THE EFFECT OF KDEL AND PPI GENES CO-EXPRESSION ON THE ANTI-TOXOPLASMA scFV ACTIVITY IN TRANSGENIC *Nicotiana tabacum* CULTIVAR SR1

University of Malaya

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FACULTY OF SCIENCE
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THE EFFECT OF KDEL AND PPI GENES CO-EXPRESSION ON THE ANTI-TOXOPLASMA scFV ACTIVITY IN TRANSGENIC *Nicotiana tabacum* CULTIVAR SR1

ABSTRACT

Toxoplasmosis is a disease caused by infection of a parasitic protozoan known as *Toxoplasma gondii*, which has economic implications due to neonatal loss and abortion in livestock animals. In human adults, it is usually asymptomatic but can cause complications in immunocompromised persons and pregnant women which leads to congenital effects on newborn babies. As a therapeutic approach to combat the disease, biopharmaceutical products, such as recombinant antibody targeting to *T. gondii* tachyzoite, has been introduced as an alternative to drugs and vaccines. Innovative approaches for the expression and production of these novel biopharmaceutical products which increased complexity towards disease targets have been the subject of recent investigations. Plants have now emerged as a preferred host to express and produce these recombinant proteins as it provides advantages in term of efficiency, cost, scalability, safety and compatibility as compared to prokaryotes and mammalian cell lines. Thus, in this study, the target antibody, an anti-*Toxoplasma* scFv encoded by the *TP60* gene was transformed into leaf explants of *Nicotiana tabacum* cv. SR1 using *Agrobacterium tumefaciens* strain LBA4404. The constructs were transformed in a series of experiments in tandem with different enhancer elements comprising proteinase inhibitors or a KDEL retention sequence or combinations of both elements in order to assess their ability to stabilize and enhance the scFv production. Two different proteinase inhibitors that have been used in this study were Oryzacystatin inhibitor (OCPI) (a cysteine inhibitor) and Bowman-Birk inhibitor (BBI) (a trypsin and chymotrypsin

inhibitors). β-glucuronidase (GUS) and green fluorescence protein (GFP) assays as well as PCR results confirmed the presence of transgenes in the tobacco genome that was stably inherited into T_1 generation. For Western blot analysis, the selected lines of transgenic tobacco plants transformed with pTP60 construct in combination with BBI-KDEL (pTP60BBIKDEL) elements was found to produce the highest anti-*Toxoplasma* scFv recombinant antibody accumulation at the expected size of ~ 54 kDa (homodimer formation) which was at first observed at ~ 27 kDa (monomer) in T_0 tobacco plants. No expression was detected in non-transformed plants. The results were also confirmed at the mRNA level using real-time PCR analysis with the BBI-KDEL tandem constructs expressing *TP60* gene approximately 19-fold higher as compared to plants transformed with the pTP60 construct in the absence of any proteinase inhibitor and/or KDEL. Interestingly, the introduction of all of these elements did not or only negligibly affected the growth and development of the transgenic tobacco plants as compared to the wild-type. Our results confirmed the ability of plants to function as bio-factories for recombinant protein production and showed that the use of proteinase inhibitor and KDEL elements may be useful in improving the yield of the targeted antibody proteins.

Keywords: *Agrobacterium*-mediated transformation, plant molecular farming, recombinant protein, single-chain fragment antibody (scFv), toxoplasmosis

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KESAN EKSPRESI BERSAMA GEN KDEL DAN PPI TERHADAP AKTIVITI ANTI-TOXOPLASMA scFV PADA *Nicotiana tabacum* KULTIVAR SR1 TRANSGENIK

ABSTRAK

Toxoplasmosis merupakan penyakit yang disebabkan oleh jangkitan protozoan parasit yang dikenali sebagai *Toxoplasma gondii*, yang mempunyai implikasi kepada ekonomi akibat kehilangan neonatal dan keguguran haiwan ternakan. Di kalangan manusia dewasa, penyakit ini biasanya tidak menunjukkan sebarang gejala jangkitan tetapi boleh mengakibatkan komplikasi pada mereka yang mempunyai immunisasi yang lemah serta wanita hamil yang boleh membawa kepada kesan kongenital terhadap bayi yang baru dilahirkan. Sebagai pendekatan terapeutik bagi memerangi penyakit ini, produk biofarmaseutikal seperti antibodi rekombinan yang disasarkan kepada *T. gondii* tachyzoite telah diperkenalkan sebagai alternatif kepada ubat-ubatan dan vaksin. Pendekatan inovatif terhadap ekspresi dan penghasilan produk biofarmaseutikal yang baharu ini telah mempunyai peningkatan kompleksiti terhadap penyakit yang disasarkan dan menjadi subjek penyelidikan terkini. Kini, tumbuhan telah muncul sebagai hos pilihan dalam mengekspresi dan menghasilkan protein rekombinan kerana kelebihannya dari segi kecekapan, kos, skala, keselamatan dan keserasian berbanding titisan sel prokariot dan mamalia. Oleh itu, dalam kajian ini, antibodi sasaran iaitu scFv anti-*Toxoplasma*, yang dikodkan oleh gen *TP60* telah ditransformasi ke dalam eksplan daun *Nicotiana tabacum* kultivar SR1 dengan menggunakan *Agrobacterium tumefaciens* strain LBA4404. Konstruk-konstruk telah ditransformasi dalam satu siri eksperimen bersama dengan unsur penambah berbeza yang terdiri daripada perencat proteinase atau jujukan pengekalan KDEL

atau gabungan kedua-dua unsur tersebut bagi menilai keupayaan mereka dalam menstabil dan meningkatkan penghasilan scFv. Dua perencat proteinase yang telah digunakan dalam kajian ini ialah perencat *Oryzacystatin* (OCPI) (perencat *cystein*) dan perencat *Bowman-Birk* (BBI) (perencat *trypsin* dan *chymotrypsin*). Ujian β-glucuronidase (GUS) dan protein pendarfluor hijau (GFP) serta keputusan PCR mengesahkan kehadiran transgen dalam genom tembakau yang telah diwariskan kepada generasi T_1. Bagi analisis pemendapan *Western*, barisan tumbuhan tembakau transgenik yang telah ditransformasi dengan konstruk pTP60 bersama dengan unsur BBI-KDEL (pTP60BBIKDEL) didapati dapat menghasilkan pengumpulan antibodi scFv anti-*Toxoplasma* tertinggi pada saiz jangkaan ~ 54 kDa (pembentukan homodimer) yang pada pertama kalinya dilihat pada saiz jangkaan ~ 27 kDa (monomer) di dalam tumbuhan tembakau T_0. Tiada ekspresi yang dikesan pada tumbuhan yang tidak ditransformasi. Keputusan ini juga disahkan pada tahap mRNA dengan menggunakan analisis *real-time* PCR di mana konstruk tandem BBI-KDEL menunjukkan ekspresi gen *TP60* kira-kira 19 kali ganda lebih tinggi berbanding dengan tumbuhan yang ditransformasi dengan konstruk pTP60 sahaja tanpa sebarang unsur penambah. Menariknya, pengenalan semua unsur penambah ini tidak atau hanya memberi kesan yang boleh diabaikan terhadap tumbesaran dan perkembangan tumbuhan tembakau transgenik apabila dibandingkan dengan tumbuhan bukan transgenik. Hasil kajian kami mengesahkan keupayaan tumbuhan untuk berfungsi sebagai kilang bio bagi pengeluaran protein rekombinan dan juga menunjukkan bahawa penggunaan unsur penambah mungkin berguna dalam meningkatkan hasil protein antibodi yang disasarkan.

Kata kunci: Transformasi *Agrobacterium*-pengantara, tumbuhan pertanian molekul, protein rekombinan, antibodi serpihan rantaian tunggal (scFv), toxoplasmosis

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TABLE OF CONTENTS

Abstract iii

Abstrak v

Acknowledgements vii

Table of Contents viii

List of Figures xiii

List of Tables xvii

List of Symbols and Abbreviations xix

List of Appendices xxiii

CHAPTER 1: INTRODUCTION 1

1.1 Background study 1

1.2 Objectives 3

1.2.1 General objective 3

1.2.2 Specific objectives 3

1.3 Hypothesis 3

CHAPTER 2: LITERATURE REVIEW 4

2.1 Toxoplasmosis 4

2.1.1 *Toxoplasma gondii* strains 5

2.1.2 Symptoms of infection 6

2.1.3 Control of toxoplasmosis 7

2.2 Expression system for recombinant protein production 7

2.2.1 Types of different expression systems 8

2.3 Plant molecular farming 10

2.4 Therapeutic proteins 12

University of Malaya

2.4.1 Antibodies 13

2.4.1.1 Single-chain variable fragment antibodies 13

2.5 Challenges and strategies of plant as protein expression system 15

2.5.1 Co-expression with proteinase inhibitors 17

2.5.2 Targeting to specific ER-organelle in plant tissue (KDEL) 18

2.6 *Agrobacterium*-mediated transformation 21

2.6.1 *Agrobacterium* strains used for recombinant protein expression 21

2.6.2 Importance of *Agrobacterium* in recombinant protein production 22

2.7 Expression vectors used for *Agrobacterium*-mediated transformation 25

2.8 Tobacco as a plant host 27

CHAPTER 3: MATERIALS AND METHODS 30

3.1 Maintenance of *in vitro Nicotiana tabacum* cv. SR1 30

3.1.1 Preparation of culture medium 30

3.1.2 Germination of tobacco seeds 30

3.1.3 Maintenance of tobacco plantlets 31

3.2 Endogenous test of tobacco leaf disc towards hygromycin 32

3.3 *Agrobacterium tumefaciens* strain LBA4404 33

3.3.1 Preparation of YEB and YEA media 33

3.3.2 Preparation of *A. tumefaciens* presence with different plasmid constructs 33

3.3.2.1 Gene sequencing and BLAST analysis 35

3.3.2.2 Plasmid extraction 35

3.3.2.3 PCR verification of *Agrobacterium* colonies 36

3.3.2.4 Agarose gel electrophoresis 39

3.3.2.5 Bacterial stock storage (glycerol stock) 40

3.4 *Agrobacterium*-mediated transformation into tobacco leaf disc 41

University of Malaya

3.4.1 Selection of transformants 41

3.5 Transient assays of putative transformed shoots 42

3.5.1 GUS assessments 42

3.5.1.1 GUS histochemical staining 42

3.5.1.2 GUS fluorometry assay 43

3.5.2 GFP visualization 43

3.6 Acclimatization and phenotypic assessment of transgenic tobacco plants 44

3.7 Assessment of putative tobacco transformants by PCR analysis 44

3.7.1 Total genomic DNA extraction 44

3.7.2 Polymerase chain reaction (PCR) 45

3.8 Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis 48

3.8.1 Gel cassette assembly 48

3.8.2 Preparation of the gel 48

3.8.3 Preparation of protein samples 50

3.8.3.1 Bradford's quantification 50

3.8.4 Electrophoresis of SDS-PAGE 50

3.8.5 Staining and de-staining protein gel with Coomassie blue 51

3.8.6 Western blot analysis 51

3.8.7 Protein purification using Dynabeads® 52

3.9 Verification of transgenic plant via real-time PCR analysis 53

3.9.1 RNA extraction and DNase treatment 53

3.9.2 Reverse-transcription PCR (RT-PCR) 55

3.9.3 Real-time PCR 57

3.10 Semi-quantitative RT-PCR 59

3.11 Statistical analysis 59

University of Malaya

CHAPTER 4: RESULTS **60**

4.1 Endogenous test of *N. tabacum* cv. SR1 leaf discs towards antibiotics 60

4.2 Confirmation of *A. tumefaciens* harboring binary vector and gene of interest 65

4.2.1 Sequencing and BLAST analysis 73

4.3 Transformation of *N. tabacum* cv. SR1 leaf discs mediated by *A. tumefaciens* 81

4.3.1 Effects of different plasmid constructs towards regeneration of putative transformed shoots 81

4.3.2 Confirmation of putative transformed shoots using transient assays 86

4.3.2.1 GUS histochemical staining 86

4.3.2.2 GUS fluorometry assay 89

4.3.2.3 GFP qualitative assay 93

4.3.3 Verification of putative transformed shoots (T_0) through PCR analysis 95

4.4 Assessment of gene stability T_1 generation of transgenic tobacco 100

4.4.1 Effect of different plasmid constructs on seed germination 100

4.4.2 Verification of transgenic seedlings (T_1) through PCR analysis 103

4.5 Acclimatization of transgenic tobacco plants 105

4.6 Phenotypic assessment on T_1 tobacco progenies 107

4.7 Real-time PCR analysis 108

4.7.1 RNA extraction and DNase treatment of T_1 tobacco progenies 108

4.7.2 Relative expression of *TP60* gene using real-time PCR 116

4.8 Recombinant anti-*Toxoplasma* protein expression in transgenic tobacco plants 124

4.8.1 Recombinant protein purification 124

CHAPTER 5: DISCUSSION **130**

5.1 Introduction 130

University of Malaya

5.2 Transformation of tobacco with recombinant plasmid constructs....................... 131

5.3 Confirmation of putative transformed shoots 132

5.4 Effects of different plasmid constructs on the phenotype of transgenic tobacco plants 134

5.4.1 T_1 seed germination 134

5.4.2 Stem height and flowering period 135

5.5 Co-expression of proteinase inhibitor and KDEL increased the relative expression of p*TP60* gene and protein accumulation 136

5.6 Homodimer formation of recombinant anti-*Toxoplasma* protein in T_1 tobacco plants 138

CHAPTER 6: CONCLUSION 140

REFERENCES 143

LIST OF PUBLICATIONS AND PAPERS PRESENTED 161

APPENDIX 162

University of Malaya

LIST OF FIGURES

Figure 2.1: Sexual and asexual life cycles of *T. gondii* life cycles. 5

Figure 2.2: Different host systems available for the production of recombinant proteins 9

Figure 2.3: Domain architecture of antibodies and antibody fragments.................... 15

Figure 2.4: Binary vector, pCAMBIA 1304. 26

Figure 3.1: Internode parts of tobacco plants used for sub-culturing 32

Figure 3.2: Schematic diagram of constructed plasmids used in this study. 34

Figure 4.1: Percentage of survival explants on media containing hygromycin at different concentrations after one month of culture 61

Figure 4.2: 10 kb of isolated plasmid containing gene of interest 66

Figure 4.3: PCR verification of empty plasmid (without insert) using a pair of GFP primer 67

Figure 4.4: PCR verification of extracted plasmid using a pair of 1304sk primer 67

Figure 4.5: PCR verification of extracted plasmid using a pair of 1304sk primers... 68

Figure 4.6: PCR verification of extracted plasmid using a pair of F-1304sk and R-GFP primers 69

Figure 4.7: PCR verification of extracted plasmid using a pair of F-1304sk and R-GFP primers 70

Figure 4.8: PCR verification of extracted plasmid using a pair of F-35S and R_1-1304sk primers 71

Figure 4.9: PCR verification of extracted plasmid using a pair of F-35S and R_1-1304sk primers 72

Figure 4.10: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and GFP. 74

Figure 4.11: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and KDEL.. 76

Figure 4.12: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and BBI proteinase inhibitor. 78

Figure 4.13: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and OCPI proteinase inhibitor. 80

University of Malaya

Figure 4.14: Number of regenerated shoots versus different constructs. 85

Figure 4.15: GUS histochemical staining of putative transformed tobacco shoots (~10 week-old) on TSM-selection media. 88

Figure 4.16: Quantitative assessment of GUS activity with fluorogenic substrates. 4-MUG which would hydrolyzed to form 4-MU protein product in the presence of *gus*A gene encoded for β-glucuronidase enzyme..........90

Figure 4.17: Standard curve of fluorescence intensity versus known 4-MU concentration 91

Figure 4.18: Standard curve of known concentration BSA using Bradford's assay quantification...92

Figure 4.19: GUS activity, (pmole per min) in 100 mg of putative transformed shoots transformed with different plasmid constructs............................92

Figure 4.20: Visualization of expanded putative transformed shoots with pTP60 construct under a confocal laser scanning microscope (40X-oil immersion objective lens).. 94

Figure 4.21: Genomic DNA of selected putative transformed tobacco shoots harboring different plasmid constructs after 10 weeks of selection........96

Figure 4.22: Genomic DNA of selected putative transformed tobacco shoots harboring different plasmid constructs after 10 weeks of selection........97

Figure 4.23: PCR confirmation of putative transformed shoots using F-RGFP primers with the expected size of 759 bp...98

Figure 4.24: PCR confirmation of putative transformed shoots using F-$R_1$1304sk primers with the expected size of ~ 900 bp..99

Figure 4.25: PCR confirmation of putative transformed shoots using F-$R_1$1304sk primers with the expected size of ~ 1.5 kb..100

Figure 4.26: The percentage of T_1 seeds germination on 7th days of germination. ...102

Figure 4.27: Germination of wild-type (1) and pTP60 T_1 seedlings (2) on selection media after 4 weeks of germination. 102

Figure 4.28: PCR confirmation of T_1 seedlings using F-RHPT1 primers with an expected size of 559 bp 104

Figure 4.29: PCR confirmation of T_1 seedlings using F-RHPT1 primers with an expected size of 559 bp 105

Figure 4.30: The growth stages of transgenic *Nicotiana tabacum* cv. SR1 within 3 months of acclimatization. 106

University of Malaya

Figure 4.31: 1 µg of non-treated total RNA isolated from 40-day old acclimatized T_1 tobacco progenies 109

Figure 4.32: 100 ng of DNase-treated RNA isolated from 40-day old acclimatized T_1 tobacco progenies 110

Figure 4.33: 1 µg of non-treated and 100 ng of DNase-treated RNA isolated from 40-day old acclimatized T_1 tobacco progenies 110

Figure 4.34: 1 µg of non-treated and 100 ng of DNase-treated RNA isolated from 40-day old acclimatized tobacco wild-type 111

Figure 4.35: Dissociation curve of different primer sets, L25 and FV60 for *NtL25* internal reference gene and *TP60* recombinant gene respectively at 60 °C annealing temperature (derivative reporter versus temperature). 116

Figure 4.36: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 3, 12, and 16 harboring pTP60 construct. 118

Figure 4.37: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 2, 1, and 14 harboring pTP60BBIKDEL construct. 119

Figure 4.38: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 7, 8, and 6 harboring pTP60KDEL construct. 120

Figure 4.39: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 1, 13, and 15 harboring pTP60OCPIKDEL construct. 121

Figure 4.40: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 5, 6, and 7 harboring pTP60BBI construct. ... 122

Figure 4.41: The expression levels of *TP60* gene in T_1 tobacco progenies, comparing between representative lines of different plasmid constructs with the highest expression of anti-*Toxoplasma* gene at mRNA level.. 123

Figure 4.42: Standard curve of known concentration BSA using Bradford's assay quantification 125

Figure 4.43: 20 µg of unpurified total soluble protein extracted from wild-type and a transgenic *N. tabacum* cv. SR1 representative from each construct (T_0) after 40-days of acclimatization. 126

Figure 4.44: Detection of recombinant anti-*Toxoplasma* scFv antibody, TP60 from unpurified total soluble protein on the selected T_0 tobacco plants and wild-type after 40-days of acclimatization using mouse monoclonal anti–E epitope tag and IgG anti-mouse with HRP conjugated by Western blot analysis. 127

University of Malaya

Figure 4.45: SDS-PAGE of purified recombinant anti-*Toxoplasma* scFv antibody protein using DyNabeads™ from *N. tabacum* cv. SR1 representative lines harboring different plasmid constructs (T_1) after 40-days of acclimatization..128

Figure 4.46: Stable expression of purified recombinant anti-*Toxoplasma* scFv antibody protein from *N. tabacum* cv. SR1 representative lines harboring different plasmid constructs (T_1) after 40-days of acclimatization..129

University of Malaya

LIST OF TABLES

Table 2.1: Plant based vaccines and antibodies in clinical development or on market 12

Table 2.2: Strategies used for enhancing the expression of transgenes in plants 16

Table 2.3: Proteinase inhibitors used for *in vitro* assays. 18

Table 2.4: Impact of subcellular targeting on recombinant protein yield in transgenic plant systems. 20

Table 2.5: Studies addressing post-inoculation environmental effects on *Agrobacterium*-mediated transient expression 25

Table 3.1: Components involved in PCR mixtures used for screening (iNtRON Biotechnology, South Korea) 36

Table 3.2: Thermal cycling conditions using i-Taq^{TM} DNA polymerase (iNtRON Biotechnology, South Korea) 37

Table 3.3: List of primers that were used for PCR screening of *Agrobacterium* transformants 38

Table 3.4: Concentrations of agarose gel used for different sizes and types of nucleic acid 40

Table 3.5: List of primers that were used for T_1 seedlings screening and real-time PCR 42

Table 3.6: 12 % (v/v) of resolving gel (SDS-PAGE) 49

Table 3.7: 4 % (v/v) of stacking gel (SDS-PAGE) 49

Table 3.8: RapidOut DNA removal reaction mixture 55

Table 3.9: Reverse-transcriptase PCR reaction mixture 56

Table 3.10: DNase treated-RNA preparation 56

Table 3.11: Thermal cycling condition of reverse-transcriptase PCR 57

Table 3.12: 5X Real-time PCR reaction mixtures 58

Table 3.13: Thermal cycle condition for real-time PCR 58

Table 4.1: Assessment on minimal concentration of hygromycin in *N. tabacum* cv. SR1 leaf discs 63

University of Malaya

Table 4.2: The list of primer combinations used for the confirmation of insert.........66

Table 4.3: The effect of different plasmid constructs towards the regeneration of putative transformed shoots on selection media (TSM-containing 20 mg/L hygromycin) after one month of culture 82

Table 4.4: Assessment on stem height of T_1 independent lines (20 days of post acclimatization) and flowering period on transgenic and wild-type tobacco plants 108

Table 4.5: $OD_{A260/280}$ and $OD_{A260/230}$ readings of non-transformed and T_1 tobacco progenies before DNase-treatment 112

Table 4.6: $OD_{A260/280}$ and $OD_{A260/230}$ readings of non-transformed and T_1 tobacco progenies DNase-treated RNA. 114

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
µg	:	Microgram
HRP	:	Horseradish peroxidase
BAP	:	6-Benzylaminopurine
BBI	:	Bowman-Birk proteinase inhibitor
bp	:	Base pair
BSA	:	Bovine serum albumin
µL	:	Microlitre
cDNA	:	Complementary deoxyribonucleic acid
Ct	:	Threshold
cv	:	Cultivar
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTPs	:	Deoxyribonucleoside triphosphates
EDTA	:	Ethylenediaminetetraacetic
EtBr	:	Ethidium bromide
g	:	Gram
dH_2O	:	Distilled water
$K_3[Fe(CN)_6]$	:	Potassium ferricyanide
$K_4[Fe(CN)_6]$	:	Potassium ferrocyanide
kb	:	Kilo base pair
L	:	Litre
LB	:	Luria-Bertani
M	:	Molar

mg	:	Milligram
RNA	:	Ribonucleic acid
MSO	:	Murashige and Skoog (without hormones added)
MW	:	Molecular weight
Na_2HPO_4	:	Sodium hydrogen phosphate
NaH_2PO_4	:	Sodium dihydrogen phosphate
NAA	:	l-Naphthaleneacetic acid
NaOH	:	Sodium hydroxide
$NaPO_4$	:	Sodium phosphate
NCBI	:	National Centre for Biotechnology Information
°C	:	Degree Celsius
PCR	:	Polymerase Chain Reaction
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
qRT-PCR	:	Quantitative real-time PCR
scFv	:	Single-chain variable fragment
SDS	:	Sodium dodecyl sulphate
CaMV	:	Cucumber mosaic virus
TBE	:	Tris-Borate-EDTA
V	:	Volt
v/v	:	Volume per volume
w/v	:	Weight per volume
OCPI	:	Oryzacystatin proteinase inhibitor
KDEL	:	ER-targeting gene
ER	:	Endoplasmic reticulum
mAb	:	Monoclonal antibody

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pAb	:	Polyclonal antibody
PAGE	:	Polyacrylamide gel electrophoresis
HCl	:	Hydrochloric acid
mM	:	Millimolar
ng	:	Nanogram
mL	:	Millilitre
µM	:	Micromolar
$MgSO_4$	:	Magnesium sulphate
LD	:	Lethal dosage
nm	:	Nanometer
GEB	:	GUS extraction buffer
GAB	:	GUS assay buffer
CSB	:	Carbonate stop buffer
MUG	:	4-Methylumbelliferyl b-D-glucuronide
$Na_2CO_3.H_2O$	:	Sodium carbonate monohydrate
4-MU	:	7-Hydroxy-4-methylcoumarin
dH_2O	:	Distilled water
h	:	Hour
min	:	Minute
RT-PCR	:	Reverse-transcription PCR
TEMED	:	Tetramethyethylenediamine
APS	:	Ammonium persulfate
[ ]	:	Concentration
PPI	:	Plant proteinase inhibitor(s)
T_1/ F_1	:	Self-pollinated of T_0 progeny lines
GUS	:	β-galactosidase

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GFP	:	Green fluorescent protein
X-gluc	:	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
TRM	:	Tobacco root induction media
TSM	:	Tobacco shoot induction media
m	:	Metre
s	:	Second
µmol	:	Micromole
cm	:	Centimetre
µmol	:	Micromole
pH	:	Potential hydrogen, representing alkalinity or acidity
YEB	:	Yeast extract broth medium
RT	:	Room temperature
OD_{600}	:	Optical density at 600 nm
Fc	:	Fragment, crystallizable receptor on antibody structure
TBST	:	Tris-buffered saline (Tween 20 added)
IgG	:	Immunoglobulin G
dT	:	Deoxythymine
UV	:	Ultraviolet
RQ	:	Relative quantification
pmole	:	Picomole
rRNA	:	Ribosomal RNA
A260/280	:	Absorbance at 260 nm and 280 nm ratio
A260/230	:	Absorbance at 260 nm and 230 nm ratio
kDa	:	Kilodalton
∞	:	Infinity

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LIST OF APPENDICES

Appendix A : Stock solutions and culture media 162

Appendix B : Bacterial culture media preparation 166

Appendix C : Stock solution for molecular works 168

Appendix D : Buffer used for GUS qualitative and quantitative assays 169

Appendix E : Buffer solutions used for protein work 173

Appendix F : Putative shoots regeneration assessment 176

Appendix G : GUS fluorometry assay 179

Appendix H : Percentage of T_1 seeds germination 181

Appendix I : Stem height assessment of T_1 tobacco plants 184

Appendix J : Flowering period (day) of T_1 tobacco progenies 187

Appendix K : Real-time PCR (qRT-PCR) analysis comparing between different construct of a representative line 190

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CHAPTER 1: INTRODUCTION

1.1 Background study

Toxoplasmosis is a severe disease caused by protozoan parasite, *Toxoplasma gondii.* This disease is predominant in birds and mammals, including humans. In human, the symptoms usually can be seen in people with weakened immunity. Toxoplasmosis can cause complication to pregnant women that may lead to congenital effects on newborn babies due to direct contact with saliva and faeces of the infected cats (Dubey et al., 2012; Flegr et al., 2014). Consuming uncooked meat and unpasteurized milk from infected livestock animals could also be the contributory factors. This infectious disease has economic implications due to neonatal loss and abortion in livestock animals. Thus, the production of the therapeutic proteins would be of great value to treat this disease besides drugs. Other than vaccines, antibody, an immune system against non-self-antigens in living organisms could serve as an effective therapeutic agent to treat toxoplasmosis. Recombinant antibody fragments such as single-chain variable fragment (scFv) have become popular therapeutic alternatives due to minimal size with the retained paratope specificity which is important for the recognition and elimination of the specific antigen (Ario de Marco, 2011; Ahmad et al., 2012).

As compared to other expression systems, plants have recently emerged as a preferred host as the level of recombinant protein accumulation in selected plant tissues has increased. This is due to advantages provided by plants with lower risk of pathogen contamination, capability to perform post-translational modification and multimeric assembly capability especially for antibody production (Tschofen et al., 2016).

However, there are some limitations in using plants as host of recombinant protein production. Most importantly, the accumulations of foreign protein in higher plants are

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very limited due to the localization of transgene in plant system and intracellular degradation caused by proteolytic enzymes, known as proteases. Recombinant proteins that interfered by proteases may lead to lower insolubility, altered integrity, and higher heterogeneity of the end-protein product (Doran, 2006; Benchabane et al., 2008).

In order to overcome these limitations and subsequently improved the level of desired foreign protein in plant system, this study was undertaken to investigate the expression level of recombinant anti-*Toxoplasma* scFv antibody expressed with or without the companion of different elements; proteinase inhibitors (BBI and OCPI) as well as ER-targeting gene (KDEL). In this study, an anti-*Toxoplasma* single-chain variable fragment (scFv) antibody encoded by *TP60* gene with or without the presence of different proteinase inhibitor and/or KDEL elements were transformed into *Nicotiana tabacum* cv. SR1 mediated by *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA 1304 containing a *mgfp*5*:gus*A fusion reporter genes and a selectable marker for hygromycin B (*hpt*II) driven by the CaMV 35S promoter. The presence of reporter genes in regenerated plants was determined based on the expression of GUS and GFP transient assays. Further confirmation of the presence of recombinant gene was verified using PCR and Western blot in both T_0 and T_1 generations. The relative expression of anti-*Toxoplasma* scFv gene also has been examined using real-time PCR in the selected lines of T_1 tobacco plants in order to assess the effect of *TP60* in the companion of plant proteinase inhibitor (PPI) and/ or KDEL. Besides, the phenotypic assessments of T_1 tobacco plants, such as percentage of germination, stem height, and flowering period have also been carried out. Findings of this study may help to improve the yield and quality of the targeted protein, which may serve as a foundation for future research in therapeutic production in plants.

1.2 Objectives

1.2.1 General objective

To investigate the effects of KDEL and PPI genes in improving the production of an anti-*Toxoplasma* scFv antibody encoded by *TP60* gene in *N. tabacum* cv. SR1.

1.2.2 Specific objectives

i. To introduce constructed gene cassettes containing *TP60* gene with and without the presence of KDEL and PPI genes into *N. tabacum* cv. SR1 by *Agrobacterium*-mediated transformation method.
ii. To analyze the expression level and stability of TP60 protein expressed in the presence or absence of KDEL and PPI genes in *N. tabacum* cv. SR1 at both mRNA and protein levels.
iii. To investigate the effects of expressing TP60 with the presence of KDEL and/ or PPI genes towards the growth and development of transgenic tobacco plants.

1.3 Hypothesis

Co-expression of KDEL and PPI genes could potentially increase the accumulation and production of an anti-*Toxoplasma* recombinant scFv antibody in *N. tabacum* cv. SR1 without interfering the endogenous gene regulations which responsible for growth and development in transgenic tobacco plants.

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CHAPTER 2: LITERATURE REVIEW

2.1 Toxoplasmosis

Toxoplasma gondii, a protozoan parasite which belongs to the family of Sarcocystidae is a causative agent to a disease called toxoplasmosis. It infects a large proportion of the world's population (Petersen, 2007) but is an uncommonly clinically significant disease (Montoya & Liesenfeld, 2004). Tachyzoites and bradyzoites are *T. gondii* oocysts responsible to cause infection in human.

According to Centre for Disease Control and Prevention (CDC), over 60 million people in United States are infected with this parasite (Friger, 2014). In Brazil, a very high rate of *T. gondii* infection was reported in humans especially congenitally infection with the estimation of 1 infected child per 1000 birth (Dubey et al., 2012). While in Malaysia, about 30-40 % of immunocompetent people showed positive screening of *T. gondii* in their blood serum without any symptoms of infection (Saidi, 2015).

Toxoplasmosis may not cause any harm to perfectly healthy people. However, people with compromised immunity, pregnant women and certain individuals are at high risk for severe or life-threatening complications. The infection mostly acquired due to direct contact with infected cat's saliva or their faeces (Mitchell et al., 1990; Luft & Remington, 1992; Rorman et al., 2006). This disease may also be transmitted through food (uncooked meat and unpasteurized milk) as well as untreated water. For pregnant women, the transmission of tachyzoites to the foetus occurs via the placenta following primary maternal infection. In rare cases, toxoplasmosis may be transmitted through a blood transfusion or a transplanted organ (Kotton et al., 2009; Singh & Sehgal 2010). In several risk factor or case-control studies, an increased risk of primary infection by *T.*

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gondii was also associated by eating unwashed fruits and vegetables besides raw meat (Kapperud et al., 1996; Berger et al., 2009; Liu et al., 2009).

Domestic cats which belong to the family of Felidae are the definitive host while warm-blooded animals including humans are the intermediate host for the propagation of *T. gondii* parasites. *T. gondii* involves both sexual (cats) and asexual (warm-blooded animals and human) life cycles (Figure 2.1).

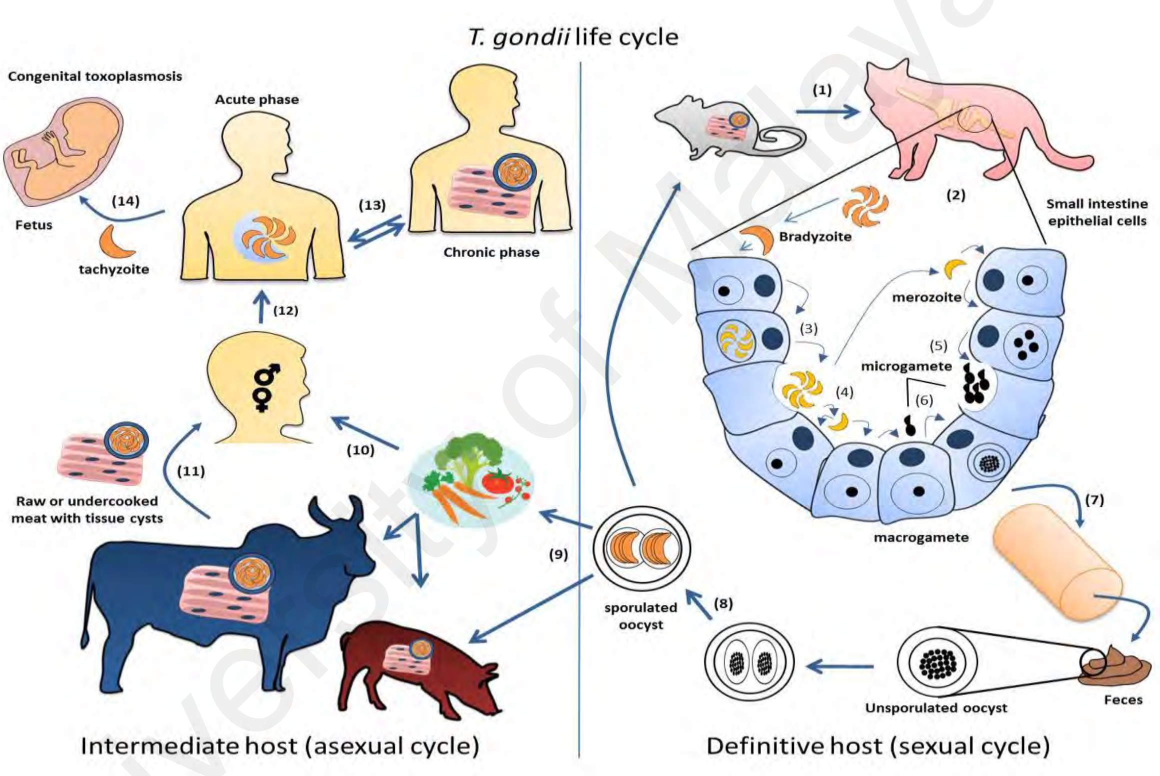

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Figure 2.1: Sexual and asexual life cycles of *T. gondii* life cycles. Source from Duque et al. (2013).

2.1.1 *Toxoplasma gondii* strains

T. gondii was discovered by Nicolle and Manceaux in 1908 from blood, spleen, and liver of a North African rodent, *Ctenodactylus gondii* and named a year later in 1909.

There are three major genotypes of *T. gondii* (type I, type II, and type III) which differ in pathogenicity effect and prevalence in human. The pathogenicity is classified

into two different categories; virulent (type I) and non-virulent (type II and III). As summarized by Maubon et al. (2008), type I was rarely isolated but highly virulent for a longer time compared to type II and III (Saeij et al., 2005). As reported by Souza and Morampudi (2011), type I *T. gondii* was also significantly high in multiplication rate in an immortalized small intestinal human epithelial cell line but low in interconversion from tachyzoite to bradyzoite. However, in Europe and United States, type II non-virulent strain is responsible for most cases of congenital toxoplasmosis (Lindsay & Dubey, 2011).

Among these three major strains, RH strain (type I) is the first human strain which is only found in severe cases of human toxoplasmosis and caused 100% lethality when tested in laboratory mice (Ajzenberg, 2010). The first isolated RH strain was from a patient who died of toxoplasmosis encephalitis in 1939 (Sabin, 1941).

2.1.2 Symptoms of infection

Symptoms for immunocompromised people are brain inflammation, headache, seizure, lung infection, cough and fever, shortness of breath, eye infection, blurry vision and eye pain. However, very mild or no symptoms of infection can be seen with immunocompetent people (Halonen & Weiss, 2013). The symptoms may include fever, swollen lymph nodes, headache, muscle aches and pains as well as sore throat which can last at least a month before fully recovered.

Approximately, 10-20 % of pregnant women infected with *T. gondii* become symptomatic (Montoya & Remington, 1996). Severe symptoms in pregnant women can be seen in newborn baby if the mother was infected with this parasite during first trimester of pregnancy (gestation). *T. gondii* parasite may transmit to fetus in placenta

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resulting in congenital toxoplasmosis in children showing symptoms of brain and nervous system problems (Petersen, 2007).

2.1.3 Control of toxoplasmosis

Currently, there are drugs available to treat toxoplasmosis, such as pyrimethamine, spiramycin and sulfadiazine (Elsheikha, 2008), which may be associated with side effects.

Over the years, there is increasing awareness in many countries in producing therapeutic proteins such as vaccines and antibodies. At present, only one commercial vaccine "Toxovax" based on live attenuated S48 strain has been licensed for use to avoid congenital infection in ewes (Buxton & Innes, 1995). However, this vaccine is expensive and has a short shelf-life. Furthermore, it may also revert to a pathogenic strain and therefore it is not safe for human use (Kur et al., 2009). There is currently no licensed vaccine available for humans. Due to chances of allergicity, specific therapeutic antibodies production is a potential approach for treating the post-infection diseases.

2.2 Expression system for recombinant protein production

With the advent of recombinant DNA technology, cloning and expression of numerous mammalian genes in different systems have been explored to produce many biopharmaceutical products, such as vaccines and antibodies, for human and animals in the form of recombinant proteins. The selection of expression system becomes crucial due to productivity, bioactivity, and physiochemical characteristics of the target protein.

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2.2.1 Types of different expression systems

The production of recombinant proteins has been widely carried out in bacteria expression system. This is due to several advantages such as rapid propagation and low cost required as well as the availability of established methods for genetic manipulation (Snustad & Simmons, 2010). However, there are several disadvantages in producing recombinant proteins in bacteria, such as lack of post-translational modifications. In addition, bacteria are unable to secrete the protein product into the extracellular medium due to protein misfolding, aggregation, and intracellular accumulation that led to the formation of inclusion body of the target protein (Rosano & Ceccarelli, 2014). However, recently, it also has been reported that this inclusion body is reversible and the aggregates protein can be recovered by using mild solubilization process (Singh et al., 2015) or IB-tag fused target protein (Jong et al., 2017) in order to examine the expression of the insoluble protein which involved the laborious downstream processes.

In contrast, Khurana et al. (2010) showed that bacteria are capable to express the properly folded functional globular HA1 domain of H1N1 vaccines, which has protected ferret against H1N1 pandemic influenza virus. This suggested that bacteria host is capable to express the recombinant protein. However, toxic components from bacteria may contaminate the final protein product and this becomes a major issue particularly in the production of recombinant protein intended for therapeutic use.

Eukaryotic organism was then established in order to overcome the problem of the prokaryotic system since it shares many molecular, genetic and biochemical features. Yeast is a lower eukaryote organism capable of secreting glycosylate protein product as compared to bacteria. It has rapid propagation ability, is inexpensive to grow, and capable to perform post-translational modification. This organism has been widely used to express different heterologous protein for almost 25 years (Hitzeman et al., 1981).

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This system is well characterized to express a number of proteins including pharmaceutical products and diagnostics purposes (Glick et al., 2010). This system has successfully produced hepatitis B vaccines (DiMiceli et al., 2006) and Hantavirus vaccines (Antoniukas et al., 2006). However, this organism carried out different protein folding and post-translational modification due to hyper-mannosylation which is not usually appropriate for the production of human therapeutic and diagnostic purposes.

Another eukaryotic organism as a host for protein production is mammalian cells. Mammalian cells are capable to perform proper protein folding, post-translational modification and assembly of the protein products which is important for complete biological activity (Khan, 2013). Besides, this system also promotes signal synthesis, process, and secretion of the glycosylate protein products. However, cost of production of the products using these cell systems is high because of the slow growth and expensive nutrient requirement. The choice of expression system invariability influences the character, quantity and cost of a final product (Figure 2.2).

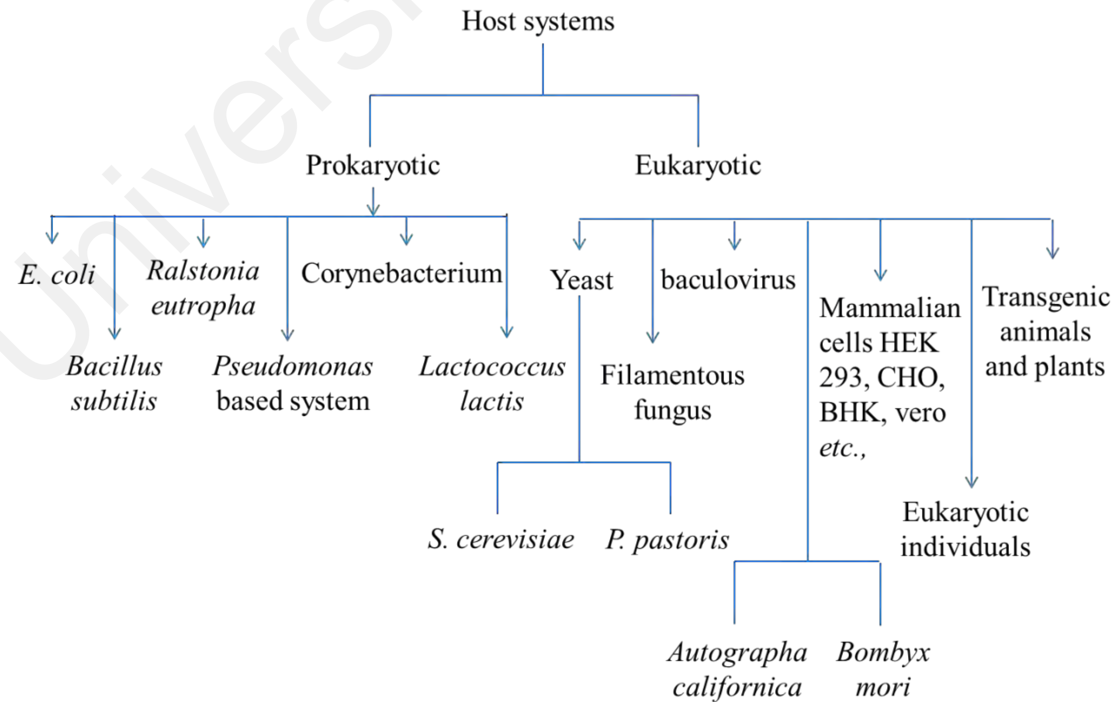

Figure 2.2: Different host systems available for the production of recombinant proteins. Adopted from Gomes et al. (2016).

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2.3 Plant molecular farming

In the last few years, plants have become an increasingly importance platform for recombinant protein production due to the unique characteristics provided by plants compared to microbial host and animal cell culture. There is a resurgence in interest to use plants as host recently as the level of foreign protein accumulation is high in selected plant tissue. This may lead to the potential of low-cost of biomass production, free of pathogen contamination, ease of combining gene by crossing and rapid scale-up (Egelkrout et al., 2011).

Besides, ethical problems associated with transgenic animals can be avoided when using plant as host for recombinant protein production. As reported by United States Food and Drug Administration (FDA), maize is generally recognized as safe for ingestion and thus can be used as a dormant carrier, appropriate for drug delivery (Ubalua, 2009).

As compared to other conventional expression systems, such as mammalian cell cultures and bacteria, plant promotes several advantages in terms of cost efficiency, product safety, and scalability of recombinant protein products. Several biopharmaceutical products have been successfully produced in plants, such as antibodies, vaccines, protein allergens, enzymes and enzyme inhibitors, coagulation factors, cytokines and hormones (Hiatt et al., 1989; Mason et al., 1992; Ruggiero et al., 2000; Kirk & Webb, 2005; Twyman et al., 2005; Floss et al., 2007; Lienard et al., 2007; Obeme et al., 2011; Ma et al., 2015).

Furthermore, plants have the ability to perform post-translational modifications that are required for producing functional mammalian proteins (Stoger et al., 2002; Breyer et al., 2009). In the industry of antibody productions, as initially reported by Hiatt et al. (1989), transgenic tobacco was capable to perform dimeric assembly of functional

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antibodies and positive expression of heavy and light-chains with biological activity in the plant system itself. The multimeric assembly was shown to perform through cross pollination between two separate plants carrying heavy and light-chains producing F_1 with full-length IgG (Hiatt et al., 1989; Hein et al., 1991; Ma et al., 1994) or through co-transformation on a single expression cassette (During et al., 1990) and two different genes (Chen & Wu, 2005).

Production of numerous complex functional mammalian proteins, such as human serum proteins, growth regulators, antibodies, and vaccines in plants have been reported (Obeme et al., 2011). For example, several therapeutic recombinant proteins against ebola virus (Maxmen, 2012; Merlin et al., 2014; Sack et al., 2015), high-immunodeficiency virus (HIV) (Niemer et al., 2014), West-Nile virus (Lai et al., 2014; Chen, 2015), H1N1 virus and its derivatives (Shoji et al., 2013; Cummings et al., 2014; Takeyama et al., 2015) and dengue virus (Kim et al., 2015; Amaro et al., 2015; Dent et al., 2016) have been published.

The field of plant-based biologics has made significant progress by addressing technical and regulatory issues that have positioned plants as a commercially attractive approach for developing and manufacturing vaccines and antibodies as shown in Table 2.1.

In addition, the cost of downstream processes of recombinant protein produced by edible part of plants can be eliminated in oral vaccines production through direct consumption (Thanavala et al., 2005). Examples of plants used to produce are tomato and banana (Gaur et al., 2012). Moreover, plant oral vaccines can also induce mucosal and humoral immune response in intestine (Holmgren et al., 2005).

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Table 2.1: Plant based vaccines and antibodies in clinical development or on market.

Product	Plant host	Expression system	Indication	Route of administration	Product development stage
Vaccines					
E. coli LT-B	Potato Maize	Transgenic Transgenic	Diarrhea	Oral	Phase 1 Phase 1
Norwalk virus CP	Potato	Transgenic	Diarrhea	Oral	Phase 1
HBsAg	Potato Lettuce	Transgenic Transgenic	Hepatitis B	Oral	Phase 1 Phase 1
Rabies virus GP/NP	Spinach	Transient (viral vector)	Rabies	Oral	Phase 1
Newcastle disease virus HN	Tobacco cell suspension	Transgenic	Newcastle disease (poultry)	Subcutaneous	USDA approved (not marketed)
Personalized anti-idiotype scFVs	*Nicotiana benthamiana*	Transient (viral vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase 1
Personalized anti-idiotype dcFVs	*Nicotiana benthamiana*	Transient (magnICON vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase 1 (ongoing)
H5N1 influenza HA VLP	*Nicotiana benthamiana*	Transient (agrobacterial binary vector)	H5N1 "avian" influenza	Intramuscular	Phase 1 (ongoing) Phase 2 (Health Canada approved and enrolling volunteers)
H5N1 influenza HAI1	*Nicotiana benthamiana*	Transient (launch vector)	H5N1 "avian" influenza	Intramuscular	Phase 1 (initiation expected in fall 2010)
H1N1 influenza HAC1	*Nicotiana benthamiana*	Transient (launch vector)	H1N1 "swine" influenza	Intramuscular	Phase 1 (ongoing)
Antibodies					
Anti-CD20	Duckweed	Transgenic	Non-Hodgkin's lymphoma, rheumatoid arthritis	Intravenous	Pre-clinical
Anti-Streptococcus surface antigen I/II	Tobacco	Transgenic	Dental caries	Topical	Phase 2; EU approved
Anti-αCCR5	*Nicotiana benthamiana*	Transient (magnICON vector)	HIV	Topical	Pre-clinical
Anti-HIV gp120	Maize *Nicotiana benthamiana*	Transgenic Transient (agrobacterial binary vector)	HIV	Topical	Pre-clinical
Anti-HBsAg scFV	Tobacco	Transgenic	Hepatitis B vaccine purification	Not applicable	On market (in Cuba)

Adopted from Yusibov et al. (2011).

2.4 Therapeutic proteins

There are two major categories of biotechnology-derived drugs, which are antibodies and vaccines. Each biopharmaceutical drug is functional at different times and targets. These recombinant proteins have already been synthesized in living organisms, such as bacteria, yeast, mammalian cell cultures and plants. However, the production of therapeutic proteins through plant host systems offers a lower production cost and lower risk of contamination compared to mammalian cells (Yao et al., 2015).

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2.4.1 Antibodies

Antibodies comprise of the principal effectors of the adaptive immune system. The ability of antibodies to bind an antigen with high degree of affinity and specificity has led to the ubiquitous use in a variety of scientific and medical disciplines. There are three forms of antibodies such as single-chain variable fragment (scFv) and monovalent antigen binding fragments (Fab) and single-domain antibodies VHH with several different classes, such as IgG, IgA, IgE, IgM, and IgY/ IgD depending on species.

Monoclonal antibody were first studied by Cesar Milstein and Georges Khőhler in 1975 from B cell hybridomas of mice and this discovery subsequently won the Nobel Prize in Medicine in 1984 (Milstein, 1985; Khőhler, 1985). The advantages brought by monoclonal antibody (mAb), such as high in monospecificity, homogeneity, and consistency, have been used as the antibody-based therapy agent for many diseases (Lipman et al., 2005). The monospecificity provided by mAb is useful in evaluating changes in molecular conformation, protein-protein interactions, and phosphorylation as well as in identifying single members of protein families. However, the production of mAb is time consuming, costly and laborious as compared to polyclonal antibodies (pAb).

2.4.1.1 Single-chain variable fragment antibodies

Generally, immunoglobulin (IgG) molecules consist of two identical heavy and light chains that are joined together by di-sulphide and non-covalent bonds. For single-chain variable fragment antibodies, it is made of a non-covalent heterodimer comprised of one heavy and light chain joined together with peptide linker. Therefore, the size of scFv is smaller compared to immunoglobulin even though the specificity is retained (Ahmad et al., 2012) (Figure 2.3).

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As reported by Baneyx in 1999, scFv is already successfully been expressed in *Escherichia coli* with good folding properties due to its small size, cheap and fast technique which can be used in multi-plexed cloning, expression, and purification. In addition, tumor-specific single-chain variable fragment antibody (T84.66) has been transiently expressed in tobacco leaves *Nicotiana tabacum* cv. Petite Harvana SR1 (Vaquero et al., 1999) which already undergo clinical trials. In 2010, a group of researchers from State University of New York used this antibody to detect the colorectal cancer xenograft in mouse model (Urva & Balthasar, 2010).

The small size of the scFv molecule is an attractive candidate due to ease in penetrating the tissue or tumor, reduced immunogenicity, and also ease of cloning it in bacteria for genetic engineering (Hagemeyer et al., 2009; Ahmad et al., 2012).

For instance, scFv with intact paratope has been successfully expressed in both prokaryotes and eukaryotes, such as bacteria (Yim et al., 2014), mammalian cells (Jäger et al., 2012), yeasts (Ferrara et al., 2012), and insects (Kurasawa et al., 2012). Recently, a recombinant 62-71-3 monoclonal antibody has been developed in plant against rabies prophylaxis in human (Both et al., 2013).

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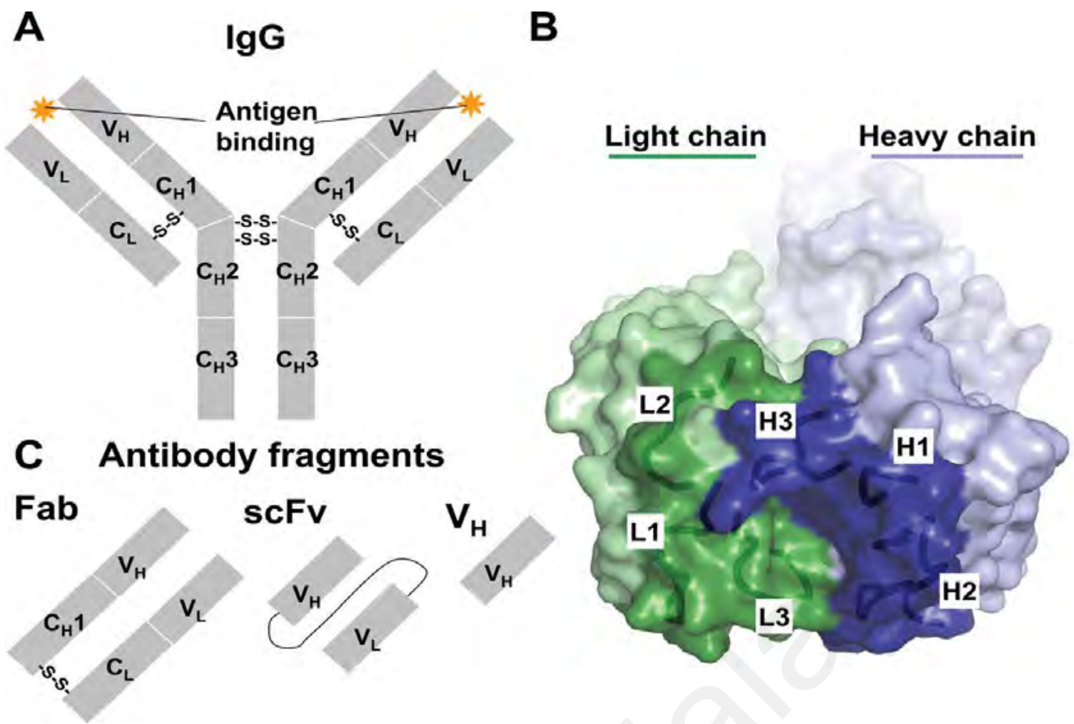

Figure 2.3: Domain architecture of antibodies and antibody fragments. A) Conventional antibody class IgG; B) Surface representation of a Fab with heavy chain (blue) and light chain (green); and C) Antibody fragments: Fab, single-chain variable fragment, and variable heavy chain domain.
Source from Ganesan et al. (2010).

2.5 Challenges and strategies of plant as protein expression system

Although plant expression systems have many advantages over the other systems, they also have significant challenges in terms of quality and yield of recombinant protein products. Naturally, endogenous protein is high in heterogeneity, often giving a complex mixture of variants in recombinant protein products (Faye et al., 2005). In relation to that, optimizations of transgene transcription and translation in plants have been carried out, including elucidation and modulation characteristics of the plant cell machinery (Gomord & Faye, 2004; Faye et al., 2005). Therefore, understanding various post-translational steps of the whole protein synthesis and assembly of nascent protein backbone is essential to ensure the functionality of the recombinant proteins.

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Another challenge in plant system is the presence of proteolytic mechanisms or defense systems for their vital metabolic functions against foreign particles. The interference of this defense systems contribute to the elimination of misfolded proteins and the selective recycling of amino acids from short-lived proteins. As a consequence, this may lead to lower production of biologically active proteins (Doran, 2006; Goulet & Michaud, 2006).

There are many strategies have been done by researchers in order to increase the yield and quality of the recombinant protein produced from plants (Table 2.2). Common approach to overcome the challenges of unwanted proteolysis *in planta* involves the introduction of element(s); such as, targeting the transgene to the specific sub-cellular compartments such as endoplasmic reticulum (ER), chloroplast, or apoplast and co-expression of recombinant proteins with proteinase inhibitors.

Table 2.2: Strategies used for enhancing the expression of transgenes in plants.

1. Transformation and integration:		
Attachment of SAR to DNA	Increased expression of GUS gene in tobacco cells up to 140-fold	Allen et al., 1996
2. Transcription		
Addition of *Myb* and leucine zipper	Increased expression	Boulikas, 1994 (Hurst, 1994)
Hybrid promoter (Mac), of CaMV 35S and mannopine synthetase	*GUS* gene expression was increased 3–5 fold in leaves and 10–15 fold in hypocotyls and roots in tobacco and tomato plant, resp.	Comai et al., 1990
Addition of Intron 2 and 6 of maize alcohol dehydrogenase-1	Increase the expression of CAT (Chloramphenicol acetyl transferase) gene by 12-fold and 20-fold respectively in maize protoplasts	Mascarenhas et al., 1990
3. Translation		
Changing initiation codon context GGUUUAUGU to CCUCCAUGU	Increased expression	Gallie, 1993
Codon optimization in tobacco and tomato plants	Increase expression of cryIA (b) (*Bacillus thuringiensis*) up to 100-fold	Perlak et al., 1991
4. Final yield or protein accumulation		
Targeting to sub-cellular compartments e.g. apoplast	10^4-fold higher Expression of human epidermal growth factor (hEGF) was obtained in tobacco	Wirth et al., 2004

Adopted from Desai et al. (2010).

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2.5.1 Co-expression with proteinase inhibitors

As an alternative approach to modulate the unwanted proteolytic activities on the targeted recombinant protein, the use of recombinant proteinase inhibitors could prove their functionality by the reactivity against specific endogenous proteases. As previously reported, proteinase inhibitors might protect the recombinant protein levels in leaves with negligibly affected the plant growth and development (Faye et al., 2005). Some examples of proteinase inhibitors and substrates that have been used are shown in Table 2.3.

Michaud et al. (2005) reported that tomato cathepsin D inhibitor (CDI) showed an increase in total soluble protein (TSP) level (20-35 %) in leaves of transgenic potato lines accumulating this inhibitor in cytosolic compartments. Besides, Bowman-Birk trypsin inhibitor from soybean also has successfully stabilized recombinant antibodies secreted by roots of transgenic tobacco plants by co-secreting the inhibitor in the medium (Komarnytsky et al., 2006). As reported by Van der Vyver et al. (2003), the co-expression of Oryzacystatin I (cysteine proteinase inhibitor) produced higher total soluble protein in tobacco leaf tissue than expected.

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Table 2.3: Proteinase inhibitors used for *in vitro* assays.

Substrates/inhibitors	Abbreviations
Synthetic fluorogenic substrates	
Ala-Ala-Phe-methylcoumarin	AAF-MCA
Succinyl-Ala-Ala-Pro-Phe-methylcoumarin	*Suc*-AAPF-MCA
Succinyl-Leu-Leu-Val-Tyr-methylcoumarin	*Suc*-LLVY-MCA
Benzoyl-Arg-methylcoumarin	*Bz*-R-MCA
Arg-methylcoumarin	R-MCA
MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2	MOCAc
Protein substrates	
Ribulose 1,5-bisphosphate carboxylase/oxygenase	Rubisco
Neomycin phosphotransferase II	NPTII
Inhibitors	
Cathepsin D inhibitor (from tomato)	CDI
Oryzacystatin I (from rice)	OCI
Corn cystatin II (from corn)	CCII
Bowman-Birk trypsin inhibitor (from soybean)	BBTI
Bovine α1-antitrypsin	α1-AT
Bovine pancreatic trypsin inhibitor (aprotinin)	Aprotinin (Apro)

Source from Rivard et al. (2006).

2.5.2 Targeting to specific ER-organelle in plant tissue (KDEL)

Recombinant protein targeting to specific cell compartments has been recognized as one of the key factors in determining the quality, yield, and stability of protein products (Wandelt et al., 1992; Schouten et al., 1996; Gomord et al., 1997). Likewise, organelles carry out their specific function in regulating specific metabolic machinery and processes in cells (Table 2.4). For example, endoplasmic reticulum (ER) in cytosol plays a large role in the synthesis of large, complex proteins and amino acids. Rough ER with ribosomes on the surface functions to assemble amino acids to form specific proteins which are essential to carry out cellular activities.

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Targeting recombinant protein to ER has been proposed to improve the stability and yield of several proteins (Ma et al., 2003; Vitale & Pedrazzini, 2005). At the biochemical level, the low abundance of proteolytic enzymes and the presence of molecular chaperons in the ER, together with an oxidizing status favoring disulfide bond formation, make this organelle a suitable destination for several proteins susceptible to rapid turnover or showing a complex folding pathway (Nuttall et al., 2002; Faye et al., 2005). Several proteins of medical and industrial interests have been tested showing similar tendencies of stable protein production, such as human interleukin-4 (Ma et al., 2005), SARS coronavirus S protein antigen (Pogrebnyak et al., 2005), the synthetic silk-like protein DR1B (Yang et al., 2005), and recombinant phytase from *Aspergillus niger* (Peng et al., 2006).

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Table 2.4: Impact of subcellular targeting on recombinant protein yield in transgenic plant systems.

Protein	Transformed species	Plant organ	Yield*						Reference
			Cytosol	Endoplasmic reticulum	Vacuole	Apoplasm	Plastid	Nucleus	
Antibodies									
ScFv anti-cutinase	*Nicotiana tabacum*	Leaf	0	100		1			Schouten *et al.* (1996)
ScFv anti-oxazolone	*N. tabacum*	Leaf		10–20		1			Fielder *et al.* (1997)
		Seed		5, 22		1			
ScFv anti-oxazolone	*Solanum tuberosum*	Tuber		1		1			Artsaenko *et al.* (1998)
ScFv anti-dihydroflavonol 4-reductase	*Petunia hybrida***	Petal	1	2, 30					De Jaeger *et al.* (1999)
		Leaf	1	20, 60					
BiscFv 2429	*N. tabacum*	BY-2 cells	Traces	10		1			Fischer *et al.* (1999)
FAb MAK33	*Arabidopsis thaliana*	Leaf/seed		1		1			Peeters *et al.* (2001)
scFv anti-carcinoembryonic	*N. tabacum*	Leaf		25		1			Stoger *et al.* (2002)
Ab anti-carcinoembryonic	*N. tabacum***	Leaf		2–6		1			Vaquero *et al.* (2002)
Ab 14D9 κ chain	*N. tabacum*	Leaf		8		1			Petruccelli *et al.* (2006)
Ab 14D9 γ chain				4		1			
Vaccines									
Escherichia coli heat-labile enterotoxin B	Zea mays	Seed	1	100	20 000	3300	7	21	Streatfield *et al.* (2003)
Hepatitis B surface antigen	*N. tabacum*	BY-2 cells	1	1.4		1.8			Sojikul *et al.* (2003)
Japanese cedar pollen allergens	*Oryza sativa*	Seed	0	4–6		1			Takagi *et al.* (2005)
Medical proteins									
Human epidermal growth factor	*N. tabacum*	Leaf	1			10 000			Wirth *et al.* (2004)
Human growth hormone	*N. benthamiana*†	Leaf	1			1000	10		Gils *et al.* (2005)
Agronomic/industrial proteins									
Vicilin	*N. tabacum*	Leaf		100		1			Wandelt *et al.* (1992)
	Medicago sativa	Leaf		20		1			
Equistatin	*S. tuberosum*	Leaf	1	5					Outchkourov *et al.* (2003)
Silk-like protein	*A. thaliana*	Leaf	1	13	0	5			Yang *et al.* (2005)
		Seed	1	7.8	5.4	0			

Adopted from Benchabane et al. (2008).

In addition, ER performs late post-translational modification in downstream processes such as formation of complex glycans, addition of a lipid moiety or the proteolytic removal of a propeptide sequence (Gomord & Faye, 2005; Faye et al., 2005).

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2.6 *Agrobacterium*-mediated transformation

2.6.1 *Agrobacterium* strains used for recombinant protein expression

A. tumefaciens is a soil-borne pathogen, gram-negative, rod shaped, aerobic and motile bacterium found in the rhizosphere (a region around the roots of the plants) where it normally survives on nutrients released from plant roots (Slater et al., 2008).

A. tumefaciens is a causative agent of crown-gall disease; an economically important disease of many plants dependent on the ability of to transfer bacterial genes into the plant genome (Slater et al., 2008). The concept of crown-gall formation through "tumor-inducing principle" had been proposed referring to *Agrobacterium*-mediated transformation in which stably transferred to and propagated in the plant genome (Braun, 1947).

There are many strains of *Agrobacterium* that have been used for transformation purposes, such as LBA4404, C58C1, GV3101, and EHA105. It has been reported that *Agrobacterium* strain GV1301 produced higher recombinant protein of influenza virus hemagglutinin production compared to strain C58C1 and LBA4404 through Agro-infiltration method (Shamloul et al., 2014). On the other hands, Yadav et al. (2014) reported that *Agrobacterium* strain LBA4404 showed highest GUS protein expression in *Bacopa monnieri* (L.) Pennell with 6.01 µmol 4-MU/min/mg compared two other strains tested, EHA105 and GV3101, through stable transformation method. Recently, a new *Agrobacterium* strain has been discovered by a group of researchers from The Ohio State University and has been nominated as JTND strain (Benzle et al., 2014). This strain has been isolated from the soil of soybean field that possessed enhanced transformation attributes for soybean. Based on GFP expression analysis, they found

out that this strain capable to produce 10-100 fold improvement in soybean transformation over EHA105 strain. In 2017, this project has been patented.

It may be concluded that the selection of *Agrobacterium* strains could be crucial in dictating the expression and production either transient or stable for plant transformation.

2.6.2 Importance of *Agrobacterium* in recombinant protein production

A. tumefaciens belongs to the family of *Rhizobiaceae*, has been used as a vector to develop transgenic plants of agronomic and horticulture importance (Stanton et al., 2003). Gene transfer mediated by *Agrobacterium* has introduced traits into crop plants, dependent on the combined action between bacteria and plant genotypes (Sheeba et al., 2010). Several factors need to be considered in the design and implementation of any plant transformation mediated by *Agrobacterium* sp. which are types of plant tissue, vector used to deliver the transgene into plant genome, and strain of *Agrobacterium* used (Slater et al., 2008).

Agrobacterium-mediated transformation provides an invaluable system in studying host-pathogen interaction due to its unique ability to transfer bacterial DNA into plant genome (Kumar & Rajam, 2007). *A. tumefaciens* provides advantages as a transfer system (vector) in plant transformation due to its simplicity, precision, integration of DNA sequence with defined ends. It also linked transfer of genes of interest along with transformation marker. Besides, *Agrobacterium* is commonly used to perform stable transformation due to high amount of single copy insertions (multiple cloning sites) and the ability to transfer long stretches of T-DNA (Veluthambi et al., 2003). *A. tumefaciens* becomes widely used tool in plant biotechnology and it is worth looking at the biology of crown-gall disease. The bacteria can infect the plant at the wounded site via

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chemotaxis, in response to chemical released from damaged plant cells (Slater et al., 2008). *Agrobacterium* are natural engineers that are able to transform or modify mainly dicotyledonous and monocotyledonous plants. Interestingly, this indirect transformation method may also achieve an economically feasible for the upstream production and downstream processing of the recombinant proteins (Fujiuchi et al., 2016).

In terms of recombinant protein production in plants, *Agrobacterium*-mediated transformation provides a promising approach to produce vaccine antigens and therapeutic proteins in a large-scale production. Besides stable transformation, transient protein production has become an alternative approach and well-chosen technique by researchers. This is due to the capability of this system to produce high level of recombinant protein expression and accumulation within a short period of time (Plesha et al., 2009) by introducing bacterial binary vectors or recombinant plant viral vectors into plant tissues (Yusibov et al., 2008).

Agro-infiltration is the common technique used for transient recombinant protein production. This is due to the fact of intracellular space within one-third of plant leaf, possibly replaced the air in this cavities with *Agrobacterium*-carrying transgenes suspension allowing the effective access or transfer of T-DNA into plant tissues (Grimsley et al., 1986; Vaghchhipawala et al., 2011; Gleba et al., 2014). A successful expression also was achieved by co-expressing both recombinant protein gene harbored in plant expression vector with p19 or p23 viral RNA silencing suppressor in *N. benthamiana* resulted in earlier accumulation and increases in production for about 15-25% of targeted influenza virus hemagglutinin recombinant protein (Shamloul et al., 2014). In addition, most advanced transient expression system is based on the unique vector in which combining both plant viruses and binary plasmid and delivered by agro-infiltration (Gleba et al., 2005; Musiychuk et al., 2007). There are several successful applied of this unique vector based on *Tobacco mosaic virus* (TMV) against pathogens

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such as *Bacillus anthracis* (Chichester et al., 2007), human papilloma virus (Massa et al., 2007), smallpox virus (Golovkin et al, 2007) and Ebola virus (Qiu et al., 2014).

Vacuum infiltration is one of the agro-infiltration methods that may result in similar yield and temporal expression pattern for several recombinant proteins compared to that of syringe infiltration. This suggested that these two agro-infiltration methods are mutually transferrable. Due to the large-scale production capability provided by vacuum infiltration over syringe infiltration, a fully automated vacuum system has been developed with the capability to agro-infiltrate up to 1.2 tons of plant biomass per day, allowing the production up to 75 g of mAb-based therapeutics per greenhouse lot (Rivera et al., 2012; Gleba et al., 2014; Chen et al., 2014). Besides, agro-infiltration also has been used in order to evaluate the stable transgenic plants harbored the recombinant proteins by infiltrating a complementing construct and inducible vector followed by crossing. Progeny from this cross were later screened by ethanol induction (Werner et al., 2011).

Agrobacterium-mediated transformation provides a series of complex biological-interaction system starting from the transfer of T-DNA from *A. tumefaciens* to protein biosynthesis and accumulation in leaf tissue. In addition, the dynamic environmental control during both pre and post-inoculation processes at an appropriate time also provide an important factor in order to obtain a high yield the recombinant production from plants (Fujiuchi et al., 2016). Several studies have examined the effect of light intensity, temperature, and humidity during post-inoculation process on recombinant protein content (Table 2.5).

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Table 2.5: Studies addressing post-inoculation environmental effects on *Agrobacterium*-mediated transient expression.

Plant species	Platform	Inoculation method	Recombinant protein	Subcellular localization	Focused environmental factor	Reference
Nicotiana tabacum	Whole plant	Vacuum	2G12, DsRed	Apoplast (2G12), plastids (DsRed)	Temperature	Buyel and Fischer (2012) and Buyel (2013)
N. tabacum	Detached leaf	Coculture	GUS	Cytosol	Temperature	Dillen et al. (1997)
Phaseolus acutifolius	Calli					
Nicotiana benthamiana *N. tabacum*	Whole plant	Syringe	Luciferase	Cytosol	Temperature, light intensity	Cazzonelli and Velten (2006)
P. acutifolius	Calli	Coculture	GUS	Cytosol	Temperature, light intensity	De Clercq et al. (2002)
Lactuca sativa	Leaf disk	Vacuum	GUS	Cytosol	Temperature, light intensity	Joh et al. (2005)
Allium sativum	Calli	Coculture	GUS	Cytosol	Temperature	Kondo et al. (2000)
N. benthamiana	Whole plant	Vacuum	Hemagglutinin	ER	Temperature, light intensity	Matsuda et al. (2012)
N. benthamiana	Whole plant	Syringe	Brome mosaic virus coat protein	Cytosol	Temperature, humidity	Moon et al. (2014)
Rose	Detached petal	N/A[a]	GUS	Cytosol	Temperature	Yasmin and Debener (2010)
Sunflower	Detached leaf	Vacuum	Endoglucanase, endoxylanase	Apoplast	Temperature	Jung et al. (2015)
Arabidopsis thaliana	Root segment	Coculture	GUS	Cytosol	Light intensity	Zambre et al. (2003)
P. acutifolius	Calli					
N. benthamiana	Detached leaf	Vacuum	Endoglucanase	Apoplast	Temperature, light intensity, humidity	McDonald et al. (2014)
N. tabacum	Whole plant	Coculture after spraying	GUS	Cytosol	Light intensity	Escudero and Hohn (1997)
N. benthamiana	Cultured cell	Coculture	GUS	Cytosol	Light intensity	Larsen (2011)
N. benthamiana	Whole plant	Syringe	GFP	Cytosol	Temperature, light intensity	Patil and Fauquet (2015)
N. benthamiana	Detached leaf	Vacuum	Alpha-1-antitrypsin	Apoplast	Humidity	Plesha (2008)
N. benthamiana	Detached leaf	Vacuum	Hemagglutinin	ER	Humidity	Fujiuchi et al. (2016)

[a]*Not available.*

Source from Fujiuchi et al. (2016).

2.7 Expression vectors used for *Agrobacterium*-mediated transformation

Plant expression vector plays the vital role in expressing the transgene, transformed into plant host. Through indirect transformation system, vector is required which can be replicated in *Agrobacterium* and promotes the initiation of T-DNA transfer with the help of virulent genes on chromosomes and/or other plasmid (Vaghchhipawala et al., 2011). The earliest transcriptional vector that has been used for *Agrobacterium*-mediated transformation was pCAMBIA or pBIN19. Interestingly, this vector has demonstrated the superiority of transient as well as stable expression in terms of speed and recombinant protein yield over the traditional protein expression in transgenic plants (Cañizares et al., 2005; Lico et al., 2008; Chen & Lai, 2013).

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Therefore, in this study, a binary vector, pCAMBIA 1304 was used as the backbone of the constructs. *A. tumefaciens* harboring pCAMBIA 1304 containing *mgfp*5:*gus*A fusion reporter gene and *hpt*II (hygromycin-resistance gene) as selectable marker driven by cauliflower mosaic virus 35S promoter (CaMV 35S) was used for transformation (Figure 2.4).

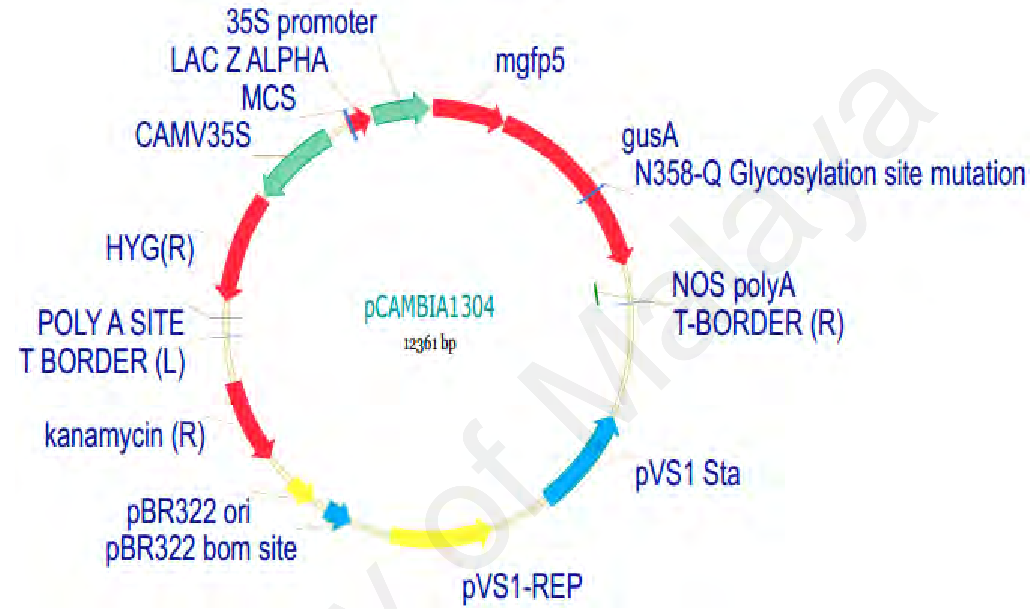

Figure 2.4: Binary vector, pCAMBIA 1304. Source from www.cambia.org

There are some features of Ti plasmid that allow transformation of T-DNA segment which contained one or more T-DNA regions, a *vir* region, an origin of replication, a region that enabling conjugative transfer, and genes for the catabolism of opines (Slater et al., 2008).

Besides the mentioned vector, there were also several designed vectors for transient and stable transformations have been constructed by researchers. As been reported by Shah et al. (2013), tobacco mosaic virus (TMV) vectors (pPZPTRBO and pJLTRBO) and pEAQ-*HT* result a strong GFP expression with or without the presence of viral vector, p19. In addition, it also has been reported that *N. benthamiana* infiltrated with GV3101 harboring pBID4-GFP transiently expressed high levels of GFP (Shamloul et

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al., 2014) which is similar to the GFP yield reported for *N. benthamiana* infiltrated with *Agrobacteria* carrying pICH-GFPSYS viral vector (up to 80 % of total soluble protein) (Marillonnet et al., 2005).

Reporter genes (*mgfp*5-*gus*A) were used to enable transformed cells to be transiently assessed (Slater et al., 2008). β-glucuronidase enzyme encoded by *gus*A gene was firstly developed in plant expression system by Jefferson (1987). This gene can be assessed through quantitative (fluorometry) and qualitative (histochemical staining) assays using 4-methylumbelliferyl-β-D-glucuronide (MUG) and 5-bromo-4-chloro-3-indolyl glucuronidase (X-gluc) substrates respectively. Besides, green fluorescence protein (GFP) encoded by *mgfp*5 was firstly found in jellyfish, *Aequorea victoria* by Osamu Shimomura in 1960s (Shimomura et al., 1962; Shimomura & Johnson, 1969). GFP also provides several advantages over GUS such as non-destructive in which no substrate required; the expression can be assessed in vivo through direct visualization without cell lysis and biochemical analysis; rapid discrimination of non-transformants, as well as capable to be exported to specific cell compartments, such as cytoplasm (Tanenbaum et al., 2014); ER (Samali et al., 2010); and mitochondria (Xu et al., 2011) through fused protein with the target peptides. As been reported by Joensuu et al. (2010), the ER-targeted GFP-hydrophobin fusion significantly enhanced the accumulation of GFP, with the concentration of the fusion protein reaching 51 % of total soluble protein, besides induced the formation of large novel protein bodies in *Nicotiana benthamiana*.

2.8 Tobacco as a plant host

Although many different crop species have been used to produce a wide range of vaccines, antibodies, biopharmaceuticals and industrial enzymes, tobacco is the established platform for green bioreactors and offers several unique advantages over

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other plant species. Tobacco is known as one of the conventional models since it amicable to genetic modification and has become the primary vehicle for proof-of-concept work in recombinant protein production for the last 20 years (Llop-Tous et al., 2011). Tobacco is a leafy plant, having high biomass yield (more than 100,000 kg per hectare) (Sheen, 1983) with high soluble protein levels compared to other model and crop species. Besides, tobacco also offers short life cycle (~3 months for flowering) and various ways of expressing protein of interests, such as transient (Leuzinger et al., 2013) and stable nuclear (Lee & Ko, 2017) or chloroplastic (Wang et al., 2015) genome-based expression.

Out of 52 *Nicotiana* varieties, *Nicotiana tabacum* cv. I 64 produced highest expression of recombinant erythropoietin and interleukin-10, in addition to producing a large amount of biomass and a relatively low quantity of alkaloids (Conley et al., 2011). *Nicotiana tabacum* and *Nicotiana benthamiana* are the most common varieties that have been used especially for recombinant protein production. Besides that, Shamloul et al. (2014) reported that *N. excelsiana* (*N. benthamiana* x *N. excelsior*) was a promising host due to ease of infiltration, high level of reporter protein production, and about two-fold higher biomass production under controlled environmental conditions.

In addition, *N. tabacum* cv. BY-2 cells suspension also provide an alternative bio-production platform for pharmaceutical proteins (Xu et al., 2007; Fu et al., 2009; Xu et al., 2010; Holland et al., 2010) due to several advantages over whole plant-system such as; rapid growth, less prone to variations due to control condition in bioreactor system, few regulatory and environmental compliance obstacles, and lacks of exogenous protein which may interfere the end-protein products (Xu et al., 2011).

Due to biosafety issue, tobacco is the most promising host as for recombinant protein productions since the expression platform is based on leaves which reducing the

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potential for gene leakage into the environment through pollen or seed dispersal from flowers. Besides that, tobacco is a non-food and non-feed crop which has minimized the regulatory barriers by eliminating the risk of plant-made protein entering the food chain (Rymerson et al., 2002; Twyman et al., 2003). Inedible characteristic of this tobacco plants due to the presence of nicotine also restrict from the formation of oral vaccines (Ubalua, 2009). However, cultivar 81V9 is one of the tobacco varieties with low-nicotine and alkaloid which capable to overcome the edible issue after maize and tomato (Menassa et al., 2001).

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CHAPTER 3: MATERIALS AND METHODS

3.1 Maintenance of *in vitro Nicotiana tabacum* cv. SR1

3.1.1 Preparation of culture medium

Murashige and Skoog (MS) basal medium (MSO) (Murashige & Skoog, 1962) has been used to maintain *in vitro* tobacco (*Nicotiana tabacum* cv. SR1) and seed germination, whereas MS basal media containing different plant growth regulators were used for shoot and root regeneration (TSM and TRM respectively) (Appendix A).

To prepare the MSO medium, MS basal salt and an appropriate volume of macronutrient, micronutrient, vitamin, and iron solutions from a prepared stock solution (Appendix A) were mixed well. For TSM and TRM media, 1 mg/L 6-benzylaminopurine (BAP) and/ or 0.1 mg/L 1-naphthaleneacetic acid (NAA) (Duchefa, Germany) were added to MSO (Appendix A).

All media contained 30 g/L of sucrose and 0.1 g/L myo-inositol (Sigma-Aldrich, USA). pH was adjusted to 5.7 ± 0.5 by adding 1 M of HCl and/or 1 M NaOH. Gelrite® (2.5 g/L; Duchefa, Germany) was added to the medium prior to autoclaving at 121 °C for 20 min. Approximately 30 mL and 25 mL of autoclaved media were poured into a culture jar and a 90 mm diameter Petri dish, respectively.

3.1.2 Germination of tobacco seeds

Seeds of *N. tabacum* cv. SR1 were previously donated by Academia Sinica Beijing (Lin, 2011). Seeds of tobacco were placed in a sterile universal container (~ 100 seeds per replicate) and sterilized with 70 % (v/v) technical grade ethanol. The seeds were

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soaked for few seconds by swirling evenly and rinsed with sterile dH_2O. The seeds were then soaked in 25 % (v/v) Clorox® for about 10-20 min on an incubator shaker, ~ 150 rpm followed by at least six times rinsing with sterile dH_2O to remove excessive ethanol and Clorox®. The imbibition was carried out by one time soaking the seeds in sterile dH_2O for at least 10 min. The imbibed seeds were then transferred onto MSO media by using sterilized forceps.

The culture plates were sealed with parafilm and incubated at 25 ± 3 ℃ under 16 h light and 8 h dark with a light intensity of 20.3 µmol m^{-2} s^{-1} for about 2 weeks before being transferred to new fresh media. Culture plates were assessed for contamination at 2 day interval.

T_1 seeds were germinated on MSO media-containing 20 mg/L hygromycin to select the transformants for subsequent experiments and/or transferred to TRM selection media for *in vitro* rooting. The percentage of T_1 seeds germination was assessed after 7 days of germination.

3.1.3 Maintenance of tobacco plantlets

Meristematic tissue of approximately 12-13 week-old of germinated tobacco plantlets (at least two internodes per plantlet) were cut and transferred to new fresh MSO-media (Figure 3.1). The tobacco plantlets were maintained on MSO media under 16 h light and 8 h dark for about 4 weeks.

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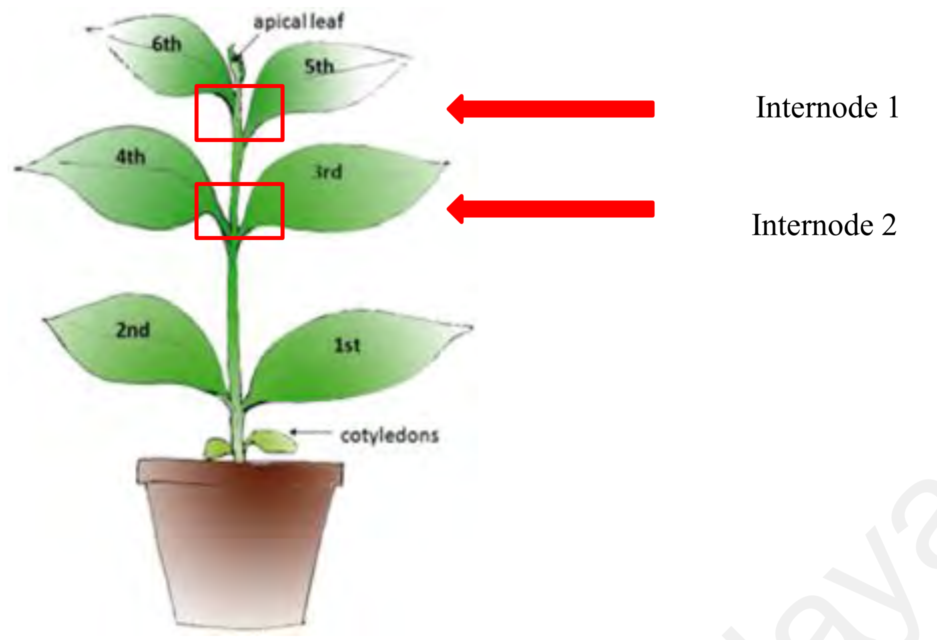

Figure 3.1: Internode parts of tobacco plants used for sub-culturing.

3.2 Endogenous test of tobacco leaf disc towards hygromycin

Endogenous test of tobacco leaf disc towards hygromycin was investigated. About 1 month-old tobacco leaves were cut into small pieces of ~ 1 cm^2 (9 explants per replicate) and cultured on TSM media containing different concentrations of hygromycin (0, 10, 20, 30, 40, and 50 mg/L). All explants were incubated at room temperature under 16 h light and 8 h dark.

The percentage of regenerated shoots from each explant was assessed after 4 weeks of culture. This experiment was conducted in triplicate.

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3.3 *Agrobacterium tumefaciens* strain LBA4404

3.3.1 Preparation of YEB and YEA media

YEB (Yeast Extract Broth) medium (1 L) was prepared by adding nutrient broth, yeast extract, sucrose and $MgSO_4$ with appropriate amounts (Appendix B) and topped up with dH_2O. pH was adjusted to 7.0 ± 0.5 by adding 1 M NaOH and/or 1 M HCl and autoclaved at 121 °C for 20 min. Autoclaved media was stored at 4 °C until used. In order to prepare YEA (Yeast Extract Agar), 1.5 % (w/v) of Oxoid™ agar technical (Thermo Fisher Scientific, USA) was added prior to autoclaving. The prepared YEB and YEA media were then aliquoted in needed volume and added with 100 mg/L kanamycin and 50 mg/L rifampicin (Duchefa, Germany) (Appendix B).

3.3.2 Preparation of *A. tumefaciens* presence with different plasmid constructs

A. tumefaciens harboring different plasmid constructs (Figure 3.2) was obtained from a previous researcher who previously conducted an experiment using different clone of anti-*Toxoplasma* recombinant protein, pToxo130 (Go, 2013). The bacterial stock was streaked on YEA medium containing 100 mg/L kanamycin and 50 mg/L rifampicin. The culture plates were incubated at 28 ± 1 °C for 2 days in darkness. After two days of incubation, the *Agrobacterium* colonies were isolated and transferred to grid-plate with new fresh media (library plate) following incubation at 28 ± 1 °C for overnight in darkness. Colonies from each construct were picked and inoculated into 10 mL of YEB medium containing same antibiotics at same concentration as in solid medium and incubated at 28 ± 1 °C for overnight and shaken at 120 rpm in darkness. This overnight culture was then verified for its insert as described in Sections 3.3.2.1 and 3.3.2.2.

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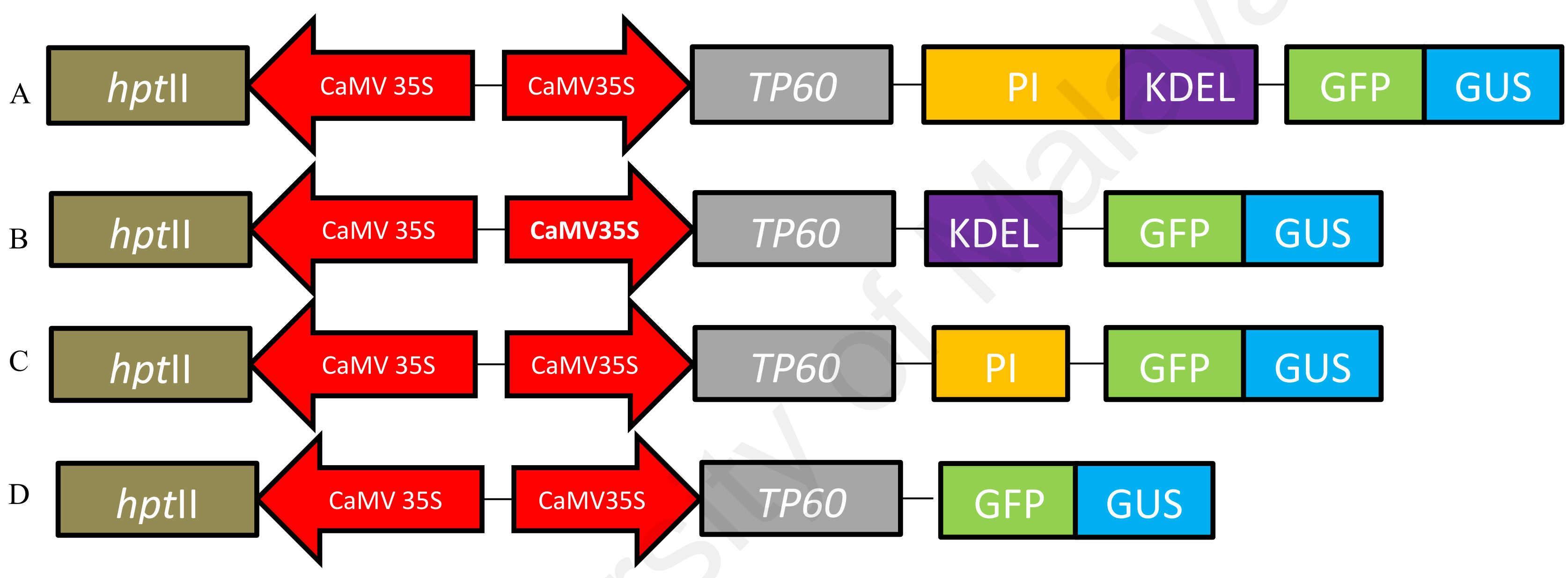

Figure 3.2: Schematic diagram of constructed plasmids used in this study. A) pTP60BBIKDEL or pTP60OCPIKDEL; B) pTP60KDEL; C) pTP60BBI; and D) pTP60. PI: proteinase inhibitor (BBI); KDEL: gene targeting to ER; *TP60*: anti-*Toxoplasma* gene fused with E-epitope tag (GAPVPYPDPLEPR); GFP: Green fluorescent protein; GUS: β-glucuronidase; *hpt*II: hygromycin phosphotransferase. These constructs were driven by CaMV 35S promoter.

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3.3.2.1 Gene sequencing and BLAST analysis

To ensure the correct sequence and reading frame between all inserted fragments which consists of anti-*Toxoplasma* recombinant protein gene (*TP60*), proteinase inhibitor gene and/or ER-retention sequence signal, the plasmids obtained from previous researcher were extracted, amplified and sequenced.

The chromatograms of the sequencing results were analyzed using FinchTV Software (Geospiza, Inc., UK) (Mishra et al., 2010). The sequences were later analyzed using ClustalW2 (Multiple Sequence Alignment) software. The analyzed sequences were then translated into amino acid sequences using http://web.expasy.org/translate/ online software (Gasteiger et al., 2003) followed by protein BLAST (BlastP) using National Center for Biotechnology Information database (Johnson et al., 2008) https://www.ncbi.nlm.nih.gov/. Anti-*Toxoplasma* (scFv) recombinant antibody encoded by *TP60* gene was tagged with E-epitope tag.

3.3.2.2 Plasmid extraction

Plasmid extraction was carried out using QIAPrep Spin Miniprep Kit (Qiagen, USA) according to manufacturer's manual. Five mL of overnight culture bacteria (from Section 3.3.2) was centrifuged and the pellet was re-suspended in 250 µL buffer P1. Another 250 µL of buffer P2 was then added and mixed by inversion of the tubes 4-6 times. About 350 µL of buffer N3 was added to the mixture, mixed and centrifuged at 13,000 rpm for 10 min at RT. The supernatant was carefully pipetted and transferred into QIAprep Spin Column and centrifuged for 1 min. The flow-through was discarded. Buffer PB (500 µL) was added and centrifuged at 13,000 rpm for 1 min to remove trace amount of nuclease activity from the *Agrobacterium.* The QIAprep Spin Column was then added with 750 µL buffer PE followed by centrifugation at 13,000 rpm for 1 min.

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To remove residual wash buffer or ethanol, an additional centrifugation was performed for 1 min at 13,000 rpm. Finally, the plasmid was eluted with 30 µL buffer EB and incubated for at least 20 min following centrifugation at 13,000 rpm for 1 min. The eluate was stored at –20 °C for subsequent use.

3.3.2.3 PCR verification of *Agrobacterium* colonies

At the beginning, the PCR reaction mixtures were prepared as described in Table 3.1. The thermal cycling condition is stated in Table 3.2. PCR amplification was conducted using PCR Thermocycler Biometra Tpersonal (Biometra, Germany).

Table 3.1: Components involved in PCR mixtures used for screening (iNtRON Biotechnology, South Korea).

No.	Components	1X
1.	10X Reaction Buffer with $MgCl_2$	1.0 µL
2.	10 mM dNTP mix	1.0 µL
3.	10 µM Forward Primer	0.5 µL
4.	10 µM Reverse Primer	0.5 µL
5.	i-Taq DNA Polymerase	0.1 µL
6.	DNA template (20-100 ng)	1.0 µL
7.	Sterile dH_2O	5.9 µL
Total volume per reaction		**10.0 µL**

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Table 3.2: Thermal cycling conditions using i-Taq^{TM} DNA polymerase (iNtRON Biotechnology, South Korea).

Condition	Temperature, °C	Time	Number of cycle
Initial denaturation	94	2 min	1
Final denaturation	94	10-20 s	30
Annealing	Depend on primer sets	10-20 s	
Initial extension	72	1 min	
Final extension	72	5 min	1
Final hold	4	∞	1

The oligonucleotide primers used in PCR amplification for transformed bacterial colonies were lists in Table 3.3. The primers were designed by using Primer3 software.

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Table 3.3: List of primers that were used for PCR screening of *Agrobacterium* transformants.

Primer set	Sequence	Length, bp	Annealing temperature, ℃	PCR product size
F-35S R-35S	5’-TCCTACAAATGCCATCATTG -3’ 5’-GAAGGATAGTGGGATTGTGC-3’	20 20	52	400 bp
F-1304sk R_1-1304sk	5’-GAGAGAACACGGGGGACTC-3’ 5’-GTGCCCATTAACATCACCATC-3’	19 21	58	pTP60 (914 bp) pTP60KDEL (935 bp) pTP60BBI (1520 bp) pTP60BBIKDEL (1541 bp) pTP60OCPIKDEL (1578 bp)
F- GFP R-GFP	5’- TCATTTGGAGAGAACACGGGGGAC-3’ 5’-TGGGGTTTCTACAGGACGTAAACT-3’	24 24	50.8	759 bp

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PCR screening was carried out to confirm the presence of insert harbored within T-DNA region of binary vector, pCAMBIA 1304. The success and integrity of PCR amplification were analyzed by agarose gel electrophoresis described in Section 3.3.2.3. For sequencing purposes, the PCR product that obtained after amplification was purified using QIAquick Gel Extraction Kit (Qiagen, USA) according to manufacturer's protocol. 3 volumes of buffer QG were added to 1 volume of excised gel. The mixture was incubated at 50 °C for 10 min to ensure the complete dissolved of the gel. Vortex was performed for every 2-3 min interval during incubation. Ratio 1: 1 isopropanol to mixture of gel was added following transfer of mixture into QIAquick spin column and centrifugation at 13,000 rpm for 1 min to ensure the binding of DNA on the membrane of column. The flow-though was discarded. Due to the purpose of sequencing, another 500 µL of buffer QG was added into the spin column and centrifuged at 13,000 rpm for 1 min. The flow-though was discarded and 750 µL of the buffer PE were added to the spin column. Centrifugation for 1 min at high speed was carried out. The flow-though was discarded followed by additional centrifugation for 1 min at highest speed to remove residual wash buffer. After that, the spin column was transferred into a new 1.5 mL microcentrifuge tube. A total of 30 µL of buffer EB was added and incubated 20 min following centrifugation at highest speed for 1 min. The eluate was stored at -20 °C.

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3.3.2.4 Agarose gel electrophoresis

Different concentrations of agarose gel were prepared for different types and sizes of nucleic acid samples as mentioned in Table 3.4.

Table 3.4: Concentrations of agarose gel used for different sizes and types of nucleic acid.

Types of nucleic acid	Product size, bp	Concentration of agarose gel, % (w/v)
DNA	<500	2.0
DNA	500-700	1.5
DNA/ RNA	700-1500	1.0
Genomic DNA/ Plasmid	>10,000	0.8

The desired amount of agarose powder was weighted and dissolved in 1X TBE buffer (Appendix C). The mixture was heated for about 2 min using microwave. The prepared solution need to be cooled before adding with 1 µL of ethidium bromide (EtBr). EtBr was added in agarose gel solution in order to ensure the intercalation of DNA with EtBr while running though the gel for viewing purposes. PCR product (5 µL) or nucleic acid samples were pipetted and mixed with Fermentas 6X loading dye (Axon Scientific, Malaysia) in the ratio of 1:5. The samples were then loaded into wells and electrophoresed at 120 V for 25 min. It was then viewed and analyzed using Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA).

3.3.2.5 Bacterial stock storage (glycerol stock)

The confirmed *Agrobacterium* was kept stock by mixing 600 µL overnight cultures with 400 µL sterilized glycerol under sterile conditions into 1.5 mL microcentrifuge tube and stored at –80 °C until use.

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3.4 *Agrobacterium*-mediated transformation into tobacco leaf disc

Approximately 3 to 4 week-old tobacco plantlets were used for transformation. Tobacco leaves were cut into ~ 1 cm^2 (10 explants per plate) and pre-cultured on TSM media under 16 h light and 8 h dark at RT for 2 days. An *Agrobacterium* with different plasmid constructs (pTP60, pTP60KDEL, pTP60BBI, pTP60BBIKDEL, pTP60OCPIKDEL) (as stated in Figure 3.2) and pCAMBIA 1304 (backbone construct) were inoculated separately in 10 mL YEB medium containing 100 mg/L kanamycin and 50 mg/L rifampicin. The inoculant was cultured for overnight at 28 ± 1 °C and shaken at 120 rpm, in darkness. Prior to transformation, the optical density (OD_{600}) of *Agrobacterium* overnight cultures was measured using Eppendorf BioPhotometer (Eppendorf, USA). The bacterial culture reached OD_{600} 0.8-1.0 was centrifuged at 5,000 rpm for 10 min, 4 °C. The pellet was re-suspended in 10 mL of co-culture media (Appendix A). The pre-cultured explants were infected with *Agrobacterium* cultures and incubated for 10 min with gentle shaking in darkness. After that, the inoculated explants were blotted on sterilized C-fold tissue towel (Scott, USA) and air dried before transferred to TSM media for co-cultivation for 3 days in darkness at RT. Each construct was replicated 3 times with 3 different biological and technical replicates.

3.4.1 Selection of transformants

After co-cultivation, the explants were rinsed with sterile dH_2O containing 250 mg/L cefotaxime for 5 times, each time 5 min, to ensure the cleanliness of explants from *Agrobacterium* residues. The explants were blotted on sterilized C-fold tissue towel and air dried in a laminar air flow. The dried explants were then transferred to TSM selection media containing 20 mg/L hygromycin and 250 mg/L cefotaxime and kept in 16 h light and 8 h dark photoperiod. The TSM selection media was changed at one

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week-interval until the formation of shoots was observed. The shoots regeneration rate towards different constructed plasmids was assessed after 7th weeks of transformation. The well-developed shoots were excised separately from the explants and transferred to MSO selection media. GUS histochemical staining and GFP visualization were performed on 10th week old putative transformed shoots. Hygromycin-resistant plantlets were transferred to TRM selection media for in vitro rooting purposes.

3.5 Transient assays of putative transformed shoots

3.5.1 GUS assessments

3.5.1.1 GUS histochemical staining

The putative transformed shoots (~10 week-old) survived on TSM selection media were isolated and stained with histochemical reagents (Appendix D) which contained 0.1 M sodium phosphate, 10.0 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.0 mM X-glucuronide (Duchefa, Germany) and 0.1 % (v/v) Triton X-100 (Sigma-Aldrich, USA). Samples were incubated in darkness at 37 °C for 3 days. After 3 days of culture, the stained putative transformants were washed with 70 % (v/v) ethanol and fixed in formalin-acetic acid-alcohol (FAA) fixative tissue solution (Appendix D). All samples were then viewed under a Stereomicroscope (Olympus, Japan) and adapted digital camera (Nikon, Japan) to examine the bluish-colour of GUS spots.

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3.5.1.2 GUS fluorometry assay

Approximately 100 mg of putative transformed shoots were ground into fine powder in the presence of liquid nitrogen and transferred into 2 mL microcentrifuge tubes. Two hundred μL of GUS extraction buffer (GEB) was added and kept on ice (Appendix D). The mixture was centrifuged at 12,000 rpm for 5 min, 4 °C to separate debris and supernatant. Ten μL of the supernatant was pipetted out for Bradford's quantification (Section 3.8.3.1). The remaining 200 μL was transferred to a new 1.5 mL microcentrifuge tube (100 μL per tube) used as replicates. Another 700 μL of GEB was added into each of the tubes. The mixture was incubated at 37 °C for 5 min. Then, 200 μL of GUS assay buffer (GAB) was added followed by incubation at 37 °C for 90 min (optimized time point) in darkness. After the incubation, 100 μL of the mixture was transferred to 900 μL of 0.2 M carbonate stop buffer (CSB) to stop the enzyme-substrate reaction. One mL of the sample was transferred into a clean glass cuvette and assayed for fluorescent signal using DyNA Quant 200 Fluorometer (Hoefer, Inc., San Francisco, CA). The quantification of GUS activity was taken according to 4-methyumbelliferone (4-MU) standard curve. The reading was measured at excitation and emission wavelength of 365/455 nm and calculated using the following formula:

$$\text{GUS activity (pmole min}^{-1}\text{ mg}^{-1}) = \frac{\text{pmole of cleavage product, 4MU, released}}{\text{(Unit of time) x (Unit of protein)}}$$

3.5.2 GFP visualization

The expanded leaves of approximately 10th week-old putative transformed shoots were used for GFP visualization. The GFP expression was observed using a confocal laser scanning microscopy, CLSM (LEICA TCS SP5 II) with 40X oil-immersion

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objectives. GFP expression was excited at wavelength 488 nm and detected though a filter for fluorescence wavelength of 550 nm. Confocal images from the selected section within upper and lower epidermis were merged using software (LAS AF version 2.3.1 build 5194 SP5). Non-transformed leaves were used as negative control to adjust the parameter of auto-fluorescence exclusion.

3.6 Acclimatization and phenotypic assessment of transgenic tobacco plants

Positive transformed plantlets (~ 12 week-old) with well-developed roots were washed thoroughly under running tap water and immersed in 1 % (w/v) IMAS-THIRAM 80 fungicide (Endona Corp, Malaysia) for at least 10 s before transferred to soil in a polythene bag. The transformed plantlets were grown at the Plant Biotechnology Facility (University of Malaya, Malaysia) under 16 h photoperiod at 25 ± 3 ^{o}C. T_1 seeds were collected for gene-stability expression study. In addition, the phenotypes of at least three individuals of T_1 positive seedlings of each line were examined and recorded.

3.7 Assessment of putative tobacco transformants by PCR analysis

3.7.1 Total genomic DNA extraction

Ten and four weeks old of putative transformed tobacco plantlets, T_0 and T_1 respectively were used for DNA extraction. The total genomic DNA from tobacco leaves was isolated using DNeasy Plant Mini Kit (Qiagen, USA) according to manufacturer's manual. About 100 mg of ground tissue were transferred to a 1.5 mL microcentrifuge tube which contained 400 µL of AP1 buffer with 4 µL of 10 mg/mL RNase A. The mixture was vortexed vigorously and incubated at 65 °C for an hour.

After that, 150 µL of AP2 buffer were added and immediately mixed by inverting the tubes following the incubation on ice for 5 min to precipitate the detergents, proteins and polysaccharides. The lysate was centrifuged at 14,000 rpm for 5 min. The clear lysate was transferred to QIAshreddar mini spin column and centrifuged at 14,000 rpm for 2 min. The flow-through fraction was then transferred to a new 1.5 mL microcentrifuge tube and 1.5 volume of AP3/E buffer was added and mixed well. The mixture was directly transferred to DNeasy mini spin column and centrifuged at 8,000 rpm for 1 min. After centrifugation, the flow-though was discarded and the DNeasy mini spin column was transferred to a new 2 mL collection tube. Five hundred µL of AW buffer was added and centrifuged at 8,000 rpm for 1 min. The flow-though was discarded followed by another addition of AW buffer and centrifuged at 14,000 rpm for 2 min. Finally, 30 µL of AE buffer were added directly onto the middle of the membrane in the spin column and incubated at RT for 20 min. Then, centrifugation was performed at 8,000 rpm for 1 min. The isolated genomic DNA was electrophoresed on agarose gel. The isolated DNA was stored at -20°C until use.

3.7.2 Polymerase chain reaction (PCR)

PCR analysis was performed to confirm the presence of transgene in the putative transformants (T_0 and T_1). The isolated genomic DNA was used as template. The presence of transgene for all plasmid constructs in putative transformants were amplified using 2 different sets of primers for T_0, F-1304sk & R_1-1304sk; F-GFP & R-GFP (Table 3.3) and T_1, F-HPT1 & R-HPT1 (Table 3.5). The expected size of the PCR product for each plasmid amplified using F-1304sk & R_1-1304sk primers was listed in Table 3.3. However, regardless of different plasmid constructs amplified using other sets of primer mentioned in both Table 3.3 and 3.5 is the same. Thermal cycle

conditions were set as described in Table 3.2 using PCR recipe as described in Table 3.1. The PCR products were electrophoresed on agarose gel mentioned in Table 3.4.

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Table 3.5: List of primers that were used for T_1 seedlings screening and real-time PCR.

Primer set	Sequence	Length, bp	Annealing temperature, °C	PCR product size
F-HPT1 R-HPT1	5’- AGATGTTGGCGACCTCGTATTG-3’ 5’-GTTTATCGGCACTTTGCATCGGC-3’	22 23	56	559 bp
F-FV60 R-FV60	5’-TGGAGTCCCTGATCGCTTCACTGGC-3’ 5’-GCTCCAGCTTGGTCCCAGCACCG-3’	25 23	60	149 bp
F-L25 R-L25	5’-GGTTGCCAAGGCTGTCAAGTCAGG-3’ 5’-CCTTCCAGGTGCACTAATACGAGGG-3’	24 25	60	139 bp

*FV60 and L25 primer sets were used for real time PCR while HPT1 primer set was used for T_1 seedlings PCR screening. The expected size of PCR products for each primer is same for all plasmids.

University of Malaya

3.8 Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

3.8.1 Gel cassette assembly

Spacer plate (1.0 mM) and short plate were cleaned using Kimtech Delicates KimWipes® (Kimberly-Clark, USA) and 70 % (v/v) ethanol before use. The plates were assembled according to manufacturer's manual (BioRad, CA, US). A short plate was placed on top of spacer plate and inserted into the green casting frame on a flat surface and was clamped by the casting frame. The plates were checked in order to be level on the bottom then put into the casting stand.

3.8.2 Preparation of the gel

All reagents except tetramethyethylenediamine (TEMED) and ammonium persulfate (APS) (BioRad, US) were mixed together for 12 % (v/v) resolving gel layer (Table 3.6). Once the resolving gel was ready, the TEMED and APS were then added and mixed by gently swirling following the transfer in between the glass plates in the casting chamber until about 2 cm inch below the short plate. Isopropanol was added on top of the gel. After the gel polymerized, the isopropanol was gently removed by absorption to Kimtech Delicates KimWipes® (Kimberly-Clark, USA) with prior to pouring the upper layer of stacking gel.

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Table 3.6: 12 % (v/v) of resolving gel (SDS-PAGE).

Components	Solution per gel
dH_2O	1.65 mL
1.5 M Tris-HCl	1.25 mL
10 % (w/v) SDS	25.00 µL
30 % (v/v) Acrylamide/ Bis solution, 37:5:1	2.00 mL
10 % (w/v) ammonium persulfate, APS	75.00 µL
TEMED	6.00 µL

For the preparation of 4 % (v/v) stacking gel, all reagents except TEMED and APS were mixed together (Table 3.7). Once the resolving gel was ready, the TEMED and APS were quickly added and mixed by gently swirling following the transfer in between the glass plates over the resolving gel in the casting chamber. The comb was inserted into the opening between the glass plates for well formation. Once the stacking gel was polymerized, the comb was gently removed.

Table 3.7: 4 % (v/v) of stacking gel (SDS-PAGE).

Components	Solution per gel
dH_2O	1.70 mL
1.0 M Tris-HCl	315.00 µL
10 % (w/v) SDS	25.00 µL
30 % (v/v) Acrylamide/ Bis solution, 37:5:1	415.00 µL
10 % (w/v) ammonium persulfate, APS	12.50 µL
TEMED	6.00 µL

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3.8.3 Preparation of protein samples

Forty-day old of transgenic tobacco plantlets were used for protein expression study (3rd to 7th leaf positions from main stem) (Robert et al., 2013). One hundred µL of 1X PBS buffer (Appendix E) was added to 100 mg of ground leaf tissue (1:1 ratio), mixed and centrifuged at 13,000 rpm, 4 °C for 5 min. The clear supernatant was collected and transferred to a new 1.5 mL microcentrifuge tube. About 10 µL of the protein extract was used for Bradford's quantification (Section 3.8.3.1). The quantified protein samples were mixed with 5X samples buffer (Appendix E) followed by heating at 100 °C for 5 min before loading into the well of casted polyacrylamide gel. The quantification of total protein was performed according to standard curve bovine serum albumin (BSA), 1 mg/mL.

3.8.3.1 Bradford's quantification

Quantification of total soluble proteins was assayed according to Bradford (1976) using Bio-Rad Bradford Dye (Hercules, CA, USA) and 1 mg/mL BSA as a protein standard.

3.8.4 Electrophoresis of SDS-PAGE

The gel cassette was removed from the casting frame stand and placed in Mini-PROTEAN Tetra Cell electrophoresis tank (BioRad, CA, US) with the short plates faced inside. Electrophoresis running buffer (1X) (Appendix E) was filled into the tank in the compartment and outside of the frame. The tank was then covered with the lid aligning the Mini-PROTEAN Tetra cell (BioRad, CA, US) appropriately. At first, the samples were run at 80 V to properly stack the protein samples. Once the blue indicator

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entering the separating gel, the protein samples were run at 100 V until the blue indicator of 5X sample buffer reached the bottom part of the gel.

3.8.5 Staining and de-staining protein gel with Coomassie blue

The gel was pre-fixed with fixing solution (Appendix E) for 30 min followed by staining with 0.25 % (w/v) Coomassie blue staining for 2-4 h (Appendix E) until a uniform blue color was observed on the gel. The gel was de-stained for 2-4 h in a de-staining solution (Appendix E). The reaction was stopped by rinsing and soaking the gel in ultrapure H_2O.

3.8.6 Western blot analysis

Total protein samples were prepared as described in Section 3.8.3 separated by SDS-PAGE (Section 3.8.4) before Western blot analysis. The run gel was transferred on nitrocellulose membrane, Amersham ECL Sensitive Nitrocellulose membrane (GE Healthcare, USA) using trans-blot Mini PROTEAN (BioRad, CA, USA). The transfer was performed using FlashBlot Transfer Buffer (Advansta, USA) for 18 min at 55 V according to manufacturer's manual. The membrane was washed with 1X TBST (Appendix E) for 5 min at 60 rpm. The blotted membrane was then blocked using blocking buffer (Appendix E) at RT for an hour. Next, the membrane was washed again with 1X TBST for 5 min followed by probing with 1: 1000 mouse monoclonal anti E-tag antibody (Abcam, USA) in blocking buffer. The membrane was incubated for overnight at 4 °C with gentle agitation. On the next day, the membrane was then washed with 1X TBST for 3 times, each time 5 min, at higher speed and probed with 1: 2000 rabbit anti-mouse IgG, Fc target antibody conjugated with HRP (Abcam, USA) in blocking buffer at RT for 90 min with gentle agitation. After 3 times washed with 1X

University of Malaya

TBST, WesternBright ECL chemiluminescent HRP substrate (Advansta, USA) was added evenly at least 0.125 mL per cm^2 on membrane (according to manufacturer's manual) and incubated for 1 min with gentle shaking at RT according to manufacturer's manual. The membrane was blotted dry using KimWipes® and exposed for at least 1 min under white light using ChemiDoc Imager (BioRad, CA, USA).

3.8.7 Protein purification using Dynabeads®

Protein purification was performed for T_1 tobacco plant samples using Dynabeads® M-280 Sheep anti-Mouse IgG (Thermo Fisher Scientific, USA) according to manufacturer's manual. The beads in vial were vortexed for 1 min and the desired volume of beads was aliquoted into a new 1.5 ml microcentrifuge tube (2 µg Ig/ 50 µL beads) per sample. One mL of washing buffer [Ca^{2+} and Mg^{2+} free 1X PBS supplemented with 0.1 % BSA and 2 mM EDTA] was added. The mixture was re-suspended by inverting the tube for few times. The tube was placed on the magnet rack, DynaMag (Thermo Fisher Scientific, USA) for 1 min and the supernatant was discarded. This step was repeated twice. To allow the binding of target Ig (primary antibody) to the magnetic beads, 2 µg of target Ig (anti E-tag; Abcam, USA) (optimized) was added to 50 µL of pre-washed beads and dissolved in 1 mL of washing buffer. The mixture was tilted gently and rotated using HulaMixer (Thermo Fisher Scientific, USA) for overnight at 4 °C to allow the perfect coupled of beads-IgG. The tube containing coupled beads-IgG was then placed on a magnetic rack for 2 min to separate coupled beads-IgG from the washing buffer. The supernatant was discarded. The tube was removed from the magnet followed by the addition of 1 mL washing buffer. The steps were repeated twice. About 1.25 µg of total soluble protein per 50 µL of pre-washed beads were added to the final volume of 250 µL, diluted in 1X PBS containing Ig-coupled beads. The mixture was mixed by tilting gently at 4 °C for

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overnight to increase the binding affinity of antigen binding to Ig-coated beads. After incubation, the tube was placed on a magnetic rack for 2 min and the supernatant was transferred to a new 1.5 mL centrifuge tube. The tube was removed from the magnetic rack and 1 mL of washing buffer was added to the mixture followed by slow tilting. This step was repeated twice. In order to elute off the target samples and IgG from the beads, 50 µL of 0.1 M citrate (Appendix E) (pH 2-3) was added to the Ig-coupled beads. The mixture was mixed well by tilting and rotating for 2 min. The tube was placed the magnetic rack and the supernatant containing purified of target protein was transferred to a new 1.5 mL centrifuge tube. This step was repeated twice and the eluate was pooled together. To avoid co-elution of the target IgG, the cross-linked of antibody to the beads was performed using BS3 reagent (Appendix E) (Thermo Fisher Scientific, USA) according to manufacturer's manual. The purified protein was subjected for SDS-PAGE and Western blot analysis.

3.9 Verification of transgenic plant via real-time PCR analysis

3.9.1 RNA extraction and DNase treatment

The RNA of tobacco plantlets (T_1) was isolated using RNeasy Plant Mini Kit (Qiagen, USA) according to manufacturer's manual. About 100 mg of fresh transgenic tobacco leaves (~ 3 months of germination) was ground into fine powder using a mortar and pestle in the presence of liquid nitrogen. About 450 µL of RLT buffer containing 1 % (v/v) fresh β-mercaptoethanol was added and the mixture was vortexed vigorously before incubation at 56 °C for 3 min. The lysate was transferred to the QIAshredder spin column sitting in 2 mL collection tube and centrifuged for 2 min at maximum speed. The flow-through fraction was pipetted and transferred to a new 1.5 mL

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microcentrifuge tube without disturbing the cell debris. Then, 0.5 volumes of chilled absolute ethanol were added to the cleared lysate.

The mixture was mixed and transferred to RNeasy mini spin column (~ 675 µL) followed by centrifugation at 10,000 rpm for 15 s. The flow-though was discarded. About 700 µL of buffer RW1 was added onto the RNeasy column and centrifuged at 10,000 rpm for 15 s. The flow-though and the collection tube were discarded. The RNeasy column was transferred to new 2 mL collection tube followed by pipetting 500 µL of buffer RPE onto the RNeasy column. Centrifugation was performed at 10,000 rpm for 15 s. The flow-though was discarded. This step was repeated twice following centrifugation at maximum speed for 2 min. Full speed centrifugation for 1 min was performed to remove any residual ethanol. The RNeasy column was then transferred to a new 1.5 mL microcentrifuge tube and added with 30 µL of RNase-free water onto the RNeasy membrane. The mixture was incubated for 20 min at RT before centrifuged at 10,000 rpm for 1 min. Then, the eluate was analyzed by agarose gel electrophoresis described in Section 3.3.2.3 and stored at –80 °C until use. The quantification of RNA was quantified using BioPhotometer (Eppendorf, USA). The quantification was measured using 50X dilution (1 µL samples with 49 µL RNase-free water. After measuring the RNA concentration, DNase treatment was carried out using RapidOut DNA Removal Kit (Thermo Fisher Scientific, USA) to remove the remaining DNA. The reaction mixture was prepared as showed in Table 3.8.

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Table 3.8: RapidOut DNA removal reaction mixture.

Components	Working concentration	Volume, µL
RNA samples	2 µg	Concentration dependent
10X DNase buffer with $MgCl_2$	1X	1
DNase I, RNase-free	1U	0.5
Water, nuclease-free	-	Adjusted to the final volume of 10 µL

The tube containing reaction mixture was mixed and incubated at 37 °C for 30 min. Then, DNase-treated RNA was quantified using BioPhotometer (Eppendorf, USA) and assessed by agarose gel electrophoresis described in Section 3.3.2.3.

3.9.2 Reverse-transcription PCR (RT-PCR)

The reverse-transcription PCR was performed prior to quantitative real-time analysis. This cDNA conversion was carried out using RevertAid H minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA). The reaction mixture was prepared according to manufacturer's protocol. The reaction mixture was prepared as described in Table 3.9 and aliquoted into prepared tube as prepare describe in Table 3.10.

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Table 3.9: Reverse-transcriptase PCR reaction mixture.

Components	Volume (1X reaction)
5X reaction buffer	4 µL
RiboLock RNase Inhibitor (2U/ µL)	1 µL
10 mM dNTP mix	2 µL
RevertAid H Minus M-MuLV Reverse Transcriptase (200U/ µL)	1 µL
TOTAL VOLUME	**20 µL**

Table 3.10: DNase treated-RNA preparation.

Components	Volume (1X reaction)
DNase-treated RNA	Concentration dependent, 1 µg
Oligo $(dT)_{18}$ primer	1 µL
Nuclease-free H_2O	Topped up to 12 µL
TOTAL VOLUME	**12 µL**

The mixture was gently mixed and briefly centrifuged before loaded into PCR Thermocycler Biometra Tpersonal (Biometra, Germany). The thermal cycling condition of reverse-transcription PCR as in Table 3.11. The cDNA was stored at – 20 °C for short storage and – 80 °C for longer storage.

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Table 3.11: Thermal cycling condition of reverse-transcriptase PCR.

Temperature	Time, (min)
42 ℃	60
70 ℃	5
4℃	∞

The cDNA was stored at -20℃ for short storage and -80 ℃ for longer storage till use.

3.9.3 Real-time PCR

The quantitative PCR was carried out using Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR machine. Firstly, the primer sets, F-FV60 & R-FV60 (target gene) and F-L25 & R-L25 (internal reference gene) described in Table 3.5 was examined though dissociation programs in order to check the binding affinity and specificity of the primers. FV60 was used to amplify the recombinant protein gene (*TP60*), mainly to compare the expression level due to the effect by different proteinase inhibitors and/or KDEL. *Nicotiana tabacum* ribosomal large subunit 25, *NtL25* was used as the internal reference gene control (Schmidt & Delaney, 2010). The reaction mixture was prepared as described in Table 3.12. Three replicates of each primer set and non-template control were prepared and assayed with the following dissociation thermal condition (Table 3.13). This dissociation curve was performed together with samples in order to ensure the free-contamination or non-specific binding of primer which will give false-positive results.

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Table 3.12: 5X Real-time PCR reaction mixtures.

Components	Working concentration	Volume, µL
2X Power SYBR® Green PCR Master mix	1X	5.0
F primer, 10 µM	0.5 µM	0.5
R primer, 10 µM	0.5 µM	0.5
cDNA template, 100 ng/µL	100 ng	1.0
Nucleases-free water	-	3.0

The transgenic samples were analyzed using comparative expression study (relative quantification, RQ) to determine the effect of proteinase inhibitors and/or KDEL (BBI, KDEL, BBIKDEL, or OCPIKDEL) towards expression of target gene, *TP60*. Each sample was prepared in triplicate including technical and biological. The PCR reaction mixture was prepared as described in Table 3.12 and thermal cycle condition was described in Table 3.13.

Table 3.13: Thermal cycle condition for real-time PCR.

Stage	Temperature, °C/ Time	Cycle
1	95/ 10 min	1
2	95/ 1 min	40
	60/ 15 sec	

Then, the Ct value obtained from Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR software was collected and recorded. The data were then analyzed

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manually using Microsoft Excel 2010 with calculation of expression $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

The non-template control was used in triplicate for every run samples to ensure free-contamination or unspecific binding of primers which lead to false-positive expression. All data of comparative expression were used to compare the gene expression level in between lines and constructed plasmids.

3.10 Semi-quantitative RT-PCR

For semi-quantitative RT-PCR, 100 ng cDNA was used as template following normal PCR amplification (1 U/µL; iNtRON Biotechnology, Gyeonggi-do, South Korea) at the annealing temperature of 60 °C for both target and internal reference gene primers. The amplicon was visualized on an EtBr agarose gel electrophoresis under UV Gel Pro Imager (Media Cybernetics, USA).

3.11 Statistical analysis

The statistical analysis was performed using SPSS 16.0 (SPSS Inc., USA) at 5 % confident level. The analysis was conducted in triplicate and statistically analyzed using one-way analysis of variance (ANOVA). The mean values of different plasmid constructs were subjected to Duncan's multiple comparison.

However, for number of shoot regeneration and GUS fluorometry analysis, Kruskal-Wallis (non-parametric) test has been carried out due to not normally distributed across samples tested ($p < 0.05$).

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CHAPTER 4: RESULTS

4.1 Endogenous test of *N. tabacum* cv. SR1 leaf discs towards antibiotics

In this study, endogenous test is the minimal shoot regeneration response of leaf disc explants of *N. tabacum* cv. SR1 on regeneration media containing hygromycin (selection agent) after one month of culture. The common method used in most publications is based on the surviving explants over the total number of explants cultured on selection media. However, this does not take into account the number of shoots regenerated from the surviving explants. Hence, in this study, shoot regenerants were scored on explants (producing the least number of shoots) when cultured on media containing hygromycin. This is important in order to achieve the highest number of shoots regenerated rather than percentage of surviving explants on selection media.

At 10 mg/L and 20 mg/L of hygromycin, the percentage of explants that survived were 98 % (LD_{10}) and 80 % (LD_{20}), respectively, after one month of culture. At 30 mg/L, the percentage of surviving explants decreased to 50 % (LD_{50}) followed by 3 % (LD_{90}) at 40 mg/L and 0 % (LD_{100}) at 50 mg/L hygromycin. This showed that explants were able to overcome the effect of antibiotics at certain concentration allowing chances of survival after four weeks on culture plate. The percentage of surviving explants was inversely proportional to the hygromycin concentrations (Figure 4.1). This assessment method is commonly used in many publications, such as in *Citrullus vulgaris* (Suratman et al., 2010) and *Pisum sativum* (Svabova et al., 2005).

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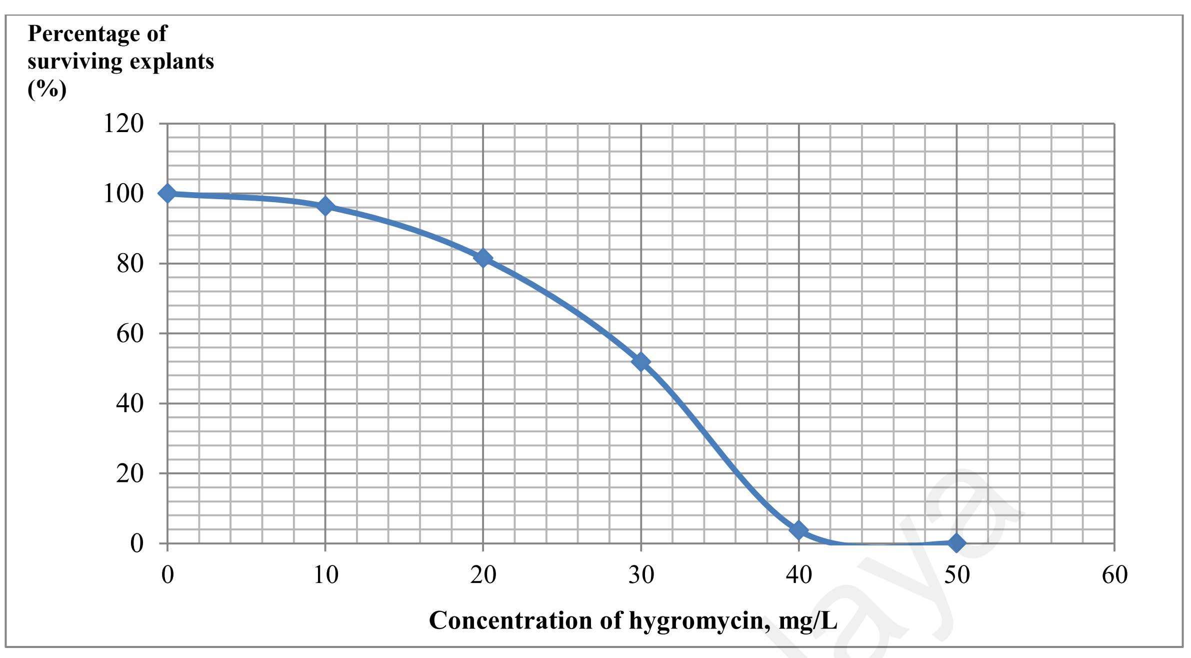

Figure 4.1: Percentage of survival explants on media containing hygromycin at different concentrations after one month of culture

At 0 mg/L, 100 % of the shoots regenerated at the cut edges and the surface of each leaf explant cultured on media. However, the shoots regeneration and necrotic area out of the total surface of explants decreased to about half (50 %) (LD_{50}) due to the selective pressure on media containing 10 mg/L hygromycin. At 20 mg/L hygromycin, the shoot regeneration area was lesser compared to the necrotic area and decreased to about 20 % (LD_{80}) (less than half of 50 %). In addition, no shoots regenerated on media-containing hygromycin at concentrations 30, 40 and 50 mg/L (LD_{100}). The whitish coloration formed at the edges of the leaf discs which were directly exposed to the antibiotics in the media showed that the explants were naturally intolerant to high concentration of hygromycin. Results are shown in Table 4.1.

Thus, 20 mg/L hygromycin (LD_{80}) was chosen for the selection of transformants based on the minimal number of shoots regenerated. Higher concentrations were not chosen because the transformants may not be able to survive due to stringent selection pressure.

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This result was in contrast to the earlier report by Pathi et al., (2013), where transgenic tobacco plants were selected at 30 mg/L hygromycin where 100 % non-transformants were eliminated. On the other hands, the obtained results were similar as reported by Suratman et al. (2010) where 5 mg/L (LD_{80}) of hygromycin was chosen for the selection of *Citrullus vulgaris* transformants.

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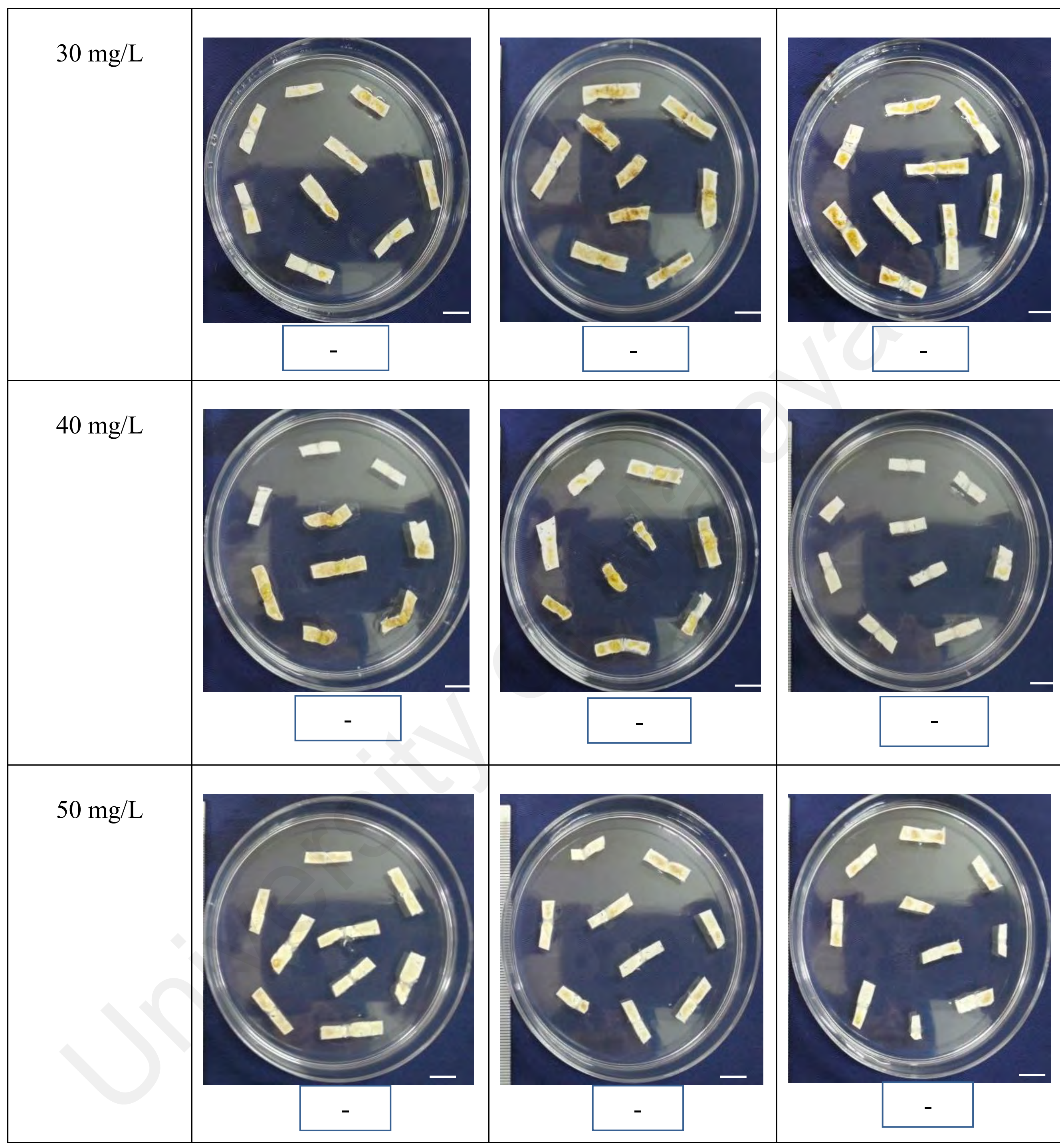
Table 4.1: Assessment on minimal concentration of hygromycin in *N. tabacum* cv. SR1 leaf discs

Concentration of hygromycin	Replicate 1	Replicate 2	Replicate 3
0 mg/L	++++	++++	++++
10 mg/L	++	++	++
20 mg/L	+	+	+

Table 4.1, continued.



30 mg/L	-	-	-
40 mg/L	-	-	-
50 mg/L	-	-	-

Key:		
	++++	Highest responsive explants forming shoot (100 %)
	++	Equal responsive explants forming shoots to necrotic areas (50 %)
	+	Less responsive explants forming shoots to necrotic areas (20 %)
	-	All of the explants undergo necrosis. None of the explants forming shoots

4.2 Confirmation of *A. tumefaciens* harboring binary vector and gene of interest

In this study, modified pCAMBIA 1304 with additional restriction sites, *Avr*II and *Afe*I was used as a backbone binary vector harboring five different constructs (Figure 3.2) (Go, 2013).

Plasmid constructs were successfully isolated from overnight culture of *Agrobacterium* and shown in Figure 4.2 with the expected size of ≥ 10 kb. Figures 4.3, 4.4, and 4.5 verified the presence of target and *mgfp*5 gene accordingly within T-DNA region of the pCAMBIA 1304. The *mgfp*5 gene presence in *Agrobacterium* colonies with empty vector was amplified using GFP primers at the expected size of 759 bp (Figure 4.3). *Agrobacterium* colonies contained gene of interest (Figure 3.2 (A-D) were amplified using 1304sk primers with the expected size of target gene ~ 900 bp (*TP60* encoded for anti-*Toxoplasma* scFv antibody and/or with KDEL) (Figure 4.4) and ~ 1.5 kb (*TP60* together with proteinase inhibitors with or without KDEL; BBI, BBIKDEL and OCPIKDEL) (Figure 4.5). The exact size of each plasmid using 1304sk primers mentioned in Table 3.3.

The presence of insert within T-DNA region of pCAMBIA 1304 was double confirmed by using another pair of primers flanking between insert and *mgfp*5 gene (F-1304sk and R-GFP) (Figures 4.6 and 4.7 at the expected size of ~1.6 and 2.3 kb, respectively) and between CaMV 35S promoter and target (F-35S and R_1-1304sk) (Figures 4.8 and 4.9 at the expected size of ~ 1.3 kb and ~ 1.9 kb, respectively). The expected size of each plasmid constructs using different combination of primers mentioned showed in Table 4.2.

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Table 4.2: The list of primer combinations used for the confirmation of insert.

Primer set	Plasmid constructs	PCR product size
F-1304sk & R-GFP	pTP60	1673 bp
	pTP60KDEL	1694 bp
	pTP60BBI	2279 bp
	pTP60BBIKDEL	2300 bp
	pTP60OCPIKDEL	2337 bp
F-35S & R_1-1304sk	pTP60	1314 bp
	pTP60KDEL	1335 bp
	pTP60BBI	1920 bp
	pTP60BBIKDEL	1941 bp
	pTP60OCPIKDEL	1978 bp

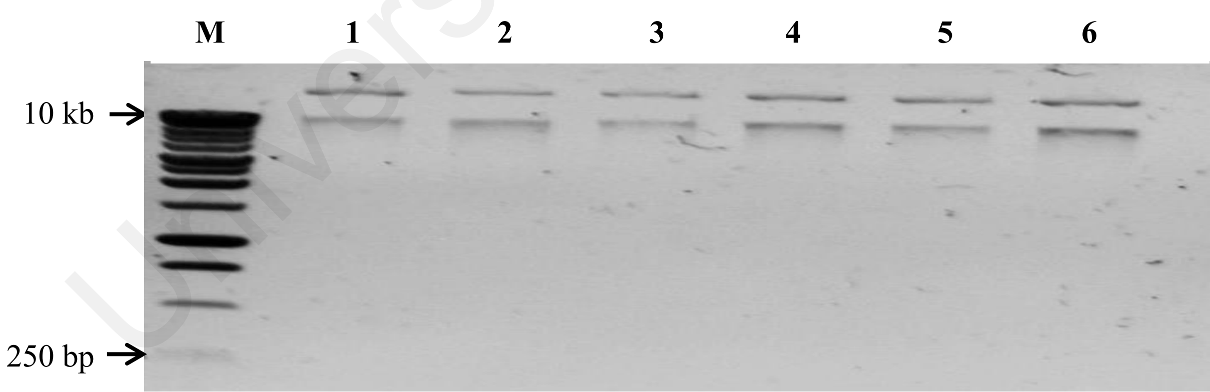

Figure 4.2: 10 kb of isolated plasmid containing gene of interest.

Lane M : VC 1 kb DNA ladder from Vivantis, Malaysia

Lanes 1-6 : Extracted plasmid of pCAMBIA 1304 containing gene of interest

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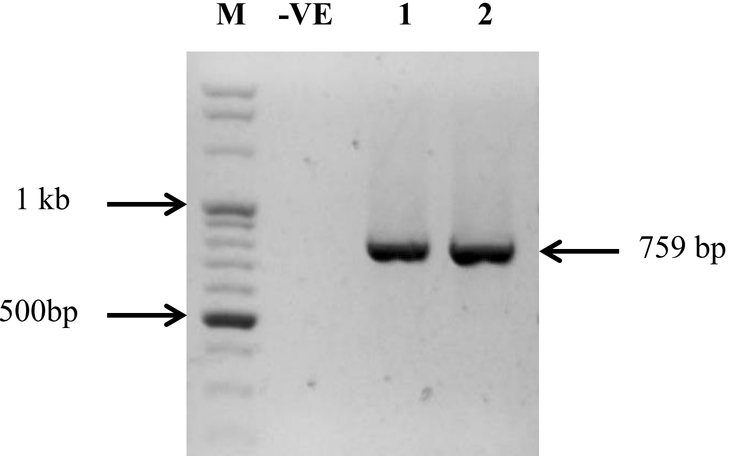

Figure 4.3: PCR verification of empty plasmid (without insert) using a pair of GFP primer.

Lane M : Perfect™ 100 bp DNA ladder from Eur_x, Poland

Lane –VE : Negative control, without plasmid template

Lane 1-2 : Isolated plasmid with empty vector, pCAMBIA 1304

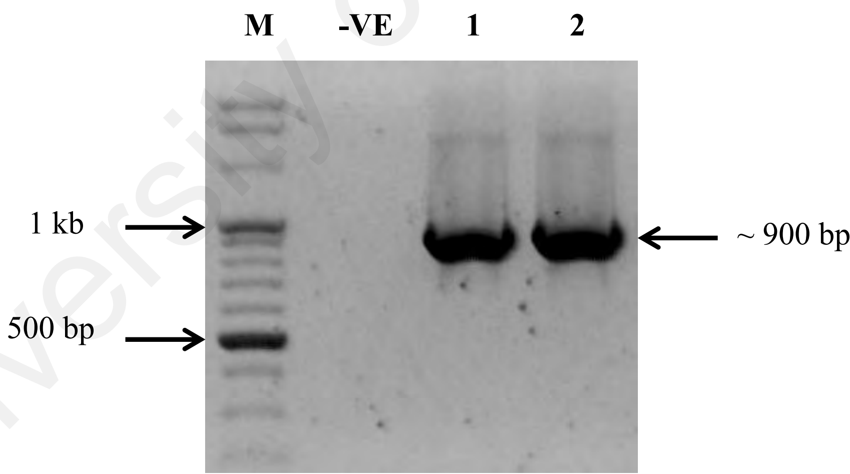

Figure 4.4: PCR verification of extracted plasmid using a pair of 1304sk primer.

Lane M : Perfect™ 100 bp DNA ladder from Eur_x, Poland

Lane –VE : Negative control, without plasmid template

Lane 1 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene, pTP60 construct (914 bp)

Lane 2 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody and ER-targeting gene, pTP60KDEL construct (935 bp)

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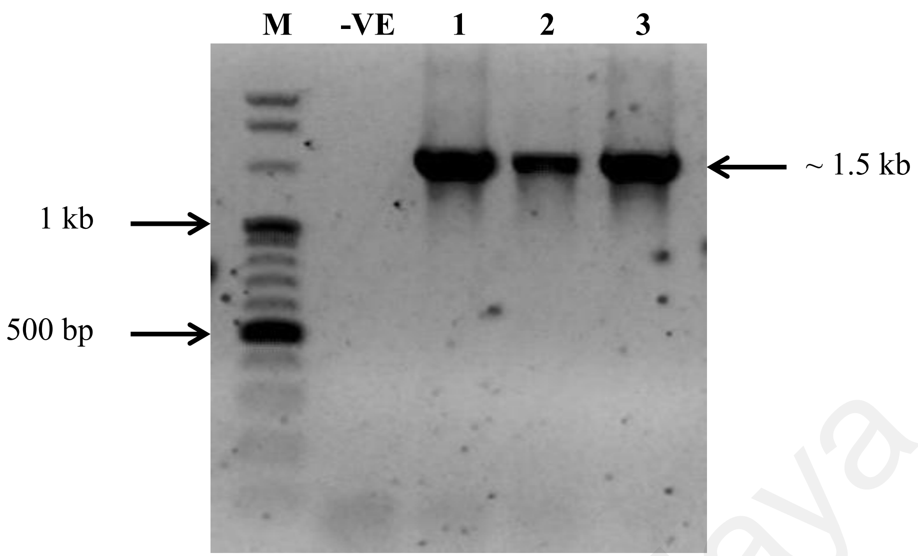

Figure 4.5: PCR verification of extracted plasmid using a pair of 1304sk primers.

Lane M : Perfect™ 100 bp DNA ladder from Eur_x, Poland

Lane –VE : Negative control, without plasmid template

Lane 1 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI, pTP60BBI construct (1520 bp)

Lane 2 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60BBIKDEL construct (1541 bp)

Lane 3 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60OCPIKDEL construct (1578 bp)

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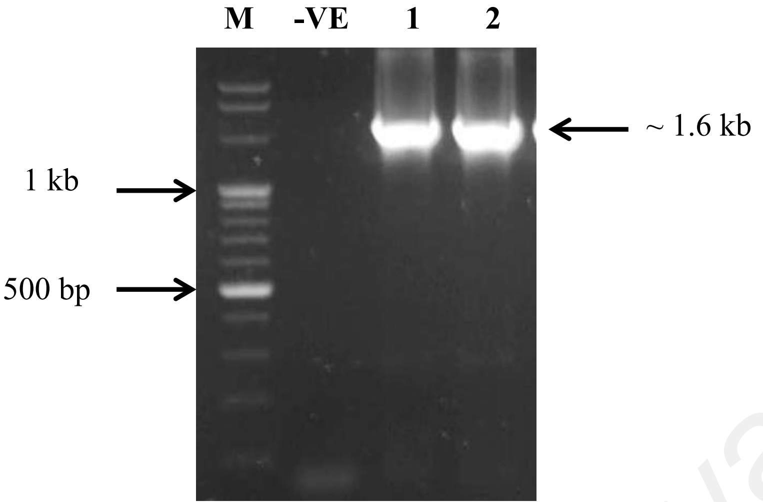

Figure 4.6: PCR verification of extracted plasmid using a pair of F-1304sk and R-GFP primers.

Lane M	:	Perfect™ 100 bp DNA ladder from Eur_x, Poland
Lane –VE	:	Negative control, without plasmid template
Lane 1	:	Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene, pTP60 construct (1673 bp)
Lane 2	:	Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody with ER-targeting gene, pTP60KDEL construct (1694 bp)

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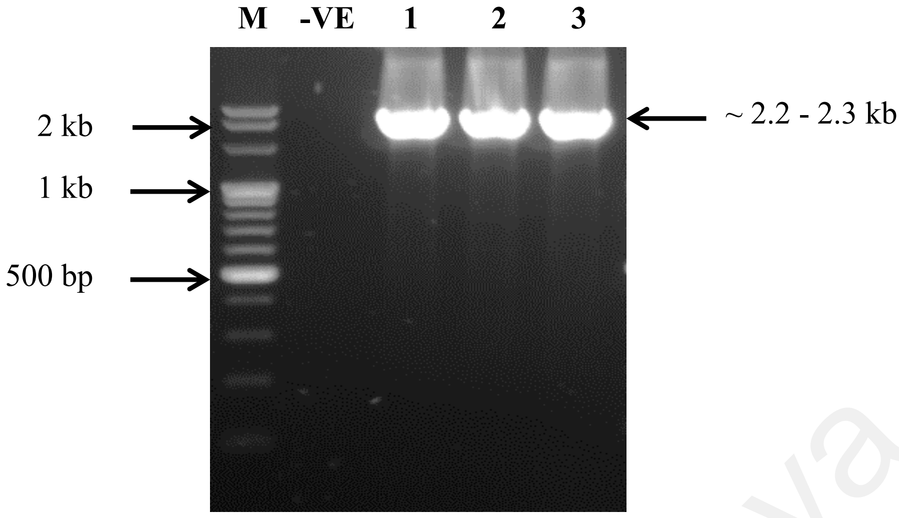

Figure 4.7: PCR verification of extracted plasmid using a pair of F-1304sk and R_1-GFP primers.

Lane M	:	Perfect™ 100 bp DNA ladder from Eur_x, Poland
Lane –VE	:	Negative control, without plasmid template
Lane 1	:	Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI, pTP60BBI construct (2279 bp)
Lane 2	:	Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60BBIKDEL construct (2300 bp)
Lane 3	:	Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60OCPIKDEL construct (2337 bp)

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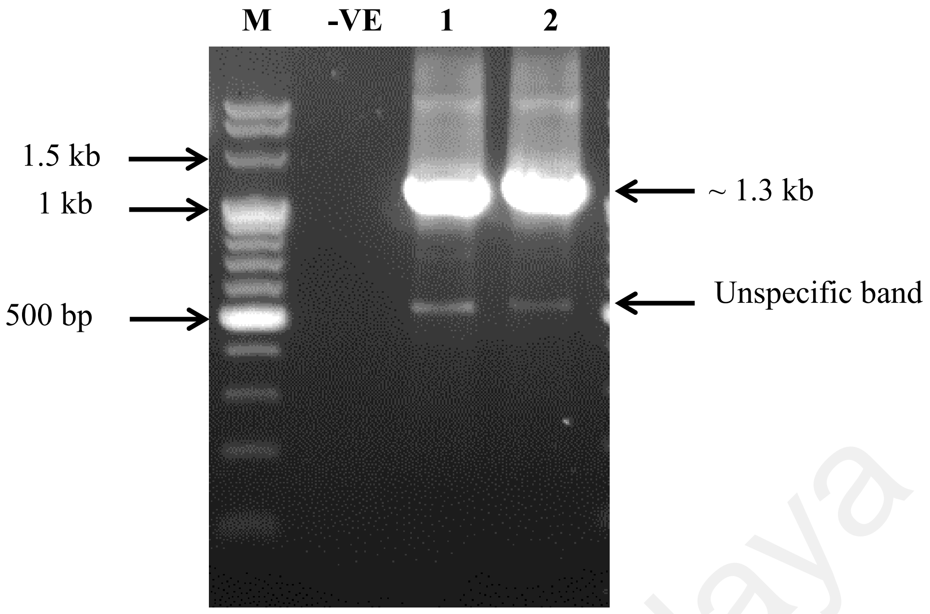

Figure 4.8: PCR verification of extracted plasmid using a pair of F-35S and R_1-1304sk primers.

Lane M : Perfect™ 100 bp DNA ladder from Eur_x, Poland

Lane –VE : Negative control, without plasmid template

Lane 1 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene, pTP60 construct (1314 bp)

Lane 2 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody with ER-targeting gene, pTP60KDEL construct (1335 bp)

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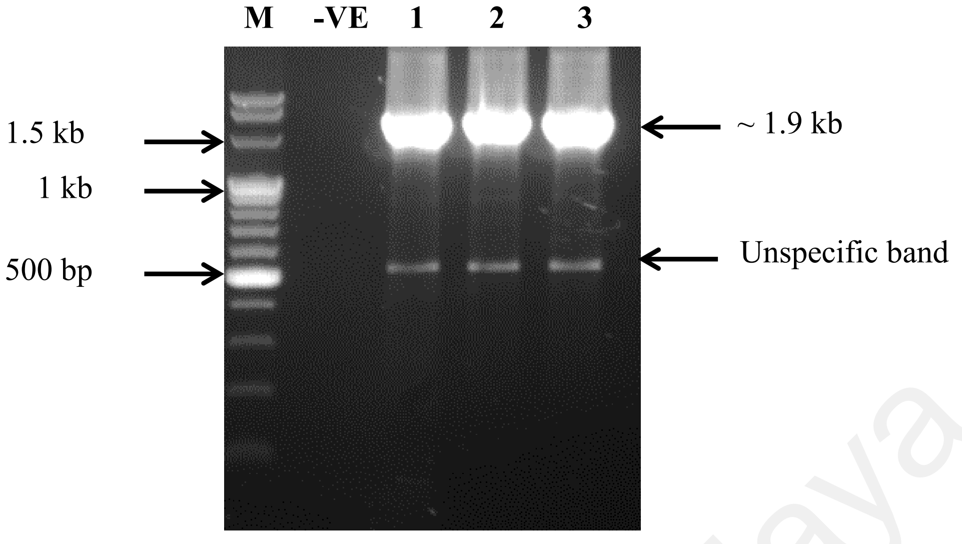

Figure 4.9: PCR verification of extracted plasmid using a pair of F-35S and R_1-1304sk primers.

Lane M : Perfect™ 100 bp DNA ladder from Eur_x, Poland

Lane –VE : Negative control, without plasmid template

Lane 1 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI, pTP60BBI construct (1920 bp)

Lane 2 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60BBIKDEL construct (1941 bp)

Lane 3 : Extracted plasmid of pCAMBIA 1304 containing anti-*Toxoplasma* (scFv) recombinant antibody gene with PI-KDEL, pTP60OCPIKDEL construct (1978 bp)

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4.2.1 Sequencing and BLAST analysis

Results as shown in Figures 4.10, 4.11, 4.12, and 4.13 confirmed the correct sequences for all constructs pTP60, pTP60KDEL, pTP60BBI/pTP60BBIKDEL, and pTP60OCPIKDEL respectively, harbored within T-DNA region of pCAMBIA 1304 using a primer pair of F-1304sk and R-GFP.

The BLAST protein result for pTP60-*mgfp*5 is shown in Figure 4.10. Based on the result obtained, target gene sequence of anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (97 % maximum identity) with anti-*T. gondii* tachyzoite single-chain variable fragment antibody [synthetic construct] (accession no.: AEX30825.1) with query coverage of 45 % (Figure 4.10) fused to E-epitope tag (GAPVPYPDPLER). Besides that, this result also showed that GFP protein is an unfused to target protein with the highest domain similarity for about 96 % of maximum identity with mGFP* hexaHis tagged in pCAMBIA vector. This confirmed that the target gene harbored within T-DNA region of pCAMBIA 1304.

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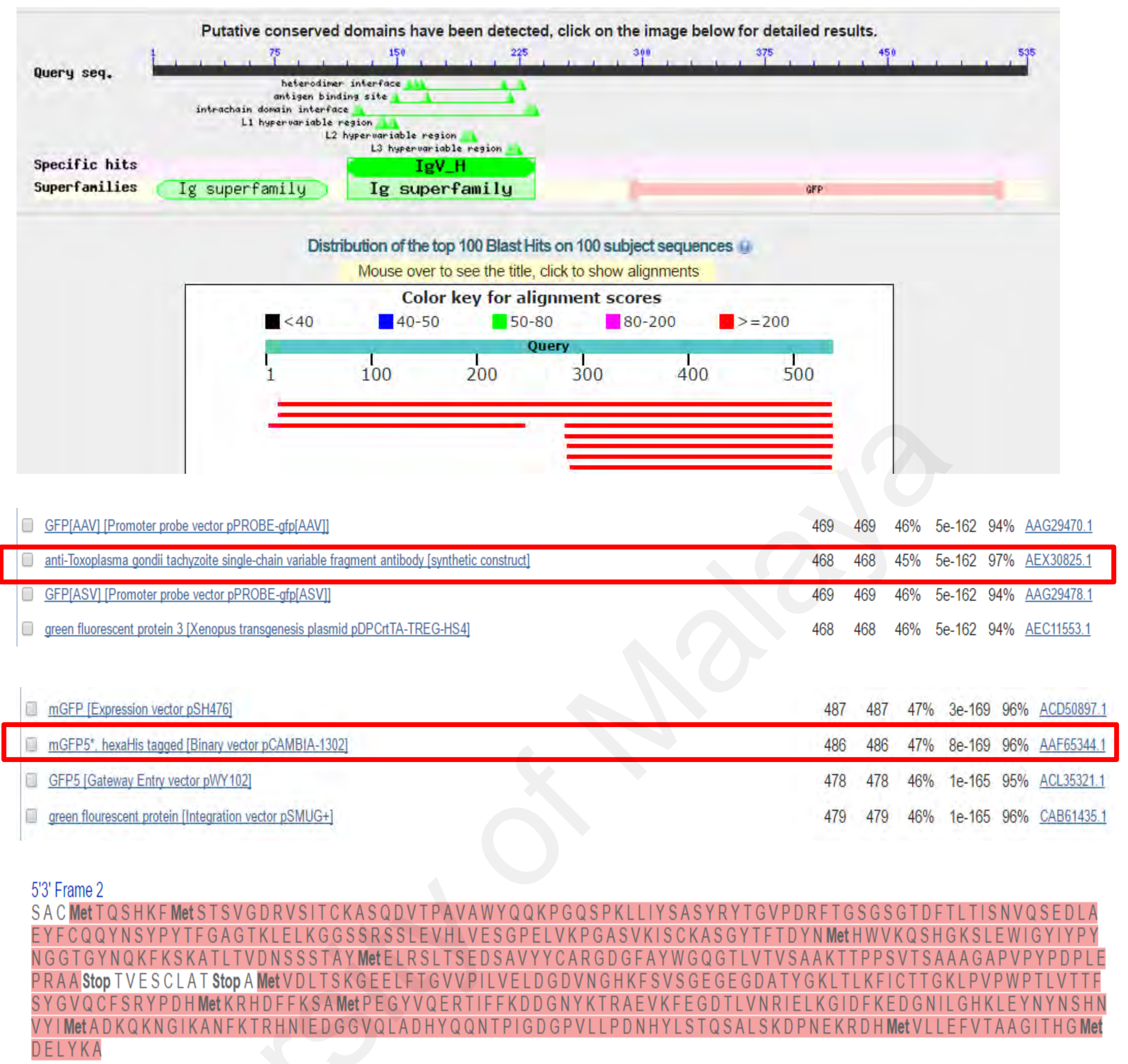

Figure 4.10: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and GFP. Anti-*Toxoplasma* (scFv) recombinant protein and conserved domain showed highest similarity (97 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody [synthetic construct] (accession no.: AEX30825.1) with query coverage of 45 % and unfused to GFP protein domain showed highest similarity (96 % maximum identity) with mGFP* hexaHis tagged [Binary vector pCAMBIA-1302] (accession no.: AAF65344.1) with query coverage 47 % of whole sequence.

The BLAST protein result for pTP60KDEL shown in Figure 4.11. Based on the result obtained, target gene sequence of anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (97 % maximum identity) with anti-*T. gondii* tachyzoite single-chain variable fragment antibody and scFv antibody TP60 [synthetic construct] (accession no.: AEX30825.1 and AHA85991.1 respectively) with query coverage of 75 % and 72 % respectively of whole sequence fused to E-epitope tag (GAPVPYPDPLER). Besides, this result also showed SEKDEL (plant microsomal retention peptide sequence) which represents ER retention sequence unfused to the target gene.

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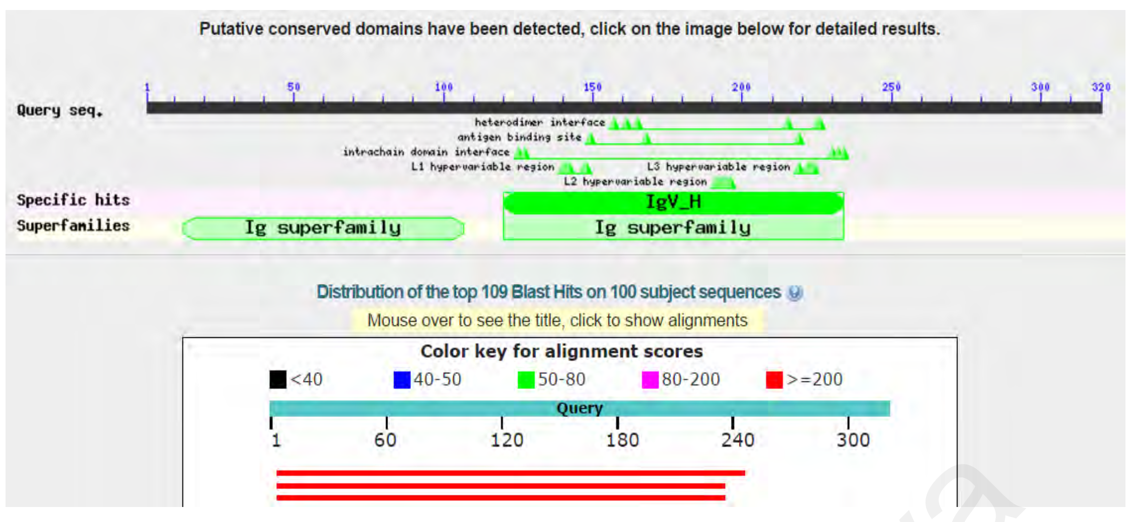

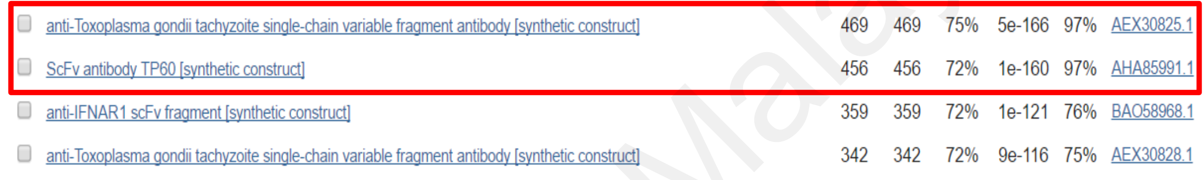

Description						
anti-Toxoplasma gondii tachyzoite single-chain variable fragment antibody [synthetic construct]	469	469	75%	5e-166	97%	AEX30825.1
ScFv antibody TP60 [synthetic construct]	456	456	72%	1e-160	97%	AHA85991.1
anti-IFNAR1 scFv fragment [synthetic construct]	359	359	72%	1e-121	76%	BAO58968.1
anti-Toxoplasma gondii tachyzoite single-chain variable fragment antibody [synthetic construct]	342	342	72%	9e-116	75%	AEX30828.1

5'3' Frame 2
SAC Met TQSHKF Met STSVGDRVSITCKASQDVTPAVAWYQQKPGQSPKLLIYSASYRYTGVPDRFTGSGSGTDFTLTISNVQSEDLA
EYFCQQYNSYPYTFGAGTKLELKGGSSRSSLEVHLVESGPELVKPGASVKISCKASGYTFTDYN Met HWVKQSHGKSLEWIGYIYPY
NGGTGYNQKFKSKATLTVDNSSSTAY Met ELRSLTSEDSAVYYCARGDGFAYWGQGTLVTVSAAKTTPPSVTSAAAGAPVPYPDPLE
PRAA Stop TVESCLA Met SEKDEL Stop PRPW Stop I Stop LVKEKNFSLELSQFLLN Stop Met

Figure 4.11: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and KDEL. Anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (97 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody and scFv antibody TP60 [synthetic construct] (accession no.: AEX30825.1 and AHA85991.1 respectively) with query coverage of 75 % and 72 % respectively of whole sequence. SEKDEL sequence represents ER retention sequence.

The BLAST protein result for pTP60BBI/ pTP60BBIKDEL shown in Figure 4.12. Based on the result obtained, target gene sequence of anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (95 % and 96 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody and scFv antibody TP60 [synthetic construct] (accession no.: AEX30825.1 and AHA85991.1 respectively) with query coverage of 56 % and 54 %, respectively, of whole sequence fused to E-epitope tag (GAPVPYPDPLER). Besides

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that, this result also showed the unfused BBI protein domain showed highest similarity (93 % maximum identity) with Bowman-Birk type proteinase inhibitor DE-4 like [*Musa acuminata* subsp. malaccensis (accession no.: XP_009413787.1) with query coverage 28 % of whole sequence. For pTP60BBIKDEL construct, the SEKDEL sequence could not be amplified due to poly-A at 3' region which led to DNA polymerase slippage.

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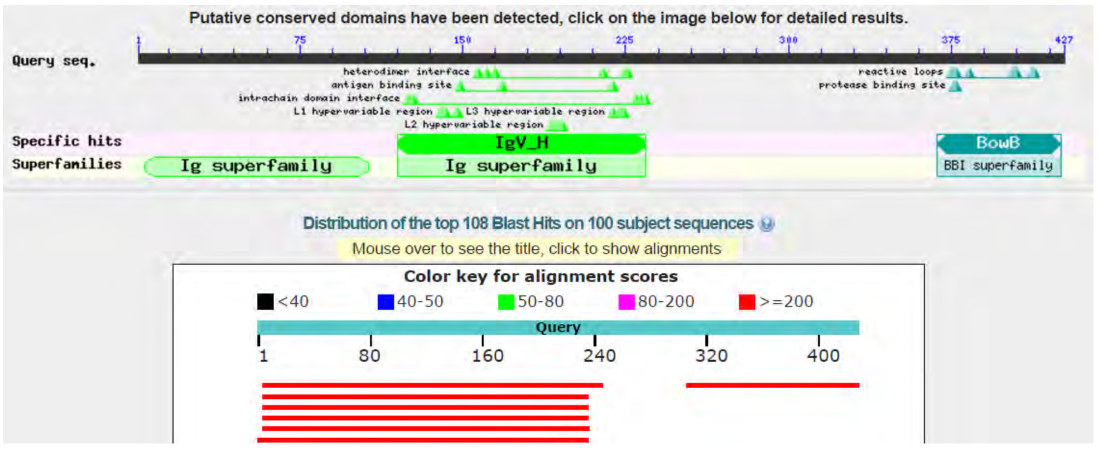

Description						Accession
anti-Toxoplasma gondii tachyzoite single-chain variable fragment antibody [synthetic construct]	461	461	56%	6e-161	95%	AEX30825.1
ScFv antibody TP60 [synthetic construct]	447	447	54%	3e-155	96%	AHA85991.1
anti-IFNAR1 scFv fragment [synthetic construct]	350	350	54%	2e-116	74%	BAO58968.1
anti-Toxoplasma gondii tachyzoite single-chain variable fragment antibody [synthetic construct]	334	334	54%	9e-111	73%	AEX30828.1
Chain A, Three-dimensional Structure Of Anti-ampicillin Single Chain Fv Fragment	326	326	54%	2e-107	73%	1H8O_A
P4A8 (ScFv)-PE38 [synthetic construct]	246	246	50%	6e-72	60%	AIL25363.1
single-chain variable fragment antibody [synthetic construct]	235	235	54%	7e-72	51%	AOR08286.1
PREDICTED: Bowman-Birk type proteinase inhibitor DE-4-like [Musa acuminata subsp. malaccensis]	228	228	28%	8e-71	93%	XP_009413787.1
FAPA scFvFc [synthetic construct]	243	243	61%	1e-70	50%	ALE27897.1
single chain variable fragment antibody [synthetic construct]	230	230	55%	1e-69	53%	ANV22114.1
scFv [synthetic construct]	229	229	55%	2e-69	53%	ALE27898.1

5'3' Frame 1

Met TQSHKF **Met** STSVGDRVSITCKASQDVTPAVAWYQQKPGQSPKLLIYSASYRYTGVPDRFAGSGSGTGFTLTISNVQSEDLAEYF
CQQYNSYPYTFGAGTKLELKGGSSRSSLEVHLVESGPELVKPGASVKISCKASGYTFTDYN **Met** HWVKQGHGKSLEWIGYIYPYNGG
TGYNQKFKSKATLTVDNSSSTAY **Met** ELRSLTSEDSAVYYCARGDGFAYWGQGTLVTASAAKTTPPSVTSAAAGAPVPYPDPLEPRA
A **Stop** TVESCLAKALQTQGRAAETWQRKKEAKARIEDAD **Met** RYN **Met** VVFSLVL **Met** VAAAFFASATTTASSSHPELRSALSTKGHEEDG
EGVGERSRQRRTWPCCDRCGGCTKSTPPQCQCQD **Met** VRSCHPSCRHCVRSPLSVSPPLYQC **Met** DRIPNYCRR

Figure 4.12: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and BBI proteinase inhibitor. Anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (95 % and 96 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody and scFv antibody TP60 [synthetic construct] (accession no.: AEX30825.1 and AHA85991.1 respectively) respectively with query coverage of 56 % and 54 % respectively of whole sequence. Unfused BBI protein domain showed highest similarity (93 % maximum identity) with Bowman-Birk type proteinase inhibitor DE-4 like [*Musa acuminata* subsp. malaccensis (accession no.: XP_009413787.1) with query coverage 28 % of whole sequence.

The BLAST protein result for pTP60OCPIKDEL is shown in Figure 4.13. Based on the result obtained, target gene sequence of anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (96 % and 97 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody (accession no.: AEX30825.1) and scFv antibody TP60 [synthetic construct] (accession no.: AHA85991.1) with query coverage of 59 % and 57 %, respectively, of whole sequence. This result also showed the unfused OCPI (CY) superfamily protein domain showed highest similarity (94 % maximum identity) with cysteine proteinase inhibitor 1 [*Oryza sativa* japonica group] (accession no.: XP_015621143.1) with query coverage 32 % of whole sequence fused with SEKDEL sequence which represents ER retention sequence.

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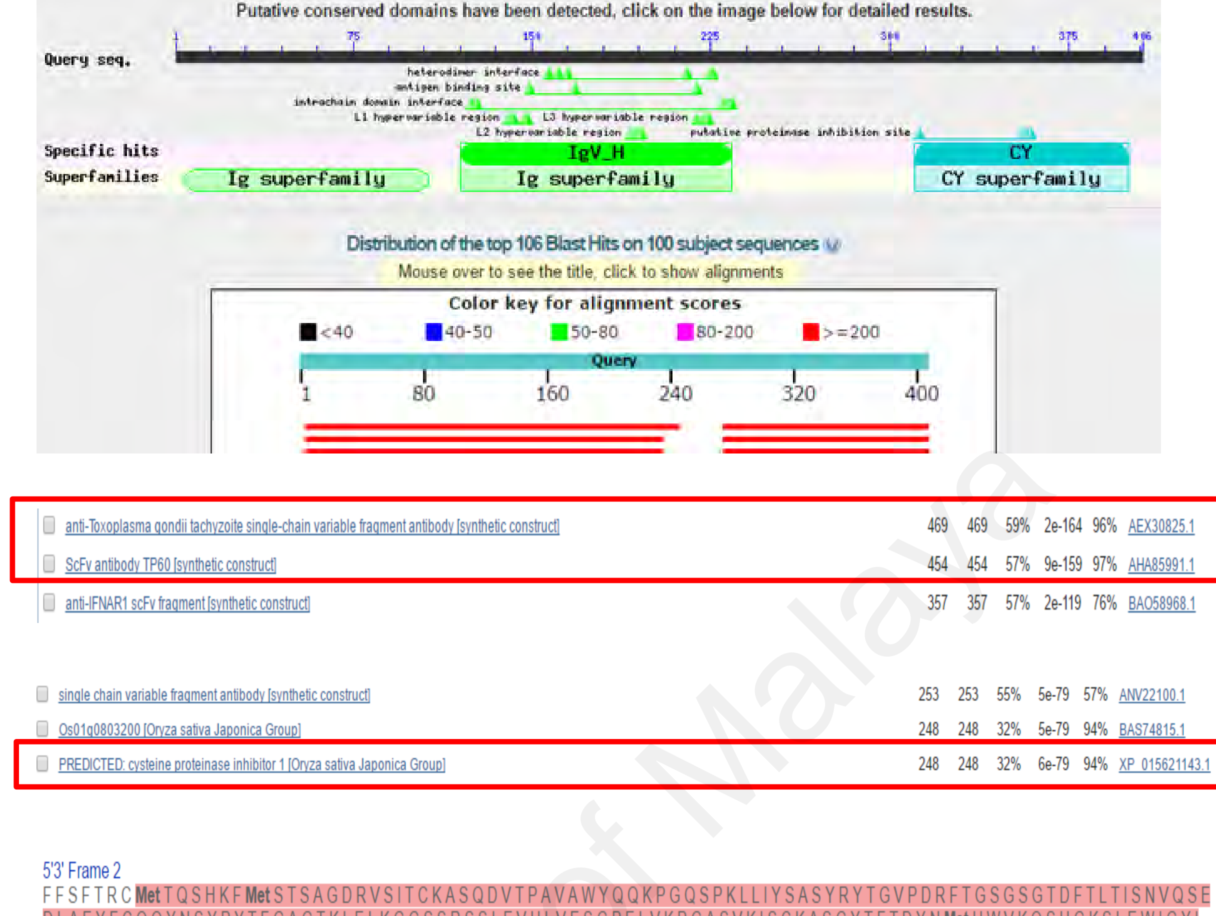

Figure 4.13: BlastP search result of anti-*Toxoplasma* (scFv) recombinant protein and OCPI proteinase inhibitor. Anti-*Toxoplasma* (scFv) recombinant protein conserved domain showed highest similarity (96 % and 97 % maximum identity) with anti-*Toxoplasma gondii* tachyzoite single-chain variable fragment antibody (accession no.: AEX30825.1) and scFv antibody TP60 [synthetic construct] (accession no.: AHA85991.1) with query coverage of 59 % and 57 % respectively of whole sequence. Unfused OCPI (CY) superfamily protein domain showed highest similarity (94 % maximum identity) with cysteine proteinase inhibitor 1 [*Oryza sativa* japonica group] (accession no.: XP_015621143.1) with query coverage 32 % of whole sequence. SEKDEL sequence represents ER retention sequence.

4.3 Transformation of *N. tabacum* cv. SR1 leaf discs mediated by *A. tumefaciens*

4.3.1 Effects of different plasmid constructs towards regeneration of putative transformed shoots

All non-transformed tobacco explants were able to regenerate showing the highest score (+++++) in shoot regeneration on TSM media in the absence of antibiotics. Certain edge of the explants also formed calluses due to the presence of auxin and cytokinin in the media. In addition, least number of shoots regenerated on media containing antibiotics especially on the mid-vein of the explants which might be due to the explants' least exposure of the to the antibiotic which led to the low selection pressure (20 % false positives) (Table 4.1 and Figure 4.1). LD_{80} (20 mg/L) has been selected instead of LD_{100} (at the minimum of 30 mg/L) to ensure the survival of all possible putative transformed shoots. This is to avoid the stringent selection of antibiotics that would inhibit the growth of transformants.

Based on the scoring results in Table 4.2, explants that have been transformed with vector, pCAMBIA 1304 without any inserts showed higher number of regenerated shoots as compared to those transformed with genes of interest (recombinant protein in the presence of proteinase inhibitor and/ or KDEL (KDEL, BBI, BBIKDEL and OCPIKDEL). This shows that the vector did not interfere with shoot regeneration.

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Table 4.3: The effect of different plasmid constructs towards the regeneration of putative transformed shoots on selection media (TSM-containing 20 mg/L hygromycin) after one month of culture.

Constructs	Replicate 1	Replicate 2	Replicate 3
Non-transformed (MS media)	+++++	+++++	+++++
Non-transformed (Selection media)	-	+	+
pCAMBIA 1304	+++++	+++++	++++

Table 4.3, continued.

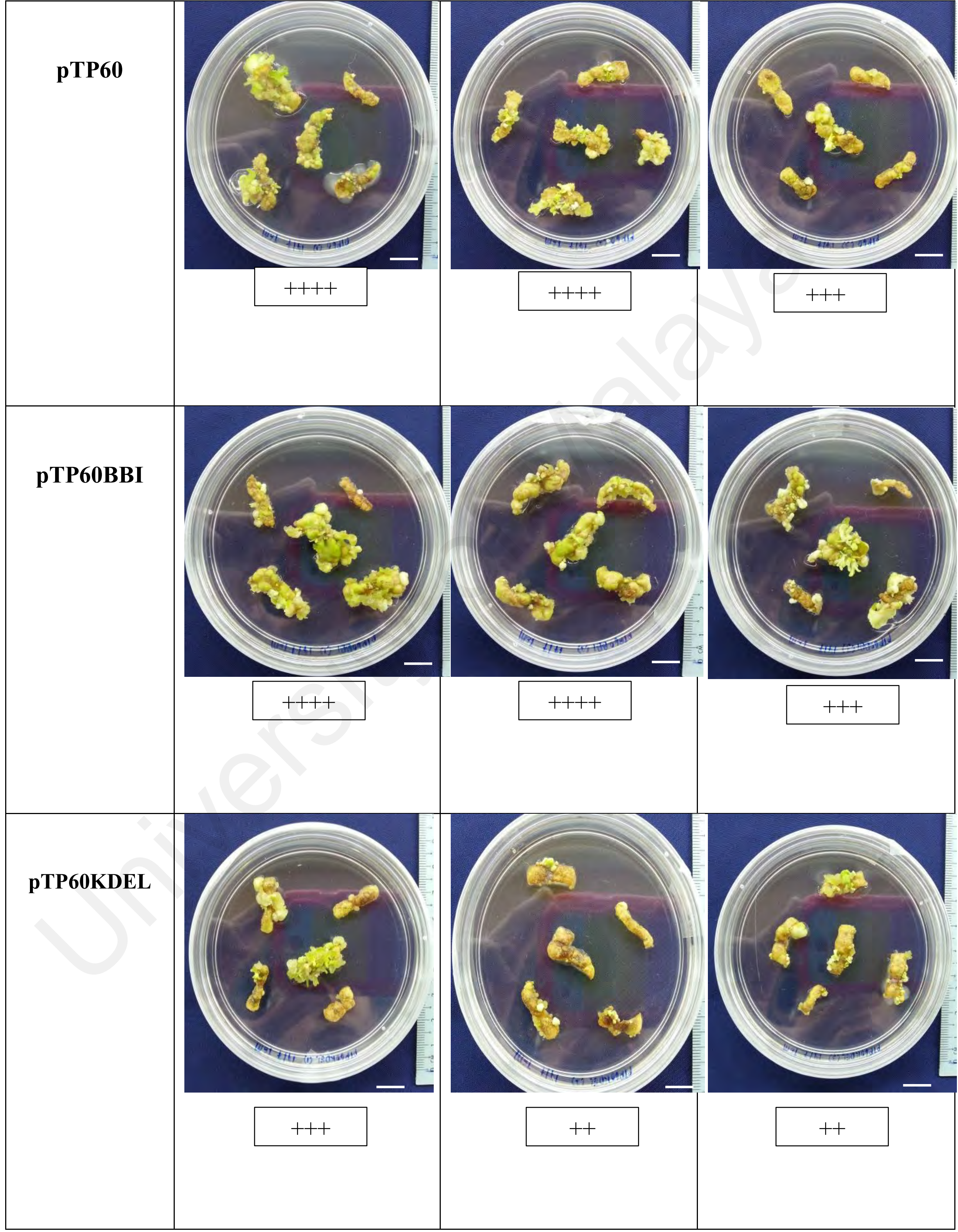
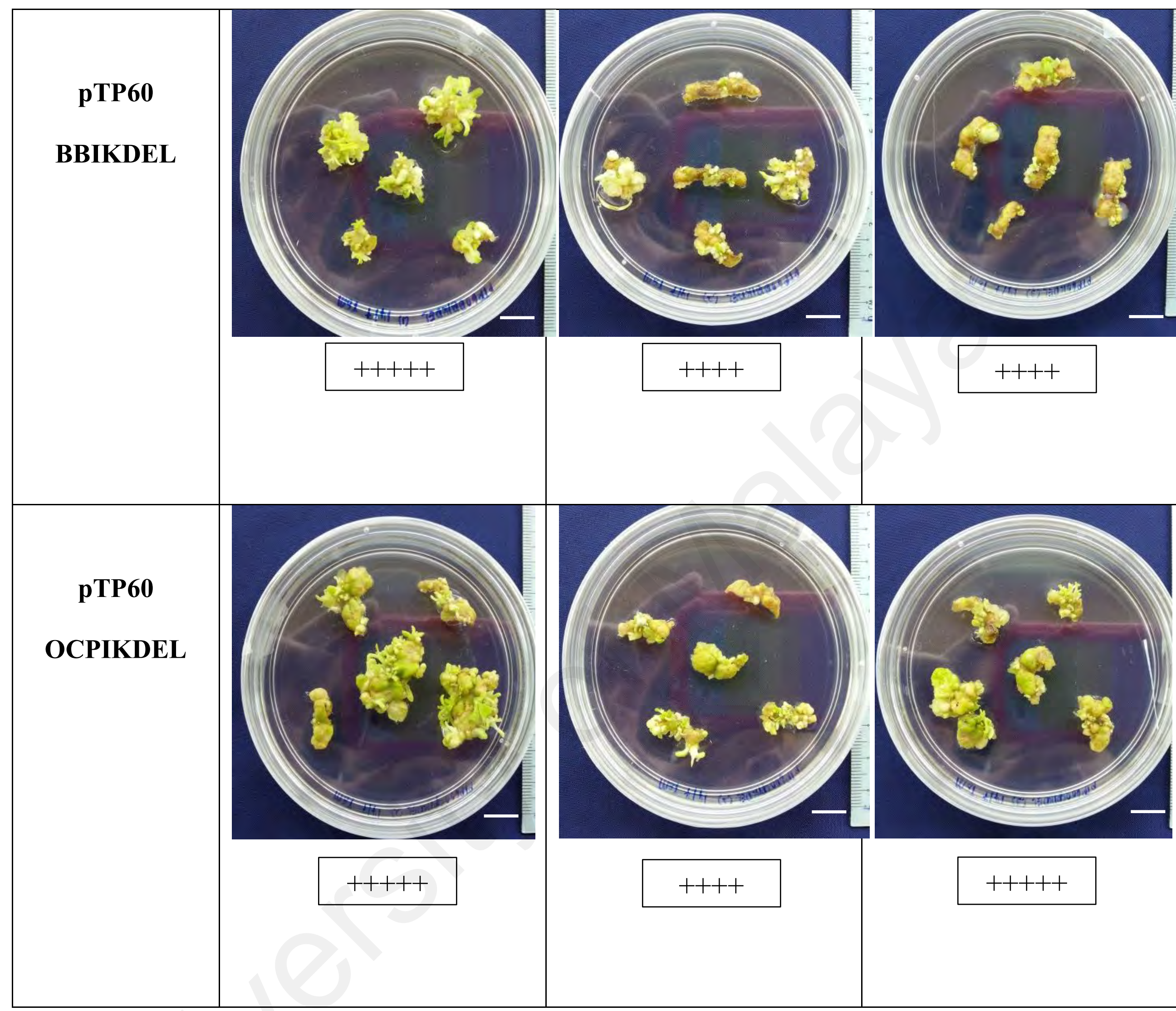


Table 4.3, continued.



Key:

+++++	Highest score of responsive explants
++++	Higher score of responsive explants
+++	Lower score of responsive explants
++	Low score of responsive explants
+	Lowest of responsive explants
-	None responsive explants

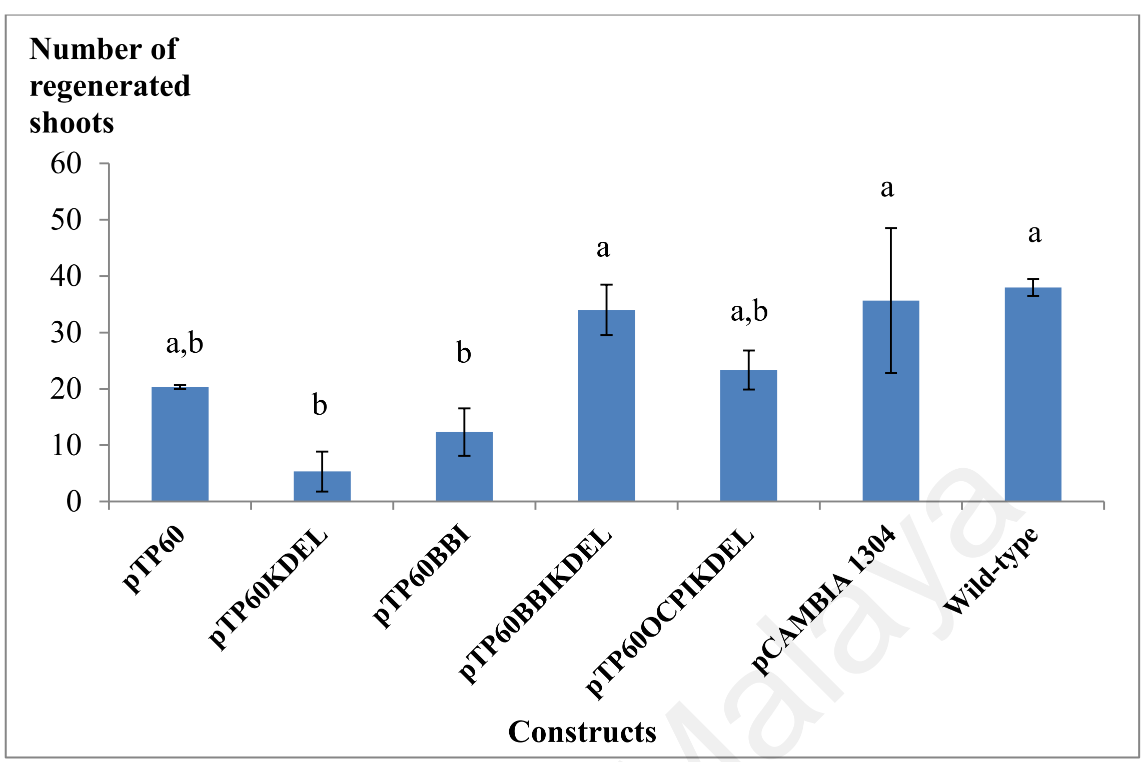


Figure 4.14: Number of regenerated shoots versus different constructs. Significant difference ($p < 0.05$) between putative transformed shoots according non-parametric test, Kruskal-Wallis and Duncan test. Bar indicates the standard error (SE).

After 10 weeks of selection, the number of shoots regenerated (~1 cm length) from explants (5 explants per replicate, triplicate) were recorded and analyzed statistically using one-way ANOVA and Duncan Multiple range test at significance level of $p <$ 0.05 (Appendix F). Based on Figure 4.14, results showed that explants transformed with empty vector, pCAMBIA 1304 without gene of interest produced (35.67 ± 12.86) shoots followed by descending shoots number of pTP60BBIKDEL (34 ± 4.16), pTP60OCPIKDEL (23.33 ± 3.48), pTP60 (20.33 ± 0.33), pTP60BBI (12.33 ± 4.18), and pTP60KDEL (5.33 ± 3.53) on selection media.

In addition, the co-expression of *TP60* gene with only BBI or KDEL element showing a significant different in the regeneration of shoots compared to transformation with pCAMBIA 1304. The co-expression of *TP60* gene in the combination of both BBI and KDEL showing higher in shoot regeneration compared to the element on its own

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and even as good as wild-type. This result also similar for the explants transformed with only *TP60* gene or the co-expression of *TP60* gene with OCPI and KDEL elements in which no significant different was observed compared to wild-type.

This could be concluded that recombinant gene of anti-*Toxoplasma* with the companion of proteinase inhibitor and ER- retention signal (OCPIKDEL and BBIKDEL) do not affect the plant growth especially in shoots regeneration.

4.3.2 Confirmation of putative transformed shoots using transient assays

4.3.2.1 GUS histochemical staining

GUS histochemical staining is a qualitative assay for transient gene expression and also allows tissue-and cell specific localization. This assay was conducted *in situ* with the chromogenic substrate (X-gluc) resulting in deposition of an insoluble blue-precipitate. Regardless of T-DNA truncation during the transformation, the formation of insoluble blue-precipitate indicates the localization of the transgene due to the successful of transformation. GUS assay was conducted on the expanded leaves of ~ 10 week-old putative transformed shoots.

Based on Figure 4.15, the regenerated shoots from explants transformed with empty vector, pCAMBIA 1304 (positive control) produced the most intense blue-GUS spots per expanded leaf area (Figures 4.15a-b). The blue intensity of regenerated shoots from explants transformed with pTP60 (Figure 4.15c) and pTP60KDEL (Figure 4.15d) were similar as pCAMBIA 1304 putative transformants, even though the number of blue-GUS spot observed per expanded leaf of these constructs are lesser compared to the positive control.

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However, putative transformed shoots of pTP60BBI, pTP60OCPIKDEL, and pTP60BBIKDEL produced least expression of blue-GUS coloration with less intensity compared to the mentioned construct (Figures 4.15e-g). No GUS expression was observed on the expanded non-inoculated regenerants confirmed the successful transformation due to the absence of endogenous GUS gene in *N. tabacum* cv. SR1. The different GUS expression between constructs might be due to the distance of *gus*A gene from promoter after the inserts. The larger the insert size, the lower the GUS expression. Although the GUS expression of control and putative transformants with different constructs was different, quantification of GUS expression is still needed to further verify its expression.

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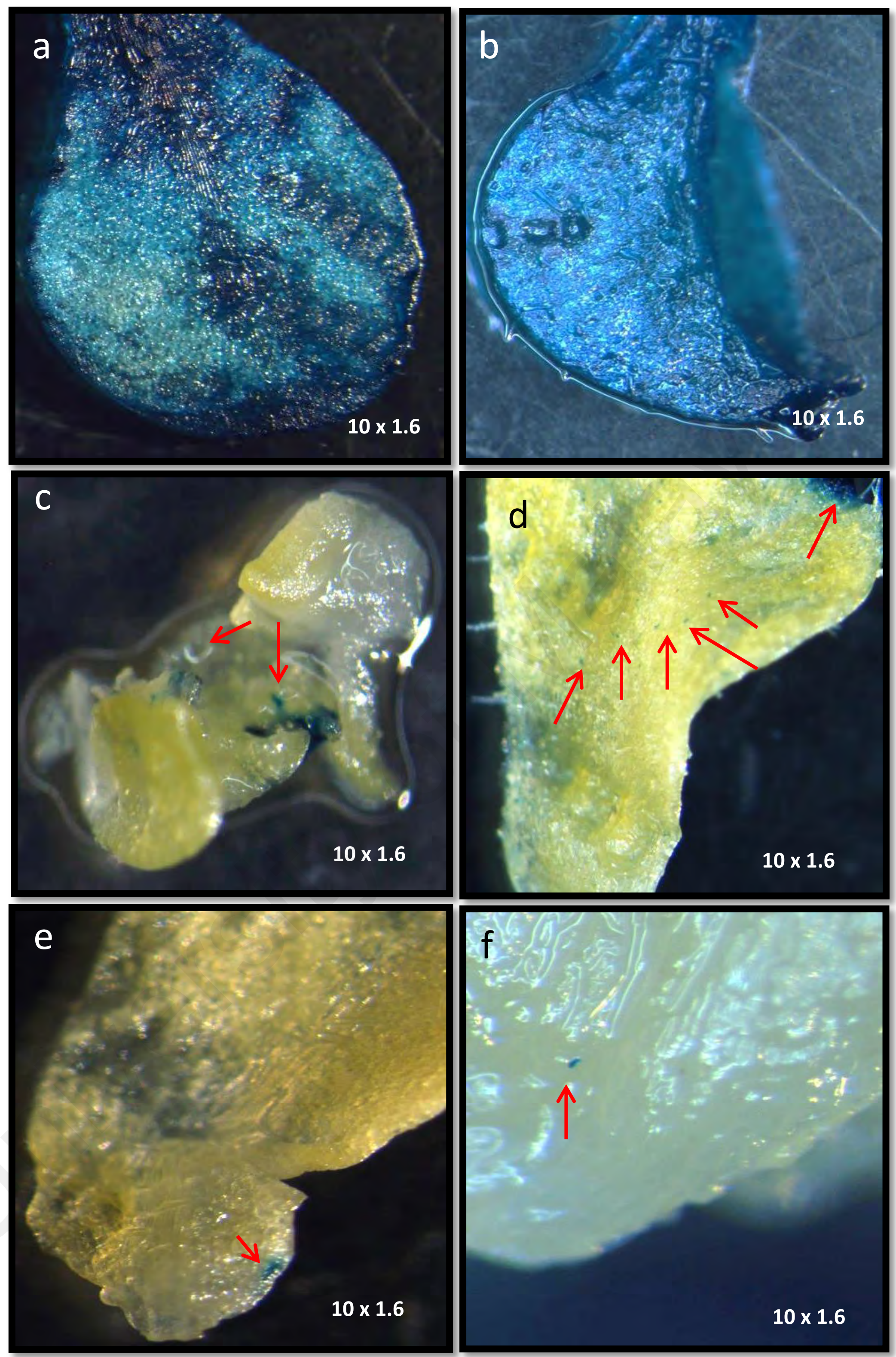

Figure 4.15: GUS histochemical staining of putative transformed tobacco shoots (~ 10 week-old) on TSM-selection media.

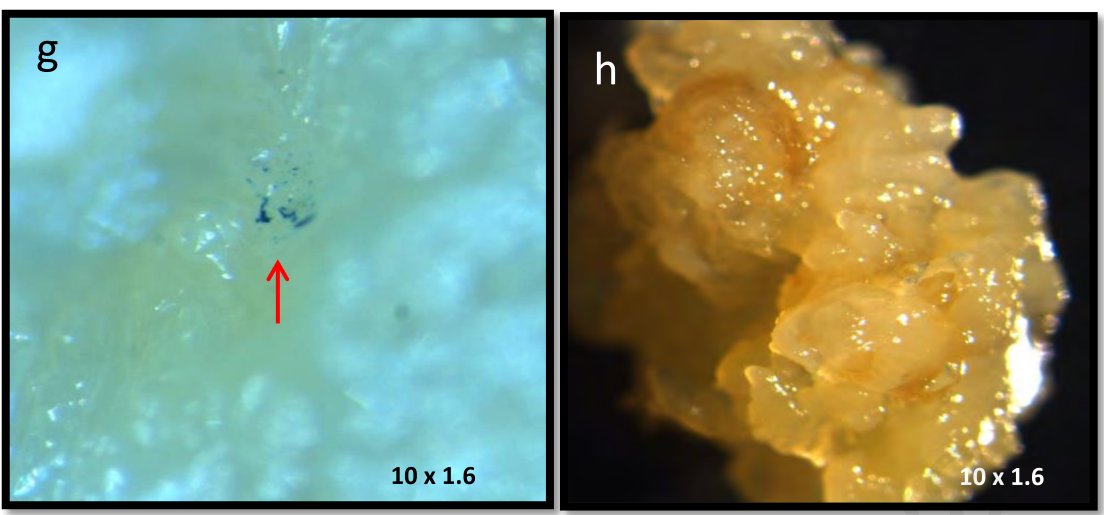

Figure 4.15, continued.

a and b : Inoculated with *A. tumefaciens* harbored with empty vector, pCAMBIA 1304

c : Inoculated with *A. tumefaciens* harbored with pTP60 construct

d : Inoculated with *A. tumefaciens* harbored with pTP60KDEL construct

e : Inoculated with *A. tumefaciens* harbored with pTP60BBI construct

f : Inoculated with *A. tumefaciens* harbored with pTP60OBBIKDEL construct

g : Inoculated with *A. tumefaciens* harbored with pTP60OCPIKDEL construct

h : Non-inoculated regenerant; as negative control

4.3.2.2 GUS fluorometry assay

GUS fluorometry assay is a quantitative assessment of GUS activity with fluorogenic substrates, 4-MUG which would be hydrolyzed to form 4-MU protein product in the presence of β-glucuronidase enzyme encoded by *gus*A gene (Figure 4.16). The reading of GUS activity was calculated over the total protein concentration in 100 mg samples. The total and 4-MU protein concentrations were calculated based on standard curve obtained from known concentrations of BSA and 4-MU (Figures 4.17 and 4.18 respectively).

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Figure 4.16: Quantitative assessment of GUS activity with fluorogenic substrates. 4-MUG which would hydrolyzed to form 4-MU protein product in the presence of *gus*A gene encoded for β-glucuronidase enzyme.
Adopted from http://biochemistry.wur.nl/pbc/gus-jenway/info3.html

Based on Figure 4.19, 4-MU protein products expression was significantly higher in pCAMBIA 1304 putative transformants with 179.07 ± 0.010 pmole 4-MU min^{-1} mg^{-1} followed by pTP60 (26.76 ± 0.015 pmole 4-MU min^{-1} mg^{-1}), pTP60KDEL (19.58 ± 0.006 pmole 4-MU min^{-1} mg^{-1}), pTP60BBI (7.41 ± 0.002 pmole 4-MU min^{-1} mg^{-1}), and pTP60BBIKDEL (6.00 ± 0.001 pmole 4-MU min^{-1} mg^{-1}). The least 4-MU protein product was expressed by pTP60OCPIKDEL putative transformants with (3.62 ± 0.001 pmole 4-MU min^{-1} mg^{-1}). All samples were corresponding to the wild-type as a baseline reading. The reading was taken in three biological replicates (Appendix G). The expression observed in wild-type might be due to the fluorescent of chloroplast under same excitation and emission wavelength as GUS gene.

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The reading obtained from the quantitative data of GUS expression correlated to the GUS qualitative assay. The expression obtained in wild-type samples might be due to the interference of auto-fluorescence signal from subcellular organelles or other pigments within plant cells which excited and emitted at the same wavelength as GUS protein.

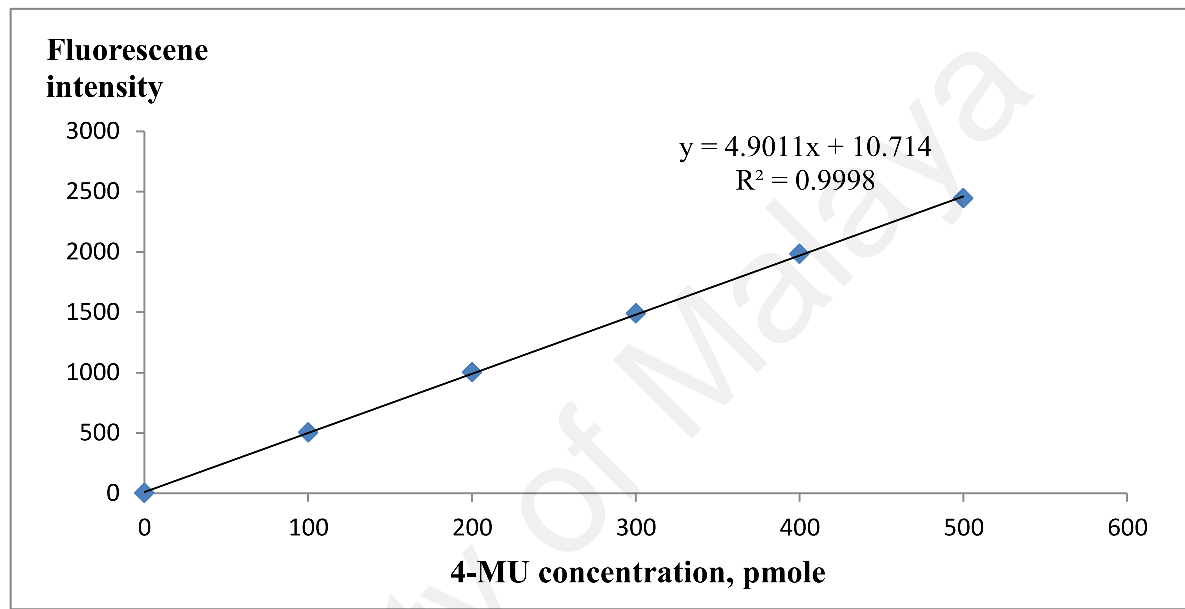

Figure 4.17: Standard curve of fluorescence intensity versus known 4-MU concentration.

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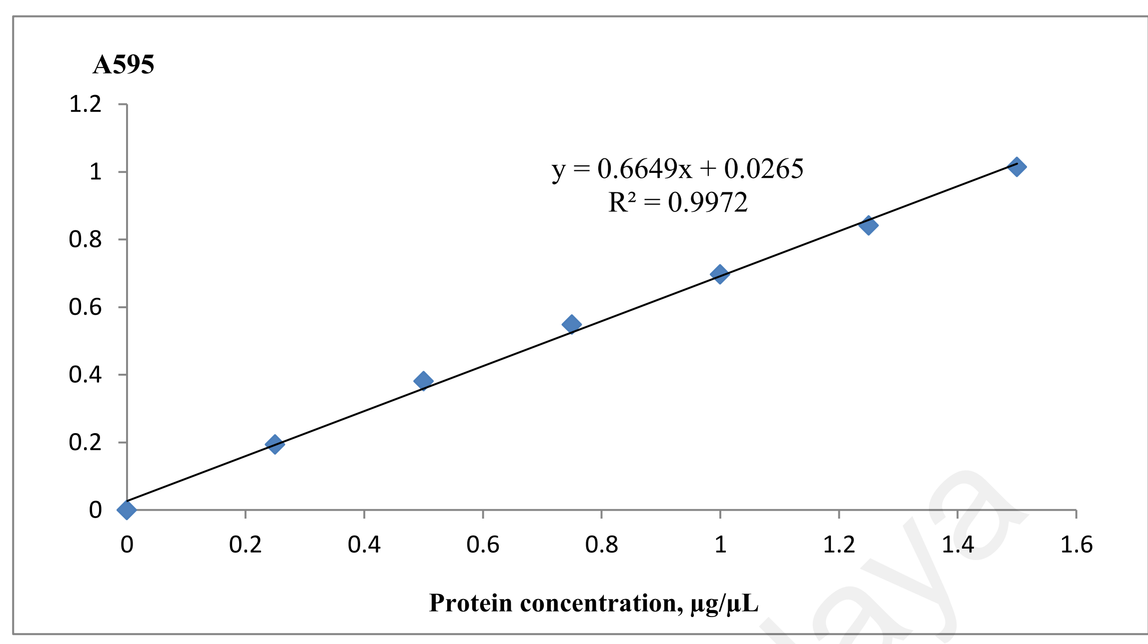

Figure 4.18: Standard curve of known concentration BSA using Bradford's assay quantification.

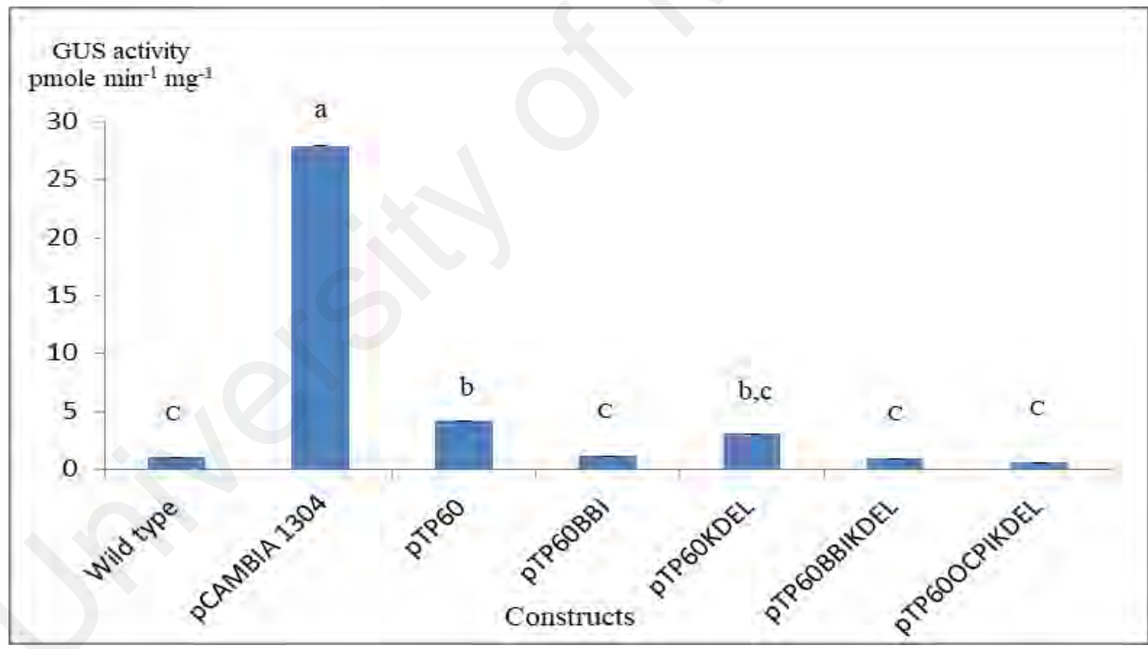

Figure 4.19: GUS activity, (pmole per min) in 100 mg of putative transformed shoots transformed with different plasmid constructs. All sample readings were taken in response to the wild-type as negative control. Significant difference ($p < 0.05$) between putative transformed shoots according to non-parametric test, Kruskal-Wallis and Duncan test. Bars indicate the standard error (SE).

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4.3.2.3 GFP qualitative assay

Green fluorescent protein encoded by *mgfp*5 gene is a reporter gene that has widely been used due to several advantages provided by this gene compared to GUS reporter gene. *GFP* reporter gene is easier to be screened and non-destructive compared to *GUS*.

The expectation of green fluorescence signal observed on the expanded putative transformed leaves was correlated with the presence of *mgfp*5 gene. Positive green fluorescence signal of GFP on putative transformed *N. tabacum* cv. SR1 shoots within merged images of upper and lower epidermis using software (LAS AF version 2.3.1 build 5194 SP5) are shown in Figure 4.20. The green fluorescence signal formation was observed on the expanded leaf of putative transformed shoots under excitation and emission wavelength of 488 nm and 550 nm, respectively, using confocal laser scanning microscope. The non-transformed region of the same expanded putative transformed shoots showed red coloration (Figure 4.20a) due to auto-fluorescence under UV light in the presence of chlorophyll background signal (Goldman et al., 2003; Zhou et al., 2005).

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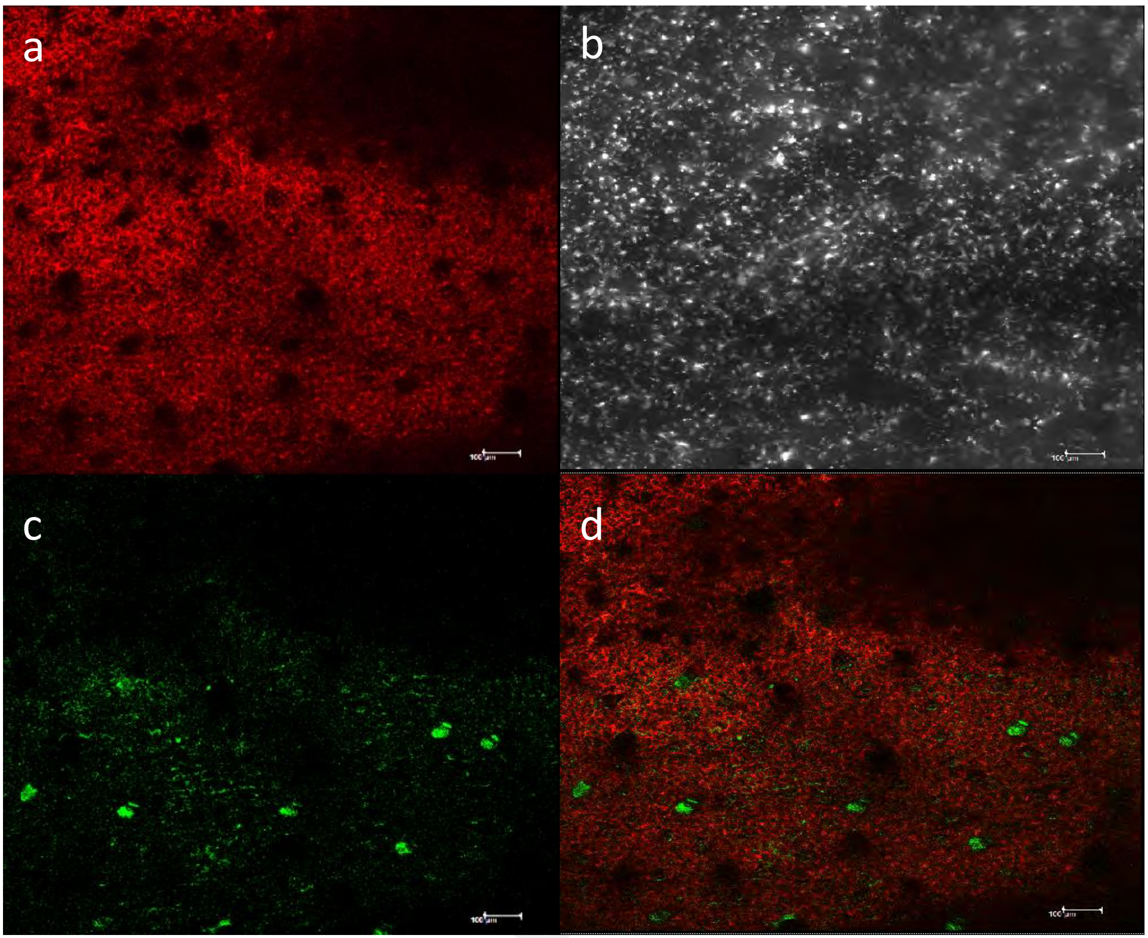

Figure 4.20: Visualization of expanded putative transformed shoots with pTP60 construct under a confocal laser scanning microscope (40X-oil immersion objective lens). Scale bar represents 100 µm.

a : Expanded leaf under CLSM at UV wavelength

b : Expanded leaf under CLSM at bright field state

c: : Expanded leaf under CLSM at GFP ex/em wavelength of 488/550 nm

d : Overlaid images of a and c

4.3.3 Verification of putative transformed shoots (T_0) through PCR analysis

Approximately 10-week-old putative transformed tobacco shoots (T_0) harboring different plasmid constructs were selected and screened using PCR analysis. Total genomic DNA that has been isolated from representative lines shown in Figure 4.21 and Figure 4.22.

Isolated genomic DNA of representative lines from each constructs were screened using PCR analysis with specific primers (Table 3.3). The putatively transformed tobacco shoots with an empty vector was screened using F-RGFP primer. The detection of about 759 bp amplified fragments confirmed the presence GFP within T-DNA region (Figure 4.23). Meanwhile, F-$R_1$1304sk primers were used to screen putatively transformed shoots present with different plasmid constructs (pTP60, pTP60KDEL, pTP60BBI, pTP60BBIKDEL, and pTP60OCPIKDEL). Detection of about ~ 900 bp and ~ 1.5 kb (Figures 4.24 and 4.25, respectively) confirmed the amplified fragment of anti-*Toxoplasma* gene with the companion of different proteinase inhibitor and/or KDEL.

Genomic DNA from wild-type SR1 tobacco plantlets was used as negative control. Plasmid DNA from *A. tumefaciens* was isolated and used as positive technical control. No amplification was detected in wild-type tobacco plantlets using both of the mentioned primers indicated the successful of the transformation.

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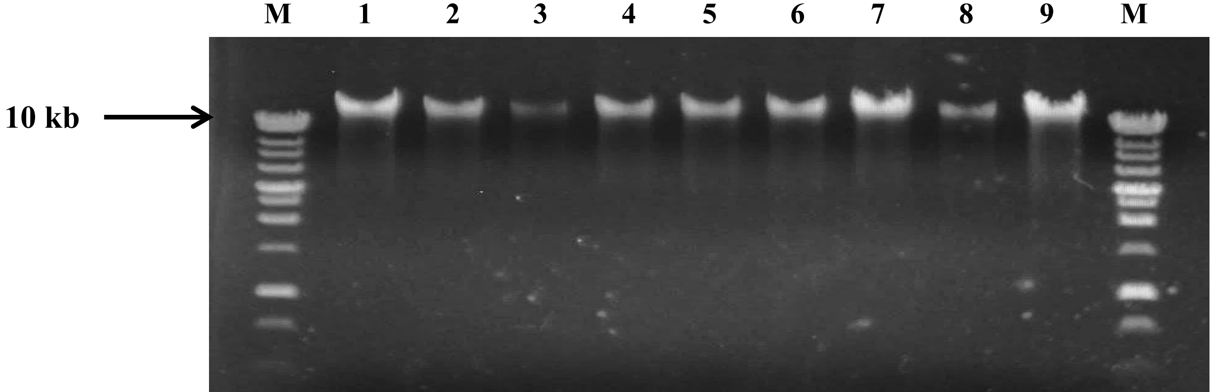

Figure 4.21: Genomic DNA of selected putative transformed tobacco shoots harboring different plasmid constructs after 10 weeks of selection.

Lane M : Perfect Plus^{TM} 1 kb DNA ladder (EUR$_{x,}$ Poland)

Lanes 1-2 : Non-transformed SR1 tobacco plantlets

Lanes 3-4 : Putative transformed shoots harboring pCAMBIA 1304

Lanes 5-6 : Putative transformed shoots harboring pTP60 construct

Lanes 7-9 : Putative transformed shoots harboring pTP60KDEL construct

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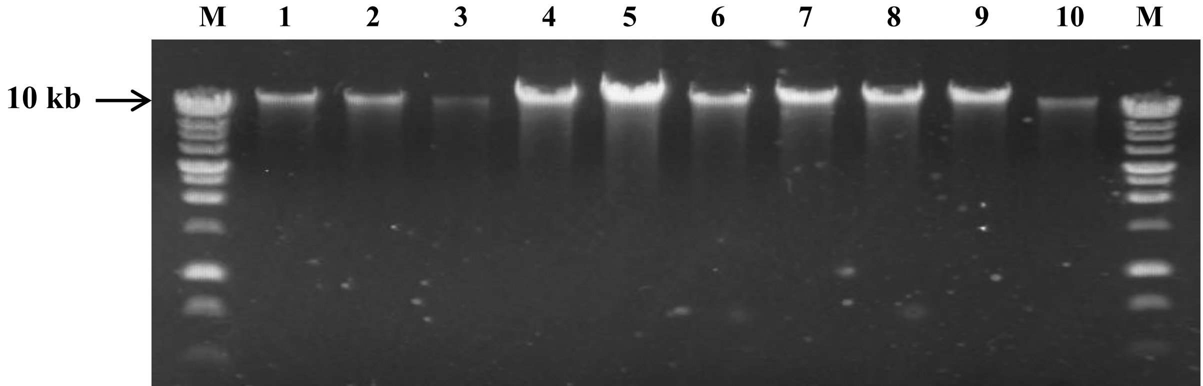

Figure 4.22: Genomic DNA of selected putative transformed tobacco shoots harboring different plasmid constructs after 10 weeks of selection.

Lane M : Perfect Plus™ 1 kb DNA ladder (EUR_x, Poland)

Lane 1 : Non-transformed SR1 tobacco plantlets

Lanes 2-3 : Putative transformed shoots harboring pCAMBIA 1304

Lanes 4-5 : Putative transformed shoots harboring pTP60BBI construct

Lanes 6-7 : Putative transformed shoots harboring pTP60BBIKDEL construct

Lanes 8-10 : Putative transformed shoots harboring pTP60OCPIKDEL construct

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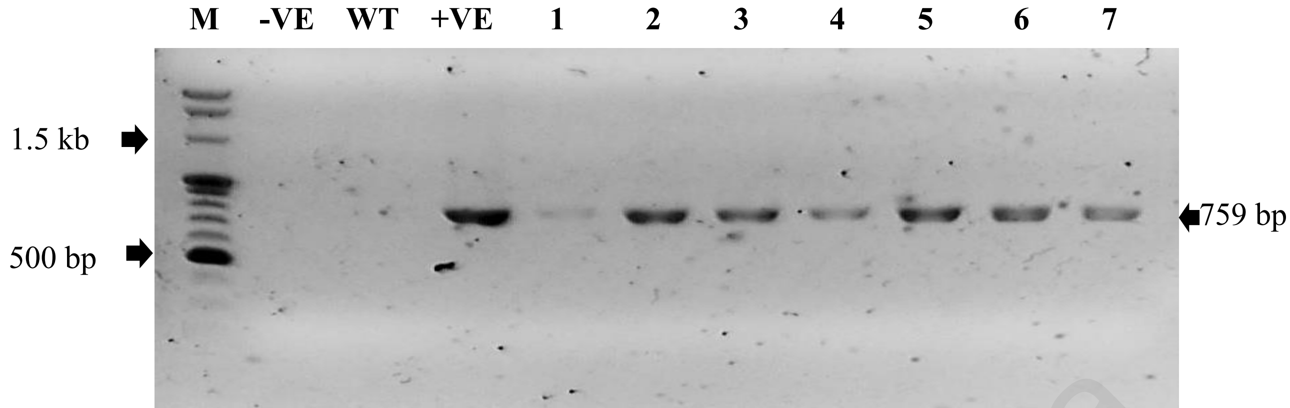

Figure 4.23: PCR confirmation of putative transformed shoots using F-RGFP primers with the expected size of 759 bp.

Lane M : Perfect™ 100 bp DNA ladder (EUR_x, Poland)

Lane -VE : Negative technical control

Lane WT : Negative biological control (wild-type)

Lane +VE : Positive control

Lanes 1-7 : Putative transformed shoots harboring pCAMBIA 1304

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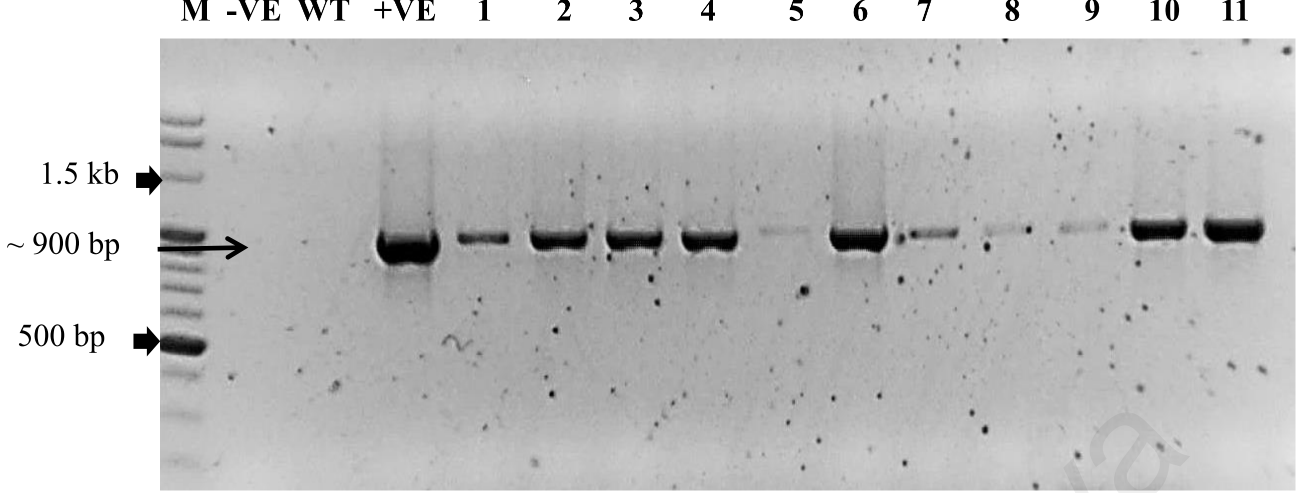

Figure 4.24: PCR confirmation of putative transformed shoots using F-$R_1$1304sk primers with the expected size of ~ 900 bp.

Lane M : Perfect™ 100 bp DNA ladder (EUR_x)

Lane -VE : Negative technical control

Lane WT : Negative biological control (wild-type)

Lane +VE : Positive control

Lanes 1-5 : Putative transformed shoots harboring pTP60 construct

Lanes 6-11 : Putative transformed shoots harboring pTP60KDEL construct

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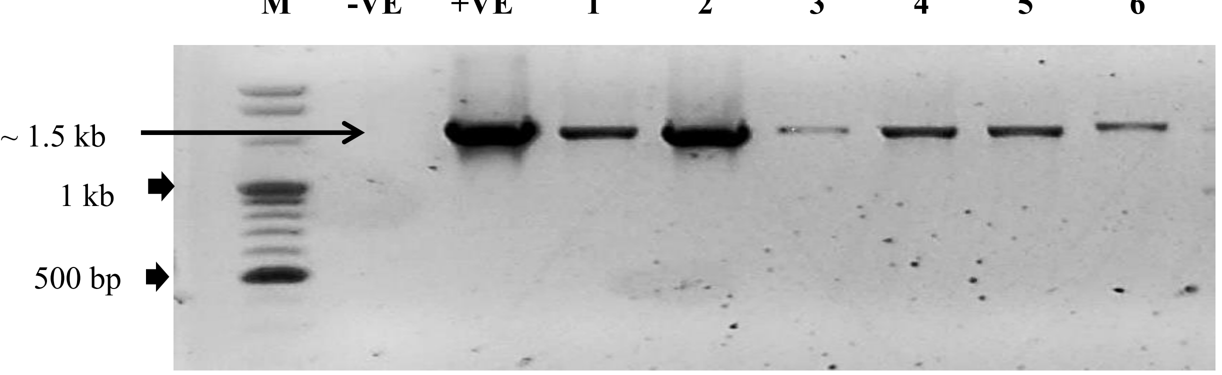

Figure 4.25: PCR confirmation of putative transformed shoots using F-$R_1$1304sk primers with the expected size of ~ 1.5 kb.

Lane M : Perfect™ 100 bp DNA ladder (EUR_x)

Lane -VE : Negative technical control

Lane +VE : Positive control

Lanes 1-2 : Putative transformed shoots harboring pTP60BBI construct

Lanes 3-4 : Putative transformed shoots harboring pTP60BBIKDEL construct

Lanes 5-6 : Putative transformed shoots harboring pTP60OCPIKDEL construct

4.4 Assessment of gene stability T_1 generation of transgenic tobacco

4.4.1 Effect of different plasmid constructs on seed germination

T_1 seedlings that have been transformed with different constructs were cultured on selection media for one week before recording their percentage of germination (Figure 4.26) (Appendix H). The percentage of germination was significantly lowest (65 % ± 2.03) in transgenic seeds transformed with the empty vector pCAMBIA 1304 compared to wild-type and other constructs. There was a possibility that there was a delay in germination (after 7 days) since the observation was only made within 7 days of

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culturing period on selection media. Low germination could be due to selective pressure caused by hygromycin.

Constructs with proteinase inhibitor and KDEL elements, pTP60BBIKDEL and pTP60OCPIKDEL recorded the highest percentage of germination (90 % ± 1.45) whereas T_1 seeds with pTP60BBI alone and pTP60KDEL were 85 % ± 1.76 and 75 % ± 1.20 respectively. The presence of proteinase inhibitors together with KDEL were shown to improve the germination (Yan et al., 2009) possibly by preventing the degradation of endogenous protein involved in plant growth and development against foreign proteins. Besides that, this mechanism also may be due to naturally isolated proteinase inhibitors from plants (regardless of the plant species) which may not be affecting the plant growth. Interestingly, the percentage of germination for T_1 seeds with pTP60 alone was similar with the wild-type germinated on non-selection media 83 % ± 0.88 and 80 % ± 1.67 respectively. This indicated that the anti-*Toxoplasma* protein did not interfere with the growth of the tobacco plant itself which is in accordance to previous study (Ahmad et al., 2012).

T_1 seedlings were assessed after one month of germination in selection media. In the presence of hygromycin in the media, the wild-type seedlings grew with abnormal phenotype (pale green color cotyledon without root formation) (Figure 4.27a) while the phenotype of the transgenic seedlings became normal plants (Figure 4.27b) which might be due to the selective pressure caused by antibiotic.

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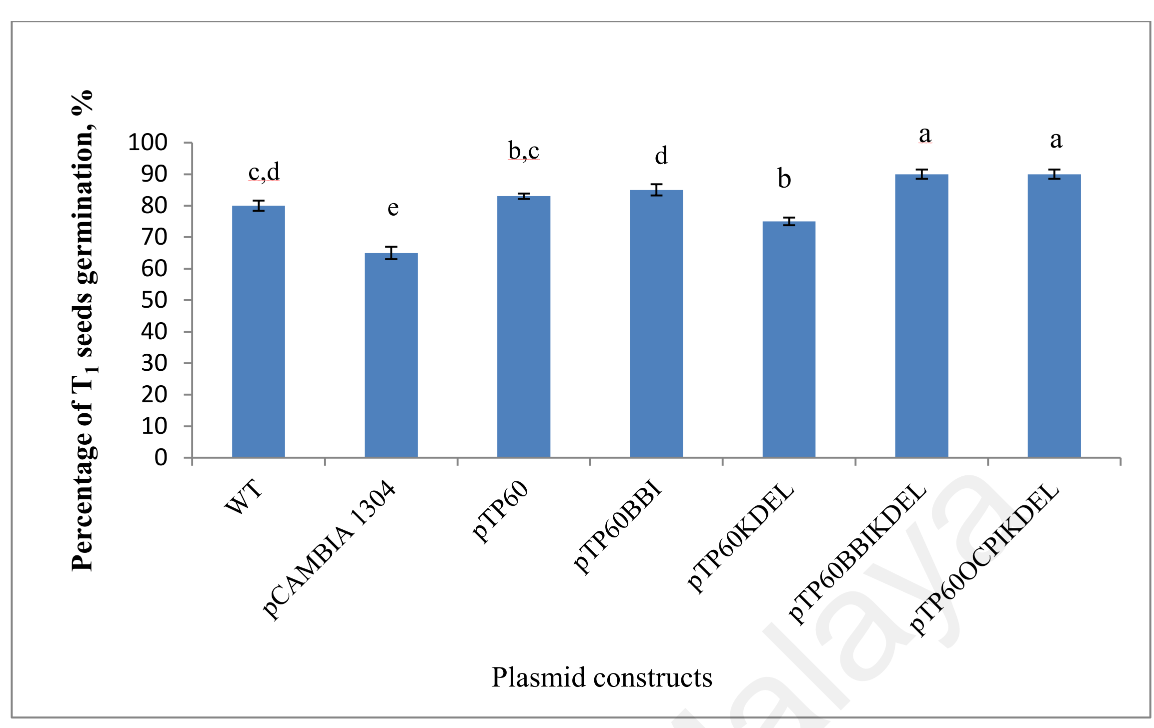

Figure 4.26: The percentage of T_1 seeds germination on 7th days of germination Same letter indicate not significantly different based on one-way ANOVA followed by Duncan's multiple range test at 95 % confident interval level. Experiment was conducted in triplicate for each constructs. Bar indicates the standard error (S.E).

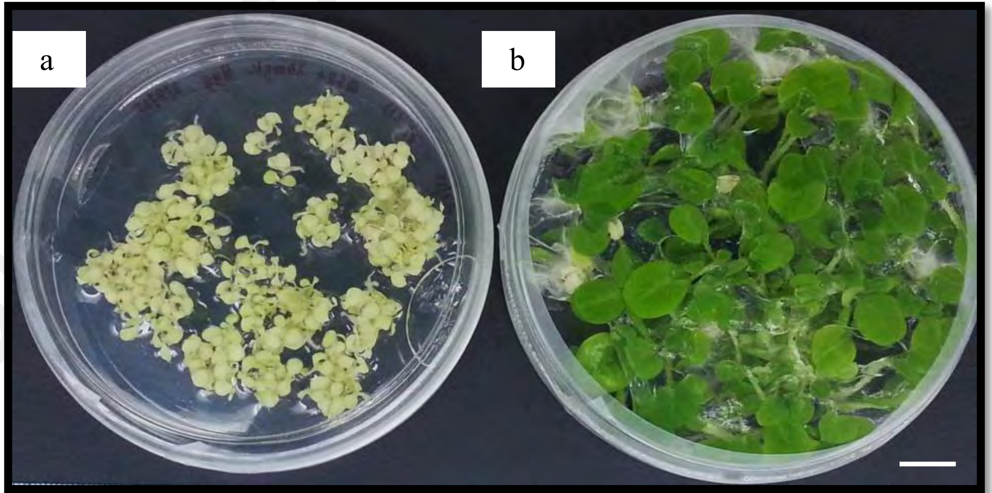

Figure 4.27: Germination of wild-type (a) and pTP60 T_1 seedlings (b) on selection media after 4 weeks of germination. Bar = 1 cm.

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4.4.2 Verification of transgenic seedlings (T_1) through PCR analysis

Approximately, 4 to 6 weeks old of germinated hygromycin-resistant T_1 seedlings were screened using PCR analysis. The stable integration and heritable hygromycin-resistant gene, *hpt*II in T_1 plant genome was confirmed using specific primers, HPT1. Detection of a 559 bp amplified fragments in randomly selected seedlings with different constructs confirmed the presence of transgene in T_1 generation (Figures 4.28 and 4.29).

Similar result was observed in the positive control; neither non-template nor wild-type tobacco showed the similar band at the expected size. At least 20 individuals from three different lines of the different constructs were screened.

No band was observed for germinated T_1 seedlings harboring pTP60BBI construct (Figure 4.29, Lane 1). This is might be due to false-positive because the hygromycin concentration use for selection was at LD_{80}, with 20 % survival rate of non-transformed seedlings. All false-positive seedlings were discarded.

Antibiotic-resistance selection marker gene was designed to locate on the left border (LB) of Ti plasmid to avoid that truncation event in the T_1 generation. The left border of T-DNA may not be inherited in subsequent generation after T_0 (Kemski et al. 2013). T_1 seeds have been produced and obtained according to self-pollination of T_0 tobacco plants, which the confirmation of the insert, *TP60* with the companion of different proteinase inhibitors and/ or KDEL was previously carried out.

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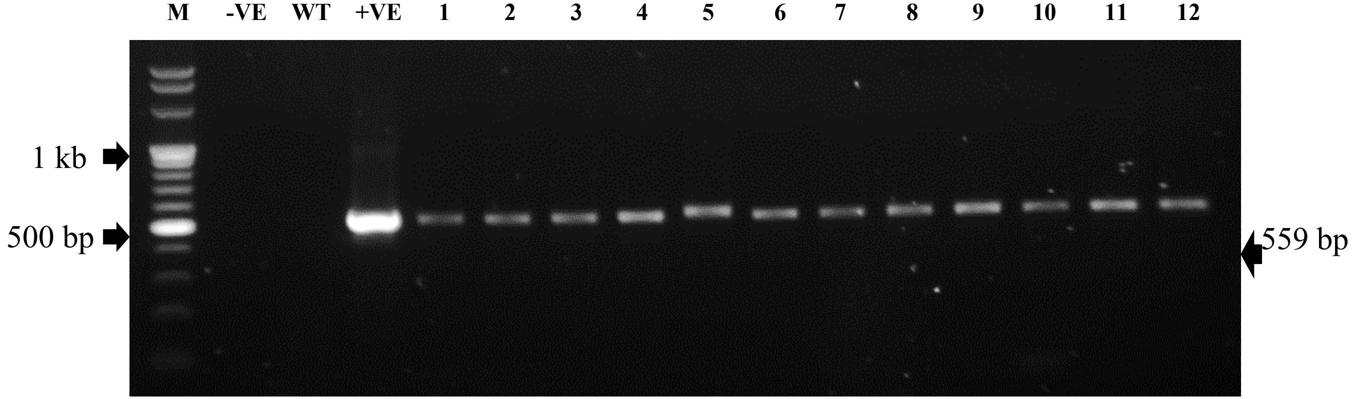

Figure 4.28: PCR confirmation of T_1 seedlings using F-RHPT1 primers with an expected size of 559 bp.

Lanes M	: Perfect™ 100 bp DNA ladder (Eur_x, Poland)
Lane –VE	: Negative technical control
Lane WT	: Wild-type
Lane +VE	: Positive control
Lane 1-4	: Germinated T_1 seedlings harboring pCAMBIA 1304 construct
Lane 5-8	: Germinated T_1 seedlings harboring pTP60 construct
Lane 9-12	: Germinated T_1 seedlings harboring pTP60KDEL construct

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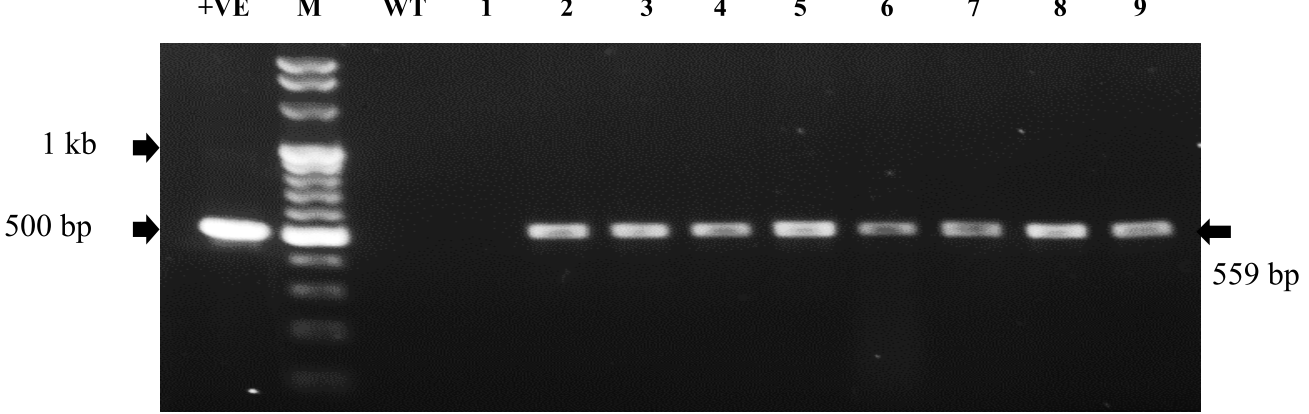

Figure 4.29: PCR confirmation of T_1 seedlings using F-RHPT1 primers with an expected size of 559 bp.

Lane M : Perfect™ 100 bp DNA ladder (Eur_x, Poland)
Lane WT : Wild-type
Lane +VE : Positive control
Lanes 1-3 : Germinated T_1 seedlings harboring pTP60BBI construct
Lanes 4-5 : Germinated T_1 seedlings harboring pTP60BBIKDEL construct
Lanes 6-9 : Germinated T_1 seedlings harboring pTP60OCPIKDEL construct

4.5 Acclimatization of transgenic tobacco plants

At least three individuals (from three different lines of each construct) of PCR positive T_0 tobacco plants and five T_1 transgenic tobacco progenies of each line were acclimatized in transgenic greenhouse (Plant Biotechnology Facility, University of Malaya, Malaysia). The development of transgenic *N. tabacum* cv. SR1 from vegetative to reproductive stages are shown in Figure 4.30. Seeds from transgenic plants were ready for collection within 3 months of acclimatization.

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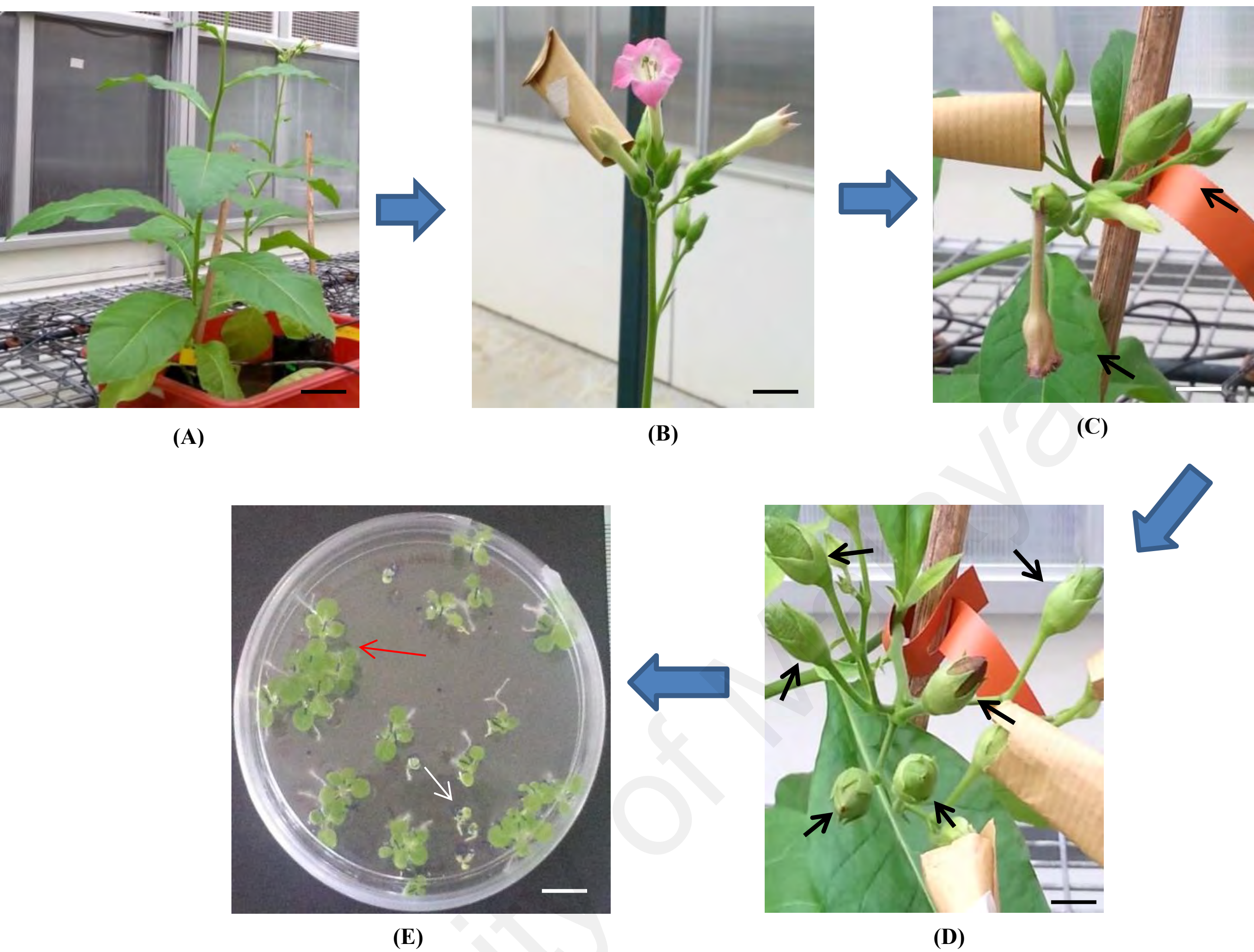

Figure 4.30: The growth stages of transgenic *Nicotiana tabacum* cv. SR1 within 3 months of acclimatization. A) 5 weeks-old acclimatized transgenic tobacco plants; B) Acclimatized transgenic tobacco lines producing flowers; C) Formation of seed pods (black arrows), 4-6 days post-pollination; D) Dried seed pods (black arrows) ready for collection after sepals and stems turned brown; E) 2 weeks-old of germinated transgenic seeds on selection media. Red arrow represents the putative transformed seedlings; while white arrow represents the false-positives showing phenotype abnormality. Bar = 1 cm.

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4.6 Phenotypic assessment on T_1 tobacco progenies

The effect on phenotypic changes of T_1 tobacco progenies transformed with different plasmid constructs were assessed and tabulated in Table 4.3.

Transgenic tobacco plant harboring pTP60 construct showed the highest stem height (11.00 ± 1.22) compared to the others with different plasmid constructs. However, there was no significant different between the wild-type and the former. These non-phenotypic difference in stem height indicated that this anti-*Toxoplasma* protein was not involved in the development of the tobacco plant itself which may be a useful characteristics in biopharming (Appendix I).

The flowering day of wild-type and transformed tobacco were analyzed. The results showed that the flowering day of wild-type tobacco plants was 65 days ± 5.49 (Table 4.4). On the other hand, the earliest flowering day was observed in T_1 tobacco progenies harboring pTP60BBI plasmid construct (45 days ± 2.67). It was also observed that T_1 tobacco progenies presence of pTP60KDEL, pTP60BBIKDEL, and pTP60OCPIKDEL plasmid constructs gave a similar period for the plant to flower in a range of 51-52 days, which non-significantly difference to the one in the presence of pCAMBIA 1304 plasmid construct (49 days ± 3.28) (Appendix J). Interestingly, anti-*Toxoplasma* recombinant protein alone showed non-significant different to wild-type (62 ± 2.08) which is an important factor for biopharming purposes. It may be concluded that the introduction of anti-*Toxoplasma* recombinant protein doesn't affect the flowering period of tobacco plants as compared to wild-type.

Table 4.4: Assessment on stem height of T_1 independent lines (20 days of post acclimatization) and flowering period on transgenic and wild-type tobacco plants.

Constructs	Stem height (cm)	Flowering period (day)
pTP60	11.00 ± 1.22^{a}	$62 \pm 2.08^{a,b}$
pTP60KDEL	3.75 ± 0.92^{c}	$52 \pm 2.33^{b,c}$
pTP60BBI	4.27 ± 0.52^{c}	45 ± 2.67^{c}
pTP60BBIKDEL	$5.31 \pm 1.27^{b,c}$	51 ± 2.96^{c}
pTP60OCPIKDEL	3.27 ± 0.58^{c}	$52 \pm 2.33^{b,c}$
pCAMBIA 1304	4.84 ± 1.59^{c}	49 ± 3.28^{c}
Wild-type	$6.60 \pm 1.43^{a,b}$	65 ± 5.49^{a}

The results represent the mean ± standard error (SE) of three replicated experiments. Different letters indicate significant difference between samples at $p < 0.05$.

4.7 Real-time PCR analysis

4.7.1 RNA extraction and DNase treatment of T_1 tobacco progenies

Leaves of approximately 40-day acclimatized T_1 tobacco progenies were harvested for the downstream expression analysis of anti-*Toxoplasma* gene at mRNA and protein levels. Total RNA of positive transformants was isolated using RNeasy Plant Mini Kit (Qiagen, USA) prior to real-time PCR. The isolated RNA was quantified and listed in Table 4.5. The RNA isolated products (before and after DNase treatment) were electrophoresed on 1 % EtBr stained agarose gel, as shown in Figures 4.31-4.34.

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Detection of bands (28S and 18S rRNA) confirmed successful RNA isolation in regards to both the purity and integrity of RNA. DNase treatment was carried out in order to remove the remaining genomic DNA which may interfere in the expression.

The quantification of RNA before and after DNase treatment was also carried out prior to cDNA conversion. After DNase treatment, the RNA concentration showed a reduction which corresponds to the quantified RNA yield (about 10-fold change) before DNase treatment Table 4.6.

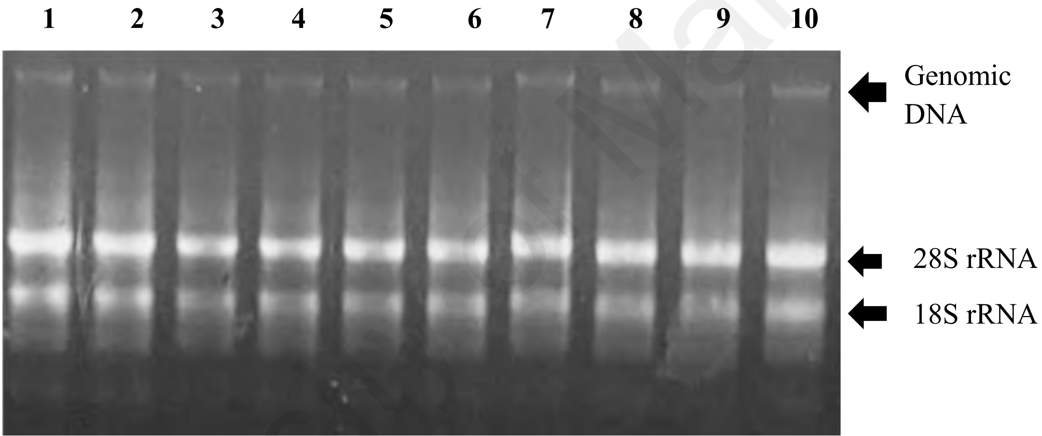

Figure 4.31: 1 µg of non-treated total RNA isolated from 40-day old acclimatized T_1 tobacco progenies.

Lanes 1-3	: T_1 tobacco progenies harboring pTP60 construct
Lanes 4-6	: T_1 tobacco progenies harboring pTP60KDEL construct
Lanes 7-9	: T_1 tobacco progenies harboring pTP60BBI construct
Lane 10	: T_1 tobacco progenies harboring pTP60BBIKDEL construct

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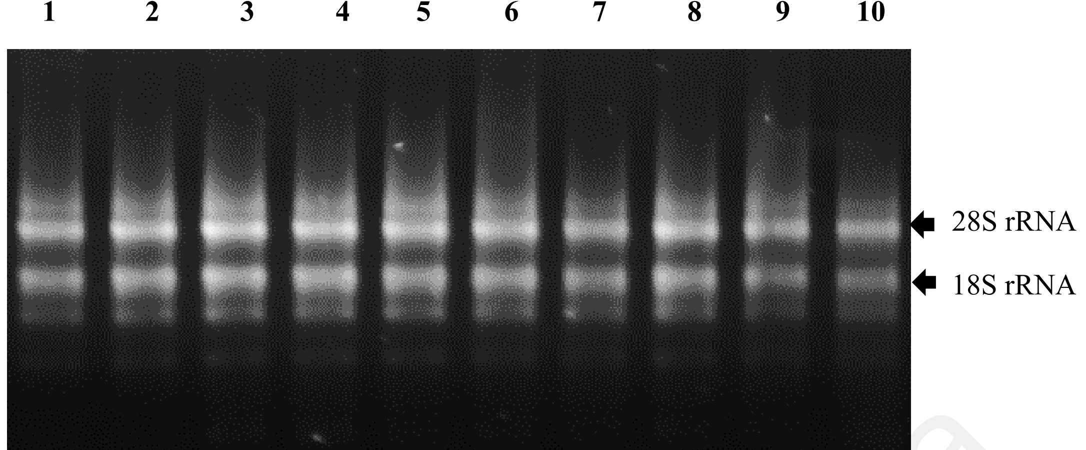

Figure 4.32: 100 ng of DNase-treated RNA isolated from 40-day old acclimatized T_1 tobacco progenies.

Lanes 1-3 : T_1 tobacco progenies harboring pTP60 construct

Lanes 4-6 : T_1 tobacco progenies harboring pTP60KDEL construct

Lanes 7-9 : T_1 tobacco progenies harboring pTP60BBI construct

Lane 10 : T_1 tobacco progenies harboring pTP60BBIKDEL construct

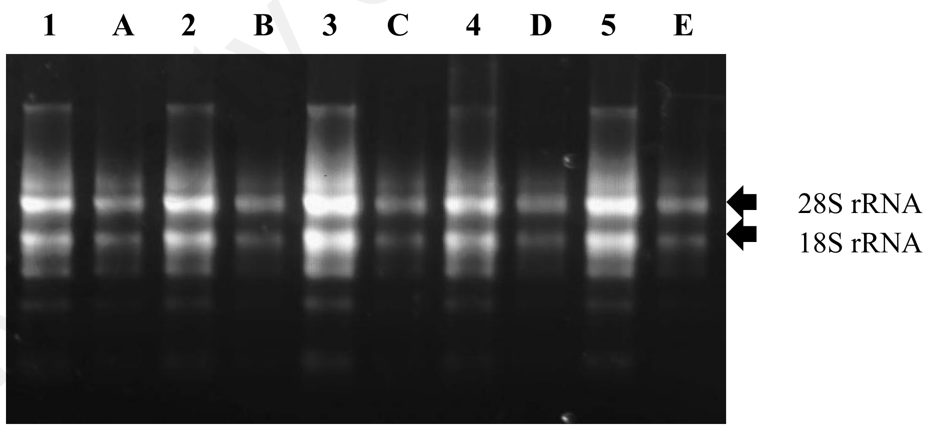

Figure 4.33: 1 μg of non-treated and 100 ng of DNase-treated RNA isolated from 40-day old acclimatized T_1 tobacco progenies.

Lane 1-2 : Non-treated RNA, T_1 tobacco progenies harboring pTP60BBIKDEL construct

Lane 3-5 : Non-treated RNA, T_1 tobacco progenies harboring pTP60OCPIKDEL construct

Lane A-B : Treated RNA, T_1 tobacco progenies harboring pTP60BBIKDEL construct

Lane C-E : Treated RNA, T_1 tobacco progenies harboring pTP60OCPIKDEL construct

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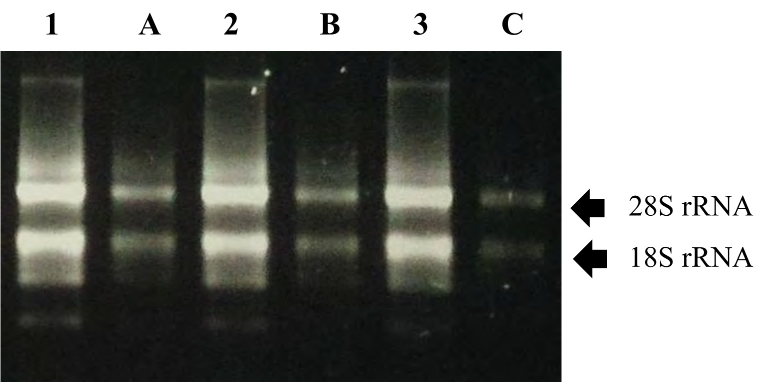

Figure 4.34: 1 µg of non-treated and 100 ng of DNase-treated RNA isolated from 40-day old acclimatized tobacco wild-type.

Lane 1-3 : Non-treated RNA, Wild-type tobacco plants

Lane A-C : Treated RNA, Wild-type tobacco plants

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Table 4.5: $OD_{A260/280}$ and $OD_{A260/230}$ readings of non-transformed and T_1 tobacco progenies before DNase-treatment.

Samples	Concentration, ng/µL	Purity		Samples	Concentration, ng/µL	Purity	
		OD A260/280	**OD A260/230**			**OD A260/280**	**OD A260/230**
Non-transformed (1)	1369	1.98	1.74	pTP60BBI (5)	1422.1	1.90	2.26
Non-transformed (2)	1019.0	1.90	2.34	pTP60BBI (6)	1171.5	1.97	2.25
Non-transformed (9)	1231.6	2.00	2.01	pTP60BBI (7)	1125.8	2.07	2.44
pTP60 (3)	1824.9	1.95	2.10	pTP60BBIKDEL (1)	1281.3	1.96	2.25
pTP60 (12)	970.2	1.92	2.15	pTP60BBIKDEL (2)	1384.1	1.98	2.35
pTP60 (17)	1157.1	1.92	2.47	pTP60BBIKDEL (14)	2296.6	2.02	1.93

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Table 4.5, continued.

pTP60KDEL (6)	1791.2	1.96	2.54	pTP60OCPIKDEL (1)	2647.9	2.01	2.37
pTP60KDEL (7)	1263.9	2.06	2.22	pTP60OCPIKDEL (13)	1810.7	2.00	2.05
pTP60KDEL (8)	2018	2.01	2.27	pTP60OCPIKDEL (15)	1524.8	2.00	2.45

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Table 4.6: $OD_{A260/280}$ and $OD_{A260/230}$ readings of non-transformed and T_1 tobacco progenies DNase-treated RNA.

Samples	Concentration, ng/µL	Purity		Samples	Concentration, ng/µL	Purity	
		OD A260/280	OD A260/230			OD A260/280	OD A260/230
Non-transformed (1)	143.5	2.16	1.65	pTP60BBI (5)	161.7	2.06	2.22
Non-transformed (2)	181.9	2.16	2.21	pTP60BBI (6)	134.8	2.07	2.44
Non-transformed (9)	130.27	2.02	1.47	pTP60BBI (7)	174.5	2.01	2.37
pTP60 (3)	206.2	2.17	1.89	pTP60BBIKDEL (1)	123.9	2.02	2.48
pTP60 (12)	215.2	1.89	2.01	pTP60BBIKDEL (2)	160.4	2.11	1.45
pTP60 (17)	209.2	2.10	1.78	pTP60BBIKDEL (14)	142.13	2.24	1.68

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Table 4.6, continued.

pTP60KDEL (6)	159.03	1.96	1.50	pTP60OCPIKDEL (1)	231.33	2.02	1.93
pTP60KDEL (7)	42.37	2.00	1.87	pTP60OCPIKDEL (13)	149.6	2.04	2.19
pTP60KDEL (8)	125.6	2.34	1.76	pTP60OCPIKDEL (15)	117.7	2.00	2.01

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4.7.2 Relative expression of *TP60* gene using real-time PCR

Prior to qRT-PCR analysis, a dissociation curve for both reference (*N. tabacum* ribosomal large subunit 25, *NtL25*) and target gene (anti-*Toxoplasma*, *TP60*) primers, L25 and FV60 respectively was performed. The dissociation curves for both primers in the screened samples showed a single peak, indicating the specificity of the primer designed (Table 3.4) and dissociated at a specific temperature confirming the single amplicon being produced (Figure 4.35).

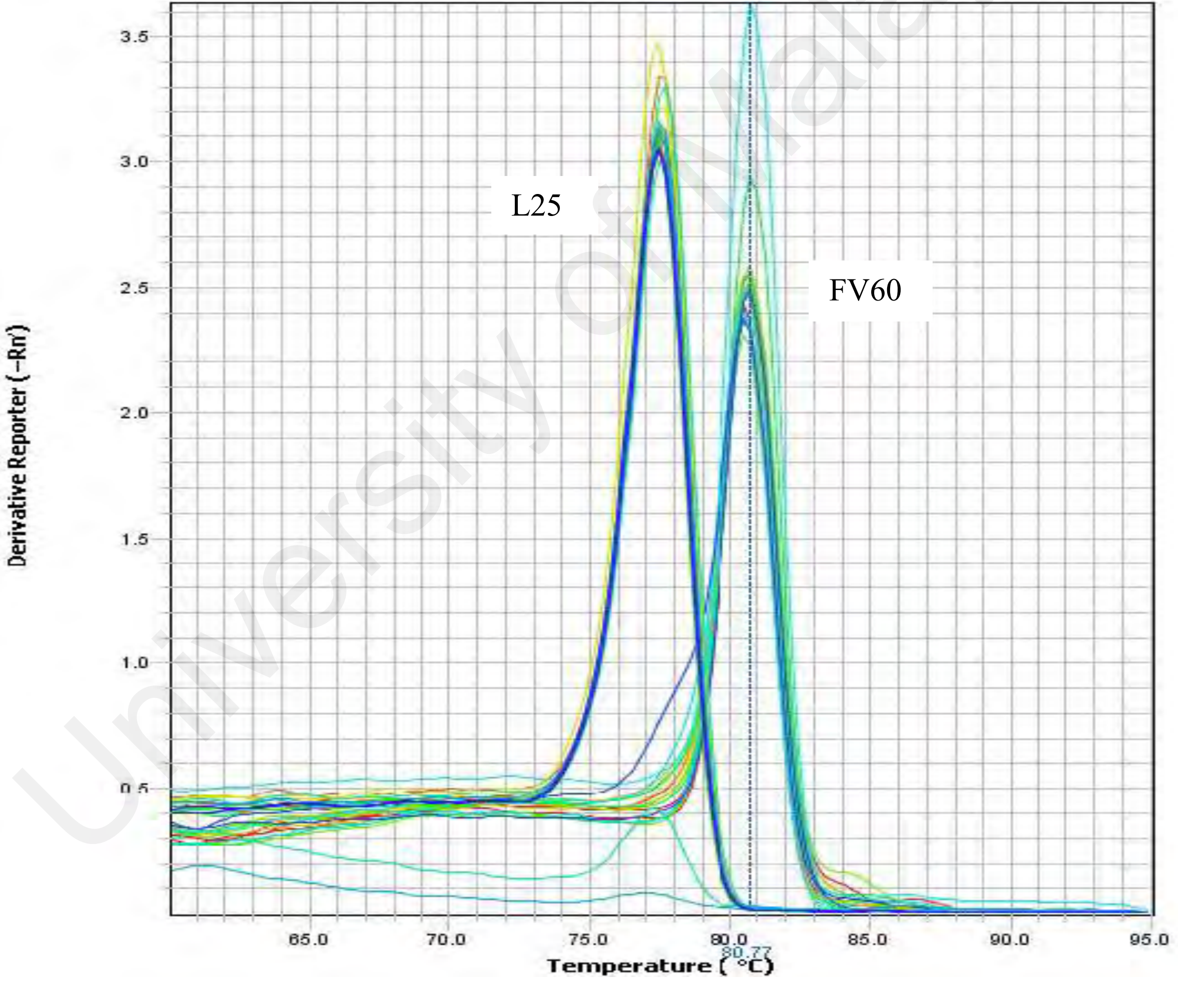

Figure 4.35: Dissociation curve of different primer sets, L25 and FV60 for *NtL25* internal reference gene and *TP60* recombinant gene respectively at 60 °C annealing temperature (derivative reporter versus temperature).

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Three lines for each construct (pTP60, pTP60KDEL, pTP60BBI, pTP60BBIKDEL, and pTP60OCPIKDEL) were used in this study. A representative from each line was selected based on the highest expression of anti-*Toxoplasma* gene at the mRNA level based on relative quantification by using qRT-PCR (tested in 3 different individuals). The list of T_1 tobacco progeny lines used for each construct was tabulated in Table 4.5 and Table 4.6.

The generated data from relative quantification was analyzed using Microsoft Excel. The results are summarized in Figure 4.36 to Figure 4.40. The transgenic line from each construct showing the highest expression of *TP60* gene was selected. The best transgenic line from each construct was re-analyzed by qRT-PCR in order to compare the expression level of *TP60* gene between different constructs. The expression data was recalculated and the data was presented in Figure 4.41. Based on Figure 4.41, the expression level of anti-*Toxoplasma* gene, *TP60* with the companion of proteinase inhibitor and KDEL (pTP60BBIKDEL) showed the highest expression with a 19-fold change, followed by pTP60KDEL (10-fold), pTP60OCPIKDEL (8-fold), and pTP60BBI (2-fold) compared to anti-*Toxoplasma* gene in the presence of neither proteinase inhibitor nor KDEL (Appendix K).

Semi-quantitative data for each construct showed correlation in intensity with the quantified expression from each line for each construct (Figure 4.36 to Figure 4.41).

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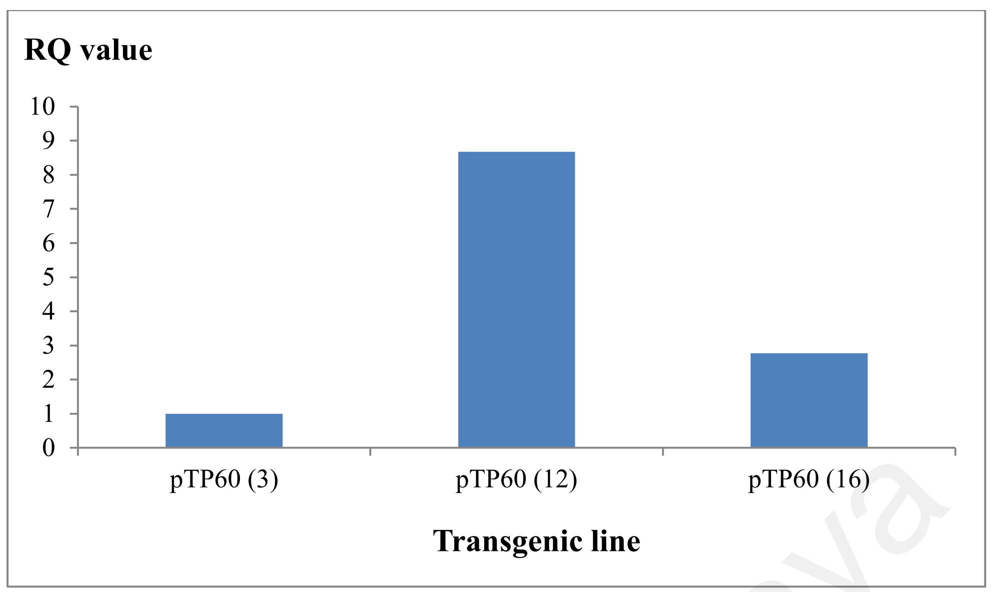

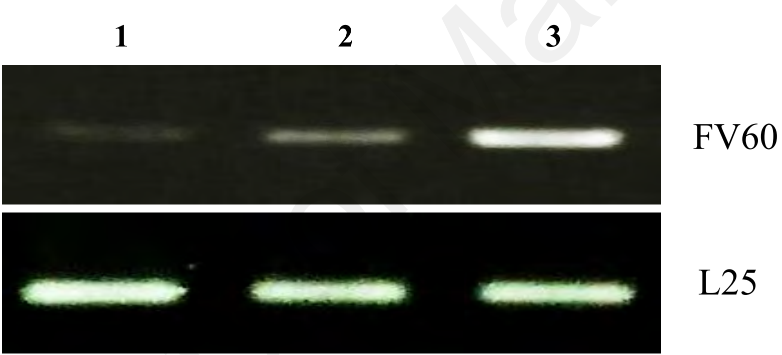

Figure 4.36: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 3, 12, and 16 harboring pTP60 construct. RQ value = Relative expression value on Y axis represents transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60 (3), 2) pTP60 (16), and 3) pTP60 (12). Experiment was conducted in 3 technical replicates.

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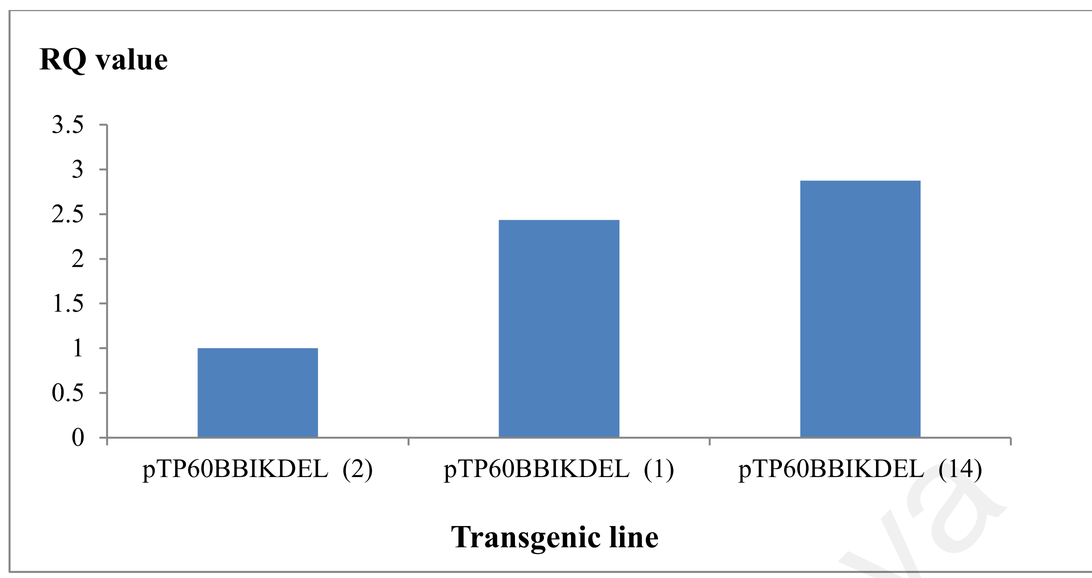

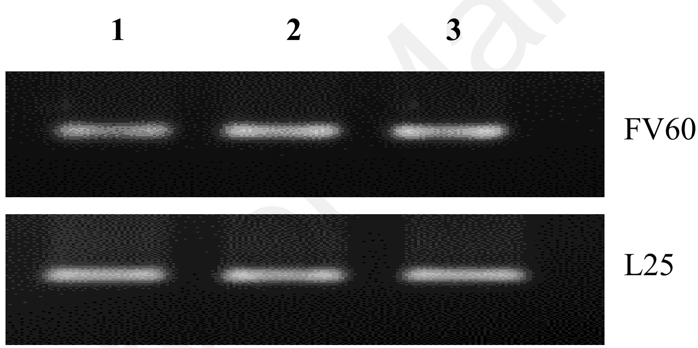

Figure 4.37: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 2, 1, and 14 harboring pTP60BBIKDEL construct. RQ value = Relative expression value on Y axis represents transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60BBIKDEL (2), 2) pTP60BBIKDEL (1), and 3) pTP60BBIKDEL (14). Experiment was conducted in 3 technical replicates.

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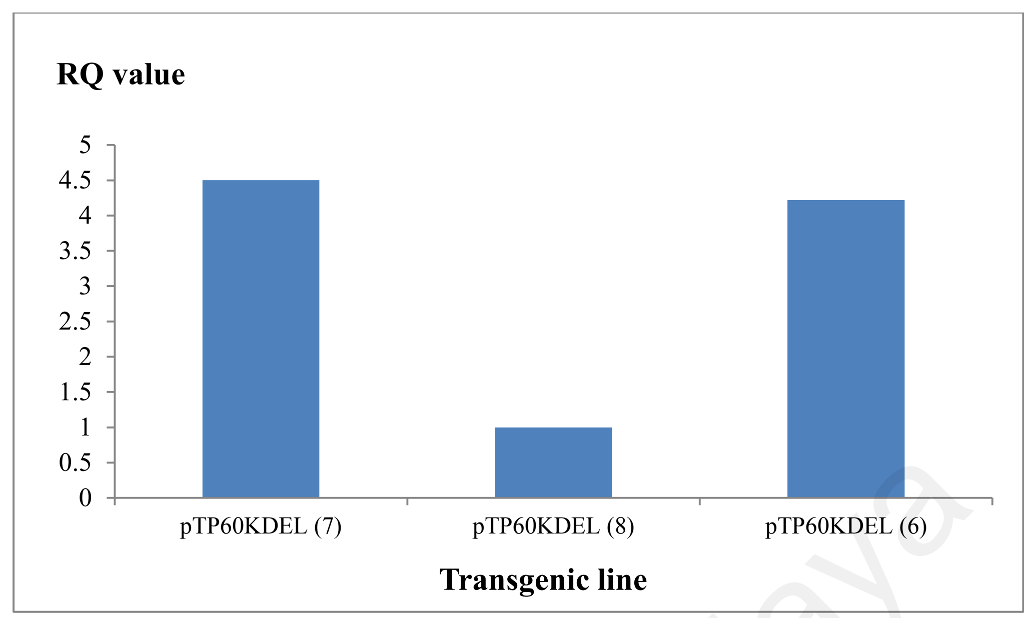

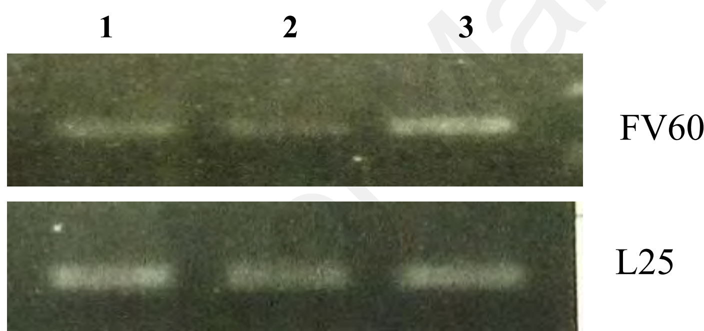

Figure 4.38: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 7, 8, and 6 harboring pTP60KDEL construct. RQ value = Relative expression value on Y axis represents transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60KDEL (7), 2) pTP60KDEL (8), and 3) pTP60KDEL (6). Experiment was conducted in 3 technical replicates.

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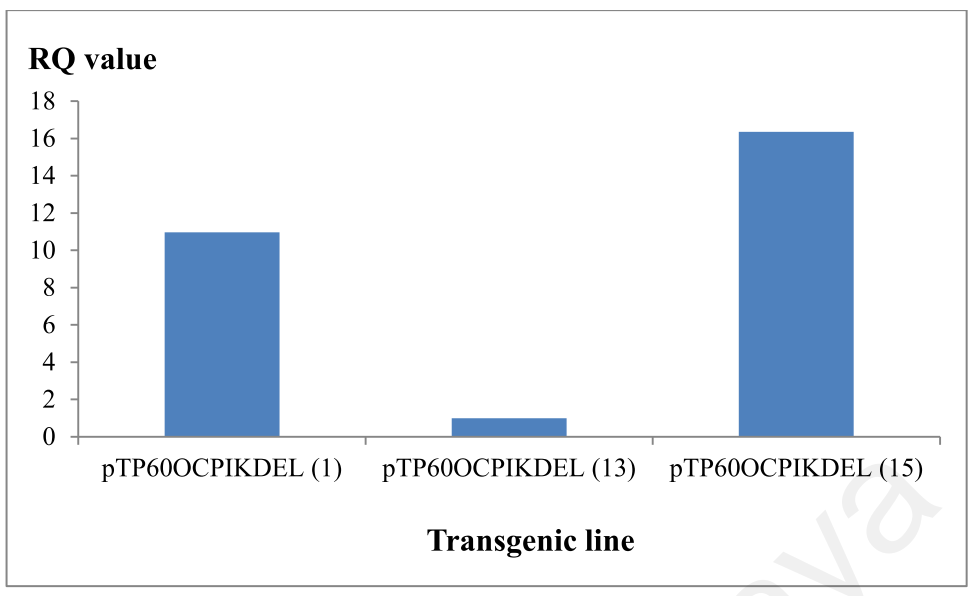

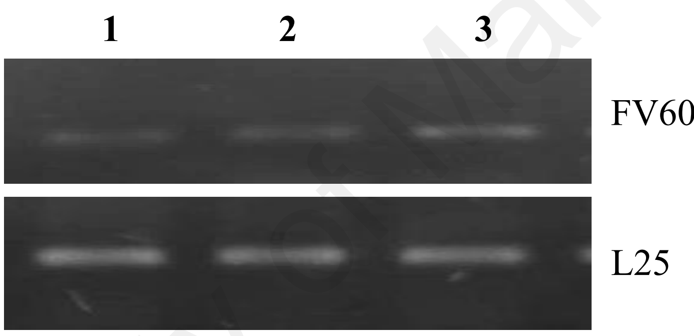

Figure 4.39: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 1, 13, and 15 harboring pTP60OCPIKDEL construct. RQ value = Relative expression value on Y axis represents transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60OCPIKDEL (1), 2) pTP60OCPIKDEL (13), and 3) pTP60OCPIKDEL (15). Experiment was conducted in 3 technical replicates.

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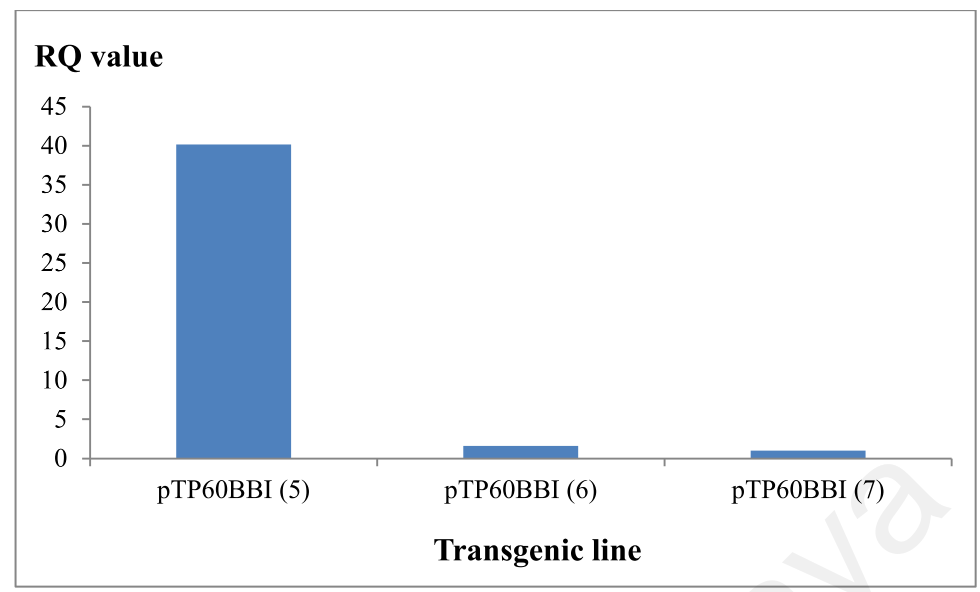

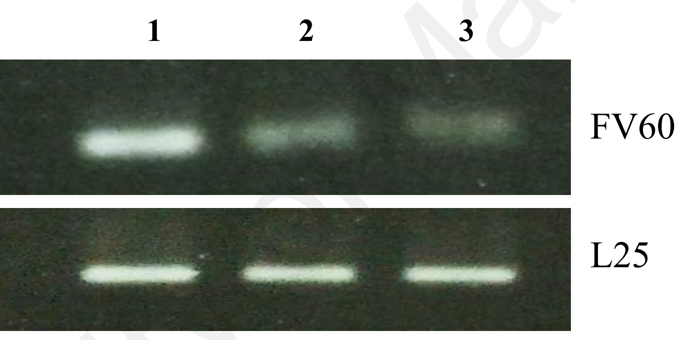

Figure 4.40: The expression levels of *TP60* gene in T_1 tobacco progenies compared between lines 5, 6, and 7 harboring pTP60BBI construct. RQ value = Relative expression value on Y axis represents transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60BBI (5), 2) pTP60BBI (6), and 3) pTP60BBI (7). Experiment was conducted in 3 technical replicates.

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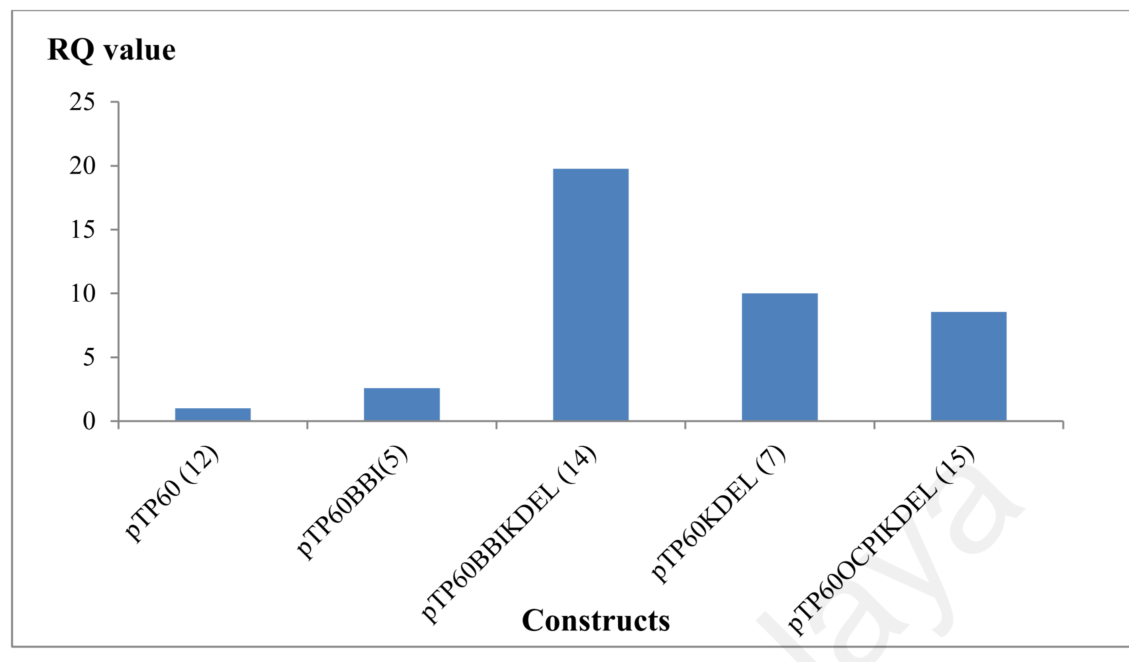

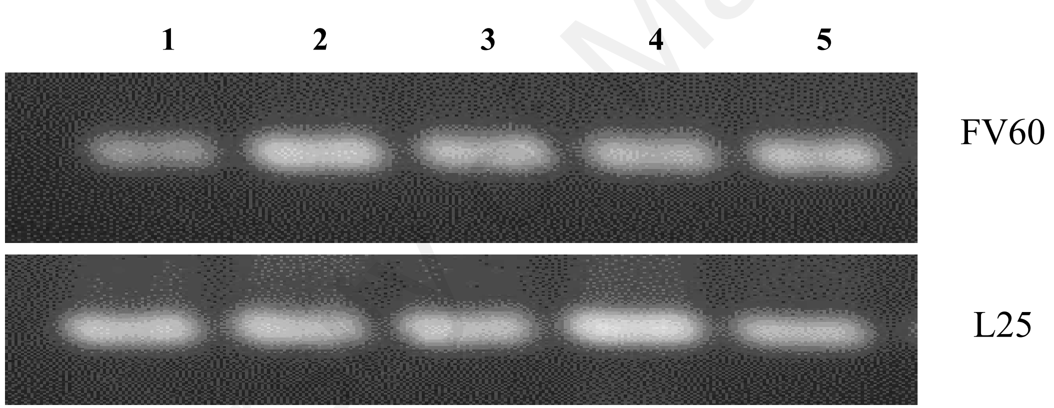

Figure 4.41: The expression levels of *TP60* gene in T_1 tobacco progenies, comparing between representative lines of different plasmid constructs with the highest expression of anti-*Toxoplasma* gene at mRNA level**.** RQ value = Relative expression value on Y axis of independent lines transcript level relative to *NtL25*. Semi-quantitative expression; 1) pTP60 (12), 2) pTP60BBIKDEL (14), 3) pTP60KDEL (7), 4) pTP60BBI (5), and 5) pTP60OCPIKDEL (15). Experiment was conducted in triplicate.

4.8 Recombinant anti-*Toxoplasma* protein expression in transgenic tobacco plants

4.8.1 Recombinant protein purification

Initially, the total protein was extracted from transgenic tobacco plants (T_0) and (T_1) followed by protein purification, SDS-PAGE and Western blot analysis. The total protein extracts were quantified according to BSA standard curve (Figure 4.42) and the integrity of the protein was determined by SDS-PAGE analysis (Figure 4.43). Based on the results obtained as shown in Figure 4.44, T_0 tobacco plants confirm the expression of the recombinant protein at the expected size of ~ 27 kDa.

In this study, the targeted recombinant protein was purified using Dynabeads® M-280 Sheep anti-Mouse IgG (ThermoFisher Scientific, USA) (Section 3.8.7) (Figure 4.45). A clear band at the size approximately of 60 kDa is suggested to be non-specific protein eluate from the purified protein product was detected in all the selected samples. This suggested the stability of the anti-*Toxoplasma* recombinant protein. No band was observed in wild-type lane. However, there were apparent dimers formed of the detected protein (~ 54 kDa instead of ~27 kDa) (Figure 4.46).

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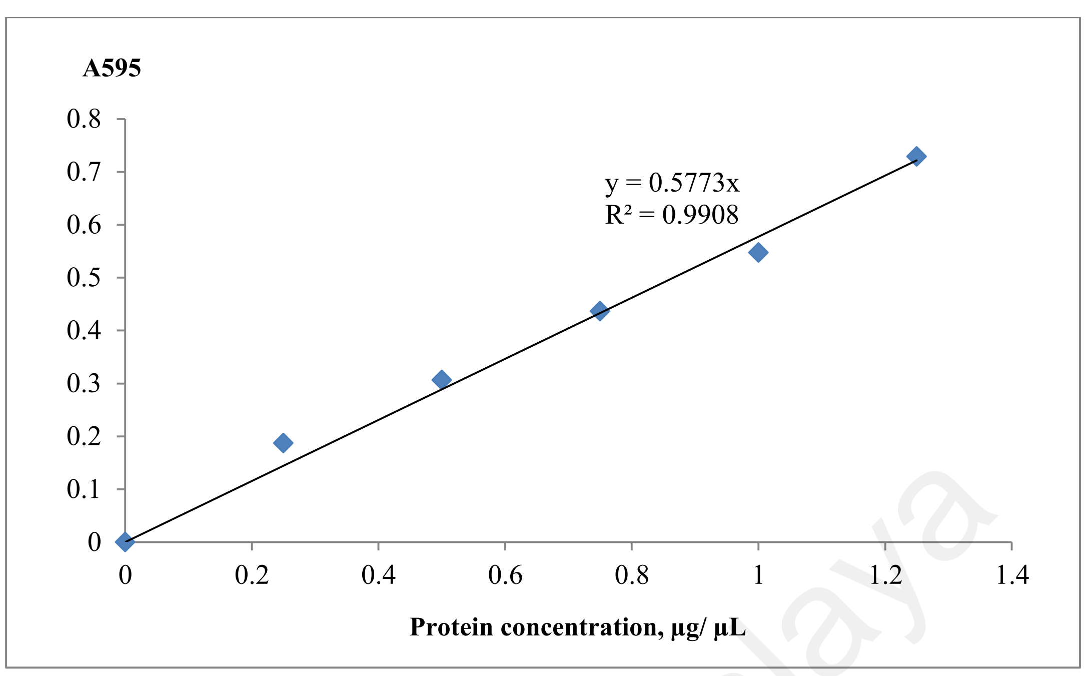

Figure 4.42: Standard curve of known concentration BSA using Bradford's assay quantification.

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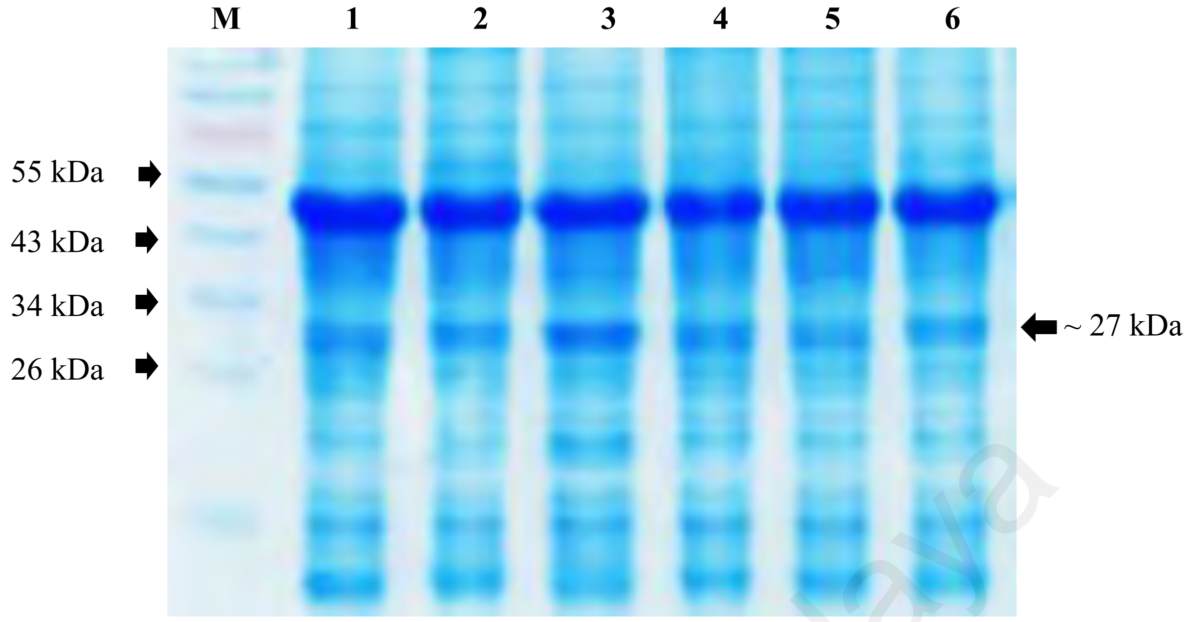

Figure 4.43: 20 µg of unpurified total soluble protein extracted from wild-type and a transgenic *N. tabacum* cv. SR1 representative from each construct (T_0) after 40-days of acclimatization. The expected size of recombinant anti-*Toxoplasma* scFv antibody protein is ~ 27 kDa.

Lane M : Page Ruler™ Pre-stained Protein Ladder, 10-180 kDa (Thermo Scientific, USA)

Lane 1 : Non-transformed tobacco plants (wild-type)

Lane 2 : Transgenic tobacco harboring pTP60 construct

Lane 3 : Transgenic tobacco harboring pTP60KDEL construct

Lane 4 : Transgenic tobacco harboring pTP60BBI construct

Lane 5 : Transgenic tobacco harboring pTP60BBIKDEL construct

Lane 6 : Transgenic tobacco harboring pTP60OCPIKDEL construct

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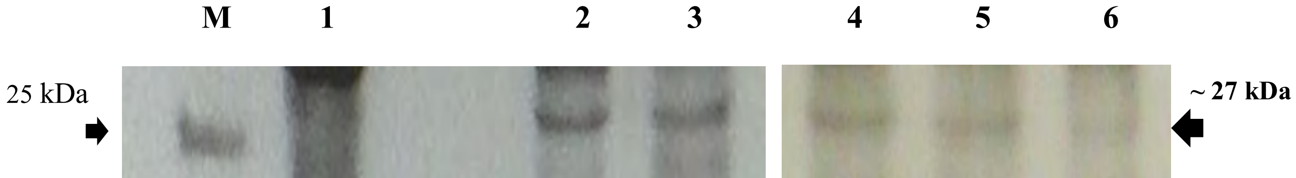

Figure 4.44: Detection of recombinant anti-*Toxoplasma* scFv antibody, TP60 from unpurified total soluble protein on the selected T_0 tobacco plants and wild-type after 40-days of acclimatization using mouse monoclonal anti–E epitope tag and IgG anti-mouse with HRP conjugated by Western blot analysis. The expected band size is ~ 27 kDa.

Lane M : PageRuler™ Plus Pre-stained Protein Ladder, 10-250 kDa (Thermo Scientific, USA)

Lane 1 : Non-transformed tobacco plants (wild-type)

Lane 2 : Transgenic tobacco harboring pTP60BBIKDEL construct

Lane 3 : Transgenic tobacco harboring pTP60OCPIKDEL construct

Lane 4 : Transgenic tobacco harboring pTP60BBI construct

Lane 5 : Transgenic tobacco harboring pTP60KDEL construct

Lane 6 : Transgenic tobacco harboring pTP60 construct

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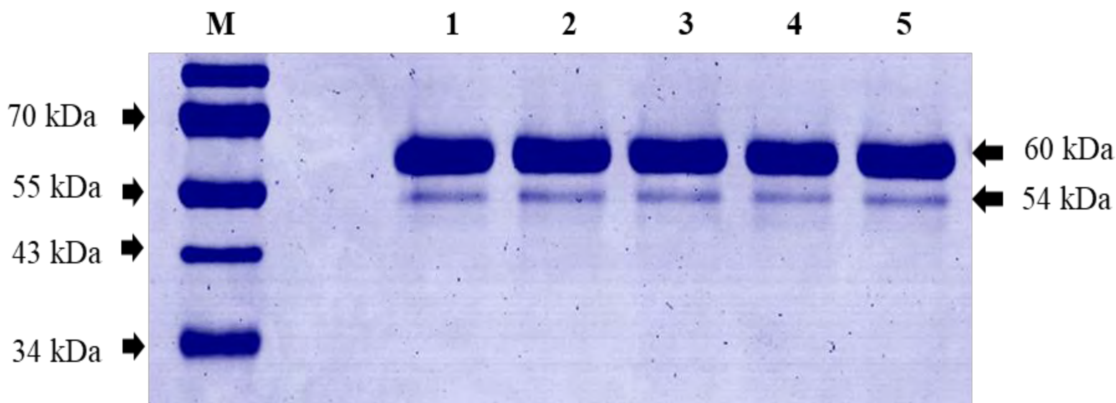

Figure 4.45: SDS-PAGE of purified recombinant anti-*Toxoplasma* scFv antibody protein using DyNabeads™ from *N. tabacum* cv. SR1 representative lines harboring different plasmid constructs (T_1) after 40-days of acclimatization.

Lane M : PageRuler™ Pre-stained Protein Ladder, 10-180 kDa (Thermo Scientific, USA)

Lane 1 : T_1 tobacco progeny harboring pTP60 construct (line 12)

Lane 2 : T_1 tobacco progeny harboring pTP60KDEL construct (line 7)

Lane 3 : T_1 tobacco progeny harboring pTP60BBI construct (line 5)

Lane 4 : T_1 tobacco progeny harboring pTP60BBIKDEL construct (line 14)

Lane 5 : T_1 tobacco progeny harboring pTP60OCPIKDEL construct (line 15)

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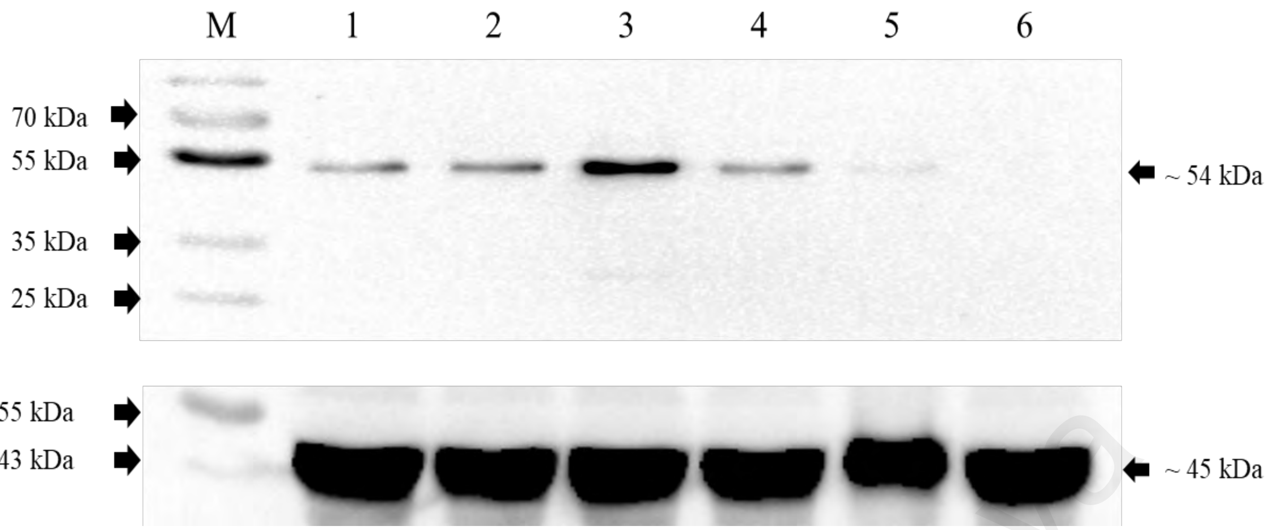

Figure 4.46: Stable expression of purified recombinant anti-*Toxoplasma* scFv antibody protein from *N. tabacum* cv. SR1 representative lines harboring different plasmid constructs (T_1) after 40-days of acclimatization. The expected protein size is at ~ 54 kDa (dimer formation). Actin-11 protein used as loading control at the predicted size of ~ 45 kDa.

Lane M: PageRuler™ Plus Pre-stained Protein Ladder, 10-250 kDa (Thermo Scientific, USA). Ladder used for loading control is Page Ruler™ Pre-stained Protein Ladder, 10-180 kDa (Thermo Scientific, USA)

Lane 1 : T_1 tobacco progeny harboring pTP60BBI construct (line 5)

Lane 2 : T_1 tobacco progeny harboring pTP60KDEL construct (line 7)

Lane 3 : T_1 tobacco progeny harboring pTP60BBIKDEL construct (line 14)

Lane 4 : T_1 tobacco progeny harboring pTP60OCPIKDEL construct (line 15)

Lane 5 : T_1 tobacco progeny harboring pTP60 construct (line 12)

Lane 6 : Wild-type tobacco plant

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CHAPTER 5: DISCUSSION

5.1 Introduction

The parasitic protozoan, *T. gondii* is a causal agent of toxoplasmosis which severely affects mammals including humans and birds. In human, this disease may cause complications in immunodeficient individuals and pregnant women which may lead to congenital disease of the newborn babies. In addition, long term period of this untreated disease can cause neonatal loss and abortion in livestock animals which may be devastating to the husbandry sector.

This study was carried out to control toxoplasmosis through diagnosis and therapeutic approaches by expressing the recombinant single-chain variable fragment (scFv) antibody against toxoplasmosis. The recombinant antibody was derived from mouse pre-cloned scFv gene (Lim, 2012) and subsequently transformed into tobacco plants by using a plant expression vector as a backbone (Go, 2013). This scFv has the same specificity as in parental antibody, IgG due to the presence of specific paratope against specific target. The small and simple size functional antibody with both heavy and light chains connected by a peptide linker (15-20 amino acids) to form a polypeptide chain was a breakthrough (Tran et al., 2013; Lai et al., 2014). In addition, scFv also provides other advantages such as increase the density of antigen-binding sites and selectivity towards target antigen as well as rapid in antigens detection even in low concentration of complex samples (Shen et al., 2005).

Besides the scalability and compatibility properties of plant-derived pharmaceutical proteins due to similar machinery and glycosylation pattern as in human, ethical issues and human-pathogenic contamination that are often associated with animal or bacterial cell culture systems could be avoided.

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However, plant system may also exhibit some drawbacks, including higher heterogeneity of the end recombinant protein product, low in solubility and expression level due to the interference of endogenous proteases. Hence, in this study, different proteinase inhibitor and/ or ER-targeting gene (KDEL) were co-expressed with anti-*Toxoplasma* recombinant scFv antibody in order to enhance and stabilize the antibody production in the model tobacco host plant, *N. tabacum.*

5.2 Transformation of tobacco with recombinant plasmid constructs

Hiatt et al., (1989) first reported on the production of functional IgG antibody from plants obtained from crossing two transgenic tobacco plants, each expressing heavy and light chains. Prior to crossing, *A. tumefaciens*-mediated transformation was performed on separate events. Following that, Vaquero et al. (1999), has successfully developed functional therapeutic scFv and IgG of a tumor-specific antibody (T84.66) in *N. tabacum* cv. Petite Harvana SR1 using agroinfiltration technique. In years later, ELPylated haemagglutinins as neutralizing antibodies against H5N1 viruses was produced in mice (Phan et al., 2014), whereas production of stable HIV-neutralizing human monoclonal antibody 2G12 was from *N. tabacum* (Ma et al., 2015). Additionally, there are some scFv undergoing the clinical test, i.e. scFv targeted to P97 antigen against Melanoma disease and bispecific scFv for B-cell tumor which was undergoing preclinical stage and phase I clinical trials respectively (Pucca et al., 2011).

In this study, standard *Agrobacterium*-mediated transformation protocol was used (Go, 2013). On selection media, all hygromycin-resistant shoots regenerated from leaf discs transformed with different plasmid constructs were assessed. Based on the results obtained, explants that have been transformed with pTP60BBIKDEL showed 7-fold higher shoot regeneration compared to the explants that have been transformed with

only pTP60KDEL. It was also observed that no significant differences between putative transformants transformed with pTP60, pTP60BBIKDEL, pTP60OCPIKDEL as well pCAMBIA 1304 with wild-type. It could be concluded that recombinant anti-*Toxoplasma* gene and the proteinase inhibitor with or without ER-targeting gene did not affect the plant growth especially in shoot regeneration even though pTP60BBI and pTP60KDEL alone gave a significant difference in the analysis.

It was also found that more shoots regenerated from the wounded edge compared to the middle of the explants and would increase the chances of obtaining putative transformed shoots. Furthermore, since the regenerated shoots were in direct contact with hygromycin, the probability of attaining false-positives would be reduced. It was also observed that the middle part of the explants produced callus and led to low chances of forming shoots. This result was in accordance with Khan et al. (2015).

5.3 Confirmation of putative transformed shoots

In this study, putative transformed shoots were verified using transient and molecular assays. Transient assays utilizing GUS (qualitative and quantitative) and GFP visualization have been carried out on the randomly selected putatively transformed shoots harboring different plasmid constructs.

GUS (*gus*A) gene has been extensively used in plant transformation and considered as a good marker for monitoring transformation efficiency (Jefferson et al., 1987). This is due to the capability of GUS gene catalytically hydrolyzed a broad range of substrates through different applications due to the distinct chromogenic, spectrophotometric and fluorogenic properties of their aglycone forms (Kim et al., 2006). The observed GUS-blue spots and GUS quantification through fluorometry assay showed initially

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promising results as an indicator of successful transfer of transgene. In this study, the lowest expression of *GUS* gene was shown in putative transformed shoots in the presence of pTP60BBI, pTP60OCPIKDEL*,* and pTP60BBIKDEL (~ 1.5 kb insert in size) compared to pTP60 and pTP60KDEL (~ 900 bp insert in size). On the other hand, putative transformed shoots with the empty vector showed the highest GUS expression (Figures 4.16 and 4.19). This might be due to the position of the *GUS* gene away from the backbone promoter CaMV 35S leading to reduce gene regulation. In addition, *GUS* gene expression also is a good indicator to explain the expression of unfused recombinant gene within the T-DNA region even by using only a constitutive promoter belongs to the backbone vector.

Besides GUS, GFP is also a reliable reporter gene that is suitable to determine the successful transfer of T-DNA. It is a simple, convenient, and non-destructive visualization assay. The putative transformed shoots were then examined for green fluorescence to determine the expression of the *mgfp*5 gene (Figure 4.20). The non-transformed region (Figure 4.20a) within the same expanded putative transformed shoots showed red coloration due to auto-fluorescence under UV light in the presence of chlorophyll background signal (Goldman et al., 2003; Zhou et al., 2005). The region in the presence of GFP gene fluoresced under specific excitation/emission wavelength 488/550nm (Figure 4.20b). However, the GFP expression diminished over a period of time indicating transient gene expression. Decreasing GFP expression is also influence by the age of the leaves. According to previous publications, following the recovery of a new putative transgenic plant, GFP fluorescence was usually visible in new emerging shoots, young tissues or organs, whereas weak signal was observed in the older ones (Zhou et al., 2004), such as in *Medicago truncatula* var. A17 (Kamaté et al., 2000) and *Verbena officialis* L. (Tamura et al., 2003).

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PCR analysis was carried out in order to verify the presence of heritable *hpt*II gene and the integration of T-DNA into T_1 tobacco progenies. For the latter, the screening of T_1 transformants was carried out by detecting *hpt*II gene in the left border (LB) part of the pCAMBIA 1304. *hpt*II gene located on the left border (LB) of Ti plasmid could be nicked during the transfer of T-DNA from *Agrobacterium* to plant cells causing truncation to occur which has been documented by Kemski et al. (2013).

5.4 Effects of different plasmid constructs on the phenotype of transgenic tobacco plants

The percentage of T_1 seed germination, stem height, and flowering period were assessed in order to determine the effect of plasmid constructs towards plant growth and development (Figure 4.26 and Table 4.4).

5.4.1 T_1 seed germination

The percentage of seed germination was significantly lowest (65 % ± 2.03) in transgenic seeds transformed with the empty vector compared. Contrastingly, construct with elements of BBI-KDEL and OCPI-KDEL showed the highest germination percentage (90 % ± 1.45) but germination of T_1 seeds present with BBI and KDEL elements alone were significantly low ($p < 0.05$). The presence of proteinase inhibitors was shown to improve the germination and it has been reported that protease inhibitor is involved in regulating the endogenous proteinases during germination of lettuce seeds and for the formation of a sulphur reservoir during dormancy (Yan et al., 2009). Sulphur reservoir results from the accumulation of amino acids rich in sulphur such as cysteine which helps in overcoming dormancy during germination. Furthermore, the levels of T_1 seed germination percentages in the presence of pTP60 construct and wild-type were

significantly similar indicating that the anti-*Toxoplasma* protein did not interfere with the growth of the tobacco plant itself.

However, the transgenic seeds showed delayed germination than wild-type after 14 days of culture on media containing with and without hygromycin respectively (no data were recorded). The delayed in germination might be due to selective pressure on transgenic seeds to adapt to the antibiotic-containing media. As previously reported by Eluk et al. (2016), the introduction of antibiotics, such as enrofloxacin, oxytetracycline, penicillin, and tylosin, on culture media delayed germination in seeds of *Glycine max*, *Helianthus annus*, *Sorghum bicolor*, *Triticum aestivum*, and *Zea mays*, showing a phytotoxic action on crop growth.

As been reported by Rivard et al. (2006), in order to prevent the proteolytic degradation towards the recombinant proteins by endogenous proteases, tomato cathepsin D inhibitor (CDI) has been co-expressed with the recombinant proteins. Surprisingly, there were non-significant difference in the morphology of transgenic *Solaneum tuberosum* in terms of height, germination time, number of leaves, stem diameter and internode length. This suggested that proteinase inhibitors were not interfered in plant growth and development regardless the types of proteinase inhibitor.

5.4.2 Stem height and flowering period

Acclimatized T_1 plantlets were assessed in terms of stem height and flowering period (Table 4.4). The data obtained for stem height was comparable between both transgenic and wild-type (control) plants in T_1 generation. The significant difference was observed for T_1 tobacco progeny presence of at least one element (proteinase inhibitor with or without KDEL). On the other hand, T_1 tobacco progeny transformed with anti-*Toxoplasma* construct only (pTP60) did not impose a detectable penalty on growth and

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development of the tobacco plant itself as compared to wild-type which is pertinent in using tobacco as a factory for biopharming (Commandeur et al., 2003; Ahmad et al., 2012). As been reported by Hartl et al. (2010), serine proteinase like inhibitor (BBI in this case), was commonly found in many species which ruled out the possibility of affecting primary growth in *Solanum americanum.* In addition, serine proteinase-like inhibitor could defend *Solanum nigrum* against general herbivores without influencing the plant growth and development.

The flowering duration was observed to be faster in T_1 tobacco progeny compared to wild-type (65 days ± 5.49). T_1 tobacco plant harboring pTP60BBI showed the earliest flowering onset (45 days ± 2.67). In plants, flowering is commonly linked regulated by environmental factors, such as photoperiod, temperature, and humidity. Most factors responsible for non-photoperiodic flowering are due to stress (Takeno, 2012). In this study, the introduction of foreign gene into the tobacco plant genome caused stress to the transgenic plants. Thus, leading to faster flowering period compared to normal tobacco plants.

In previous studies, it has been reported that the introduction of proteinase inhibitor genes did not affect the developmental phenotype in other plant species. In the previous study, it was also found that there was no significant difference in flower diameter (Sin et al., 2006), number of reproductive units (Zavala et al., 2004b), plant growth (Zavala et al., 2004a; Xie et al., 2007), or trichome morphology and density (Liu et al., 2006; Luo et al., 2009) might be due to effector-specificity.

5.5 Co-expression of proteinase inhibitor and KDEL increased the relative expression of p*TP60* gene and protein accumulation

The co-expression of proteinase inhibitors and KDEL-encoding transgenes in plants is an alternative way to enhance protein expression. This approach may provide *in situ*

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stabilization of the recombinant protein against enzymatic reaction by endogenous proteases and migration to specific sub-cellular compartments such as ER.

Despite the occurrence of different proteases with distinct modes of action and specificities towards peptide bonds reported to be active in leaf extracts (van der Valk & van Loon, 1988; Gonçalves et al., 2016), single broad-spectrum inhibitors, such as BBI and OCPI, may effectively protect co-expressed recombinant proteins in leaf tissue. As previously reported in 2008, the introduction of serine-like BBI had increased the accumulation of a transgenic recombinant human granulocyte-macrophage colony stimulating factor by decreasing the protease activity in transformed rice callus (Kim et al., 2008). Besides, the pharmaceutically important cytokine, human interleukin 2 was also found to be protected by the co-expression of both BBI and silk protease inhibitors (Redkiewicz et al., 2012). In 2012, transgenic tobacco plants that constitutively express the OCPI had significantly increased glutathione reductase activity and the amount expressed in transgenic tobacco plants by inhibiting cysteine protease activity which was shown to be lowered in abundance (Pillay et al., 2012).

Transgenes that are targeted for expression within the ER also showed improvement in the yield of recombinant proteins (Benchabane et al., 2008). The KDEL or SEKDEL retention sequence (Ser-Glu-Lys-Asp-Glu-Leu) was first described by Munro and Pelham (1987) has been used in this study. De Wilde et al. (2013) showed that VHH-Fc and scFv-Fc accumulation levels of 1 % or more in *Arabidopsis thaliana* seeds triggered an unfolding protein response and enhanced expression of genes involved in protein folding, glycosylation, protein translocation, degradation, and vesicle trafficking was observed. Hence, the combination of both of these strategies was investigated in this study to potentially facilitate better expression of the targeted antibody.

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Based on the Western blot analysis (Figure 4.44 and Figure 4.46), the results showed that the highest protein production and accumulation of anti-*Toxoplasma* scFv antibody was achieved in the presence of co-expressed combination BBI-KDEL elements. The result obtained was correlated to the expression at the mRNA level (Figure 4.41).

The data suggest that the combination of PI and KDEL had likely mitigated the effect of proteinase and had thus enhanced the production of antibody. This finding validates the use of these construct combinations for expression of the TP60 recombinant antibody and may be useful for future application in designing plant based systems for large scale production of recombinant proteins.

5.6 Homodimer formation of recombinant anti-*Toxoplasma* protein in T_1 tobacco plants

Detection of anti-*Toxoplasma* recombinant scFv, TP60 using an anti E-epitope tag fused protein (Figure 4.45) confirmed the expression of the protein at different accumulation levels as a ~ 54 kDa dimer protein in T_1 tobacco plants instead of the expected ~ 27 kDa of scFv protein size which was at first observed in T_0 tobacco plants. The dimeric formation of TP60 might be due to a serine residue in the linker peptide (GGSSRSS) that could be involved in dimer formation of the disulphide band under reducing conditions (Schouten et al., 2002). Dimerization of recombinant proteins produced from *Nicotiana tabacum* cv. Xanthi due to a cysteine residue present in the linker peptide of the recombinant protein has been reported (Dobhal et al., 2013). This dimeric formation of TP60 might increase the effectiveness and inhibition toward the *T. gondii* tachyzoite antigen which causes the *Toxoplasma* disease. As in a previous study, dimerized anti-^{Nb}TNF-V_HH_{CK} proteins produced from *Nicotiana benthamiana* blocked the TNFα-activity more effectively than monomeric anti-^{Ec}TNF-V_HH_{CK} protein produced in *Escherichia coli* (Geirsberg et al., 2010). No band was observed in the lane

for non-transformed tobacco plant. Recombinant scFv antibody of T_1 tobacco plants in combination with the BBIKDEL elements showed highest protein accumulation compared to the other elements and were correlated with the gene transcript quantification at mRNA level.

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CHAPTER 6: CONCLUSION

This study was conducted in order to assess the transgene expression of anti-*Toxoplasma* recombinant scFv antibody encoded by *TP60* at mRNA and protein levels. The expression of *TP60* was also been assessed at mRNA level in T_1 generation. In order to improve the amount of desired recombinant protein in transgenic tobacco plants, this study was undertaken by co-expressing *TP60* gene in combination with different elements, namely proteinase inhibitors (BBI or OCPI) and ER-retention sequence (KDEL). The gene was tandemly expressed with either one of this elements or combination of a proteinase inhibitor with KDEL retention sequence.

Different plasmid constructs, namely pTP60, pTP60KDEL, pTP60BBI, pTP60BBIKDEL and pTP60OCPIKDEL, were transformed into *N. tabacum* cv. SR1 mediated by *A. tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1304 containing a *gusA*-m*gfp*5 fusion reporter genes encoded for GUS and GFP, respectively, and a selectable marker for hygromycin B (*hpt*II) genes driven by the CaMV 35S promoter. Quantitative GUS assay showed that the non-significant difference of pTP60BBI, pTP60BBIKDEL, and pTP60OCPIKDEL constructs generated at least 20-fold low expression while, pTP60 and pTP60KDEL constructs generated at least 7-fold lower expression compared to the empty vector which generated significantly highest in GUS expression. This observation was correlated to the least expression of GUS blue spot(s) on the expanded putative transformed shoots due to the presence of gene insert. No GUS-blue spots and green fluorescence signal were observed on non-transformed region of the putative transformed shoots.

Based on the results obtained from the Western blot analysis, the accumulation of the recombinant TP60 protein was highest in the presence of BBIKDEL elements followed

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by OCPIKDEL, BBI, and KDEL in both T_0 and T_1 generations. This result can be concluded due to the lowest expression of TP60 protein with the absence of either one of these elements. The recombinant protein was first detected in the T_0 at ~ 27 kDa but then observed as homodimer at ~ 54 kDa in the T_1 generation.

In order to support the observation of recombinant protein accumulation derived from different constructs, the expression of *TP60* transcript was analyzed in the T_1 generation. In this analysis, three lines for each construct were evaluated and it was found that the highest relative expression of *TP60* was with the companion of BBI-KDEL that showed 19-fold higher expression compared to the other constructs. This result correlated with the expression of TP60BBIKDEL at the protein level.

According to the statistical data analysis on phenotypic assessments, data obtained were comparable between both transgenic and control plants in T_1 generation in terms of number of putative shoots regeneration, stem height, and flowering initiation time. The significant difference was observed for T_1 tobacco progeny presence of BBIKDEL or OCPIKDEL element. Transformation with anti-*Toxoplasma* constructs without any additional elements (pTP60) and stably inherited in T_1 tobacco progeny did not interfere in growth and development of the tobacco. The phenotypic characteristic observed was similar and no significant difference to wild-type; which is pertinent in using tobacco as a factory for biopharming However, in the presence of proteinase inhibitor (BBI or OCPI) with or without the companion of KDEL elements, the percentage of T_1 seeds germination was significantly higher compared to without proteinase inhibitors.

This study demonstrated that the production of recombinant anti-*Toxoplasma* protein in plants is feasible and can be enhanced using specific elements. This complements the advantages of the plant system which includes expression of eukaryotic proteins without the need of downstream modifications, such as chemical refolding associated with

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prokaryotic systems or purification against pathogen. This technology can thus be exploited for the production of pharmaceutically important proteins and diagnostic antibodies which will have a wide impact on the medical biotechnology sector.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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Fatin Iffah Rasyiqah. (2016, Dec). *Enhancement of recombinant protein production through different enhancer elements approach in Nicotiana tabacum.* Paper presented at the 21st Biological Sciences Graduate Congress 2016. University of Malaya, Kuala Lumpur, Malaysia.

University of Malaya