

**EFFECTS OF THE EXPRESSION OF THE BACTERIAL
YEFM-YOEB_{SPN} CHROMOSOMAL TOXIN-ANTITOXIN
SYSTEM IN *ARABIDOPSIS THALIANA***

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**FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

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THALIANA***

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ABSTRACT

Toxin-antitoxin (TA) systems are extensively found in bacteria as well as in archaea where they play diverse roles in important cellular functions. Bacterial TA systems usually comprise of a pair of genes encoding a stable toxin and its cognate labile antitoxin and are located in the chromosome or in plasmids. Chromosomally-encoded TA systems are involved in a variety of cellular processes including as part of the global stress response of bacteria, and as mediators of programmed cell death as well as biofilm formation, in which the activation of the toxins usually leads to cell death or dormancy. The genome of the human pathogen *Streptococcus pneumoniae* contains up to 10 putative TA systems and among these, the *yefM-yoeB_{Spn}* locus has been well studied and demonstrated to be biologically functional. Overexpression of the *yoeB_{Spn}* toxin has been shown to lead to cell stasis and eventually cell death in its native host cell as well as in *E. coli*. Several toxins of TA systems have been heterologously expressed in eukaryotic systems including yeasts, zebrafish, frog embryos and human cell lines where they have been shown to be lethal. However, there has been no report on the functionality of any bacterial TA systems in plants. In this study, a two-component 17- β -estradiol-inducible expression system was utilized to investigate the heterologous expression of the *yoeB_{Spn}* toxin along with its cognate *yefM_{Spn}* antitoxin in *Arabidopsis thaliana*. The coding sequence of the *yoeB_{Spn}* toxin was cloned as a translational fusion with Green Fluorescent Protein and *A. thaliana* was transformed via floral dip using *Agrobacterium tumefaciens*-mediated transformation method. Transgenic *A. thaliana* were allowed to grow on selection media until T₂ generation. Induced expression of the *yoeB_{Spn}* toxin-*GFP* fusion transgene apparently triggered apoptosis and was lethal in *A. thaliana*. To investigate if expression of the *yefM_{Spn}* could mitigate the toxicity of *yoeB_{Spn}* in *A. thaliana*, transgenic plant carrying *yefM_{Spn}* was first constructed and then cross-pollinated with transgenic plant containing the *yoeB_{Spn}*-*GFP* transgene. The *yefM_{Spn}* \times *yoeB_{Spn}*-*GFP* hybrid transgenic plants obtained were allowed to grow until maturity on selection media. When co-expressed in *A. thaliana*, the YefM_{Spn} antitoxin was found to neutralize the toxicity of YoeB_{Spn}-GFP. Interestingly, the inducible expression of both the *yefM_{Spn}* antitoxin and *yoeB_{Spn}* toxin-*GFP* fusion transgenes in transgenic hybrid plants resulted in larger rosette leaves, taller plants with more inflorescence stems and increased silique production. The detailed mechanism by which co-expression of *yoeB_{Spn}*-*GFP* and *yefM_{Spn}* led to enhanced plant growth remains to be elucidated. In their original bacterial hosts, YefM_{Spn} forms a tight protein complex with YoeB_{Spn} and this TA complex binds to the operator site overlapping the *yefM-yoeB_{Spn}* promoter to repress its transcription. It is possible that the YefM-YoeB_{Spn} complex in *A. thaliana* binds to certain regions of the plant genome leading to the enhanced growth phenotype. To our knowledge, this is the first demonstration of a prokaryotic antitoxin neutralizing its cognate toxin in plant cells. The functional lethality of the YoeB_{Spn} toxin enables it to be harnessed for a potential novel plant cell ablation system.

ABSTRAK

Sistem Toksin - antitoksin (TA) terdapat secara meluas di dalam bakteria dan juga arkea di mana sistem ini memainkan pelbagai peranan dalam fungsi-fungsi sel yang penting. Sistem TA di dalam bakteria biasanya terdiri daripada sepasang gen yang mengkodkan toksin yang stabil dan antitoksin yang goyah dimana gen ini terletak sama ada di kromosom atau plasmid. Sistem TA yang dikodkan dalam bakteria kromosom terlibat dalam perbagai proses selular termasuk tindak balas tekanan global, program sel mati dan pembentukan biofilem, dimana pengaktifan toksin biasanya membawa kepada kematian sel atau dorman. Genom patogen manusia *Streptococcus pneumoniae* mengandungi 10 sistem TA yang telah dikenal pasti dan diantaranya ialah, *yefM-yoeB_{Spn}*. Sistem TA *yefM-yoeB_{Spn}* telah dikaji dengan teliti dan terbukti tindak-balas ekspresi yang berlebihan oleh toksin *yoeB_{Spn}* menyebabkan sel stasis dan akhirnya membawa kepada kematian dalam kedua-dua sel tuan rumah dan juga *E. coli*. Beberapa jenis toksin daripada sistem TA yang berlainan telah dimasukkan ke dalam sistem eukariot seperti yis, ikan zebra, embrio katak dan sel manusia dimana toksin ini telah terbukti boleh menyebabkan kematian dalam sel-sel tersebut. Walau bagaimanapun, tiada sebarang laporan mengenai fungsi sistem TA bakteria ini dalam tumbuhan. Dalam kajian ini, dua komponen sistem induksi 17- β -estadiol telah digunakan untuk mengkaji kesan gabungan ekspresi diantara toksin *yoeB_{Spn}* bersama dengan antitoksin *yefM_{Spn}* dalam *Arabidopsis thaliana*. *yoeB_{Spn}* telah diklon sebagai gabungan translasi dengan Green Fluorescent Protein dan ditransformasi ke dalam *Arabidopsis thaliana* melalui dip bunga dengan menggunakan kaedah pengantara transformasi *Agrobacterium*. Transgenik *A. thaliana* dibenarkan untuk tumbuh dalam media pemilihan sehingga generasi T₂. Ia jelas menunjukkan bahawa gabungan *yoeB_{Spn}* toksin-GFP boleh menyebabkan apoptosis dan membawa kepada kematian dalam *A. thaliana*. Untuk mengkaji dengan lebih lanjut sama ada ekspresi *yefM_{Spn}* boleh meneutralkan ketoksikan *yoeB_{Spn}* dalam *A. thaliana*, pendebungaan silang antara tumbuhan transgenik yang membawa antitoksin *yefM_{Spn}* dan tumbuhan transgenik yang membawa gabungan *yoeB_{Spn}* toksin-GFP telah dilakukan. Tumbuhan hibrid *yefM_{Spn}* \times *yoeB_{Spn}*-GFP yang mengandungi kedua-dua *yoeB_{Spn}*-GFP dan *yefM_{Spn}* toksin dan antitoksin dibenarkan untuk tumbuh dalam media pilihan sehingga tempoh matang. Keputusan fenotip jelas menunjukkan bahawa antitoksin *yefM_{Spn}* boleh meneutralkan ketoksikan *yoeB_{Spn}*-GFP apabila kedua-dua diekspreskan dalam *Arabidopsis*. Yang menariknya, ekspresi kedua-dua antitoksin *yefM_{Spn}* dan gabungan *yoeB_{Spn}* toksin-GFP dalam *Arabidopsis* hibrid transgenik menyebabkan daun roset yang lebih besar dan tumbuhan yang lebih tinggi dengan peningkatan dari segi pengeluaran silique. Mekanisme terperinci dimana gabungan ungkapan *yoeB_{Spn}*-GFP dan *yefM_{Spn}* dalam meningkatkan pertumbuhan tumbuhan masih belum dapat dijelaskan dan merupakan subjek untuk penyelidikan lanjut. Di dalam bakteria itu sendiri, YefM_{Spn} membentuk kompleks protein yang ketat dengan YoeB_{Spn} dan kompleks TA ini melekat kepada laman web pengendali yang bertindih dengan promoter *yefM-yoeB_{Spn}* untuk menghalang transkripsi daripada berlaku. Oleh itu, terdapat kemungkinan bahawa kompleks YefM-YoeB_{Spn}-GFP dalam *A. thaliana* melekat kepada kawasan-kawasan tertentu dalam genom tumbuhan yang membawa kepada pertumbuhan dalam fenotip tumbuhan. Untuk pengetahuan kita, ini adalah demonstrasi pertama daripada antitoksin prokariot meneutralkan toksin yang seumpamanya di dalam sel tumbuhan. Ketoksikan YoeB_{Spn} yang membawa kepada kematian boleh dimanfaatkan untuk mewujudkan sistem ablasi dalam tumbuhan.

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TABLE OF CONTENTS

Abstract	iii
Abstrak	iv
Acknowledgements	v
Table of Contents	vi
List of Figures	xiii
List of Tables.....	xvii
List of Symbols and Abbreviations.....	xviii
List of Appendices	xxi
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	4
2.1 Bacterial Toxin-Antitoxin systems	4
2.1.1 Type I TA Systems	6
2.1.2 Type II TA systems	7
2.1.3 Type III TA systems	9
2.1.4 Type IV TA systems	11
2.1.5 Type IV TA systems	12
2.2 Functions of TA systems	12
2.3 <i>Streptococcus pneumoniae</i> and its TA systems	18
2.4 Applications of TA systems in biotechnology	21

2.4.1	Tools for molecular biology	21
2.4.2	Suitability of antibacterial target	24
2.4.3	Gene therapy against viral infection	26
2.4.4	Functionality of TA systems in eukaryotic cells	27
2.4.5	Selection of high-transgene expressing mammalian cell pools	28
2.5	Genetic Restriction Use Technologies (GURT): Male sterility	29
2.6	<i>Agrobacterium tumefaciens</i> -mediated transformation	32
2.7	Approaches of multi-transgene-stacking in plants	35
2.7.1	Sexual crossing approach	36
2.7.2	Sequential transformation	37
2.7.3	Co-transformation	38
2.8	<i>Arabidopsis thaliana</i> as a plant model	40
2.9	Inducible expression system in plant	41
2.9.1	The AlCR/AlcA ethanol inducible gene expression system	42
2.9.2	The dexamethasone-inducible expression system	44
2.9.3	XVE/OlexA β -estradiol inducible expression system	46
CHAPTER 3: MATERIALS AND METHODS		49
3.1	Plant growth	49
3.2	Bacterial strains and growth conditions	49

3.3	Plasmid vectors and isolation of plasmid DNA	49
3.4	Primer design	51
3.5	Polymerase Chain Reaction	54
3.6	Agarose gel electrophoresis	55
3.7	Purification of DNA fragments from agarose gels	55
3.8	Restriction enzyme digestion	56
3.9	Ligation	57
3.10	Cloning of DNA fragments into Gateway® pENTR-D-TOPO vector	57
3.11	Preparation of competent <i>E. coli</i> cells and transformation	58
	3.11.1 Preparation of chemically-induced <i>E. coli</i> competent cells	58
	3.11.2 Transformation of chemically-induced <i>E. coli</i> competent cells	59
	3.11.3 Screening of transformed cells	59
3.12	Cloning of transgenes into the Gateway® plant binary destination vectors	60
3.13	<i>Agrobacterium tumefaciens</i> LBA 4404 competent cell preparation	61
3.14	Transformation of recombinant constructs into <i>Agrobacterium tumefaciens</i> using freeze and thaw method	62
3.15	Transformation of recombinant constructs into <i>Arabidopsis thaliana</i> using floral dip protocol	63
3.16	Selection of <i>A. thaliana</i> transformants	63

3.17	Genomic DNA extraction from <i>A. thaliana</i>	64
3.18	Detection of transgene by PCR amplification of <i>A. thaliana</i> genomic DNA	65
3.19	Southern Blotting	65
3.20	Induction for transgene expression in transgenic <i>A. thaliana</i> using 17- β -estradiol	69
3.21	Phenotypic analysis of transgenic <i>A. thaliana</i> after induction	70
3.22	Quantitative real-time reverse transcriptase PCR (qRT-PCR)	70
3.22.1	Total RNA extraction	70
3.22.2	RNA quantification	71
3.22.3	DNase I treatment	71
3.22.4	Reverse transcriptase-PCR (RT-PCR)	72
3.22.5	Quantitative real-time RT-PCR (qRT-PCR)	73
3.23	Apoptosis DNA fragmentation assay	73
3.24	Construction and analysis of hybrid transgenic <i>A. thaliana</i> co-expressing YefM _{Spn} and YoeB _{Spn} -GFP	74
3.24.1	Cross-pollination of transgenic <i>A. thaliana</i>	74
3.24.2	Selection of hybrid transgenic seeds	75

3.24.3	Expression analysis on transgenic hybrid <i>A. thaliana</i> harbouring <i>yefM_{Spn}</i> and <i>yoeB_{Spn}-GFP</i>	75
3.24.4	Phenotypic analysis on transgenic hybrid <i>A. thaliana</i>	76
3.25	Statistical analysis	76
CHAPTER 4: RESULTS.....		79
4.1	PCR amplification of <i>yoeB_{Spn}-GFP</i> fusion, <i>yefM_{Spn}</i> antitoxin, <i>GFP</i> and CaMV 35S promoter	79
4.1.1	Ligation of <i>yoeB_{Spn}</i> and <i>GFP</i> coding sequences	81
4.2	Cloning of <i>yoeB_{Spn}-GFP</i> , <i>yefM_{Spn}</i> , <i>GFP</i> and CaMV 35S into Gateway® pENTR-D-TOPO vector	83
4.2.1	Plasmid extraction and validation of the recombinant constructs pENTR_ <i>yoeB_{Spn}-GFP</i> , pENTR_ <i>yefM_{Spn}</i> , pENTR_ <i>GFP</i> and pENTR_CaMV 35S	85
4.3	Development of plant expression constructs of pMDC221_ <i>yoeB_{Spn}-GFP</i> , pMDC160_ <i>yefM_{Spn}</i> , pMDC221_ <i>GFP</i> and pMDC150_35S	87
4.3.1	Plasmid extraction and validation of pMDC recombinant constructs	90
4.4	Transformation of <i>Agrobacterium tumefaciens</i> with pMDC221_ <i>yoeB_{Spn}-GFP</i> , pMDC160_ <i>yefM_{Spn}</i> , pMDC221_ <i>GFP</i> and pMDC150_35S	92
4.5	Transformation of <i>A. thaliana</i> with the recombinant constructs	94
4.6	Phenotypic observation of transgenic and control <i>A. thaliana</i>	98

4.7	Expression analysis of <i>yoeB_{Spn}</i> -GFP and <i>yefM_{Spn}</i> in transgenic <i>A. thaliana</i>	101
4.8	DNA fragmentation assay	105
4.9	Crosses of T ₁ transgenic AtYoeBGFP plants with T ₁ transgenic AtYefM plants to produce <i>yefM_{Spn}</i> × <i>yoeB_{Spn}</i> -GFP hybrid lines	107
4.10	Expression of <i>yefM_{Spn}</i> and <i>yoeB_{Spn}</i> -GFP in the hybrid plants after induction with 17-β-estradiol	109
4.11	Induced expression of <i>yefM_{Spn}</i> and <i>yoeB_{Spn}</i> -GFP in hybrid <i>A. thaliana</i>	110
CHAPTER 5: DISCUSSION		115
5.1	Development of plant expression constructs using Gateway® cloning technology	116
5.2	Expression of <i>yoeB_{Spn}</i> toxin in <i>Arabidopsis thaliana</i> leads to plant death	118
5.3	<i>yefM_{Spn}</i> antitoxin is able to neutralize the <i>yoeB_{Spn}</i> toxin-GFP fusion in <i>Arabidopsis</i> after induction with 17-β-estradiol	122
5.4	Induced co-expression of <i>yefM_{Spn}</i> and <i>yoeB_{Spn}</i> -GFP enhanced growth in hybrid <i>Arabidopsis</i>	123
5.5	Prediction of possible DNA binding site of the YefM-YoeB _{Spn} -GFP protein complex in <i>A. thaliana</i> genome	126
5.6	Biotechnological applications for the heterologous expression of the <i>yefM</i> - <i>yoeB_{Spn}</i> genes in plants	135

CHAPTER 6: CONCLUSION	137
References	140
List of Publications and Papers Presented	176
Appendix	179

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LIST OF FIGURES

Figure 2.1: Schematic representations of the currently known TA classes	5
Figure 2.2: Functions of plasmid-encoded TA systems	13
Figure 2.3: TA systems are involved in a broad range of cellular processes which are summarized in this diagram	17
Figure 2.4: Application of TA systems for DNA cloning	23
Figure 2.5: Possible ways of inducing artificial activation of TA systems	25
Figure 2.6: Major steps in the process of T-DNA transfer and integration	34
Figure 2.7: Diagram depicting the constructs that make up the <i>alc</i> -derived gene-expression system	43
Figure 2.8: Structure of the trans-acting factor and cis-acting element in a typical GVG system	46
Figure 2.9: A typical two component XVE-based system for β -estradiol-inducible expression	47
Figure 3.1: The blot transfer setup used in Southern Blotting	68
Figure 4.1: PCR product of <i>yoeB_{Spn}</i> (255 bp) amplified from pET28a_HisYefMYoeB and separated by electrophoresis on a 1% agarose gel	79
Figure 4.2: PCR product of <i>yefM_{Spn}</i> (255 bp) amplified from pET28a_HisYefMYoeB and separated by electrophoresis on a 1% agarose gel	80

Figure 4.16: (A) Agarose gel electrophoresis of undigested plasmids extracted from <i>E. coli</i> TOP10 harbouring different recombinant pMDC vectors	91
(B) PCR confirmation for each extracted recombinant pMDC plasmid using gene-specific primers	91
Figure 4.17: Colony PCR of <i>A. tumefaciens</i> transformed with pMDC221_yoeB _{Spn} -GFP	92
Figure 4.18: Colony PCR of <i>A. tumefaciens</i> transformed with pMDC160_yefM _{Spn}	93
Figure 4.19: Colony PCR of <i>A. tumefaciens</i> transformed with pMDC221_GFP	93
Figure 4.20: Colony PCR of <i>A. tumefaciens</i> transformed with pMDC150_35S	94
Figure 4.21: Map of the recombinant constructs used in this study	95
Figure 4.22: PCR confirmation from four-week-old T ₂ transgenic AtYoeBGFP and AtGFP plants	97
Figure 4.23: Detection of the yefM _{Spn} antitoxin in transformed <i>A. thaliana</i>	98
Figure 4.24: Effects of yoeB _{Spn} -GFP expression in <i>A. thaliana</i> over a time course of 9 days	99
Figure 4.25: GFP fluorescence image of rosette leaves of T ₂ <i>A. thaliana</i>	100
Figure 4.26: Four-week-old T ₂ transgenic <i>A. thaliana</i> plants harbouring the yefM _{Spn} transgene 3 days, 6 days and 9 days post-induction (dpi) with 17-β-estradiol	101
Figure 4.27: Transcript analysis of T ₂ transgenic AtYoeBGFP and AtYefM from line 1 after induction with 17-β-estradiol	102

Figure 4.28: Agarose gel electrophoresis of RT-PCR products with <i>Actin</i> -specific primer of T ₂ transgenic AtYoeBGFP and AtYefM from line 1 after induction with 17-β-estradiol	103
Figure 4.29: The levels of <i>yoeB_{Spn}</i> toxin and <i>yefM_{Spn}</i> antitoxin transcripts in transgenic plants from day 1 – day 7 after 17-β-estradiol induction as determined by qRT-PCR	104
Figure 4.30: DNA extracted from <i>A. thaliana</i> and electrophoresed for 3 h on 1.8% agarose	106
Figure 4.31: Detection of <i>yefM_{Spn}</i> and <i>yoeB_{Spn}-GFP</i> transgenes in <i>yefM_{Spn} × yoeB_{Spn}-GFP</i> hybrids of transgenic <i>A. thaliana</i>	108
Figure 4.32: The relative expression levels of <i>yefM_{Spn}</i> and <i>yoeB_{Spn}-GFP</i> transcripts in transgenic hybrid <i>A. thaliana</i>	110
Figure 4.33: Morphology of the <i>yefM_{Spn} × yoeB_{Spn}-GFP</i> transgenic hybrid and control plants at 9 weeks	112
Figure 4.34: Silique phenotypes in induced and non-induced plants at 9 weeks after germination	114

LIST OF TABLES

Table 2.1: The cellular targets of some of the TA toxins	18
Table 3.1: Recombinant plasmids constructed in this study	51
Table 3.2: A list of oligonucleotide primers used in this study	52
Table 3.3: PCR conditions using <i>Pfu</i> DNA polymerase	55
Table 3.4: Single Restriction enzyme digestion setup	56
Table 3.5: Double Restriction enzyme digestion setup	57
Table 3.6: Colony PCR reaction setup	60
Table 3.7: PCR conditions using GoTaq [®] Green Master Mix	60
Table 3.8: Selection of transgenic <i>A. thaliana</i>	64
Table 3.9: PCR reaction setup for amplification of <i>A. thaliana</i> genomic DNA	65
Table 3.10: Genomic DNA elimination reaction components	72
Table 3.11: Reverse-transcription reaction components	72
Table 4.1: Transgenic <i>A. thaliana</i> transformed with different recombinant constructs ...	96
Table 5.1: Possible binding sites for the YefM-YoeB _{Spn} -GFP complex in the <i>Arabidopsis thaliana</i> genome based on the 27-nucleotides binding site for YefM-YoeB _{Spn} in <i>S. pneumoniae</i> as determined by DNase I footprinting	129

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
µg	:	Microgram
µl	:	Microlitre
µm	:	Micrometer
µM	:	Micromolar
β	:	Beta
A	:	Adenine (in DNA base sequence)
<i>A. thaliana</i>	:	<i>Arabidopsis thaliana</i>
bp	:	Base pair
°C	:	Degree celcius
CaCl ₂	:	Calcium chloride
CaMV	:	Cauliflower mosaic virus
cDNA	:	Complementary deoxyribonucleic acid
Ct	:	Cycle threshold
CTAB	:	Cetyltrimethylammonium bromide
C	:	Cytosine (in DNA base sequence)
DNA	:	Deoxy ribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxy ribonucleotide triphosphate
dpi	:	Day post-induction
<i>E. coli</i>	:	<i>Escherichia coli</i>
EDTA	:	Ethylene diamine tetra acetic acid
e.g.	:	Exempli gratia
G	:	Guanine (in DNA base sequence)

g	:	Gram
GFP	:	Green Fluorescent Protein
GUS	:	β -glucuronidase
HCl	:	Hydrochloric acid
hr	:	hour
i.e.	:	Id est
kb	:	Kilo bases
L	:	Liter
LB	:	Luria bertani
M	:	Molar
mg	:	Milligram
MgCl ₂	:	Magnesium chloride
MgSO ₄	:	Magnesium sulphate
Min	:	Minute
ml	:	Milliliter
mM	:	MilliMolar
MS	:	Microsoft
NaCl	:	Sodium chloride
ng	:	Nanogram
nm	:	Nanometer
OD	:	Optical density
PCR	:	Polymerase chain reaction
qRT-PCR	:	Quantitative real-time reverse transcriptase PCR
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rpm	:	Revolutions per minute

RT-PCR	:	Reverse-transcriptase PCR
s	:	Second
SDS	:	Sodium dodecyl sulphate
sp.	:	Species
spn	:	<i>Streptococcus pneumoniae</i>
SPSS	:	Statistical package for social sciences
T	:	Thymine (in DNA base sequence)
TBE	:	Tris-borate EDTA
TE	:	Tris-EDTA
Tris-HCl	:	Trisaminomethane hydrochloride
U	:	Unit
UV	:	Ultra-violet
V	:	Voltage
v/v	:	Volume per volume
w/v	:	Weight per volume

LIST OF APPENDICES

Appendix A: Vector maps used in this study	179
Appendix B: Total RNA extracted from transgenic T2 AtYoeBGFP and wild type plants following 17- β -estradiol treatment.....	182
Appendix C: Total RNA extracted from transgenic T2 AtYefM and wild type plants following 17- β -estradiol treatment	183
Appendix D: Concentration and purity reading of extracted RNA from transgenic <i>A. thaliana</i> harboring pMDC150_35S:pMDC221_yoeBGFP and control plants	184
Appendix E: Concentration and purity reading of extracted RNA from transgenic <i>A. thaliana</i> harboring pMDC150_35S: pMDC160_yefM Spn and control plants	185
Appendix F: Melt curve analysis taken at the end of qRT-PCR reactions	186
Appendix G: Agarose gel electrophoresis of total RNA extracted from transgenic hybrid and wild type <i>A. thaliana</i> after induction with 17- β -estradiol	187
Appendix H: Concentration and purity reading of extracted RNA from transgenic hybrid <i>A. thaliana</i> and control plants	188
Appendix I: Transgenic plants expressing yoeB Spn showed characteristic DNA fragmentation patterns indicative of apoptosis. No such fragmentation was observed in the 17- β -estradiol-induced transgenic hybrid plants	189
Appendix J: Ratio (length/width) of the yefM Spn \times yoeB Spn -GFP transgenic hybrid and control plants at 9 weeks.	190

University of Malaya

CHAPTER 1: INTRODUCTION

Toxin-antitoxin (TA) systems were discovered in the early 1980s in bacterial plasmids where they function in maintaining the stable segregation of plasmids. It was only in the mid-1990s that chromosomal homologues of plasmid-encoded TA systems were found and following this, that their characteristics and cellular functions were extensively studied especially in the past two decades (Gerdes et al., 2005; Chan et al., 2014). TA systems are extensively found in bacteria as well as in archaea, but so far, not in eukaryotes (Pandey and Gerdes, 2005; Hayes and van Melderen, 2011; Goeders and van Melderen, 2014). Typically, they consist of two genes: one encoding the antitoxin and the other encoding the toxin. In general, toxins are activated under stress or other conditions that prevent the continuation of antitoxin synthesis, thus liberating the toxin to act on its target. Currently, TA systems are classified into five types, depending to the nature and mode of action of the antitoxin (Unterholzner et al., 2013; Goeders and van Melderen, 2014). Type II TA systems are prevalent in bacterial genomes and are the most widely studied (Yamaguchi et al., 2011; Leplae et al., 2011; Unterholzner et al., 2013; Bertram and Schuster, 2014; Hayes and Kędzierska, 2014). In type II TA systems, the antitoxin and toxin are both proteins and the antitoxin blocks the toxicity of the toxin by binding avidly to the toxin to form an inactive complex (Yamaguchi et al., 2011).

When encoded on plasmids, TA systems were known to play a role in maintaining plasmid stability through a process called post-segregational killing (Gerdes et al., 1986), or addiction (Yarmolinsky, 1995). While the role of TA systems located on plasmids is quite clear, the role of chromosomally-encoded TA systems remained enigmatic for some time (Unterholzner et al., 2013). Chromosomal TA systems have been proposed to function or mediate in a variety of cellular processes such as those related to the global stress response (Christensen et al., 2001),

programmed bacterial cell death (Engelberg-Kulka and Glaser, 1999), maintenance of mobilomes (Rowe-Magnus et al., 2003; Szekeres et al., 2007), persistence (Gerdes and Maisonneuve, 2012), biofilm formation (Harrison et al., 2009; Soo and Wood, 2013), niche colonization (Norton and Mulvey, 2012), virulence (Ren et al., 2012), and phage abortive infection system (Fineran et al., 2009; Dy et al., 2014).

Most known type II TA toxins function as RNases or endoribonucleases (Christensen et al., 2001; Nariya and Inouye, 2008; Jørgensen et al., 2009; Yamaguchi and Inouye, 2009), whereas other toxins target essential cellular components such as DNA gyrase (Van Melderen, 2001), cell wall (Mutschler et al., 2011), and EF-Tu elongation factor (Castro-Roa et al., 2013). Some of these bacterial toxins have been demonstrated to be functionally active when expressed in eukaryotic systems. They have been proposed to have potential biotechnological application in the control of cellular growth in eukaryotic cells particularly in preventing the accidental escape of genetically-modified cells (Kristoffersen et al., 2000). The RelE toxin of *E. coli* was demonstrated to be functional in the yeast *Saccharomyces cerevisiae* where induction of the toxin gene in transformed yeast cells inhibited growth (Yamamoto et al., 2002). Expression of the RelE toxin and the Kid toxin were also shown to trigger apoptosis in a human osteosarcoma cell line (Yamamoto et al., 2002) and in HeLa cells (de la Cueva et al., 2003) respectively. These findings eventually led to the development of a method using the Kis-Kid TA system to select for mammalian cells with a stable and high level expression of transgenes (Nehlsen et al., 2010).

The genome of the Gram-positive human pathogen *Streptococcus pneumoniae* harboured up to 10 putative type II TA systems (Chan et al., 2012). Out of these, four have been identified as functional, namely *relBE2*, *yefM-yoeB_{Spn}*, *pezAT* and *phd-doc* (Chan et al., 2013; Chan et al., 2014). The *yefM-yoeB_{Spn}* system has been well-studied and demonstrated to be biologically functional with overexpression of the *yoeB_{Spn}* toxin

gene leading to cell stasis and eventually cell death in its native host cell as well as in *E. coli* (Nieto et al., 2007; Chan et al., 2011). However, until now there have been no reports on the functionality of any bacterial TA system in plants. The main objective of this study is thus to determine the functionality of the pneumococcal YoeB_{Spn} toxin and YefM_{Spn} antitoxin in *Arabidopsis thaliana* as a model plant using a 17-β-estradiol-inducible two-component plant expression system.

The specific objectives of this study were:

1. To construct the relevant recombinant vectors required for the heterologous inducible expression of the *yoeB_{Spn}* toxin gene and the *yefM_{Spn}* antitoxin gene in *A. thaliana*: the responder vector pMDC221 carrying the *yoeB_{Spn}* toxin gene as a *yoeB_{Spn}-GFP* (YoeB_{Spn}-Green Fluorescent Protein) translational fusion and pMDC160 carrying the *yefM_{Spn}* antitoxin gene;
2. To obtain transgenic *A. thaliana* lines that contained the *yoeB_{Spn}-GFP* and *yefM_{Spn}* transgenes;
3. To determine if the expression of the *yoeB_{Spn}* toxin-*GFP* fusion transgene is lethal in *A. thaliana*;
4. To obtain hybrid *A. thaliana* that contained both the *yoeB_{Spn}-GFP* and *yefM_{Spn}* transgenes through cross-pollination of transgenic *A. thaliana* lines ; and
5. To determine the functionality of the YefM_{Spn} antitoxin in neutralizing the toxicity of YoeB_{Spn} in *A. thaliana*.

CHAPTER 2: LITERATURE REVIEW

2.1 Bacterial Toxin-Antitoxin Systems

Toxin-antitoxin (TA) systems were first discovered in the mid 1980's encoded on low copy number plasmids where they function to mediate the stable maintenance of the plasmid by post-segregational killing (Ogura and Hiraga, 1983). These genetic loci were known as 'addiction modules' since they cause the death of cells in which the plasmids were lost thereby causing the cells to become 'addicted' to the presence of these loci (Jensen and Gerdes, 1995). An addiction module usually comprise of two genes: one gene which encodes for the toxic protein and another encoding for its relatively less stable cognate antitoxin. Subsequent to their discovery on plasmids, pairs of genes homologous to these 'addiction modules' were found on the chromosome of *E. coli* (Aizenmann et al., 1996) and various other bacteria (Gerdes, 2013; Pandey and Gerdes, 2005) where they have evolved to mediate various cellular functions (Unterholzner et al., 2013; Chan et al., 2015).

Most bacteria are now known to harbor several TA systems, and for each of these systems, its antitoxin counterpart renders the encoded toxin inactive under normal conditions. Many studies have shown that both toxin and antitoxin genes are usually co-transcribed and the proteins are co-expressed to form a tight complex (Engelberg-Kulka and Glaser, 1999). Under stressful conditions, transcription of the TA locus is usually disrupted, and the remaining antitoxins in the cell will be degraded by cellular proteases at a faster rate than their cognate toxins. Upon antitoxin degradation, the toxin will be freed and therefore induces cell stasis or death (Gerdes et al., 2005).

In general, the antitoxin protein is usually smaller than the toxin protein. However, there are a few exceptions like the antitoxins encoded by the *higBA* and *hipBA* TA systems (Engelberg-Kulka and Glaser, 1999; Tian et al., 1996; Black et al., 1991). The distance between both toxin and antitoxin-encoding genes varies among the

various TA systems. In some cases, both genes overlap by one nucleotide (in which case the stop codon of the antitoxin gene overlaps with the start codon of the toxin gene) (Pandey and Gerdes, 2005).

A wide variety of TA systems have been discovered particularly in the past decade and currently, TA systems are grouped into classes I, II, III, IV and V according to the nature and action of the antitoxin to neutralize the toxin (Figure 2.1).

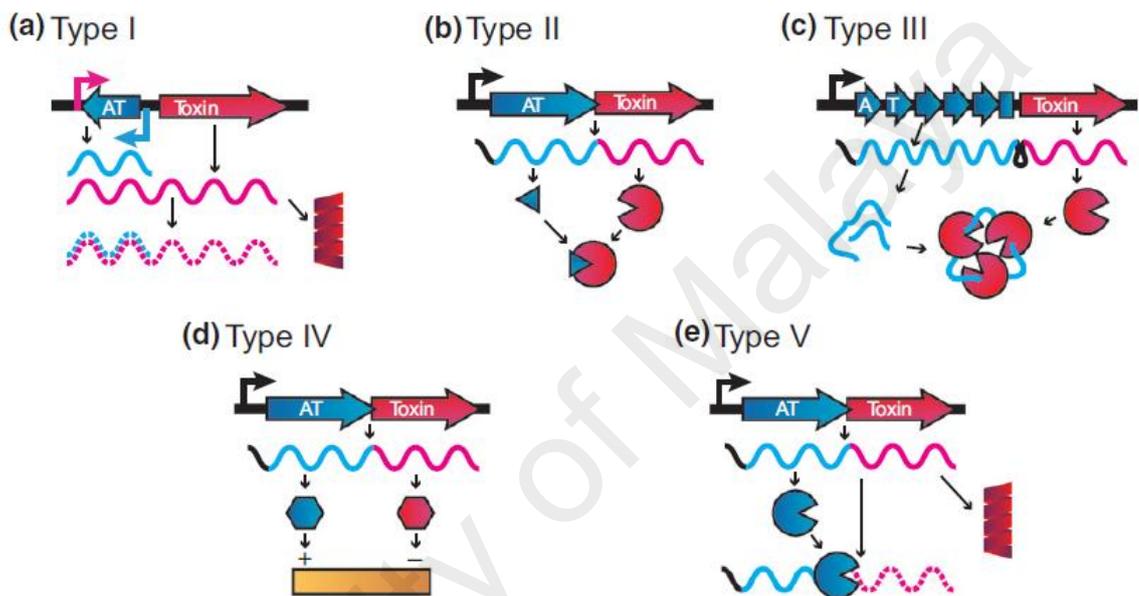


Figure 2.1: Schematic representations of the currently known TA classes. Toxin genes and proteins are illustrated in red, antitoxin (AT) in blue, DNA as sinus curves. (a) Type I TA systems. Toxin and RNA-antitoxin (antisense RNA) are transcribed separately. RNA-antitoxin binds to mRNAs for toxin to form a duplex inhibiting toxin translation. (b) Type II TA systems. Antitoxin and toxin mRNAs are synthesized from the same promoter and both are translated into proteins. Antitoxin forms a tight complex with toxin to inhibit toxin activity. The TA complex autoregulates the operon: The antitoxin itself usually functions as an autorepressor, but more weakly than the TA complex. (c) Type III TA systems. The toxin protein binds to antitoxin RNA, thereby inhibiting the toxicity. (d) Type IV TA systems. The toxin protein inhibits the target molecules (orange box), whereas the antitoxin molecules counteract these effects by binding to the same target molecules. (e) Type V TA systems. The mRNA of the small toxin-encoding ORF is cleaved by the antitoxin, which functions as a toxin-specific ribonuclease. Diagram was obtained from Schuster and Bertram, (2013).

2.1.1 Type I TA systems

In Type I TA systems, the toxin gene expression is controlled by an RNA antitoxin located adjacent to the toxin gene but transcribed in reverse orientation (i.e., antisense RNA) and therefore inhibits the translation of the toxin mRNA, a process called RNA interference (Yamaguchi et al., 2011). Examples of this system are chromosomally located operons found in *Escherichia coli*, such as *tisAB* (Vogel et al., 2004) and *symER* (Kawano et al., 2007) as well as the plasmid-encoded loci *hok-sok* and *parB* of *E. coli* (Gerdes et al., 1986). All these systems have different roles and functions. Generally, all toxins (except the SymE toxin) from type 1 TA systems are small hydrophobic proteins, each comprising less than 60 amino acids that contain a potential transmembrane domain (Fozo et al., 2008). They act by introducing pores into the cell membrane, which then leads to weakening ATP synthesis. As a result, replication, transcription and translation may be inhibited, eventually leading to cell death.

The *hok-sok* of plasmid R1 was the first type I TA system to be discovered due to its ability to stabilize heterologous replicons in *E. coli* (Gerdes et al., 1986). The *hok/sok* TA system was known to mediate plasmid maintenance through a process called post-segregational killing (PSK), a common phenomenon in some protein-regulated TA systems (Bravo et al., 1987; Jaffe et al., 1985; Gerdes and Maisonneuve, 2012). This TA system has three genes: ‘host killing’ (*hok*) encodes a highly toxic transmembrane protein that permanently disrupts the cell membrane (Gerdes et al., 1986); ‘modulation of killing’ (*mok*) overlaps with *hok* and is required for *hok* translation; ‘suppression of killing’ (*sok*) encoded an antisense RNA in *cis* that blocks translation of *mok*. Because translation of *hok* relies on *mok* translation, the *sok* antisense RNA indirectly inhibits *hok* translation by inhibiting *mok* translation (Thisted and Gerdes, 1992). The *sok* antisense RNA is very unstable and is easily degraded when

the R1 plasmid is lost from the cell. Under these conditions, the more stable *hok* mRNA is translated, and the resulting Hok protein kills the cells that are no longer carrying the plasmid (Gerdes and Wagner, 2007).

In addition to the *hok/sok* TA system, the chromosomally-encoded *symER* in *E. coli* has also been characterized as a type I TA system (Kawano et al., 2007). SymR, is a cis-encoded sRNA in *E. coli* and tightly controls the synthesis of SymE toxin, a SOS-induced protein which also depends on the degradation by the Lon protease. In response to DNA damage, SymE is proposed to play a role in recycling RNAs damaged by agents that induce the SOS response. The SymE toxin acts as an mRNA interferase where the toxin binds to ribosomes in order to exert its activity. Moreover, overproduction of the toxin inhibits cell growth, reduces protein synthesis and increases RNA degradation (Kawano et al., 2007). While other antitoxins from previously characterized TA systems are rapidly degraded (such as the *sok* antisense RNA that is extremely unstable), an interesting difference was reported where the *symR* antitoxin RNA was found to be quite stable and in this case, the toxin was instead the target of Lon protease (Kawano et al., 2007).

2.1.2 Type II TA systems

Among all TA systems, the most studied are those that belong to type II TA systems. These usually comprise of two co-transcribed genes that encode an unstable antitoxin and a stable toxin. Antitoxins of this class are proteins and they bind to the proteic toxins through direct protein-protein interactions. The first TA system identified that belonged to this system was *ccdAB* on the low copy number F plasmid of *E. coli* (Ogura et al., 1983). In this TA system, the CcdB acts as a toxin whereas CcdA acts as an antitoxin. The CcdB toxin kills by interfering with the function of the bacterial DNA gyrase, an essential enzyme that causes negative supercoiling of the DNA. This will

cause double-stranded DNA breaks, followed by induction of the SOS response (Karoui et al., 1983; Bernard and Couturier, 1992; Baharoglu and Mazel, 2014)) and ultimately cell death.

Another TA system that belongs to this class is *parDE*. It was discovered on the broad host range, low copy number plasmid RK2 (also known as RP4) in Gram-negative bacteria. Its function is known to maintain the stability of the RK2 plasmid in its host cells (Saurugger et al., 1986; Gerlitz et al., 1990; Roberts et al., 1990; Deghorain et al., 2013). The ParE protein is a toxin that inhibits cell growth, causes extensive cell filamentation and eventually leads to cell death (Roberts et al., 1994; Johnson et al., 1996; Deghorain et al., 2013). In vitro, ParE hinders *E. coli* DNA gyrase in the presence of ATP and converts the supercoiled plasmid DNA to a singly cleaved linear form (Jiang et al., 2002; Deghorain et al., 2013). However, addition of the ParD antitoxin can prevent and reverse the inactivation of gyrase by ParE (Jiang et al., 2002).

Chromosomally encoded type II TA systems are now known to be nearly ubiquitous in bacteria and archaea (Leplae et al., 2011). However, the function of chromosomally-encoded TA systems is varied and was the subject of intense debate. One of the first described and well-characterized chromosomally-encoded TA system was the *Escherichia coli*-encoded *mazEF* (toxin MazF and antitoxin MazE). The MazEF system was found to mediate cell death under a wide variety of stresses, including nutritional stress, short-term antibiotic exposure, high temperature and oxidative shock (Van Melderen and De Bast, 2009). Under stress conditions, the MazE antitoxin is rapidly degraded by an ATP-dependant protease thus releasing the MazF toxin (Melderen and De Bast, 2009). The released MazF toxin prevents translation by cleaving RNAs, resulting in cell death or growth arrest (Christensen et al., 2003; Kolodkin-Gal and Engelberg-Kulka, 2006; Van Melderen and De Bast, 2009). Numerous homologs of MazEF have been discovered in various bacterial genomes

(Leplae et al., 2011). More recently, a MazEF homolog was reported in the probiotic bacterium, *Bifidobacterium longum* and experiments showed that MazEF_{Bif} was induced under acid stress condition. Overexpression of MazF_{Bif} was toxic to *E. coli* which resulted in severe growth inhibition or cell death and its toxicity could be neutralized by the co-expression of its cognate antitoxin MazE_{Bif} (Wei et al., 2015).

Research into another *E. coli*-encoded TA system, *relBE*, showed that activation of the RelE toxin under conditions of nutritional stress led to cell growth arrest instead of cell death (Christensen and Gerdes, 2003). The *relE* gene is located downstream of *relB* and upstream of *relF*. The *relF* gene is a *hok* homolog, and overproduction of the RelF protein led to rapid interruption of cell growth, arrest or respiration and collapse of the cell membrane potential (Gerdes et al., 1986). Later on, the *relF* gene was designated *hokD* (Pedersen and Gerdes, 1999). Based on the analysis of the *relBE* TA system, *relE* was shown to encode a very potent inhibitor of cell growth and that cell growth inhibition was because of inhibition of translation (Gotfredsen and Gerdes, 1998; Pedersen et al., 2002). In contrast to the MazF toxin, the RelE toxin does not target free mRNA but cleaves mRNA in the ribosomal A site with codon specificity (Christensen and Gerdes, 2003). In addition to that, Gotfredsen and Gerdes (1998) have shown that the *relBE* of *E. coli* K-12 had the genetic organization of a type II TA system: (i) *relE* encodes a cytotoxin that is lethal to host cells; (ii) *relB* encodes an antitoxin that neutralize the lethality of the *relE*-encoded toxin; and (iii) the RelB antitoxin autoregulates the *relBEF* operon at the level of transcription.

2.1.3 Type III TA systems

The ability of bacteria to develop resistance to phage infection led to the identification of type III TA systems (Fineran et al., 2009). The *toxIN* operon is a type III TA system encoded on the cryptic plasmid pECA1039 that was isolated from

Pectobacterium atrosepticum strain SCRI1039 (previously named *Erwinia caratova*), a plant pathogen, with homologues found in several genera of both Gram-negative and Gram-positive bacteria (Fineran et al., 2009). One gene on pECA1039, named *toxN*, was shown to encode a protein with 31% identity to the abortive infection protein AbiQ, present in *Lactococcus lactis* W37 (Fineran et al., 2009; Emond et al., 1998).

Unlike type I TA systems, where the antitoxin and toxin interact as RNAs, or type II TA systems, where they interact as proteins, in a type III TA system, the ToxN toxin is directly inhibited by binding to the RNA antitoxin (ToxI) forming an RNA-protein complex (Blower et al., 2009; Fineran et al., 2009; Blower et al., 2012). The ToxN toxin functions as an endoribonuclease and a short palindromic repeat which precedes the *toxN* gene functions as a transcriptional terminator, controlling both antitoxin RNA and toxin transcript levels. During phage infection, changes in host transcription or translation or the degradation of bacterial DNA could change the ToxI:ToxN ratio, resulting in the release of active toxin that eventually cleave cellular RNAs (Fineran et al., 2009; Cook et al., 2013).

In Gram-positive and Gram-negative bacteria, several type III TA systems were identified which not only showed similarity to ToxN, but also consisted of putative RNA antitoxin sequences (Fineran et al., 2009). For instance, the putative ToxIN homolog from *Bacillus thuringiensis* was shown to act as a TA system in kill/rescue assays in *E. coli* (Fineran et al., 2009). Based on a thorough search using the structural information obtained from ToxN, 37 putative Type III TA loci were discovered and further grouped into three distinct families (Blower et al., 2012). Using a sequence-based search with representative of these three families, a further 125 putative type III TA systems were identified and each of these three families functioned as TA systems in kill/rescue assays. Interestingly, at least two of these families confer resistance to phages, although this might not be their sole function (Blower et al., 2012). Type III

TA systems are not as widespread as type I or type II TA systems, but it is possible that the number of type III TA systems will increase as more are discovered in the future. Blower et al., (2012) reported that active type III TA systems are far more distinct than previously known, and suggested that more remain to be elucidated.

2.1.4 Type IV TA systems

Among all classes of TA systems in which the toxin and antitoxin interact either at the RNA or the protein level, only the components of type IV TA systems do not directly interact. In Type IV TA systems, the proteic antitoxin interferes with binding of the toxin to its substrate rather than inhibiting the toxin directly by protein-protein interaction as in type II systems. The YeeU/YeeV (also known as CtdA/CtdB) system of *E. coli* falls into this class of TA system. The YeeV toxin functions by disrupting the polymerization of MreB and FtsZ proteins, which are the homologues of eukaryotic actin and tubulin, respectively (Van den Ent et al., 2001; Van den Ent et al., 2010), leading to disruption of cytoskeleton assembly. The YeeU antitoxin counteracts YeeV by binding and stabilizing the MreB and FtsZ polymers thereby reversing the toxic action of YeeU (Masuda et al., 2012a).

Similarly, another type IV TA system of *E. coli*, *cptA/cptB* (*ygfX/ygfY*) was also reported by Masuda et al., (2012b). The putative toxin, YgfX was shown to cause cell growth inhibition and led to significant changes in the cellular morphology of *E. coli*. When the YgfX toxin was induced, the cells were initially elongated and subsequently became inflated in the middle. However, the co-expression of YgfY antitoxin was shown to be able to neutralize the YgfX toxicity, indicating that YgfY is an antitoxin of YgfX. In this report, YgfX is the first *E. coli* TA systems shown to be associated with membrane. Like the YeeV toxin, YgfX was shown to inhibit cell division by interfering with the polymerization of essential bacterial cytoskeletal proteins, FtsZ

and MreB. Based on these results, Masuda et al., (2012b) proposed to rename YgfX and YgfY as CptA and CptB (for Cytoskeleton Polymerization inhibiting Toxin A and B), respectively.

2.1.5 Type V TA systems

The *ghoS/ghoT* TA system of *E. coli* was recently designated as a type V TA system (Wang et al., 2012). The activity of the *ghoT* toxin gene is regulated post-transcriptionally by the GhoS antitoxin which functions as an endoribonuclease with specificity for the GhoT toxin mRNA, thereby preventing toxin translation. The unique mechanism of how this toxin is inactivated makes it a principal distinctive criterion for the type V TA system, which is otherwise genetically similar to type II TA loci. In addition to that, as compared to type II TA systems, GhoS is stable and not a transcriptional regulator of its own operon. Overexpression of GhoT damages cell membrane and resulted in reduced cellular levels of ATP. As a result, GhoT has been demonstrated as a membrane lytic peptidase that causes ghost cell formation (lysed cells with damaged membranes). This new TA system was also found in *Shigella*, *Salmonella*, *Citrobacter* and *Proteus* spp. (Wang et al., 2012).

2.2 Functions of TA systems

The function of plasmid-encoded TA systems is clear. When TA systems were first discovered on plasmids, they were known to play a role in maintaining plasmid stability through a process called post-segregational killing (Gerdes et al., 1986), or addiction (Yarmolinsky, 1995). During cell division, if the plasmid that harboured a TA system fails to segregate to both daughter cells, then the destiny of the siblings is greatly different. In the daughter cells which did not inherit the plasmid, the unstable antitoxin is degraded by cellular proteases while the stable toxin remains and acts on its cellular target resulting in killing or inhibition of cellular growth (Figure 2.2A). In

contrast, the plasmid-containing daughter cells remained viable through the continued expression of the antitoxin gene (Gerdes et al., 1986; Thisted et al., 1994) thereby out-competing any plasmid-free daughter cells that developed. In addition, plasmid-encoded TA systems are also necessary for mediating the exclusion of co-existent compatible plasmids (Cooper and Heinemann, 2000). During conjugation, cells containing two plasmids from the same incompatibility group can be produced but cannot be securely preserved in the same host. The plasmid that harboured a TA system will be maintained through postsegregational killing whereas the loss of the other plasmid without a TA system will not affect the cell (Figure 2.2B). After several rounds of conjugation and subsequent exclusion, the plasmid which harboured a TA system can surpass the second plasmid from the bacterial population (Cooper and Heinemann, 2000; Unterholzner et al., 2013).

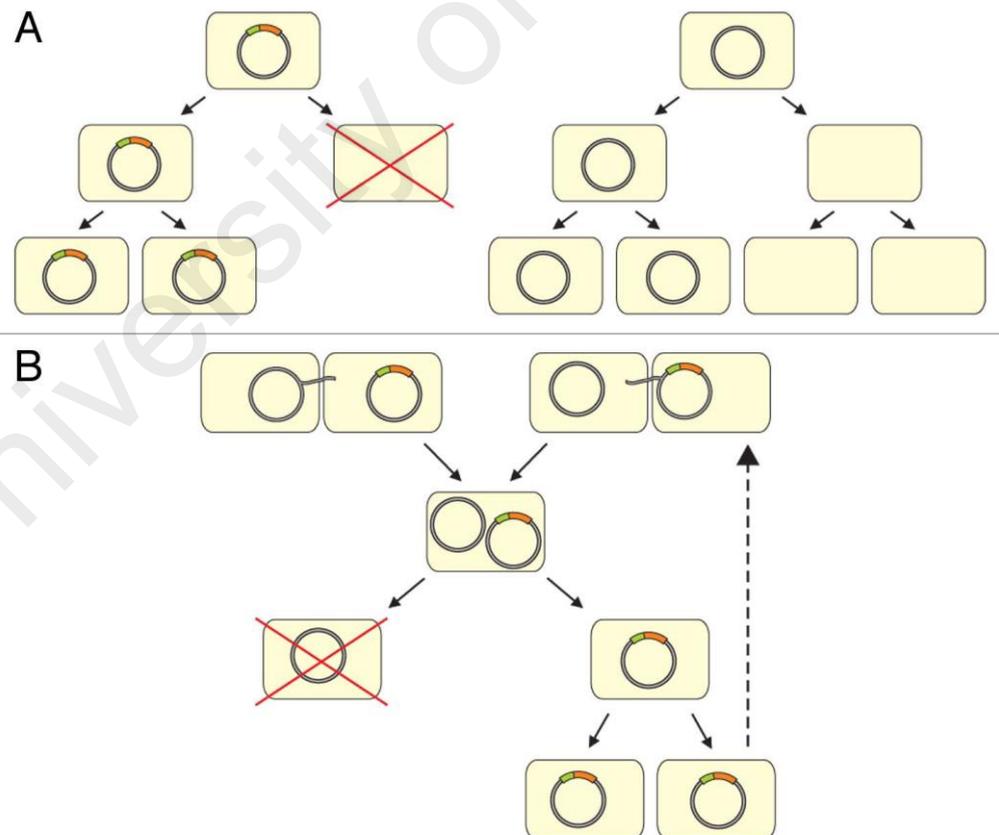


Figure 2.2: Functions of plasmid-encoded TA systems. (A) Stabilization of plasmids by post segregational killing. (B) Exclusion of co-existing incompatible plasmids. Diagram obtained from Unterholzner et al., (2013).

While the role of TA systems located on plasmids is quite clear, the role of chromosomally-encoded TA systems remained enigmatic for a long time (Unterholzner et al., 2013). There have been a number of reports on the functionality of these TA systems such as involvement in protection against invading DNAs (i.e. plasmids and phages). Bacteria have developed various phage-exclusion mechanisms including abortive infection, during which the bacteriophage-infected cells commit suicide to prevent the spread of phages in the bacterial population. For example, the *hok-sok* type I TA system of plasmid R1 has been shown to exclude T4 phages in *E. coli* and this observation led to the conclusion that *hok-sok* decreased the T4 burst size, increased the time to form mature phages and increased the time to cell lysis (Pecota and Wood, 1996). Similarly, Hazan and Engelberg-Kulka, (2004) have demonstrated that the chromosomal *mazEF* type II TA system from *E. coli* induced abortive infection upon P1 bacteriophage attack. Although *mazEF* caused lethality in cells upon phage growth, this was advantageous to the bacterial culture as it caused P1 phage to be excluded from the bacterial population and thus protecting the cells.

TA systems have also been implicated in bacterial persistence and have been well reported (Lewis, 2010; Gerdes and Maisonneuve, 2012; Wen et al., 2014). Persistence can be described as a phenotypic variant of bacterial or unicellular fungal cells that are much less sensitive to antibiotics than most other cells in an isogenic population, leading to antibiotic tolerance (Lewis, 2010). Involvement of TA systems in the development of bacterial persistence has further been validated by mutagenesis studies. Deletion of the *tisAB*, *mqsRA* or *hipAB* operons dramatically affected the level of persistence (Keren et al., 2004; Dorr et al., 2010; Kim and Wood, 2010). The effect is more distinct in stationary-phase or biofilm cells where a higher frequency of persistence formation is observed. The functional redundancy of several type II TA systems in some bacterial genomes meant that deletion of a single TA operon did not

always result in the complete withdrawal of persister formation. Maisonneuve et al., (2011) demonstrated that upon antibiotic treatment, there was a progressive decrease in survival when up to ten endoribonuclease-encoding TA systems were disrupted in *E. coli*, resulting in a 100- to 200-fold reduction in persistence formation.

Other possible functions of chromosomally encoded TA systems include regulation of biofilm formation and action as global regulators (Wang and Wood, 2011). It is well known that bacteria often grow in dense, multicellular communities called biofilms (Sauer and Camper, 2001). Biofilm formation is a well-controlled developmental process with unique steps including initial attachment to the surface, maturation of the biofilm and detachment of cells and dispersal (Hall-Stoodley and Stoodley, 2002). Many infectious diseases are correlated with the biofilm formation capability of pathogenic bacteria (Von Rosenvinge et al., 2013). Some examples of biofilm-associated infections include those that are associated with *Pseudomonas aeruginosa* (Mulcahy et al., 2014) and *Mycobacterium tuberculosis* (Bjarnsholt, 2013). However, a direct role for TA systems in biofilm formation has long been debated. The *E. coli*-encoded MqsR toxin that forms a type II TA system with its cognate antitoxin, MqsA, was the first TA system that was reported to directly regulate biofilm formation (Ren et al., 2004; Kasari et al., 2010). Further evidence of the role of TA systems in biofilm formation was obtained by studying an *E. coli* strain named $\Delta 5$ that had five of the most-studied TA systems deleted (Tsilibaris et al., 2007). This strain lacks the TA pairs MazF/MazE, ChpBI/ChpBK (of the *chpB* locus) RelE/RelB, YoeB/YefM, and YafQ/DinJ. It was reported that these five deletions had no impact on the stress response of cells (Tsilibaris et al., 2007); however, Ren et al., (2004) had earlier reported that TA systems were important for biofilm formation based on their microarray results. Upon deletion of these five TA systems, biofilm formation

decreased after 8 hours but increased after 24 hours in rich medium at 37°C (Ren et al., 2004).

TA systems are not only involved in general bacterial physiological processes such as the general stress response, persistence and biofilm formation but also have a direct effect on the pathogenicity of bacteria (reviewed by Wen et al., 2014). Georgiades and Raoult, (2011) discovered unexpected correlation between the number of TA systems in the genome and the virulence capacity of bacteria. Most pathogenic bacteria acquire antibiotic resistance and virulence genes in large mobile elements known as resistance or pathogenicity islands and TA systems could be found in some of these genomic islands (Ma et al., 2013). Pathogenic bacteria frequently employ suicide mechanisms, in which the dead cells benefit the population that survived. This mechanism was found to be controlled by TA systems which are related to DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis and ATP synthesis (Yamaguchi et al., 2011). In *Haemophilus influenzae* (a human pathogen that causes respiratory tract infections and is the most common cause of recurrent otitis media), deletion of VapBC TA homologues resulted in strong reduction of virulence in tissue and animal models for otitis media (Ren et al., 2012). Another notable finding was reported by Audoly et al., (2011) which involved VapC in *Rickettsia*: VapC was expressed and released in the cytoplasm upon chloramphenicol treatment and caused apoptosis in the host cell, but was not released in untreated infected cells. A further demonstration showed that VapC toxicity was related to its RNase activity (Audoly et al., 2011).

In general, toxin proteins of TA systems function by disrupting a broad range of cellular targets (Figure 2.3). Most identified toxins from TA systems are proteins whose activity usually leads to the inhibition of cell growth by interfering with cellular processes such as DNA replication, translation, cell division and ATP synthesis (Table

2.1). Some toxins may also interfere with the synthesis of the bacterial cell wall (Mutschler & Meinhart, 2011; Yamaguchi et al., 2011).

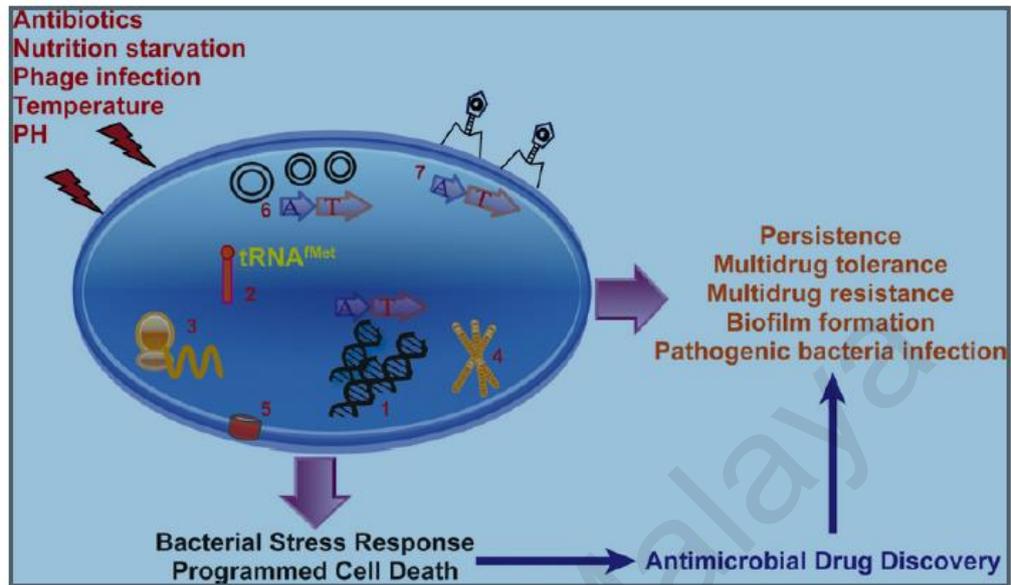


Figure 2.3: TA systems are involved in a broad range of cellular processes which are summarized in this diagram: 1) DNA replication. 2) tRNA-related translation. 3) Macromolecular synthesis. 4) Cytoskeletal polymerization. 5) Cell wall disruption. 6) Plasmid maintenance. 7) Phage infections. Figure obtained from Wen et al., (2014).

Table 2.1: The cellular targets of some of the TA toxins. Table adapted from Wen et al., (2014).

Targets of toxins		
Involved cellular process	Target	Toxin–antitoxin systems
DNA replication	DNA gyrase	CcdA-CcdB ParD-ParE
tRNA synthesis	Glutamyl-tRNA synthetase (GltX)	HipA-HipB
Macromolecular synthesis	Ribosome-dependent tRNA ^{fMet} Ribosome-independent	RelB-RelE Doc-Phd VapB-VapC MazE-MazF MqsA-MqsR
Cell wall synthesis	UDP-N-acetylglucosamine (UNAG)	ω - ϵ - ζ
Membrane disruption	Inner membrane	Sok-hokTisB-IstR
Phage infections		ToxN-ToxI
Cytoskeletal polymerization	MreB and FtsZ	CbtA-CbtE

2.3 *Streptococcus pneumoniae* and its TA systems

The Gram-positive bacterium, *Streptococcus pneumoniae* (the pneumococcus) is the common cause of respiratory tract infections and has been associated with outstanding morbidity and mortality (Chan et al., 2012). *S. pneumoniae* is an important cause of sepsis, meningitis, pneumonia and otitis media. Every year, it has been estimated that 14.5 million episodes of serious pneumococcal disease occurred, resulting in the deaths of nearly 2 million children below 5 years, which is more than AIDS, tuberculosis and malaria combined (O'Brien et al., 2009).

Up to 10 putative type II pneumococcal TA systems have been identified from a bioinformatics search of available pneumococcal genomes (Chan et al., 2012) and out of these, 4 have been identified as functional, namely *relBE2*, *yefM-yoeB*, *pezAT* and *phd-doc* (Chan et al., 2013; Chan et al., 2014).

One of the best-studied type II TA is the *relBE* family, originally found in the genome of *E. coli* K12 (Gotfredsen and Gerdes, 1998; Gronlund and Gerdes, 1999; Christensen and Gerdes, 2004; Cherny et al., 2007; Overgaard et al., 2008; Hurley et al., 2011). The genome of *S. pneumoniae* harboured two *relBE* homologs, designated *relBE1* and *relBE2* (Nieto et al., 2006). In the case of *relBE2*, the *relE2_{spn}* expression was toxic to both *S. pneumoniae* and *E. coli* which resulted in cell growth inhibition in the latter host, however it could be rescued by its cognate *relB2_{spn}* antitoxin, resulting in normal cell growth (Nieto et al., 2006). Overexpression of *relBE1* was found to be not toxic in *E. coli* as well as *S. pneumoniae*, suggesting that this homolog was likely non-functional (Nieto et al., 2006; Chan et al., 2014).

The pneumococcal *yefM-yoeB* chromosomal TA locus was first identified based on its similarity with the *axe-txe* TA system of multidrug-resistance and non-conjugative plasmid pRUM in *E. faecium* (Grady and Hayes, 2003). It was shown that the expression of the *txe* toxin gene inhibited protein synthesis in the cell but did not affect DNA or RNA synthesis (Halvorsen et al., 2011). YefM belongs to the Phd family of antitoxin, whereas YoeB is a homolog of the RelE toxin rather than Doc, the cognate toxin of Phd (Kumar et al., 2008). YoeB is a well-folded protein whereas YefM is a natively unfolded antitoxin, lacking in secondary structure even at low temperatures or in the presence of a stabilizing agent (Cherny and Gazit, 2004). The *E. coli*-encoded YefM-YoeB forms a trimeric YefM₂YoeB complex with the YefM dimer having a symmetrical N-terminal globular structure while the C-terminus is natively unfolded in the absence of YoeB but undergoes a disorder-to-order transition upon YoeB binding (Kamada and Hanaoka, 2005). The pneumococcal *yefM-yoeB_{spn}* was shown to be a functional TA system with overexpression of the *yoeB_{spn}* toxin gene leading to cell death in both *S. pneumoniae* and *E. coli* (Nieto et al., 2007). In *E. coli*, homologous YoeB toxins have been shown to be endoribonucleases and their activity neutralized by

tight binding with their cognate YefM antitoxin, leading to inhibition of the YoeB toxin activity (Nieto et al., 2007).

Epsilon/Zeta (ϵ/ζ) is a unique three-component TA system first discovered in plasmid pSM19035 from *Streptococcus pyogenes* whereby Epsilon functions solely as an antitoxin to the Zeta toxin whereas Omega (ω), encoded upstream from ϵ/ζ functions as the transcriptional regulator to the operon (Meinhart et al., 2001). Homologues of ϵ/ζ were discovered in the genome of *S. pneumoniae* and were designated *pezAT* (pneumococcal epsilon-zeta). No homologue of the ω regulator was found in *S. pneumoniae*, instead the PezA antitoxin functions as a transcriptional autorepressor much like other two-component TA systems (Khoo et al., 2007). While the mode of action of most other TA toxins was known, the toxin activity of the ζ /PezT toxins had remained mysterious until recently (Gerdes, 2013). Unlike any other TA systems, neither the primary amino acid sequence nor any tertiary structure contributed adequate information to define detailed knowledge of its toxic activity. Nevertheless, the toxic mechanism of ζ /PezT toxins was suggested to be different from other TA toxins as its active site contained a characteristic kinase-fold or Walker A motif for ATP/GTP binding (Meinhart et al., 2003; Gerdes, 2013). The ζ /PezT toxin was subsequently shown to kill bacterial cells by impairing the integrity of the bacterial cell wall, particularly affecting a conserved component, uridine diphosphate-*N*-acetylglucosamine (UNAG), which is essential for synthesis of the peptidoglycan layer (Mutschler et al., 2011). Thus ζ /PezT toxins are the first TA toxins recognized to harm bacterial cell wall synthesis.

Recently, *S. pneumoniae* was shown to encode a fourth functional TA system, known as *phd-doc*, in addition to the three well-characterized TA systems namely *relBE2*, *yefM-yoeB* and *pezAT*. This TA system comprises of the Phd (prevents host death) antitoxin and the Doc (death on curing) toxin and is one of the

smallest families of TA systems which was first discovered as a plasmid addiction module on *E. coli* bacteriophage P1 (Lobočka et al., 2004). P1-encoded Doc was reported to be a kinase and phosphorylates the EF-Tu elongation factor on a conserved threonine residue and therefore inhibited protein synthesis which ultimately kills bacterial cells (Cruz et al., 2014). Similarly, the pneumococcal Doc was shown to exhibit toxicity to its natural host and co-expression of its cognate Phd antitoxin was able to neutralize the toxicity of Doc, indicating that Phd-Doc is a functional TA (Chan et al., 2014).

2.4 Applications of TA systems in Biotechnology

2.4.1 Tools for molecular biology

TA systems have been demonstrated to be very useful in the further development and improvement in recombinant DNA technology and heterologous protein expression. The first company that marketed a TA system-based molecular biology kit was Delphi Genetics SA (Stieber et al., 2008). In DNA cloning, the most common problem is the difficulty in inserting a heterologous fragment into a linearized vector. Strategies based on TA systems were used to resolve this problem. The first of these systems was developed using the CcdA/CcdB TA pair from the plasmid F of *E. coli* (Bernard et al., 1994). The *ccd* system is composed of two genes, *ccdA* and *ccdB* which encode small proteins: the CcdA antitoxin and the CcdB toxin. The CcdB protein acts as a poison because it selectively targets the *E. coli* DNA gyrase, a bacterial topoisomerase II. The *ccdB* selection method (Bernard et al., 1994) was commercially developed by Delphi Genetics which eventually sold the license of this technology to Invitrogen Inc. It has proven to be a successful approach for constructing positive selection vectors. The *ccdB* toxin gene, that is inactivated upon insertion of foreign DNA (Schuster and Bertram, 2013), allowed only insert-containing clones to grow (Figure 2.4A). The principle of this strategy involves site-specific recombination to

insert a gene of interest into the vector and the recombinants are selected by the replacement of the toxin gene by the gene of interest (Schuster and Bertram, 2013).

The *ccdA/ccdB* module was subsequently used in the StablyCloning™ system which was commercialized by Invitrogen (Stieber et al., 2008). In this system, the vector contains a truncated inactive *ccdA* antitoxin. Insertion of a 14 bp sequence to the 5' end of the DNA fragment to be cloned, for instance by including the sequence in the PCR primer will restore the active antitoxin, which is capable of neutralizing the toxin that has been introduced into the genome of the host cell (Stieber et al., 2008). As a result, only cells that contained the vector with a correct insert can form colonies (Figure 2.4B). An advantage of using this system is that there is no need to include antibiotic resistance genes in the DNA vector, as reconstituted CcdA functions as the selective marker for plasmid maintenance (Gerdes, 2013). An additional advantage of this procedure is the speed of the whole cloning procedure, which is 1 hour until plating (Stieber et al., 2008).

The *ccdA/ccdB* system has also been successfully incorporated into the Gateway™ cloning system that is based on the recombination system of phage λ . The fragment to be cloned is flanked by *attB1* and *attB2* sequences that are attached by PCR, producing a PCR fragment containing these two sites. Subsequently, an *in vitro* recombination reaction is performed between the PCR fragment and the vector containing the *attP1* and *attP2* sites from *E. coli*. To differentiate between vectors containing the insert and an empty vector, the *ccdB* toxin is placed in between the *attP1* and *attP2* sites in the empty vector, ensuring that cells that are transformed with the empty vector do not survive (Figure 2.4C).

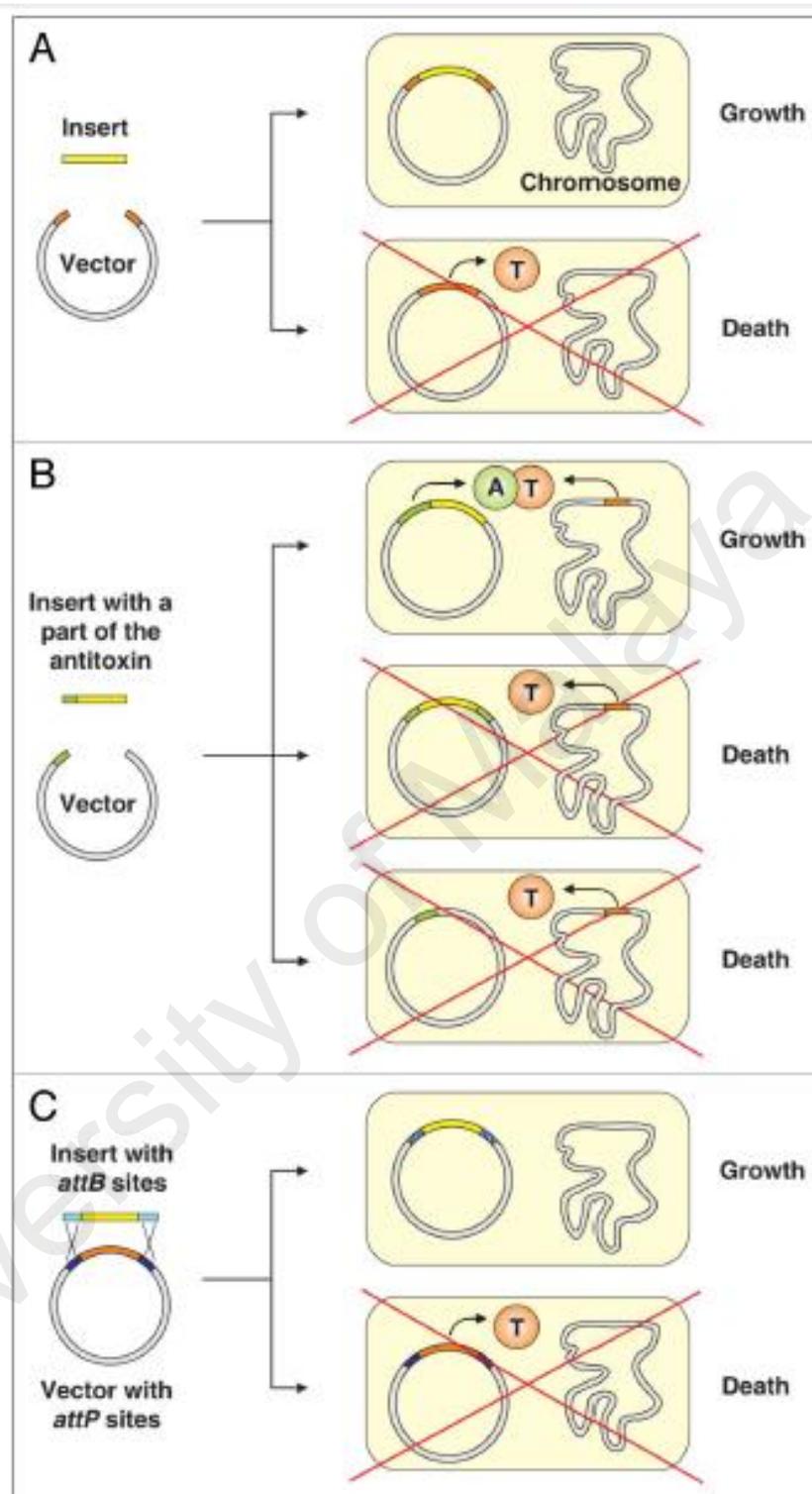


Figure 2.4: Application of TA systems for DNA cloning. (A) Insertion of the gene of interest destroys the toxin gene and allows the bacteria to growth. (B) Strategy of the StablyCloning™ system. (C) Principle of the selection used in the Gateway Cloning™ system. Diagram obtained from Unterholzner et al., (2013).

2.4.2 Suitability as antibacterial target

The evolution and development of pathogenic bacteria that have become resistant to multiple antibiotics through lateral gene transfer have increased the need for novel antimicrobials. TA systems have no human homologs and appear to be present in most of the important bacterial pathogens. In addition, TA systems are known to have a great potency to inhibit growth or even kill cells and therefore, they have been proposed as potential targets for development of antibacterial drugs (Williams and Hergenrother, 2012; Chan et al., 2015). The most straightforward approach for toxin activation would be a drug that directly targets the TA system proteins to either disrupt preformed complexes or to prevent their formation in the first instance (Figure 2.4). Due to its inherent antibacterial activity, TA toxins could be used for killing cells directly or to support antibiotic treatments.

Alternatively, activation of the cellular proteases by Lon or Clp would lead to antitoxin degradation, thereby release the toxin and allowing it to kill the host cell (Figure 2.4). This is in view of the observation that in response to cellular stress, activation of certain toxins requires increased expression of Lon or Clp proteases, which are responsible for the degradation of a few antitoxins (Christensen et al., 2001). Antitoxins are susceptible to degradation by cellular proteases and therefore must be constantly replenished in cells to prevent their toxic partners from functioning. In this case, activation of these proteases could serve as an indirect mechanism for toxin activation within the cell. Such mechanism was observed in Lon overproduction conditions in *E. coli*, whereby the YoeB toxin was activated due to degradation of the cognate YefM antitoxin, resulting in mRNA cleavage and cell lethality (Christensen et al., 2004).

Apparently, both direct and indirect strategies could activate the toxin from the inert TA complex to kill the bacterial cell (Figure 2.5). However, there are two main

areas that need to be taken into consideration to achieve this successful application. The first one would be an understanding of which TA pairs could be used as ideal targets of an artificial activator and once the specific TA target systems have been identified, the next one would be to identify which molecules need to be activated that could lead to toxin-mediated cell death. The choice of TA pair is important as the activation of some toxins could lead to cells entering a dormant state (i.e., becoming persister cells) that leads to antibiotic tolerance instead (Chan et al., 2015).

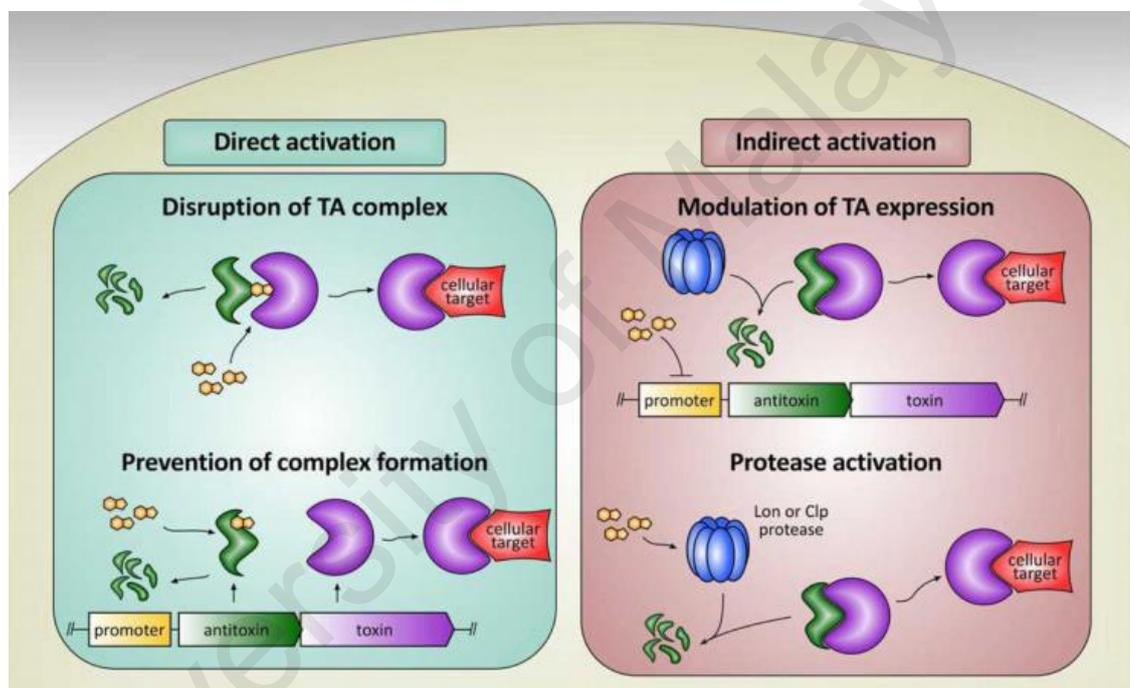


Figure 2.5: Possible ways of inducing artificial activation of TA systems. The orange fused ring that acts as toxin activator can activate the TA complex in either a direct or indirect way. Direct activation (left) can be achieved by either interfering the TA complex (top) or prevention of complex formation (bottom). Indirect activation (right) may take place either by regulating the expression of the TA complex at the promoter (top) or by activating cellular proteases (bottom) responsible for antitoxin degradation. Diagram obtained from Williams and Hergenrother, (2012).

2.4.3 Gene therapy against viral infection

One of the challenges in modern medicine is the fight against viral infections. While vaccination is considered to be the most effective method to curb viral infections, for some viral pathogens causing major health problems such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), no effective vaccine has yet been produced. Therefore, another promising use of TA systems would be in gene therapy against viral infections. In a recent work, MazF and MazE have been used to implement novel antiviral strategies against HCV and HIV (Park et al., 2012). The genome of these viruses consist of NS3 serine protease and PR aspartyl protease which are essential for HCV and HIV replication, respectively, and hence, have been used as targets for drug development. In the research carried out by Park et al., (2012), they utilized the protease activity to activate a toxic protein instead of targeting these proteases to prevent viral infection. Therefore, a retroviral vector was constructed containing the *E. coli* MazE antitoxin that was fused to MazF toxin using linkers having a specific protease cleavage site for either HIV PR (HIV-1 protease) or NS3 protease (HCV protease). These fusion proteins formed a stable dimer to inactivate the ACA (sequence)-specific mRNA interferase activity of MazF. However, when these fusion proteins were incubated with their corresponding proteases, the MazE antitoxin was cleaved from the fusion proteins, thereby releasing active MazF toxin, enabling the cleavage of single-stranded RNA in vitro (Park et al., 2012). Likewise, when MazF was fused to a neutralizing fragment of MazE via an NS3-cleavable linker, expression of this fusion protein did not affect healthy cells but killed HCV-infected cells and cells expressing NS3 (Shapira et al., 2012).

2.4.4 Functionality of TA systems in eukaryotic cells

Some TA systems have been demonstrated to be functionally active when expressed in eukaryotic cells. They have been proposed to have potential application in the control of cellular growth in eukaryotic cells particularly in preventing the accidental escape of genetically-modified cells (Kristoffersen et al., 2000). The RelE toxin of *E. coli* has been shown to be functional in the yeast *Saccharomyces cerevisiae* where induction of the toxin gene in transgenic yeast cells inhibited their growth (Kristoffersen et al., 2000). Expression of the RelE toxin was also shown to trigger apoptosis in human osteosarcoma cells, although the neutralizing effect of RelB was not evaluated (Yamamoto et al., 2002). To prevent accidental escape of genetically-modified cells, RelE toxin may be expressed continuously in these cells, while production of RelB antitoxin would only be allowed in a controlled environment, and not in cells that may escape from these.

Another report has shown that the bacterial Kid toxin from plasmid R1 was able to prevent proliferation in eukaryotic cells including yeasts, oocytes of *Xenopus laevis* and HeLa cells, whilst its cognate Kis antitoxin protects these cells from the lethal action of Kid (de la Cueva-Mendez et al., 2003). This demonstrated that expression of Kid and Kis could be regulated to kill particular cells, including tumour cell lines, in a selective way. To achieve this, *kid* and *kis* genes can be expressed under the control of promoters that are, respectively, induced and repressed in these cells, and that have the inverse behavior in normal cells. Another interesting exploration of TA systems in eukaryotes was exemplified by Slanchev et al., (2005), in which the *kid* toxin was expressed in the primordial germ cells of the male zebrafish embryos, while somatic cells were protected by *kis* antitoxin expression, producing sterile male fish. All this approaches could have value in future research into cellular differentiation, organogenesis or degenerative disorders (de la Cueva-Mendez et al., 2003).

Similar results were obtained subsequently when the *E. coli* MazF toxin, which is also a chromosomal homolog of Kid, was demonstrated to inhibit protein synthesis by cleavage of cellular mRNA and induced apoptosis in human cells (Shimazu et al., 2007). This showed that the mRNA interferase activity of MazF (and presumably also of Kid) is responsible for its lethal effect in human cells. In addition to that, MazF-induced apoptosis required pro-apoptotic protein BAK and its upstream regulator NBK/BIK and results showed that cells deficient in BAK were resistant to cell death despite of the inhibition of protein synthesis in these cells (Shimazu et al., 2007). Therefore, this study demonstrated an attractive possibility of developing a Single Protein Production (SPP) system in mammalian cells, which would have great potential for the structural characterization of human proteins by in-cell Nuclear Magnetic Resonance (NMR) spectroscopy (Shimazu et al., 2007). One of the drawbacks of in-cell NMR spectroscopy in bacteria is its difficulty in studying post-translational protein modifications. With the development of a eukaryotic SPP system, this would be useful to overcome this limitation. Thus, similar observations reported by de la Cueva-Mendez et al., (2003) and Shimazu et al., (2007) provided an interesting clue of how a human cell-based SPP system could be produced.

2.4.5 Selection of high-transgene expressing mammalian cell pools

To generate stably expressing cell lines in eukaryotic cells, one of the major drawbacks is gene silencing (Unterholzner et al., 2013). Integration often occurs randomly and to identify the transgene that have integrated in chromosomal locations with high expression levels itself is time consuming. Since high expression often reduces the growth rate, cells with low or no expression are selected over time (Unterholzner et al., 2013). To circumvent these problems, the Kid-Kis TA system has been exploited to enable easy selection of clones whereby the transgene is tightly co-

expressed with the Kis antitoxin in cells that expresses the Kid toxin. This allows enrichment of mammalian cells expressing high levels of the transgene. Indeed, this strategy enabled selection of cells with strong transgene expression and this was observed for several weeks (Nehlsen et al., 2010). Other TA toxins that were shown to be effective in mammalian cell lines which included RelE (Andreev et al., 2008) and MazF (Shimazu et al., 2007) might be useful for similar techniques.

2.5 Genetic Restriction Use Technologies (GURT): Male Sterility

Currently, there are many genetic strategies that could be used to restrict the spread of transgenes, these are generally known as Genetic Use Restriction Technologies (GURT). Some technologies that would be useful as GURTs include reducing seed shattering (Konishi et al., 2006), or enabling the seeds that contain the transgene to be separated mechanically by, for example, seed size, seed weight or seed colour. One example of GURT is the development of a male-sterility system. Male sterility can be defined as the inability of pollen to fertilize the ovum which can be caused by defects in structure or function of the reproductive organs. A variety of different plant male-sterility systems have been developed (Goldberg et al., 1993; Schnable and Wise, 1998; Li et al., 2004; Kausch et al., 2010), most of these are similar to the Barnase/Barstar system and require a tapetal- or pollen-specific promoter fused to a toxin gene.

Different types of sterility genes are classified as cytotoxic genes which are used for producing sterility. They include ribonuclease genes such as the bacterial *barnase* gene cloned from a soil bacterium, *Bacillus amyloquefaciens* (Mariani et al., 1990; Goldberg et al., 1993; Goetz et al., 2001; Lemmetyinen et al., 2001), diphtheria toxin A chain (DTA) (Thorsness et al., 1991; Kandasamy et al., 1993) and the ribosome inactivating protein (RIP) (Cho et al., 2001). Mariani et al., (1990) used a tobacco tapetum-specific gene promoter (TA29) to regulate the expression of the bacterial

barnase gene in tobacco and oilseed plants to produce male sterility. Expression of the *barnase* gene disrupted the tapetal cells and successfully prevented pollen formation and resulted in male sterile plants. Tapetum is the tissue and innermost wall layer of the micro-sporangia in angiosperm plants (Goldberg et al., 1993). It functions in the development of male gametophyte (microspore) by providing enzymes, nutrients and wall material, first by secretion and eventually by degeneration (Zhu et al., 2008). Tapetum degradation is a programmed cell death (PCD) event with typical diagnostic features of cell shrinkage, mitochondria and cytoskeleton degeneration, nuclear condensation, oligonucleosomal cleavage of DNA, vacuole rupture and endoplasmic reticular swelling (Papini and Brighigna, 1999; Wu and Cheung, 2000; Li et al., 2006). Tapetal PCD that occurs at a specific developmental stage is essential for pollen fertility, and early or delayed disruption of the timing of PCD could result in pollen abortion or male sterility. However, male fertility could be restored by improving tapetum-specific expression of the *barstar* gene, which is also obtained from *B. amyloliquefaciens* and introduced into the other parental line. The two-component Barnase-Barstar system is ideal and capable of delivering male sterility through differential expression from these promoters. The Barnase-Barstar system has been tested in many crops including Brassica (Roque et al., 2007), cabbage (Lee et al., 2003), tomato (Roque et al., 2007) and wheat (Block et al., 1997); however, issues such as leaky expression of the *barnase* gene and difficulty to obtain restoration lines of the *barstar* gene and biosafety concerns related with the use of the bacterial cytotoxic gene in food crops are the key challenges associated with its applicability (Bisht et al., 2007).

Liu et al., (2008) created chimeric promoters to regulate expression of another toxin gene, DT-A in the plant reproductive organs. Liu and Liu (2008) found that the second intron of Arabidopsis AGAMOUS (AG) could induce gene expression in stamens and carpels specifically, which would be useful for engineering sterility. Later,

Liu's group (Yang et al., 2010; 2011) used the second intron/enhancer fragments of tobacco and petunia AG to produce novel but highly effective chimeric promoters. These gene promoters confer similar carpel- and stamen-specific expression without any leaky activity in vegetative tissues. The expression of the DT-A gene, driven by these promoters led to complete sterility but normal growth and development of vegetative organs (Yang et al., 2010; 2011)

A second group of genes that can be used to produce male sterility are the ones involved in specific metabolic pathways in higher plants. For example, a study by Yui et al., (2003) clearly demonstrated that inhibition of mitochondrial pyruvate dehydrogenase (PDH) activity in anther tapetum, which is essential for the operation of the tricarboxylic acid cycle, could result in male sterility. In addition to that, over-expression of β -ketothiolase via integration of the transgene into the chloroplast genome of tobacco was able to produce the male sterile phenotype (Ruiz and Daniell, 2005). This was reportedly the first engineered cytoplasmic male-sterility in plants and offered a new tool for transgene containment for both nuclear and organelle genomes (Ruiz and Daniell, 2005). Besides PDH and β -ketothiolase, expression of bacterial enoyl-CoA hydratase/lyase enzyme and an unedited *atp9* gene also has been shown to cause male sterility (Worrall et al., 1992; Hernould et al., 1993; Tsuchiya et al., 1995; Hernould et al., 1998). Both ATP9 and the enoyl-CoA hydratase/lyase enzyme are involved in the proton channel of ATP synthase by re-routing the phenylpropanoid pathway. These metabolic genes are known as endogenous genes and are not toxic to either plants or animals when expressed. Therefore, biosafety concerns over transgenic plants can be minimized as compared to plants harbouring the *barnase* transgene. Nevertheless, the potential of using these metabolic genes to produce male sterility is still much lower than that of using toxin genes such as *barnase* (reviewed by Ding et al., 2014).

2.6 *Agrobacterium tumefaciens*-mediated transformation

Plant transformation started in the early 1980's and refers to the introduction and integration of 'foreign' DNA in plant cells and the consequent regeneration of transgenic plants (Newell, 2000). Plant transformation mediated by *Agrobacterium tumefaciens*, which is a Gram negative, rod-shaped, aerobic bacteria that dwells in the soil, has become one of the most widely-used methods to introduce foreign genes into plant cells. *A. tumefaciens* naturally infects the wounded sites in dicotyledonous plants leading to the formation of crown gall tumours (Gelvin, 2010; Lacroix and Citovsky, 2013). The evidence that indicated *A. tumefaciens* as the causative agent of the crown gall was first discovered a century ago (Smith and Townsend, 1907; Hwang et al., 2015).

Genetic transformation mediated by *A. tumefaciens* was first reported in the 1980's (De Block et al., 1985). Transgenic tobacco expressing foreign genes was first produced through this transformation procedure. *Agrobacterium*-mediated transformation has advantages over other transformation methods. The transgenic plants obtained are generally fertile and the foreign genes are often transmitted to their progenies in a Mendelian manner (Aldemita and Hodges, 1996).

During infection by *A. tumefaciens*, a segment of DNA, designated the T-DNA, is transferred from the bacterium into the plant cell (Figure 2.6). Not all *A. tumefaciens* is capable of this trans-kingdom DNA transfer. Only strains that harbour tumour-inducing (Ti) plasmids are capable of this transfer, the T-DNA of which will be incorporated into the host genome leading to crown gall disease (Nester et al., 1984; Binns and Thomashaw, 1988; Bourras and Meyer, 2015). The Ti plasmid typically contains two distinct regions known as the T-DNA region and the *vir* region that is essential for T-DNA transfer. The T-DNA consists of 25 bp direct repeats that flank the T-DNA region, designated as left and right borders (Gelvin, 2003). The T-DNA region

contained genes that encode proteins involved in the biosynthesis of plant-type hormones and opines (Zupan et al., 2000). Meanwhile, the *vir* region that is also resident on the Ti-plasmid is not transferred to the host cell but is responsible for processing and transporting the T-DNA into the plant cell (Zupan et al., 2000). Since the T-DNA is driven only by demarcating the left and right borders and not by any other DNA sequence, basically any type of DNA can be placed between the borders and utilized for plant transformation (Zupan et al., 2000 and as reviewed by Krenek et al., 2015).

The stable transformation of plants generated using *Agrobacterium*-mediated transfer can be transmitted to the next generation, therefore providing a platform for the development of fully transgenic plants, in which a T-DNA copy is integrated into its genome in every single cell. Such plants exhibit consistent and stable expression of the transgene that allows for the temporal and spatial control of the transgene expression level (Chen et al., 2004; Clough and Bent, 1998; Harwood, 2012; Hiei et al., 2014). For the past three decades, the development of *Agrobacterium*-based stable transformation protocol has been given more attention to establish reliable protocols for various plant species including food crops (Clough and Bent, 1998; Hiei et al., 1994). Although best known among *Agrobacterium* species is *A. tumefaciens*, another pathogenic *Agrobacterium* species *A. rhizogenes*, the causative agent for hairy root (root mat) disease (Kersters and De Ley, 1984; Farrand et al., 2003; reviewed by Platt et al., 2014) has also been used for plant transgenic studies. The fundamental mechanism of pathogenesis is the same for each of these species: DNA transfer from the bacterium to the host plant leads to integration and expression of a portion of a large plasmid [Ti-(tumor inducing) or Ri-(root inducing) plasmid] originally extant in the bacterium (reviewed by Gelvin, 2012). Despite the availability of other methods for plant transformation such as protoplast transformation or biolistics, *Agrobacterium*-mediated

transformation is still the preferred choice since plants harboring single transgene copies can be more easily obtained (Komari et al., 2004).

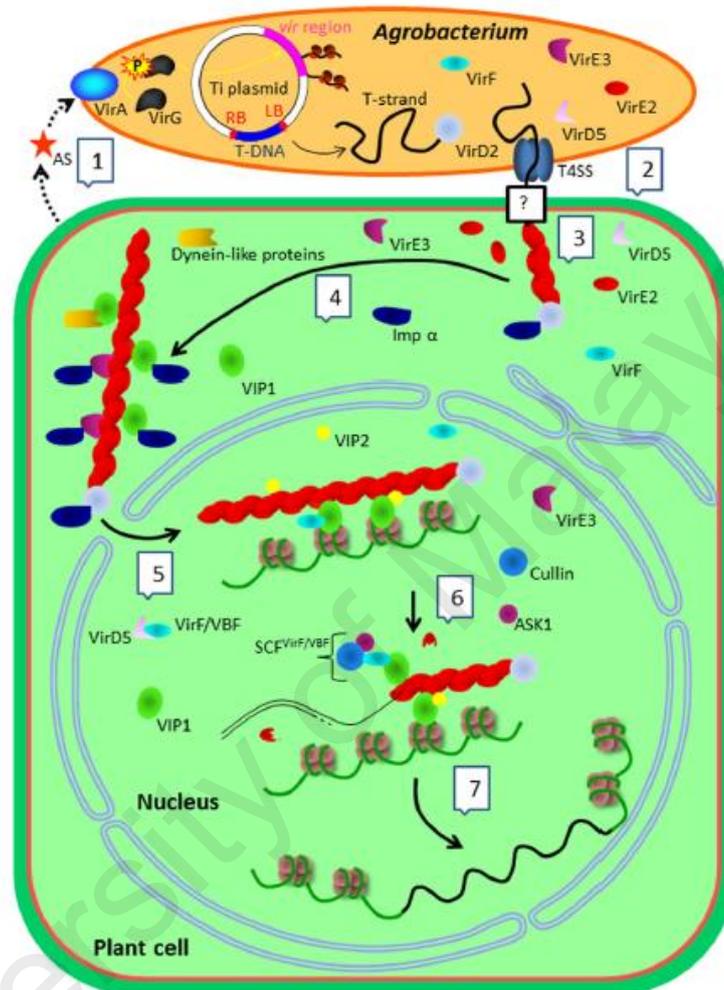


Figure 2.6: Major steps in the process of T-DNA transfer and integration. Step 1: Phenolic compounds, such as acetosyringone (AS), secreted by wounded plant tissues activate *Agrobacterium tumefaciens vir* gene expression via the VirA-VirG sensor, resulting in generation of a mobile single-stranded T-DNA copy (T-strand). Step 2: The T-strand and VirD2 forms a complex and is transported to the host cell cytoplasm via the bacterial type 4 secretion system (T4SS), which also transports into the host cell four other bacterial virulence effectors (VirD5, VirE2, VirE3, and VirF). Step 3: In the host cytoplasm, the mature T-complex is assembled by cooperative binding of VirE2 molecules along the T-strand molecule. Step 4: The T-complex is directed into the nucleus via interactions with the host cell proteins such as *importin a*, VIP1 (or the bacterial VirE3), and dynein-like proteins, such as DLC3. Step 5: In the nucleus, the T-complex is targeted, presumably by interactions between VIP1 and the host chromatin,

to the integration site. Step 6: The associated proteins are removed by proteasomal degradation via the SCF^{VirF/VBF} pathway mediated by VirF or its host functional analog VBF. Step 7: The T-strand is converted to a double-stranded form and randomly integrated into the host genome by the host DNA repair machinery. Diagram obtained from Lacroix and Citovsky, (2013).

2.7 Approaches for multi-transgene-stacking in plants

The introduction and expression of foreign genes in plants by genetic transformation has become a popular method for many species (François et al., 2002). By manipulating plant genomes, crops can be engineered to produce enhanced nutritional value and to be resistant to biotic and abiotic stresses. ‘Stacking’ or ‘pyramiding’ offers many advantages including the potential to provide durable multitoxin resistance to particular pests and for engineering multiple resistance to multiple pathogens, also in addition to combination with herbicide tolerance (reviewed by Halpin, 2005). Besides that, manipulation of metabolic pathways through overexpression of a specific gene has also been introduced to improve agronomic characteristics (Stark et al., 1992). However, most metabolic processes that are targeted for manipulation rely on the connection between numerous genes, and flux through biochemical pathways is often co-ordinated with that of competing pathway. Hence, the effective metabolic engineering will only be successful by controlling multiple genes on the same, or interconnected, pathways. For instance, the production of ‘Golden rice’ was successfully engineered with three carotenoid biosynthesis genes to allow it to produce provitamin A (Ye et al., 2000). However, efficient provitamin A absorption may require the resorbable iron content to be enhanced, potentially requiring the introduction of another three genes (Ye et al., 2000 and reviewed by Halpin, 2005)

A number of approaches have been taken to combine multiple transgenes in one plant (multi-transgene-stacking) with varying degree of success. These methods include sexual crosses, sequential transformation and co-transformation (François et al., 2002).

2.7.1 Sexual crossing approach

In the sexual crossing approach, two plants are crossed to obtain progeny that carry the desired traits from the two parents. In the case of transgenic plants, the first gene is introduced in one of the parents, whereas the second gene is transformed into the other parent. If both parents are homozygous for the transgene they are carrying, then all of the progeny of the cross will contain both transgenes. However, if the parents are hemizygous for the transgene, only 25% of the progeny from their crossing will contain the two transgenes (François et al., 2002; Docheva et al., 2003).

The main advantage of sexual crossing is that the method is technically simple as it involves the transfer of pollen from one parent to the female reproductive organ of the other. Furthermore, optimal expression of each transgene can be screened from the transgenic population of each parent, therefore promoting the succession of two optimally expressed transgenes (François et al., 2002).

However, sexual crossing has also some drawbacks. Apart from being time-consuming, the two transgenes in the lines resulting from the cross will always reside on different loci, which complicates further breeding through conventional methods. In addition, heterozygous plants that are vegetatively propagated such as perennial fruit crops and potato cannot be crossed since the (desired) heterozygous nature of the genetic background will be altered due to recombination during meiosis (Gleave et al., 1999).

Numerous examples for sexual crosses to incorporate multiple transgenic traits into a cultivar have been described in detail. The biodegradable plastic

polyhydroxybutyrate (PHB) was successfully produced in the leaves of alfalfa plants by integration of two to three bacterial genes (Poirier et al., 1992) whereas the biosynthesis of co-polymer of PHB requires four to six genes (Poirier, 1999). The transgenic plants accumulated PHB as granules which were similar in size and appearance to the bacterial PHB granules. Another good example for combining genes involved in metabolic pathways was reported by Bizily et al., (2000). The two genes, encoding enzymes from a bacterial enzymatic pathway for organic mercury detoxification were combined by sexual crossing between two transgenic *Arabidopsis thaliana*. The genes were isolated from a bacterium that can be found from mercury contaminated environment and contributed to methylmercury detoxification (Bizily et al., 2000; Summers, 1986; Maestri and Marmiroli, 2011).

2.7.2 Sequential transformation

Sequential transformation, also known as repeated transformation or re-transformation has also been proven to be one of the feasible strategies and is defined as the repetitive insertion of transgenes into a plant. In this method, the first transgene is introduced into a plant, the second transgene is introduced subsequently in the plant resulting from the first transformation event, and so on (François et al., 2002).

By this method, multiple transgenes can be incorporated into heterozygous plants that are vegetatively propagated (e.g., perennial fruit crops, potato, many ornamentals). In comparison to sexual crossing, the sequential introduction of transgenes allows the maintenance of the elite genotype and will not lead to the loss of the desirable combination of existing traits due to recombination (Gleave et al., 1999). On the other hand, the foremost limitation of this method is that the transgenes introduced are not linked, thus they may be segregated apart in subsequent generations, requiring the maintenance and screening of a larger progeny population (Hohn et al., 2001; Hare and Chua, 2002). Another problem associated with sequential

transformation method is that it requires a variety of selectable marker genes so that a different one can be used for each transformation (Hohn et al., 2001; Hare and Chua, 2002).

Rosati et al., (2003) demonstrated that induced anthocyanin synthesis through sequential transformation with the genes for dihydroflavonol 4-reductase from *Antirrhinum majus* and anthocyanidin synthase from *Matthiola incana* could modify flower colour in *Forsythia*, an early spring-flowering shrub. The double transformants exhibited a unique bronze-orange petal colour caused by the de novo accumulation of cyanidin-derived anthocyanins over the carotenoid yellow background of the wild-type (Rosati et al., 2003). Similarly, Singla-Pareek et al., (2003) showed that the introduction of a two-gene glyoxalase pathway into tobacco by sequential transformation led to enhanced salinity tolerance with the double transgenics responding better under salinity stress than plants harbouring either of the single transgene.

2.7.3 Co-transformation

Co-transformation methods are used for the simultaneous introduction of multiple genes into the cell followed by the integration of those genes in the cell genome (François et al., 2002; Taverniers et al., 2008). One method is by transformation with a plasmid construct carrying multiple genes of interest, or it can also be done by co-transformation with different plasmids or multiple DNA fragments carrying different transgenes and introduced into the plant cell (Taverniers et al., 2008).

The main advantage of co-transformation is that having multiple genes of interest on one plasmid construct can result in single integration of the transgenes in a single transformation event as opposed to sequential transformation, which requires multiple and time-consuming transformation events. At least with the single-plasmid co-transformation method, linked integration of the transgene into the plant genome is

guaranteed, which facilitates further breeding (François et al., 2002). On the other hand, co-transformation of different plasmids carrying different transgenes requires construction of different expression cassettes which is technically simple and easier as it is done independently on different plasmids (Komari et al., 1996).

Despite the above advantages, co-transformation also has limitations. For instance, the technical restriction of co-transformation involving multiple plasmids has not been addressed. The achievement of this multi-transgene-stacking method relies on the frequency of which two or more independent transgenes are transferred to the plant cell and integrated into the cell genome. Chen et al., (1998) discovered that co-transformation efficiency was correlated with the ratio at which the plasmids were mixed with respect to the selectable marker gene when two separate plasmids were co-transformed. They found that the percentage of first generation transgenic plants expressing both transgenes was doubled when the amount of target gene was increased from a 1:1 to 1:12 molar ratio with respect to the selectable marker gene. Another drawback of the co-transformation method is the difficulty in assembling complex plasmids with multiple gene cassettes in a single-plasmid co-transformation (François et al., 2002). The lack of unique restriction cloning sites, the loss of direct selection, as well as the relatively low efficiency for ligation of inserts into larger vectors, are some of the technical limitations when three or more foreign genes are sub-cloned into a transformation vector using this method (Lin et al., 2003). In addition, the condition that promote co-integration might also favour the integration of high copy numbers of transgenes, which normally integrate as repeat structures and this could possibly lead to transgene silencing in subsequent generations (Muskens et al., 2000).

Many examples of the use of co-transformation have been reported in literature. Among those, Ye et al., (2000) had exploited a combination of single plasmid and multiple-plasmid co-transformations mediated by *Agrobacterium tumefaciens* whereby

the entire β -carotene biosynthetic pathway was introduced into carotenoid-free rice endosperm in a single transformation event with three vectors. The oil-rich aleurone layer from rice that turns rancid upon storage is usually removed leaving the remaining edible part of rice grains, the endosperm, which lacks several essential nutrients including β -carotene (provitamin A). Therefore, recombinant DNA technology rather than conventional breeding was utilized to improve the nutritional value of rice in this respect. Immature rice embryos were co-inoculated with two separate binary vectors, one carrying two of the three genes required for β -carotene production, and the second vector carrying the genes coding for the third biosynthetic enzyme and an antibiotic resistance gene as selectable marker. During selection from the 60 regenerated lines, 12 lines contained the three β -carotene biosynthetic genes and the selectable marker gene. Due to the yellowish colour of the rice grains i.e. indicating carotenoid formation, it was called 'golden rice' (Ye et al., 2000).

2.8 *Arabidopsis thaliana* as a plant model

Of all the known species of flowering plants, *A. thaliana* stands out as the most thoroughly studied (Koornneef and Meinke, 2010). *A. thaliana*, known as *Thale cress*, is a small flowering weed that grows well in a temperate climate. *A. thaliana* belongs to the mustard family (*Brassicaceae*) which includes cultivated species such as cabbage and radish. *A. thaliana* is a small plant which can be grown on as little as 1 cm² of soil and has a generation time of only 4 – 6 weeks (Somerville and Koornneef, 2002; Koornneef and Meinke, 2010). Over the years, *A. thaliana* has been used in numerous plant genetics research and has become a well-studied organism of the plant kingdom (Meyerowitz and Somerville, 1994). *A. thaliana* is a plant model of choice because of its short generation time (four to five weeks), large number of offspring and well-known genetic system (Leutwiler et al., 1984). In addition, each plant can produce hundreds to thousands of seeds, thus making it very suitable for experimental studies. For a complex

multicellular eukaryote, *A. thaliana* has a relatively small genome of approximately 135 Mbp (Arabidopsis Genome Initiative, 2000). Previously, it was long thought to have the smallest genome of all flowering plants (Leutwiler et al., 1984), but the smallest flowering plant genomes are now considered to belong to plants of the genus *Genlisea*, order *Lamiales*, with *Genlisea tuberosa*, a carnivorous plant which has a genome size of approximately 61 Mbp (Fleischmann et al., 2014). However, *A. thaliana* was the first plant to have its genome sequenced and is now a popular model plant for understanding the molecular biology of many plant traits, including flower development and light sensing (Coelho et al., 2007; Coen and Meyerowitz, 1991; Sullivan and Deng, 2003). Following early studies on transgenic plant experiments in the 1980s (Fraley et al., 1983), many plant species including *A. thaliana* have been genetically modified for production of useful proteins (Guan et al., 2013). Using *A. thaliana* expression system offers few advantages such as the possibility of post-translational modifications and also low cost of production (Fisher and Schillber, 2004).

A. thaliana can be stably transformed by floral dipping or transiently transformed by agro-infiltration with *A. tumefaciens* (Zhang et al., 2006; Lee and Yang, 2006). When overexpressing heterologous genes in this organism using stable transformants, the main limitation would be the long culture cycle, lasting two months between generations of plant seeds, as compared to only 30 to 50 min for bacteria.

2.9 Inducible expression system in plants

Inducible gene expression is a robust tool to study and engineer genes whose overexpression could be detrimental for the host organism. Several systems for induction of transgene expression in plants have been constructed and described, mainly in tobacco, rice, *Arabidopsis*, tomato and maize (Borghi, 2010). Ideally, an inducible expression system makes use of a chemical compound that is easily applied to the

transgenic plants and specific gene expression can be induced immediately. Furthermore, gene expression should be absent or extremely low in the absence of inducer and the level of expression should be adjustable by varying the inducer concentration (Borghini, 2010).

Inducible systems are usually based on two components which are a chimeric transcription factor and binding sites for the driver to control the expression of the gene of interest (Borghini, 2010). The chimeric transcription factor can specifically bind to tightly controlled promoters, only after the induction, and is generally called a driver or activator. Meanwhile, the second component that contains the binding sites is generally referred to as reporter or responder, which is usually flanked by a minimal 35S promoter (Borghini, 2010). To obtain strong and ubiquitous expression, the activator can be cloned either under the control of the CaMV 35S promoter or tissue-specific promoters. For plant transformation purpose, activator and responder can either be in the same cassette or split into two different vectors. Below is the description of three different inducible systems that are commonly used: AICR/AlcA (ethanol inducible); GVG (dexamethasone inducible) and XVE/OlexA (beta-estradiol inducible).

2.9.1 The AICR/AlcA ethanol inducible gene expression system

The *alc* gene switch is considered to be one of the best chemically-inducible gene expression systems for use in plants (Padidam, 2003; Moore et al., 2006) because of its utility in both the laboratory and field (Li et al., 2005). This system uses the ethanol inducible genetic system that controls the cellular response to ethanol in the fungus *Aspergillus nidulans*. In *A. nidulans*, the *alcR* gene encodes a transcription factor, ALCR that regulates the activation of several structural genes, such as *alcA* and *aldA*, which encode alcohol dehydrogenase I (ADH1) and aldehyde dehydrogenase (AldDH), respectively (Salter et al., 1998; Roslan et al., 2001; Li et al., 2005)

The ethanol-inducible gene expression system consists of two transcription units (Figure 2.7). The first one is the *alcR* expression cassette (p35S:*alcR*), which is constructed by cloning a constitutive CaMV 35S promoter (the promoter can be replaced with cell/tissue/developmental-stage-specific promoter) with an *A. nidulans* *alcR* gene placed downstream. The other one is the target gene expression cassette (*palcA*+mini-p35S:target gene), which consists of a minimal 35S promoter with the upstream activator region of the *alcA* promoter (*palcA*) and a target gene. When ethanol is absent, no expression of the target gene takes place as the ALCR protein expressed from the first unit cannot bind to the *palcA* promoter located in the second unit. Upon ethanol treatment, ALCR interacts with ethanol, resulting in its conformational change and becoming active. The activated ALCR then binds to the specific *cis* element in the *palcA* promoter, and directs the transcription of the downstream target gene (Caddick et al., 1998).

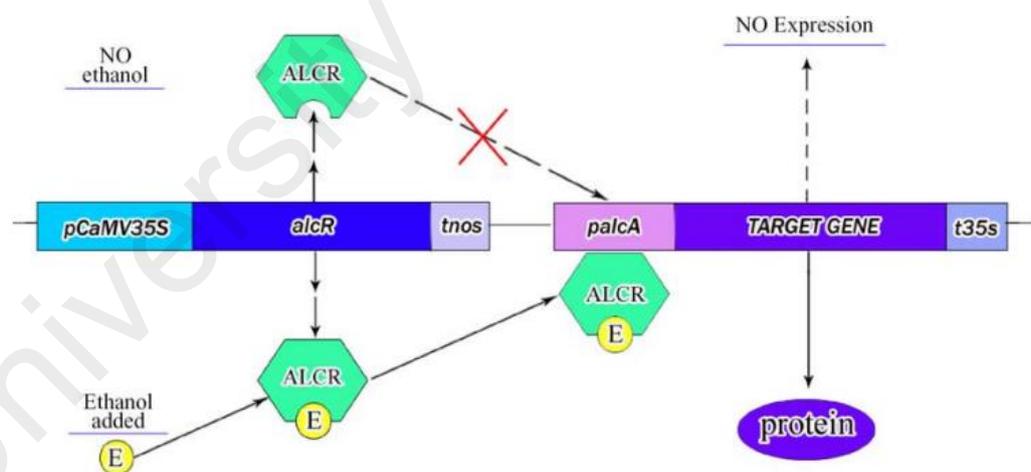


Figure 2.7: Diagram depicting the constructs that make up the *alc*-derived gene-expression system. The construct consists of full CaMV 35S promoter (p35S) driving the expression of ALCR which is positioned upstream of the NOS terminator (*tnos*). The chimaeric promoter *alcA*-35S contains two ALCR binding sites of the *Aspergillus nidulans* *alcA* promoter and a 35S minimal promoter. The target gene is under the control of the inducible *alcA* promoter and terminated by the 35S terminator. Diagram was obtained from Li et al., (2005).

Compared with other gene expression systems, the ethanol-inducible gene expression system has several advantages. The construct of the system is relatively simple and it is composed of two main elements, which are the *alcR* gene and the *alcA* promoter. Besides that, the *alcA* promoter possess high induction, has a rapid but reversible onset of action, and gives sufficient levels of expression to result in phenotypic effects (Caddick et al., 1998; Roslan et al., 2001). As ethanol is cheap, readily available, biodegradable, and environmentally safe to the plants, this system is considered to have a great potential for field application, especially with the possible development of a non-volatile inducer (Corrado and Karali, 2009). Although extremely useful, this system is not without drawbacks. For instance, Caddick et al., (1998) reported basal expression of reporter genes under control of the *alc* switch in seedlings grown on agar in the absence of exogenous ethanol. Such background activity could be a problem if the transgene inhibits plant growth. Another potential disadvantage is that alcohol that is generally used as the exogenous chemical inducer is volatile and hence difficult to manage in a large-scale agricultural context, as large volumes of chemicals may be lost during spraying (Li et al., 2005).

2.9.2 The dexamethasone-inducible expression system

The dexamethasone-inducible expression system utilizes the sequence of the ligand-binding domain of the glucocorticoid receptor (GR), which is fused to the gene of interest, usually encoding a transcription factor (Lloyd et al., 1994; Sablowski and Meyerowitz, 1998; Simon et al., 1996). In the absence of steroid-ligand dexamethasone (DEX), the chimeric protein remains in the cytoplasm where it is secreted by the HSP90 protein complex, and cannot enter the nucleus (Picard, 1993). On the other hand, the chimeric protein is released from its interaction with HSP90 and can travel to the nucleus and exert its function upon addition of DEX. In addition to being inducible,

transgene activity can be tissue-specific or ubiquitous, depending on the promoter used to drive the chimeric transgene (Rossignol et al., 2014).

One of the earliest examples of the dexamethasone-inducible expression system is the GVG inducible system (Figure 2.8) which relies upon a chimeric transcription factor that contains a yeast **G**AL4 DNA-binding domain, the Herpes **V**P16 activation domain and the rat **g**lucocorticoid receptor (Aoyama and Chua, 1997). A major advantage of the GVG system in plants is the fact that the concentrations of GR used are non-toxic and have no detectable adverse physiological effects on plants, thus no pleiotropic effects caused by the induction of the target genes. To maintain this advantage, all the other components in the GVG system were also obtained from non-plant sources (Aoyama and Chua, 1997). Although this system has shown its potential for a number of transgenes (Aoyama and Chua, 1997; Geng and Mackey, 2011; Gonzalez et al., 2015), some limitations have been reported. These include the possibility of inducing phenotypic abnormalities and the unintentional activation of endogenous gene expression in different plant species (Amirsadeghi et al., 2007; Kang et al., 1999). Furthermore, the stability over generations of Gal4-mediated gene expression in plants has also been questioned (Galweiler et al., 2000; Zuo and Chua, 2000).

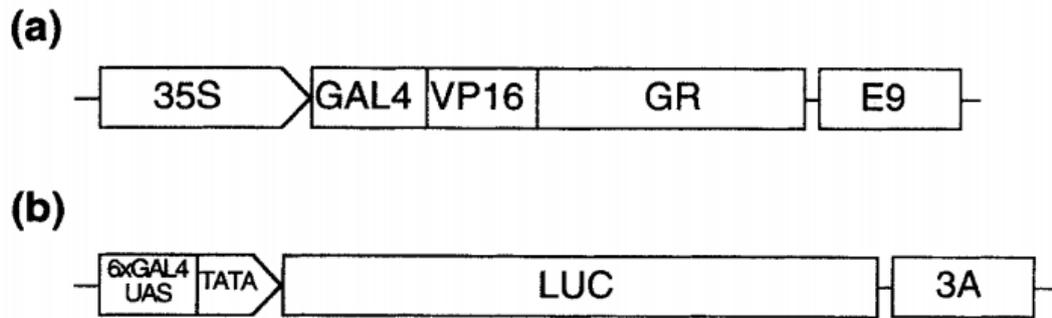


Figure 2.8: Structure of the trans-acting factor and cis-acting element in a typical GVG system. (a) Structure of the *GVG* gene. The DNA fragment spanning the chimeric transcription factor *GVG* was cloned between the CaMV 35S promoter and the poly(A) addition sequence of the pea ribulose biphosphate carboxylase small subunit *rbcS-E9*. (b) Structure of the *Luc* reporter gene. The DNA fragment spanning the luciferase gene is preceded by a promoter consisting of six copies of the GAL4 UAS and the -46 to +1 region of the 35S promoter and flanked at the 3' end by the poly(A) addition sequence of the pea *rbcS-3A*. Diagram was obtained from Aoyama and Chua, (1997).

2.9.3 XVE/OlexA β -estradiol inducible expression system

The XVE chimeric transcription activator has been effectively used in inducible expression systems for flowering plants and is comprised of the DNA-binding domain of the bacterial repressor *LexA*, the transcriptional activation domain *VP16* and the carboxyl region of the human *estrogen* receptor (Zuo et al., 2000). The XVE transcription factor is only activated in the presence of 17- β -estradiol, which then binds to the *LexA* operator and recruits RNA polymerase II to the *lexA* promoter to induce transgene expression (Figure 2.9). This estrogen-inducible system has shown tight regulation and high inducibility in *Arabidopsis*, tobacco and rice (Okuzaki et al., 2011; Zuo et al., 2000). This system is able to overcome the limitation of the GVG system and has been successfully used for chemical-regulated site-specific DNA excision (by combining the XVE system with the site-specific DNA recombination *cre/loxP* system)

in Arabidopsis (Zuo et al., 2001). Despite having the same trans-activating VP16 sequence, the estrogen-inducible system did not elicit any defense response in Arabidopsis nor caused growth retardation in Arabidopsis or rice (Okuzaki et al., 2011; Zuo et al., 2000).

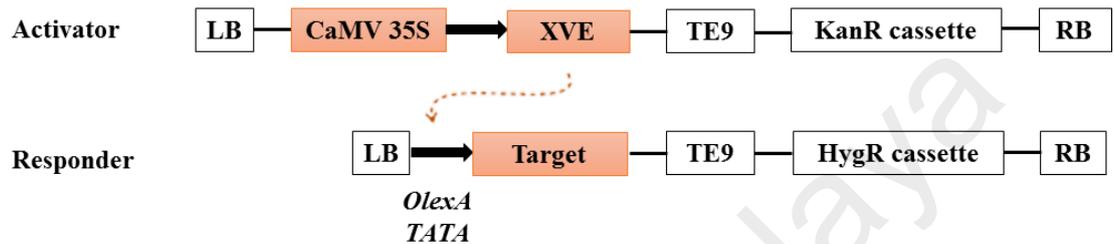


Figure 2.9: A typical two component XVE-based system for β -estradiol-inducible expression. The T-DNA region of activator and responder vectors are shown. LB, left border; RB, right border; *KanR*, kanamycin resistance gene; *HygR*, hygromycin resistance gene; *OlexA TATA*, XVE-responsive promoter; CaMV 35S, promoter.

One of the advantages of using this system is its usefulness in studying the function of genes that cannot be examined by their overexpression in plants, such as lethal genes (Xu and Dong, 2007) or genes affecting morphology or causing growth defects (Petrasek et al., 2006; Klimaszewska et al., 2010). Besides that, the advantages of the XVE system could also be attributed to several factors. Firstly, since the estrogen receptor binds to estradiol with a very high affinity (0.05 nM; Mueller-Farnow and Egner, 1999), the XVE chimeric transactivator can thus be activated by a relatively low concentration of inducer. Secondly, the DNA-binding domain of LexA does not have the same structure to any known eukaryotic factors (Oertel-Buchheit et al., 1992; Zuo et al., 2000), thereby reducing the possibilities that XVE may bind to endogenous plant *cis* elements.

Brand et al., (2006) introduced one of the currently available systems for tissue- and cell-type-specific gene expression by using two-component systems. In this system,

treatment with inducers, 17- β -estradiol promotes the transcription factor binding to and activating the target promoter to allow for temporal control of gene activation in addition to the spatial control afforded by tissue-specific promoters. The system is tightly regulated with no detectable transactivating activity in the absence of an inducer (Zuo et al., 2000 and Brand et al., 2006). Hence, Brand et al., (2006) has successfully demonstrated that despite containing a cytotoxin gene such as diphtheria A-chain toxin (DT-A), transgenic plants could develop normally in the absence of inducer.

University of Malaya

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant growth

Arabidopsis thaliana ecotype Columbia 0 (Arabidopsis Biological Resource Center) plants were used in this study. The plants were grown on soil in the Plant Biotech Facility (a Genetic Modification of Plants Biosafety Level 2 (GP BSL2) containment facility), University of Malaya, under a 16 hours light and 8 hours dark cycle at $20 \pm 1^\circ\text{C}$. For plant transformation using floral dip (Section 3.15), the plants were grown until bolting stage.

3.2 Bacterial strains and growth conditions

Escherichia coli DH5 α and TOP10 (purchased from Invitrogen, USA) were used as hosts for the various cloning vectors and recombinant plasmids constructed in this study. *E. coli* strains were grown overnight in Luria Bertani (LB) media supplemented with the appropriate antibiotics at 37°C . LB media were prepared from Lennox L agar or Lennox L broth base powder (Invitrogen, USA) using deionised distilled water according to the manufacturer's instructions and sterilized by autoclaving at 121°C and 15 psi for 20 min.

3.3 Plasmid vectors and isolation of plasmid DNA

The plasmid vectors and recombinants constructed and used in this study are listed in Table 3.1. The plant inducible expression vectors, pMDC150, pMDC160 and pMDC221 were obtained from Mark Curtis and his team at the University of Zurich (Brand et. al., 2006).

The DNA fragment containing the *S. pneumoniae* *yefM-yoeB*_{Spn} locus for this research was obtained from plasmid pET28a_HisYefMYoeB which was previously constructed (Chan et al., 2011). The CaMV 35S promoter and *GFP* gene were obtained from the pCAMBIA1304 plasmid (CAMBIA Labs, Australia). These and other

plasmids used in this research were isolated from their *E. coli* hosts using the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega, USA) as follows. One full loop of overnight bacterial colony grown on agar was thoroughly resuspended in 250 µl of Cell Resuspension solution in a microfuge tube before adding 250 µl of Cell Lysis solution and mixed by inverting the tube four times. This was followed by the addition of 10 µl of Alkaline Protease solution and incubating the tube at room temperature for 5 min before mixing in 350 µl of Neutralization solution. The tube was then centrifuged at $14,000 \times g$ for 10 min at room temperature. The cleared lysate obtained after centrifugation (approximately 850 µl) was transferred into a spin column that was placed on top of a collection tube which was then subjected to centrifugation at $14,000 \times g$ for 1 min at room temperature. The flow-through was discarded and the column was reinserted into the collection tube. The column was then washed with 750 µl of Column Wash solution containing 95% ethanol followed by a final wash using 250 µl of Column Wash solution. For the first wash, the column along with the collection tube was centrifuged at $14,000 \times g$ for 1 min whereas for the final wash, centrifugation was carried out at $14,000 \times g$ for 2 min. Prior to elution, the spin column was transferred to a new microfuge tube and the plasmid DNA was eluted by adding 100 µl of nuclease-free water to the spin column. Lastly, the tube was centrifuged at $14,000 \times g$ for 1 min at room temperature and the spin column was discarded from the microfuge tube. Plasmid DNAs isolated were stored at -20°C until further use.

Table 3.1: Recombinant plasmids constructed in this study

Recombinant constructs	<i>E. coli</i> host strain	Primer pairs used for PCR amplification
pMDC150_35S	Top10	CaMV 35S Forward & CaMV 35S Reverse
pMDC160_yefM _{Spn}	Top10	<i>yefM_{Spn}</i> Forward & <i>yefM_{Spn}</i> Reverse
pMDC221_yoeB _{Spn} GFP	Top10	<i>yoeB_{Spn}</i> Forward & <i>GFP</i> Reverse
pMDC221_GFP	Top10	<i>GFP</i> Forward & <i>GFP</i> Reverse

3.4 Primer design

All primers used in this study were designed using the Primer3 software (available online from <http://bioinfo.ut.ee/primer3-0.4.0/>) whereas the relevant properties of each primer, including melting temperature and secondary structure formation were analysed using Sequence Manipulation Suite, SMS (available online from <http://www.bioinformatics.org/sms2/>). The primers were designed with reference to the sequence of the *S. pneumoniae* R6 genome (NCBI accession number: NC_003028) for the *yefM-yoeB_{Spn}* locus and pCAMBIA 1304 (NCBI accession number: AF234300.1) for the GFP and CaMV 35S promoter coding sequences. Primers used in this study are listed in Table 3.2.

In this study, primers were designed mainly for PCR amplification which was used for cloning, screening transformed plants and quantitative real time RT-PCR (qRT-PCR) to determine the level of specific transcripts in the cell. Primer pairs for PCR were designed depending on several factors including: (a) the length for primer pairs was 18 - 25 nucleotides and with similar melting temperature ($\pm 3^{\circ}\text{C}$); (b) the GC content was ensured to be between 40 – 60% with C and G nucleotides distributed uniformly throughout the primer; (c) more than three G or C nucleotides at 3'-end of the primer were avoided as it can result in multiple annealing at the non-target sites (Rychlik, 1995). In addition, the primer pairs were carefully designed not to contain

complementary within themselves; that is, they should not form hairpins (with estimated melting temperature $<35^{\circ}\text{C}$, primer-dimers and homodimers under the PCR annealing conditions (Rychlik and Rhoads, 1989; Rychlik, 1995; Skerra, 1992). For cloning, some primers were designed with certain restriction sites incorporated into the 5' end so that the resulting amplified products could be cleaved by specific restriction enzymes and subsequently ligated as one fragment. In addition, the primers were designed such that the CACC sequence was added just upstream of the AUG start codon for the forward primers of each of the target genes studied to enable incorporation into Invitrogen's pENTR-D-TOPO entry vector. For screening positive transgenic plants, primers were designed based on the full length of the target genes transformed into the plants. For quantitative real-time RT-PCR, primers were designed such that the amplicon size was in the range of 100 – 200 bp in length.

Table 3.2: A list of oligonucleotide primers used in this study

Primer name	Primer sequence (5'→3')	Size of amplified product (bp)
<u>A. Primers for cloning into plant binary destination vectors</u>		
i. <u>Genes of interest: <i>yoeB_{Spn}</i></u> yoeB _{Spn} Forward yoeB _{Spn} Reverse	CACCATGCTACTCAAGTTTA <u>GGATCCGTAATGATCTTTAAA</u>	252
ii. <u>Genes of interest: <i>yefM_{Spn}</i></u> yefM _{Spn} Forward yefM _{Spn} Reverse	CACCATGGAAGCAGTCCTT TCACTCCTCAATCACATGGA	255
iii. <u>Genes of interest: <i>GFP</i></u> GFP Forward GFP Reverse	<u>GGATCCATGGTAGATCTGA</u> TTATAATCCCAGCAGCTGTT	732

Table 3.2, Continued

Primer name	Primer sequence (5'→3')	Size of amplified product (bp)
A. <u>Primers for cloning into plant binary destination vectors</u>		
iv. <u>Genes of interest: CaMV 35S</u> CaMV 35S Forward CaMV 35S Forward	CACCGCGTATTGGCTAGAGCAG AGAGATAGATTTGTAGAGAGAG ACTGG	808
B. <u>Primers for screening transgenic plants</u>		
i. <u>Genes of interest: <i>yoeB_{Spn}-GFP</i></u> Trans yoeB Forward Trans GFP Reverse	ATGCTACTCAAGTTTACAGAA TTAAGCTTTGTATAGTTCATCCA	990
ii. <u>Genes of interest: <i>yefM_{Spn}</i></u> Trans yefM Forward Trans yefM Reverse	ATGGAAGCAGTCCTTTACT TCACTCCTCAATCACATGGA	255
iii. <u>Genes of interest: <i>GFP</i></u> (control) Trans GFP Forward Trans GFP Reverse	ATGGTAGATCTGACTAGTAAAG GA TTAAGCTTTGTATAGTTCATCCA	732

Table 3.2, Continued

<p><u>C. Primers for quantitative real time RT-PCR</u></p>		
<p>i. Genes of interest: <u><i>yoeB_{Spn}-GFP</i></u> <u>q-YG-Forward</u> <u>q-YG-Reverse</u></p>	<p>GGACGACGGGA ACTACAAGA CGGCCATGATGTATACGTTG</p>	<p>168</p>
<p>ii. Genes of interest: <u><i>yefM_{Spn}</i></u> q-yefM-Forward q-yefM-Reverse</p>	<p>AGCCTTTGACGGTGGTCAATAA AGCACGGACTTGAGCCATTC</p>	<p>149</p>
<p>iii. Genes of interest: <u><i>Actin</i></u> q-Actin-Forward q-Actin-Reverse</p>	<p>CCAGTGGTCGTACAACCGGTAT ACCCTCGTAGATTGGCACAGT</p>	<p>180</p>

3.5 Polymerase chain reaction (PCR)

Both the *yoeB_{Spn}* toxin and *yefM_{Spn}* antitoxin gene sequences were amplified from the plasmid construct pET28a_HisYefMYoeB (Chan *et al.*, 2011) using gene-specific primers (Table 3.2) with PCR conditions as shown in Table 3.3. The *Bam*HI restriction site was included at the 5' end of the *yoeB_{Spn}* reverse primer and the 5' end of the *GFP* forward primer (Table 3.2) to enable construction of the *yoeB_{Spn}-GFP* fusion product. The CaMV 35S promoter and *GFP* gene were amplified from pCAMBIA 1304 using the same PCR conditions.

Pfu DNA polymerase was used in generating all fragments for DNA cloning as it has 3'-5' exonuclease proof-reading activity which enables it to correct nucleotide incorporation errors during PCR amplification (Lundberg *et al.*, 1991). The optimal composition of buffers and solutions used to perform PCR were 2 mM dNTP mix (dATP, dCTP, dGTP and dTTP), 1 µM forward and reverse primers each, 1.25 U *Pfu* DNA Polymerase (Fermentas, Lithuania) and 10 × *Pfu* Buffer with 3 mM MgSO₄. The set of conditions shown in Table 3.3 (except for initial denaturation of the DNA

template) was repeated for 29 cycles, followed by a final extension at 72°C for three minutes.

Table 3.3: PCR conditions using *Pfu* DNA polymerase

PCR step	Temperature (°C)	Duration (min)
Initial denaturation	95	2
Denaturation	95	1
Annealing	51-57*	1
Extension	72	1
Final extension	72	3

* Annealing temperature depended on the primer pair that was used

3.6 Agarose gel electrophoresis

DNA fragments (plasmid DNA, PCR products or digested DNAs) were separated by gel electrophoresis on 1% (w/v) agarose gel prepared in 1 × TBE buffer (90 mM Tris base, 90 mM boric acid, 0.2 M EDTA) containing 0.1 µg/mL ethidium bromide. The samples were mixed with 1 × bromophenol blue loading dye onto the relevant wells of the casted agarose gel along with an appropriate DNA size marker (100 bp or 1 kb DNA ladder; Fermentas, Lithuania). The loaded gel was subjected to electrophoresis in 1 × TBE buffer with a voltage of 120 V applied until the dye front had reached the edge of the gel. The visualization of DNA fragments on the gel was performed by exposing the gel to long-wave ultraviolet-light (302 nm) and the image photographed using the ChemiImager™ System (Alpha Innotech, USA).

3.7 Purification of DNA fragments from agarose gels

DNA fragments (plasmid DNA, PCR product or digested DNAs) were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, the DNA fragment was excised from the agarose gel under long-wave UV light (302 nm) using a clean and sharp scalpel. Then,

the gel slice was weighed in a 1.0 ml tube. Five volumes of Buffer PB was added to one volume of the DNA sample and mixed. Then, a QIAquick spin column was placed in a 2 ml collection tube to bind DNA, prior to centrifugation for 30 – 60 s. Next, the flow through was discarded and the QIAquick column was placed back into the same tube. For washing, 750 μ l of Buffer PE was added to the QIAquick column and followed by centrifugation at $10,000 \times g$ for 30 – 60 s. The flow through was then discarded and the QIAquick column was placed back in the same tube before subjected to centrifugation for an additional 1 min. A total of 33 μ l of water was added to the center of the QIAquick membrane and the column was centrifuged for 1 min at $10,000 \times g$ to elute DNA. Then, the DNA was visualized by staining with 0.1 μ g/mL of ethidium bromide on a 1% agarose gel for validation (Section 3.6).

3.8 Restriction enzyme digestion

Restriction enzymes from New England Biolabs, USA, were used to digest plasmids or PCR products for recombinant plasmid construction. Digestions were carried out in sterile microfuge tubes for 3 hours at 37°C under the conditions indicated in Table 3.4. For double digestion, the same reaction mixture was carried out by using a common buffer as recommended by the manufacturer and listed in Table 3.5. The digested PCR products of plasmids were separated by 1% agarose gel electrophoresis and excised out from the gel for purification (section 3.7).

Table 3.4: Single Restriction enzyme digestion setup

Component	Volume (μ l)
DNA	1 μ g
10 \times NEB Buffer 3	4
10 \times BSA	4
<i>Bam</i> HI, 10 units	2
Total reaction volume	40

Table 3.5: Double Restriction enzyme digestion setup

Component	Volume (μl)
DNA	1 μg
10 \times NEB Buffer 4	5
10 \times BSA	5
<i>Asc</i> I, 10 units	2
<i>Pac</i> I, 10 units	2
Water, nuclease-free	To 50 μl
Total reaction volume	50

3.9 Ligation

The *Pfu* polymerase-amplified and the *Bam*HI-digested PCR products (*yoeB_{Spn}* and *GFP*) were ligated using T4 DNA Ligase (Promega, USA) to produce a *yoeB_{Spn}-GFP* fusion gene. The ligation was carried out in a reaction volume of 40 μl containing 1 \times ligation buffer, 2 μl of T4 DNA ligase (20 Units/ μl), digested purified PCR products (*yoeB_{Spn}* and *GFP*) in a molar ratio of 1:1. The overall concentration of digested purified PCR products was between 1 – 5 $\mu\text{g/ml}$ for efficient ligation and the ligation was carried out at 4°C overnight. After ligation, PCR was carried out from 2 μl of the overnight ligation reaction mixture as a template using the *yoeB_{Spn}* Forward and *GFP* Reverse primers to produce a *yoeB_{Spn}-GFP* fusion fragment. The resulting PCR product was then subjected to electrophoresis on 1% agarose gel and the expected 990 bp fusion product was excised from the gel and purified (Section 3.7).

3.10 Cloning of DNA fragments into Gateway® pENTR-D-TOPO vector

The gel-purified *yoeB_{Spn}-GFP* ligated product was cloned into Gateway® pENTR-D-TOPO (Invitrogen, USA) vector using pENTR™ Directional TOPO® Cloning Kit (Invitrogen, USA). The reaction setup consisted of 4 μl of ligated *yoeB_{Spn}-GFP* DNA (0.1 $\mu\text{g}/\mu\text{l}$), 1 μl of salt solution (1.2 M NaCl and 0.06 M MgCl₂) and 1 μl

(15 – 20 ng/μl) of the TOPO vector. The reaction mixture was mixed gently and incubated for 5 min at room temperature. The reaction was then transformed into chemically competent *E. coli* TOP10 cells (section 3.11). The *Pfu* polymerase amplified and purified-*yefM_{Spn}* antitoxin gene, -*GFP* gene and -CaMV 35S promoter were directly cloned into pENTR-D-TOPO using the same protocol as above and transformed into chemically competent *E. coli* TOP10 cells using the same methods.

3.11 Preparation of competent *E. coli* cells and transformation

E. coli cells were made chemically competent for transformation using CaCl₂ as described by Sambrook et al., (1989).

3.11.1 Preparation of chemically-induced *E. coli* competent cells

One colony of *E. coli* TOP10 was inoculated into 10 ml sterile LB broth and incubated overnight at 37°C in a shaking incubator at 250 rpm. A 400 μl aliquot of the overnight culture was then inoculated into 40 ml sterile LB broth in a 200 ml flask and the culture was allowed to grow at 37°C with shaking at 250 rpm until reaching an OD₆₀₀ of 0.3-0.4. The cells were then placed on ice before harvesting in a refrigerated centrifuge at 6500 × *g* and at 4°C for 10 min. The supernatant was discarded and the cell pellet was then gently resuspended in 20 ml of ice-cold 0.1 M CaCl₂. The resuspended cells were kept on ice for 30 min before they were harvested by centrifugation at 6500 × *g* and at 4°C for 10 min. The resulting cell pellet was then resuspended in 4 ml of ice-cold CaCl₂ solution (85 mM CaCl₂ containing 15% glycerol) using chilled tips and 200 μl of the resuspended competent cells were then aliquoted into pre-chilled 1.5 ml tubes to be stored at -80°C until further use.

3.11.2 Transformation of chemically-induced *E. coli* competent cells

Frozen competent *E. coli* cells in 1.5 ml tubes were taken from -80°C storage and thawed on ice for 15 min. DNA for transformation such as plasmid vectors or 3 µl from the TOPO mixture (Section 3.9) was added to the competent cells and incubated on ice for 30 minutes. Heat shock was then performed on the cells by subjecting them to 42°C for 1 min, immediately followed by placing the cells on ice for another 2 min. Subsequently, 1 ml of sterile SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added to the cells and then incubated at 37°C in an orbital shaker at 250 rpm for 1.5 h. After incubation, a 150 µl aliquot of the cell mixture was plated on an LB agar plate supplemented with the appropriate antibiotics for selection of the transformed cells and the plate incubated overnight at 37°C.

3.11.3 Screening of transformed cells

To screen for *E. coli* transformants that harboured the required recombinant plasmid, single colonies were selected from the overnight incubated selection LB agar plate, picked up by sterile toothpick and streaked onto fresh LB agar plate containing the appropriate antibiotics to form a backup library of the potential recombinant clones that were screened. The agar plates were incubated overnight at 37°C whereas the toothpick that was used to pick up the colony was dabbed into a 0.2 ml PCR tube containing 10 µl of distilled water. The PCR tube with the picked colony was boiled at 95°C for 10 min and used as a template for colony PCR. Colony PCR was performed using GoTaq® Green Master Mix (Promega, USA) in a total volume of 25 µl. The PCR reaction setup and cycling conditions are listed in Tables 3.6 and 3.7, respectively. The positive colonies were chosen for plasmid extraction and DNA sequencing for further verification.

Table 3.6: Colony PCR reaction setup

Component	Volume (μ l)
GoTaq [®] Green Master Mix	12.5
Forward primer, 10 μ M	1
Reverse primer, 10 μ M	1
DNA (200 ng)	3
Distilled water	7.5
Total	25

Table 3.7: PCR conditions using GoTaq[®] Green Master Mix

PCR step	Temperature ($^{\circ}$ C)	Duration (min)
Initial denaturation	95	2
Denaturation	95	1
Annealing	51-57, depending on the primer sets	1
Extension	72	1
Final extension	72	5

3.12 Cloning of transgenes into the Gateway[®] plant binary destination vectors

The XVE-based two-component inducible gene expression system was utilized in this study. The activator vector (pMDC150) and the responder vectors (pMDC160 and pMDC221) were developed to enable conditional gene expression in selected plant tissues or cell types (Brand *et. al.*, 2006). The *yoeB_{Spn}-GFP*, *yefM_{Spn}* and CaMV 35S promoter fragments that had been cloned into the Gateway entry vectors (section 3.9) were transferred into Gateway[®] pMDC221, pMDC160 and pMDC150 respectively, using LR recombination reaction (Invitrogen, USA). In a sterile 1.5 ml tube, 7 μ l (about 50-150 ng) of each entry clone (pENTR-D-*yoeB_{Spn}GFP*, pENTR-D-*yefM_{Spn}* or pENTR-D-35S) and 1 μ l (150 ng/ μ l) of the appropriate destination vector (pMDC221, pMDC160 or pMDC150) were added at room temperature and mixed. The LR

Clonase™ Enzyme (2 µl) was then added to the reaction and mixed well by vortexing. The reaction was incubated at room temperature overnight. On the next day, 1 µl of 2 µg/µl Proteinase K solution was added to the mixture and vortexed briefly before incubation at 37°C for 10 min. A total of 5 µl from the reaction mixture was then used to transform chemically-induced *E. coli* competent cells (section 3.11.2). Positive transformants were screened by colony PCR (section 3.11.3) using gene-specific primers and validated by sequencing. The positive control used in this study, pMDC221_GFP containing only the GFP was also produced using the same methods as mentioned above.

3.13 *Agrobacterium tumefaciens* LBA 4404 competent cell preparation (Holster et al., 1978)

A single colony of *Agrobacterium tumefaciens* strain LBA 4404 was streaked out on an LB agar plate containing 50 µg/ml rifampicin and grown at 28°C for 2-3 days. A single colony obtained was then used to inoculate 5 ml of LB broth containing 50 µg/ml rifampicin and grown at 28°C overnight with shaking at 200 rpm. On the next day, a 50 ml of LB broth without antibiotic was inoculated with 0.5 ml (1:100 dilution) of the overnight culture and grown again overnight in an incubator-shaker at 28°C and with shaking at 200 rpm. In the following morning, the culture was chilled on ice for 30 min prior to centrifugation for 10 min at 4°C and at 6500 × g to pellet the cells. After centrifugation, the supernatant was discarded and the cell pellet was gently resuspended in 5 ml of ice cold 20 mM CaCl₂. After another centrifugation step as above, the pellet was gently resuspended in 1 ml of ice cold CaCl₂ solution (20 mM CaCl₂ containing 12.5% glycerol). The resuspended cells were aliquoted in 200 µl batches into pre-chilled 1.5 ml tubes and kept in -80°C for further use.

3.14 Transformation of recombinant constructs into *Agrobacterium tumefaciens* using freeze and thaw method (Holsters et al., 1978)

Prior to plant transformation, the recombinant constructs, pMDC221_yoeB_{Spn}GFP, pMDC221_GFP, pMDC160_yefM_{Spn} and pMDC150_35S were transformed separately into competent *Agrobacterium tumefaciens* LBA4404 using the freeze and thaw method (Holsters et al., 1978). Frozen competent *A. tumefaciens* cells in 1.5 ml tubes were taken from -80°C storage and thawed on ice for 15 min. Approximately 500 ng of plasmid DNA was added to the thawed *A. tumefaciens* competent cells (section 3.13) and the tubes were placed on ice for 30 min. The contents of the tubes were mixed by flicking the tubes a few times before the tubes were frozen in liquid nitrogen for 5 min. The cells were subsequently subjected to heat shock at 37°C for 5 min, then immediately placed on ice for another 5 min. The cells were then mixed with 1 ml sterile LB broth and incubated at 28°C in an orbital shaker at 200 rpm for 4 h. The cells were then plated out on LB agar plates (about 100 – 200 µl per agar plate) containing the selective antibiotics. The plates were incubated at 28°C for two to three days until presumptive transformed colonies were visible. These colonies formed were then picked and checked for the presence of the recombinant plasmids by colony PCR using gene-specific primers (section 3.11.3).

3.15 Transformation of recombinant constructs into *Arabidopsis thaliana* using floral dip protocol (Davis et al., 2009)

Single colonies of *A. tumefaciens* containing each recombinant construct (i.e. pMDC150_35S, pMDC221_yoeB_{Spn}-GFP, pMDC160_yefM_{Spn} and pMDC221_GFP) were inoculated into 50 ml LB broth containing the appropriate antibiotics (50 µg/ml spectinomycin and 50 µg/ml rifampicin for pMDC150_35S; 100 µg/ml ampicillin and 50 µg/ml rifampicin for pMDC221-derived recombinants and 100 µg/ml ampicillin and

50 µg/ml rifampicin for pMDC160_*yefM_{Spn}*) and grown at 28°C for 2 days with shaking at 200 rpm. At the end of the incubation period, the entire 50 ml culture was poured into 200 ml LB broth without any antibiotics and grown for another 8 hours at 28°C with shaking at 200 rpm. The *A. tumefaciens* cultures containing the recombinant activator were combined with cultures containing the appropriate recombinant responder into the same beaker and 200 µl of Silwet-77 was added into the cultures. The recombinant activator – responder combinations used in this study were as follows: (1) pMDC150_35S + pMDC221_*yoeB_{Spn}*-*GFP*; (2) pMDC150_35S + pMDC221_*GFP* (as experimental control); and (3) pMDC150_35S + pMDC160_*yefM_{Spn}*.

Flowers of healthy six-week-old *A. thaliana* plants were subsequently dipped in the *A. tumefaciens* culture solution containing the recombinant activator-responder combinations. Inoculation was performed by dipping aerial parts of the plants for a few seconds. After dipping, the plants were covered with plastic bags to ensure high humidity for overnight. The plants were grown under the same condition (section 3.1) until maturity phase and seeds were harvested for selection. For each recombinant activator-responder combination, five different *A. thaliana* plants were used in the floral dip transformation.

3.16 Selection of *A. thaliana* transformants

Seeds harvested from transformed *A. thaliana* plants were selected under antibiotic or herbicide selection (Table 3.8). For selection of transgenic *A. thaliana* on the antibiotics kanamycin and hygromycin, seeds were stratified for 3 days at 4°C before sowing on the soil. The germinated seeds were grown for about 1 week before spraying with a mixture of 50 mg/l kanamycin and 50 mg/l hygromycin. The spraying of the antibiotic mixture was performed at 3-day intervals for 2 weeks.

For selection of transgenic *Arabidopsis* on the antibiotic kanamycin and the herbicide Basta (glufosinate), the concentration used was 50 mg/l and 0.25 mg/l, respectively. The same selection methods as above were used. All positive transformants were grown in a GP BSL2 facility until T₂ generation.

Table 3.8 Selection of transgenic *A. thaliana*

Recombinant activator-responder combination	Antibiotics/herbicide selection
pMDC150_35S and pMDC221_ <i>yoeB_{Spm}GFP</i>	kanamycin and hygromycin
pMDC150_35S and pMDC221_ <i>GFP</i>	kanamycin and hygromycin
pMDC150_35S and pMDC160_ <i>yefM_{Spm}</i>	kanamycin and Basta

3.17 Genomic DNA extraction from *A. thaliana*

Genomic DNA was extracted from fresh leaf tissues of four-week-old plants using the CTAB method (Roger and Bendich, 1994) with some modifications. The samples were ground to a fine powder under liquid nitrogen using a mortar and pestle. 1 ml of CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8, 20 mM EDTA, 2% PVP 40 and 1.4 M NaCl) was added to the samples followed by addition of 20 µl of 2% β-mercaptoethanol and vortexed to mix. RNA was removed from the samples by addition of 4 µl of 10 mg/ml RNase A, mixed by short vortexing for 5 s, and incubating the samples at 55°C for 30 min with inversion 2-3 times during incubation. Following incubation, 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the samples prior to centrifugation (8000 × g) at room temperature for 15 min. The aqueous phase was transferred to a new 1.5 ml tube and the above step was repeated until a clear supernatant was obtained. 1 volume of chloroform: isoamylalcohol was then added to the aqueous phase and centrifuged at room temperature (8000 × g) for 15 minutes. Next, the aqueous phase was carefully transferred into a new 1.5 ml tube and mixed with 1

volume of ice-cold isopropanol. The samples were incubated at -20°C for 3 hours and were then centrifuged ($8000 \times g$) at 4°C for 30 min to pellet the DNA. The supernatant was carefully removed without disturbing the pellet. Next, the pellet was washed with 70% ethanol and centrifuged for another 5 min at 4°C . Finally the pellet was air-dried prior to adding 30 μl of sterile distilled water to elute the DNA. The quality and concentration of the DNA obtained were checked by gel electrophoresis (section 3.6) and Nanodrop spectrophotometer, respectively.

3.18 Detection of transgene by PCR amplification of *A. thaliana* genomic DNA

Presence of the transgenes in the transgenic *A. thaliana* was confirmed by PCR-amplification with gene specific primers (Table 3.2) using extracted genomic DNA as the template. Each PCR reaction was carried out in a total volume of 25 μl using GoTaq[®] Green Master Mix (Promega, USA) as indicated in Table 3.9. PCR conditions were as indicated in Table 3.6.

Table 3.9: PCR reaction setup for amplification of *A. thaliana* genomic DNA

Component	Volume (μl)
GoTaq [®] Green Master Mix	12.5
DNA (0.1 μg)	1
Forward primer, 10 μM	1
Reverse primer, 10 μM	1
Distilled water	9.5

3.19 Southern blotting

Southern hybridization was performed to confirm the integration and copy number of *yoeB_{Spn}-GFP* and *yefM_{Spn}* transgenes in the T₂ generation *A. thaliana* plants. The specific probe used to hybridize the transgene was labelled according to the instructions in the DIG DNA labelling and detection Kit application manual (Roche, USA). As the first step, 16 μl of *Taq* polymerase-amplified and 1 μg purified DNA

templates (*yoeB_{Spn}-GFP* and *yefM_{Spn}*) were denatured by heating in boiling water bath for 10 min and quickly chilling on ice. Following this, 4 µl of 5 × Dig-High Prime was added to the denatured DNA, mixed and centrifuged briefly. The mixtures were incubated overnight at 37°C. In the following morning, the reaction was stopped by heating to 65°C for 10 min.

To determine the efficiency of the labelled probes, a series of dilutions of Dig-labelled DNA was applied to a small strip of positively charged nylon membrane (Roche, USA) with defined dilutions of DIG-labelled control DNA as standards. The nucleic acids on the membrane were fixed by baking at 80°C for 30 minutes. This was followed by transferring the membrane into a plastic container containing 20 ml of 1 × Maleic Acid buffer (0.1 M Maleic Acid, 0.15 M NaCl; pH adjusted to 7.5) and incubated under shaking for 2 min at room temperature. The membrane was then incubated with agitation in 10 ml of 1 × Blocking solution (100 ml 10 × Blocking Stock solution dissolved in 900 ml Maleic Acid buffer) for 30 min, followed by incubation in 10 ml of an antibody solution (150 mU/ml of anti-Digoxigenin-AP diluted in Blocking solution) for 30 min. For the washing step, the membrane was washed twice with Washing buffer (0.1 M Maleic Acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20), each for 15 min. Finally, the membrane was equilibrated in Detection buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) for 5 min prior to incubation in 2 ml freshly prepared Colour Substrate solution (40 µl Nitro Blue Tetrazolium (NBT) / 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)) in the dark. The reaction was stopped when the desired spot intensity was achieved.

Total genomic DNA (20 µg) from both wild-type and transgenic *A. thaliana* were digested with *EcoRI* (New England Biolabs, USA) at 37°C for 48 h. Electrophoresis of DNA samples were then carried out in 0.7% agarose gel at 50 V until

the DNA bands were well separated. Ethidium bromide was not used in the preparation of agarose gel to avoid uneven background problem.

Next, the DNA on the membrane was agitated in Depurination solution (250 mM HCl) for 10 min at room temperature or until the bromophenol blue dye turned yellow. The Depurination solution was then discarded and the gel was rinsed twice with distilled water. This was followed by immersing the gel in Denaturation solution (0.5 M NaOH and 1.5 M NaCl) twice, each for 15 mins with constant shaking. Again, the solution was discarded and the gel was rinsed with distilled water twice. The gel was then submerged in Neutralization solution (0.5 M Tris-HCl; pH 7.5 and 1.5 M NaCl) twice, each for 15 min under constant agitation. Prior to transferring DNA to a membrane, the gel was equilibrated for at least 10 min in $20 \times$ SSC (3 M NaCl, 300 mM Sodium Citrate; pH 7.0).

For transferring DNA to the nylon membrane, the setup for Southern blotting was constructed as follows. A piece of Whatmann 3MM paper soaked with $20 \times$ SSC was placed atop a 'bridge' that rests in a shallow reservoir of $20 \times$ SSC. The gel containing the DNA was placed atop the soaked sheet of Whatmann 3MM paper and a sterile pipette was rolled over the sandwich to remove all air bubbles that formed between the gel and paper. This was followed by placing a piece of dry positively charged nylon membrane on the DNA-containing surface of the gel. The air bubbles were eliminated using a sterile pipette as mentioned above. The Southern blot setup was completed by adding a dry sheet of Whatmann 3MM paper, a stack of paper towels, a glass plate and a 200 – 500 g weight. The completed blot transfer 'sandwich' is shown in Figure 3.1. The blot was left overnight at room temperature to ensure complete transfer of the DNA to the membrane.

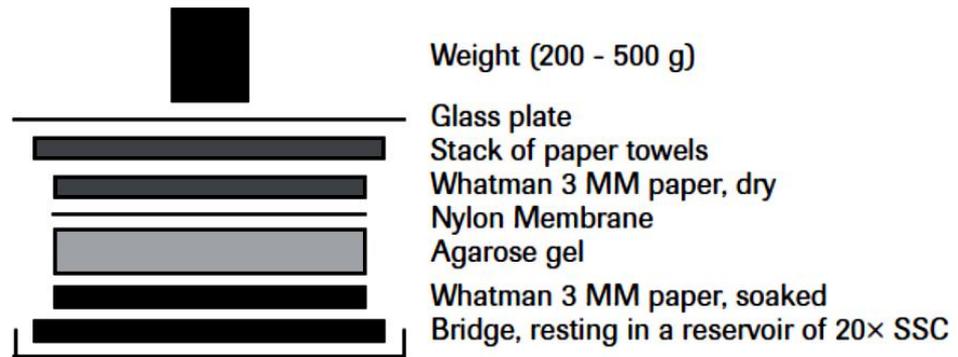


Figure 3.1: The blot transfer setup used in Southern Blotting. The diagram was obtained from the DIG DNA labelling and detection Kit application manual (Roche, USA).

Upon completion, the membrane was washed briefly with $2 \times \text{SSC}$ and the transferred DNA was fixed to the membrane by baking at 80°C for 2 h. The membrane was used immediately for prehybridization. A total of 10 ml and 12 ml of Dig Easy Hyb solution for prehybridization and hybridization, respectively, were preheated to 46°C in a water bath. This was followed by transferring the membrane into 10 ml of pre-heated Dig Easy Hyb solution and incubated for 30 min under gentle agitation. While waiting for prehybridization to complete, approximately 25 ng/ml of Dig-labelled DNA probe was denatured by boiling for 5 min and cooled immediately on ice. For hybridizing the Dig-labelled DNA probe to DNA on the membrane, the denatured probe was added into 12 ml pre-warmed Dig Easy Hyb and mixed by inversion to form the hybridization solution. The membrane was then incubated overnight with gentle shaking at 46°C . Following incubation, the hybridization solution was poured out and the membrane was washed twice, each time for 5 min with Low Stringency buffer ($2 \times \text{SSC}$ containing 0.1% SDS) at room temperature. During this step, the High Stringency buffer ($0.1 \times \text{SSC}$ containing 0.1% SDS) was preheated at 65°C in a shaking water bath. The Low Stringency buffer was poured out after finished washing, followed by the addition of

High Stringency buffer to a membrane. The membrane was incubated twice, each time for 15 min under constant agitation at 65°C before undergoing immunological detection.

All the incubation steps described in the following passage were performed at room temperature with gentle agitation. Immediately after the stringency washes, the membrane was rinsed briefly in washing buffer. The membrane was then incubated in Blocking solution for 30 min, followed by 30 min in Anti-Dig-AP conjugation solution. The membrane was then washed twice, each for 15 min with Washing buffer. The membrane was then equilibrated in Detection buffer for 3 min before incubated in the dark using freshly prepared Color-substrate solution for about 16 h. The hybridization bands that were developed were visualized by development of colour on the membrane and the reaction was stopped with distilled water when the desired bands were obtained. Images of the hybridization bands on the membrane were captured using 8-megapixel iSight camera.

3.20 Induction for transgene expression in transgenic *A. thaliana* using 17- β -estradiol

To induce the expression of the respective transgenes in the transgenic *A. thaliana* lines obtained, 100 μ M 17- β -estradiol (in the presence of 0.02% Tween-20; Curtis and Grossniklaus, 2003) was applied using an artist paint brush (Brand et al., 2006) from a stock solution of 20 mM 17- β -estradiol (Sigma Aldrich, USA) prepared in DMSO. Following 17- β -estradiol application, the plants were covered with plastic overnight to ensure high humidity.

For RNA extraction, the rosette leaves were harvested every 24 hours after induction for 7 days, frozen by immersion in liquid nitrogen and then stored at -80°C.

3.21 Phenotypic analysis of transgenic *A. thaliana* after induction

After induction, transgenic *A. thaliana* transformed with pMDC150_35S / pMDC221_*yoeB_{Spn}GFP* and pMDC150_35S / pMDC160_*yefM_{Spn}* were monitored daily for 2 weeks to observe the effects of the expression of the *yoeB_{Spn}* toxin and *yefM_{Spn}* antitoxin, respectively. GFP expression was monitored in transgenic plant samples transformed with pMDC150_35S / pMDC221_*yoeB_{Spn}-GFP* on days 1, 3, 6, 8 and 9 after induction and observed by confocal microscopy under a Leica DMIRE2 microscope equipped with a Leica TCS SP5 II laser scanning device. Images were taken using a camera lenses brand ZEISS and analysed using LAS AF SP5-II software.

3.22 Quantitative real-time reverse transcriptase PCR (qRT-PCR)

3.22.1 Total RNA extraction

A maximum of 1 mg of plant tissues was ground using mortar and pestle to a fine powder under liquid nitrogen. The total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocols as follows: A total of 450 μ l of Buffer RLT (1% β -mercaptoethanol added as instructed in the protocol) was added to the frozen tissues and the tube was vortexed vigorously. This was followed by applying the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at $8000 \times g$. The supernatant was then transferred to a new microfuge tube without disturbing the cell-debris pellet at the bottom of the tube and half volume of ethanol was added to the clear lysate, mixed by pipetting. The sample was then applied to an RNeasy mini column placed in a 2 ml collection tube which was then subjected to centrifugation at $15000 \times g$ for 1 min. The flow-through was discarded and the column was reinserted into the collection tube. A total of 700 μ l of Buffer RW1 was added to the RNeasy spin column and centrifuged for 1 min at $8000 \times g$. The flow-through was then discarded prior to addition of 500 μ l of

Buffer RPE to the RNeasy column and centrifuged for 2 min at $10000 \times g$. For elution, the RNeasy column was transferred to a new 1.5 ml collection tube. Then, 30 μ l of RNase-free water was added directly to the column, incubated for 1 min at room temperature, and centrifuged at $10,000 \times g$ for 1 min. The column was discarded and the eluted RNA was visualized by electrophoretic separation on a 1% agarose gel and stained with 0.1 μ g/ml Ethidium bromide (section 3.6).

3.22.2 RNA quantification

The quantity of extracted RNA samples (section 3.21.1) was measured using a Nanodrop 2000/2000C spectrophotometer (Thermo Scientific). RNA concentration and purity were determined by measuring the ratio of the UV absorbance at 260 nm and 280 nm. Individual samples were measured in triplicate and the average was taken. OD_{260} of 1.0 is approximately equivalent to 40 μ g/ml single-stranded RNA. The ratio of $OD_{260}/OD_{280} = 1.8$ to 2.0 was considered as pure RNA (Instruction manual, Qiagen, Germany).

3.22.3 DNase I treatment

To remove any DNA contamination in the extracted RNA samples, 1 μ g of each of the RNA samples were treated with DNase I that is provided in the QuantiTect[®] Reverse Transcription Kit (Qiagen, Germany). RNA samples were thawed on ice whereas gDNA Wipeout Buffer, and RNase-free water were thawed at room temperature (15 - 25°C). Each solution was mixed by flicking the tubes and centrifuged briefly to collect any residual liquid from the sides of the tubes. The genomic DNA elimination reaction was prepared on ice and according to Table 3.10. The reaction mixture was then incubated at 42°C for 2 min and then was placed immediately on ice. The DNase-treated RNA samples were kept at -20°C until further use.

Table 3.10: Genomic DNA elimination reaction components

Component	Volume / reaction	Final concentration
gDNA Wipeout Buffer, 7×	2 μ l	1×
Template RNA	1 μ g	
RNase-free water	Variable	
Total volume	14 μl	-

3.22.4 Reverse transcriptase-PCR (RT-PCR)

Complimentary DNA (cDNA) was synthesized from 1 μ g of RNA in two steps using a QuantiTect[®] Reverse Transcription Kit (Qiagen, Germany). Master Mix (20 μ l) was set-up as advised by the manufacturer and shown in Table 3.11. To initiate reverse transcription, the reaction mixture was incubated at 42°C for 15 min followed by inactivation at 95°C for 3 min.

Table 3.11: Reverse-transcription reaction components

Component	Volume / reaction	Final concentration
Quantiscript Reverse Transcriptase	1 μ l	
Quantiscript RT Buffer, 5×	4 μ l	1×
RT Primer Mix	1 μ l	
Template RNA (entire genomic DNA elimination reaction from Section 3.22.3)	14 μ l	
Total volume	20 μl	-

To determine the presence of the relevant cDNA prior to proceeding to quantitative real-time RT-PCR (section 3.22.5), PCR (section 3.5) was performed using GoTaq[®] Green Master Mix (Promega, USA) in a total reaction volume of 25 μ l (Table 3.7). All the primer sequences used here were as listed in Table 3.2. Each gene was

assayed on three biological replicates and *A. thaliana Actin* cDNA was used as the reference. PCR conditions were as indicated in Table 3.7.

3.22.5 Quantitative real-time RT-PCR (qRT-PCR)

Gene expression levels were determined using QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, USA). Each reaction consisted of 0.5 µM of both forward and reverse primers, 25 ng of cDNA as template, and 1 × SYBR Green Master mix (Applied Biosystem, USA) in a final volume of 20 µl. The primers that were used for amplifying the cDNA are listed in Table 3.2. The reaction settings consisted of an initial denaturation step of 5 min at 94°C followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, and 15 s at 72°C. The *A. thaliana Actin* gene was used as the reference to normalize gene expression across the samples (Livak and Schmittgen, 2001). Fluorescence readings were taken at the end of each cycle and the specificity of amplification as well as the absence of primer dimers was confirmed with a melting curve analysis at the end of each reaction. Fluorescence and Ct values were exported and analysed in MS Excel (Microsoft, USA). Relative expression of each *yoeB_{Spn}-GFP* and *yefM_{Spn}* cDNA was obtained by dividing the average number of each transcript copies by the copy number of *A. thaliana Actin* (endogenous control) for the same tissue. The lowest transcript level (day 1 after induction) was then set to a value of 1 and subsequently expression levels were reported relative to this number.

3.23 Apoptosis DNA fragmentation assay

Internucleosomal DNA fragmentation has been widely shown to be a typical sign of apoptotic cell death (Wyllie, 1980). In this study, DNA fragmentation in the induced transgenic *A. thaliana* was investigated using an Apoptotic-Ladder Kit (bioPLUS, USA) as follows. Genomic DNA was extracted from plant tissues 6, 12 and 24 h after induction (section 3.20) using the kit with some modifications. The sample (2

– 5 mg tissue) was ground to a fine powder under liquid nitrogen using a mortar and pestle. A total of 30 μ l of DNA Stripping Buffer and 10 μ l of Proteinase K solution were added to the sample, mixed and incubated at 55°C for 3 – 4 h. The tube was then allowed to cool to room temperature. This was followed by the addition of 150 μ l of Precipitation Buffer and inverting the tube a few times to mix. The tube was then centrifuged at 15,000 $\times g$ for 10 min to pellet the precipitate. The supernatant obtained after centrifugation was transferred to a clean tube, followed by the addition of 500 μ l of ethanol and inverted a few times to mix. The mixture was incubated at -20°C for 1 h and then subjected to centrifugation at 15,000 $\times g$ for 10 min to recover the nucleosomal DNA precipitate. The supernatant was discarded and centrifuged again for 10 s to remove any remaining supernatant. The pellet obtained after centrifugation was allowed to evaporate and dry by a brief incubation at 35°C - 40°C. Extracted DNA samples were resuspended in 10 - 15 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and incubated at 35°C until the DNA pellet was fully hydrated. The sample was then electrophoretically separated on a 1.8% agarose gel at 60 V for 2 hrs.

3.24 Construction and analysis of hybrid transgenic *A. thaliana* co-expressing YefM_{Spn} and YoeB_{Spn}-GFP

To investigate if the YefM_{Spn} antitoxin is able to counteract the toxicity of the YoeB_{Spn} toxin in *A. thaliana*, hybrid transgenic *A. thaliana* co-expressing both YefM_{Spn} and YoeB_{Spn}-GFP were constructed by cross-pollinating *A. thaliana* harbouring *yefM_{Spn}* and *A. thaliana* harbouring *yoeB_{Spn}-GFP*.

3.24.1 Cross-pollination of transgenic *A. thaliana*

Two different transgenic *A. thaliana* from the T₁ generation harbouring pMDC150_35S/pMDC160_*yefM_{Spn}* and pMDC150_35S/pMDC221_*yoeB_{Spn}GFP* were grown under antibiotic or herbicide selection (Table 3.8; section 3.16) until the bolting

stage, for cross-pollination. The flower of each transgenic *A. thaliana* harbouring pMDC150_35S/pMDC160_yefM_{Spn} and pMDC150_35S/pMDC221_yoeB_{Spn}GFP were chosen as ovule donor and pollen donor, respectively. To start cross-pollination, unopened flower buds were sliced open lengthwise and emasculated with sterilized forceps. Mature pollen from the donor plant was transferred onto the stigmas of emasculated plants by brushing its anthers. The cross-pollinated flowers were marked and wrapped with small plastic bags to prevent additional pollination from other pollen sources. The plants were grown under normal growth conditions (section 3.1) until the seeds were matured and ready to be collected. Eleven lines of hybrid plants were produced from the cross-pollination.

3.24.2 Selection of hybrid transgenic seeds

After cross-pollination, the seeds harvested were grown under the appropriate antibiotic and herbicide selection (section 3.16). The presumptive hybrid transgenic *A. thaliana* plantlets that survived were transferred to soil in the greenhouse and grown for another 4 weeks before proceeding with further analysis. Genomic DNA was isolated (section 3.17) from each of the surviving transgenic plants and PCR (section 3.18) was carried out using gene-specific primers (Table 3.2) to confirm the presence of both the yoeB_{Spn} toxin and yefM_{Spn} antitoxin transgenes in the hybrid transgenic *A. thaliana*.

3.24.3 Expression analysis on transgenic hybrid *A. thaliana* harbouring yefM_{Spn} and yoeB_{Spn}-GFP

qRT-PCR was carried out (section 3.22) to study the expression of yefM_{Spn} and yoeB_{Spn}-GFP in the transgenic hybrid *A. thaliana* using the primers q-yefM-F/R and q-yoeB-F/R, respectively, as listed in Table 3.2.

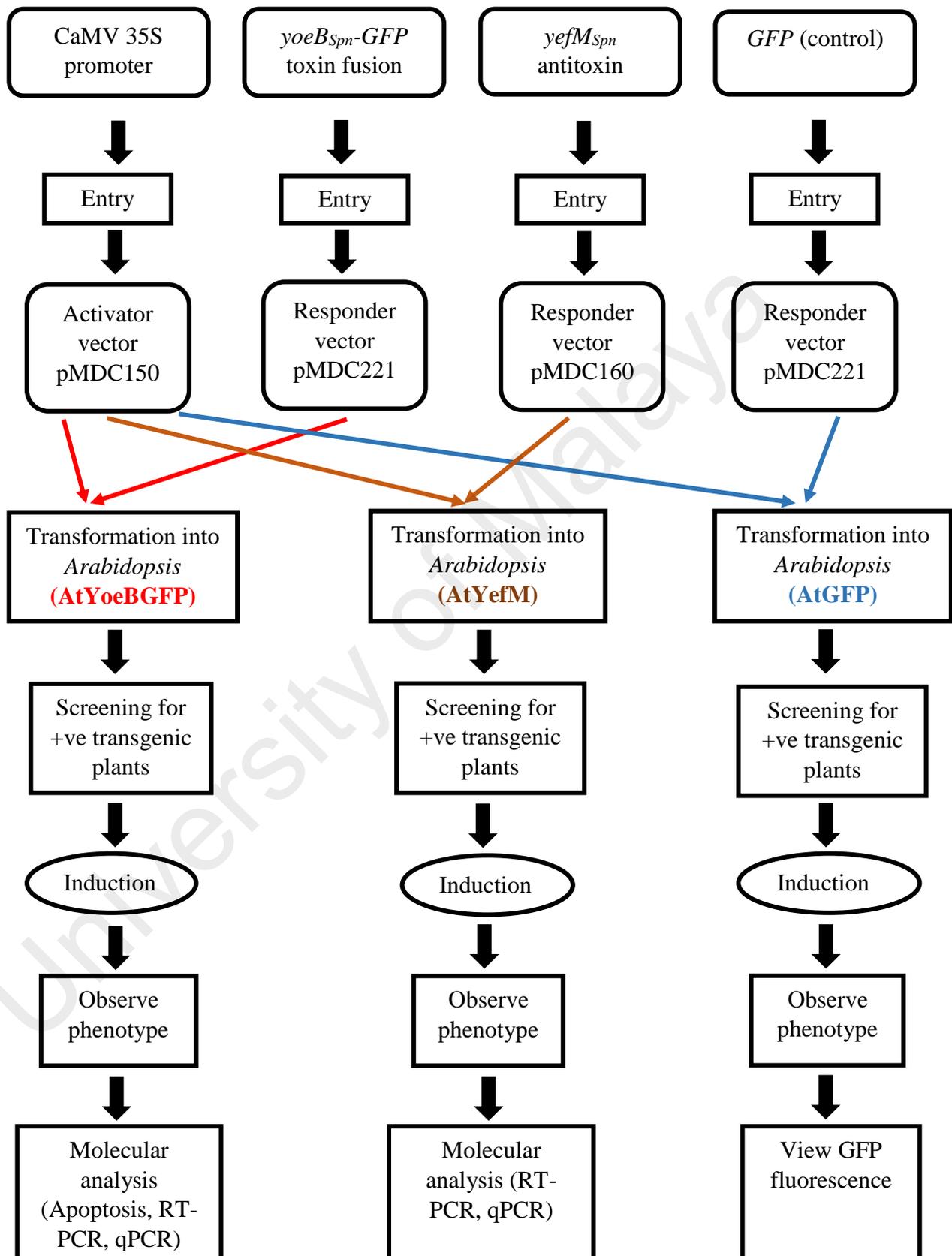
3.24.4 Phenotypic analysis on transgenic hybrid *A. thaliana*

After induction with 17- β -estradiol, phenotypic measurements of induced, non-induced and wild-type plants were recorded after the plants were fully matured, in which all siliques were fully formed (usually after six to eight weeks after the seeds have germinated) (section 3.20). Phenotypic measurements ($n=20$ for each group) included fresh and dry weight, length of rosette leaves, height (measured as the length from the soil to the top of each plant), number of inflorescence stems formed in each plant, number of branches bearing siliques, length of silique and total number of silique harvested per plant.

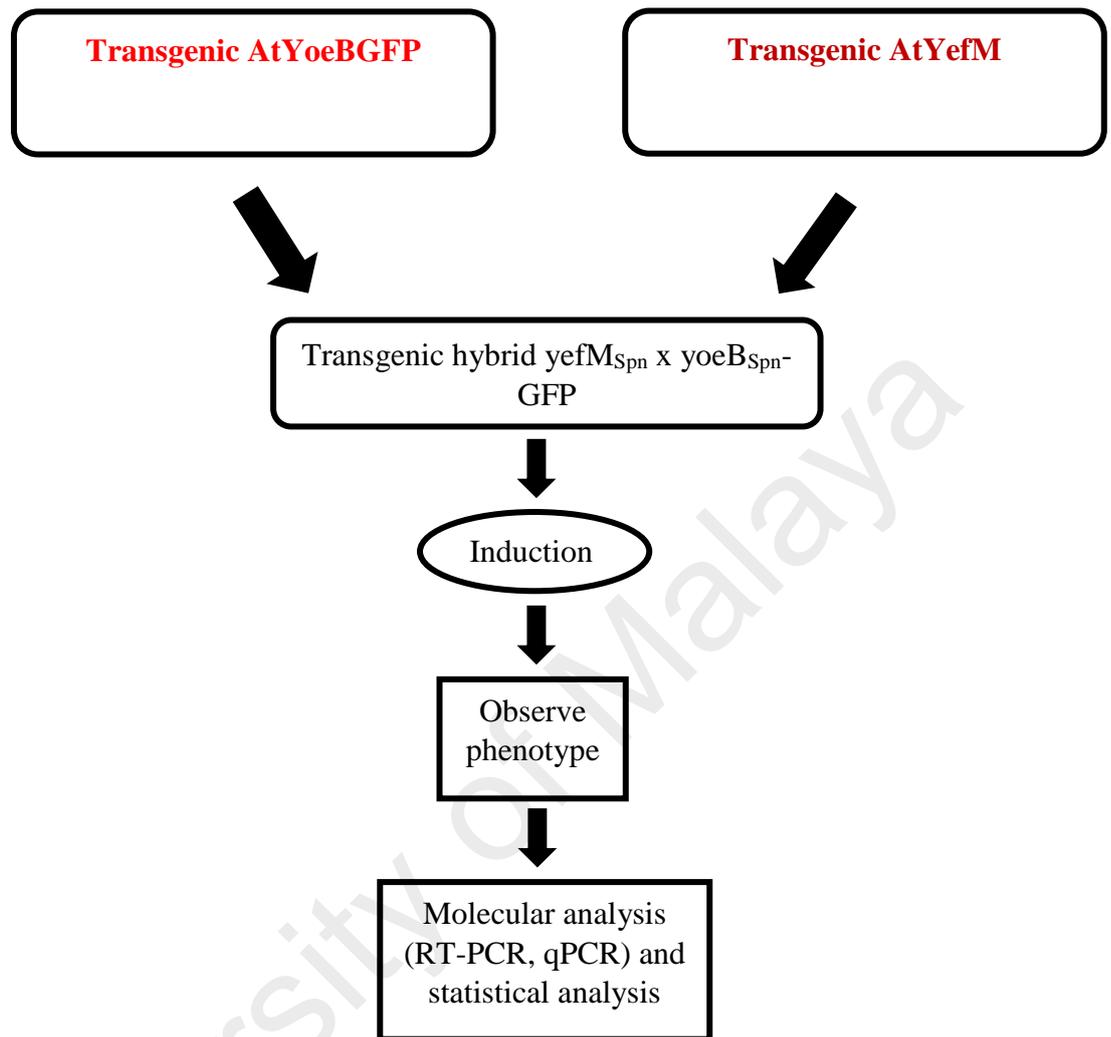
3.25 Statistical analysis

Data was analysed using ANOVA with IBM SPSS statistics 16.0 software. Significant difference from the control value(s) was determined at $P < 0.05$ levels. All reported data represent the mean \pm SD of at least three independent experiments.

Summary of the experimental workflow of this study



Continued



CHAPTER 4: RESULTS

4.1 PCR amplification of *yoeB_{Spn}*-*GFP* fusion, *yefM_{Spn}* antitoxin, *GFP* and CaMV 35S promoter

The first step in constructing the recombinant expression vectors for creating the desired transgenic *Arabidopsis thaliana* was to obtain the required heterologous DNA fragments, namely the *yoeB_{Spn}*, *yefM_{Spn}*, *GFP* and CaMV 35S promoter fragments. These DNA fragments were obtained by PCR amplification as detailed in the Materials and Methods (section 3.5) and were validated by electrophoresis on 1% agarose gels (Figures 4.1 – 4.4). The presence of intact bands of the expected sizes for *yoeB_{Spn}* (Figure 4.1), *yefM_{Spn}* (Figure 4.2), *GFP* (Figure 4.3) and the CaMV 35S promoter (Figure 4.4) was observed. The *yefM_{Spn}*, *GFP*, and CaMV 35S amplified products were then cloned into the Gateway pENTR-D-TOPO cloning vector.

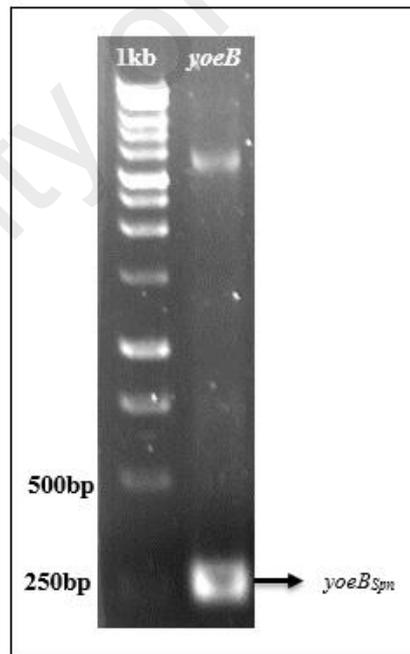


Figure 4.1: PCR product of *yoeB_{Spn}* (255 bp) amplified from pET28a_HisYefMYoeB and separated by electrophoresis on a 1% agarose gel. *yoeB*: *yoeB_{Spn}* toxin gene; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

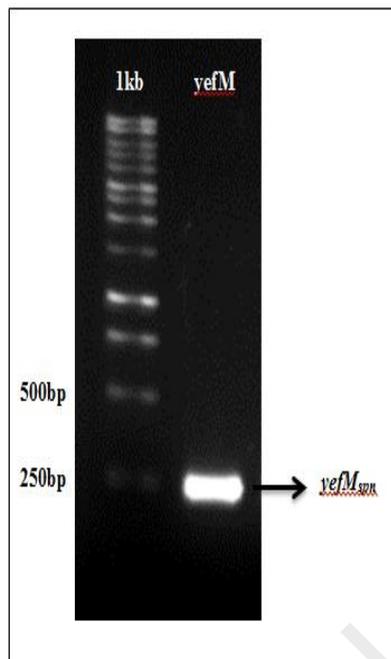


Figure 4.2: PCR product of *yefM_{Spn}* (255 bp) amplified from pET28a_HisYefMYoeB and separated by electrophoresis on a 1% agarose gel. *yefM*: *yefM_{Spn}* antitoxin gene; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

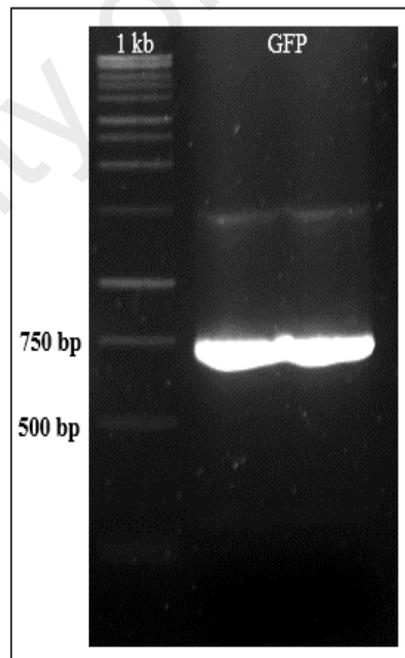


Figure 4.3: PCR product of GFP (732 bp) amplified from pCAMBIA 1304 and separated by electrophoresis on a 1% agarose gel. GFP: Green Fluorescent Protein; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

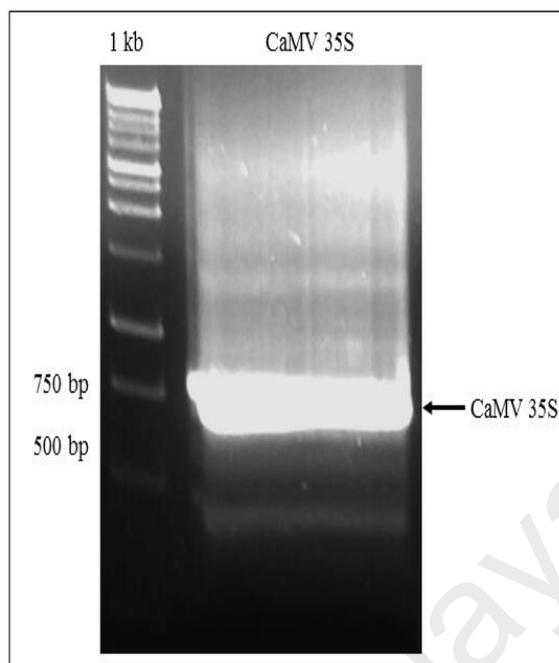


Figure 4.4: PCR amplification of CaMV 35S promoter (800 bp) from pCAMBIA 1304 separated by electrophoresis on a 1% agarose gel. CaMV 35S: Cauliflower Mosaic Virus 35S Promoter; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

4.1.1 Ligation of *yoeB_{Spn}* and *GFP* coding sequences

To construct the *yoeB_{Spn}-GFP* fusion, the *yoeB_{Spn}* and *GFP* DNA fragments, which had been obtained by PCR-amplification using primers incorporating *Bam*HI sites at the 5'-ends, were digested with *Bam*HI, separated on agarose gels (Figure 4.5) and purified. The purified *Bam*HI-digested *yoeB_{Spn}* and *GFP* fragments were ligated overnight and the ligated product was PCR-amplified using the *yoeB_{Spn}* forward primer and the *GFP* reverse primer (section 3.5). The resulting amplified product of 990 bp (Figure 4.6) was purified and cloned into the Gateway pENTR-D-TOPO cloning vector.

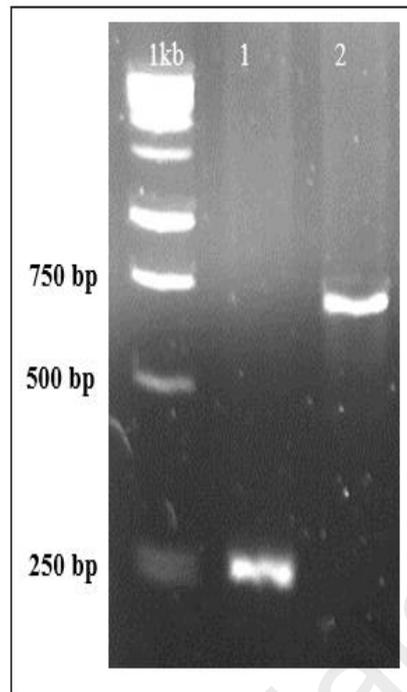


Figure 4.5: Agarose gel electrophoresis of *Bam*HI-digested amplified products. Lane 1: *yoeB_{Spn}* (255 bp); lane 2: *GFP* (732 bp); 1 kb: 1 kb DNA ladder as marker (Fermentas, Canada).

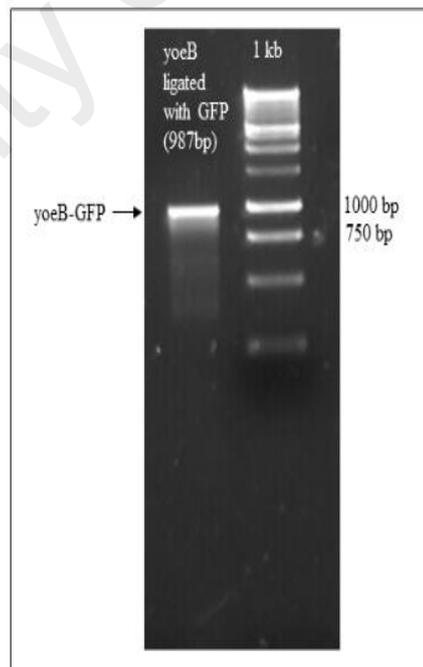


Figure 4.6: Agarose gel electrophoresis of the PCR product of ligated *yoeB_{spn}-GFP* (~990 bp). 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

4.2 Cloning of *yoeB_{Spn}-GFP*, *yefM_{Spn}*, *GFP* and CaMV 35S into Gateway® pENTR-D-TOPO vector

Out of 47 colonies screened by PCR after the transformation of *yoeB_{Spn}-GFP* into the Gateway pENTR-D-TOPO, only 29 colonies showed the expected size of 1141 bp after 1% agarose gel electrophoresis of their respective amplified products (Figure 4.7).

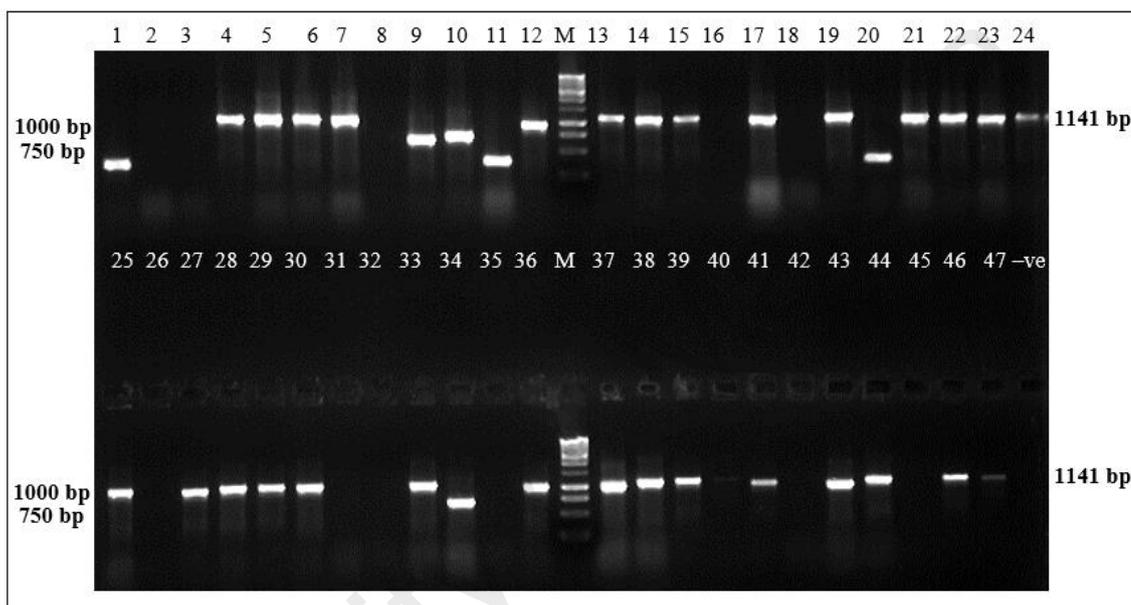


Figure 4.7: Colony PCR of pENTR_*yoeB_{Spn}-GFP* ligated products. Lanes 1-47: PCR of bacterial colonies with M13 Forward and GFP Reverse primers. Lane –ve: negative control, PCR carried out without bacterial colony; M: 1 kb DNA ladder marker (Fermentas, Canada).

Cloning of the *yefM_{Spn}* amplified fragment into pENTR-D-TOPO resulted in a total of 16 colonies of transformed *E. coli* TOP10 cells that showed positive results following agarose gel electrophoresis, as indicated by the presence of an amplified DNA band of the expected size of 406 bp (Figure 4.8). Meanwhile, six colonies (lanes 3, 6, 10, 16, 18 and 21) of *E. coli* TOP10 cells from the transformation of pENTR_*GFP* showed the desired result, i.e. the presence of an amplified band of the expected size of 883 bp after agarose gel electrophoresis (Figure 4.9). Following transformation of

pENTR_CaMV 35S, all 11 colonies of *E. coli* TOP10 cells showed the presence of an amplified band of the expected size of 951 bp after agarose gel electrophoresis (Figure 4.10).

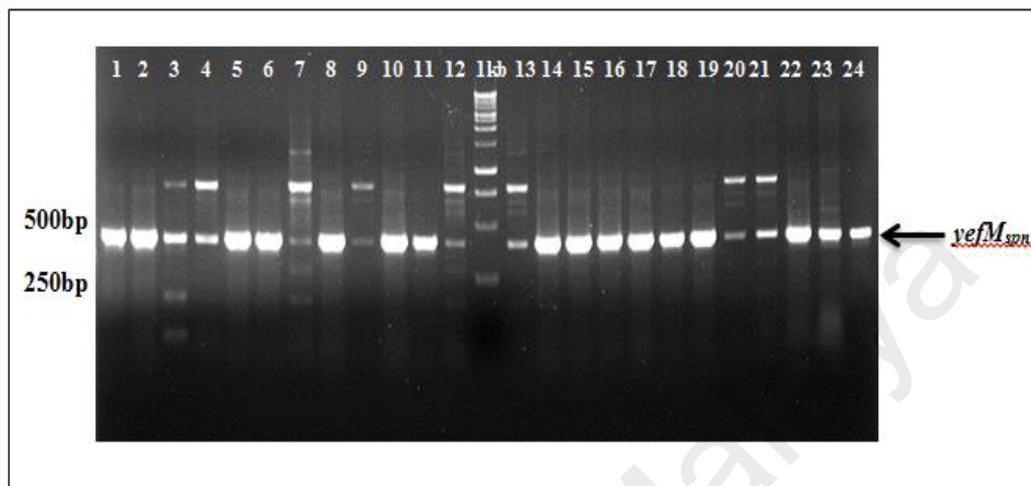


Figure 4.8: Colony PCR of pENTR_*yefM_{Spn}* transformants. Lanes 1-24: PCR of bacterial colonies with M13 Forward and *yefM* Reverse primers. 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

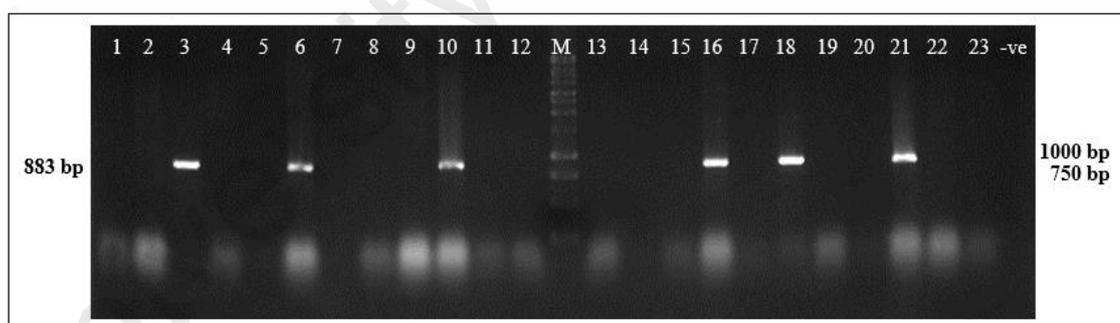


Figure 4.9: Colony PCR of pENTR_*GFP* transformants. Lanes 1-23: PCR of bacterial colonies with M13 Forward and *GFP* Reverse primers. Lane -ve: negative control, PCR carried out without bacterial colony; M: 1 kb DNA ladder marker (Fermentas, Canada).

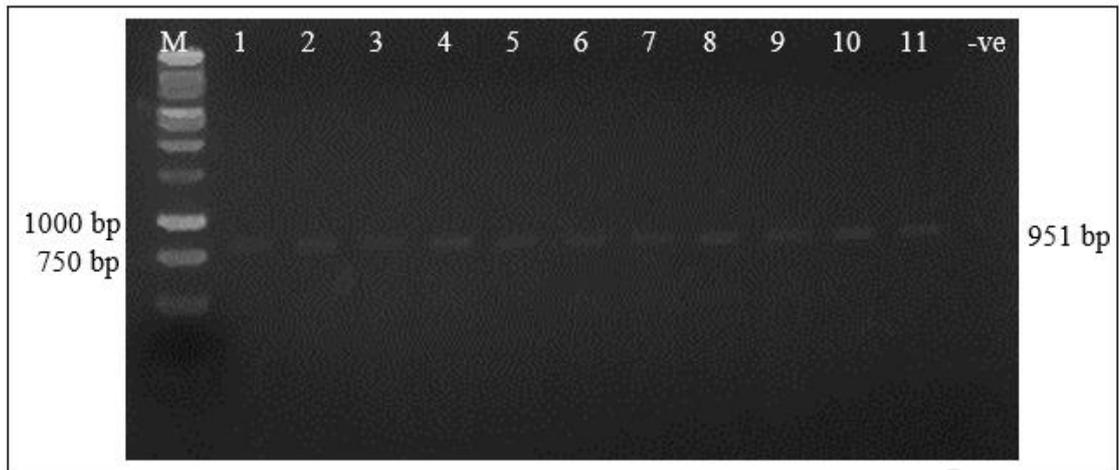


Figure 4.10: Colony PCR of pENTR_CaMV35S transformants. Lanes 1-11: PCR of bacterial colonies with M13 Forward and CaMV 35S Reverse primers. Lane -ve: negative control, PCR carried out without bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

4.2.1 Plasmid extraction and validation of the recombinant constructs

pENTR_yoeB_{Spn}-GFP, pENTR_yefM_{Spn}, pENTR_GFP and pENTR_CaMV 35S

Plasmids were extracted from two randomly-chosen clones of each of the four pENTR-D-TOPO recombinant constructs, i.e., pENTR_yoeB_{Spn}-GFP, pENTR_yefM_{Spn}, pENTR_GFP and pENTR_CaMV 35S. The plasmids were electrophoresed on 1% agarose gel as depicted in Figure 4.11(A). The extracted plasmids were then subjected to PCR for validation of their recombinant status.

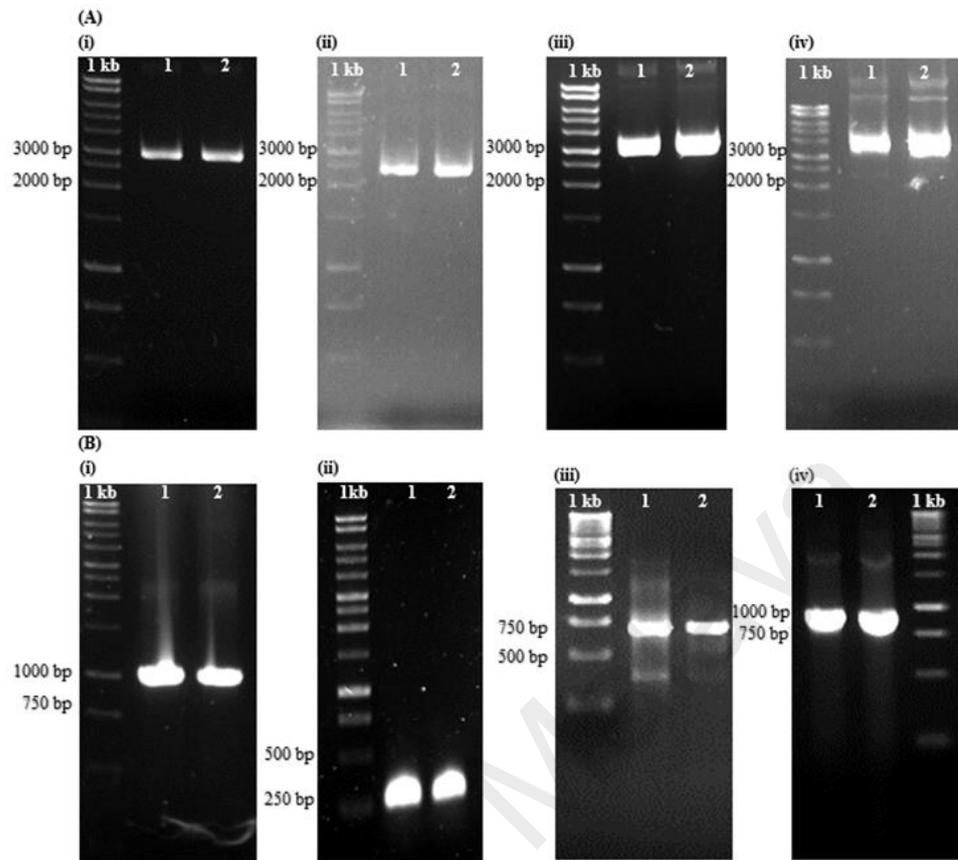


Figure 4.11: (A) Undigested plasmids extracted from *E. coli* TOP10 harbouring different recombinant constructs. (i) pENTR_yoeBSpn-GFP; (ii) pENTR_yefMSpn; (iii) pENTR_GFP and (iv) pENTR_CaMV 35S. (B) PCR confirmation from the extracted plasmids using gene-specific primers. (i) pENTR_yoeBSpn-GFP; (ii) pENTR_yefMSpn; (iii) pENTR_GFP and (iv) pENTR_CaMV 35S. 1 and 2 represent two different plasmids randomly extracted from each recombinant construct. 1 kb: 1 kb DNA ladder (Fermentas, Canada).

PCR performed using the extracted plasmids as template showed the expected sizes of 990 bp for pENTR_yoeBSpn-GFP, 255 bp for pENTR_yefMSpn, 732 bp for pENTR_GFP and 800 bp for pENTR_CaMV 35S (Figure 4.11B). The plasmids were then sequenced by conventional Sanger sequencing and sequence analysis showed all the extracted and PCR-amplified products had been successfully inserted into the Gateway® pENTR-D-TOPO vector in the desired orientation and without any mutations in the respective genes.

4.3 Development of plant expression constructs of pMDC221_*yoeB_{Spn}*-GFP, pMDC160_*yefM_{Spn}*, pMDC221_GFP and pMDC150_35S

The Gateway recombinant entry clones were used to transfer the CaMV 35S fragment into pMDC150, the *yoeB_{Spn}*-GFP and GFP fragments into pMDC221 and the *yefM_{Spn}* fragment into pMDC160 as detailed in section 3.12.

For recombinant construct pMDC221_*yoeB_{Spn}*-GFP, colony PCR resulted in bands at the expected size of 990 bp after agarose gel electrophoresis (Figure 4.12). However, out of the 23 colonies that were randomly selected, only two colonies (lane 4 and lane 21 of Figure 4.12) gave distinct bands and were subsequently chosen for plasmid extraction. Meanwhile, for recombinant construct pMDC160_*yefM_{Spn}*, only 6 colonies grew on the selective plates. The presence of an amplicon of the expected size (about 255 bp) was clearly visible from all 6 colonies after agarose gel electrophoresis (Figure 4.13) and colonies corresponding to samples shown in lanes 1 and 2 were chosen for plasmid extraction. The positive control vector pMDC221_GFP was produced using the same method; however, only two colonies (lane 2 and lane 8 of Figure 4.14) indicated that the GFP sequences had been successfully inserted into pMDC221 vector, producing a band of 732 bp after agarose gel electrophoresis (Figure 4.14). These two clones were chosen for subsequent plasmid extraction.

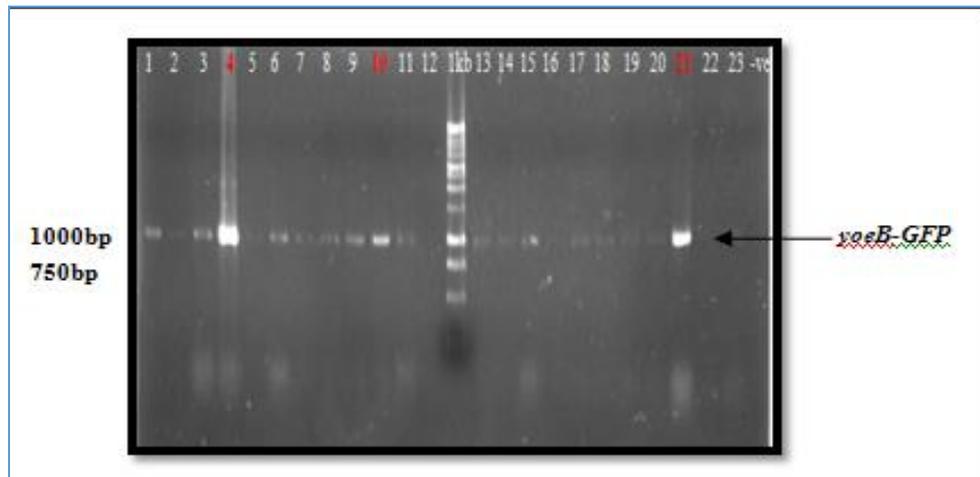


Figure 4.12: Colony PCR of pMDC221_ *yoeB_{Spn}*-GFP transformants. Lanes 1-23: PCR of bacterial colonies with *yoeB_{Spn}* Forward and GFP Reverse primers. Lane -ve: negative control of PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

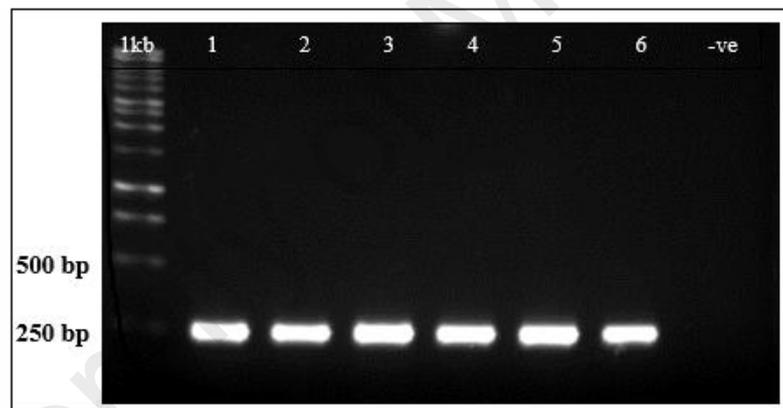


Figure 4.13: Colony PCR of pMDC160_ *yefM_{Spn}* transformants. Lanes 1-6: PCR of bacterial colonies with *yefM_{Spn}* Forward and *yefM_{Spn}* Reverse primers. Lane -ve: negative control of PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

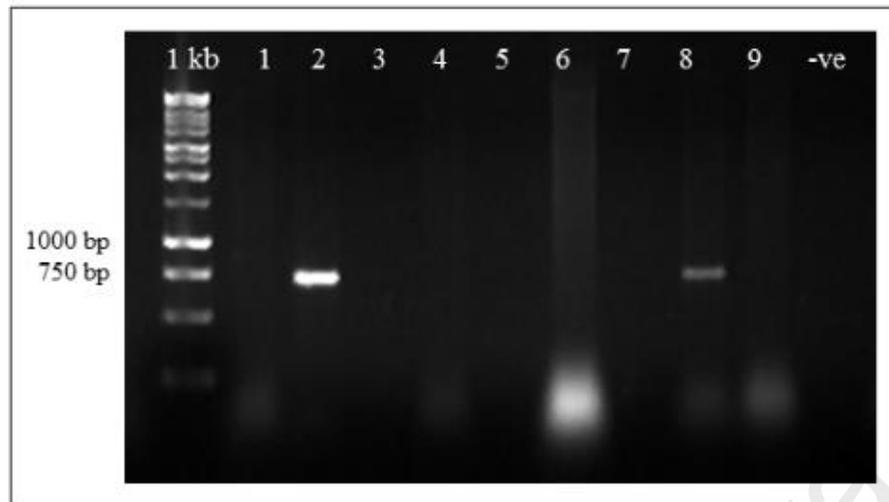


Figure 4.14: Colony PCR of pMDC221_GFP transformants. Lanes 1-9: PCR of bacterial colonies with GFP Forward and GFP Reverse primers. Lane -ve: negative control of PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

The CaMV 35S promoter was cloned upstream of the XVE element, producing the activator T-DNA fragment in pMDC150_35S. The amplified CaMV 35S promoter consists of two repeated regions (double enhancer), of about 800 bp. After the LR clonase reaction, the presence of an amplicon of the expected size (about 800 bp) was clearly visible after agarose gel electrophoresis (Figure 4.15). Since some colonies showed an additional band on agarose gel, only colonies 1 and 9 (that clearly showed single amplified bands) were chosen for plasmid extraction.

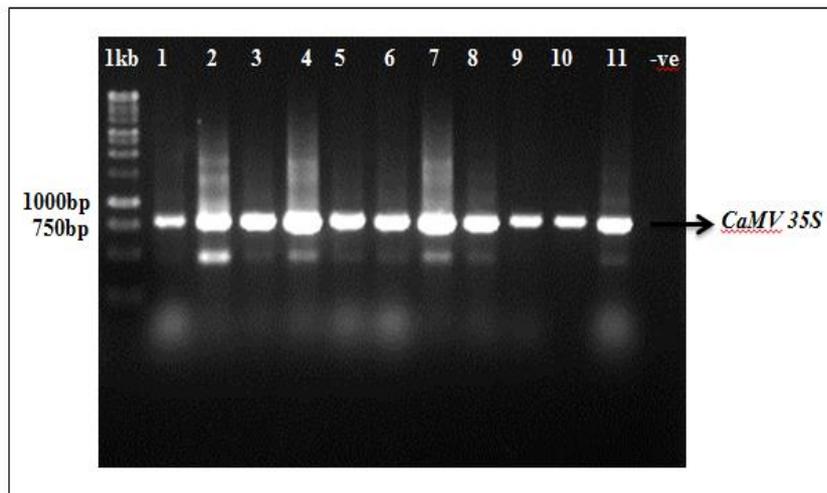


Figure 4.15: Colony PCR of pMDC150_ 35S transformants. Lanes 1-11: PCR of bacterial colonies with CaMV35S Forward and CaMV35S Reverse primers. Lane –ve: negative control of PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

4.3.1 Plasmid extraction and validation of pMDC recombinant constructs

Electrophoresis of plasmids extracted for each recombinant construct (from the two PCR-positive clones) showed the presence of bands, indicating that the plasmids had been successfully extracted (Figure 4.16A). Vector maps for each of the plasmids used in this study are shown in Appendix A.

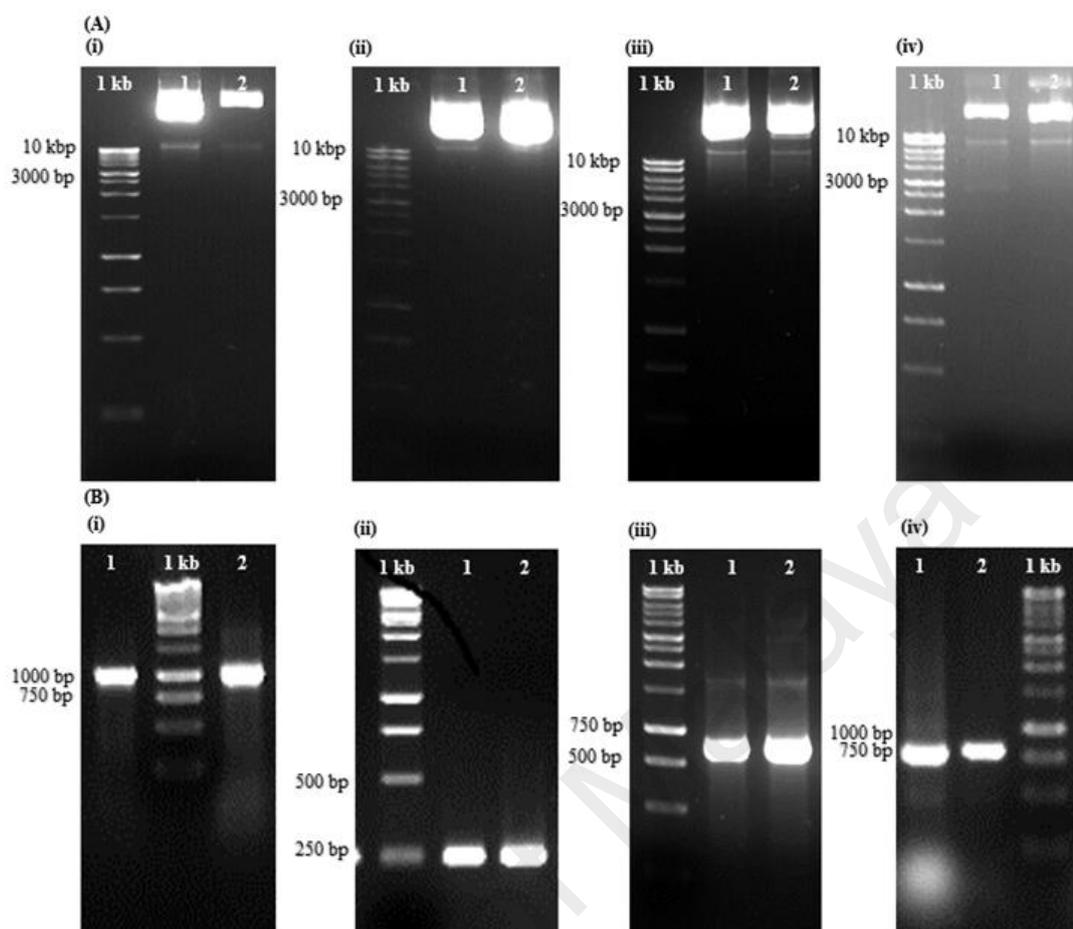


Figure 4.16: (A) Agarose gel electrophoresis of undigested plasmids extracted from *E. coli* TOP10 harbouring different recombinant pMDC vectors. (i) pMDC221_yoeBSpn-GFP; (ii) pMDC160_yefMSpn; (iii) pMDC221_GFP and (iv) pMDC150_35S. (B) PCR confirmation for each extracted recombinant pMDC plasmid using gene-specific primers. (i) pMDC221_yoeBSpn-GFP (990 bp); (ii) pMDC160_yefMSpn (255 bp); (iii) pMDC221_GFP (732 bp) and (iv) pMDC150_35S (800 bp). 1 and 2 represent two different plasmids from each recombinant construct. 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

PCR using the extracted plasmids as a template showed that each plasmid harboured the correct insert as indicated by amplicons of the expected sizes on agarose gels (Figure 4.16B). The recombinant plasmids obtained were also sequenced and analysis showed all the extracted and PCR-positive plasmids were correctly cloned in between the *attR* sites of each pMDC vector and had the desired DNA sequences.

4.4 Transformation of *Agrobacterium tumefaciens* with pMDC221_yoeB_{Spn}-GFP, pMDC160_yefM_{Spn}, pMDC221_GFP and pMDC150_35S

The recombinant pMDC constructs, namely pMDC221_yoeB_{Spn}-GFP, pMDC160_yefM_{Spn}, pMDC221_GFP and pMDC150_35s, were each transformed into *A. tumefaciens* LBA 4404 and colonies formed on selective LB agar plates were randomly picked for colony PCR. All picked colonies produced amplicons of the expected sizes i.e., 990 bp for pMDC221_yoeB_{Spn}-GFP (Figure 4.17), 255 bp for pMDC160_yefM_{Spn} (Figure 4.18), 732 bp for pMDC221_GFP (Figure 4.19) and 800 bp for pMDC150_35S (Figure 4.20). This indicated that the plasmids had been successfully transformed into *A. tumefaciens*.

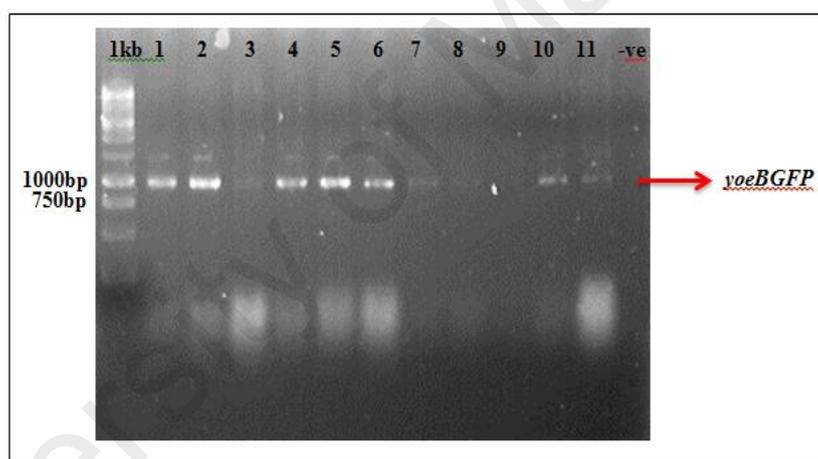


Figure 4.17: Colony PCR of *A. tumefaciens* transformed with pMDC221_yoeB_{Spn}-GFP. Lanes 1-11: PCR of bacterial colonies with yoeB_{Spn} Forward and GFP Reverse primers. Lane -ve: negative control, PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

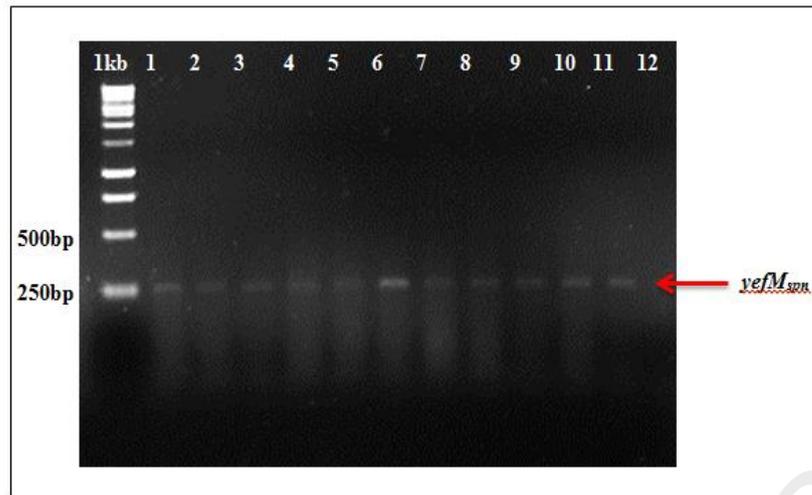


Figure 4.18: Colony PCR of *A. tumefaciens* transformed with pMDC160_*yefM_{Spn}*. Lanes 1-11: PCR of *A. tumefaciens* colonies with *yefM_{Spn}* Forward and *yefM_{Spn}* Reverse primers. Lane 12: negative control, PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

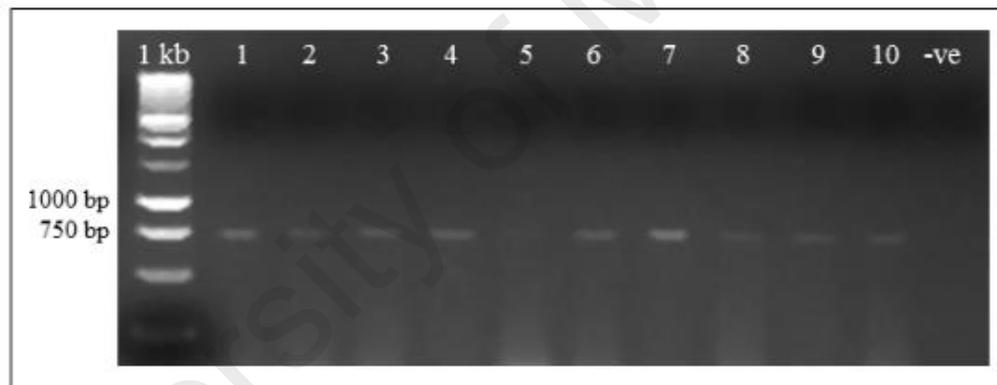


Figure 4.19: Colony PCR of *A. tumefaciens* transformed with pMDC221_*GFP*. Lanes 1-10: PCR of bacterial colonies with *GFP* Forward and *GFP* Reverse primers. Lane -ve: negative control, PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

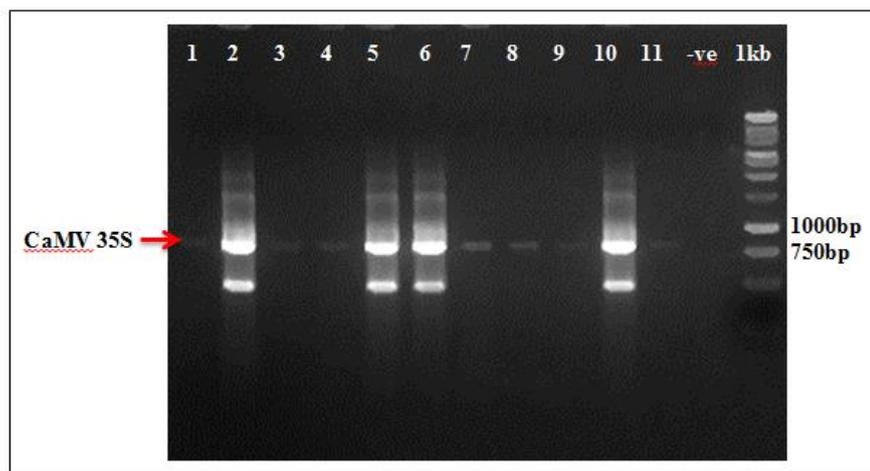


Figure 4.20: Colony PCR of *A. tumefaciens* transformed with pMDC150_35S. Lanes 1-11: PCR of bacterial colonies with CaMV 35S Forward and CaMV 35S Reverse primers. Lane -ve: negative control, PCR carried out without any bacterial colony; 1 kb: 1 kb DNA ladder marker (Fermentas, Canada).

4.5 Transformation of *A. thaliana* with the recombinant constructs

The transformed *A. tumefaciens* strains were used to transfer the relevant constructed transgenes into *A. thaliana*. In this study, *A. thaliana* plants were transformed with three pairs of recombinant constructs that consisted of the activator vector (pMDC150_35S) with each of the responder vectors (pMDC160_*yefM*_{Spn}, pMDC221_*yoeB*_{Spn}-*GFP* and pMDC221_*GFP*) as shown in Figure 4.21. *A. thaliana* transformed with each set of recombinant constructs were named as shown in Table 4.1. Plants surviving on selective media were self-pollinated and analysis was carried out at the T₂ generation.

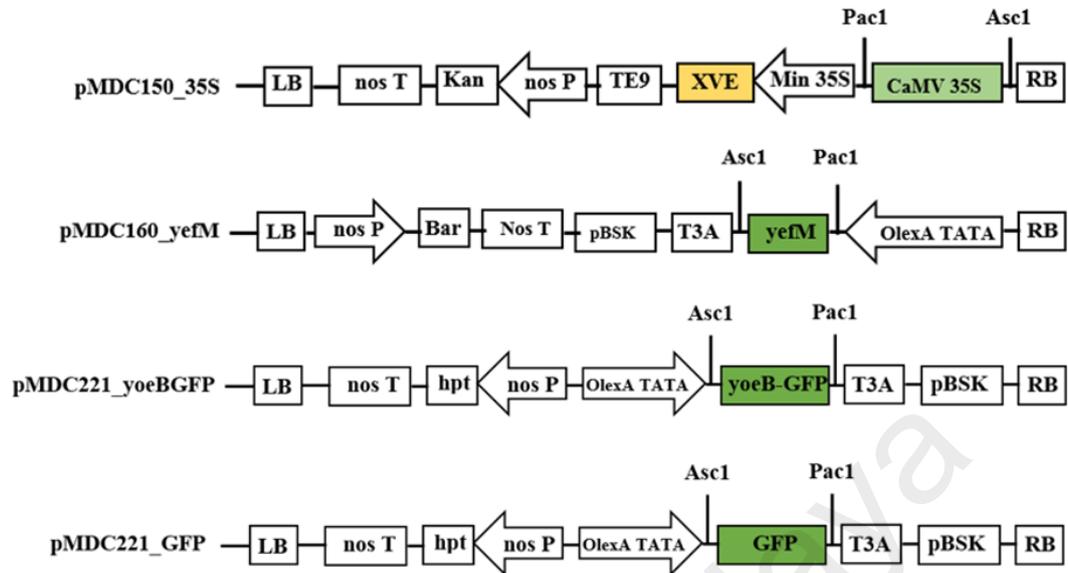


Figure 4.21: Map of the recombinant constructs used in this study. The T-DNA region of activator vector pMDC150_35S and responder vectors pMDC160_*yefM*_{Spn}, pMDC221_*yoeB*_{Spn}-GFP and pMDC221_GFP are shown. LB, left border; RB, right border; *nosT*, *nos* terminator; *Bar*, Basta resistance gene; *Kan*, kanamycin resistance gene; *nosP*, *nos* promoter that drives the expression of the marker gene for plant selection; *OlexA TATA*, XVE-responsive promoter; *AscI* and *PacI*, unique restriction sites flanking the gene of interest; TE9, transcription terminator; pBSK, pBlueScript backbone. Note that the diagram is not drawn to scale.

Table 4.1: Transgenic *A. thaliana* transformed with different recombinant constructs

Recombinant constructs used for transformation	Transgenic Arabidopsis	Total T₀ plants obtained	Total T₁ lines analyzed	Total T₂ lines produced	Total T₂ seeds analyzed
pMDC150_35S / pMDC221_yoeB _{Spn} -GFP	AtYoeB-GFP	5	5	4	100
pMDC150_35S / pMDC160_yefM _{Spn}	AtYefM	5	5	3	68
pMDC150_35S / pMDC221_GFP	AtGFP (control)	3	3	3	55

In transgenic AtYoeBGFP T₂ plants, the presence of a 732 bp-amplicon after PCR using *GFP*-specific primers of extracted plant DNA indicated the presence of the *yoeB_{Spn}-GFP* fusion transgene, and thus, the successful integration of the transgene into the *A. thaliana* plants (Figure 4.22A). An amplicon at the same size was also present in the positive control AtGFP plants (transformed with pMDC150_35S / pMDC221_GFP) and was absent in the negative control (wild-type) plant (Figure 4.22A, lanes 12 and lane -ve, respectively). Similarly, when using *yoeB_{Spn}*-specific primers, the presence of a *yoeB_{Spn}*-specific band was also observed in the transgenic AtYoeBGFP plants (Figure 4.22B).

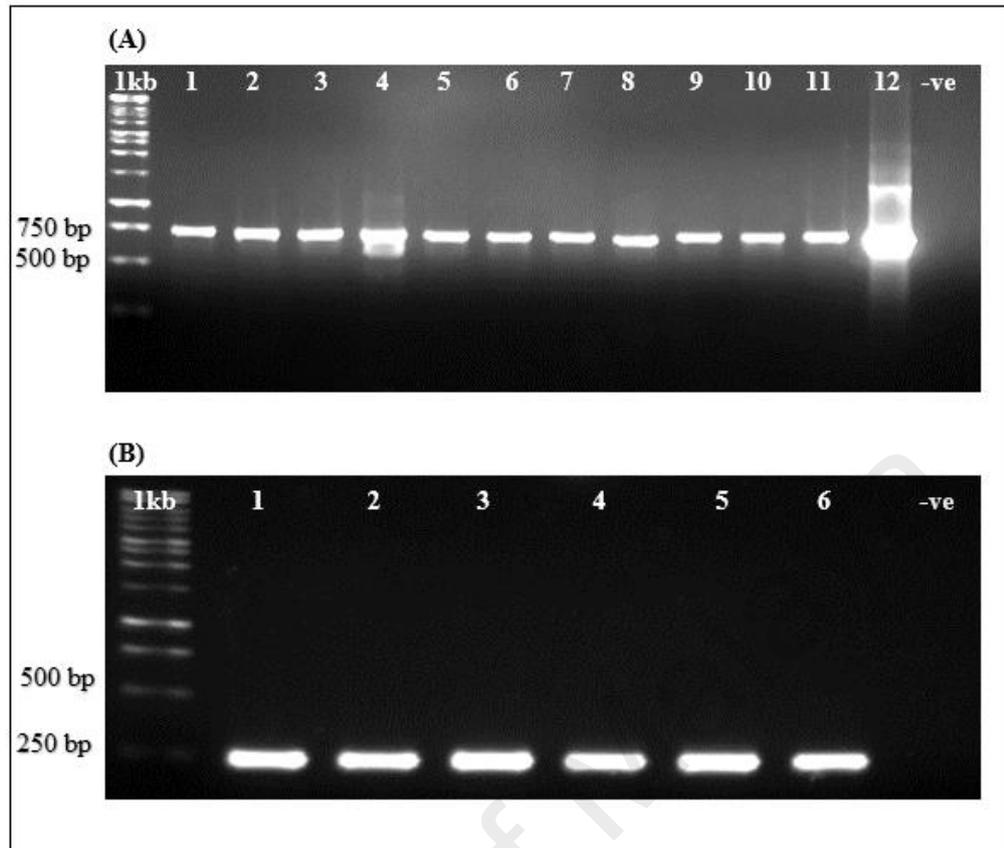


Figure 4.22 PCR confirmation from four-week-old T₂ transgenic AtYoeBGFP and AtGFP plants. (A) Templates were total DNA that were screened by PCR using *GFP*-specific primers (lanes 1–3: transgenic line 1; lanes 4–5: transgenic line 2; lanes 6–8: transgenic line 3; lanes 9–11: transgenic line 4) or with AtGFP as the template (lane 12) showing the expected amplified product of 732 bp. (B) Amplification of *yoeB_{Spn}* toxin using *yoeB_{Spn}*-specific primers showing the expected amplified product of 255 bp (Lanes 1–2: transgenic line 1; lanes 3–4: transgenic line 2; lanes 5–6: transgenic line 3). Lane -ve: DNA from a wild-type *A. thaliana* as negative control; 1 kb: 1 kb DNA ladder marker (Fermentas).

In plants transformed with vectors pMDC150_35S / pMDC160_yefM_{Spn}, a total of 68 Basta- and kanamycin-resistant transgenic T₂ lines were produced. All of the T₂ progeny tested contained the expected 255 bp amplified product corresponding to the *yefM_{Spn}* DNA fragment indicating that the transgene was successfully integrated into the transgenic *A. thaliana* genome.

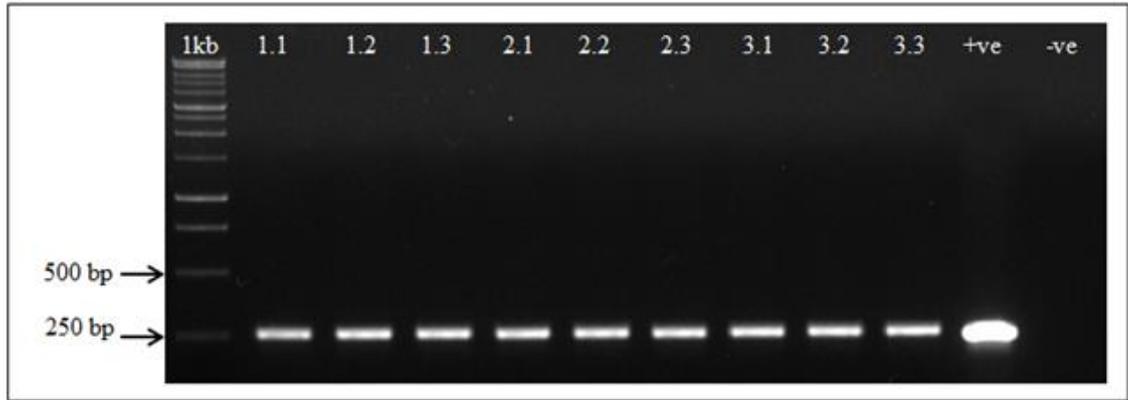


Figure 4.23: Detection of the *yefM_{Spn}* antitoxin in transformed *A. thaliana*. Extracted DNA from transformed plants of 3 different T₂ lines was each used as template for PCR (Lanes 1.1-1.3: transgenic line 1; lanes 2.1-2.3: transgenic line 2; lanes 3.1-3.3: transgenic line 3) using *yefM_{Spn}*-specific primers. Lane +ve: pMDC160_*yefM_{Spn}*. Lane -ve: wild-type *A. thaliana* Col 0. 1 kb: 1 kb DNA ladder marker (Fermentas). Molecular sizes are indicated.

4.6 Phenotypic observation of transgenic and control *A. thaliana*

Prior to 17- β -estradiol induction, all the transgenic AtYoeBGFP and AtYefM plants grown in selective media did not show any obvious phenotypic difference when compared with untransformed control *Arabidopsis* plants of the same age (n = 20 for each of 4 transgenic lines). In transgenic AtYoeBGFP plants, induction with 17- β -estradiol in four-week-old plants at the T₂ generation resulted in plant defects and tissue necrosis by the 3rd day after induction, followed by plant death by the 9th day after induction (Figure 4.24A). Leaves of the transgenic plants showed severe discoloration and distortion over time and were completely discoloured on the 9th day after induction (Figure 4.24A, 9 dpin), whereas no such abnormalities were observed in the mock-induced transgenic AtYoeBGFP *A. thaliana* that were sprayed with ethanol instead of 17- β -estradiol. Similar results were observed for all four transformed plant lines. The mock-induced plants grew normally and were able to produce flowers (Figure 4.24B, 9 dpin). Likewise, the AtGFP transgenic plants (transformed with the construct lacking

YoeB) did not show abnormalities following 17- β -estradiol induction (Figure 4.24C), indicating that the plant death was likely due to the expression of the *yoeB_{Spn}* toxin.

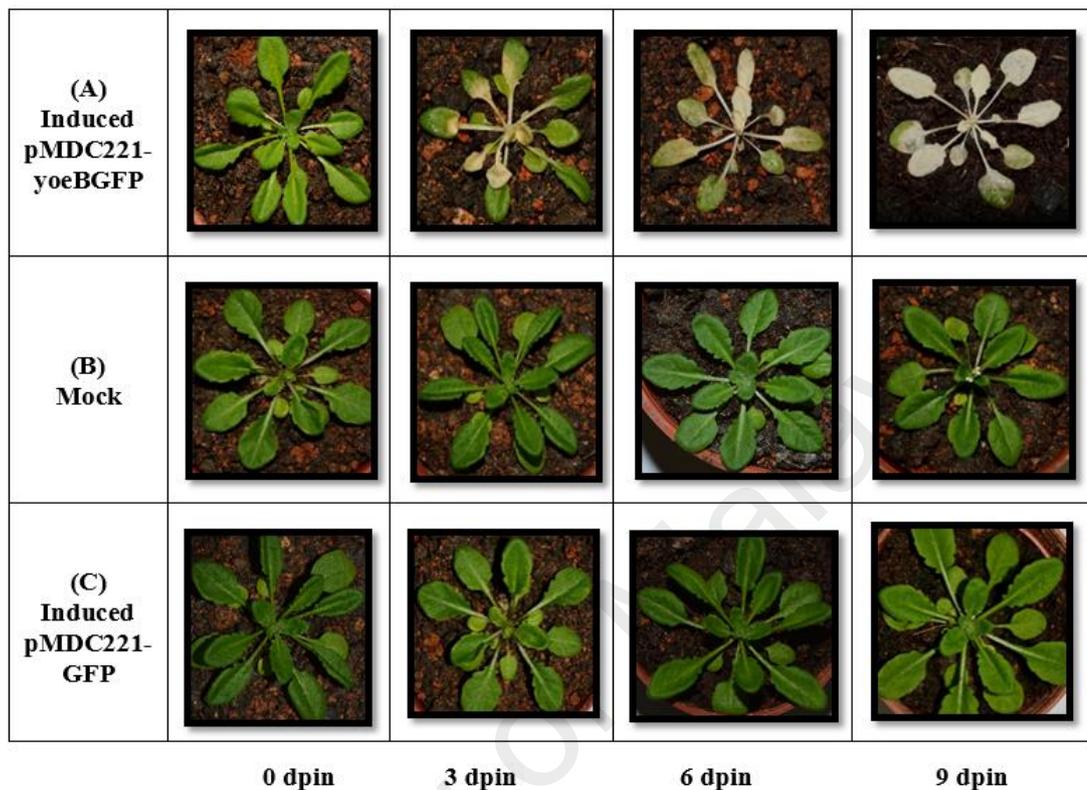


Figure 4.24: Effects of *yoeB_{Spn}*-GFP expression in four-week-old *A. thaliana* over a time course of 9 days. (A) 17- β -estradiol induced AtYoeBGFP plants, (B) AtYoeBGFP plants mock induced (with ethanol), (C) 17- β -estradiol induced AtGFP plants. dpin, days post-induction.

When the leaves of the AtYoeBGFP plants were examined under fluorescence confocal microscope, GFP fluorescence was detectable in all parts of the leaves 24 hours after 17- β -estradiol induction (Figure 4.25). Leaves showed GFP fluorescence at days 3 and 6 after induction, and even at days 8 and 9, after plant death (Figure 4.25E-F). Distortion of the newly emerging rosette leaves and necrotic symptoms in all leaves were observed from day 3 onwards, as shown in the same figure.

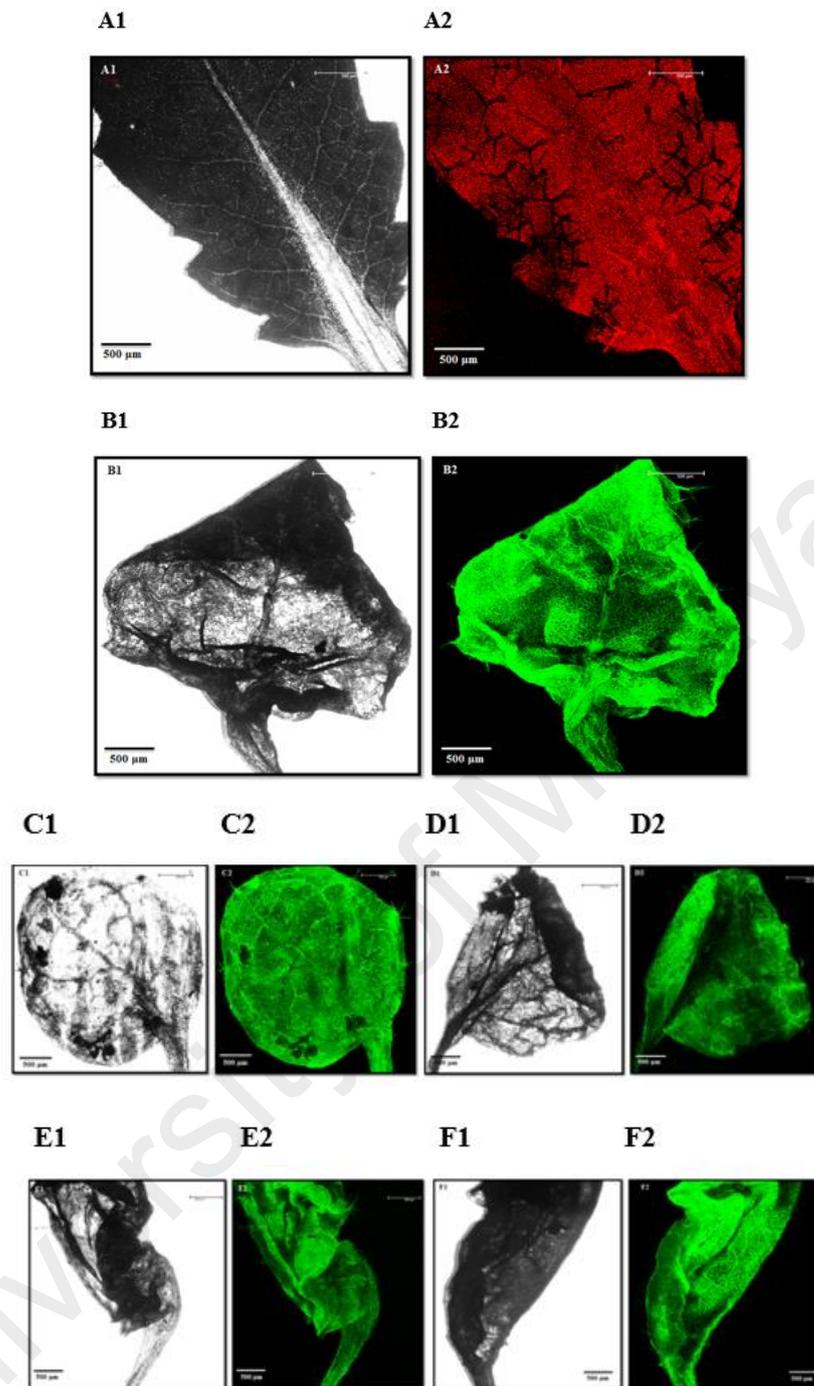


Figure 4.25: GFP fluorescence image of rosette leaves of T₂ *A. thaliana*. AtYoeBGFP plants were induced with 100 μM 17-β-estradiol at 4 weeks after selection. (A) no induction; (B) 1 day after induction; (C) 3 days after induction; (D) 6 days after induction; (E) 8 days after induction; and (F) 9 days after induction. 1 and 2 represent bright field and fluorescent images, respectively. Scale bars = 500 μm.

In AtYefM plants, a similar experiment was carried out to study if there were any phenotypic changes caused by *yefM_{Spn}* expression. After induction, the growth of the T₁ and T₂ transgenic plants was not distinguishable from that of the wild-type and non-induced transgenic AtYefM *A. thaliana* (Figure 4.26). There were no morphological differences in the shape and appearance of the rosette leaves even after 9 days of induction for all plant groups, indicating that *yefM_{Spn}* expression did not cause any observed detrimental effect on the plants. Similar results were observed for all 3 independent transgenic lines (n = 20).

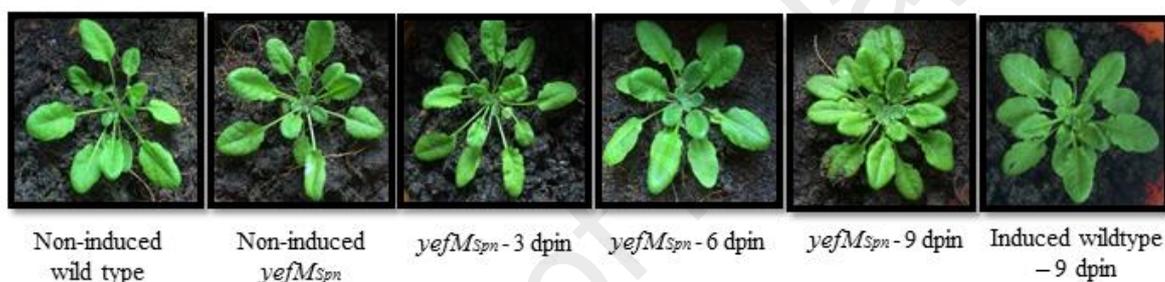


Figure 4.26: Four-week-old T₂ transgenic *A. thaliana* plants harbouring the *yefM_{Spn}* transgene 3 days, 6 days and 9 days post-induction (dpi) with 17- β -estradiol. Also depicted are non-induced wild-type *A. thaliana*, non-induced transgenic *A. thaliana* (*yefM_{Spn}*) and induced wild-type *A. thaliana* applied with 17- β -estradiol at 9 days post-induction.

4.7 Expression analysis of *yoeB_{Spn}-GFP* and *yefM_{Spn}* in transgenic *A thaliana*

To monitor the expression levels of the *yoeB_{Spn}-GFP* and *yefM_{Spn}* genes following their induction in the transgenic *A. thaliana*, total RNA was extracted and quantitative real-time RT-PCR was performed. Intact total RNA extracted from *A. thaliana* rosette leaves showed 28S rRNA band intensity about twice that of the 18S rRNA (Appendices B and C). The concentration of each extracted RNA sample is listed in Appendices D and E.

The PCR products amplified from *yoeB_{Spn}-GFP* cDNA using the *q-yoeB₁* primers showed positive results after agarose gel electrophoresis, as indicated by the presence of a DNA band of the expected size of 168 bp (Figure 4.27A). Meanwhile, the PCR product amplified from *yefM_{Spn}* cDNA using *q-yefM₁* primers also showed the desired result, i.e. the presence of a band of the expected size of 149 bp after agarose gel electrophoresis (Figure 4.27B).

In this study, the expression of the *Actin* housekeeping gene was used as the reference to normalize gene expression levels across the samples (Vandesompele et al., 2002). Figure 4.28 shows the *Actin* RT-PCR products from both transgenic AtYoeBGFP and AtYefM plants.

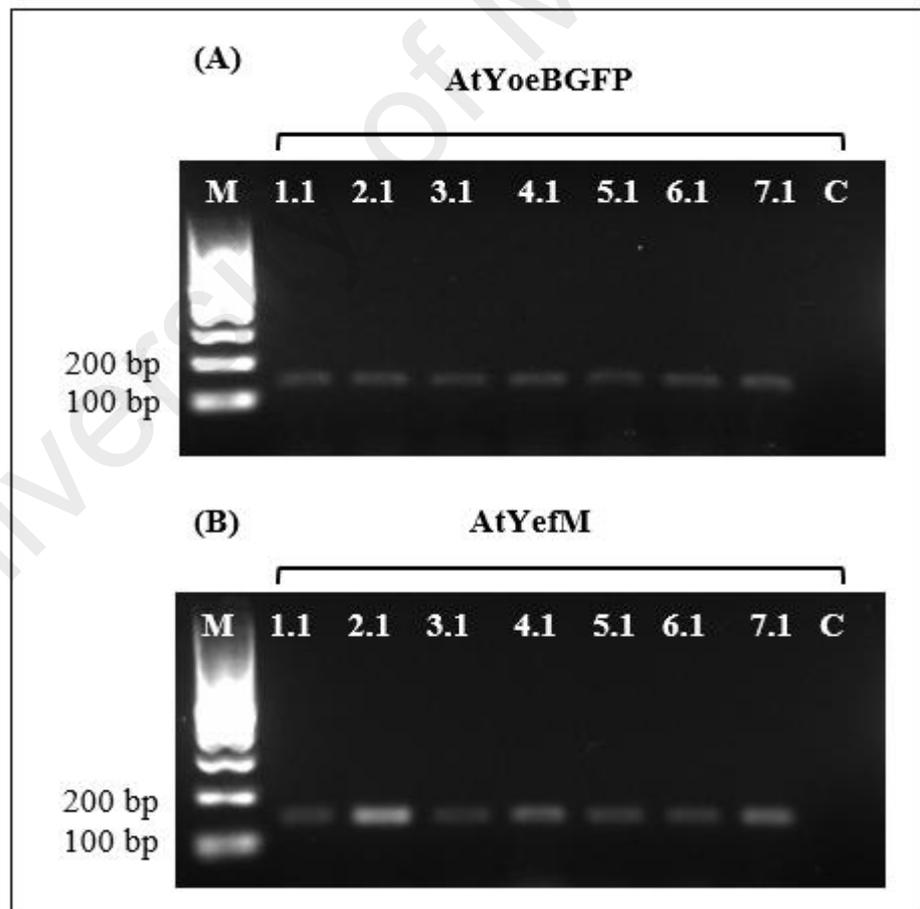


Figure 4.27: Transcript analysis of T₂ transgenic AtYoeBGFP and AtYefM from line 1 after induction with 17- β -estradiol. (a) Agarose gel of amplified products from RT-PCR of AtYoeBGFP with the *q-yoeB₁* primer set showing the expected 168 bp

band; (b) RT-PCR of AtYefM with the *q-yefM_I* primer set showing the expected 149 bp band. Lanes 1.1-sample from day 1 after 17- β -estradiol induction; lanes 2.1: sample from day 2; lanes 3.1: sample from day 3; lanes 4.1: sample from day 4; lanes 5.1: sample from day 5; lanes 6.1: sample from day 6; lanes 7.1: samples from day 7; lane C: wild-type. M: 100 bp DNA ladder marker (Fermentas).

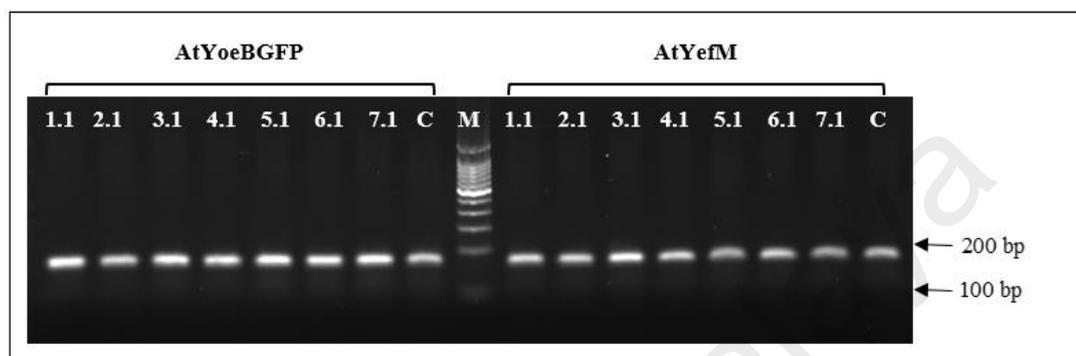


Figure 4.28: Agarose gel electrophoresis of RT-PCR products with *Actin*-specific primer of T₂ transgenic AtYoeBGFP and AtYefM from line 1 after induction with 17- β -estradiol. Lanes 1.1-sample from day 1 after 17- β -estradiol induction; lanes 2.1: sample from day 2; lanes 3.1: sample from day 3; lanes 4.1: sample from day 4; lanes 5.1: sample from day 5; lanes 6.1: sample from day 6; lanes 7.1: samples from day 7; lane C: wild-type. M: 100 bp DNA ladder marker (Fermentas). All samples showed the expected 180 bp *Actin* amplified product.

The same cDNA templates and primers used in RT-PCR were used to quantify the expression levels of the specific genes from day 1 until day 7 after 17- β -estradiol induction using qRT-PCR. Melt curve analysis showed the absence of primer dimers for each primer set used (Appendix F). qRT-PCR analysis revealed that the expression levels of the *yoeB_{Spn}* toxin in AtYoeBGFP plants as well as the *yefM_{Spn}* antitoxin in AtYefM plants peaked at 3 days after 17- β -estradiol induction after which the levels decreased to day 1 levels by the 6th day after induction (Figure 4.29).

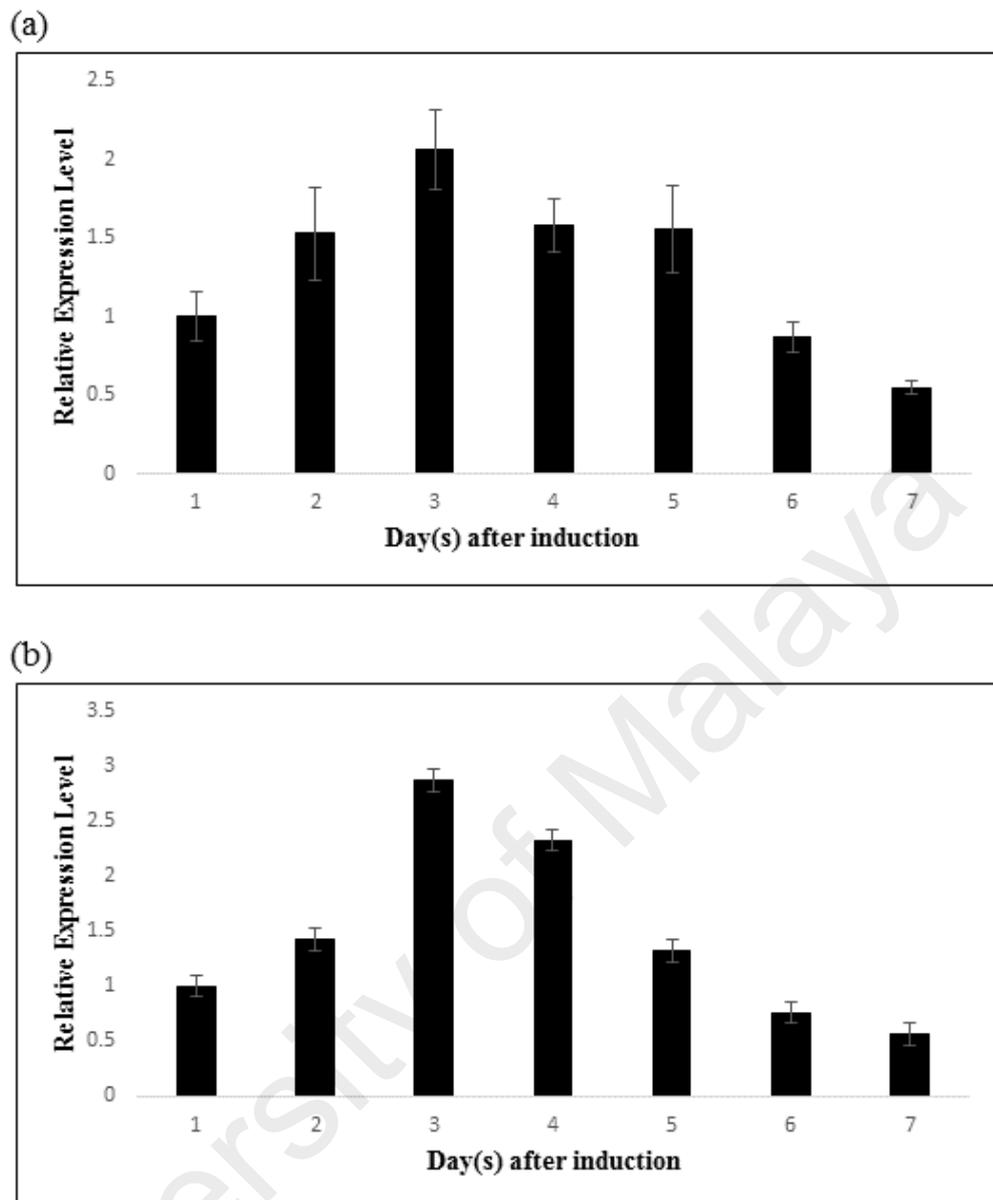


Figure 4.29: The levels of *yoeB_{Spn}* toxin and *yefM_{Spn}* antitoxin transcripts in transgenic plants from day 1 – day 7 after 17- β -estradiol induction as determined by qRT-PCR. (a) *yoeB_{Spn}-GFP* expression levels in AtYoeBGFP plants; (b) *yefM_{Spn}* expression levels in AtYefM plants. Each bar represents the mean and standard deviation for 3 biological replicates which were chosen randomly from each T₂ transgenic plants line 1, line 2 and line 3. Data were normalized to the endogenous *A. thaliana* actin gene and the sample taken at day 1 was set to 1.0. Relative expression of each *yoeB_{Spn}-GFP* and *yefM_{Spn}* cDNA was obtained by dividing the average number of each transcript copies by the copy number of *A. thaliana* Actin for the same tissue

(Vandesompele et al., 2002). Error bars represent the standard deviation from the RT-PCR amplification of 3 biological replicates of *A. thaliana* rosette leaves total RNA samples.

4.8 DNA fragmentation assay

Phenotypic observation had indicated that expression of the YoeB_{Spn} toxin is lethal in *A. thaliana* AtYoeBGFP (Figure 4.24). To investigate the possibility that the lethality of YoeB_{Spn} is due to activation of apoptosis, a DNA fragmentation assay was carried out on 17- β -estradiol-induced *A. thaliana* samples. Agarose gel electrophoresis of the extracted genomic DNA indicated that DNA fragmentation was evident 24 h after induction where distinct oligonucleosomal DNA fragments ranging from 180 – 200 bp were observed in all the sampled plants (Figure 4.30c), suggestive of apoptosis. These oligonucleosomal fragments were not evident in extracted genomic DNA 6 h and 12 h after induction but smearing of DNA was observed (Figure 4.30b). Such fragmentation was also not observed in the non-induced transgenic AtYoeBGFP plant where the genomic DNA isolated formed a single high molecular weight band following gel electrophoresis (Figure 4.30a).

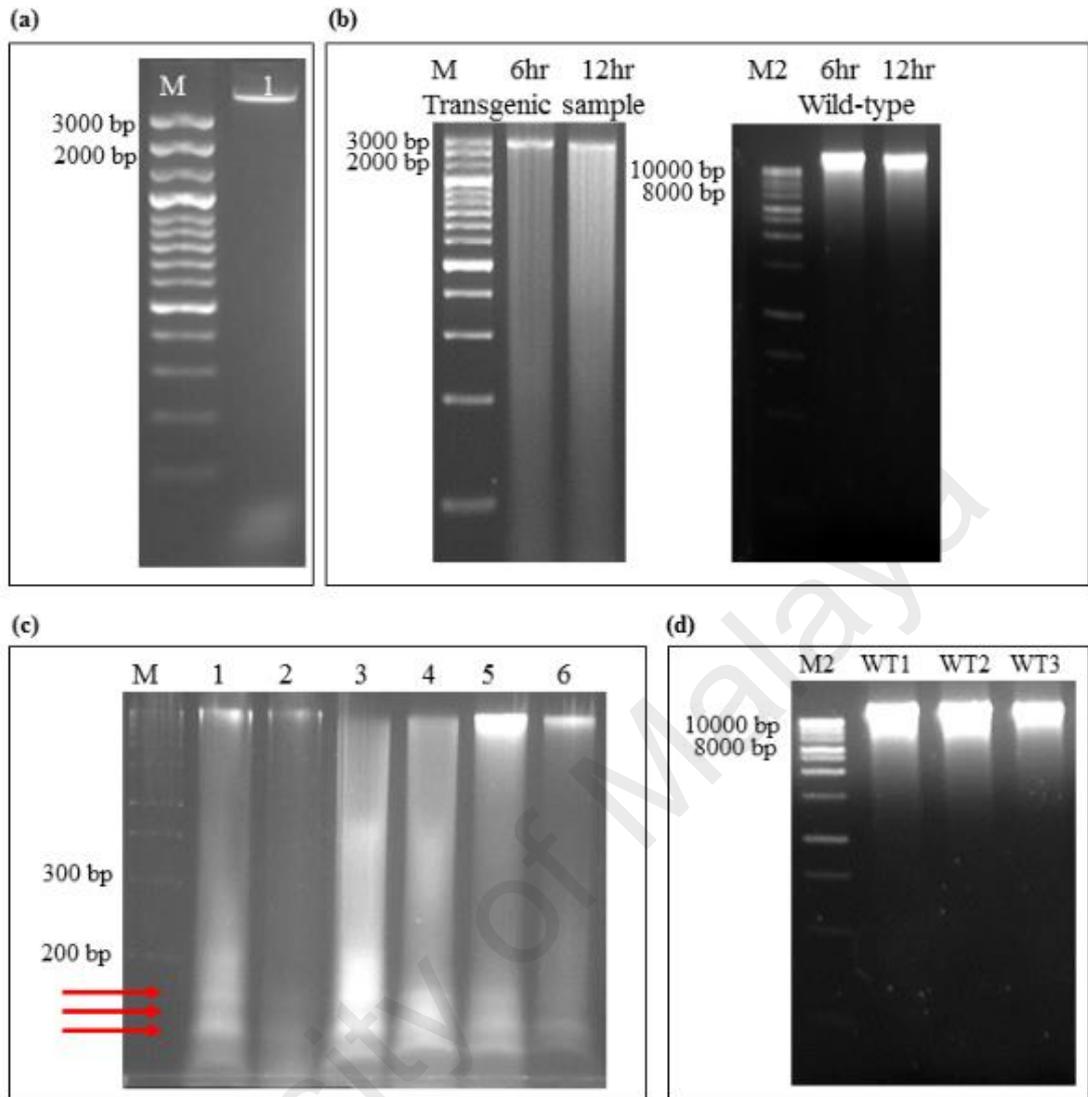


Figure 4.30: DNA extracted from *A. thaliana* and electrophoresed for 3 h on 1.8% agarose. (a) Lane 1: DNA from non-induced AtYoeBGFP plant. (b) DNA isolated from AtYoeBGFP plants (left) and wild-type plants (right) at 6 and 12 hours after 17- β -estradiol induction. (c) DNA isolated from transgenic plants 24 hours after induction with 17- β -estradiol; lanes 1-6: AtYoeBGFP plants. (d) DNA isolated from wild-type plants 24 hours after induction with 17- β -estradiol; lanes WT1-WT3: wild-type. M: 100 bp DNA ladder marker (Fermentas, USA); M2: 1 kb DNA ladder marker (Fermentas, USA). Arrows indicate DNA laddering or fragmentation suggestive of apoptosis.

4.9 Crosses of T₁ transgenic AtYoeBGFP plants with T₁ transgenic AtYefM plants to produce *yefM_{Spn}* × *yoeB_{Spn}-GFP* hybrid lines

All T₀ transgenic AtYoeBGFP and AtYefM plants were capable of self-pollination and produced normal seeds. To investigate if the YefM_{Spn} antitoxin is able to counteract the lethal effects of the YoeB_{Spn} toxin in *A. thaliana*, transgenic hybrids capable of co-expression of both *yefM_{Spn}* and *yoeB_{Spn}-GFP* were created by cross-pollination (section 3.24). From the crosses between the T₁ AtYoeBGFP plants with T₁ AtYefM plants, a total of 237 hybrid plants from 6 different lines survived under dual antibiotic-herbicide selection. These hybrids were named *yefM_{Spn}* × *yoeB_{Spn}-GFP* hybrids and three different plants were chosen from each line for PCR to confirm the integration of *yefM_{Spn}* (Figure 4.31a) and *yoeB_{Spn}-GFP* (Figure 4.31b), sequences. Genomic DNA from these PCR-positive plants was also examined by Southern hybridization and all tested hybrid transgenic plants showed DNA hybridisation signals, indicating the likely integration of the *yefM_{Spn}* (Figure 4.31c) and *yoeB_{Spn}-GFP* (Figure 4.31d) transgenes in the genome of the transgenic hybrid lines. No hybridization band was observed in the genomic DNA sample from wild-type plants indicating no non-specific binding of the *yoeB_{Spn}-GFP* probe to the wild-type *A. thaliana* genomic DNA (Figure 4.31d).

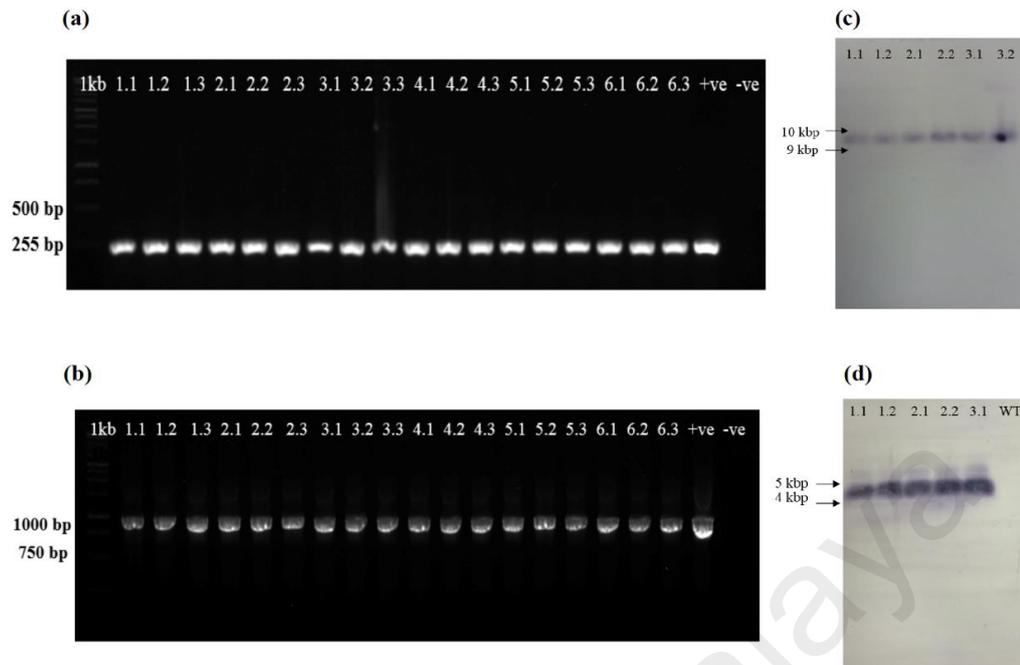


Figure 4.31: Detection of *yefM_{Spn}* and *yoeB_{Spn}-GFP* transgenes in *yefM_{Spn} × yoeB_{Spn}-GFP* hybrids of transgenic *A. thaliana*. (a) PCR detection using *yefM_{Spn}*-specific primers (lanes 1.1-1.3: hybrid line 1; lanes 2.1-2.3: hybrid line 2; lanes 3.1-3.3: hybrid line 3; lanes 4.1-4.3: hybrid line 4; lanes 5.1-5.3: hybrid line 5, lanes 6.1-6.3: hybrid line 6) showing the expected amplified product of 255 bp; lane +ve: pMDC160_*yefM_{Spn}*; lane -ve: wild-type plants; 1 kb: 1 kb DNA ladder marker (Fermentas). (b) PCR detection of the hybrid *Arabidopsis* plants using *yoeB_{Spn}GFP*-specific primers (lanes 1.1-1.3: hybrid line 1; lanes 2.1-2.3: hybrid line 2; lanes 3.1-3.3: hybrid line 3; lanes 4.1-4.3: hybrid line 4; lanes 5.1-5.3: hybrid line 5, lanes 6.1-6.3: hybrid line 6) and showing the expected amplified product of 990 bp; lane +ve: pMDC221_*yoeB_{Spn}-GFP*; lane -ve: wild-type plants; 1 kb: 1 kb DNA ladder marker (Fermentas). (c) Southern hybridization using *yefM_{Spn}* as a probe on *EcoRI* digested genomic DNA (1.1, 1.2: hybrid line 1; 2.1, 2.2: hybrid line 2; 3.1, 3.2: hybrid line 3); (d) Southern hybridization using *GFP* as a probe (1.1, 1.2: hybrid line 1; 2.1, 2.2: hybrid line 2; 3.1: hybrid line 3, WT: non-transgenic plant).

4.10 Expression of *yefM_{Spn}* and *yoeB_{Spn}-GFP* in the hybrid plants after induction with 17- β -estradiol

Before induction, the *yefM_{Spn}* \times *yoeB_{Spn}-GFP* hybrid plants grown in selective media did not show any signs of abnormality and no expression of either transgene was detected by RT-PCR (Figure 4.32a). The RT-PCR analysis with total RNAs extracted from rosette leaf tissues after induction (Figure 4.32, Appendices G and H) using q-*yefM1* and q-*yoeB1* primers confirmed the transcription of both genes from day 1 until day 7 after germination (Figure 4.32a). Using the same total RNA extracted from the rosette leaves from the same plants, the transcript levels of *yefM_{Spn}* and *yoeB_{Spn}-GFP* were determined by qRT-PCR. The transcript levels of *yefM_{Spn}* and *yoeB_{Spn}-GFP* each increased over the first 3 days, after which they decreased with *yefM_{Spn}* showing higher relative expression levels than *yoeB_{Spn}-GFP* from day 2 post-induction (Figure 4.32b).

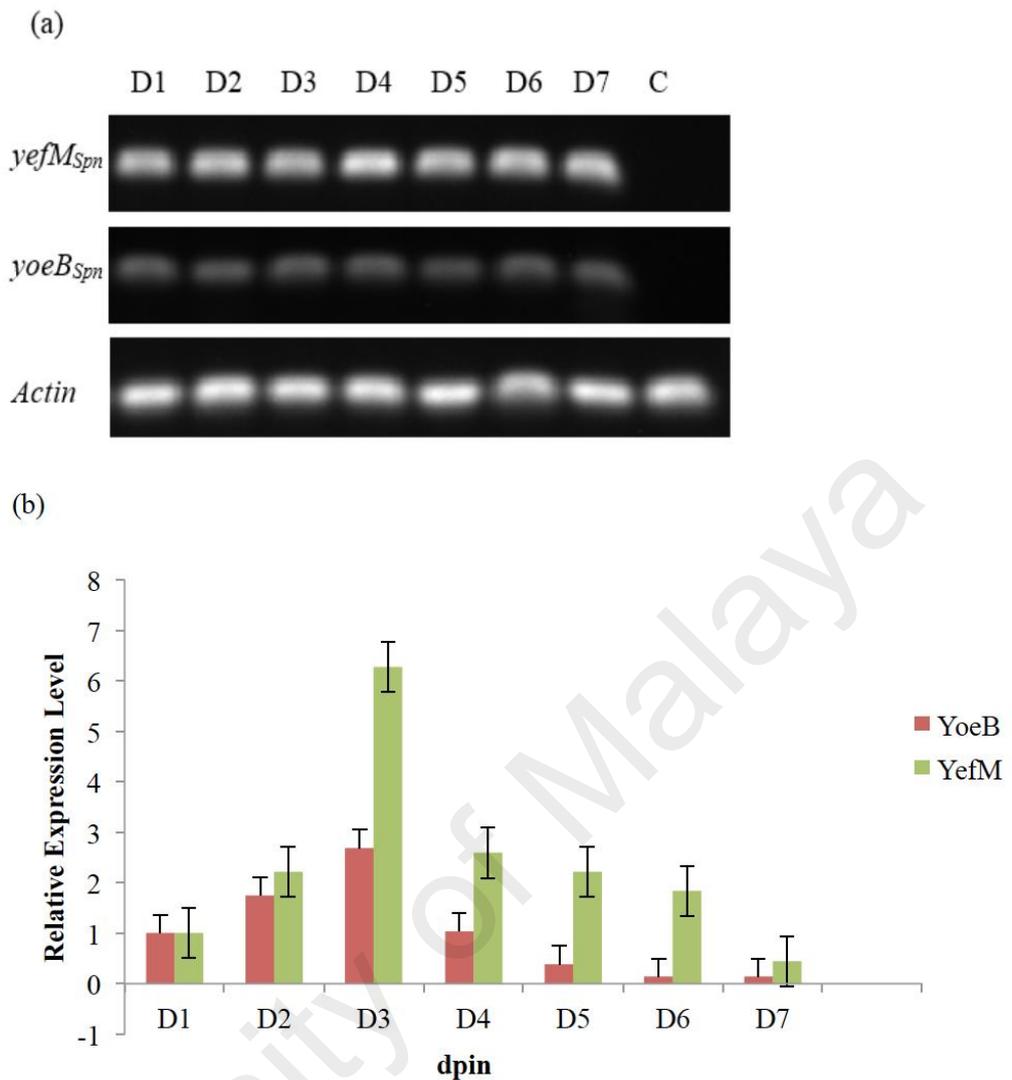


Figure 4.32: The relative expression levels of *yefM_{Spn}* and *yoeB_{Spn}-GFP* transcripts in transgenic hybrid *A. thaliana*. (a) Agarose gel following RT-PCR to detect the transgene transcript in the hybrid plants harbouring *yefM_{Spn}* and *yoeB_{Spn}-GFP* transgenes. (b) qRT-PCR analysis of *yefM_{Spn}* with q-yefM₁ primers and *yoeB_{Spn}-GFP* with q-yoeB₁ primers in hybrid plants. Data were normalized to the endogenous *A. thaliana* *Actin* gene and the sample at day 1 after induction was set to 1.0. Error bars represent the standard deviation from the RT-PCR amplification of 3 biological replicates of *A. thaliana* rosette leaves total RNA samples.

4.11 Induced expression of *yefM_{Spn}* and *yoeB_{Spn}-GFP* in hybrid *A. thaliana*

Before induction, the growth of transgenic AtYefM plants, transgenic AtYoeBGFP plants and *yefM_{Spn} × yoeB_{Spn}-GFP* hybrid plants was similar to that of the untransformed control plants (Figure 4.33). To observe the growth pattern and possible

differences in morphology among the induced hybrid plants and control plants (induced and non-induced *yefM_{Spn}* transgenic plants, induced and non-induced *yoeB_{Spn}-GFP* transgenic plants, non-induced *yefM_{Spn} × yoeB_{Spn}-GFP* hybrid plants and wild-type plants), 100 μM 17-β-estradiol was applied on the 4-week-old hybrid plants and were allowed to grow until the full stage of maturity. By the 7th day post-induction, transgenic AtYoeBGFP had died (Figures 4.24, 4.33 and 4.34). However, hybrid transgenic plants co-expressing both the *yoeB_{Spn}-GFP* fusion and the *yefM_{Spn}* antitoxin gene remained healthy, indicating that co-expression of the *yefM_{Spn}* antitoxin was able to neutralize the lethality of the *yoeB_{Spn}* toxin (Figure 4.33a). Transgenic plants expressing *yoeB_{Spn}* showed characteristic DNA fragmentation patterns indicative of apoptosis (Figure 4.34C and Appendix I). No such fragmentation was observed in the 17-β-estradiol-induced transgenic hybrid plants (Appendix I).

Interestingly, in all 3 independent hybrid lines, the plants induced to express both *yefM_{Spn}* and *yoeB_{Spn}-GFP* displayed increased growth in terms of both height and rosette leaf size. At the full stage of maturity (i.e., 9 weeks post planting), the growth of each rosette leaf in the hybrid plants exceeded that of the leaves from the non-induced and induced control plants (i.e., *yefM_{Spn}*, *yoeB_{Spn}-GFP* and wild-type plants); both the petiole length and width of the rosette leaves were greater and significantly increased in the induced hybrid plants (Figure 4.33b and Appendix J).

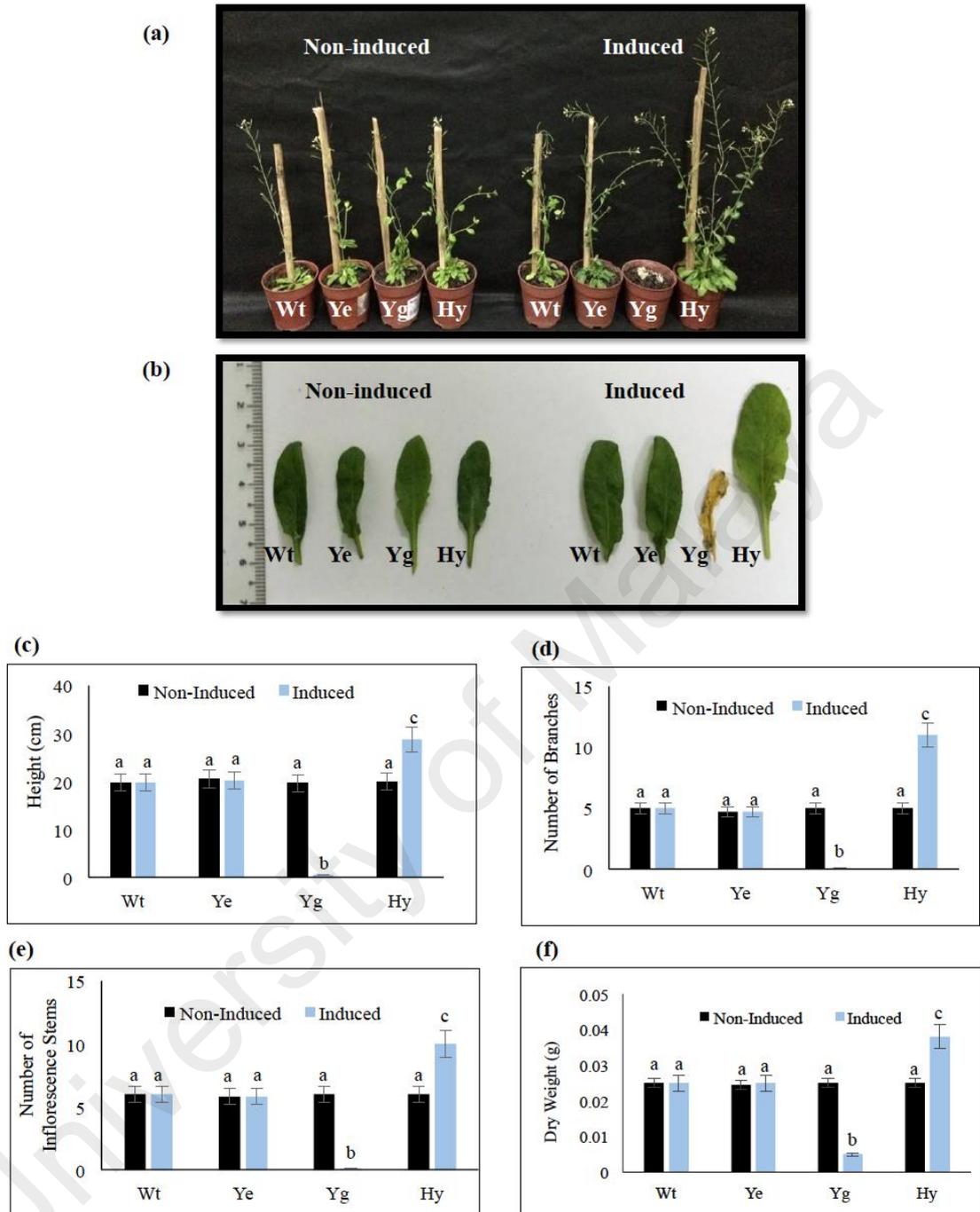


Figure 4.33: Morphology of the *yefM_{Spn}* × *yoeB_{Spn}-GFP* transgenic hybrid and control plants at 9 weeks. (a) Non-induced and induced plants at 9 weeks after planting. Plants were induced with 17- β -estradiol 4 weeks after planting and allowed to grow until full stage of maturity. (b) The 7th leaf of the rosette of the hybrid plants. (c) Total height for each plant measured from the soil to the top of the plant. (d) Number of branches bearing siliques in each plant. (e) Number of inflorescence stems formed in each plant. (f) Dry weight of each group of plants. Wt: wild-type *A. thaliana*; Ye: transgenic *A. thaliana* (*yefM_{Spn}*); Yg: *A. thaliana* (*yoeB_{Spn}-GFP*); Hy: transgenic *A.*

thaliana (*yefM_{Spn}* × *yoeB_{Spn}-GFP*) hybrid. The data in (c) to (f) are shown as the mean ± standard deviation (*n*=20) for 3 biological replicates which were chosen randomly from each transgenic hybrid line 1, line 2 and line 3. Different letters above the bars indicate significantly different means [*P* < 0.05 as analysed by one-way ANOVA (Tukey used as post hoc test)].

At 9 weeks post-planting (which corresponds to 5 weeks after induction), hybrid plants were taller with a significant increase in the numbers of branches, inflorescence stems and notable difference in their dry weight over those of all the control plants (Figure 4.33 and Appendix J). The differences in the length of siliques were however, not significant (Figures 4.34a and 4.34b). Nevertheless, the number of siliques per induced hybrid plants was significantly higher (up to 50%) than that of all control plants (Figure 4.34c), except for the *yoeB_{Spn}-GFP* transgenic plants that had died after the first week of induction and therefore, no measurement could be recorded.

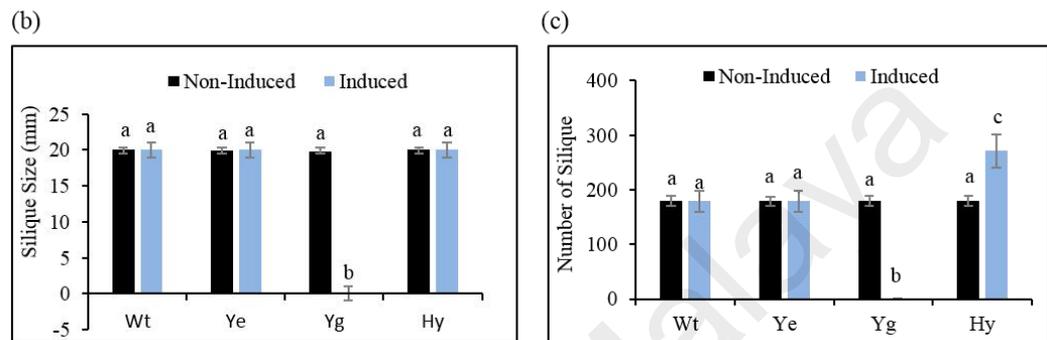
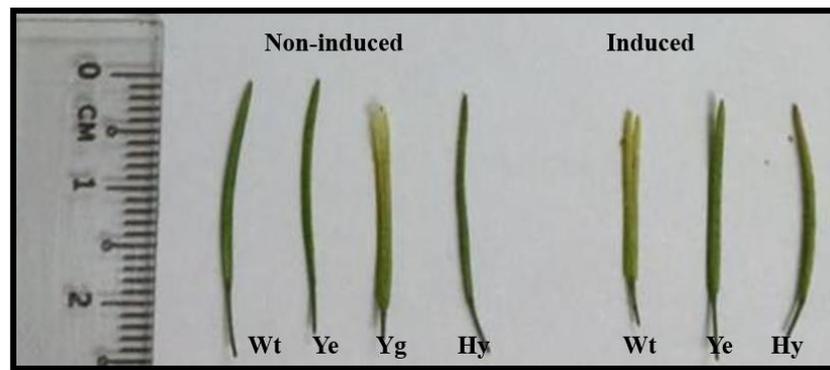


Figure 4.34: Silique phenotypes in induced and non-induced plants at 9 weeks after germination. (a) Silique size of non-induced and induced plants. **(b)** Comparison of mean silique size harvested from each non-induced and induced plant. **(c)** Mean number of siliques harvested from non-induced and induced plants. Wt: wild-type *A. thaliana*; Ye: *A. thaliana* (*yefM_{Spn}*); Yg: *A. thaliana* (*yoeB_{Spn}-GFP*); Hy: *A. thaliana* (*yefM_{Spn} × yoeB_{Spn}-GFP*) hybrid. Data in (b) and (c) are presented as the mean \pm standard deviation ($n=20$) for 3 biological replicates which were chosen randomly from each transgenic hybrid line 1, line 2 and line 3. Significance values were determined by one-way ANOVA (Tukey used as post hoc test with $P < 0.05$).

CHAPTER 5: DISCUSSION

In recent years, TA systems have captivated a rising interest because of their abundance in the genomes of bacteria and archaea despite the limited knowledge of their physiological functions. For instance, at least 33 TA systems have been identified in *E. coli* K12, with several being well characterized (Yamaguchi et al., 2011) whereas in *Streptococcus pneumoniae*, only four chromosomally-encoded TA systems have been studied in detail, namely *relBE2*, *pezAT*, *yefM-yoeB_{Spn}* and *phd-doc* (Chan et al., 2013; Chan et al., 2014). Among these, the *yefM-yoeB* TA systems are among the best studied and have been described in various bacteria, including *E. coli* (Grady and Hayes 2003; Christensen et al., 2004), *S. pneumoniae* (Nieto et al., 2007; Chan et al., 2011), *M. tuberculosis* (Kumar et al., 2008), *Staphylococcus aureus* (Yoshizumi et al., 2009; Larson and Hergenrother 2014), *Staphylococcus equorum* (Nolle et al., 2013) and *Streptomyces* sp. (Sevillano et al., 2012). The *yefM-yoeB_{Spn}* TA system has been shown to be functional in its native *S. pneumoniae* host as well as in *E. coli* where over-expression of the *yoeB_{Spn}* toxin gene, which encodes an endoribonuclease, was found to be inhibitory to cellular growth, leading to reduced cell viability (Chan et al., 2011;; Nieto et al., 2007). However, until now there have been no reports on the functionality of this bacterial TA system in plants or any other eukaryotic systems. Therefore, it was of interest to investigate if the YoeB_{Spn} toxin would function in plants, much as other TA systems which have been demonstrated to be functional in eukaryotic systems such as yeast, zebrafish, frogs and human cell lines (Yamamoto et al., 2002; de la Cueva Méndez et al., 2003; Slanchev et al., 2005; Nehlsen et al., 2010).

The plant selected for this study was *Arabidopsis thaliana*, which is a model plant as it is small in size with a short generation time and easily grown at high density in a plant growth room (Meyerowitz and Somerville, 1994). Therefore, *A. thaliana* was

used to explore the effects of the expression of the *yoeB_{Spn}* toxin-*GFP* fusion gene, followed by the co-expression of the *yefM_{Spn}* antitoxin and *yoeB_{Spn}* toxin-*GFP* fusion as a proof of function in plants. To our knowledge, this is the first reported demonstration of the functional expression of a prokaryotic TA system in plant cells.

5.1 Development of plant expression constructs using Gateway® cloning technology

To achieve the aims of this study, a two-component XVE-based inducible expression system that was developed for controlled expression of heterologous genes in *A. thaliana* (Brand et al., 2006) was utilized. Four recombinant constructs (namely pMDC221_*yoeB_{Spn}*-*GFP*, pMDC160_*yefM_{Spn}*, pMDC221_*GFP* and pMDC150_35S) were successfully engineered using the Gateway® cloning technology (Figure 4.21). Cloning of the CaMV 35S promoter in the activator vector pMDC150 enabled the constitutive expression of the XVE transcriptional activator while the *yoeB_{Spn}*, *yefM_{Spn}* and *GFP* transgenes were cloned in the respective responder vectors under the control of the XVE-responsive promoter. The transgenes would thus be expressed when induced with 17- β -estradiol, which activates the XVE transcriptional activator (Brand et al., 2006). This system was chosen as it was possible that expression of the *yoeB_{Spn}* toxin gene would be lethal in plants, hence a strictly inducible expression system would be needed. Brand et al. (2006) demonstrated the stringency of the two-component Gateway compatible system using pMDC150_35S activator lines co-transformed with the responder pMDC221 containing the cytotoxic diphtheria A-chain. The DT-A gene has been reported to be lethal when expressed in cells due to the inhibition of protein synthesis (Collier, 1967). In the absence of the 17- β -estradiol inducer, the transgenic plant lines containing both the pMDC150_35S activator and pMDC221-DT-A responder did not show any phenotypic changes (Brand et al., 2006). However, signs of

cell death were observed in all plant lines after induction with 17- β -estradiol, which demonstrated the tight regulation of genes that are under the control of the XVE-responsive promoter (Brand et al., 2006). Similar results were obtained in this study when the lethal gene of interest (*yoeB_{Spn}* toxin-GFP fusion) was functionally expressed in transgenic plants upon induction with 17- β -estradiol. In the absence of 17- β -estradiol, no obvious phenotypic changes were observed and no GFP fluorescence was detected in transgenic AtYoeBGFP plants (Figures 4.24 and 4.25), clearly demonstrating that the *yoeB_{Spn}*-GFP fusion gene inserted into the responder vector (i.e. pMDC221) was tightly regulated by the XVE-responsive promoter.

In this study, a strong constitutive promoter, i.e., the CaMV 35S promoter, which consisted of a double enhancer region, was used to enable optimal and high level gene expression for the XVE activator in *A. thaliana*. Kay et al., (1987) reported that duplication of the CaMV 35S enhancer led to an approximately tenfold increase in its activity while a reduction in the number of the enhancer elements led to a decrease in enhancer activity, indicating that the number of enhancer sequence elements is an important determinant CaMV 35S enhancer function (Kay et al., 1987).

In this work, the green fluorescent protein (GFP) gene was used as a reporter gene and was translationally fused with the *yoeB_{Spn}* toxin gene, which enabled the monitoring of the transgene expression *in vivo*. GFP, unlike another frequently used reporter gene β -glucuronidase (GUS), can be assayed in living cells and tissues, thus allowing each transgenic plant expressing GFP to be monitored over a time course (Ghim et al., 2010). The non-destructive nature of the GFP assay -and the measurement of its activity have enabled the gene to be successfully used as a reporter to study the transcriptional activities of a wide range of hosts, including plants (Ghim et al., 2010; Casper & Holt, 1996).

5.2 Expression of *yoeB_{Spn}* toxin in *Arabidopsis thaliana* leads to plant death

In the absence of 17- β -estradiol, transgenic AtYoeBGFP plants were not detectably different from the wild-type plants (Figure 4.33a). This observation indicated that despite containing a toxin gene, the transgenic plants could develop normally in the absence of induction. However, upon induction for expression, the transgenic AtYoeBGFP rosette leaves became distorted with severe discoloration starting at 3 days after induction and leading eventually to the death of the entire plant by day 8 (Figure 4.26A). The lethality of YoeB_{Spn} or other YoeB toxins has not been demonstrated in eukaryotes before. Nevertheless, other endoribonuclease TA system toxins such as MazF, RelE and Kid have been shown to be lethal when expressed in eukaryotic cells such as yeast, frogs, zebrafish and human cell lines (Kristoffersen et al., 2000; Yamamoto et al., 2002; de la Cueva-Méndez et al., 2003; Slanchev et al., 2005; Shimazu et al., 2007). In bacteria, Nieto et al. (2007) had shown that overproduction of YoeB_{Spn} under control of the arabinose-inducible P_{BAD} promoter inhibited cell growth and colony formation when tested in different strains of *E. coli*. Similarly, a recent study by Zheng et al., (2015) demonstrated that overproduction of the *Streptococcus suis*-encoded YoeB toxin led to drastic growth inhibition in *E. coli*.

The detection of the *yoeB_{Spn}-GFP* transcripts in AtYoeBGFP for up to 7 days after induction (Figure 4.27A) confirmed that the *yoeB_{Spn}-GFP* fusion gene was expressed. However, RNA extraction was not possible at day 8 because all the plants were dead by day 8. Therefore, it is obvious from these findings that the YoeB_{Spn} toxin is lethal to *A. thaliana* and that the gene can remain in the transgenic plant genome without any adverse effects until its expression is induced by the inducer.

The presence of GFP fluorescence in the transgenic AtYoeBGFP plants under confocal microscope showed that the *yoeB_{Spn}-GFP* transgene was functionally expressed. The YoeB_{Spn}-GFP protein is stable in transgenic AtYoeBGFP as the

fluorescence was observed even at days 8 and 9, after plant death (Figure 4.25). GFP is known as a stable protein and could continue to emit fluorescence long after the host has died (Ghim et al., 2010). Transgenic AtGFP plants expressing only GFP did not show any signs of leaf distortion and discolouration (Figure 4.24C) as compared to transgenic AtYoeBGFP plants, strongly supporting that the lethality of the expressed YoeB_{Spn}-GFP fusion protein is due to the YoeB_{Spn} toxin_GFP fusion protein and not to GFP *per se*.

This is the first demonstration on the functionality of the YefM-YoeB_{Spn} bacterial TA system in plants. However, the exact mechanism by which YoeB_{Spn} exerts its lethality in plants is still unclear. The mechanisms of YoeB action in its native hosts and the heterologous expression of related TA toxins in other cells, may provide some indication of the likely mechanism of action in plants: In *E. coli*, the YoeB_{Eco} functions as an endoribonuclease that catalyzes mRNA cleavage by binding to the 70S ribosome and cleaving mRNA at the second position of the A-site codon, thus inhibiting translation (Christensen et al., 2004; Zhang and Inouye, 2009; Larson and Hergenrother, 2014). The heterologous expression of another *E. coli*-encoded endoribonuclease toxin, RelE, which also triggers mRNA cleavage in a ribosome-dependent mode, in human osteosarcoma cell line U2OS led to apoptosis as indicated by DNA laddering and morphological changes such as membrane blebbing and chromatin condensation (Yamamoto et al., 2002). Another endoribonuclease toxin, Kid (of the *kis-kid* TA system) was also shown to trigger apoptosis when expressed in human HeLa cells, causing widespread cell death after 3 days of Kid expression and total cell death after 15 days (de la Cueva-Méndez et al., 2003). In this study, DNA fragmentation assays carried out on DNA extracted from the transgenic *A. thaliana* expressing YoeB_{Spn}-GFP showed the presence of oligonucleosomal DNA ladders 24 h after 17- β -estradiol induction (Figure 4.30c), which are early markers of the apoptotic pathway, a result similar to that found when the RelE

and Kid endoribonuclease toxins were expressed in human cell lines (Yamamoto et al., 2002; de la Cueva-Méndez et al., 2003).

Oligonucleosomal cleavage is one of the common characteristics observed during animal apoptosis and has also been observed in plant cells undergoing programmed cell death (PCD). Plant cells have a form of PCD called apoptotic-like PCD (AL-PCD) which results in “corpse morphology” where the cytoplasm is condensed away from the cell wall and nuclear DNA is fragmented, similar to the apoptotic morphology seen in animal cells, however the pathways are not thought to be very similar (Liu and Bassham, 2012; Howell, 2013). For instance, cell condensation has been observed in plant PCD, but it is not accompanied by cell fragmentation and formation of the so-called cellular apoptotic bodies, which characterize apoptosis (Hiraga et al. 2010; Lytvyn et al., 2010). In animal cells, mitochondrial damage and caspase-like activation are an important trigger of apoptosis, but there are no clear reports of the same pathways in plants (Liu and Bassham, 2012; Howell, 2013; Michaeli, 2015). Notable differences between PCD in plants and animal apoptosis are the presence of a cell wall that prevents the dead plant cell from being engulfed by adjacent cells (Liu and Bassham, 2012; Wang and Bayles, 2013) and the central role played by caspases in apoptosis in mammalian cells, while true caspase homologs have not been found in plants (Reape and McCabe, 2010; Reape et al., 2015). However, there is extensive evidence for the involvement of caspase-like activities functioning during plant PCD (Reape and McCabe, 2010; Liu and Bassham, 2012). For these reasons, the DNA fragmentation assay that was used in this study could only be helpful in diagnosing an early stage of apoptosis. How much is known on the sequence of events that occurs after this initial DNA laddering and how similar it is to apoptosis, is a subject for further investigation. Although sequencing of the Arabidopsis genome has not revealed the existence of caspase orthologs, the genome does contain metacaspase encoding sequences which have been shown to be involved in developmental PCD of the

Norway spruce (Suarez et al., 2004; Bozhkov et al., 2005) and in stress induced PCD in *Arabidopsis* (He et al., 2008).

Although eukaryotes have 80S ribosomes in the nucleus, the ribosome found in the chloroplasts and mitochondria of higher plants consists of large and small subunits bound together with proteins into one 70S particle (Wilson and Doudna, 2012). These organelles are believed to be originated from bacteria and as such their ribosomes are similar to those of bacteria (Berk, 2000; reviewed by Yang et al., 2012). Therefore, it can be speculated that if the YoeB_{Spn} toxin is able to enter either or both of these organelles, it could bind to 70S ribosomes in the chloroplasts and/or mitochondria and cleave the mRNA, which may be a trigger of the apoptotic pathway in the transgenic plants, eventually leading to cell death. When mitochondria perceive stimuli, for example death signals within the cell, mitochondria will be triggered to release two main groups of pro-apoptotic proteins (which are cytochrome c and apoptogenic proteins) from the intermembrane space (IMS) into the cytosol (Otera and Mihara, 2012; Reape et al., 2015). The release of these components leads to the activation of downstream caspases and induces apoptosis in eukaryotic cells. However, it is unlikely that any organelle (such as mitochondria) will act alone during PCD and therefore, further insight into the role of chloroplasts in plant PCD has been explored in a series of studies using *Arabidopsis* suspension cultures (Doyle et al., 2010; Gutiérrez et al., 2014). It has been reported that chloroplasts may have some important role in regulating PCD in plants, as with the mitochondria, the chloroplast is also one of the major site of reaction oxygen species (ROS) production and ROS are known to act as regulators of PCD in both animal and plant cells (De Pinto et al., 2012). Based on the findings of the current study and reports in the literature, it is thus possible that expression of the YoeB_{Spn} endoribonuclease toxin in *A. thaliana* led to the activation of the plant apoptotic pathway. How YoeB_{Spn} triggers apoptosis is unknown at this point; future studies are required to further identify the

pathways involved and molecular mechanism of their action. AL-PCD in transgenic *A. thaliana* expressing YoeB_{Spn}-GFP could be induced to investigate if there is mitochondrial involvement in the cell death process and specifically to see if cytochrome c is released from the mitochondria to the cytosol.

5.3 *yefM_{Spn}* antitoxin is able to neutralize the *yoeB_{Spn}* toxin-GFP fusion in *Arabidopsis* after induction with 17- β -estradiol

It has been reported that the lethality of the YoeB_{Spn} toxin in its native host cell as well as in *E. coli* could be neutralized by tight binding with its cognate antitoxin, YefM_{Spn} (Nieto et al., 2007). Therefore, it was of interest to investigate whether co-expression of the *yefM_{Spn}* antitoxin gene along with the *yoeB_{Spn}* toxin-GFP fusion in *A. thaliana* could neutralise the lethal effects of the *yoeB_{Spn}* toxin-GFP fusion protein. In this study, the *yoeB_{Spn}* toxin-GFP fusion and *yefM_{Spn}* antitoxin genes were inducibly expressed by 17- β -estradiol through pMDC221 and pMDC160-derived recombinants, respectively (Figure 4.21). The use of two different responder vectors in this study (with two different selection markers) was a strategy to overcome the difficulty in selecting for transgenic hybrid plants after cross-pollination between an *Arabidopsis* line containing the *yoeB_{Spn}*-GFP transgene and an *Arabidopsis* line carrying the *yefM_{Spn}* transgene.

Several possible approaches are available to generate single plants carrying multiple transgenes, including sexual crossing, sequential transformation and co-transformation (François et al., 2002; Que et al., 2010). In this study, sexual crossing was chosen as the method of selection to produce hybrid plants containing both the *yoeB_{Spn}* toxin-GFP fusion and *yefM_{Spn}* antitoxin constructs, as this approach is simple and allows the analysis of combinations of different existing transgenic lines without

the need to produce new transgenic plants (François et al., 2002; Kalunke et al., 2013). In sexual crossing, each of the transgenes in the hybrid reside at identical chromosome position as in their parent line. This allows direct comparison between the hybrid plants to each transgenic population of parents and hence, the data obtained would be more reliable. Using another approach, for example sequential transformation which involves repetitive insertion of transgenes into a plant, the hybrid produced will probably not be in the same locus as the single line and therefore resulting in weaker comparisons due to the possibility of positional effects.

In this study, it was shown that induced co-expression of the *yefM_{Spn}* and *yoeB_{Spn}-GFP* transgenes enabled the hybrid plants to thrive (Figure 4.33a). This finding indicates that the *yefM_{Spn}* antitoxin was able to neutralize the lethality of the *yoeB_{Spn}* toxin in Arabidopsis. The *yefM_{Spn}-yoeB_{Spn}* TA is characterized as a type II TA system in which the antitoxin functions to prevent the lethal action of the toxin mainly through tight binding with the toxin and masking its active site (Hayes and van Melder, 2011; Nolle et al., 2013). It is reasonable to suggest that co-expression of YefM_{Spn} antitoxin and YoeB_{Spn} toxin-GFP fusion proteins led to the formation of antitoxin-toxin complexes in plants which abrogates the toxicity of YoeB_{Spn}. Nevertheless, this could only be inferred, and has not yet been proven in *A. thaliana* so would be interesting to investigate in future studies.

5.4 Induced co-expression of *yefM_{Spn}* and *yoeB_{Spn}-GFP* enhanced growth in hybrid Arabidopsis

In the absence of the 17- β -estradiol inducer, the growth of the transgenic *yefM_{Spn}* \times *yoeB_{Spn}-GFP* hybrid plants was similar to that of the wild-type plants, and the transgenic induced *yefM_{Spn}* plants. Interestingly, when *yefM_{Spn}* and *yoeB_{Spn}-GFP* were

expressed together in the hybrid plants, unexpected phenotypic effects in the growth and morphology of the transgenic *Arabidopsis* were observed (Figures 4.33 and 4.34). Commonly, the phenotype observed for any transgenic construct may be a direct consequence of transgene expression, although there is also a possibility of other, uncharacterized interactions between transgenic material and the host genome resulting in changes in transgenic plants (François et al., 2002; Douglas and Halpin, 2010; Christova and Batchvarova, 2015). In this study, the major alterations in phenotypes were observed as larger rosette leaves, taller plants with more inflorescence stems and increased silique production in the induced hybrid lines compared to all control plants (i.e., wild-type, transgenic induced and non-induced AtYefM, transgenic induced and non-induced AtYoeBGFP and non-induced hybrid). Based on the comparison with these control plants, this apparent enhanced growth could be attributed to co-expression of the *yefM_{Spn}* antitoxin and *yoeB_{Spn}* toxin-GFP fusion genes and was unlikely to be due to other factors, such as the position of transgene integration as the locus of integration is the same in the hybrid and single-transgene-carrying plant lines. This suggestion is also supported by the observation that three independent hybrid lines displayed the same enhanced growth phenotypes. A possible explanation for the enhanced growth observed is that the larger rosette leaves provide more of the photosynthate needed for higher number of inflorescence stems and seed development, thereby leading to an increase in silique production. The reasons and mechanisms underlying the increase in leaf size are still not clear, but some of the possible pathways that could be affected are plant hormones, water-use efficiency, mineral uptake and photosynthetic efficiency.

Plant hormones such as gibberellin (GA) regulate growth and development in plants by controlling the expression of genes involved in these processes (Kusaba et al., 1998). To find out if any of the major plant hormones are affected by the YefM-YoeB_{Spn}-GFP protein complex, measurements of free and conjugate forms of these

hormones could be quantified in the transgenic hybrid plants by gas chromatography-mass spectrometry (GC-MS).

In addition to plant hormones, it is also possible that enhanced growth in the induced hybrid plants were due to their enhanced ability to obtain water and/or mineral nutrients from the soil (Dubouzet et al., 2013). Presumably, the induced co-expression of *yefM_{Spn}* and *yoeB_{Spn}-GFP* could have an impact on plant water use, which was not present in all control plants. In this study, the significant increase in the dry weight was also reflected by the increased growth of the hybrid plants (Figure 4.33F). In future, it would be valuable to carry out further research such as measuring nutrient uptake capability between induced hybrid, transgenic induced and non-induced AtYefM, transgenic induced and non-induced AtYoeBGFP and wild-type plants in order to confirm this possibility.

Another reason for the unexpected enhanced growth phenotype observed in this study is a possible increase in photosynthetic efficiency. At this juncture, it could not be ruled out that the induced hybrid plants had a higher photosynthesis rate which led to enhanced growth. To determine this, in-depth study such as measuring chlorophyll content would be helpful to study photosynthetic efficiency in induced hybrid plants and other control plants.

In both plants and animals, proteins are targeted to desired subcellular compartments, such as the chloroplast, mitochondrion or the nucleus. In the nucleus for instance, proteins are targeted by specific nuclear localization signals (NLSs). These proteins, such as those regulating transcription, are required in the nucleus and need to be specifically transported from the cytoplasm into the nucleus (Puchta and Fauser, 2013). The YefM_{Spn} and YoeB_{Spn}-GFP proteins are 84 and 329 amino acid residues, with YefM_{Spn} having an estimated molecular weight of 9.7 kDa whereas the predicted

molecular weight of the YoeB_{Spn}-GFP fusion protein is 37.6 kDa (as determined by ProtParam). As far as can be ascertained from the prediction of NLS in both YefM_{Spn} and YoeB_{Spn}-GFP using cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), neither the YefM_{Spn} nor the YoeB_{Spn}-GFP proteins contained any recognizable NLS. However, the estimated size of the putative TA complex is still within the size limit to allow transit through the nuclear membrane (i.e., 90-110 kDa; Wang and Brattain, 2007). How the YefM_{Spn} and YoeB_{Spn}-GFP proteins transit through the nucleus is unknown but further studies, for example, by fusing YefM-YoeB_{Spn}-GFP to a NLS could be carried out to enable monitoring using fluorescence and confocal microscopy.

5.5 Prediction of possible DNA binding site of the YefM-YoeB_{Spn}-GFP protein complex in *A. thaliana* genome

In *S. pneumoniae*, the YefM_{Spn} antitoxin protein also functions as a transcriptional autorepressor by binding to a palindrome sequence that overlaps the promoter for the *yefM-yoeB_{Spn}* operon (Chan et al., 2011). The YoeB_{Spn} toxin functions as a co-repressor by enhancing the binding of YefM_{Spn} to its operator site when it is in a YefM-YoeB_{Spn} protein complex. This indicated that the YefM-YoeB_{Spn} TA complex binds more strongly to the DNA target compared to YefM_{Spn} antitoxin alone (Chan et al. 2011). Since the YefM-YoeB_{Spn} protein complex has DNA-binding capabilities, and it is thus, possible that binding of the YefM-YoeB_{Spn}-GFP protein complex to certain sections of the Arabidopsis genome in the transgenic hybrids could have led to the enhanced growth phenotype (as indicated in Figures 4.33 and 4.34) by affecting biosynthetic routes in plant development such as increase in plant hormone production. Known binding sites for a DNA-binding protein could be used to identify additional

sites for this particular protein, and thereby identify further genes regulated by this protein complex (Wasserman and Fickett, 1998).

To explore the possibility that the *A. thaliana* genome contained similar sequences to the native YefM-YoeB_{Spn} binding sites, the 27-nucleotide binding sequence of the protein complex obtained through DNase I footprinting assays (Chan et al. 2011) was used as the query in a BLASTN search of the *A. thaliana* genome sequence (<http://www.ncbi.nlm.nih.gov/genome/?term=arabidopsis+thaliana>). As this prediction was using only a single sequence of the protein complex to represent the specificity of a DNA-binding protein, it was not possible to use score matrix-type motif, which could be more accurate in the sense that a score is assigned to each possible base at each position in the binding sites. In addition, the current prediction method employed might not even cover all the binding sites that went into creating it. In higher eukaryotes such as plants, the binding sites can occur upstream, downstream, or in the introns of the genes that they regulate; in addition, they can be close to or far away from regulated gene(s). For all these reasons, it can be very difficult to find possible binding sites using relatively simple sequence-searching tools like BLASTN (Cliften et al., 2001; Bulyk, 2004).

Based on the BLASTN search, a total of ten Arabidopsis loci were found to have 18 – 21 nucleotide matches to the 27-nucleotide YefM_{Spn}-binding motif (Table 5.1). Nevertheless, none of these loci appeared to be within gene promoter or enhancer regions. Following are the description of various predicted targets and functions.

The highest nucleotide identity (of 21 out of 27 nucleotides) was found within the *ARPC5* gene (GI: 240256243) which encodes one of seven subunits of the plant Arp2/3 protein complex. This complex is involved in the control of actin polymerization in cells and is an important component of plant cell growth and development. However,

the exact role of the protein encoded by *ARPC5* gene, has yet to be determined. Li et al. (2003) demonstrated that the Arp2/3 protein complex plays a role in cell morphogenesis through its role in cell polarity establishment and polar cell expansion in *Arabidopsis*. Similar functional roles for this complex have also been reported in yeast but not in any multicellular organisms.

Another matching sequence (with identity for 20 out of 27 nucleotides) was found to belong to a gene encoding an ubiquitin-specific protease, UBP13 (GI: 2402544121). This gene was reported to be involved in circadian clock and photoperiodic flowering-regulation in *Arabidopsis* by altering the proteasomal degradation pathway (Cui et al., 2013). In plants, the circadian clock coordinates various aspects of development and promotes adaptation to the environment (McClung 2011; Nagel and Kay, 2012). In addition to the role in regulation of the circadian clock and flowering, Ewan et al. (2011) reported that UBP13 was also required for immunity against virulent *Pseudomonas syringae* in tomato. Hence, it seems likely that UBP13 targets key factors in the processes described above and regulates protein levels by counteracting ubiquitin-mediated degradation.

Table 5.1: Possible binding sites for the YefM-YoeB_{Spn}-GFP complex in the *Arabidopsis thaliana* genome based on the 27-nucleotides binding site for YefM-YoeB_{Spn} in *S. pneumoniae* as determined by DNase I footprinting (Chan et al., 2011)

Accession No.	Chromosome No.	Identities (out of 27 nucleotides)	Gene	Features	Possible Molecular Function(s)
gi/240256243 ref/NC_003075.7	4	21	<i>ARPC5</i>	Actin related protein 2/3 complex, subunit 5A	Regulation of actin polymerization and together with an activating nucleation – promoter factor (NPF)
gi/240255695 ref/NC_003074.8	3	20	Representative gene model – At3g31051.1	Hypothetical protein	Involved in transcription or purifying selection
gi/240254412 1 ref/NC_003070.9	1	20	<i>UBP13</i>	Ubiquitin carboxyl terminal hydrolase related protein	Cystein type peptidase activity; Ubiquitin – dependent protein catabolic process
gi/240256493 ref/NC_003076.8	5	19	1.MATE (Multidrug and toxic compound extrusion) 2.Representative gene model – At2g16870	1.MATE efflux family protein 2.TIR-NBS-LRR class disease resistance protein	1. Citrate exudation into the rhizosphere, drug transmembrane transporter activity. 2. ADP/ATP binding

Table 5.1, Continued

Accession No.	Chromosome No.	Identities (out of 27 nucleotides)	Gene	Features	Possible Molecular Function(s)
gi/240256243 ref/NC_003075.7	4	19	Representative gene model – At4g37095.1	Hypothetical protein	Gene codes for small protein either involved in transcription or purifying selection
gi/240255695 ref/NC_003074.8	3	19	<i>T21J18-100</i>	Polynucleotide adenylyltransferase domain and RNA recognition motif containing protein	Nucleotide binding nucleotidyltransferase activity
gi/240254678 ref/NC_003071.7	2	19	TT12/DTX14 Representative gene model – At3g59030	MATE efflux family protein (F3L12.8)	Citrate exudation into the rhizosphere, drug transmembrane transporter activity.
gi/240254678 ref/NC_003071.7	2	19	<i>TT12/DTX14</i> Representative gene model – At3g59030	MATE efflux family protein	Citrate exudation into the rhizosphere, drug transmembrane transporter activity.

Table 5.1, Continued

Accession No.	Chromosome No.	Identities (out of 27 nucleotides)	Gene	Features	Possible Molecular Function(s)
gi/240254421 ref/NC_003070.9	1	19	1.Representative gene model – At1g34930 2. <i>LAX3</i>	1. Plant thionin family protein 2. Auxin influx carrier <i>LAX3</i>	1. Cystein rich protein with toxic and antimicrobial properties involved in plant defense mechanism. 2. Promotes lateral root emergence and is involved in phototaxis arrangement of leaves and stem
gi/240254678 ref/NC_003071.7	2	18	<i>APC2</i> Representative gene model – At2g04660.1	Anaphase promoting complex subunit – 2	A highly conserved ubiquitin protein ligase involved in cell cycle regulation and also promotes protein binding

What is interesting in the predictions for the possible binding sites for the YefM-YoeB_{Spn}-GFP complex within the Arabidopsis genome sequence (Table 5.1) is that two of the matching sequences (with identities of 19 out of 27 nucleotides) belonged to genes encoding multidrug and toxic compound extrusion (MATE) efflux family proteins (GIs: 240256493 and 240254678). MATE proteins are a newly characterized group of secondary active transporters that are present abundantly in all domains of life. However, our knowledge of their function in plants is still limited. Tiwari et al., (2014) reported that overexpression of either OsMATE1 or OsMATE2 in Arabidopsis resulted in pleiotropic phenotypes such as accelerated growth rate of rosette leaves, early flowering and increased number of lateral roots. These findings strongly suggest that MATEs are involved in plant growth and development as well as conferring defense mechanism against biotic stress (Tiwari et al., 2014).

The NBS-LRR protein family consists of proteins that confer disease resistance, and these proteins were named after the domains they typically contain: i.e., the nucleotide binding sites (NBS) and the leucine-rich repeat (LRR), which are abundant in plant species (Ellis et al., 2000). Based on BLASTN search, this highly conserved gene family showed a 19 out of 27 nucleotide identity with the YefM-YoeB_{Spn} binding site (GI: 240256493). In recent years, an increasing number of reports have shown the overexpression of NBS-LRR genes in plants to induce resistance to pathogens: *A. thaliana* NBS-LRRs were found to be required for resistance to multiple pathogens such as *Pseudomonas syringae*, *Ralstonia solanacearum* and *Colletotrichum higginsianum* (Gassmann et al., 1999; Deslandes et al., 2002; Narusaka et al., 2009). In addition, a similar report in melon showed NBS-LRRs to provide resistance against *Fusarium oxysporum* and papaya ring-spot virus (Brotman et al., 2013). The gene that encodes a

plant thionin family protein (GI: 24025442) was also found to share identity of 19 out of 27 nucleotides to the YefM-YoeB_{Spn} binding site. Thionins are cysteine-rich antimicrobial peptides which have been discovered in many plant species (Epple et al., 1997). Due to the fact that thionins, in general, are considered toxic to animals (Bohlmann, 1994), their use has not been extended to crop improvement. Hoshikawa et al. (2012) reported that transgenic potato plants expressing thionin genes isolated from *Brassicaceae* species showed enhanced resistance to gray mold (*Botrytis cinerea*).

Another binding prediction that showed nucleotide identities for 19 out of 27 (Table 5.1) was found within an Auxin influx carrier LAX3 gene (GI: 24025442). So far, all of the AUX1/LAX family members are crucial for the regulation in vascular transport (Péret et al., 2012), leaf positioning (Bainbridge et al., 2008) and root stem patterning (Lewis et al., 2011). It is interesting to compare the enhanced growth in transgenic YefM-YoeB_{Spn}-GFP expressing hybrid plants (Figures 4.33 – 4.34) with that reported for LAX3 in *Medicago truncatula* by Revalska et al., (2015) whereby overexpression of LAX3 led to a bigger leaf size, multiple secondary root branches, increased number of seed pods as well as increased seed number in the legume. In the same study, Revalska et al., (2015) also reported that up-regulation of LAX3 and down-regulation of its orthologs caused the abnormal phenotypes in transgenic lines. Whether the expression of LAX3 was influenced by the YefM-YoeB_{Spn} complex in the hybrid *Arabidopsis* and whether this would lead to growth enhancement would require further studies.

In general, the possible binding sites for the YefM-YoeB_{Spn} complex identified in the *Arabidopsis* genome are related to genes involved in plant cell growth and development, circadian clock and photoperiodic flowering regulation as well as genes

conferring defence against biotic stress and a wide spectrum of plant pathogens. Whether these are truly binding sites for the YefM-YoeB_{S_{pn}} complex in the Arabidopsis genome, and the mechanism that leads to enhanced growth is still unknown. Perhaps binding of the TA complex represses the transcription of these genes which perturbs other components essential for growth and development of the plant. Clues to the eventual cause of the pleiotropic effects observed in the co-expression of the *yefM_{S_{pn}}* antitoxin gene and the *yoeB_{S_{pn}}* toxin gene may be found using sequence alignment and 3D-structure homology modelling in future studies. In addition, comparison of transcriptomes would also be beneficial to be included in future studies to allow the identification of genes that are differentially expressed which led to enhanced growth in the transgenic hybrid plants. Molecular docking of the protein complex and predicted active site residues responsible for enhanced growth could also be done in order to initiate structural studies of this protein complex. A yeast two-hybrid assay may be utilized to obtain more insights on the physical interaction between the YefM antitoxin and the YoeB_{S_{pn}}-GFP toxin fusion, particularly in eukaryotic cells. This would entail the determination of which protein domains are responsible for interaction and under what conditions do these interactions take place (Field and Sternglanz, 1994; Chen and Song, 2013). Finally, in-depth studies using molecular models for protein-DNA binding along with gel retardation assays might give us further insights into the DNA-binding capabilities of the YefM-YoeB_{S_{pn}}-GFP complex on each of the putative binding sites in the Arabidopsis genome. These would hopefully yield some knowledge about the possible mechanism of growth enhancement observed in the transgenic Arabidopsis hybrids.

5.6 Biotechnological applications for the heterologous expression of the *yefM-yoeB_{Spn}* genes in plants

One of the concerns for the widespread use of transgenic plants in the open field is the spread of their pollen into the environment. To address this concern, male-sterile transgenic plants were developed (reviewed by Daniell, 2002) and one of the earliest approaches to engineering male sterility was proposed by Mariani et al., (1990) which involved tapetal-specific expression of a ribonuclease gene, *barnase*, to cause complete male sterility. Its antidote, called *barstar*, restored fertility to hybrid plants by forming highly stable complexes that could inhibit Barnase from functioning (Mariani et al., 1992). Expression of *barnase*, under the control of a tissue-specific promoter, enabled it to be expressed and destroyed the reproductive organs, thereby conferring sterility to the transgenic plants (Mariani et al., 1990; Goldman et al., 1994; Bisht et al., 2015). Although not a TA system, Barnase has been shown to have similar folding as the RelE and YoeB TA toxins from *E. coli* (Takagi et al., 2005). Although *yefM-yoeB* TA system is widely present, its mechanism however, is not fully understood in eukaryotic system. In eukaryotes, the complete lack of TA systems as opposed to their ubiquitous presence in bacteria and archaea has opened a new pathway to explore the potential of TA systems to ablate specific cells in plant. In this study, expression of YoeB_{Spn} toxin was shown to cause lethality in *A. thaliana* and this could form the platform of an inducible plant cell ablation system where it would have potential application in biotechnology such as tissue specific expression to ablate pollen formation for the development of male sterile plants for containment of transgenic plants and to prevent hybrid seed production in the field. Likewise, the expression of its cognate YefM_{Spn} antitoxin could be used to restore fertility in the hybrid plants specifically for their seed production. The conditional expression of the *yoeB_{Spn}* toxin gene could also be used to remove any

unwanted genetically-modified plants arising from accidental out-crossing events, without danger to unmodified plants. Other promoters and regulatory systems could be used instead of the XVE-based system to reduce the cost as the use of 17- β -estradiol as the inducer for toxin expression may not be commercially viable due its relatively high cost.

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CHAPTER 6: CONCLUSION

Bacterial toxin-antitoxin (TA) systems play important roles in prokaryotic cells particularly in the general stress response of bacteria. The genome of the Gram-positive human pathogen *Streptococcus pneumoniae* harbours several putative TA systems including *yefM-yoeB_{Spn}* which is one of the four systems that has been demonstrated to be biologically functional (Chan et al., 2014). In recent years, several TA toxins have been found to be functional in eukaryotic cells (reviewed in Yeo et al., 2016). However, no such studies have been carried out in plant systems. In this study, a two-component XVE-based 17- β -estradiol-inducible expression system was utilized to investigate the functionality of the *yefM_{Spn}* antitoxin and *yoeB_{Spn}* toxin from *S. pneumoniae* in *Arabidopsis thaliana* as a model plant system.

The expression of *yoeB_{Spn}* toxin was confirmed in transgenic *A. thaliana* plants using GFP as a reporter system. Significantly, the expression of the YoeB toxin is lethal in *Arabidopsis* plants, resulting in plant cell death within seven days of 17- β -estradiol induction. This finding demonstrated that the *yoeB_{Spn}* toxin gene can remain in the transgenic plant genome without any adverse effects until its expression is induced by the inducer. Expression of YoeB_{Spn} apparently triggers apoptosis in *A. thaliana* as was observed for the RelE and Kid endoribonuclease toxins in human cell lines. How YoeB_{Spn} triggers apoptosis is unknown at this point; perhaps the degradation of mRNA in the nucleus due to the endoribonuclease activity of YoeB_{Spn} sets the cells down a particular apoptotic pathway, such as had been discovered for the MazF endoribonuclease toxin in human T-Rex-293 cells (Shimazu et al., 2007). Future studies are required to further identify the pathways involved and molecular mechanism of their action. AL-PCD in transgenic *A. thaliana* expressing YoeB_{Spn}-GFP could be

induced to investigate if there is mitochondrial involvement in the cell death process and specifically to see if cytochrome c is released from the mitochondria to the cytosol.

The toxic effect of YoeB_{Spn} was able to be neutralized through co-expression of its cognate YefM_{Spn} antitoxin. It was also shown that co-expression of the *yefM_{Spn}* antitoxin and *yoeB_{Spn}* toxin genes did not lead to any detrimental effects in the hybrid transgenic Arabidopsis. Interestingly, the induced expression of *yefM_{Spn}* and *yoeB_{Spn}-GFP* constructs when together in hybrid plants, led to unexpected pleiotropic effects with increased growth (increased height, dry weight, length of rosette leaves, number of siliques) observed in the hybrid transgenic *A. thaliana*. It is speculated that the formation of the YefM-YoeB_{Spn} TA complex, which is a strong transcriptional repressor in its bacterial host (Chan et al., 2011), might have activated or repressed certain genes involved in Arabidopsis growth and development. In future, comparing the transcriptome profiles of non-induced and 17- β -estradiol-induced transgenic hybrids and determining the expression levels of the putative target genes by qRT-PCR, would hopefully yield insights into the possible mechanism of growth enhancement observed in the transgenic hybrids. In addition, comparison of transcriptomes would also be beneficial to be included in future studies to allow the identification of genes that are differentially expressed which led to enhanced growth in the transgenic hybrid plants.

To our knowledge, this is the first demonstration of a prokaryotic antitoxin neutralizing its cognate toxin in plant cells. The detailed mechanism by which co-expression of *yoeB_{Spn}-GFP* and *yefM_{Spn}* led to enhanced plant growth remains to be elucidated and is a subject for further research. Nevertheless, the unexpected enhanced growth phenotype of the transgenic hybrid plants is an attractive motivation to pursue research along this line for potential biotechnological applications. The demonstration of the functionality of the YefM-YoeB_{Spn} TA system in Arabidopsis also mark an

important milestone towards the development of a bio-containment strategy for preventing the accidental release of genetically modified plants to the environment, for the development of male sterile plants for hybrid seed production as well as the development of a novel cell ablation system for functional studies in plants. At this point, the use of YoeB_{Spn} or any other bacterial TA toxins for the ablation of specific plant cells has yet to be carried out. Nevertheless, the potential is there and in future, it would be good to explore the possibility of developing male sterile plants by using tissue-specific promoters to express the *yoeB_{Spn}* toxin gene.

University of Malaya

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Journal Papers (ISI-cited)

1. **Abu Bakar, F.**, Yeo, C. C. and Harikrishna, J. A. (2015). Expression of the *Streptococcus pneumoniae* yoeB Chromosomal toxin gene causes Cell Death in the model plant *Arabidopsis thaliana*. *BMC biotechnology*, 15(1): 1.
2. **Abu Bakar, F.**, Yeo, C. C. and Harikrishna, J. A. (2016). Neutralization of Bacterial YoeBSpn Toxicity and Enhanced Plant Growth in *Arabidopsis thaliana* via Co-Expression of the Toxin-Antitoxin Genes. *International Journal of Molecular Sciences*, 17(4): 321.
3. Yeo, C. C., **Abu Bakar, F.**, Chan, W. T., Espinosa, M. and Harikrishna, J. A. (2016). Heterologous Expression of Toxins from Bacterial Toxin-Antitoxin Systems in Eukaryotic Cells: Strategies and Applications. *Toxins*, 8(2): 49.
4. Ng, S. L., Harikrishna, J. A., **Abu Bakar, F.**, Yeo, C. C. and San Cha, T. (2016). Heterologous expression of the *Streptococcus pneumoniae* yoeB and pezT toxin genes is lethal in *Chlorella vulgaris*. *Algal Research*, 19:21-29.

Conference Papers

1. **Abu Bakar, F.**, Yeo, C. C., & Harikrishna, J. A. (2011). Expression of the Bacterial *yoeB_{Spn}* Chromosomal Toxin Gene in the Model Plant *Arabidopsis thaliana*. 9th Malaysia Genetics Congress, Pullman Hotel Kuching, Malaysia. (Poster).
2. **Abu Bakar, F.**, Yeo, C. C., & Harikrishna, J. A. (2012). Engineering of Plant Expression Construct Harboring Bacterial *yoeB_{Spn}* Chromosomal Toxin Gene in the Model Plant *Arabidopsis thaliana*. MSMBB Postgraduate Research Award 2012, Research Management & Innovation Complex (RMIC) University of Malaya. (Poster).
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