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EXPRESSION OF HAEMAGGLUTININ-NEURAMINIDASE ENVELOPE PROTEIN OF NEWCASTLE DISEASE VIRUS STRAIN AF2240 IN *CENTELLA ASIATICA* (PEGAGA) EMBRYOGENIC CALLI THROUGH OPTIMIZED PARTICLE BOMBARDMENT METHOD

LAI KOK SONG

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MASTER OF SCIENCE
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By

LAI KOK SONG

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

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

EXPRESSION OF HAEMAGGLUTININ-NEURAMINIDASE ENVELOPE PROTEIN OF NEWCASTLE DISEASE VIRUS STRAIN AF2240 IN *CENTELLA ASIATICA* (PEGAGA) EMBRYOGENIC CALLI THROUGH OPTIMIZED PARTICLE BOMBARDMENT METHOD

By

LAI KOK SONG

June 2008

Chairman: Professor Maziah Mahmood, PhD

Faculty: Biotechnology and Biomolecular Sciences

Centella asiatica is a locally important medicinal plant. It is non-toxic, high in medicinal values, and can serve as a good candidate for genetic manipulation. However, to date no transformation protocol has been developed to fully utilize the potential of this plant. Therefore, this research is to establish an efficient particle bombardment transformation protocol for *C. asiatica* embryogenic calli. In addition, an attempt to express the haemagglutinin-neuraminidase (HN) protein from Newcastle disease virus (NDV) strain AF2240 in *C. asiatica* embryogenic calli were carried out using the developed transformation system. The HN protein can serve as a potential vaccine candidate for Newcastle disease (ND) in poultry. The induced embryogenic calli revealed the presence of extracellular matrix layer (ECM) during the microscopy studies. Particle bombardment transformation protocol was developed using the green fluorescent protein (GFP) as reporter. A total of eight parameters mainly different target distance, helium pressure, gold particles size, chamber vacuum

pressure, number of bombardment, precipitation agents, post-bombardment incubation time, and plasmid DNA concentration were identified and successfully optimized. Based on the established protocol, transformations of *C. asiatica* embryogenic calli were performed using the constructed recombinant pMDC32'HN and HBT95:sGFP(S65T)-NOS'HN plasmids. Genomic PCR analysis revealed the presence of HN transgene in the transformed lines. Unfortunately no protein bands were detected during SDS-PAGE and western blotting, indicating low or no HN protein expression. Transformation using recombinant HBT95:sGFP(S65T)-NOS'HN plasmid resulted in very low GFP expression as compared to the positive control. Nonetheless, the mRNA transcripts were detected in the RT-PCR analysis. Positive signal from the dot blot assay further confirmed the presence of the HN protein expression in the transformed lines.

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

EXPRESSI PROTEIN HAEMAGGLUTININ-NEURAMINIDASE DARI VIRUS PENYAKIT NEWCASTLE STRAIN AF2240 UNTUK KALUS EMBRYOGENIK *CENTELLA ASIATICA* (PEGAGA) MELALUI KAEDAH MIKROPROJEKTIL BEDILAN YANG DIOPTIMASIKAN

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LAI KOK SONG

June 2008

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Fakulti: Bioteknologi dan Sains Biomolekul

Centella asiatica merupakan tumbuhan ubatan tempatan yang penting. Ia tidak bertoksik, kaya dengan nilai ubatan, dan boleh bertindak sebagai calon manipulasi genetik yang baik. Malangnya, hingga kini tiada protokol transformasi yang dihasilkan dalam memaksimunkan potensi tumbuhan ini. Justeru itu, penyelidikan ini bermatlamat untuk menghasilkan protokol mikroprojektil bedilan transformasi yang cekap untuk kallus embryogenik *C. asiatica*. Tambahan pula, cubaan untuk mengekspreskan protein haemagglutinin-neuraminidase (HN) strain virus penyakit Newcastle (NDV) AF2240 dalam kalus embryogenik *C. asiatica* juga dijalankan berdasarkan sistem protokol yang dihasilkan. Protein HN boleh digunakan sebagai calon vaksin yang berpotensi untuk penyakit Newcastle (ND) dalam pertenakkan. Kalus embryogenik yang diinduksikan menunjukkan kehadiran lapisan matrik luaran (ECM) semasa kajian mikroskop. Protokol mikroprojektil bedilan transformasi telah dihasilkan dengan menggunakan

green fluorescent protein (GFP) sebagai sistem pelapor. Sejumlah lapan parameter yang terdiri dari jarak tisu sasaran, tekanan helium, saiz partikel emas, tekanan ruang vakum, bilangan bedilan, bahan pengikatan, masa pos-bedilan, dan kepekatan DNA plasmid telah dikenalpasti dan berjaya dioptimasikan. Berdasarkan protokol yang dihasilkan, transformasi dijalankan pada kalus embryogenik *C. asiatica* dengan menggunakan plasmid pMDC32'HN dan HBT95:sGFP(S65T)-HN. Analisi PCR genomik menunjukkan kehadiran transgen HN dalam transforman. Malangnya, tiada produk protein yang dikesan semasa SDS-PAGE dan blot western menunjukkan rendah atau ketiadaan ekspressi protein HN. Transformasi menggunakan plasmid HBT95:sGFP(S65T)-HN memberi ekpressi GFP yang rendah berbanding pada kawalan positif. Walaubagaimanapun, transkrip mRNA berjaya dikesan dalam analisis RT-PCR. Keputusan positif dari pemblotan titikan menpastikan kehadiran ekspressi protein HN pada transforman.

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I certify that an Examination Committee has met on 25 September 2008 to conduct the final examination the of Lai Kok Song on his Master of Science thesis entitled "Expression of Haemagglutinin-neuraminidase Envelope Protein from Newcastle Disease Virus Strain AF2240 in *Centella asiatica* (Pegaga) Embryogenic Calli Through Optimized Particle Bombardment Method" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

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

____

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LIST OF ABBREVIATIONS

2'4 D	2,4-dichlorophenoxy acetic acid
5'-UTL	5' untranslated leader
Ads	adenine sulfate
APS	ammonium persulfate
BA	6-benzyladenine
BDMA	benzyldimethylamine
BSA	bovine serum albumin
ºC	Celsius
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
cm^2	centimetre square
DDSA	dodecenlysuccinic anhydride
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
F	fusion (glycoprotein)
FAA	formaldehyde-acetic acid-ethanol
GFP	green fluorescent protein
GE	gene end
g	gram
GS	gene start

h	hour
HN	haemagglutinin-neuraminidase (glycoprotein)
IBA	indole-3-butryic acid
Ig G	immunoglobulin G
kb	kilobase
kDa	kilo dalton
Kn	kinetin
kPa	kilopascal
L	litre
LB	Luria-Bertani
M	molar
mA	milliampere
$MgCl_2$	magnesium chloride
mg	milligram
min	minute
mL	millilitre
mm	millimeter
mM	millimolar
mm Hg	millimetre of mercury
MNA	2-methyl-4-nitro-aniline
MS	Murashige and Skoog
mRNA	messenger ribonucleic acid
NAA	naphthalene acetic acid
NaOH	sodium hydroxide
NDV	Newcastle disease virus

ng	nanogram
Nm	nanometer
NP	nucleoprotein
P	phosphoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomol
s	second (time)
scFv	single-chain variable fragment
SCW	silicon carbide whisker
SDS-PAGE	sodium dodecyl sulphate-PAGE
SEM	scanning electron microscope
RBC	red blood cell
RE	restriction enzyme
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
TAE	40 mM Tris-Cl(pH 7.4), 20mM sodium acetate, 1 mM EDTA
T-DNA	transfer deoxyribonucleic acid
TDZ	thidiazuran
TE	10 mM Tris-Cl (pH 8.0), 1 mM EDTA
TEM	transmission electron microscope
TEMED	N,N,N',N'-tetramethylethylenediamine
TPBS	Tris phosphate buffer saline

µg	microgram
µL	microlitre
µm	micrometer
µM	micro Molar
v/v	volume /volume
w/v	weight/volume

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CHAPTER 1

INTRODUCTION

The term plant genetic engineering has long conveyed a highly efficient and precise process for the manipulation of plant genomes (Ow, 2007). This technology has become a versatile platform for cultivar improvement, expression and production of valuable proteins, and as well as studying gene function in plant. Recently, much effort has been channeled to develop the transformation system for medicinal and aromatic plants. In our country, *Centella asiatica* (*C. asiatica*) is an important medicinal plant that is grown for commercialization purposes. *C. asiatica* is non-toxic, easily grown, high in medicinal values, and can serve as a good platform for genetic engineering. Unfortunately, to date no efficient transformation protocol has been developed and genetic manipulation been carried out to fully exploit this essential medicinal herb. The aims of this research study are to develop an efficient particle bombardment transformation protocol for *C. asiatica* embryogenic calli and to express HN protein from Newcastle disease virus strain AF2240 based on the establish transformation protocol.

Newcastle disease (ND) is a worldwide economic problem in poultry industry caused by NDV. The continued presence of ND causes economic losses to the industry in terms of production (such as in death, loss of body weight and impaired egg production) and control (such as quarantine, mass slaughter and disinfection) (Wong, 2004). The current protocol for NDV vaccination utilizes

both inactivated and live viruses for the control of ND in poultry. Although both types of vaccination produce excellent immunity among the flocks, the drawback of these vaccines still remain the major obstacle in the industry. Live vaccine will cause disease in the presence of the complicating infections, while it is often difficult to ensure complete inactivation of the virus which could remain as risk in inactivated vaccines (Wong, 2004). Studies had shown that one of the enveloped proteins of NDV known as haemagglutinin-neuraminidase (HN) protein is the potential candidate as immunogen for the development of NDV subunit vaccine. Passive immunization with either polyclonal or monoclonal antibodies to this protein is able to confer protection in chickens against NDV challenge (Reynolds and Maraqa, 2000). Thus many attempts had been applied to express the HN protein in different systems such as baculovirus and animal systems for the production of NDV subunit vaccine. Despite the successful expression and promising immunogenicity of the HN protein, they are still too costly for commercial production. As a result, a more feasible host protein expression system is needed to ensure the continuous supply of subunit vaccine for the control of NDV.

Extensive research over the past two decades has shown that plant can provide a new platform for the expression of recombinant proteins. Its protein synthesis pathway appears to be well conserved especially in glycosylation and post-translation modification enabling them for the production of various pharmacokinetics and biological active recombinant proteins. Moreover, plant is considered to be much safer than both animals and microbes because they generally lack human pathogens, endotoxin, and oncogenic sequences

(Commandeur, 2003). This system also provides lower cost of production and rapid scale up unlike the current available systems. Hence, plant can serve as a better and more feasible alternative system for the expression of recombinant proteins.

Thus the specific objectives of this study are:

1. to study the histochemical and morphological features of the target tissue, *C. asiatica* embryogenic calli;

2. to optimize transformation conditions for particle bombardment of *C. asiatica* embryogenic calli and to construct recombinant plasmids carrying the HN gene of NDV strain AF2240;

3. to bombard, select, verify the integration of transgene using PCR and to express the HN protein of NDV strain AF2240 in *C. asiatica* embryogenic calli.

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CHAPTER 2

LITERATURE REVIEW

2.1 Newcastle Disease

Newcastle disease (ND) is a worldwide economic problem in poultry industry caused by Newcastle disease virus (NDV), a prototype *Avulavirus* in the subfamily *Paramyxovirinae* (De Leeuw and Peeters, 1999). Outbreaks of ND were first reported in poultry from Java, Indonesia and Newcastle-upon-Tyne in 1926 (Bruce et al., 2000). NDV has a wide host range with 27 of the 50 orders of birds reported to be infected by the virus (Kaleta and Baldauf, 1988). The NDV isolates can be divided into three main pathotypes: lentogenic, mesogenic, and velogenic depending on the severity of disease produced by the isolate.

Mildly virulent lentogenic strain usually causes mild respiratory infection in young chicks but not in adult birds. Mesogenic strain is more virulent and can induce mild disease with mortality accruing primarily in young chickens. Meanwhile the highly virulent velogenic strain induces severe diseases and mortality to birds of all ages. Differential diagnosis of NDV involves electron microscopic identification, hemagglutination inhibition with polyclonal NDV specific antisera, use of the ELISA, oligonucleotide probes, and viral genomic RNA fingerprint analysis (Bruce et al., 2000) The current protocol for NDV vaccination utilizes both inactivated and live viruses for the control of ND in poultry. Various routes of vaccination were applied such as injection, inhalation