UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF AN AGROBACTERIUM-MEDIATED TRANSFORMATION SYSTEM AND IN VITRO REGENERATION PROTOCOL FOR RICE (ORYZA SATIVA SP. INDICA VAR.) MR219

CHIN WAI HOE

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Agricultural Biotechnology

September 2008

DEDICATIONS

to :

My mother (Wong Pow Yoong)
My brother (Chin Shee Hoe)

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of
Master Science of Agrobiotechnology

ESTABLISHMENT OF AN AGROBACTERIUM-MEDIATED TRANSFORMATION SYSTEM AND IN VITRO REGENERATION PROTOCOL FOR RICE (ORYZA SATIVA SP. INDICA VAR.) MR219

By

CHIN WAI HOE

July 2008

Chairman :Associate Professor Datin Dr. Siti Nor Akmar Abdullah, PhD

Faculty : Agriculture

This study consisted of several parts which include development of tissue culture and regeneration system for local *indica* rice MR 219 variety, establishment of *Agrobacterium*-mediated transformation system and molecular analysis to confirm introduction of oil palm leaf-specific promoter in putative rice transformants

Different concentrations of 2-4,D (0, 1.5, 3.0, 4.5, 7.5 and 10mg/l) were tested for embryogenic and nodular calli induction from scutellum region of *indica* rice using MS medium supplemented with 500 mg/L proline, 500 mg/L casein hydrolysate, 30 g/L sucrose and 2.5 g/L gelrite and it was shown that 3.0mg/l 2-4,D was the best concentration to use.

Different concentrations of 6-benzylaminopurine (BAP) (1.0, 2.0, 4.0, 6.0 mg/l) alone or in combination with 0.5mg/l naphthalene acetic acid (NAA) and two different concentrations of Kinetin (1.0 and 2.0 mg/l) in MS media in the presence of 500 mg/L proline, 500 mg/L casein hydrolysate, 30 g/L sucrose and 6.0 g/L gelrite were used to determine the most suitable plant growth regulators for regeneration of rice plants. The results showed that BAP 6.0 mg/l alone is the best condition for multiple shoot formation from desiccated rice calli.

Plasmid pCAMBIA 1301 is a binary vector having hygromycin resistant gene (*hpt*) as selectable marker gene in the T-DNA region. The minimal inhibitory concentration of hygromycin was determined by testing different concentrations of hygromycin (10, 20, 30, 50, 70 ,90mg/l) for survival of rice embryogenic callus. Hygromycin at 50 mg/l which gave 53.34% retarded growth of calli but with minimal browning was chosen as the most suitable for selection of putative transformants. This experiment together with the other tissue culture experiments were conducted and arranged in a Completely Randomized Design (CRD).

The oil palm leaf-specific gene promoter was cloned individually into binary vector pCambia 1301 carrying β-glucuronidase (GUS) reporter gene after removal of the CaMV 35S promoter and the recombinant plasmids produced were transferred into *Agrobacterium tumefaciens* strain EHA105 and C58.

Agrobacterium tumefaciens strain EHA 105 and C58 shown to contain oil palm leaf-specific promoter based on PCR analysis were used to transform rice calli.

Calli subjected to heat and centrifugation treatments were found to be sucessfuly transformed based on GUS histochemical analysis. Different concentrations of antibiotics on the MS medium including carbenicilin (250, 500, 800, 1000, 1500, 1800 and 2000 mg/l), cefotaxime (250, 500, 800 mg/l), timentin (200,300 mg/l) either alone or in combination were not successful in eliminating *Agrobacterium* after transformation. PPM (plant preservative mixture) was found to be the best chemical to remove excessive *Agroabcterium*. Calli were subsequently transferred to regeneration medium (MS salts gelled with 500 mg/L proline, 500 mg/L casein hydrolysate, 30 g/L sucrose and 6 g/L gelrite, 50mg/l hygromycin B, pH 5.8) after hygromycin selection.

Successful introduction of the oil palm tissue-specific promoters in putative transformants were confirmed via PCR and real time PCR analysis using primers designed based on the oil palm leaf-specific promoter sequence. Real time PCR analysis showed that the gene copy numbers of transgenic calli were not more than 2 copies per genome. Using GUS histochemical assay it was shown that CAMV 35S promoter but not the oil palm leaf-specific promoter can drive GUS expression in transformed rice calli.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Agrobioteknologi

PEMBANGUNAN SISTEM KULTUR TISU DAN TRANSFORMASI BERPERANTARAAN AGROBAKTERIA PADI *INDICA* VARIETI MR219

Oleh

CHIN WAI HOE

July 2007

Pengerusi : Professor Madya Datin Dr. Siti Nor Akmar Abdullah

Fakulti : Pertanian

Penyelidikan ini dibahagikan kepada beberapa bahagian iaitu tisu kultur, dan regenerasi padi *indica* varieti MR 219, transformasi menggunakan *Agrobakteria* dan analisis molekular untuk mengesahkan kejayaan promoter spesifik daun kelapa sawit yang telah masukkan ke dalam padi.

2-4,D dengan konsentrasi yang berlainan (0, 1.5, 3.0, 4.5, 7.5 dan 10mg/l) telah digunakan untuk menguji induksi kalus embriogenik dan kalus nodular daripada padi *indica* dengan menggunakan MS yang telah dicampurkan dengan 500 mg/l prolin, 500 mg/l kasein hidrolisat, 30 g/l sukrosa and 2.5 g/l gelrite. Keputusan menunjukan bahawa 3.0 mg/l 2-4,D adalah konsentrasi yang terbaik untuk digunakan.

Penggunaan 6-benzylaminopurine (BAP) dengan konsentrasi yang berlainan (1.0, 2.0, 4.0, 6.0 mg/l) sahaja atau dengan kombinasi 0.5 mg/l naphthalene acetic acid (NAA) dan dua konsentrasi kinetin yang berlainan (1.0, 2.0 mg/l) dalam media MS dengan kehadiran 500 mg/l prolin, 500 mg/l kasein hidrolisat, 30 g/l sukrosa dan 6.0 g/l gelrite telah digunakan untuk menentukan jenis hormon yang paling sesuai bagi percambahan pucuk padi. Dalam penyelidikan ini, penggunan BAP sahaja dengan konsentrasi 6.0 mg/l adalah yang terbaik dalam perbentukkan pucuk berganda daripada kalus yang dikeringkan.

Plasmid pCAMBIA 1301 adalah vektor binari yang mengandungi gen rintang higromisin dalam T-DNA. Kadar minima konsentrasi perencatan higromisin telah ditentukan dengan menggunakan konsentrasi higromisin yang berlainan (10, 20, 30, 50, 70 dan 90mg/l) untuk menguji ketahanan kalus embriogenik padi. Dalam penyelidikan ini, higromisin dengan konsentrasi 50 mg/l memberikan 53.34% perencatan pertumbuhan dan kalus menunjukkan keperangan yang paling minima dan telah dipilih sebagai konsentrasi yang paling sesuai untuk pemilihan kalus yang ditransformasikan. Kesemua eksperimen dilaksanakan dan disusun mengikut Rekabentuk Rawak Lengkap.

Promoter khusus daun kelapa sawit telah diklonkan ke dalam vektor binari pCAMBIA 1301 yang mengandungi gen β-glucuronidase (GUS) selepas promoter CaMV 35S telah dikeluarkan. Plasmid rekombinan yang telah

dihasilkan dipindahkan kepada *Agrobacterium tumefaciens* jenis EHA105 and C58. Daripada analisis PCR yang telah dijalankan *Agrobakteria* yang dipastikan mengandungi promoter khusus daun kelapa sawit telah digunakan untuk transformasi kalus padi.

Kalus yang telah diuji dengan kaedah haba dan pengemparan didapati berjaya ditransformasikan berasaskan analisis histokimia GUS. Kandungan konsentrasi antibiotik yang berbeza dalam MS media termasuk carbenicilin (250, 500, 800, 1000, 1500, 1800 and 2000 mg/l), cefotaxime (250, 500, 800 mg/l), timentin (200,300 mg/l) sama ada sendiri atau bergabung, didapati tidak berjaya dalam menyingkirkan *Agrobakteria* selepas ditransformasikan. PPM telah didapati sebagai bahan kimia yang paling bagus dalam menyingkirkan *Agroabakteria* yang tidak dikehendaki. Kalus kemudian dipindahkan kepada media pertumbuhan pucuk (garam MS dengan 500 mg/l prolin, 500 mg/l kasein hidrolisat, 30 g/L sukrosa dan 6 g/l gelrite, 50mg/l higromisin B, pH 5.8) selepas pemilihan higromisin.

PCR dan analisis Real Time PCR menggunakan primer-primer yang dihasilkan berasaskan jujukan promoter khusus daun kelapa sawit menunjukkan promoter khusus daun kelapa sawit telah berjaya ditransformasikan ke dalam padi *indica* MR219. Analisis Real Time PCR juga telah menunjukan bahawa bilangan salinan gen dalam kalus transgenik adalah tidak lebih daripada dua salinan per genom. Esei histokimia GUS menunjukkan bahawa promoter CAMV 35S dan

bukan promoter khusus daun kelapa sawit boleh menggalakan pengespressan GUS dalam kalus padi yang telah ditransformasikan.

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I especially appreciate my mother and brother for their continuing encouragement and support and for their patience and understanding during my graduate studies.

I certify that an Examination Committee has met on 24th September 2008 to conduct the final examination of Chin Wai Hoe on his thesis entitled ""ESTABLISHMENT OF AN AGROBACTERIUM-MEDIATED TRANSFORMATION SYSTEM AND IN VITRO REGENERATION PROTOCOL FOR RICE (ORYZA SATIVA SP. INDICA VAR.) MR219 ""in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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Date : 29 January 2009

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree fo Master Science of Agrobiotechnology. The members of the Supervisory Committee are as follows:

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Date:12 February 2009

DECLARATION

I hereby declare that this thesis is based on my original work except quotations and citations which 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.

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CHIN WAI HOE
Date:

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LIST OF ABBREVIATION

%	per cent
°C	degree Centigrade
µl	microliter
µM	micromolar
A_{600}	Absorbance at 600 nonometers
ANOVA	analysis of variance
BAP	6-benzylaminopurine
bp	base pairs
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
cm	centimeter
CRD	completely randomized design
cv.	cultivar
ddH_2O	distilled deionized water
DNA	deoxy ribonucleic acid
DNMRT	duncan new multiple range test
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
et al.	et alia