UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF AGROBACTERIUM-MEDIATED TRANSFORMATION PROTOCOL FOR MALAYSIAN RICE (ORYZA SATIVA L.) CULTIVAR MR219 USING SHOOT APICES AS TARGET TISSUE

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ITA 2015 6

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By

CLEMENT WONG KIING FOOK

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

April 2015

A special dedication to my family and friends

For their endless support and trust

"To Accomplish Great Things, We Must Not Only Act, But Also Dream; Not Only Plan; But Also Believe." – Anatole France

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Masters of Science

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Chairman: Maziah Mahmood, PhD

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Optimization of the *Agrobacterium*-mediated transformation protocol of rice has always been the fundamental behind all transgenic research before the insertion of any gene of interest. After all, an optimized protocol often translates into improved transformation efficiency. Rice calli was the most preferred target tissue since optimization studies using this explant were more established. Nonetheless, rice shoot apices derived from germinated seedlings has proven to be a better target tissue since it by-pass lengthy and frequent subculturing and regeneration steps. The first part of the study was to investigate the explants ability to form *in-vitro* multiple shoots by using three cytokinins – KIN, BAP and TDZ. Optimal concentration of 4 mg/l of TDZ produced the most shoots (6.75), followed by 4 mg/l of KIN (5.50) and 3 mg/l BAP (5.25). The biochemical changes of the *in-vitro* shoots grown at optimal concentrations of cytokinins indicated that *in-vitro* shoots produced in KIN contain the highest total chlorophyll content (0.430 g/mg FW) as compared to BAP (0.223 g/mg FW) and TDZ (0.154 g/mg FW). TDZ-produced *in-vitro* shoots contain the highest total soluble sugar content (0.120 g/mg FW), followed by KIN (0.104 g/mg FW) and lastly, BAP (0.097 g/mg FW). There were no significance difference observed on the total soluble protein content of the *in-vitro* shoots cultured in all three cytokinins supplemented media. KIN was subsequently chosen as the suitable cytokinin due to its production of relatively high number of shoots and quality shoots with highest total chlorophyll content and moderately high total soluble sugar content. After four weeks of culturing in 4 mg/l of KIN, *in-vitro* shoots were successfully rooted for 2 weeks in 0, 0.5 and 1.0 mg/l of IBA. Hardened rice plants were acclimatized in the glasshouse with influorescence observed after two months. Minimal inhibitory concentration (MIC) of selected antibiotics and herbicide towards the growth of shoot apices was also determined. Aminoglycosidic antibiotics such as kanamycin, paromomycin and neomycin showed 100% survival of shoot apices at all tested concentrations of 0 to 500 mg/l. However, the MIC for another aminoglycosides –

geneticin (G418) was determined at 300 mg/l with 52% of survived shoot apices. Hygromycin and basta proved to be better alternatives with relatively lower MIC recorded. At concentration of 20 mg/l hygromycin, the percentage survival was 8.5% whereas 8.0% of explant survival was determined at 9 mg/l of basta. The optimization of *Agrobacterium*-mediated transformation protocol showed that strain EHA101 (38.0%, 35.0%) and EHA105 (33.0%, 27.5%) presented higher transient GFP and GUS reporter genes expression respectively as compared to LBA4404 (15.0%, 22.5%). Shoot apices derived from four days old seedlings gave the highest GFP (35.0%) and GUS (33.5%). Additional wounding of the explant with needle (18.0%, 10.0%) and scalpel (13.0%, 18.0%) did not improve the expression of GFP and GUS comparing to excised shoot apices (33.0%, 47.5%). A 30 minutes bacterial immersion time (35.0%, 33.0%) and 72 hours of co-cultivation period (38.0%, 35.0%) gave the highest transient GFP and GUS expression. The application of heat treatment for 3 minutes in addition to 30 minutes of immersion time produced 63.0% and 42.5% of shoot apices transiently expressing GFP and GUS as compared to untreated explant (34.0% GFP, 36.25% GUS expressions). A series of phenolic compounds at 100 µM was evaluated for their ability in *vir* induction. Amongst tested phenolics which include cinnamic acid, coumaric acid and ferulic acid, vanillin (32.5% and 23.5%) was selected as a potential substitute for acetosyringone (37.5%, 28.8%) in terms of percentage plant expressing GFP and GUS. Optimal concentration of vanillin was also determined at 150 µM with 47.5% and 45.0% plants expressing GFP and GUS. Pre-treatment of explants with a combination of macerase, cellulase and pectinase before bacterial immersion produced 40.0% and 35.0% explants expressing GFP and GUS. Untreated explants and explants treated with individual enzymes did not yield high expression of both reporter genes. A 60 minutes of enzyme incubation period (37.5%, 35.0%) was optimal to achieve high GFP and GUS expression as compared to untreated explants (32.5%, 28.5%). Molecular PCR analyses showed that transformation efficiencies was improved when explants were treated with heat (15.83%), vanillin (17.50%), hydrolytic enzymes (16.67%) and a combination of the three treatments (10.00%) when compared to the normal transformation protocol (5.83%). To conclude, the use of shoot apices could reduce the time needed to produce transgenic rice with superior agronomic traits. Also, the improved transformation efficiency of this protocol will allow efficient production of transgenic rice.

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

PENGOPTIMUMAN PROTOKOL *AGROBACTERIUM* TRANSFORMASI UNTUK KULTIVAR PADI (*ORYZA SATIVA* L.) MALAYSIA MR219 DENGAN MENGGUNAKAN PUCUK APEKS SEBAGAI TISU SASARAN

Oleh

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Pengoptimuman protokol transformasi padi dengan *Agrobacterium* merupakan asas dalam penyelidikan transgenik sebelum kemasukan gen asing ke dalam padi dilakukan. Protokol yang optimum adalah penting untuk menambahbaik kecekapan transformasi. Pada kebiasaannya, kalus padi telah menjadi pilihan tisu sasaran kerana kajian pengoptimuman dengan eksplan ini adalah lebih mantap. Akan tetapi, pucuk apeks padi dari benih padi yang bercambah telah terbukti untuk menjadi tisu sasaran yang lebih baik kerana ia mengurangkan masa untuk subkultur dan regenerasi. Tiga jenis cytokinin – KIN, BAP dan TDZ telah digunakan untuk mengkaji kebolehan eksplan ini untuk menghasilkan anak pucuk secara *in-vitro*. Kepekatan optima bagi 4 mg/l TDZ menghasilkan paling banyak anak pucuk (6.75), diikuti oleh 4 mg/l KIN (5.50) dan 3 mg/l BAP (5.25). Perubahan biokimia untuk anak pucuk *in-vitro* yang bertumbuh dalam kepekatan cytokinin yang optima menunjukkan anak pucuk *in-vitro* bagi KIN (0.430 g/mg FW) mengandungi jumlah kandungan klorofil yang paling tinggi berbanding dengan BAP (0.223 g/mg FW) dan TDZ (0.154 g/mg FW). Anak pucuk dalam TDZ pula mengandungi jumlah kandungan gula terlarut yang paling tinggi (0.120 g/mg FW), diikuti oleh KIN (0.104 g/mg FW) dan akhirnya, BAP (0.097 g/mg FW). Didapati tiada perubahan yang signifikan untuk jumlah kandugan protein terlarut bagi anak pucuk *in-vitro* yang bertumbuh di semua media yang sudah ditambahkan dengan ketiga-tiga jenis cytokinin. Secara keseluruhannya, KIN dipilih sebagai cytokinin yang sesuai kerana ia menghasilkan jumlah anak pucuk yang serdahana tinggi dan anak pucuk yang berkualiti dengan jumlah kandungan klorofil yang paling tinggi dan jumlah kandungan gula terlarut yang serdahana tinggi. Selepas empat minggu bertumbuh di media 4 mg/l KIN, anak pucuk *in-vitro* yang dikultur dalam media mengandungi 0, 0.5 dan 1.0 mg/l IBA telah berjaya bertumbuh akar dalam masa 2 minggu. Anak pokok padi telah disesuaikan di dalam keadaan rumah kaca dengan kemunculan bunga padi selepas dua bulan. Kepekatan minima rencatan (MIC) bagi antibiotik dan herbisid terpilih terhadap pertumbuhan pucuk apeks juga dijalankan. Antibiotik aminoglikosidik seperti kanamycin, paramomycin dan neomycin menunjukkan 100% pucuk apeks yang hidup dalam semua kepekatan yang dikaji (0 – 500 mg/l). Tetapi, kepekatan minima untuk aminoglikosid yang lain seperti geneticin (G418) telah ditentukan pada 300 mg/l dengan

52% pucuk apex yang hidup. Hygromycin dan basta dibuktikan lebih baik dengan kepekatan minima yang rendah. Dalam kepekatan minima hygromycin pada 20 mg/l, peratusan eksplan hidup adalah 8.5% manakala 8.0% eksplan hidup telah ditentukan bagi 9 mg/l basta. Pengoptimuman protokol transformasi dengan *Agrobacterium* menunjukkan bakteria jenis EHA101 (38.0%, 35.0%) dan EHA105 (33.0%, 27.5%) mencatatkan peratus ekspresi gen penanda bagi GFP dan GUS yang paling tinggi berbanding dengan LBA4404 (15.0%, 22.5%). Pucuk apeks dari anak benih bercambah yang berumur empat tahun menghasilkan peratusan ekspresi GFP (35.0%) dan GUS (33.5%) yang tertinggi. Mengenakan luka tambahan pada eksplan dengan jarum (18.0%, 10.0%) dan skapel (13.0% dan 18.0%) tidak menambahbaik peratusan ekspresi GFP dan GUS berbanding dengan pucuk apeks yang dikerat (33.0%, 47.5%). Rendaman bakteria untuk 30 minit (35.0%, 33.0%) dan ko-kultivasi selama 72 jam (38.0%, 35.0%) mencatatkan ekspresi GFP and GUS yang tertinggi. Rawatan haba selama 3 minit dengan rendaman bakteria untuk 30 minit menghasilkan 63.0% ekspresi GFP dan 42.5% ekspresi GUS jika dibandingkan dengan eksplan yang tidak dirawat (34.0% GFP, 36.25% GUS). Sekumupulan sebatian fenolik pada kepekatan 100 µM telah dipilih untuk mengkaji kebolehan setiap fenolik dalam menyebabkan ekspresi gen-gen *vir*. Dalam kalangan fenolik seperti asid cinnamic, asid coumaric dan asid ferulik yang telah dikaji, vanillin (32.5%, 23.5%) telah dipilih sebagai pengganti berpotensi untuk acetosyringone (37.5%, 28.8%) dari segi peratusan pokok yang mengekspres GFP and GUS. Kepekatan optima bagi vanillin juga telah ditentukan pada 150 µM dengan 47.5% dan 45.0% pokok menunjukkan ekspresi GFP dan GUS. Rawatan awal eksplan dengan kombinasi enzim macerase, cellulase dan pectinase sebelum rendaman bakteria mencatatkan 40.0% dan 35.0% eksplan yang mengekspres GFP dan GUS. Eksplan yang tidak dirawat dan juga eksplan yang dirawat dengan enzim secara individu tidak menunjukkan peratusan ekspresi GFP dan GUS yang tinggi. Inkubasi enzim selama 60 minit (37.5%, 35.0%) adalah optima untuk menghasilkan peratusan ekspresi GFP dan GUS yang tinggi berbanding dengan eksplan yang tidak dirawat (32.5%, 28.5%). Analisis molekular PCR menunujukkan kadar kecekapan transformasi telah bertambahbaik apabila eksplan dirawat dengan haba (15.83%), vanillin (17.50%), enzim (16.67%) dan kombinasi ketiga-tiga rawatan (10.00%) jika dibanding dengan protokol transformasi yang biasa (5.83%). Secara keseluruhannya, penggunaan pucuk apeks dapat mengurangkan masa untuk menghasilkan pokok padi transgenik yang mempunyai sifat agronomi yang baik. Selain itu, menambahbaik kecekapan transformasi juga dapat membantu dalam penghasilan pokok transgenik dengan efisien.

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

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ANOVA	analysis of variance
BAP	6-benzylaminopurine
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
CTAB	cetyltrimethylammonium bromide
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
GFP	green fluorescent protein
GUS	β-glucuronidase
gusA	β-glucuronidase A
HCl	hydrochloric acid
hptII	hygromycin phosphotransferase II
IBA	Indole-3-butyric acid
KIN	kinetin
LB	Luria-Bertani
MCE	mixed cellulose esters
mgfp	membrane-bound green fluorescent protein
MS	Murashige and Skoog basal media
NaCl	sodium chloride
Na_2EDTA	disodium salt of ethylenediaminetetraacetic acid

NaOH	sodium hydroxide
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
rpm	rotation per minute
SDS	sodium dodecyl sulphate
TDZ	thiadizuron
TBE	Tri/Borate/EDTA
TE	Tris/EDTA
Tris	tris(hydroxymethyl)aminomethane
UV-vis	ultraviolet-visible
v/v	volume per volume
w/v	weight per volume
x-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

CHAPTER 1

INTRODUCTION

Food security is still of major concern especially in nations where the staple food source came from cereal crops such as rice. Approximately 90% of rice production accounts for the daily diet of more than half of the world's population, particularly those from the Asian region (Yookongkaew et al., 2007). Currently, only an estimated of 3% of the world's farmland is dedicated to rice farming (Lipton, 2007). Considering the amount of arable land for rice cultivation is likely to be taken out for urbanization programmes to accommodate the hike in human population, opportunities to produce more rice for the people is greatly reduced (Lipton, 2007). Henceforth, increased production has been emphasized on more yields in tonnes per hectare. Besides breaking the yield barrier, other factors including climate change, soil infertility, pests and disease infestation have also impeded rice production. This has led to various government and non-government organization (NGO) interventions in sustaining and increasing rice productivity (Beddington, 2010; Siwar et al., 2014). Higher rice yield is often translated into better incomes for poor farmers, more stable price in the rice market, as well as increased purchasing power of the urban poor (Sheehy and Mitchell, 2011).

Malaysia lies in the tropical region where rice cultivation is one of the most important agricultural sectors. New rice varieties that carry high yield and stress resistant traits are continuously being developed through conventional plant breeding. However, lavish budget has to be set aside each year to import rice from neighbouring countries. In order to sustain and improve the Malaysian rice productivity, the great potential of transgenic research, as mentioned previously, cannot be overlooked (Hashim et al., 2002). *Agrobacterium* transformation of local Malaysian rice variety remains untapped. The preliminary optimization of *Agrobacterium* method using mature-seed derived embryogenic callus MR219 was only carried out recently (Zuraida et al. 2011). It was reported later that a 2.4% of transformation efficiency was recorded from somatic embryos and embryogenic calli MR 219 (Zuraida et al. 2013). The use of rice shoot apices as target tissues in *Agrobacterium*-mediated transformation has been performed for various recalcitrant *indica* rice cultivars with different levels of reported transformation efficiencies ranging from 0.4 to 13.8% which is relatively low (Padua et al., 2001; Dey et al., 2012) Up until now, there are no available reports on the *Agrobacterium*-mediated transformation study on Malaysian rice cultivars using shoot apices. In addition, no improvements have been carried out to increase the current transformation efficiency. This warrants for an optimization and improvement studies to be carried out. An optimized *Agrobacterium* transformation protocol is, therefore, essential as it is the fundamental behind all future endeavours in producing transgenic local cultivars with improved economical traits. Therefore, this study was carried out with the following objectives:

1. To determine the suitable cytokinin for multiple shoots production from MR219 shoot apices.

2. To optimize and improve the *Agrobacterium*-mediated transformation protocol for MR219 shoot apex by monitoring the transient expression GUS and GFP reporter genes.

3. To select putative transformants using hygromycin as the selectable agent.

4. To confirm transgene insertion in transformants using polymerase chain reaction (PCR).

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