



**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**

**FP 2008 33**



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**By**

**CHIN WAI HOE**

**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 Hoo)*



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  $\beta$ -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 successfully transformed based on GUS histochemical analysis. Different concentrations of antibiotics on the MS medium including carbenicillin (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 *Agrobacterium*. 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**

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**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 menunjukkan 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, penggunaan BAP sahaja dengan konsentrasi 6.0 mg/l adalah yang terbaik dalam perbentukan 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  $\beta$ -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 menunjukkan 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 certify that an Examination Committee has met on 24<sup>th</sup> 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.

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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.

---

CHIN WAI HOE  
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## TABLE OF CONTENTS

	<b>Page</b>
<b>DEDICATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	vi
<b>ACKNOWLEDGEMENTS</b>	ix
<b>APPROVAL</b>	xii
<b>DECLARATION</b>	xiii
<b>LIST OF TABLES</b>	xvi
<b>LIST OF FIGURES</b>	xviii
<b>LIST OF ABBREVIATIONS</b>	xxi
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	
2.1 The Origin of Rice	6
2.2 Tissue Culture of Rice	7
2.2.1 Somatic Embryogenesis and Regeneration	8
2.3 Plant Transformation	11
2.3.1 <i>Agrobacterium</i> -mediated Transformation	14
Cereal Crops	15
Other Monocotyledonous Plants	19
2.4 Factors Influencing the Transformation Efficiency	24
2.4.1 Explants	24
2.4.2 <i>Agrobacterium</i> Strains and Plasmids	27
2.4.3 Pretreatment and Co-culture Conditions	32
2.5 Removal of <i>Agrobacterium</i> after Transformation	30
2.6 Selectable Marker	31
2.7 Promoter Analysis	32
<b>3 PLANT REGENERATION SYSTEM OF LOCAL INDICA RICE VARIETY</b>	<b>37</b>
3.1 Introduction	37
3.2 Material and Methods	39
3.2.1 Explant Materials	39
3.2.2 Callus Induction	40
3.2.3 Medium for Determining Minimum Inhibitory Concentration (MIC) for Hygromycin B	41
3.2.4 Shoot Regeneration	41



3.2.5	Experimental Design and Statistical Analysis	45
3.3	Results and Discussion	45
3.3.1	Callus Induction	45
3.3.2	Shoot Regeneration	53
3.3.3	Determination of Minimal Inhibitory Concentration of Hygromycin B	60
3.4	Conclusion	63
<b>4</b>	<b>OPTIMISATION OF AGROBACTERIUM-MEDIATED TRANSFORMATION PROTOCOL OF INDICA MR219 RICE VARIETY AND INTRODUCTION OF VECTOR CONSTRUCT CONTAINING OIL PALM LEAF-SPECIFIC PROMOTER</b>	<b>64</b>
4.1	Introduction	64
4.2	Materials and Methods	67
4.2.1	<i>Agrobacterium</i> Strains and Culture	67
4.2.2	<i>Agrobacterium</i> Transformation: Freeze-Thaw Method	68
4.2.3	<i>Agrobacterium</i> -mediated Transformation of Rice Callus	69
4.2.4	Bacterial Inoculation	70
4.2.5	Optimization of Transformation Protocol	70
4.2.6	Assay for GUS Activity	73
4.2.7	Antibiotic Selection	73
4.2.8	Comparison between <i>Agrobacterium</i> <i>tumefaciens</i> Strain EHA 105 and C58	76
4.2.9	Regeneration of Transgenic Plants Production of Vector Constructs Containing Oil Palm Leaf-Specific Promoter Estimating Copy Number of Transgene in Transgenic Calli by Absolute Quantitative Real-time PCR	76 77 78
4.3	Results and Discussion	83
4.3.1	Transformation of <i>Agrobacterium</i> with pCAMBIA 1301	83
4.3.2	Establishment of Method for Obtaining Successfully Transformed <i>Oryza sativa</i> MR219 Rice Calli	87
4.3.3	Analysis of transformants	88
4.3.4	Transformation Efficiency of EHA 105 and C58 <i>Agrobacterium</i> Strains Using Heat and Centrifugation Method	93
4.3.5	Study to Overcome <i>Agrobacterium</i> Contamination of Calli after Co-cultivation	95
4.3.6	Production of Promoter: Reporter Gene	100

	Construct	
	4.3.7 Molecular analysis of transgenic rice calli	106
	4.4 Conclusion	117
<b>5</b>	<b>GENERAL DISCUSSION</b>	<b>120</b>
	5.1 Introduction	120
	5.2 <i>In vitro</i> Culture of Rice	121
	5.3 Transformation of Rice	123
	5.4 Molecular analysis for Integration and Expression of Genes	125
<b>6</b>	<b>CONCLUSION</b>	<b>128</b>
	<b>REFERENCES</b>	<b>130</b>
	<b>APPENDICES</b>	<b>152</b>
	<b>BIODATA OF STUDENT</b>	<b>157</b>



## LIST OF TABLES

Table		Page
2.1	Transgenic crop production area by country	12
2.2	<i>Agrobacterium</i> mediated transformation of rice	21
2.3	The progress in production of transgenic rice plant	35
3.1	Media containing different concentration of 2,4-D for callus induction from seeds of <i>Oryza sativa</i> MR219	42
3.2	Media containing different concentration of hygromycin B for determining the minimal inhibitory concentration for retarding callus growth from seeds of <i>Oryza sativa</i> MR219	43
3.3	Media containing different concentration of plant growth regulators for regeneration from callus of <i>Oryza sativa</i> MR219 seeds	44
3.4	Percentage of callus formation from <i>Oryza sativa</i> MR219 seeds based on the callus intensity at each concentration of 2,4-S tested after three weeks of culture	52
3.5	Effect of plant growth regulators on percentage of green spot, shoot regeneration from calli of <i>Oryza sativa</i> MR219 seeds after 30 days of culture	59
3.6	Effect of Hygromycin B on callus growth of <i>Oryza sativa</i> MR219 after four weeks of culture	61
4.1	The rice calli were subjected to six different experiments as indicated	72
4.2	The effects of different concentration of three different concentration of three different antibiotics (carbenicilin, cefotaxime and timentin) and plant preservative mixture (PPM) in overcoming <i>Agrobacterium</i> contamination after co-cultivation	75
4.3	Primer Pairs for absolute quantitative real time PCR	79



4.4	The effects of six different treatments in producing successfully transformed rice calli	90
4.5	The effects of different concentration of three different concentration of three different antibiotics (carbenicilin, cefotaxime and timentin) and plant preservative mixture (PPM) in overcoming <i>Agrobacterium</i> contamination after co-cultivation	96
4.6	Reproducibility of the $C_t$ measurement in replicate standard for recombinant plasmid pCAMBIA 1301 and transformed rice calli	114
4.7	Average number of transgene insertions in each transformed calli	116



## LIST OF FIGURES

Figure		Page
3.1	Pedigree of MR219 rice variety	39
3.2	Callus developed from scutellar region of <i>indica</i> rice MR219 seeds on MS basal medium supplemented with 3.0 mg/l 2, 4-D, 500 mg/l proline, 500 mg/l casein hydrolysate, 30 g/l sucrose and gelrite 2.5 g/l after 7 days of culture	48
3.3	Pictorial presentation of calli formation on proliferation medium after 21 days of culture.	49
3.4	Percentage of callus formation from <i>Oryza sativa</i> MR219 seeds placed on medium containing different 2, 4-D concentration after three weeks of culture	51
3.5	Shoot formation on MS medium supplemented with 6.0 mg/l BAP, 500 mg/l proline, 500 mg/l casein hydrolysate, 30 g/l sucrose and 6.0 g/l gelrite after four weeks of culture	62
3.6	Percentages of green spots and shoot formation from partially dehydrated calli of <i>Oryza sativa</i> MR219 placed on media containing different concentrations of BAP alone and different concentration of kinetin in combination with NAA 0.5 mg/l after four weeks of culture.	65
3.7	Effect of the minimal inhibitory concentration of hygromycin B on calli growth of <i>Oryza sativa</i> MR219 after four weeks of culture	62
4.1	Map of pCAMBIA 1301 with GUS as reporter gene	67
4.2	The oil palm leaf-specific promoter inserted into the <i>Eco</i> R1 and <i>Bgl</i> II sites after removal of the <i>CaMV</i> 35S promoter <i>pCAMBIA</i> 1301 (11Kb) to generate transcriptional configuration to the GUS gene	78
4.3	Plate setup for absolute real time PCR in gene copy number quantification	82



4.4	PCR analysis to confirm successful introduction of pCAMBIA 1301 with the GUS reporter gene into <i>Agrobacterium</i>	85
4.5	PCR analysis to confirm genotype of <i>A. tumefaciens</i> which was successful transformed with the pCAMBIA 1301	86
4.6	Pictorial presentation of calli showing GUS expression from experiment 6	91
4.7	Transformation efficiency of rice calli subjected to heat and centrifugation prior to infection with <i>Agrobacterium tumefaciens</i>	92
4.8	Comparison of the transformation efficiency of two different <i>A. tumefaciens</i> strain using the heat and centrifugation transformation method	94
4.9	<i>Agrobacterium</i> contaminations of calli were not overcome with antibiotic treatment	97
4.10	Bacteria contamination was overcome by soaking of co-cultivated calli in PPM	98
4.11	The effect of soaking calli in plant preservative mixture (PPM) in overcoming <i>Agrobacterium</i> contamination after co-cultivation	99
4.12	Cloning of oil palm leaf-specific promoter into binary vector pCAMBIA 1301	101
4.13	PCR analysis to confirm successful introduction of recombinant vector containing oil palm leaf specific promoter into <i>A. tumefaciens</i>	102
4.14	After elimination of bacteria <i>Agrobacterium tumefaciens</i> , selection of putative transformed calli on callus induction media containing 50 mg/l hygromycin to select for putative transformants	103
4.15	Transformant of calli with oil palm leaf-specific promoter subjected for histochemical GUS assay	105
4.16	PCR analysis to confirm successful introduction of recombinant oil palm leaf-specific promoter with GUS as	107



	reporter gene into rice calli	
4.17	Standard curve of recombinant plasmid pCAMBIA 1301 containing oil palm leaf-specific promoter	109
4.18	Melting curves depicting the fluorescence negative first derivative plots for the application of the standard plasmid and transgenic calli from triplicate measurements	110
4.19	Real-time PCR amplification of recombinant plasmid pCAMBIA 1301 containing oil palm leaf-specific promoter	111
4.20	Melting curves depicting the fluorescence intensity the amplified for the standard plasmid and transgene calli from triplicate measurements	112





## LIST OF ABBREVIATION

%	per cent
°C	degree Centigrade
µl	microliter
µM	micromolar
A <sub>600</sub>	Absorbance at 600 nonometers
ANOVA	analysis of variance
BAP	6-benzylaminopurine
bp	base pairs
CaCl <sub>2</sub>	calcium chloride
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
cm	centimeter
CRD	completely randomized design
cv.	cultivar
ddH <sub>2</sub> O	distilled deionized water
DNA	deoxy ribonucleic acid
DNMRT	duncan new multiple range test
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra acetic acid
<i>et al.</i>	et alia

