UNIVERSITI PUTRA MALAYSIA

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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By

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

Oleh

LAI KOK SONG

June 2008

Pengerusi: Profesor Maziah Mahmood, PhD
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 bertamatlamat untuk menghasilkan protokol mikroprojektil bedilan transformasi yang cekap untuk kallus embryogenik C. asiatica. Tambahan pula, cubaan untuk mengekspresikan protein haemagglutinin-neuraminidase (HN) strain virus penyakit Newcastle (NDV) AF2240 dalam kallus embryogenik C. asiatica juga dijalankan berdasarkan sistem protokol yang dihasilkan. Protein HN boleh digunakan sebagai calon vaksin yang berpotensi untuk penyakit Newcastle (ND) dalam pertenakan. 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 ekspresi protein HN. Transformasi menggunakan plasmid HBT95:sGFP(S65T)-HN memberi ekspresi GFP yang rendah berbanding pada kawalan positif. Walaubagaimanapun, transkrip mRNA berjaya dikesan dalam analisis RT-PCR. Keputusan positif dari pemblotan titikan menpastikan kehadiran ekspresi protein HN pada transforman.
I wish to express my deepest thanks and appreciation to my supervisor, Professor Dr. Maziah Mahmood for her guidance, time, and encouragement throughout this research project. Special thanks to my co-supervisor Professor Datin Paduka Dr. Khatijah Yusoff for her invaluable advice, support, and opportunity to carry out some stages of my research in her laboratory. My sincere appreciation is also extended to Dr. Mohd. Puad Abdullah, Dr. Janna Ong Abdullah, Dr. Parameswary Namasivayam, Associate Professor Dr. Tan Wen Siang, and Associate Professor Dr. Siti Nor Akmar Abdullah for their assistance and helpful suggestions during the research. Special appreciation goes to KUOK Foundation for providing the scholarship during my study in UPM.

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I certify that an Examination Committee has met on 25 September 2008 to conduct the final examination 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.

(LAI KOK SONG)

Date:

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<tr>
<td>2’4 D</td>
<td>2,4-dichlorophenoxy acetic acid</td>
</tr>
<tr>
<td>5’-UTL</td>
<td>5’ untranslated leader</td>
</tr>
<tr>
<td>Ads</td>
<td>adenine sulfate</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>BA</td>
<td>6-benzyladenine</td>
</tr>
<tr>
<td>BDMA</td>
<td>benzyldimethylamine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>ºC</td>
<td>Celsius</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cm$^2$</td>
<td>centimetre square</td>
</tr>
<tr>
<td>DDSA</td>
<td>dodecenlysuccinic anhydride</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>distilled water</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>F</td>
<td>fusion (glycoprotein)</td>
</tr>
<tr>
<td>FAA</td>
<td>formaldehyde-acetic acid-ethanol</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GE</td>
<td>gene end</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GS</td>
<td>gene start</td>
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h  hour
HN  haemagglutinin-neuraminidase (glycoprotein)
IBA  indole-3-butyric acid
Ig G  immunoglobulin G
kb  kilobase
kDa  kilo dalton
Kn  kinetin
kPa  kilopascal
L  litre
LB  Luria-Bertani
M  molar
mA  milliampere
MgCl₂  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), 20 mM 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
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>µM</td>
<td>micro Molar</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
<td>weight/volume</td>
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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 Maraca, 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-translocation 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.
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