporulation and germination of *Fusarium oxysporum* f.sp. *cubense*, race 1 (VCGs 01217) were carried out for four weeks. For plant inoculation bioassay, untransformed Pisang Rastali (AAB) plantlets roots sections were cut at 3 cm from the root tips. The plants dipped into three different spore concentrations (10^4, 10^6 and 10^8 spores mL^{-1}) for one hour and were then transplanted in a plastic container (8×8 cm) with sterile perlite (Fig. 1 and 2). One ml of different spore concentrations was again inoculated near the root/stem regions for double confirmation. Each concentration of spores inoculated in four replicates and was repeated for three times except for testing transgenic bananas. The total spores obtained were diluted into three different spore concentrations (2×10^4, 2×10^6 and 2×10^8 spores mL^{-1}). The plantlets were watered using hormone and sucrose free MS liquid medium. Disease development and severity were monitored and measured over for a period of four weeks. The same protocol was applied for disease development in transgenic plantlets using the minimum spores which can caused disease symptoms. Necrosis in new and old leaves tissue (yellowing) indicated Fusarium wilt symptoms occurred.

Biochemical Analyses

Hydrogen peroxide (H_2O_2) compound and phenylalanine ammonia lyase (PAL) enzyme activities were carried out at 12 h intervals for 72 h, while other enzymes (peroxidase, polyphenol oxidase, chitinase and β-1, 3-glucanase), activities were measured at four weeks after inoculation.

Hydrogen Peroxide (H_2O_2)

H_2O_2 was assayed according to the method of Jiang *et al.* (1990). Hydrogen peroxide (H_2O_2) activity was expressed in nmol g^{-1}fresh weight.

Phenylalanine Ammonia Lyase (PAL) Assay

PAL was assayed according to the method of Robert and Helmut (1992). PAL activity was expressed as changes in absorbance at 290 nm h^{-1} mg^{-1} protein.

Fig. 1: The assembly of the single banana plantlet with perlite for Fusarium bioassay assay. The pipette tip indicates the initial point where inoculum (1 mL) was applied after the each single plantlet dipped for an hour in Fusarium spores suspension

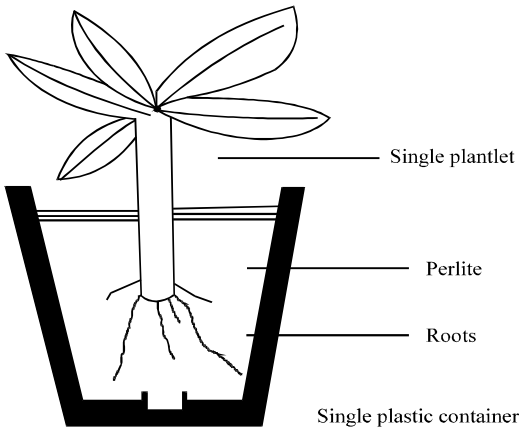

Fig. 2: Systemic assembly of cross section the single banana plantlet onto perlite for Fusarium bioassay. The roots were challenged with different numbers of variable spores. Disease development was scored after 28 days of experiments

Chitinase and β-1,3-glucanase Assays

A modified method of Tonon *et al*. (1998) was used to detect chitinase and β-1,3-glucanase activities. Chitinase activity was expressed as nkat/mg protein with N-acetylglucosamine as standard. β-1,3-glucanase activity was expressed as nkat/mg protein with glucose as standard.

Peroxidase Activity Assay

Peroxidase activity was assayed as described by Hammerschmidt *et al*. (1982) and was expressed in unit/mg protein at 470 nm. A change in 0.1 absorbance/minute/mg protein refers as one unit.

Polyphenol Oxidase Activity

Polyphenol oxidase activity was assayed as described by Mozzetti *et al*. (1995) and was expressed in unit/mg protein at 410 nm. A change in 0.1 absorbance/minute/mg protein refers as one unit.

Results and Discussion

Of six antibiotic selection agents tested, hygromycin was the best selective agent and followed by basta. Both selection agents showed an earliest sign of toxicity in fast for inhibition response at low concentration level particular in liquid medium (Table 3 and 4). Since the major transforming plasmid used in this study carries *npt11* gene that confers resistance to aminoglycoside antibiotics, it was suggested that geneticin G-418 at a concentration of 100 mg L^{-1} in solid and 50 mg L^{-1} in liquid media will be an effective selection of transformants for banana single buds. The use of alternating the solid and liquid medium appears to be more effective for selecting transformant tissues and possibility may minimise the occurrence of chimeras (non-transformed tissues). Main consideration was still on geneticin G-418 because all plasmids used in this study confers resistance to *npt 11* gene.

For microprojectile bombardment system, it was found that optimised physical parameters could achieved by bombarding twice at 1100 psi of helium pressure, 9 mm distance from stopping plate to target tissues, 28 mmHg vacuum pressure and 1 um of gold size based on GUS and GFP transient expressions. For biological parameters, single buds at 3 mm, 1.5 μg of gold per bombardment, three days preculture prior to bombardment and six days post bombardment were found to be optimal conditions for Pisang Rastali (AAB) (Sreeramanan *et al*., 2005).

For transformation using chitinase and β-1,3-glucanase genes, stable *gusA* and *gfp* genes in transformed single buds, multiple bud clumps, leaves and roots were successfully obtained (Fig. 3).

Table 3: The number of single buds which survived on selective solid media after four weeks of selection

Concentration (mg L^{-1})	Kanamycin	Geneticin G-418	Neomycin	Basta	Paromomycin	Hygromycin
0	40 (100)	40 (100)	40 (100)	40 (100)	40 (100)	40 (100)
25	27 (68)	19 (48)	35 (87)	1 (3)	32 (80)	6 (15)
50	14 (36)	5 (12)	29 (73)	0 (0)	28 (57)	0 (0)
100	1 (3)	1 (3)	24 (60)	0 (0)	23 (57)	0 (0)
150	0 (0)	0 (0)	15 (38)	0 (0)	16 (40)	0 (0)
200	0 (0)	0 (0)	7 (18)	0 (0)	8 (20)	0 (0)
250	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
300	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

For each selection, a total of 40 single buds were tested. The data were taken after four weeks on solid selective media. The numbers in parentheses indicate the percentage of surviving buds (number of single bud survived/total number buds selected)

Table 4: The number of single buds which survived on selective liquid media after four weeks of selection

Concentration (mg L^{-1})	Kanamycin	Geneticin G-418	Neomycin	Basta	Paromomycin	Hygromycin
0	40 (100)	40 (100)	40 (100)	40 (100)	40 (100)	40 (100)
25	19 (47)	4 (10)	30 (76)	1 (3)	27 (67)	2 (5)
50	4 (11)	1 (3)	27 (67)	0 (0)	14 (35)	0 (0)
100	1 (3)	0 (0)	24 (60)	0 (0)	23 (19)	0 (0)
150	0 (0)	0 (0)	15 (38)	0 (0)	1 (3)	0 (0)
200	0 (0)	0 (0)	6 (16)	0 (0)	0 (0)	0 (0)
250	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
300	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

For each selection, a total of 40 single buds were tested. The data were taken after four weeks on solid selective media. The numbers in parentheses indicate the percentage of surviving buds (number of single bud survived/total number buds selected)

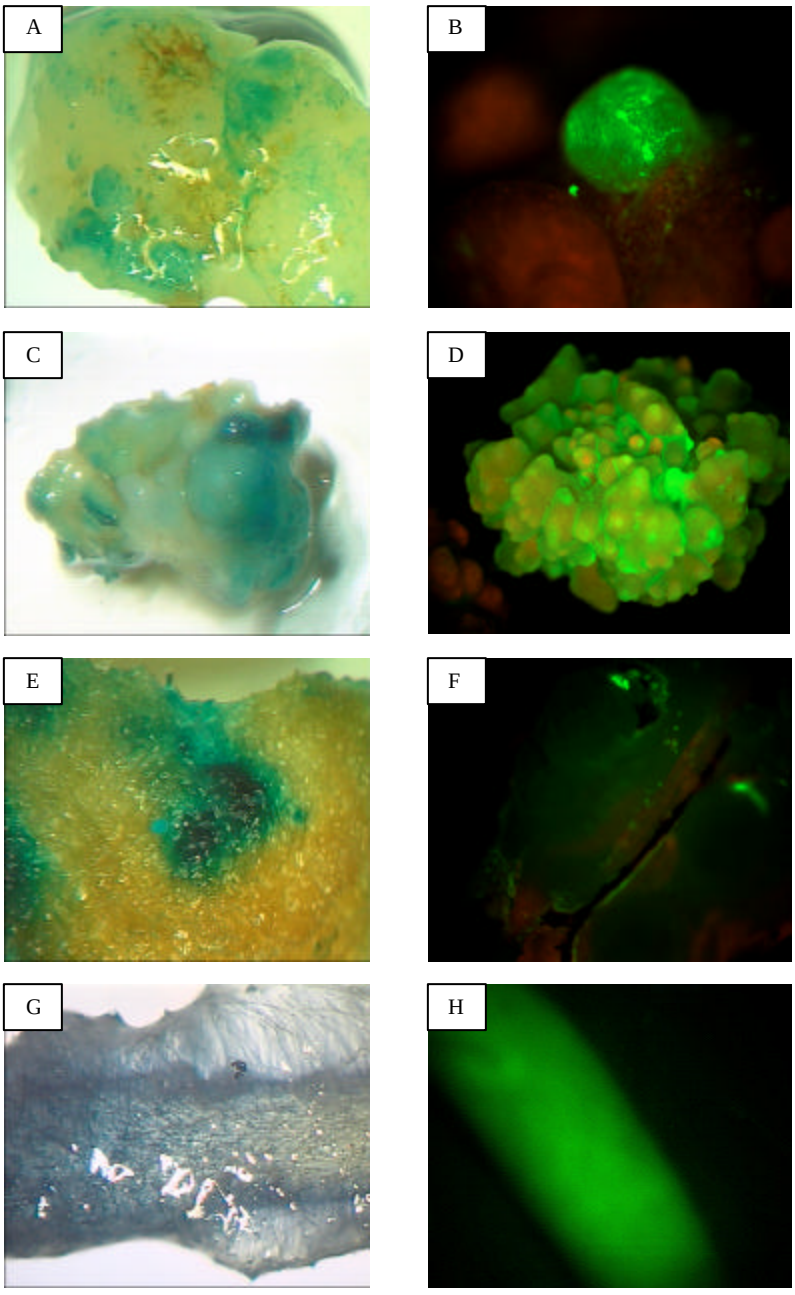

Fig. 3: Visualisation of stable *gusA* (A, C, E and G) and *gfp* (B, D, F and H) genes expression in various tissues of Pisang Rastali (AAB). A and B: single buds; C and D: multiple bud clumps (Mbcs); E and F: leaves; G and H: roots

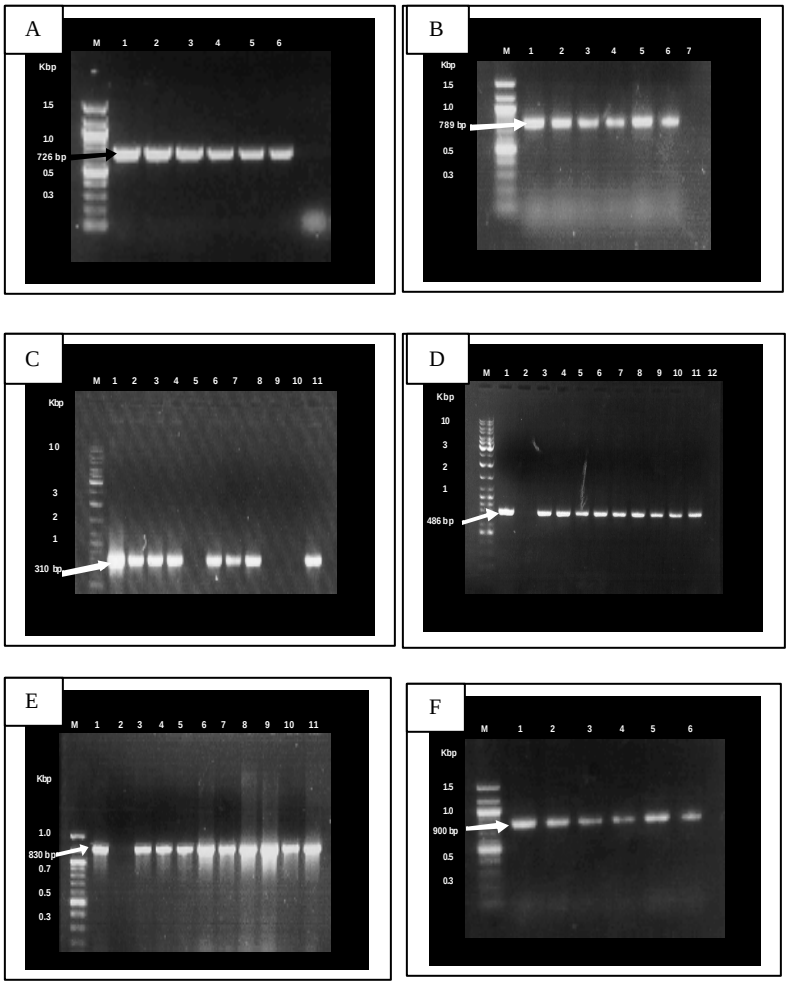

Fig. 4: PCR analyses. Amplification PCR products of 726 bp of *gfp* gene (A), 789 bp of *gusA* gene (B), 310 bp of *RCC2* (chitinase) gene (C), 486 bp of *Chi* (chitinase) gene (D), 830 bp of *Eg* (β-1,3 glucanase) gene (E) and 900 bp of *npt11* gene (F) in transgenic Pisang Rastali (AAB) plantlets selected on geneticin G-418

Integrative of the transgenes and stable of microprojectile bombardment system were assessed by PCR amplications 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 (Fig. 4a-f). Genomic Southern blot hybridization confirmed the incorporation of the RCC2, pMRC1301 and pROKLa-Eg genes in host genome between one and four inserted copies (Fig. 5a-c).

For *Fusarium* bioassay method, time course of *Fusarium oxysporum* f.sp. *cubense* (Race 1) spore production *in vitro* and the actual number of geminated spores determined. Highest spore production at 28 days with highest germination rate (62%) applied for bioassay testing of transgenic plantlets. Hydrogen peroxide (H_2O_2) and phenylalanine ammonia lyase (PAL) activities were the most sensitive and responsive compound and enzyme to *Fusarium* spore inoculation in transgenic plantlets reaching

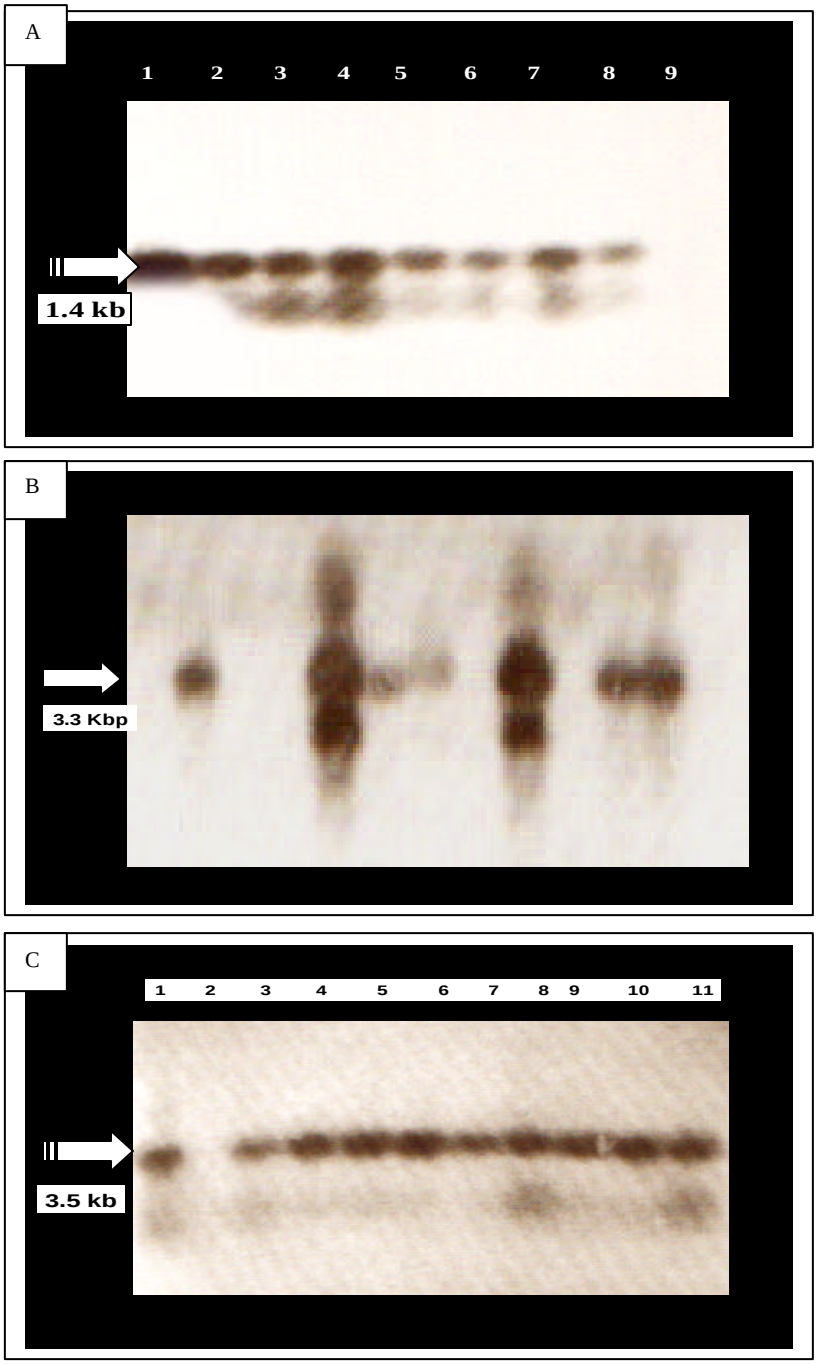

Fig. 5: Southern blot analyses. Integration patterns of the *RCC2* (Chitinase) gene (A) *Chi* (Chitinase) gene (B) and *Eg* (ß-1,3 glucanase) gene (C) in transgenic Pisang Rastali (AAB) plantlets selected on geneticin G-418

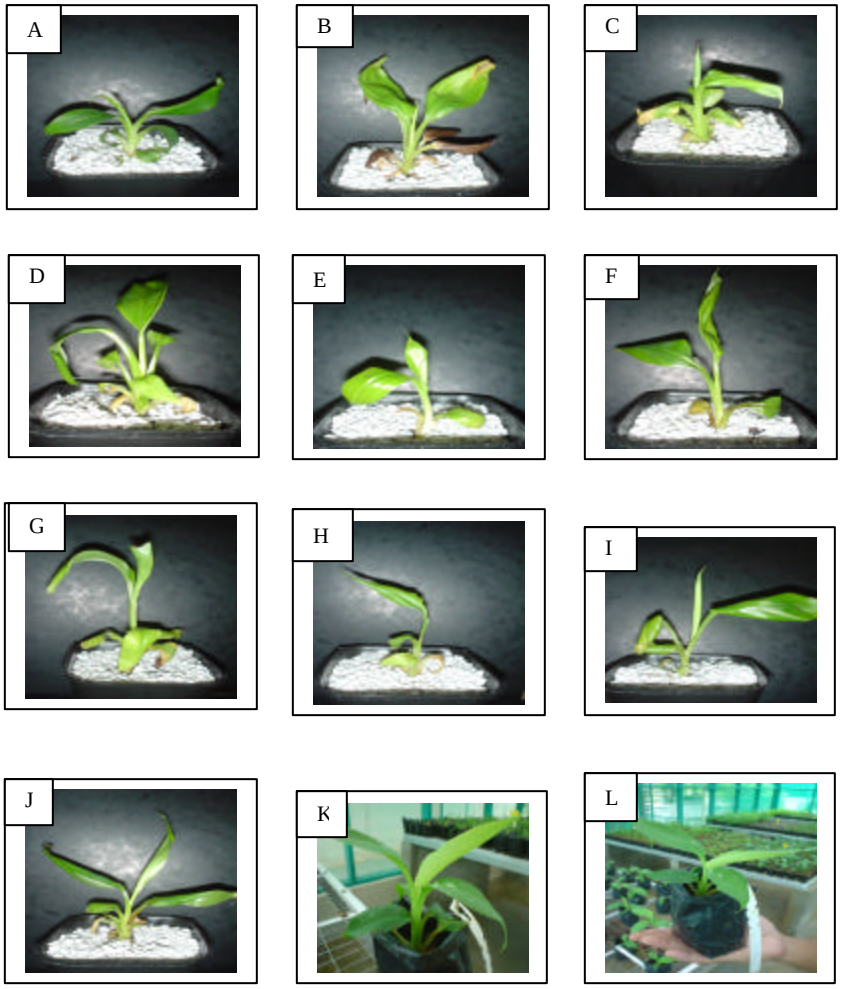

Fig. 6: Progress of Disease Symptoms (Secondary) caused by *Fusarium oxysporum* f.sp. *cubense* (Race1) on the leave of transgenic Pisang Rastali (AAB) plantlets (Particle bombardment-mediated transformation). (A) Control plantlet (No spore inoculated); (B) Control plantlet with (Spores were inoculated); (C) R2T1(19); (D) R3T1 (7); (E) \R1T2 (11); (F) R3T2 (24); (G) R2T3 (Y10); (H) R2T3 (Y11); (I) R3T4 (7); (J) R2T5 (42); (K and J) Transgenic banana in the glasshouse

significantly greater concentrations than the corresponding untransformed plantlets Transgenic Pisang Rastali (AAB) plantlets which contain both chitinase and β-1,3-glucanase genes show the higher chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase enzyme activities after 28 days compared to plantlets with a single inserted chitinase or β-1,3-glucanase gene. The involvement of peroxidase (PER) and polyphenol oxidase (PPO) in defense mechanisms in banana roots was suggested as the enzymes activity increased markedly after 28 days of inoculation. Progress of the disease symptoms caused by *Fusarium oxysporium* f.sp. *cubense* (race 1) on the leave of the transgenic Pisang Rastali (AAB) plantlets shown in Fig. 6.

Below is the summary of final evaluation obtained for leaf disease symptoms after four weeks inoculation (Fig. 6):

- Treatment 1 [*RCC2* (Chitinase gene) + *Eg* (β-1, 3 glucanase gene)] Both plants [Plant codes: R2T1 (19) and R3T1 (7)] appear healthy. However, plant code R3T1 (7) showed a slight yellow streaking in one leaf (lower part) after 25 days of inoculation.
- Treatment 2 [*Chi* (Chitinase gene) + *Eg* (β-1, 3 glucanase gene)] Both plants [Plant codes: R1T2 (11) and R3T2 (24)] appear healthy. However, both plant showed a slight discolouration in one younger leaf (upper part) before transfer into polybags.
- Treatment 3 [*RCC2* (Chitinase gene)] Both plants [Plant codes: R2T3 (Y10) and R2T3 (Y11)] showed slight streaking and yellowing of one older leaf (lower part) could be observed after 21 days of inoculation.
- Treatment 4 [*Chi* (Chitinase gene)] One yellowing of older leaf [Plant code: R3T4 (7)] could be observed after 17 days of inoculation. Slight yellow discolouration of the third and fourth leaves (upper part) beginning to appear after 22 days of inoculation.
- Treatment 5 [*Eg* (β-1, 3 glucanase gene)] This plant [Plant code: R2T5 (42)] appears slight streaking and yellowing on 25% of the total leaves after 28 days of inoculation.

Transgenic tomato plants expressing only a chitinase transgene or a β-1,3-glucanase transgene were susceptible to *Fusarium oxysporum* f.sp. *lycopersici*, whereas plants expressing both genes had significantly higher resistance than the plants expressing only chitinase or β-1,3-glucanase (Jongedijk *et al*., 1995). However, we found that combination between RCC2 (chitinase) and *Eg* (β-1,3-glucanase) genes found to be more effectives compared to combination with *chi* (chitinase) and *Eg* (β-1,3-glucanase) genes. In addition, several studies have been made on transgenic plants integrated with RCC2 chitinase gene possessed increased resistance to various fungal diseases (Asao *et al*., 1997; Nishizawa *et al*., 1999). Transgenic tobacco plants expressing a soybean β-1,3-glucan-elicitor releasing β-1,3 glucanase or the tobacco class II β-1,3 glucanase show reduced symptoms when infected with *Alternaria alternate* or the oomycetes *Phytophthora parasitica* var. *nicotiane* and *Peronospora tabacina* (Yoshikawa *et al*., 1993; Lusso *et al*., 1996). In addition, transgenic potato plants expressing soybean β-1,3-endoglucanase gene exhibit an increase in the activity of β-1,3 glucanase in transgenic plants (Borkowska *et al*., 1998).

A new tool is therefore available to contribute to the genetic improvement of local banana against Fusarium wilt disease. Although the production of non-chimaeric transgenic banana plants is at present limited to the use of embryogenic cultures, the improvement of selection techniques is envisaged for its direct application to the more readily accessible meristematic tissues (single buds used in this study), which will broaden the transformation possibilities to virtually all banana cultivars.

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