GENETIC ENGINEERING FOR TOLERANCE TO FUSARIUM WILT IN *MUSA SAPIENTUM* CV RASTALI (AAB)

By

SREERAMANAN SUBRAMANIAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Dedicated To:

My Lovingly Parents: Amma, Inthrani
And
Appa, Mr C.S.Maniam

My beloved wife: Deepa Chandramohan

Wonderful Brother and Sister: Sreeharan Subramaniam
Meena

My In-Laws: Dr and Mrs Chandramohan, Lavanya

Great Lab Friends: Vanitha Mariappan, Sunil Bhalla,
Roslí Normí, Alí Reza, Adeline Ting,
Yap Wai Sum and Chew Yern Chern.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

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Chairman: Professor Maziah Mahmood, PhD

Faculty : Biotechnology and Biomolecular Sciences

This study aims at developing tolerant Pisang Rastali (AAB) to Fusarium wilt via genetic engineering. Several considerations support that banana is a good candidate crop that could benefit from biotechnology applications.

Single buds from multiple bud clumps (Mbcs) of Pisang Rastali (AAB) were established to be suitable target tissues for the introduction of antifungal protein and reporter genes using the *Agrobacterium*-mediated and particle bombardment transformation methods. Multiple bud clumps (Mbcs) were easily micropropagated *in vitro* that provided a reliable source of potentially regenerable single bud tissues. The effectiveness of kanamycin, neomycin, geneticin G-418, paromomycin, basta and hygromycin as selection agents to inhibit growth of Pisang Rastali (AAB) was evaluated in solid and liquid media. Of six

antibiotic selection agents tested, hygromycin was the best, followed by basta and geneticin G-418. In addition, the use of liquid media has been shown to be more effective than in solid medium. Geneticin G-418 was the preferable antibiotic agent compared to kanamycin, neomycin and paromomycin since all plasmids used in this studies confers resistance to *npt11* gene.

Chemotaxis of *Agrobacterium tumefaciens* strains (EHA 101 and LBA 4404) towards wounded banana tissues has been studied using swarm agar plates. The results obtained indicate a minor role of chemotaxis in determining host specificity and suggest that it could not be responsible for the absence of tumorigenesis in banana under natural conditions. A method developed for the quantification of bacterial attachment to banana, based on the use of the *gusA* and *gfp* genes in marked *Agrobacterium* strains, is also described. Its application has demonstrated that attachment may also not be a main factor determining host specificity in *Agrobacterium tumefaciens*. In addition, optimization of several factors affecting transient *gusA* and *gfp* genes expression such as preculture period, co–cultivation period, acetosyringone concentrations, types of wounding, strains of *Agrobacterium tumefactions,* influence of single bud sizes and post cultivation period were evaluated to determine the efficiency of *Agrobacterium*-mediated transfer during the early stages of transformation in banana particularly in Pisang Rastali (AAB).

Physical and biological parameters affecting DNA delivery into Pisang Rastali (AAB) single buds have been optimised. The physical parameters tested were helium pressure, distance from stopping plate to target tissues, vacuum pressure, multiple bombardment

and gold microcarrier size. The optimised biological parameters were explant types, effect of various sizes of explants, effect of preculture treatment prior bombardment, effect of DNA concentrations and post bombardment incubation time.

Regeneration of transgenic Pisang Rastali (AAB), molecular analyses (PCR and Southern blot), analysis of chitinase and β-1,3-glucanase protein production via *Agrobacterium*-mediated transformation were carried out. DNA samples from transgenic Pisang Rastali (AAB) obtained only from selection media contained geneticin G-418 as a selectable marker and were tested positive for the presence of the chitinase (RCC2) or β-1,3 glucanase (Eg) and *npt11* coding sequences by PCR analyses. Genomic Southern blot hybridization confirmed the incorporation of the the chitinase (RCC2) or β-1,3-glucanase (Eg) gene into host genome.

Using particle bombardment system, five different treatments using different chitinases and β-1,3-glucanase genes inserted singly or in combination were carried out. Genes encoding either chitinases (RCC2 or *Chi*) or β-1,3-glucanase (Eg) or both was bombarded together with *gfp* gene (pGEM.Ubi-SgfpS65T) for early transient expressions signal. Integration of the transgenes and stablity of the particle bombardment system were confirmed by PCR amplification of *gfp*, *gusA*, RCC2 (chitinase), *Chi* (chitinase), Eg (β-1, 3-glucanase) and *npt11* genes. Genomic Southern blot hybridization confirmed the incorporation of the RCC2, *Chi* and Eg genes in host genome between one and five inserted copies in transformed plantlets. Similar to *Agrobacterium*-mediated transformation system, the chitinase and β-1,3-glucanase enzyme activity of transgenic

plantlets obtained from particle bombardment was higher than untransformed plantlets. In addition, co-bombardment of chitinase and β-1,3 glucanase genes (Treatment 1 and 2) gave higher enzyme activities compared with single gene insertion (Treatment 3, 4 and 5).

For *Fusarium* bioassay method, time course of *Fusarium oxysporum* f.sp. *cubense* (race 1 ; VCG 01217) spore production *in vitro* and the actual number of germinated spores were determined . The 28 days old *Fusarium* spores with 62% germinating capacity at $2x10^6$ spores/ml were used for bioassay testing of transgenic Pisang Rastali (AAB) plantlets. Hydrogen peroxide (H_2O_2) and phenylalanine ammonia lyase (PAL) is the most sensitive chemical compound and enzyme to *Fusarium* spores inoculation. The chitinase and β-1,3-glucanase enzyme activities of transgenic plantlets was almost four folds higher than untransformed plantlets. The peroxidase (PER) and polyphenol oxidase (PPO) enzyme activities in banana roots increased markedly up to 2-3 folds higher in transformed plants compared to untransformed plantlets after 28 days of inoculation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEJURUTERAAN GENETIK PISANG YANG TOLERAN TERHADAP LAYU FUSARIUM PADA *MUSA SAPIENTUM* CV RASTALI (AAB).

Oleh

SREERAMANAN SUBRAMANIAM

April 2005

Pengerusi: Profesor Maziah Mahmood, PhD

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Kajian ini bertujuan menghasilkan pisang Rastali (AAB) yang toleran terhadap penyakit layu Fusarium melalui kejuruteraan genetik. Beberapa kenyataan menyokong bahawa pisang adalah tanaman pertanian yang sesuai dan mendapat faedah daripada aplikasi bioteknologi.

Pucuk tisu individu daripada kelompok tisu pucuk pisang Rastali (AAB) dihasilkan bagi kesesuaian tisu sasaran untuk menerima protein antikulat dan gen pelapor dengan menggunakkan kaedah transformasi perantaraan *Agrobakterium* dan tembakan partikel. Kelompok pucuk tisu mudah dikulturkan secara *in vitro* dan berpotensi untuk regenerasi pucuk tisu secara individu. Kesesuaian kanamycin, neomycin, geneticin G-418, paromomycin, basta dan hygromycin sebagai agen pemilih dinilai dalam media pepejal dan cecair. Daripada enam agen antibiotik yang diuji, hygromycin adalah yang terbaik, diikuti dengan basta dan geneticin G-418. Selain itu, penggunaan medium cecair didapati

lebih efektif daripada medium pepejal. Geneticin G-418 merupakan agen antibiotik yang lebih sesuai berbanding dengan kanamycin, neomycin dan paromomycin kerana semua plasmid yang digunakan dalam penyelidikkan ini menunjukkan kerintangan terhadap gen *npt11*.

Kemotaksis oleh jenis *Agrobakterium tumefaciens* (EHA 101 dan LBA 4404) terhadap tisu pisang yang dilukakan telah dikaji dengan menggunakan 'swarm agar plates'. Keputusan yang diperolehi menunjukkan peranan kemotaksis adalah kecil dalam menentukan kekhususan penerima dan ini menunjukkan ia tidak bertanggungjawab bagi ketiadaan tumoregenesis pada pisang dalam keadaan semulajadi. Satu kaedah pengukuran bagi pelekatan bakteria berdasarkan penggunaan gen *gusA* dan *gfp* yang terdapat pada *Agrobakterium* juga dihasilkan. Aplikasi ini menunujukkan bahawa pelekatan bukanlah merupakan faktor utama dalam menentukan kekhususan penerima dalam *Agrobakterium tumefaciens*. Selain itu, pengoptimuman beberapa faktor yang mempengaruhi pengekspresan gen sementara *gusA* dan *gfp,* seperti tempoh para-kultur, tempoh pengeraman, kepekatan acetosyringone, jenis kecederaan, jenis *Agrobakterium tumefaciens*, pengaruh saiz tunas individu dan jangka masa selepas subkultur dinilai untuk menentukan keberkesanan pemindahan perantaraan *Agrobakterium* pada peringkat awal transformasi pisang, khususnya pisang Rastali (AAB).

Parameter fizikal dan biologi yang mempengaruhi pemindahan DNA ke dalam individu pucuk tisu pisang Rastali (AAB) telah dioptimumkan. Parameter fizikal yang diuji ialah tekanan helium, jarak antara plat penghalang dengan tisu sasaran, tekanan vakum,

tembakan berganda dan saiz pembawa mikro emas. Parameter biologi yang dioptimumkan ialah jenis tisu penerima, kesan pelbagai saiz tisu asal, kesan rawatan para-kultur sebelum penembakan, kesan kepekatan DNA dan tempoh pengeraman selepas tembakan.

Regenerasi pisang Rastali (AAB) transgenik, analisis molekul (PCR and Southern blot), analisis protein chitinase dan β-1,3-glucanase melalui transformasi perantaraan *Agrobakterium* telah dijalankan. Sampel DNA daripada pisang Rastali (AAB) transgenik diperolehi hanya daripada media pemilihan yang mengandungi geneticin G-418 sebagai penanda terpilih dan telah diuji positif bagi kehadiran gen chitinase dan β-1,3-glucanase (Eg) dan kod linear *npt11* melalui analisis PCR. Hybridasi genomik Southen blot telah membuktikan pemindahan gen chitinase (RCC2) dan β-1,3-glucanase didalam genomik penerima.

Menggunakan sistem tembakan partikel, lima rawatan berlainan menggunakan gen chitinase dan β-1,3-glucanase berlainan kombinasi dipindahkan secara tunggal dan gabungan dijalankan. Gen berkod chitinase (RCC2 atau Chi) atau β-1,3-glucanase (Eg) dan kedua-duanya sekali ditembakkan bersama dengan gene *gfp* (pGEM.Ubi-SgfpS65T) bagi mendapatkan isyarat awal pengekspresan gen sementara pada peringkat awal. Integrasi transgen dan kestabilan system tembakan partikel dibuktikan melalui amplifikasi gen *gfp*, *gusA*, RCC2 (chitinase), *Chi* (chitinase), Eg (β-1,3-glucanase) dan *npt11*. Hybridasi Southern blot genomik membuktikan kehadiran gene RCC2, Chi dan Eg pada genomik penerima antara satu hingga lima salinan dimasukkan pada anak pokok

yang ditransformkan. Sama seperti kaedah transformasi perantaraan *Agrobakterium*, aktiviti enzim chitinase dan β-1,3-glucanase pada anak pokok transgenik yang diperolehi daripada tembakan partikel adalah lebih tinggi daripada pokok yang tidak ditransformasikan. Selain itu, tembakan bersama gen chitinase dan β-1,3-glucanase (Rawatan 1 dan 2) menghasikan aktiviti enzim yang lebih tinggi berbanding kemasukkan gen tunggal (Rawatan 3, 4 dan 5).

Bagi kaedah biouji *Fusarium*, tempoh pengeraman spora *Fusarium oxysporum f.sp*. *cubense* (race 1; VCG 01217) secara *in vitro* dan bilangan sebenar percambahan spora telah ditentukan. Spora *Fusarium* pada hari ke 28 dengan keupayaan 62% percambahan pada $2x10^6$ spora/ml telah digunakan sebagai biouji pokok pisang Rastali (AAB) transgenik. Hidrogen peroxide (H_20_2) dan phenylalanine ammonia lyase (PAL) adalah bahan kimia dan enzim yang paling sensitif kepada inokulasi spora *Fusarium*. Aktiviti enzim chitinase dan β-1,3-glucanase pada anak pokok transgenik adalah hampir empat kali ganda lebih tinggi daripada pokok yang tidak ditransformasikan. Aktiviti enzim peroxidase (PER) dan polyphenol oxidase (PPO) pada akar pisang didapati dua hingga tiga kali ganda lebih tinggi daripada anak pokok yang tidak ditransformasikan selepas 28 hari inokulasi.

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I certify that an Examination Committee met on _____ to conduct the final examination of Sreeramanan Subramaniam on his Doctor of Philosophy thesis entitled "Genetic Engineering For Tolerance To Fusarium wilt in *Musa sapientum* cv Rastali (AAB)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations that have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

SREERAMANAN SUBRAMANIAM

Date:

TABLE OF CONTENTS