INTERNAL RIBOSOME ENTRY SITE FOR EXPRESSION OF INFECTIOUS BURSAL DISEASE VIRAL SEGMENTS A AND B GENES IN Arabidopsis thaliana (L.) Heynh.

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By

LIEW PIT SZE

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

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December 2015

Chair: Prof. Mohd Hair Bejo, PhD
Faculty: Veterinary Medicine

Infectious bursal disease (IBD) is an acute and highly contagious viral infection of young chickens caused by the IBD virus (IBDV). The IBDV genome consists of two segments: A and B. Segment A has two partially overlapping open reading frames that encode for viral protein VP5 and precursor polyprotein. The precursor polyprotein undergoes proteolytic cleavage to give rise to mature viral proteins VP2, VP3 and VP4. The genome segment B encodes for viral protein VP1. A growing body of evidence showed that both genome segments of IBDV contribute to the virulence and pathogenicity of the virus. As the principal control method of IBDV infection in chickens is by vaccination, the application of whole IBDV genome involving both segments A and B for vaccination become judicious and crucial. The present study was therefore undertaken to determine the feasibility of expressing both IBDV genome segments A and B of a local virus isolate in the plant production system, as part of the greater goal in development of effective poultry vaccines. Since plants offer several distinct advantages over other systems, expression of IBDV viral proteins from both genome segments were thus carried out in the chosen host plant *Arabidopsis thaliana* or commonly known as thale cress, mouse-ear cress or arabidopsis by *Agrobacterium*-mediated transformation. At the same time, internal ribosome entry site (IRES) from tobacco etch virus (TEV) and crucifer-infecting tobamovirus (CrTMV) capable of initiating translation internally of an mRNA transcript were selected to determine their usefulness in expression of IBDV genes. Prior to expression in the plant, complete nucleotide (nt) coding sequence of the UPM04/190 IBDV isolate was determined. In segment A, there were 465 nt with 154 amino acids (aa) in VP5, while the polyprotein contained 3039 nt with 1012 aa. In segment B, there were 2640 nt with 879 aa in VP1. In comparison with the published sequences on the deduced aa, it revealed nine unique aa conserved only in UPM04/190 IBDV. They were D240G, E677K, and L693H in VP1, D212N, Q249E and I264M in hypervariable region of VP2, and V616I in VP4. The VP5 has two unique substitutions at L133I and position 150 from a stop codon to arginine that led to the extension of its C-terminal. There were no unique aa substitutions found in VP3. The overall branching pattern of the phylogenetic trees clustered the UPM04/190 IBDV with very virulent IBDV. Following molecular characterisation, UPM04/190 IBDV isolate was used to construct expression plasmids. The genome segments A and B were linked by CrTMV or TEV IRES, and inserted into a plasmid vector pcDNA™-DEST40 via recombinational cloning to make an expression clone compatible for expression in wheat germ extract. In vitro transcription produced ssRNA runoff transcripts of appropriate sizes upon separation on resolving gel. Protein expression in wheat germ extract has also verified the presence of

specific protein size. The presence of VP4 protein or smaller form of VP3 protein led to the assumption that the polyprotein has been transcribed and expressed from the expression clones. In the IRES-containing expression clones, the presence of VP1 protein revealed that the IRES were functional in wheat germ extracts. Following this, plant transformation plasmids were constructed. The genes of interest were transferred into the T-DNA region of a binary plant destination vector pMDC32, after which were mobilised into *Agrobacterium tumefaciens* strain LBA4404. Transient infection to validate the expression of IBDV viral proteins was achieved in 5-day-old *Arabidopsis thaliana* seedlings by co-cultivation with *Agrobacterium* colonies carrying constructs of interest. After co-cultivation, RT-PCR specific for IBDV VP1 and VP4 genes were performed on the total plant RNA extract and detected products of expected band size upon agarose gel electrophoresis. Western blot analysis on the transiently expressed IBDV proteins also saw the expression of IBDV-specific proteins. Following transient expression assay, the same plant transformation plasmids were used for stable transformation of *A. thaliana* adult plants. Seeds from the treated T_0 generation plants were grown on selection media to screen for positive primary transformants. The positive T_1 plants were then pushed through T_2 generation to obtain T_3 seeds. Preliminary PCR screening targeting the HPT gene within T-DNA region had identified five transgene positive plants. Further PCR identification targeting both partial and full-length IBDV gene specific sequences have revealed two plants that were positive for segment A-specific genes and another two plants were positive for segment B-specific genes. The remaining one plant transformed with plasmid containing both IBDV segments linked by the CrTMV IRES was positive for both segments A- and B-specific genes. Hybridisation with polyclonal IBDV antibodies had detected the mature VP2 protein in segment A-containing plants, while hybridisation with VP1 antibodies have also found the VP1 protein in segment B-containing plants. In conclusion, the present study demonstrated the importance of using both segments A and B for characterisation of IBDV strains, especially the very virulent IBDV due to their co-operative manners in affecting the viral virulence and pathogenicity. The study revealed that plants, specifically *Arabidopsis thaliana,* were capable of supporting the processing of polyprotein and maturation of IBDV precursor VP2 protein. The IRES were also functional in *Arabidopsis* and promoted translation of downstream VP1 gene when placed in a distal cistron in a dicistronic construct. The use of IRES for gene expression in plant production system is warranted for further exploration for vaccine development, applicable not only for IBDV, but also many pathogenic agents of importance in poultry.

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

TAPAK KEMASUKAN RIBOSOM DALAMAN UNTUK UNGKAPAN GEN SEGMEN A DAN B VIRUS PENYAKIT BURSA BERJANGKIT PADA *Arabidopsis thaliana* (L.) Heynh.

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Penyakit bursal berjangkit (IBD) adalah jangkitan virus yang akut dan amat mudah berjangkit di kalangan ayam muda yang disebabkan oleh virus IBD (IBDV). Genom IBDV terdiri daripada dua segmen: A dan B. Segmen A mempunyai dua bingkai bacaan terbuka yang sebahagiannya adalah bertindih dan mereka mengekod untuk protein virus VP5 dan prekursor poliprotein. Prekursor poliprotein menjalani belahan proteolitik untuk menjana protein virus VP2, VP3 dan VP4 yang matang. Segmen B genom mengekod protein virus VP1. Semakin banyak bukti menunjukkan bahawa kedua-dua segmen genom IBDV menyumbang kepada kevirulenan virus. Disebabkan kawalan utama IBD pada ayam adalah dengan vaksinasi, penggunaan seluruh genom IBDV yang melibatkan kedua-dua segmen A dan B untuk pelalian menjadi wajar dan penting. Oleh itu, kajian ini dijalankan untuk mengkaji kemungkinan untuk mengungkapkan kedua-dua segmen A dan B genom IBDV tempatan dalam sistem penghasilan tumbuhan, sebagai salah satu matlamat utama dalam pembangunan vaksin ayam yang berkesan. Disebabkan tumbuhan menawarkan beberapa kelebihan yang ternyata lebih baik daripada sistem lain, ungkapan IBDV protein dari kedua-dua segmen genom telah dijalankan di perumah tumbuhan terpilih *Arabidopsis thaliana* atau lebih dikenali sebagai selada thale, selada tetikus telinga atau arabidopsis dengan transformasi yang dilaksanakan oleh *Agrobacterium*. Pada masa yang sama, tapak kemasukan ribosome dalaman (IRES) dari virus punaran tembakau (TEV) dan tobamovirus menjangkiti crucifer (CrTMV) yang mampu memulakan terjemahan transkrip mRNA dari kedudukan dalaman telah dipilih bagi memeriksa kelebihan mereka dalam ungkapan gen IBDV. Sebelum ungkapan dalam tumbuhan, urutan pengekodan nukleotida (nt) virus tempatan IBDV UPM04/190 telah ditentukan. Dalam segmen A, terdapat 465 nt dengan 154 asid amino (aa) di VP5 manakala poliprotein mengandungi 3039 nt dengan 1012 aa. Dalam segmen B, VP1 mengandungi 2640 nt dengan 879 aa. Perbandingan pada aa dengan urutan yang telah diterbitkan mendedahkan sembilan aa unik terpelihara hanya dalam IBDV UPM04/190. Mereka ialah D240G, E677K dan L693H di VP1, D212N, Q249E dan I264M di rantau hiper-boleh ubah VP2 dan V616I di VP4. Terdapat dua penggantian unik di VP5 iaitu pada L133I dan kedudukan 150 dari codon berhenti kepada arginina yang membawa kelanjutan kepada C-pangkalannya. Tiada sebarang penggantian aa unik pada VP3. Corak percabangan pokok filogenetik secara keseluruhan mengelompokkan IBDV UPM04/190 dengan strain IBDV yang sangat virulen (vv). Berikutan pencirian molekular, IBDV UPM04/190 telah digunakan untuk membina plasmid ungkapan. Genom segmen A dan B yang dihubungkan oleh IRES dari CrTMV atau TEV telah

dimasukkan ke dalam vektor plasmid pcDNA™-DEST40 melalui pengklonan secara penggabungan semula untuk membuat klon ungkapan bersesuaian untuk ekspresi dalam ekstrak germa gandum. Transkripsi secara in vitro menghasilkan ssRNA transkrip larian dengan saiz yang sesuai apabila diasingkan di gel pengasingan. Ekspresi protein dalam ekstrak germa gandum juga mengesahkan kehadiran saiz protein yang khusus. Kehadiran protein VP4 atau bentuk protein VP3 yang lebih kecil membawa kepada andaian bahawa poliprotein telah disalin dan diungkap daripada klon ekspresi. Dalam klon ekspresi yang mengandungi IRES, kehadiran protein virus VP1 mendedahkan bahawa IRES adalah berfungsi dalam ekstrak germa gandum. Berikutan itu, plasmid transformasi tumbuhan telah dibina. Gen berkepentingan telah dipindahkan ke kawasan T-DNA dalam vektor destinasi tumbuhan binari pMDC32. Klon ungkapan yang dihasilkan telah digerakkan ke *Agrobacterium tumefaciens* strain LBA4404. Jangkitan sementara untuk mengesahkan ungkapan protein IBDV telah dilaksanakan ke atas benih *Arabidopsis thaliana* berumur 5 hari secara penyemaian bersama dengan kumpulan *Agrobacterium* yang mengandungi plasmid penting. Selepas penyemaian bersama, RT-PCR khusus untuk IBDV gen VP1 dan VP4 telah dilakukan ke atas jumlah ekstrak RNA tumbuhan dan saiz produk yang dijangka telah dikesan selepas elektroforesis dengan gel agarose. Analisa permindahan Western pada protein IBDV yang diungkapkan daripada jangkitan sementara juga melihat ungkapan protein khusus kepada IBDV. Berikutan asai ungkapan sementara, plasmid transformasi tumbuhan yang sama juga telah digunakan untuk transformasi stabil tumbuhan dewasa *A. thaliana*. Biji benih dari tumbuhan generasi T_0 telah disemai atas media memilih untuk menyaring bagi transformants positif yang utama. Tumbuh-tumbuhan T_1 yang positif kemudiannya ditumbuh melepasi generasi T_2 untuk mendapatkan biji benih generasi T_3. Pemeriksaan awal dengan PCR yang mensasarkan gen HPT di rantau T-DNA telah mengenalpasti lima tumbuh-tumbuhan positif dengan transgene. Pengenalpastian lanjutan dengan PCR yang mensasarkan urutan tertentu pada IBDV gen dengan kepanjangan separa dan sepenuh telah mendedahkan dua tumbuh-tumbuhan yang positif bagi gen khusus untuk segmen A dan dua lagi tumbuh-tumbuhan yang positif bagi gen khusus untuk segmen B. Satu lagi tumbuhan diubah dengan plasmid yang mengandungi kedua-dua segmen IBDV yang dihubungkan oleh CrTMV IRES adalah positif bagi gen khusus untuk segmen A dan B. Penyilangan dengan antibodi poliklonal IBDV telah mengesani protein VP2 yang matang dalam tumbuh-tumbuhan yang mengandungi segmen A manakala penyilangan dengan antibodi VP1 telah mengesani protein VP1 dalam tumbuh-tumbuhan yang mengandungi segmen B. Kesimpulannya, kajian ini menunjukkan bahawa penggunaan kedua-dua segmen A dan B genom untuk pencirian IBDV, terutamanya IBDV sangat virulen, adalah penting kerana sifat berkerjasama mereka dalam mempengaruhi kevirulenan virus. Kajian ini mendedahkan bahawa tumbuh-tumbuhan, khususnya *Arabidopsis thaliana*, mampu untuk menyokong pemprosesan poliprotein dan kematangan IBDV prekursor VP2 protein. IRES yang digunakan juga ternyata berfungsi dalam *Arabidopsis* dan menterjemahkan gen VP1 di hiliran apabila ia diletakkan di cistron distal dalam plasmid dicistronic. Penggunaan IRES untuk ungkapan gen dalam sistem tumbuhan mewajarkan penerokaan lanjut bagi pembangunan vaksin, digunakan bukan sahaja untuk IBDV, tetapi juga ke atas agen patogen lain yang penting pada ayam.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF APPENDICES		xviii
LIST OF ABBREVIATIONS		xix
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	4
	2.1 Infectious Bursal Disease Virus	4
	2.1.1 Structure and Genome Organization	4
	2.1.2 Viral Polypeptides	5
	2.1.3 Strain Characterisation and Classification	8
	2.2 Infectious Bursal Disease	10
	2.2.1 History, Clinical Manifestations and Pathology	10
	2.2.2 Immunity and Immunosuppression	11
	2.2.3 Vaccination Against Infectious Bursal Disease	13
	2.3 Plant-based Vaccines	16
	2.3.1 Production of Foreign Proteins in Plant System	16
	2.3.2 Plant-based Vaccine Expression Platforms	17
	2.3.3 Expression Systems of Plant Derived Vaccines	19
	2.4 Internal Ribosome Entry Site	21
	2.4.1 General Translation Pathway in Eukaryotes	21
	2.4.2 Alternative Translation Initiation Pathway in Viral RNAs	22
	2.4.3 Plant Viral Internal Ribosome Entry Site	24
	2.4.4 Regulation of Protein Synthesis and Translation Efficiency	26
3	**CHARACTERISATION OF LOCAL ISOLATE OF INFECTIOUS BURSAL DISEASE VIRUS**	28
	3.1 Introduction	28
	3.2 Materials and Methods	30
	3.2.1 Propagation of Infectious Bursal Disease Virus	30
	3.2.2 Extraction of Viral RNA	30
	3.2.3 First-strand Complementary DNA Synthesis of Viral Segments A and B	31
	3.2.4 Polymerase Chain Reaction of Complementary DNA	31
	3.2.5 Detection and Purification of PCR Products	31
	3.2.6 Cloning and Transformation of Segment A	32
	3.2.7 Cloning and Transformation of Segment B	33
	3.2.8 Plasmid Extraction and Purification	33

3.2.9 Sequencing Verification and Sequence Analysis 34
3.2.10 Multiple Sequence Alignment and Phylogenetic Analysis 34
3.3 Results 35
3.3.1 Virus Inoculation and Propagation 35
3.3.2 Amplification and Cloning of Segments A and B 35
3.3.3 Nucleotide Sequence Analysis 37
3.3.4 Amino Acid Sequence Analysis 37
3.3.5 Phylogenetic Analysis and Sequence Homology 39
3.4 Discussion 44

4 IN VITRO EXPRESSION OF INFECTIOUS BURSAL DISEASE VIRAL SEGMENTS A AND B PROTEINS 49
4.1 Introduction 49
4.2 Materials and Methods 51
4.2.1 Internal Ribosome Entry Site Elements 51
4.2.2 Construction of Plasmids 51
4.2.3 Primer Design 51
4.2.4 Amplification of DNA Elements 51
4.2.5 Construction of Entry Clones 53
4.2.6 Single-fragment Expression Clones 54
4.2.7 Three-fragment Expression Clones 54
4.2.8 Generation of Expression Clones 54
4.2.9 Preparation of DNA Template 55
4.2.10 In vitro Transcription 55
4.2.11 Eukaryotic Cell-free Protein Expression 56
4.2.12 Denaturing Polyacrylamide Gel Electrophoresis 57
4.2.13 Western Blotting 58
4.2.14 Chromogenic Immunodetection 58
4.3 Results 59
4.3.1 Synthetic Internal Ribosome Entry Site Elements 59
4.3.2 Construction of Entry Clones 59
4.3.3 Construction and Analysis of Recombinant Expression Clones 59
4.3.4 Analysis of Runoff Transcripts 62
4.3.5 In vitro Expression of IBDV Proteins 63
4.4 Discussion 64

5 TRANSIENT EXPRESSION OF INFECTIOUS BURSAL DISEASE VIRAL SEGMENTS A AND B PROTEINS IN *Arabidopsis thaliana* 68
5.1 Introduction 68
5.2 Materials and Methods 70
5.2.1 Construction of Three-fragment Entry Clones 70
5.2.2 Double Digestion of the Entry Clones 70
5.2.3 Propagation of Plant Destination Vector 70
5.2.4 Construction of Plant Transformation Plasmids 70
5.2.5 Preparation of Chemically Competent *Agrobacterium tumefaciens* strain LBA4404 71
5.2.6 Transformation into *Agrobacterium tumefaciens* strain LBA4404 71

5.2.7 Mini Preparations of Plasmid DNA 72
5.2.8 Growth of *Arabidopsis thaliana* Seedlings for Transient Gene Expression 72
5.2.9 Preparation of *Agrobacterium tumefaciens* strain LBA4404 for Transient Assay 72
5.2.10 Fast Agro-mediated Seedling Transformation 73
5.2.11 Total RNA Extraction and Analysis 73
5.2.12 Total Plant Protein Extraction 74
5.2.13 Immunodetection of Transiently Expressed IBDV Proteins 74
5.3 Results 75
5.3.1 Constructions of Three-fragment Entry Clones 75
5.3.2 Constructions of Plant Transformation Plasmids 76
5.3.3 Transformation of *Agrobacterium tumefaciens* strain LBA4404 77
5.3.4 Transient Transformation of *Arabidopsis thaliana* 77
5.4 Discussion 81

6 STABLE TRANSFORMATION AND ANALYSES OF *Arabidopsis thaliana* EXPRESSING INFECTIOUS BURSAL DISEASE VIRUS SEGMENTS A AND B GENES 85
6.1 Introduction 85
6.2 Materials and Methods 87
6.2.1 Growth and Care of *Arabidopsis thaliana* to Flowering 87
6.2.2 Growth and Care of *Arabidopsis thaliana* to Maturity 87
6.2.3 Seed Harvest and Storage 87
6.2.4 Preparation of *Agrobacterium tumefaciens* strain LBA4404 for Transformation 88
6.2.5 *Agrobacterium tumefaciens*-mediated Stable Transformation 88
6.2.6 Screening of Primary Transformants 89
6.2.7 Selection of Transgenic *Arabidopsis thaliana* 89
6.2.8 Detection of Transgene in Transformed *Arabidopsis thaliana* 90
6.2.9 Total Plant Protein Extraction 91
6.2.10 Immunoblot Analysis of Total Plant Protein Extracts 92
6.3 Results 92
6.3.1 Growth and Transformation of *Arabidopsis thaliana* 92
6.3.2 Identification of Transgenic *Arabidopsis thaliana* 93
6.3.3 Expression of IBDV Proteins in *Arabidopsis thaliana* 98
6.4 Discussion 102

7 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 106

REFERENCES 112
APPENDICES 152
BIODATA OF STUDENT 232
LIST OF PUBLICATIONS 233

LIST OF TABLES

Table		Page
3.1	Nucleotide sequence of primers used in reverse transcription and PCR amplification of segments A and B of UPM04/190 IBDV	32
3.2	Name and type of the IBDV strains used for MSA and phylogenetic tree comparison with their respective GenBank accession numbers and geographical origin	36
4.1	Nucleotide sequences of primers with *att* sites used in PCR to generate single DNA element flanked by specific *att*B sites	53
5.1	Nucleotide sequence of primers used in one-step RT-PCR for detection of partial sequence of target genes	74
6.1	Nucleotide sequence of primers used in PCR for detection of a known plant gene and T-DNA sequence of expression plasmid	91

LIST OF FIGURES

Figure		Page
3.1	Agarose gel electrophoresis of PCR product containing fragments specific to the Segment A and B of UPM04/190 IBDV isolate	35
3.2	Schematic illustration of the predicted genomic structure of UPM04/190IBDV isolate segment A and B genome	37
3.3	Phylogenetic relationships based on complete nucleotide coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	40
3.4	Phylogenetic relationships based on VP5 coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	40
3.5	Phylogenetic relationships based on polyprotein coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	41
3.6	Phylogenetic relationships based on VP1 coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	41
3.7	Phylogenetic relationships based on VP2, VP3 and VP4 nucleotide coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	42
3.8	Phylogenetic relationships based on VP2, VP3 and VP4 amino acid coding sequences of UPM04/190 and 20 IBDV strains available from GenBank	43
4.1	Overview of the construction of three-fragment expression clones	52
4.2	Nucleotide sequence of IRES element from CrTMV and TEV	60
4.3	Agarose gel electrophoresis of PCR product containing fragments specific to the segments A and B of IBDV isolate UPM04/190	61
4.4	Agarose gel electrophoresis of PCR product containing fragments specific to the IRES elements	61
4.5	Restriction endonuclease analysis on the expression clones	62
4.6	Agarose gel electrophoresis of ssRNA runoff transcripts	62
4.7	Immunodetection of translation products from the expression clones in wheat germ extract	63
5.1	Double digestion analysis on the entry clones	76

5.2	Agarose gel electrophoresis of plant transformation plasmids extracted from the transformed *Agrobacterium tumefaciens* strain LBA4404	77
5.3	Agarose gel electrophoresis of VP4 RT-PCR product on total RNA extract from FAST assay	78
5.4	Agarose gel electrophoresis of VP1 RT-PCR product on total RNA extract from FAST assay	78
5.5	Immunodetection of IBDV segment A translation products from the plant expression clones in *Arabidopsis* seedlings	79
5.6	Immunodetection of IBDV segment B translation products from the plant expression clones in *Arabidopsis* seedlings	80
6.1	Growing *Arabidopsis thaliana* for floral dip	93
6.2	Selection of *Arabidopsis* T_1 seedlings on hygromycin-containing MS selection agar plates	94
6.3	Growing of *Arabidopsis* T_1 seedlings on MS selection agar plates	94
6.4	Further selection of *Arabidopsis* T_1 seedlings on MS selection agar plates	95
6.5	Growing of *Arabidopsis* T_1 seedlings on MS agar without antibiotics	95
6.6	Agarose gel electrophoresis of PCR amplification product for transgene detection on total gDNA extract of T_1 *Arabidopsis* plants	96
6.7	Agarose gel electrophoresis of PCR amplification product targeting partial VP4 sequence on total gDNA extract of T_1 *Arabidopsis* plants	96
6.8	Agarose gel electrophoresis of PCR amplification product targeting partial VP1 sequence on total gDNA extract of T_1 *Arabidopsis* plants	97
6.9	Agarose gel electrophoresis of PCR amplification product targeting IBDV complete segment A sequence on total gDNA extract of T_1 *Arabidopsis* plants	97
6.10	Agarose gel electrophoresis of PCR amplification product targeting IBDV complete segment B sequence on total gDNA extract of T_1 *Arabidopsis* plants	98
6.11	Agarose gel electrophoresis of PCR amplification product targeting partial VP4 sequence on total gDNA extract of T_2 *Arabidopsis* plants	99
6.12	Agarose gel electrophoresis of PCR amplification product targeting partial VP1 sequence on total gDNA extract of T_2 *Arabidopsis* plants	99

6.13 Agarose gel electrophoresis PCR of amplification product targeting partial VP4 sequence on total gDNA extract of T_3 *Arabidopsis* plants 100

6.14 Agarose gel electrophoresis of PCR amplification product targeting partial VP1 sequence on total gDNA extract of T_3 *Arabidopsis* plants 100

6.15 Immunodetection of expressed proteins from the segment A positive *Arabidopsis* T_3 seedlings 101

6.16 Immunodetection of expressed proteins from the segment B positive *Arabidopsis* T_3 seedlings 101

LIST OF APPENDICES

Appendix		Page
A	Oligonucleotide primers used for sequencing	152
B	Nucleotide BLAST results	154
C	Amino acid BLAST results	158
D	Alignment of amino acid sequences	165
E	Sequence identity matrices	176
F	Analyses on recombinant IBDV plasmids	179
G	Map of vectors	229

LIST OF ABBREVIATIONS

SYMBOLS

α	alpha
β	beta
λ	lambda
µg	microgram
µL	microliter
µM	micromolar

ABBREVIATIONS

aa	amino acids
AP	alkaline phosphatase
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAM	chorioallantoic membrane
CIRE	cap-independent regulatory elements
CITE	cap-independent translation element
CrTMV	crucifer-infecting tobamovirus
CVP(s)	chimeric virus particle(s)
ds	double-stranded
EDTA	ethylenediamine tetraacetic acid
eIF(s)	eukaryotic initiation factor(s)
EMCV	encephalomyocarditis virus
ETEC	enterotoxigenic *Escherichia coli*
Fluc	firefly luciferase
FMDV	foot-and-mouth disease virus
g	relative centrifugal force (RCF)
g/L	gram per litre
GCN2	general control non-derepressible-2
gDNA	genomic DNA
GTP	guanosine triphosphate
HBcAg	hepatitis B virus core antigen
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IL	interleukin
IRES	internal ribosome entry site
ITAF(s)	IRES trans-acting factor(s)
kDa	kilo Dalton
MDA	maternally-derived antibody
Met-$tRNA_i^{met}$	methionine charged initiator tRNA
mg/mL	milligram per millilitre
mg/L	milligram per litre
min	minute(s)
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog
NDV	Newcastle disease virus
nt	nucleotide
OD_{600}	optical density of 600 nm
ORF(s)	open reading frame(s)

P domain	projection or protrusion domain
PKR	dsRNA-dependent protein kinase
Pk(s)	pseudoknot(s)
pVP2	precursor VP2
RdRp	RNA dependent RNA polymerase
REase(s)	restriction endonuclease(s)
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
s	seconds
SDS	sodium dodecyl sulphate
SPF	specific-pathogen-free
TAE	tris acetate EDTA
T-DNA	transfer DNA
TEV	tobacco etch virus
Ti	tumour-inducing
tRNA	transfer RNA
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
VN	virus-neutralizing
VPg	genome-linked protein
vv IBDV	very virulent IBDV
w/v	weight per volume
4E-BP	eIF4E-binding protein

AMINO ACID ABBREVIATIONS

Alanine	A, Ala
Arginine	R, Arg
Aspartic Acid	D, Asp
Asparagine	N, Asn
Cysteine	C, Cys
Glutamic Acid	E, Glu
Glycine	G, Gly
Glutamine	Q, Gln
Histidine	H, His
Isoleucine	I, Ile
Leucine	L, Leu
Lysine	K, Lys
Methionine	M, Met
Phenylalanine	F, Phe
Proline	P, Pro
Serine	S, Ser
Tyrosine	Y, Tyr
Threonine	T, Thr
Tryptophan	W, Trp
Valine	V, Val

CHAPTER 1

INTRODUCTION

Stripped of bare essentials, viruses unfold a vast diversity of rebellious life and show how they could be initiated and translated. Among them, the mammalian picornaviruses were the first to go against the standard, so called 'cap-dependent' pathway and defy cap recognition for initiation of translation (Pelletier & Sonenberg, 1988; Jang *et al*., 1988). They shut down the host protein synthesis by interfering with the cap-binding proteins within the cell and thus favour the expression of viral RNA with less competition from the cellular messenger (Svitkin *et al*., 1999; Gradi *et al*., 1998). Although similar findings have later been reported from the plant kingdom, the plant virus genera were not known to shut down host plant protein synthesis (Kneller *et al*., 2006). Notwithstanding, these RNA viruses function via a sequence in their internal region and near the start codon called the internal ribosome entry site (IRES) that is capable of hijacking cellular protein factors to initiate translation of their own RNA. These IRES could exist in the 5'-untranslated region or between two open reading frames of the viral RNA. In plant RNA virus, the cap-independent translation elements can also be found in the 3'-untranslated region.

The IRES, depending on their individual requirements for the eukaryotic initiation factors (eIF), recruit the 40S ribosomal subunit to the vicinity of start codon and thus bypass the need for conventional cap-binding and scanning processes (Sonenberg & Hinnebusch, 2009). The presence of secondary and/or tertiary structures within the stretch of IRES sequence have been associated with their ability to interact with eIFs and modulate cap-independent translation (Baird *et al*., 2006). Notwithstanding, the IRES activity is influenced by the cell types, as well as the physiological status of the cell where the expression is targeted, hence the translation efficiency. In fact, the mode of internal ribosomal entry could not be inferred from one IRES to another IRES of different groups and require to be tested on a case by case basis (Pestova *et al*., 2001).

Although the working mechanism of IRES have not been fully elucidated, their ability to initiate protein translation in a cap-independent manner is very appealing to the biotechnological applications. When used in dicistronic expression vectors, the first gene can be translated by cap-dependent pathway, while that of the second gene is via IRES-dependent manner. They overcome the restriction of monocistronic mRNA in eukaryotic cells and allow the construction of polycistronic transcription unit for expression of two or more proteins (Halpin, 2005). The use of the same constitutive promoter for multiple transgenes expression, which may induce homology-dependent gene silencing in plants can also be avoided (Potenza *et al.*, 2004).

Infectious bursal disease (IBD) is an endemic viral disease of poultry in Malaysia since the first outbreak was reported in 1991 (Hair-Bejo, 1992). The disease is caused by the double-stranded RNA IBD virus (IBDV), which possesses two genome segments named A and B (Delmas *et al*., 2005). Segment A encodes for viral proteins VP2, VP3, VP4 and VP5, and segment B encodes for a single polypeptide VP1. The immunogenic dominant protein VP2, especially its hypervariable region has frequently been used for the molecular diagnosis of IBDV and evolutionary studies of the virus. However, the occurrence of natural IBDV reassortant strains (Jackwood *et al.*, 2011; Le Nouën *et al.*, 2006; Wei *et al.*, 2006) or mosaic virus from homologous recombination within segments (Jackwood, 2012a; He *et al.*, 2009; Hon *et al.*, 2008) have been documented.

Experimental studies showed that the viral segment B does influence the virulence and disease outcome (Escaffre *et al.*, 2013; Yu *et al.*, 2013; Le Nouën *et al.*, 2012). Certainly, this has called for molecular diagnosis as well as genetic studies of IBDV to be based on both genome segments in order to achieve better control of the disease.

In addition, this has signified the importance to include IBDV genome segments A and B in the vaccine preparation as vaccination is the primary control of IBD in poultry (Eterradossi & Saif, 2008). Thus far, four types of IBD vaccines are commercially available in Malaysia for prevention of IBD. The killed vaccines are mainly reserved for breeder flocks to boost and prolong humoral immunity, while the live attenuated vaccines, recombinant vector based vaccines and IBD immune complex vaccines are commonly used as first vaccination in chicks. Although the disease is considerably under controlled with the introduction of vaccination, occasional outbreaks are still reported from the field. The conventional live attenuated vaccine virus retain a certain level of pathogenicity; they could revert to wild-type and cause disease in chickens (Muskett *et al.*, 1985). In contrast, the newer generation recombinant vector based vaccines have so far showed no issues of reversion and capable to confer protection against challenge (Tsukamoto *et al.*, 2002). However, the authors used only the VP2 gene for expression, which did not prevent the development of bursal lesions upon challenge with the IBD virus. While the IBD immune complex vaccine has been shown to be efficacious (Whitfill *et al.*, 1995), the working mechanism of the vaccine remains to be fully elucidated. Therefore, there is the need for a vaccine that could be tailored and revised according to the field situation, and such vaccine must be affordable, safe and readily available.

Plant expression system is an attractive approach for production of vaccine targets as it offers not only safety, but also low cost and scalability (Santi, 2009). It is less likely to harbour microbes or prions that are pathogenic to animals and have lower risks of contamination by extraneous infectious agents. In comparison to microbial or mammalian cell systems, it is generally cheaper in plants to produce the same protein (Mett *et al.*, 2008; Giddings, 2001). The production can be scaled up simply by increasing the cultivation area (Mason *et al.*, 2002). Furthermore, plants expressing the immunogenic proteins can be consumed directly or with only minimal processing should food plants were used. Hence, the need for needles and cold chain delivery of vaccine is reduced.

The use of plants for production of target antigens can be attained by either transient or stable transformation methods. Preliminary studies to examine transgene expression in transgenic plants have often seen the use of *Arabidopsis thaliana* as the preferred prototype plants (Floss *et al.*, 2007). *A. thaliana* is a small flowering weed used widely as a model organism in plant science (Ku *et al*., 2000). The *Agrobacterium*-mediated transformation of *A. thaliana*, for both transient and stable foreign protein expression are established. Notwithstanding, given the insufficiency of data in predicting whether a host plant will work or the protein will be stably expressed at desirable level and correctly folded to function or not, the expression systems, be it stable or transient transformation, necessitate more comprehensive studies (Rybicki, 2009). Each target antigen should be examined in isolation to determine if a given system is suitable for the expression of a particular protein.

Thus far, only the VP2 gene of IBDV has been studied for its expression in a plant-based system using the stable (Wu *et al.*, 2007; Wu *et al.*, 2004a), transient (Gómez *et al.*,

2013), or chimeric viral particles (Chen *et al.*, 2012) approach. The expression of other IBDV viral proteins in plants have not been investigated yet. Furthermore, there are no studies which have incorporated segments A and/or B for expression in plants. Therefore, this study was conducted to examine the feasibility of expressing the IBDV segments A, B and both segments A and B linked by an IRES in plants.

The hypothesis of the study was that the expression of segments A, B or both the segments A and B connected by an IRES link in *A. thaliana* will be possible through both transient and stable transformation. Thus, it was envisaged that the study will provide beneficial groundwork for the greater goal in the development of plant-made vaccines for poultry.

The main objectives of the study were:

1. to characterise the UPM04/190 IBDV local isolate based on the full-length gene coding genome sequences of segments A and B,
2. to analyse the function of synthetic IRES in driving the translation of full length UPM04/190 IBDV segment gene in cell free lysates,
3. to validate the expression of binary constructs carrying UPM04/190 IBDV segments A and B, and IRES-linked segments A and B in *Arabidopsis* seedlings,
4. to generate transgenic *Arabidopsis* plants expressing viral proteins of UPM04/190 IBDV segment A, segment B and both segments A and B as linked by the IRES.

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