IN-VITRO PLANTLET REGENERATION AND *AGROBACTERIUM*-MEDIATED GENE TRANSFORMATION OF *CARICA PAPAYA* L. cv. EKSOTIKA

By

SYAIFUL BAHRI PANJAITAN

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

2006

Specially Dedicated

To

My Father and Mother:
Lokot Panjaitan and Boniyem

…….. who inspired, supported and gave me tremendous courage to be a well educated person

All teachers

…….. who crossed their paths with mine and taught me essential things in life

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

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November 2006

Chairman : Associate Professor Maheran Abdul Aziz, PhD

Faculty : Agriculture

The study was conducted at the *In Vitro* Laboratory, Institut Pertanian Persekitaran Terkawal (IPPT), Faculty of Agriculture and Molecular and Cell Biology Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor. The main objectives of the study were to develop *in-vitro* plantlet regeneration systems and to carry out preliminary studies of *Agrobacterium*-mediated gene transformation of *Carica papaya* L. cv. Eksotika.

The study consisted of *in-vitro* plantlet regeneration through organogenesis, and somatic embryogenesis using different types of explant and different concentrations and combinations of plant growth regulators whereby the most appropriate system could be applied in the genetic transformation of the crop. The experiments were arranged in a completely randomized design (CRD).

The study on organogenesis included shoot induction and rooting. On shoot induction, modified MS (Murashige and Skoog, 1962) medium supplemented with 500 mgL^{-1} (w/v) casein hydrolysate, 30 gL^{-1} (w/v) sucrose and 0.2% (w/v) phytagel was used as the basic medium, and different concentrations of BAP (0, 0.1, 1.0, and 2.0 mgL^{-1} (w/v)) in combination with 0, 0.05, 0.1 and 0.2 mgL^{-1} (w/v) NAA were applied as treatments.

A modified MS medium containing 1 mgL^{-1} (w/v) BAP combined with 0.05 mgL^{-1} (w/v) NAA was the most suitable treatment combination to induce shoot proliferation from shoot tip, leaf and petiole explants of field-grown hermaphrodite plant. The mean number of shoots produced was 73 on shoot tip explant, 47 on leaf explant and 10 on petiole explant. The treatment also successfully enhanced the percentage of explant producing shoots which were 60.24% on petiole explants, 70.07% on leaf explants and 100% on shoot tip explants.

In the rooting study, shoots obtained above were pretreated in different concentrations of IBA (0, 0.5, 1.0, and 2.0 mgL^{-1} (w/v)) for one week followed by culture on medium with or without vermiculite. Pretreatment in 1 mgL^{-1} (w/v) IBA for one week stimulated root initiation on shoots cultured on MS medium with vermiculite supplement. Pretreatment in 1 mgL^{-1} (w/v) IBA followed by culture on medium with vermiculite was most suitable for root induction on shoots derived from shoot tip, leaf and petiole. Shoots derived

from field-grown shoot tip and petiole explants produced 90% rooting while 87.50% rooting occurred on shoots derived from leaf explant. However, the treatments and methodology used for rooting still need to be modified and improved in order to produce roots of good characteristics and root quality.

Plantlet regeneration through somatic embryogenesis was successfully achieved on leaf and petiole explants derived from proliferated shoots of hermaphrodite field grown shoot tips as well as from embryogenic cell suspension culture. Modified MS medium with the macro nutrients reduced to half-strength, full-strength of other elements, 500 mgL^{-1} (w/v) glutamine, 30 gL^{-1} (w/v) sucrose, 0.2% (w/v) phytagel, and 2,4-D at 1.0, 2.5, 5.0 and 7.5 mgL^{-1} (w/v) in combination with 0, 0.001, 0.005 and 0.010 mgL^{-1} (w/v) BAP as treatments was employed for the induction of somatic embryos on leaf and petiole explants. For the establishment of cell suspension culture, two treatments which were liquid full strength MS and liquid half-strength MS medium containing 2 mgL^{-1} (w/v) 2,4-D were assessed.

Treatment containing 5 mgL^{-1} (w/v) 2,4-D was the most suitable for induction of somatic embryos from leaf explant with the highest percentage of explants that responded to form somatic embryos at 55% and mean number of somatic embryos produced at 76.56. Meanwhile, treatment containing 2.5 mgL^{-1} (w/v) 2,4-D was most suitable for production of somatic embryos on petiole explant with the highest percentage of explants that responded to form

somatic embryos at 20.50% and mean number of somatic embryos produced at 11.79.

High frequency of somatic embryo formation was achieved through cell suspension culture method. Liquid full-strength MS medium supplemented with 2 mgL^{-1} (w/v) 2,4-D significantly produced the highest percentage of somatic embryo formation in cell suspension derived from embryogenic calli of leaf or petiole explants compared to treatment consisting of liquid half-strength MS medium containing 2 mgL^{-1} (w/v) 2,4-D. 58.33 ± 5.86 cotyledonary somatic embryos were produced per mL of suspension culture derived from embryogenic calli of leaf explant in liquid full-strength MS medium supplemented with 2 mgL^{-1} (w/v) 2,4-D whereas only 35.67 ± 1.53 cotyledonary somatic embryos were obtained per mL of suspension culture established from embryogenic calli of petiole explant in the same treatment (liquid full-strength MS supplemented with 2 mgL^{-1} (w/v) 2,4-D).

Different concentrations of NAA (0, 0.01, 0.02 and 0.03 mgL^{-1} (w/v)) in combination with 0, 0.1, 0.2 and 0.3 mgL^{-1} (w/v) BAP were used as treatments for germination of somatic embryos derived from the leaf and petiole explants and from the cell suspension cultures. For somatic embryos obtained from leaf explant, treatment containing 0.02 mgL^{-1} (w/v) NAA combined with 0.1 mgL^{-1} (w/v) BAP gave significantly the highest percentage of somatic embryos germination (92.50%) and also the highest percentage of normal

plantlet formation (83%). Whereas for somatic embryos obtained from petiole explant, treatment containing 0.02 mgL^{-1} (w/v) NAA combined with 0.2 mgL^{-1} (w/v) BAP produced significantly the highest percentage of somatic embryos germination (93.25%) and also the highest percentage of normal plantlet formation (83%). Meanwhile, treatment containing 0.02 mgL^{-1} (w/v) NAA combined with 0.1 mgL^{-1} (w/v) BAP was the most suitable for germination of somatic embryos derived from cell suspension culture, whereby 92% of the somatic embryos germinated into seedlings (plantlets).

In the development of genetic transformation protocol via *Agrobacterium*-mediated gene transfer, the minimal inhibitory concentration (MIC) of hygromycin on target materials such as leaf, petiole and embryogenic calli was evaluated. The different concentrations of hygromycin tested were 2, 4, 6, 8, 10, 12 and 15 mgL^{-1} (w/v). Other parameters for genetic transformation that were evaluated, were the different bacterial concentrations (1 X 10^5, and 1 X 10^7 cfu) combined with co-cultivation periods of 5, 15 and 30 minutes.

Hygromycin at 12 mgL^{-1} (w/v) was chosen as the minimal inhibitory concentration (MIC) that caused the death of leaf explant and embryogenic calli derived from leaf and petiole explants, while 15 mgL^{-1} (w/v) hygromycin was chosen as the minimal inhibitory concentration for petiole explant.

Although plantlet regeneration via organogenesis and somatic embryogenesis and through cell suspension culture were successfully established in this study and applied in the genetic transformation of papaya, no transgenic plant was obtained. This means that further work on the establishment of an efficient genetic transformation protocol via *Agrobacterium*-mediated gene transfer for papaya cv. Eksotika need to be pursued.

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

KAEDAH REGENERASI ANAK POKOK SECARA *IN-VITRO* DAN TRANSFORMASI GEN MELALUI PERANTARA AGROBAKTERIA PADA BETIK KULTIVAR EKSOTIKA

Oleh

SYAIFUL BAHRI PANJAITAN

November 2006

Pengerusi : Profesor Madya Maheran Abdul Aziz, PhD

Fakulti : Pertanian

Kajian ini telah dijalankan di Makmal *In Vitro*, Institut Pertanian Persekitaran Terkawal (IPPT), Fakulti Pertanian dan Makmal Biologi Sel dan Molekul, Fakulti Bioteknologi dan Sains Biomolekul, Universiti Putra Malaysia, Serdang, Selangor. Matlamat utama kajian ini ialah untuk mengembangkan sistem regenerasi anak pokok secara *in vitro* dan melakukan kajian awal transformasi gen melalui perantara Agrobakteria pada betik kv. Eksotika.

Kajian ini terdiri daripada regenerasi anak pokok secara *in vitr*o melalui organogenesis dan embriogenesis soma menggunakan jenis eksplan yang berbeza dan kombinasi serta kepekatan pengawal atur pertumbuhan yang berbeza dan sistem regenerasi yang sesuai akan digunakan untuk kaedah transformasi genetik tanaman tersebut. Kajian-kajian tersebut telah disusun dalam reka bentuk berawak penuh.

Kajian organogenesis terdiri daripada induksi tunas dan pengakaran. Bagi kajian induksi tunas, media MS (Murashige dan Skoog, 1962) yang telah diubahsuai dengan menambahkan 500 mgL^{-1} (b/i) kasein hidrolisat, 30 gL^{-1} (b/i) sukrosa dan 0.2% (b/i) agar phytagel telah digunakan sebagai media asas, dan kombinasi kepekatan BAP berbeza-beza (0, 0.1, 1 dan 2 mgL^{-1} (b/i)) dengan 0, 0.05, 0.1 dan 0.2 mgL^{-1} (b/i) NAA telah digunakan sebagai rawatan.

Suatu media MS yang telah diubah suai yang mengandungi kombinasi 1 mgL^{-1} (b/i) BAP dan 0.05 mgL^{-1} (b/i) NAA merupakan kombinasi rawatan yang sesuai untuk penggandaan tunas daripada mercu pucuk daripada pokok hermafrodit yang di tanam di lapangan, daun dan tangkai daun. Purata bilangan tunas adalah sebanyak 73 pada eksplan mercu pucuk daripada pokok yang ditanam di lapangan, 47 pada eksplan daun dan sebanyak 10 pada eksplan tangkai daun. Rawatan ini juga berjaya meningkatkan peratusan eksplan yang telah menghasilkan tunas iaitu 60.24% pada eksplan tangkai daun, 70.07% pada eksplan daun dan 100% pada eksplan mercu pucuk.

Bagi kajian pengakaran, tunas yang dihasilkan di atas telah diberikan pra rawatan kepekatan IBA yang berbeza (0, 0.5, 1.0 dan 2.0 mgL^{-1} (b/i)) untuk seminggu dan kemudian dikultur pada Medium MS dengan bahan tambahan vermikulit atau tanpa vermikulit (MSO). Penggunaan 1 mgL^{-1} (b/i) IBA

sebagai pra rawatan selama satu minggu telah merangsang pembentukan akar pada tunas yang dipindahkan ke atas media MS dengan tambahan vermikulit. Kombinasi pra rawatan 1 mgL^{-1} (b/i) IBA diikuti dengan pengkulturan di atas medium mengandungi vermikulit adalah yang paling sesuai untuk induksi pengakaran pada tunas yang diperoleh daripada mercu pucuk, dari lapangan, eksplan daun dan eksplan tangkai daun. Tunas yang diperoleh daripada mercu pucuk dan tangkai daun menghasilkan 90% pengakaran manakala sebanyak 87.50% pengakaran diperoleh daripada tunas yang berasal daripada eksplan daun. Walau bagaimanapun rawatan dan kaedah yang telah diguna pakai untuk pengakaran tersebut masih perlu diubahsuai dan diperbaiki untuk mendapatkan ciri dan kualiti akar yang baik.

Pada regenerasi anak pokok melalui kaedah embriogenesis soma telah berjaya dicapai bagi dua jenis eksplan iaitu daun dan tangkai daun yang diperoleh daripada proliferasi tunas mercu pucuk pokok hermafrodit yang ditanam di lapangan dan juga daripada kultur ampaian sel. Media MS yang telah diubah suai dengan mengurangkan kadar pemakanan makro kepada setengah kuasa, unsur lain pada kuasa penuh, 500 mgL^{-1} (b/i) glutamin, 30 gL^{-1} (b/i) sukrosa, 0.2% (b/i) agar phytagel dan dengan mengandungi berbagai kepekatan 2,4-D (1.0, 2.5, 5.0 dan 7.5 mgL^{-1} (b/i)) yang dikombinasikan dengan berbagai kepekatan BAP (0, 0.001, 0.005 dan 0.010 mgL^{-1} (b/i)) telah digunakan sebagai rawatan-rawatan untuk induksi embrio soma pada eksplan daun dan tangkai daun. Manakala untuk kajian kultur

ampaian sel, dua rawatan iaitu medium cecair MS dengan kuasa penuh dan medium cecair MS dengan setengah kuasa yang masing-masing mengandungi 2 mgL^{-1} (b/i) 2,4-D telah diuji.

Rawatan yang mengandungi 5 mgL^{-1} (b/i) 2,4-D adalah paling sesuai untuk induksi embrio soma daripada eksplan daun dengan peratus paling tinggi eksplan membentuk embrio soma sebanyak 55% dan purata bilangan embrio soma yang terbentuk sebanyak 76.56. Manakala rawatan yang mengandungi 2.5 mgL^{-1} (b/i) 2,4-D adalah yang paling sesuai untuk eksplan tangkai daun dengan peratus paling tinggi eksplan yang membentuk embrio soma sebanyak 20.50% dan purata bilangan embrio soma yang terhasil sebanyak 11.79.

Pencapaian yang tinggi dalam pembentukan embrio soma telah dicapai melalui kaedah kultur ampaian sel. Rawatan medium cecair MS kuasa penuh yang mengandungi 2 mgL^{-1} (b/i) 2,4-D telah memberikan pembentukan embrio soma yang sangat bererti bagi ampaian sel yang diperoleh daripada kalus embriogenik eksplan daun dan eksplan tangkai daun berbanding dengan rawatan medium cecair MS setengah kuasa yang mengandungi 2 mgL^{-1} (b/i) 2,4-D. Sebanyak 58.33±5.86 embrio soma kotiledonari dihasilkan per mL kultur ampaian yang terbentuk daripada kalus embriogenik eksplan daun pada rawatan medium cecair MS kuasa penuh yang mengandungi 2 mgL^{-1} (b/i) 2,4-D. Manakala sebanyak 35.67±1.53 embrio soma kotiledonari

dihasilkan per mL kultur ampaian yang terbentuk daripada kalus embriogenik eksplan tangkai daun pada rawatan yang sama (medium cecair MS berkuasa penuh yang mengandungi 2 mgL^{-1} (b/i) 2,4-D).

Berbagai kepekatan NAA (0, 0.01, 0.02 dan 0.03 mgL^{-1} (b/i)) yang dikombinasikan dengan 0, 0.1, 0.2 dan 0.3 mgL^{-1} (b/i) BAP telah digunakan sebagai rawatan untuk kajian percambahan embrio soma yang diperoleh daripada eksplan daun, eksplan tangkai daun dan daripada kultur ampaian sel. Untuk embrio soma yang diperoleh daripada eksplan daun, rawatan yang mengandungi kombinasi 0.02 mgL^{-1} (b/i) NAA dengan 0.1 mgL^{-1} (b/i) BAP telah memberikan peratus percambahan tertinggi yang sangat bererti sebanyak 92.50% dan juga peratus pembentukan anak pokok normal tertinggi sebanyak 83%. Manakala bagi bagi embrio soma yang diperoleh daripada eksplan tangkai daun, rawatan yang mengandungi kombinasi 0.02 mgL^{-1} (b/i) NAA dengan 0.2 mgL^{-1} (b/i) BAP telah memberikan peratus percambahan tertinggi yang sangat bererti sebanyak 93.25% dan juga peratus pembentukan anak pokok normal tertinggi sebanyak 83%. Sementara rawatan yang mengandungi kombinasi 0.02 mgL^{-1} (b/i) NAA dengan 0.1 mgL^{-1} (b/i) BAP adalah yang paling sesuai untuk percambahan embrio soma yang diperolehi daripada kultur sel ampaian dengan 92% daripada embrio soma tersebut bercambah menjadi anak pokok.

Bagi mengembangkan suatu protokol transfromasi genetik melalui kaedah pemindahan gen menggunakan perantaraan *Agrobakterium*, kajian kepekatan perencatan minimal (MIC) hygromycin ke atas bahan sasaran transformasi genetik seperti eksplan daun, tangkai daun dan kalus embriogenik dijalankan. Berbagai kepekatan hygromycin (2, 4, 6, 8, 10, 12 dan 15 mgL^{-1} (b/i)) telah diujikan dalam kajian MIC ini. Parameter lain yang diuji untuk kajian transformasi genetik ialah kepadatan bakteria ($1x10^5$ dan $1x10^7$ cfu) yang dikombinasikan dengan masa pengkulturan bersama (5, 15 dan 30 minit) rawatan kajian.

Hygromycin pada kepekatan 12 mgL^{-1} (b/i) telah dipilih sebagai kepekatan perencatan minimal yang menyebabkan eksplan daun dan kalus embriogenik yang berasal daripada eksplan daun dan tangkai daun mati, manakala rawatan yang mengandungi 15 mgL^{-1} (b/i) hygromycin telah dipilih sebagai kepekatan perencatan minimal bagi eksplan tangkai daun.

Meskipun kaedah regenerasi anak pokok melalui organogenesis dan embriogenesis soma diikuti dengan kultur ampaian sel telah berjaya dilaksanakan untuk menyokong transformasi genetik pada betik tetapi tanaman transgenik tidak diperolehi. Ini bermakna kajian lanjut untuk mendapatkan protokol transformasi genetik yang efisien melalui pemindahan gen dengan perantaraan *Agrobakterium* masih diperlukan untuk betik kv. Eksotika.

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I certify that an Examination Committee has met on 3rd November 2006 to conduct the final examination of Syaiful Bahri Panjaitan on his Master of Agriculture Science thesis entitled "*In Vitro* Plantlet Regeneration and *Agrobacterium*-Mediated Gene Transformation of *Carica papaya* L. cv. Eksotika" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Act 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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The thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Agriculture Science. Members of the Supervisory Committee are as follows:

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Date : 2006

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.

SYAIFUL BAHRI PANJAITAN

Date : 8th December 2006

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LIST OF ABBREVIATION

ACC oxidase	1-aminocyclopropane 1-carboxylate oxidase
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
bp	Base pair
^{o}C	Degree Celsius
$CaCl_2$	Calcium chloride
Car.	Carbenicilin
cfu	Colony forming unit
cm	Centimeter
$CoCl_2$ $6H_2O$	Cobalt chloride 6-water
$CuSO_4$ $5H_2O$	Cuprum sulfate 5-water
cv.	Cultivar
2,4-D	2,4-Dichlorophenoxyacetic acid
dH_2O	Deionized sterile distilled water or Denatured water
DMSO	Dimethyl sulfoxide
DNMRT	Duncan's New Multiple Range Test
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
e.g.	Exempli gratia (for example)
et al.	Et alia

etc.	Et cetera
FDA	Fluorescein diacetate
$FeSO_4$ $7H_2O$	Ferrous sulfate 7-water
gL^{-1}	Gram per liter
GTE	Glucose, Tris Cl, EDTA solution
H_3BO_3	Boric acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
Hyg.	Hygromycin
2-iP	γ, γ-(dimethylaly) aminopurine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPPT	Institut Pertanian Persekitaran Terkawal
Kan.	Kanamycin
kbp	Kilo base pair
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
µL	Microliter
LB medium	Luria and Broth medium
$lb/inch^2$	Pound per square inch (newton)
LAF	Laminar Air Flow

M	Molar; Molarity
MARDI	Malaysia Agriculture research and Development Institute
$MgSO_4$	Magnesium sulfate
mM	Millimolar
µM	Micromolar
$\mu molm^{-2}s^{-1}$	Micromole per square per second
mgL^{-1}	Milligram per liter
$MnSO_4\ 4H_2O$	Manganese sulfate 4-water
MS medium	Murashige and Skoog medium
MSO medium	Hormone-free Murashige and Skoog Medium
NAA	α-Naphtaleneacetic acid
NaCl	Sodium chloride
Na_2EDTA	Dinatrium ethylene diamine tetraacetate
NaOH	Sodium hydroxide
$Na_2MoO_4\ 2H_2O$	Natrium molybdate 2-water
NH_4NO_3	Ammonium nitrate
OD	Optical density
p=0.05	Probability at 95%
PCR	Polymerase chain reaction
PGR	Plant growth regulator
pH	Potential Hydrogen or –Log (H)
$pPACOA_1$	Plasmid $PACOA_1$

$pPACOA_2$	Plasmid $PACOA_2$
PRSV	Papaya ring-spot virus
SD	Standard deviation
TAE	Tris-acetate/EDTA electrophoresis buffer
TFB	Buffer for preparation of competent *E. coli*
$ZnSO_4$ $7H_2O$	Zinc sulfate 7-water
%	Percent