



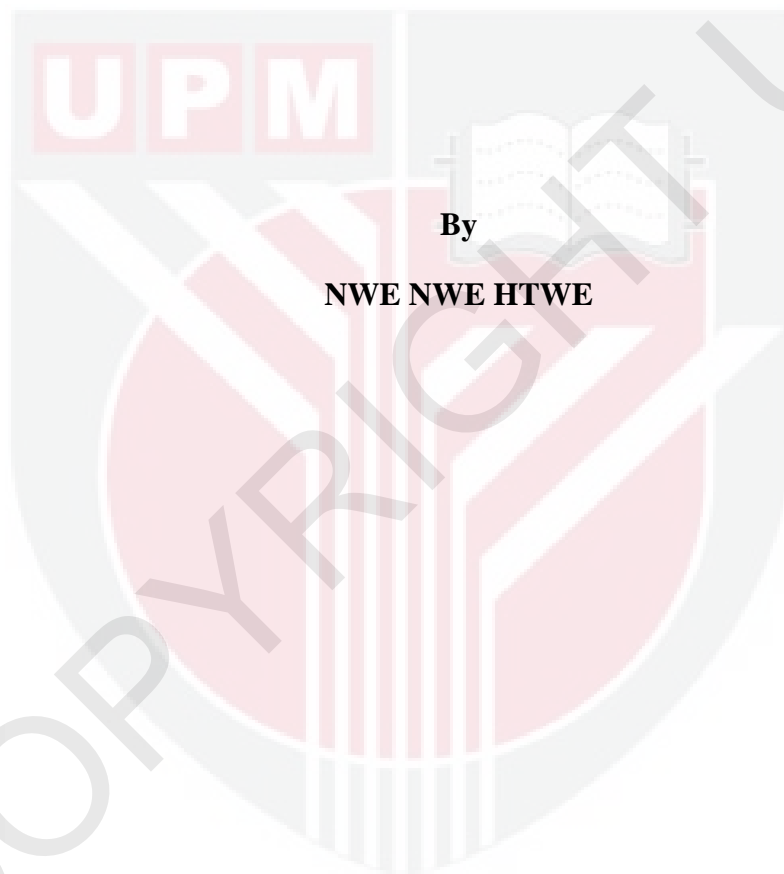
UNIVERSITI PUTRA MALAYSIA

**GENETIC TRANSFORMATION OF RICE CULTIVARS USING PARTICLE
BOMBARDMENT FOR SALINITY TOLERANCE**

NWE NWE HTWE

IB 2011 4

**GENETIC TRANSFORMATION OF RICE CULTIVARS USING
PARTICLE BOMBARDMENT FOR SALINITY TOLERANCE**



By

NWE NWE HTWE

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of
the Requirement for the Degree of Doctor of Philosophy**

July, 2011

DEDICATED TO;

MY LOVELY FATHER, U KHIN MAUNG HTWE AND MOTHER,

DAW KHIN SAW CHIT,

BROTHERS AND SISTERS,

ALSO ALL MY TEACHERS AND FRIENDS

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

**GENETIC TRANSFORMATION OF RICE CULTIVARS USING PARTICLE
BOMBARDMENT FOR SALINITY TOLERANCE**

By

NWE NWE HTWE

July, 2011

Chairman: Professor Maziah Mahmood, PhD

Faculty: Institute of Bioscience

Rice is one of the major cereal crops and staple food for feeding more than half of world population. Now we are facing with some problems for rice production and it must be increased by 60% for next 20 years. Also 30% of rice growing area in the world contains high salt to allow normal yield. Salinity stress is severely limited the plant growth and productivity. The main problem in rice growth and productivity are unfavourably affected by salt stress. Na^+ and Cl^- derived from NaCl salts contaminate the soils, which are well known as the toxic ions that damage the plant cells in ion homeostatis and osmotic effect. Genetic transformation has widely been considered as a tool for crop improvement. Transient gene expression system is a critical requirement for target gene into cell and easy for analysing the function of a particular gene. This study was mainly aimed to establish a suitable *in vitro* culture system under salinity condition of selected five Malaysian rice genotypes as well as to introduce the delta 1 Pyrroline five carboxylase (P5CS) cDNA into rice genome using particle

bombardment. This present study has highlighted some interesting findings as described below:

Callus induction is one of the significant steps for selection of suitable genotypes and identifies the most suitable medium. In this study, five selected Malaysian rice genotypes were used and investigated for *in vitro* salt stress responses. All genotypes showed that the callus-growth capacities were significantly affected by the genotypes and the culture medium. Markedly, callus was best induced on the MS medium added with 10 μ M 2,4-D and 0.4gm/L casein hydrolysate. The shoot regeneration capacity was the most effective in half MS strength with 10 μ M BAP. The genotype, MR219-4, consistently performed the best in callus induction (93.5%) as well as in plant regeneration (27%). However under salinity condition, it showed a decline in callus growth, regeneration capacity and proline accumulation. This genotype can be a good model system for studying the genetic transformation.

For genetic transformation, antibiotic resistance genes are routinely used as powerful markers for selection of transformed cells. The minimum inhibitory level of hygromycin concentration was optimized for selected mutant rice line and it was showed that 21.3% calli survived on the medium containing 45 mg/L hygromycin. Transient expression of *gfp* and *gus* gene mediated by particle bombardment is rapid and provide useful approach for visual monitoring of genetic transformation. All the results indicated that *gfp* gene expression is superior to *gus* gene expression. The optimised conditions were bombardment once at a helium pressure of 1100psi, 6cm target tissue distance, 27 inHg vacuum pressure, 1 μ m gold particle size and 1.5 μ g DNA concentration per bombardment. These optimized conditions have been used to

obtain stably transformed explants for subsequent regeneration. Sixteen transgenic rice plants expressing *gfp gus* and *hptII* transgene were obtained from MR219-4 and transformation efficiency was 2.5% for *hptII*, *gfp* and *gus*. The pBIP5CS plasmid including P5CS (Δ 1-pyrroline-5-carboxylate synthetase catalyses the first two steps of proline biosynthesis) cDNA encoding *hptII* gene conferring resistance to hygromycin was transformed to rice callus. The resultant primary transgenic plants showed more proline accumulation than non-transformed plant and transformation efficiency was 2.1%. Nine plants regenerated from hygromycin containing medium and were molecularly characterized by using PCR, RT PCR (Reverse transcriptase) and Southern Blot analysis. The result showed that P5CS cDNA had been integrated into six transgenic rice lines and proline level was increasing nine fold compared with non-transformed plants in 250mM NaCl stress. All these lines will be integrated into breeding programs for further assessment of their benefits.

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

**TRANSFORMASI GENETIK KULTIVAR PADI MENGGUNAKAN
PENEMBAKAN PARTIKEL UNTUK TOLERANSI SALINITI**

Oleh

NWE NWE HTWE

July 2011

Pengerusi: Profesor Maziah Mahmood, PhD

Fakulti: Institut Biosains

Padi merupakan salah satu tanaman bijirin utama dan makanan ruji kepada lebih dari separuh populasi dunia. Kini, kita sedang menghadapi krisis dalam penghasilan padi di mana penghasilan padi perlu dipertingkatkan sebanyak 60% dalam masa 20 tahun yang akan datang. Dalam masa yang sama, 30% tempat penanaman padi di seluruh dunia mengandungi garam yang terlampau tinggi sehingga menjejaskan penghasilan. Tekanan saliniti akan menghadkan pertumbuhan pokok dan penghasilannya. Pertumbuhan padi dan produktivitinya dipengaruhi oleh tekanan saliniti. Na^+ dan Cl^- yang terhasil daripada NaCl mencemarkan tanah, merupakan ion-ion toksik yang boleh merosakkan sel tumbuhan dari segi homeostasis ionik dan juga osmotik. Oleh itu, transformasi genetik telah menjadi pilihan utama untuk proses penambahbaikan tanaman. Sistem ekspresi gen transien merupakan keperluan kritikal untuk merangsang pergerakan gen sasaran ke dalam sel dan mempermudah analisis fungsi gen tertentu. Kajian ini dijalankan untuk menghasilkan system kultur *in vitro* di bawah kondisi saliniti di dalam lima genotaip padi dari Malaysia dan juga untuk

memperkenalkan gen delta 1 Pyrroline five carboxylase gene (P5CS) ke dalam genom padi dengan menggunakan kaedah penembakan partikel. Kajian ini telah menunjukkan penemuan yang menarik seperti yang diterangkan di bawah:

Induksi kalus merupakan satu langkah yang signifikan untuk memilih genotaip dan medium yang paling sesuai. Dalam kajian ini, lima genotaip padi Malaysia digunakan dalam kajian tekanan saliniti secara *in vitro*. Kesemua genotaip menunjukkan kapasiti pertumbuhan kalus dipengaruhi oleh jenis genotaip dan jenis kultur media. Kadar induksi kalus didapati paling tinggi dalam medium MS yang dibekalkan dengan 10 μ M 2,4-D dan 0.4gm/L kasein hidrolisat. Kadar regenerasi pucuk pula didapati paling efektif di dalam media ½ MS yang dibekalkan dengan 10 μ M BAP. Genotaip MR219-4 menunjukkan kadar pembentukan kalus (93.5%) dan regenerasi pucuk (27%) yang tertinggi. Walaubagaimanapun, kedua-dua genotaip ini menunjukkan kadar pembentukan kalus, kapasiti regenerasi dan pembentukan prolina yang rendah di bawah tekanan saliniti. Genotaip ini sesuai dijadikan sistem model bagi tujuan kajian transformasi genetik.

Dalam transformasi genetik, gen yang tahan terhadap antibiotik sering digunakan sebagai penanda yang kuat bagi memilih sel yang sudah mengalami transformasi.

Kadar rencatan minimum kepekatan higromisin dioptimumkan untuk menentukan padi mutan terpilih dan menunjukkan 21.3% kalus yang rentan di dalam media yang mengandungi 45 mg/L higromisin. Ekspresi transien gen (*gfp*) dan (*gus*) yang dibantu oleh penembakan partikel merupakan kaedah yang cepat dan berguna bagi memerhatikan transformasi gen secara visual. Kesemua keputusan telah menunjukkan

gen *gfp* lebih efektif daripada gen *gus*. Keadaan yang optimum ialah penembakan sekali di bawah tekanan helium sebanyak 1100 psi, dengan jarak sasaran 6cm, tekanan vakum sebanyak 27 mmHg, saiz partikel emas 1 μ m dan 1.5 μ g DNA untuk setiap tembakan. Keadaan optimum ini telah digunakan untuk memperolehi eksplan yang sudah mengalami transformasi yang stabil untuk regenerasi berikutnya. Enam belas tanaman padi yang mengekspres *gfp*, *gus* dan transgen *hptII* telah diperolehi daripada MR219-4 dan keberkesanan transformasi adalah 2.5% untuk *hptII*, *gfp* dan *gus*. Plasmid pBIP5CS mengandungi P5CS (enzim utama delta 1- pyrroline-5-carboxylate synthetase menjadi katalis dua langkah pertama sintesis prolin), gen yang mengkod *hptII* tahan terhadap higromisin ditransform kepada kalus padi. Tanaman transgenik primer yang terhasil menunjukkan akumulasi prolin yang lebih berbanding tanaman yang tidak ditransform di mana keberkesanan transformasi direkod sebanyak 2.1%. Sembilan tanaman telah diperolehi daripada medium yang mengandungi higromisin telah diperhatikan secara molekular dengan cara PCR, RT PCR dan analisa Southern Blot. Kandungan prolina didapati lebih di dalam tanaman transgenik di bawah tekanan saliniti sebanyak 250mM NaCl. Hasil keputusan menunjukkan cDNA P5CS telah diintegrasikan ke dalam enam padi transgenik dan kandungan prolin menunjukkan kenaikan sebanyak sembilan kali ganda berbanding dengan tanaman yang tidak ditransform di dalam 250 mM tekanan NaCl. Kesemua tanaman ini akan diserapkan ke dalam program pembiakbakaan dengan tujuan penilaian faedah yang selanjutnya.

ACKNOWLEDGEMENTS

First of all, my deepest gratitude goes to my mother and my father, the first teachers who taught to me. Especially, I am much appreciative to many other teachers since my primary school in Myanmar until my doctorate degree in Universiti Putra Malaysia.

I am grateful to the Ministry of Agricultural and Irrigation Myanma Agriculture service for the opportunity given to pursue this Degree of Doctor of Philosophy. Special thanks are also extended to Dr Khin Maung Thet Myanmar Agriculture Service, for their patience, invaluable advice and guidance and endless encouragement throughout my graduate study. I am very much delighted to thanks for the warm and cordial friendship provided by Dr Pa Pa Aung and all my lab-mates from Biotechnology laboratory Shwe nantha farm, Yangon, Myanmar.

My deepest appreciation goes to my supervisor Prof Dr Maziah mahmood for her constant guidance, inspiration, and support throughout the course of my study. She contributed the most from the first day of my arrival to today and guided me during the whole my PhD study.

I would like to extend my deep gratitude to my supervisory committee, Associate Professor Dr Ho Chai Ling, Faculty of Biotechnology and Biomolecular Science and

Associate Professor Dr Faridah Qamaruz Zaman, Faculty of Science for their scientific guidance, warm-hearted advice and patience. My graduate also goes to Prof Dr Abdul Rahman Omar, Institute of Bioscience for his invaluable advice, encouragement and support all the way. This always shows his gracious compassion and willingness to help. During my graduate research in LAB 235, I have been fortunate to work with people who are all generous in giving help. I express thank to those colleagues and friends for their friendship.

My special thanks go to the Organization for Women in Science for the Developing World (OWSDW) formerly Third World Organization for Women in Science (TWOWS) for generous financial support for my graduate study. Last, but not least, I would like to thank my parents and all my brothers and sisters for their support and endurance, which has been invaluable source of strength throughout my graduate study. Finally, I would like to thank all my friends and especially Myanmar friends from UPM, who have been helpful directly and indirectly during the course of my work.

APPROVAL

I certify that an Examination Committee has met on 13.7.2011 to conduct the final examination of NWE NWE HTWE on her Doctor of Philosophy thesis entitled "GENETIC TRANSFORMATION OF RICE CULTIVARS FOR SALINITY TOLERANCE BY PARTICLE BOMBARDMENT." 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 Doctor of Philosophy.

Members of the Thesis Examination Committee are as follows:

Chairman,

Dr Nor'aini Abdul Rahman
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Examiner 1

Professor Dr Norhani Abdullah
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Internal Examiner)

Examiner 2

Professor Dr Mohd Arif Syed
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Internal Examiner)

External examiner

Professor Dr Neera Bhalla Sarin
School of Life Science
Jawaharlal Nehru University
New Delhi, India
(External Examiner)

BUJANG KIM HUAT, PhD

Professor and Deputy Dean

School Of Graduate Studies

University Putra Malaysia

Date:

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

Maziah Mahmood, PhD

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

Ho Chai Ling, PhD

Associate Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Member)

Faridah Quamun Zaman, PhD

Associate Professor

Faculty of Science

Universiti Putra Malaysia

(Member)

HASANAH MOHD GHAZALI, PhD

Professor and Dean

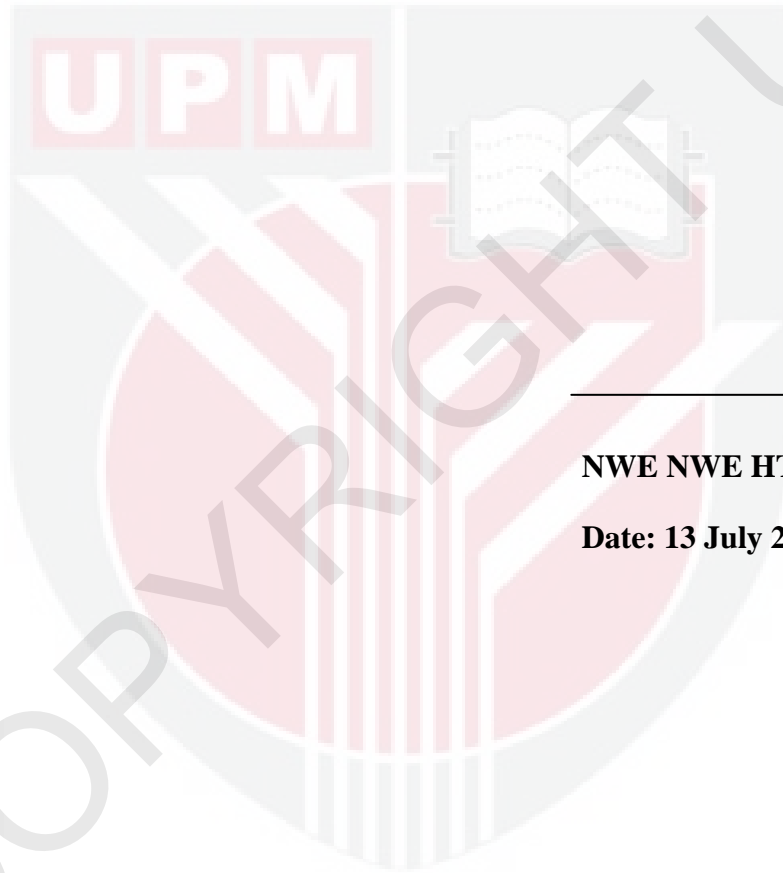
School of Graduate Studies

University Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.



NWE NWE HTWE

Date: 13 July 2011

TABLE OF CONTENTS

	Page
DEDICATION	II
ABSTRACT	III
ABSTRAK	VI
ACKNOWLEDGEMENTS	IX
APPROVAL	XI
DECLARATION	XIII
LIST OF TABLES	XIX
LIST OF FIGURES	XX
LIST OF ABBREVIATIONS	XXIV
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	6
2.1 Asian rice (<i>Oryza sativa</i>)	6
2.2 Use of genetic engineering for rice plant improvement	7
2.3 Tissue culture technique as a tool for genetic transformation	9
2.3.1 <i>In vitro</i> culture of rice	9
2.4 Genetic transformation system	13
2.4.1 DNA delivery methods	14
2.5 Screenable marker genes and selectable marker genes	17
2.5.1 Screenable marker or reporter gene	18
2.5.2 Selectable marker gene	21
2.6 Strategies to develop stress tolerance in plants	24
2.7 Development of biotic and abiotic stresses in transgenic rice plant	25

2.8 Salinity stress and plant response	26
2.8.1 Ion homeostasis	27
2.8.2 Oxidative stress management	28
2.8.3 Synthesis of compatible solutes	29
III EFFECT OF SODIUM CHLORIDE CONCENTRATIONS ON GROWTH AND PLANT REGENERATION OF RICE CALLUS CULTURES	38
3.1 Introduction	38
3.2 Materials and methods	39
3.2.1 Plant materials	39
3.2.2 The effect of culture medium and different types of auxins on callus induction	40
3.2.3 The study of the callus growth pattern	41
3.2.4 Optimisation of callus growth medium	41
3.2.5 Plant regeneration study	43
3.2.6 Optimisation for plant regeneration	44
3.2.8 Effect of salinity on callus growth and plant regeneration	45
3.2.9 Proline analysis	46
3.3 Results and discussion	47
3.3.1 Callus induction	47
3.3.2 Plant regeneration	63
3.3.3 <i>In vitro</i> responses for salinity condition	85
3.3.4 Proline accumulation	90
3.4 Conclusion	91
IV TRANSIENT EXPRESSION OF GREEN FLUORESCENT PROTEIN (<i>gfp</i>) AND β-GLUCURONIDASE (<i>gus</i>) GENES IN RICE CALLUS	93
4.1 Introduction	93
4.2 Materials and methods	94
4.2.1 Embryogenic callus for bombardment	94
4.2.2 Plasmid DNA	94
4.2.3 Histochemical <i>gus</i> staining and fluorescence microscopy	95
4.3 Results and discussion	97
4.3.1 The effect of helium pressure	97

4.3.2 The effect of vacuum pressure	99
4.3.3 The effect of gold micro carrier size	101
4.3.4 The effect of DNA quantity	103
4.3.5 Effect of preculture time	105
4.3.6 The effect of age of target tissue	107
4.3.7 Effect of CaCl ₂ and spermidine for <i>gfp</i> and <i>gus</i> gene expression	109
4.3.8 Effect of the distance from the stopping screen to the target tissue for <i>gfp</i> and <i>gus</i> gene expression	111
4.3.9 Effect of post-bombardment incubation time for <i>gfp</i> and <i>gus</i> gene expression	114
4.4 Conclusion	121

V MOLECULAR ANALYSIS OF REGENERATED PUTATIVE TRANSFORMANTS

5.1 Introduction	122
5.2 Materials and methods	124
5.2.1 Plant material, chemicals and supplies	124
5.2.2 Hygromycin sensitivity	124
5.2.3 Bacterial strains, plasmid constructs and primer design	125
5.2.4 DNA agarose gel electrophoresis	126
5.2.5 Preparation and transformation of competent cells	127
5.2.6 Plasmid extraction	128
5.2.7 Restriction enzyme (RE) digestion and polymerase chain reaction (PCR) for plasmid	129
5.2.8 Selection of transformants	130
5.2.9 <i>Gfp</i> monitoring and histochemical <i>gus</i> assays	131
5.2.10 Plant genomic DNA extraction and PCR analysis of transgene integration in host genome	131
5.2.11 Total plant RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)	133
5.2.12 Southern hybridization	134
5.3 Results and discussion	137
5.3.1 Hygromycin sensitivity	138
5.3.2 Hygromycin as the selectable agent	142
5.3.3 Selection of the transformants after bombardment	143

5.3.4 <i>Gfp</i> and <i>gus</i> gene expression of the transformants	146
5.3.5 Quantitative analysis of the transgenic plant	151
5.3.6 Accumulation of proline in transgenic plants	152
5.3.7 Genomic PCR analyses of the transformants	154
5.3.8 RT-PCR analysis	156
5.3.9 Production of transgenic plants and Southern Blotting	158
5.4 Conclusion	161
VI GENERAL CONCLUSION AND SUGGESTIONS	163
BIBLIOGRAPHY	167
APPENDICES	194
BIODATA OF STUDENT	202