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**Construction and Molecular Characterisation of an
Improved Chloroplast Transformation Vector System as
a Versatile Delivery and Expression Platform for
in-vitro Propagated *Nicotiana benthamiana***

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**Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy**

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For my parents and Bi Juin

Abstract

The objective of this study is to develop a versatile vector system for the delivery and expression of transgenes in the chloroplast genome of *N. benthamiana*. The successful advent of such a system would vastly streamline the construction process of chloroplast transformation vectors for the expression of recombinant proteins, such as vaccine candidates, in the chloroplasts of *N. benthamiana*. Transgenes targeted to the chloroplasts of higher plants are expected to be expressed at considerably higher levels as compared to nuclear expression, resulting in more significant accumulation of recombinant proteins. In this study, a 2-part chloroplast transformation vector system was developed and two new GFP vector prototypes, pEXPR-G and pEXPR-UG were generated for preliminary evaluation of functionality. The *aadA* and GFP expression cassettes of pEXPR-G and pEXPR-UG were evaluated in *E. coli* prior to actual delivery into *N. benthamiana* via particle bombardment. Particle bombardment parameters were optimised with particular emphasis on minimising excessive damage to the target tissue in order to facilitate the recovery of antibiotic resistant shoots and calli following transformation. To further evaluate the versatility of the developed system for the expression of vaccine antigens, recombinant vectors, pEXPR-HA and pEXPR-NA were constructed for the delivery of hemagglutinin (HA) and neuraminidase (NA) genes of avian influenza strain H5N1 into the chloroplast genome of *N. benthamiana*. Experimental results indicated that pEXPR-G and pEXPR-UG were fundamentally functional in *E. coli* and both the *aadA* and GFP expression cassettes were active, allowing the bacteria to withstand 500mg/l spectinomycin and express the transgene of interest at the protein level. Similar results were also observed in transplastomic *N. benthamiana* transformed with pEXPR-UG and pEXPR-NA. In essence, the developed 2-part chloroplast transformation vector system was found to be highly versatile and could be conveniently applied for the construction of transformation vectors for the delivery and expression of HA and NA in the chloroplast of *N. benthamiana*.

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Abbreviations

<i>aadA</i>	aminoglycoside 3'-adenyl transferase gene
ADR	adverse drug reaction
ANOVA	analysis of variance
BAP	6-Benzylaminopurine
Bp or bp	base pair
BSA	bovine serum albumin
Chl	<i>Chlamydomonas</i>
CIP	calf intestinal phosphatase
cpRNPs	chloroplast ribonucleoproteins
CTB	cholera toxin B
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
FDA	food and drug administration
GB	Gamborg media
GCD	glucocerebrosidase
GFP	green fluorescent protein
GOI	gene of interest
GUS	β -galacturonidase
HA	hemagglutinin
He	Helium
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
INSL/INSR	left and right insertion sequence of chloroplast vectors
IR	inverted repeat
Kb	kilo-base
kDa	kilo-Dalton
LB	left border
LSC	long single copy region of chloroplast genome
M	Molar
MCS	Multiple cloning site
MIC	minimal inhibitory concentration
MRT	multiple range test
MS	Murashige and Skoog medium
NA	neuraminidase
NAA	1-Naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
NEP	nuclear encoded polymerase
NGS	next generation sequencing
<i>NptI</i>	kanamycin resistance gene
Nt or nt	Nucleotide
OD	optical density
ORF	open reading frame
Ori	origin of replication
PCR	polymerase chain reaction
PDS	particle delivery system
PEG	polyethylene glycol
PEP	plastid encoded polymerase

<i>Prrn</i>	<i>rrn</i> operon promoter
PSII	photosystem II
<i>Ptrn^{fM}</i>	tRNA ^{fMet} operon promoter
PVDF	polyvinylidene fluoride
RB	right border
RBP	RNA-binding proteins
RBS	ribosome binding site
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
RUA	rRNA operon upstream activator
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	Shine-Dalgarno
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SiC	silicon carbide
SLF	sigma-like factor
SPP	Sodium-potassium phosphate
SSC	short single copy region of chloroplast genome
Ti	tumour-inducing
Tob	tobacco
tsp	total soluble protein
<i>uidA</i>	β -galacturonidase gene
UTR	untranslated leader region
Vit	vitamin
VLPs	virus-like particles
WHO	World Health Organisation
Wt	wild-type

Materials

M1. Chemicals, enzymes and other consumable materials

Materials	Manufacturer
Culture Media	
Glucose	Fisher Scientific
Sucrose	Duchefa Biochemie
Murashige & Skoog media	Duchefa Biochemie
A-Naphtalene Acetic Acid (NAA)	Duchefa Biochemie
6-Benzylaminopurine (BAP)	Duchefa Biochemie
Lysogeny broth / agar	Conda
Phytigel	Sigma-Aldrich
PCR Reagents	
EXT DNA polymerase	Finnzymes
Taq DNA polymerase	Promega
dNTPs mix	Fermentas
All primers	Bioneer
Restriction Enzymes/ Other Enzymes	
<i>NheI</i>	Fermentas
<i>NcoI</i>	Fermentas
<i>AflII</i>	Fermentas
<i>BspHI</i>	Fermentas
<i>BsiWI</i>	Fermentas
<i>MluI</i>	Fermentas
<i>BglII</i>	Vivantis
<i>EcoRV</i>	Vivantis
<i>PstI</i>	Promega
T4 DNA ligase	New England Biolabs
Alkaline phosphatase	New England Biolabs
Antibodies	
GFP-specific primary antibodies (rabbit)	Abcam
c-Myc-specific primary antibodies (rabbit)	Abcam
NA-specific primary antibodies (rabbit)	Abcam
RuBisCO-specific primary antibodies (chicken)	Sigma
HRP-secondary antibody against rabbit IgG	Abcam
HRP-secondary antibody against chicken IgG	Antibodies
Gel Electrophoresis / Markers & Ladders	
Agarose	Promega
10x TAE buffer	Promega
10x TAE buffer	Promega
1kb DNA ladder	Fermentas
100bp Plus DNA ladder	Fermentas
50bp Plus DNA ladder	Fermentas
RNA ladder	Invitrogen
6x DNA loading dye	Fermentas
2X RNA loading dye	Fermentas
EZ-Vision 6x DNA dye & buffer	Amresco
SYBR-green 10 000x concentrate	Invitrogen

Southern blotting	
20x SSC buffer	Invitrogen
BioNick™ Labelling System	Invitrogen
ULTRAhyb® hybridization buffer	Thermo Fisher
50x Denhardt's solution	Thermo Fisher
UltraPure™ Salmon Sperm DNA Solution	Invitrogen
Chemiluminescent Detection Module	Thermo Fisher
Nylon membrane	Ambion
SDS-PAGE / Western Blotting	
NuPAGE® Bis-tris gels	Invitrogen
NuPAGE® LDS sample buffer	Invitrogen
SimplyBlue™ SafeStain	Invitrogen
Protein ladder	Nacalai Tesque
Nitrocellulose membrane	Pall Corporation
Antibiotics	
Ampicillin	Sigma-Aldrich
Spectinomycin	Duchefa Biochemie
Kanamycin	Duchefa Biochemie
Streptomycin	Duchefa Biochemie
Rifampicin	Duchefa Biochemie
Chemical Reagents	
Acetic acid (glacial)	R&M Chemicals
EDTA	Promega
Tris-base	Promega
Lithium chloride	R&M Chemicals
Potassium acetate	Sigma-Aldrich
SDS	Promega
Sodium chloride	Sigma-Aldrich
Sodium acetate	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
Triton X-100	Fisher Scientific
Tween-20	MP Biomedicals
Glycerol	Calbiochem
UltraPure™ glycine	Invitrogen
Ammonium persulfate	Promega
Acrylamide monomer	Promega
Bisacrylamide	Promega
Organic Chemicals	
Chloroform	System
Methanol	Emsure
Ethanol (100% molecular grade)	Merck
Isopropanol (99.7%)	R & M Chemicals
Phenol: chloroform: isoamyl alcohol (25: 24: 1)	Fluka
TRIzol	Invitrogen
Consumables	
Consumables for particle bombardment	BioRad
DNA purification kit	GeneAll
Bacterial genome extraction kit	Analytik Jena
Plasmid extraction kit	GeneAll
Reverse transcription kit	Qiagen
ReadyPrep™ Protein Extraction Kit	BioRad
c-Myc immunoprecipitation kit	Clontech Laboratories

Other standard chemicals which are non-listed were purchased from the following suppliers: Merck, Sigma-Aldrich and R&M chemicals.

M2. Media

Media for Bacterial Culture (<i>E. coli</i>)					
Reagents		Lysogeny Broth (LB)	SOC*	Competent Cell Storage Solution*	Antibiotic selection media
Nutrients	Bacto Tryptone	10.0 g/l	20.0 g/l	10.0 g/l	10.0 g/l
	Yeast Extract	5.0 g/l	5.0 g/l	5.0 g/l	5.0 g/l
Salts	NaCl	10.0 g/l	5.8 g/l	10.0 g/l	10.0 g/l
	KCl	-	0.2 g/l	-	-
	MgCl ₂	-	1.0 g/l	-	-
	MgSO ₄	-	1.2 g/l	0.15 g/l	-
Other Additives	Glycerine	-	-	36 %	-
	PEG 7500	-	-	12 %	-
	D-glucose	-	0.2 %	-	-
	Ampicillin/ Spectinomycin	-	-	-	100 mg/l

* Reference: Nishimura *et al.*, 1990

The pH was adjusted to 7.0(±0.02) and sterilised by autoclaving at 121°C for 20 minutes, 15psi on liquid cycle. Competent cell storage solution was sterilised by filtering through a 0.22µm disposable filter. Alternatively, the preparation of LB liquid and solid medium can be prepared using stock powder mixtures (Conda) according to manufacturer's instructions.

M3. Buffers

Application	Buffer	Reagents	Required Volume/Mass
Chloroplast Isolation	SPP Buffer (1L)	Na ₂ HPO ₄	3.97 g
		KH ₂ PO ₄	2.99 g
		Sucrose	136.9 g
		KCl	0.745 g
DNA/RNA Gel Electrophoresis	50x TAE Buffer (1L)	Tris-base	242 g
		Acetic Acid (glacial)	57.1 ml
		EDTA	18.6 g
	10x TBE Buffer (1L)	Tris-base	108 g
		Boric acid	55 g
		EDTA	5.85 g

Total Protein Extraction	NP40 Protein Extraction Buffer* (100 ml)	Tris-base	0.61 g
		NaCl	0.88 g
		Triton X-100	1.0 ml
		ddH ₂ O	Top up to 100 ml
SDS-PAGE	2x SDS-PAGE Sample Buffer pH6.8 (100 ml)	Tris-base	1.5 g
		SDS	4 g
		Glycerol	20 ml
		B-mercaptoethanol	2 ml
		Bromophenol blue	0.02 g
	ddH ₂ O	Top up to 100 ml	
	SDS-PAGE Running Buffer (1L)	Tris-base	3.03 g
		Glycine	24 g
SDS		1.0 g	
ddH ₂ O		1000 ml	
Western Hybridisation	Transfer buffer (1L)	Tris-base	3.03 g
		Glycine	14.4 g
		Methanol	200 ml
		ddH ₂ O	800 ml
	TBS Buffer pH7.6 (1L)	Tris-base	2.4 g
		NaCl	8 g
		ddH ₂ O	1000 ml
	TBST (1L)	TBS buffer	1000 ml
Tween 20		1 ml	
Blocking Buffer (100 ml)		TBS buffer	100 ml
		Non-fat milk	5 g

*Protease inhibitor cocktails can be added fresh according to manufacturer's instructions.

Alkaline Lysis plasmid extraction solutions

Solutions for Plasmid Extraction via Alkaline Lysis	
Resuspension Buffer (Solution 1)	25mM Tris-Cl (pH8.0)
	10 mM EDTA
	50 mM D-glucose
Lysis Buffer (Solution 2)	200 mM NaOH
	1% SDS
Neutralising Buffer (Solution 3)	60ml 5M Potassium Acetate
	11.5 ml Acetic Acid
EB Buffer	Tris-Cl (pH 8.5)

Solutions were prepared using sterile autoclaved distilled H₂O and sterilised by filtering through a 0.22µm disposable filter.

RNA extraction buffers

Buffers for the selective precipitation and recovery of RNA	
Lithium chloride (LiCl)	4M LiCl solution
Tris-SDS	100mM Tris base + 0.2% SDS (w/v)
Sodium Acetate (NaAc)	3M NaAc solution

Solutions were prepared using sterile autoclaved distilled H₂O and sterilised by filtering through a 0.22µm disposable filter.

M4. Gels

DNA and RNA electrophoresis gels

Application Reagents	DNA gels	RNA gels
1x TAE buffer	According to gel size	According to gel size
Agarose powder	1% w/v of gel	1.5% w/v of gel
SYBR-green	1 μ l/30ml gel	1 μ l/30ml gel

12% Polyacrylamide gels

Application Reagents	Separating Gel (4 mini gels)	Stacking Gel (4 mini gels)
50% Acrylamide/bis (29:1)	12.0 ml	1.0 ml
1M Tris-HCL (pH8.8)	18.8 ml	-
0.375M Tris-HCL (pH6.8)	-	4.2 ml
10% SDS	0.5 ml	125 μ l
50% Sucrose	8.0 ml	-
ddH ₂ O	9.6 ml	6.3 ml
TEMED	12.5 ml	5.0 μ l
10%APS	1.25 ml	1.0 ml

*the components were mixed thoroughly in the stated order.

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Chapter 1: General Introduction

Expression of foreign genes in plants provides a valuable alternative for the production of valuable recombinant proteins on par with or better than those produced in traditional microbial and mammalian based systems. The potential economic and biosafety benefits along with the potentially high expression levels in plants adds to the fact that plant-based expression systems would likely play a key role in future recombinant protein production on an industrial scale. Recent research as well as a better understanding of the latent potential of molecular pharming has led to and an overall decrease in hostility towards genetically engineered crops over the last decade. In addition, establishment of governing bodies in addition to revised legislation regarding proper biosafety practices have also bolstered the development of several recombinant plant-based products on the commercial market. One such example is the first world marketed plant-made product, ELELYSO[®] (taliglucerase alfa or glucocerebrosidase) which was granted the approval of the U.S. Food and Drug Administration (USFDA) for injection in 2012 for the treatment of adults with a confirmed diagnosis of Type 1 Gaucher's disease. ELELYSO[®] is a recombinant enzyme produced in genetically engineered carrot cells developed by Protalix Biopharmaceutics and marketed by Pfizer Inc. in the United States. The successful commercialisation of this product has indeed heralded a new era for plant-based expression platforms. Recent research interest has shifted towards the expression of transgenes in the chloroplasts of higher plants. Introduction of foreign genes into the chloroplast genome would potentially allow the accumulation of much higher concentrations of recombinant proteins in a sustainable manner and has captured the interest of many research groups. To date, several subunit vaccines have already been successfully expressed in the chloroplasts of tobacco at appreciable concentrations with impressive levels of immunogenicity (Daniell *et al.*, 2009).

1.1. Summarised research approaches

In this study, a 2-part vector system was developed in order to generate chloroplast transformation vectors for the delivery and expression of transgenes in the chloroplasts of tobacco. Details for the construction of the completed chloroplast transformation vectors were presented in Chapter 3. In brief, the completed pEXPR chloroplast transformation vector is composed of two separate plasmids, namely, the carrier plasmid pCART and the transgene expression plasmid pGENE. The carrier plasmid, pCART, is mainly responsible for the integration of transgenes into the chloroplast genome of tobacco and contains chloroplast-specific homologous recombination sequences to direct integration into the *MunI* site between the two tRNA genes, *trnR* and *trnN*. In addition, the expression cassette for the *aadA* antibiotic resistance gene is also located on pCART under the regulation of the *P_{trnFM}* promoter allowing selective regeneration of transformed antibiotic resistant plants. The transgene expression plasmid, pGENE, is composed of the promoter of the *rrn* operon (*P_{rrn}*), as well as, the 5'-untranslated regulatory element (UTR) of the *psbA* gene to modulate the transcription and translation of the gene of interest (GOI). Chloroplast-specific promoters and the 5'-regulatory elements were amplified from isolated chloroplast genomic DNA using primers designed based on sequence information that was available for the chloroplast genome of tobacco. Suitable restriction enzyme (RE) sites were also introduced during the amplification process to facilitate assembly. The functionality of the completed pEXPR transformation vectors were tested in *E. coli* prior to delivery into tobacco as described in Chapter 4. Successful transformation of *E. coli* was evaluated based on *aadA* expression; which allowed the transformed bacteria to survive in the presence of spectinomycin. In addition, the presence of transgenic proteins in the protein extracts from transformed *E. coli* was evidenced by Western blotting assays. Once the expression of transgenes were confirmed in *E. coli*, the transformation vectors were delivered into the leaf explants of tobacco using optimised particle

bombardment parameters and protocols detailed in Chapter 5. Leaves that were used for transformation via particle bombardment were harvested from fertile resilient lines of tobacco that have been established *in-vitro*. The transformed leaf explants were regenerated on selective media containing optimised levels of plant growth regulators and antibiotics as presented in Chapter 6. Putative antibiotic resistant calli and shoots that emerged 4 to 6 weeks post-bombardment were subcultured onto fresh antibiotic media. Polymerase chain reaction (PCR) analysis was used for the screening of the putative transformants in order to distinguish true transplastomic plants from nuclear transformed plants or spontaneous mutants. Site directed integration of transgenes into the chloroplast genome was detected using a set of primers which were designed to flank the insertion site. Leaves were harvested from the PCR-positive shoots and subjected to cycling of selective regeneration to homoplastomy. In Chapter 7, the presence of the transgene was further evaluated in the chloroplast genome of the regenerated shoots via Southern blot using probes specific to the GOI. Transgene expression was also confirmed at the transcription and translation levels via reverse-transcription (RT) PCR and Western blotting assays, respectively. Both c-Myc-specific and protein-specific primary antibodies were used to verify the specificity of the immunopurification process for the isolation of c-Myc-tagged recombinant proteins from total protein samples. In general, the versatility of the 2-part chloroplast transformation vector system was subsequently evaluated via expression of the hemagglutinin (HA) and neuraminidase (NA) surface antigens of avian influenza A strain H5N1 in the chloroplast of tobacco. This proof-of-concept experiment serves as a preliminary step towards the development of a chloroplast-based expression platform for the expression of subunit vaccines against the H5N1 infection.

1.2. Problem statements

This study was rationalised to overcome the following research constraints which was imperative in order to achieve the general and specific objectives of this study. A variety of systems exist for the production of important biopharmaceutical proteins such as antibodies and vaccines. Traditional methods of vaccine production typically involve animal based systems; whereas, modern methods of vaccine production incorporate the use of modified mammalian cells, bacteria or plants as expression platforms (Daniell *et al.*, 2001). Traditional production of live-attenuated vaccines against avian influenza in embryonated chicken eggs is highly disadvantageous as there would most probably be a short supply of chicken eggs during an avian influenza pandemic (Lu *et al.*, 2005; Chen *et al.*, 2010). Although modified mammalian cell-based expression systems have the advantage of producing vaccine antigens which are virtually identical to their natural host, the high cost incurred for culturing mammalian cells is a significant drawback which severely limits its usefulness for large-scale sustainable production of vaccines. Conversely, bacteria- or yeast-based expression systems allow large-scale production of transgenic proteins, but carry the disadvantage of producing products which differ appreciably from their natural form (Daniell *et al.*, 2001). For example, proteins which are usually glycosylated in animals are not glycosylated in bacteria and may be hyper-glycosylated in yeast, resulting in poor protein expression in both systems (Harashima, 1994). Plant-based expression systems offer several advantages which are consistent with the objectives of this study, such as; rapid scalability without the requirement of cumbersome fermenters or bioreactors, minimal risk of contamination from mammalian pathogens or bacterial toxins, as well as, readily available technology for the harvesting and processing of plants and plant products on a large scale (Daniell *et al.*, 2001). Thus, in this study, plants were chosen as the ideal platform for the expression of transgenic proteins. In addition to the nuclear genome, the chloroplast

genomes of higher plants are also amendable to genetic manipulation. Expression of transgenic proteins in the chloroplast of higher plants has been proven to be an expedient method for the production and accumulation of many valuable biopharmaceuticals, antibodies and edible vaccines of up to 47% tsp (total soluble protein) (De Cosa *et al.*, 2001). Nevertheless, chloroplast transformation is a tedious process due to the absence of readily available chloroplast transformation vectors and the absolute requirement of sequence information from the chloroplast genome of the intended host. Hence, in this study, the tobacco plant was chosen as an ideal candidate as its chloroplast genome has been extensively studied and completely sequenced (Shinozaki *et al.*, 1986; Wakasugi *et al.*, 1998). Consequently, the development of a versatile chloroplast transformation vector was also required to facilitate the delivery, integration and expression of transgenes into the chloroplast genome of tobacco. Evaluation of the newly constructed chloroplast transformation vectors was performed in the prokaryotic model organism, *E. coli*. Advantages such as, rapid proliferation rates, similar susceptibility towards antibiotics and convenient transformation protocols make *E. coli* an ideal host for the preliminary evaluation of chloroplast transformation vector prototypes prior to the actual transformation of tobacco (Verma *et al.*, 2008). The GFP gene was used as an exemplar cassette for the preliminary evaluation of chloroplast transformation vectors as it provided a convenient, non-destructive means for the detection of transgene expression in both *E. coli* and tobacco. Alternative marker genes such as the β -glucuronidase gene (*uidA*) were considered to be unsuitable as it required destructive histochemical assays for the detection of results. In addition, the *uidA* gene was also native to *E. coli* which may provide biased results; as compared to GFP which was non-native in both *E. coli* and tobacco (Martins *et al.*, 1993). The HA and NA genes of avian influenza strain H5N1 was selected for expression in the chloroplasts of tobacco as a preliminary study in order to investigate the feasibility of developing a plant-based vaccine against the

infection. Avian influenza strain H5N1 is a predominant strain of influenza in birds and is predicted to be a candidate for the next influenza pandemic. The urgency of this matter is further emphasised by the startling increase in the number of confirmed cases of H5N1 infection in humans. In the first 5 months (January 1st to 31st May) of 2015, a total of 139 confirmed cases have already been reported worldwide to the World Health Organisation (WHO) as compared to only 52 confirmed cases in the entire year of 2014 (Source: http://www.who.int/influenza/human_animal_interface/EN_GIP_20150501CumulativeNumberH5N1cases.pdf?ua=1; date accessed: 01/05/2015). The successful expression of the HA and NA genes in the chloroplast of tobacco may prove to be a significant step towards the development of plant-based vaccines against the avian influenza infection.

1.3. General research objectives

The general objectives of this study are; 1) to develop a versatile 2-part chloroplast transformation vector system for the delivery and expression of a variety of genes in the chloroplast genome of *N. benthamiana*, 2) to evaluate the constructed chloroplast transformation vector prototypes in *E. coli*, 3) to optimise the particle bombardment parameters for the transformation of leaf explants of *N. benthamiana*, 4) to establish resilient lines of *N. benthamiana in-vitro* and to determine optimal concentrations of plant growth regulators and antibiotics required for the selective regeneration of transformed explants, 5) to evaluate the efficiency of the developed chloroplast transformation vectors for transgene delivery and expression in the chloroplasts of *N. benthamiana*, and 6) to evaluate the versatility of the developed 2-part chloroplast transformation vector system for the delivery and expression of other genes in the chloroplasts of *N. benthamiana*.

1.4. Significance of study

In general, the versatility of the 2-part chloroplast transformation vector system would potentially simplify chloroplast-based expression of a wide variety of recombinant proteins of both economic and therapeutic significance. A prominent example demonstrated in this study is the chloroplast-based expression of the HA and NA transgenes in tobacco. Due to the impending global impact of the H5N1 avian influenza pandemic, the H5 hemagglutinin (H5) and N1 neuraminidase (N1) genes were chosen as antigenic determinants from a locally isolated strain of H5N1 virus as a vaccine candidate for plant-based expression. The local isolate of avian influenza used in this study has almost identical protein sequences when compared to other isolates found throughout Southeast Asia (for example, in Thailand, Cambodia, Vietnam and Hong Kong) (NCBI; Viseshakul *et al.*, 2004; Puthavathana *et al.*, 2005). It is therefore hypothesised that vaccine candidates made from this local strain will potentially provide higher levels of immunoprotectivity against Southeast Asian strains of avian influenza. The successful expression of recombinant HA and NA viral antigens in the chloroplast of tobacco would have a regional impact on the advent of chloroplast-based production of vaccines against the H5N1 infection.

Chapter 2: Literature Review

2.1. Introduction to molecular pharming

The term “molecular pharming” generally refers to the production of recombinant pharmaceutical proteins in plants using modern biotechnology (Ma *et al.*, 2003; Paul *et al.*, 2013). The idea of using plants for the large-scale production of biopharmaceuticals emerged in the early 1990s as a result of a biotechnology boom. However, the non-conventional idea of genetically modifying crops for the production biopharmaceuticals was faced with many hurdles, including overinflated expectations, uncertainties in plant regulatory systems, religious controversies, anti-genetic-modification movements, and the reluctance of established pharmaceutical companies to stray from well-established production systems (Fischer *et al.*, 2013). As a result, the increasing demand for recombinant biopharmaceutical products over the past 20 years has been mainly focused on the improvement of previously established systems such as mammalian cell culture technology, animal and bacteria expression platforms. Significant improvements have been made to improve the productivity of these systems through a combination of strain and process development together with improvements in down-stream processing. However, these methods alone are insufficient to answer the demand of the rising world population. The initial goal of molecular pharming was for the large-scale production of valuable recombinant proteins at a fraction of the cost of conventional systems (Stoger *et al.*, 2014). Today, this goal is still valid, however, improved knowledge regarding the limitations and benefits of plant expression systems have led to the realisation that plants are unlikely to replace current industry standards. Nevertheless, plant expression systems hold great potential for supporting currently established systems, as well as, for the production of certain niche products that are incompatible with currently established expression systems. In

contrast to other production systems, a key feature of molecular pharming is that there is neither a standardized platform nor a single technological method for the production of recombinant proteins (Stoger *et al.*, 2014). That is to say that virtually any plant species can be used for the recombinant expression of a particular protein of interest using a variety of production and expression platforms such as whole plant tissue culture, plant cell culture, *in-vivo* cultivation, transformation of sub-cellular organelles or even only in specific organs such as leaves, roots, seeds and flowers. Depending on the type of plant and organ of interest, as well as, the desired transformation outcome, a variety of methods are also available for the delivery of transgenes (section 2.2). The field of molecular pharming has been reinvigorated with the current understanding of realistic opportunities for commercial development by significant advances that were made over the last few years (Fischer *et al.*, 2013; Fischer *et al.*, 2012; Fujiyama *et al.*, 2007). For example, an important breakthrough was achieved in 2012 when the first product of molecular pharming was approved for use in humans: a recombinant form of human glucocerebrosidase (GCD) expressed in carrot cells developed by Protalix Biotherapeutics for the treatment of the lysosomal storage disorder Gaucher's disease (Stoger *et al.*, 2014; Shaaltiel *et al.*, 2015). The expression of GCD in carrot cells would allow oral administration and provides a distinct advantage over the conventional method of enzyme replacement therapy which requires the intravenous administration of recombinant GCD every 2 weeks (Shaaltiel *et al.*, 2015). In addition, two other clinical trial applications for plant-derived insulin (produced in safflower developed by SemBioSys Genetics) and a HIV-neutralizing monoclonal antibody (produced in tobacco developed by Pharma-Planta) have also been approved in the European Union (EU) (Stoger *et al.*, 2014). These advances have finally attracted the interest of policy makers as well as key players in the pharmaceutical industry to the potential of molecular

pharming. A summary of notable plant-derived biological proteins are summarised in table 2.1.

Table 2.1: Example of large-scale plant-derived biopharmaceuticals produced by various companies over the last decade (adapted from Stoger *et al.*, 2014).

Company	Plant species	Plant organ/cells	Expression technology	Lead product
Protalix Biotherapeutics	Carrot	Suspension cells	Stable nuclear	ELELYSO® (taliglucerase alfa)
Medicago	<i>Nicotiana Benthamiana</i> Alfalfa	Leaves	Proficia (transient), Stable nuclear	Vaccine for pandemic/ Seasonal flu
Kentucky BioProcessing	<i>Nicotiana benthamiana</i>	Leaves	Transient, agroviral launch vector	Subunit vaccine for influenza
Fraunhofer IME	Tobacco	Leaves	Stable nuclear	Antibody for HIV (microbicide)
Ventria Biosciences	Rice	Seeds	Stable Nuclear	VEN100 (lactoferrin)
Synthon/Biolex Therapeutics	Duckweed	Leafy biomass	Stable nuclear LEX system	Antibody for non-Hodgkin's lymphoma
Planet Biotechnology	Tobacco	Leaves	Stable nuclear	CaroRx (cavity inhibiting treatment)
Icon Genetics	<i>Nicotiana benthamiana</i>	Leaves	MagnICON (transient)	Vaccine for non-Hodgkin's lymphoma
VAXX	Potato	Tubers	Stable nuclear	Oral subunit vaccine for Norwalk virus (NoroVAXX)
SAFC	Tobacco	Leaves	Stable nuclear	Contract manufacturing
NAIST	Strawberry	Fruits	Stable nuclear	Canine interferon alpha
Mapp Biopharmaceutical/ LeafBio	<i>Nicotiana benthamiana</i>	Leaves	MagnICON (transient)	Multiple vaccines and microbicides

Abbreviations: Fraunhofer CMB, Fraunhofer Centre for Molecular Biotechnology; Fraunhofer IME, Fraunhofer Institute for Molecular Biology and Applied Ecology; NAIST, National Institute of Advanced Industrial Science and Technology; SAFC, Sigma-Aldrich Fine Chemicals.

2.1.1. Plants as biofactories for heterologous protein expression

Within the past two decades, plant-based expression strategies have developed from being just a conceptual idea into a serious competitive alternative for the large-scale production of recombinant proteins. The first plant derived proteins for technical applications have already entered production (Hood *et al.*, 1997; Witcher *et al.*, 1998) and have been evaluated to be economically competitive against their established equivalent in the market (Kusnadi *et al.*, 1998; Evangelista *et al.*, 1998). Significant advancements have been made within the last decade leading to the production of plant-derived biopharmaceutical proteins such as vaccines, antibodies, human growth regulators, hormones and blood products leading to the advent of molecular pharming (Fischer and Emans, 2000; Giddings, 2001). The practice of molecular pharming can be defined as the application of experimental biotechnology for the genetic modification of plants in order to produce proteins and chemicals of therapeutic and commercial value. Production of these high value compounds through conventional use of animal systems and huge bioreactors in industry involves a huge cost which is a significant constraint for developing countries. The expression of therapeutic compounds in plants will not only allow cheaper production costs but also the possibility of producing novel compounds otherwise impossible via other biochemical methods. Thus, molecular pharming could prove to be a valuable tool to support the growing demand of biopharmaceuticals. In addition, plant based protein expression is also expected to be more cost effective, safer, more abundant; and heat-stable compared to conventionally produced recombinant proteins.

Today, the concept of molecular pharming has evolved further giving rise to the idea of edible vaccines. Transgenes encoding antigenic proteins such as sub-components of viral coat proteins are expressed in edible parts of plants (Richter *et al.*, 2000; Streatfield, 2006). Upon consumption, these antigenic

proteins stimulate a mucosal immune response along the gastro-intestinal tract triggering the production of antibodies. This response is parallel to that of oral subunit vaccines (Richter *et al.*, 2000; Rigano *et al.*, 2006). Simplification of immunisation procedures due to the non-invasive nature of vaccine administration (Streatfield, 2006) together with the requirement of fewer trained staff can significantly improve the prospect of vaccine delivery in developing countries (Ma *et al.*, 2003). However, this method of immunisation is not perfect and has its particular limitations such as the requirement of very high levels of expression of the antigen in the edible parts of plants (Streatfield, 2006). This ensures that the minimum threshold of antigens needed to trigger an immunogenic response is attained while still maintaining the required amount of consumed plant material within sensible amounts (Richter *et al.*, 2000). This is particularly important as some studies indicate that oral versus parenteral administration of the same antigen can require up to ten times as much material for oral dosing to attain the same magnitude of immune responses (Gerber *et al.*, 2001; Rybicki, 2009). Allergic reactions and the difficulty of controlling vaccine dosages are also major concerns associated with this method of immunisation (Daniell *et al.*, 2001). To date, edible vaccines still remain within stages of clinical trials. Although appreciable results have been obtained where efficient immunogenic responses were successfully triggered (Streatfield, 2006), further fine tuning is still required before edible vaccines can be made commercially available. Nevertheless, edible vaccines hold great potential of being highly beneficial to humans as well as domesticated animals.

2.1.2. Chloroplasts for the production of candidate vaccines

The expression of subunit vaccines in edible parts of plants may potentially allow the oral delivery of vaccines without the need of conventional methods of intravenous injections (Kamarajugadda and Daniell, 2006). Plant-expressed subunit vaccines were found to induce a mucosal response when administered orally or parenterally in animal models; and the immunised animals were also able to withstand a pathogen challenge (Verma and Daniell, 2007). One major concern is the ability of orally delivered vaccines to survive in the stomach which has been shown to cause a significant decline in efficiency. However, depending on their intended site of action, vaccines against respiratory infections such as influenza may not be affected as mucosal immunity can be induced along the upper digestive tract. Another alternative would be to encapsulate the plant-derived vaccines in a protective coating allowing its gradual release in the gut (Mor *et al.*, 1998). Within the past decade, vaccine antigens against cholera (Daniell *et al.*, 2001), tetanus (Tregoning *et al.*, 2003), CPV (Molina *et al.*, 2004), anthrax (Watson *et al.*, 2004; Koya *et al.*, 2005), plague (Daniell *et al.*, 2005), and amebiasis (Chebolu and Daniell, 2007) have been successfully expressed in transgenic chloroplast (Verma and Daniell, 2007). The cholera toxin B (CTB) is encoded by *Vibrio cholerae* and has been demonstrated to be an effective vaccine candidate (Daniell *et al.*, 2001). CTB expressed in the chloroplast was found to assemble into its correct pentameric form and assumed its fully-functional quaternary structure (Daniell *et al.*, 2001). Subsequent binding assays also showed that chloroplast expressed CTB was fundamentally functional and could act as a very efficient transmucosal carrier (Verma and Daniell, 2007). Chloroplast expressed protective agents (PA) against *Bacillus anthracis* was also found to accumulate at high levels (14.2% of tsp) and could be successfully used to protect mice against lethal doses of *Bacillus anthracis* toxin (Watson *et al.*, 2004; Koya *et al.*, 2005). The conventional method for the production of PA against *Bacillus anthracis* is derived from the

culture supernatant of *Bacillus anthracis* which may also contain contaminating traces of edema and lethal factors leading to undesired side effects. Chloroplast expression of subunit vaccines provides an alternative platform for the production of vaccine agents that are free from other contaminating factors providing a safer and more cost effective method of large-scale vaccine production.

2.1.2.1. Advantages of chloroplast transformation

Chloroplasts are a specialised subset of plastids which are mainly involved in the process of photosynthesis and are found in all photosynthetically active organisms such as green plants and eukaryotic algae. As the process of photosynthesis is known to be the only natural occurring method of carbon assimilation, chloroplasts can be considered as the primary source of the world's food productivity and vital for sustenance of all life on earth. In addition to photosynthesis, chloroplasts also perform an array of critical functions which include hydrolysis for the evolution of oxygen, sequestration of carbon, synthesis of starch, amino acids, fatty acids, pigments and also play key roles in sulphur and nitrogen metabolism (Verma and Daniell, 2007). The Russian botanist K. Merezhkovsky hypothesised that chloroplasts arose from an early endosymbiotic event between an ancient cyanobacterial ancestor and a pre-eukaryotic cell. It is interesting to note that even though the chloroplast genome is significantly smaller in size as compared to the genome of modern free-living cyanobacteria, distinct similarities are still present in both genomes (Martin *et al.*, 2002). The genome of free-living cyanobacteria is known to contain upwards of 1500 genes. In comparison, the much smaller chloroplast genome of land plants typically contain approximately 110 to 120 genes with most of the missing genes relocated to the nuclear genome of the host (Martin *et al.*, 2002). Plastids are inherited maternally in most angiosperm species (Hagemann, 2004), thus, providing a natural method of transgene containment. This is highly beneficial

for the cultivation of genetically modified crops as the transgenes cannot be disseminated via pollen to other wild-type plants, therefore posing a much lower risk factor to the environment (Daniell, 2002). In addition, genetic engineering of cytoplasmic male sterility can also be used as a further approach for transgene containment within chloroplasts (Ruiz and Daniell, 2005). Expression of therapeutic proteins in the chloroplast of plants also provides a valuable alternative to conventional animal based systems involving mammalian cell culture or microbial fermentation systems which are prone to contamination by mammalian viral vectors and human pathogens (Verma and Daniell, 2007). Chloroplasts are also able to accumulate large quantities of transgenic protein with recorded levels of up to 46% of total leaf protein following stable transgene integration and expression (De Cosa *et al.*, 2001). This is mainly due to the highly polyploid nature of the chloroplast genome where up to a 100 genome copies can exist in a single chloroplast. A photosynthetically active plant cell can contain up to 100 chloroplasts which amounts to an estimated 10 000 functional genome copies per cell (Verma and Daniell, 2007). In addition, the integration of transgenes into the chloroplast genome occurs site-specifically via two events of homologous recombination which eliminates any concern of positional effects, commonly observed during nuclear transformation (Daniell, 2002). In contrast to nuclear transformation, a high copy number is highly desirable for the transformation of chloroplasts as the gene silencing mechanism is not present within chloroplasts (Lee *et al.*, 2003). Other advantages of chloroplast-based expression are the capacity of expressing polycistronic cassettes allowing multivalent vaccines or antibodies to be expressed from a single mRNA transcript (Quesada-Vargas *et al.*, 2005). Post-transcriptional modifications such as the formation of disulfide bonds (Staub *et al.*, 2000; Ruhlman *et al.*, 2007), as well as, lipid modifications (Glenz *et al.*, 2006) are also expected to occur in the chloroplast allowing the proper folding of chloroplast expressed proteins.

2.1.2.2. Chloroplast genome organisation: concept of chloroplast transformation

The chloroplast genome of higher plants is composed of basic units of double-stranded DNA of approximately 120 to 220kb and can be found in several different configurations such as multimeric, monomeric or linear forms (Palmer, 1985; Lilly *et al.*, 2001). The circular double-stranded chloroplast genome of tobacco was first described by Shinozaki *et al.* (1986) to be approximately 155 844bp in length with characteristic 25 339bp inverted repeat (IR) regions separated by short (18 482bp) and long (86 684bp) single copy regions. An update to the sequence of the chloroplast genome of tobacco was made by Wakasugi *et al.* (1998) after several sequencing errors were discovered (Shimada *et al.*, 1990; Olmstead *et al.*, 1993) and confirmed (Wakasugi *et al.*, 1998). The sequence of the chloroplast genome of *Nicotiana tabacum* is currently deduced to be 155 939bp in length with two IR regions of 25 341bp separated by short and a long single copy regions of 18 571bp and 86 686bp, respectively (Figure 2.1).

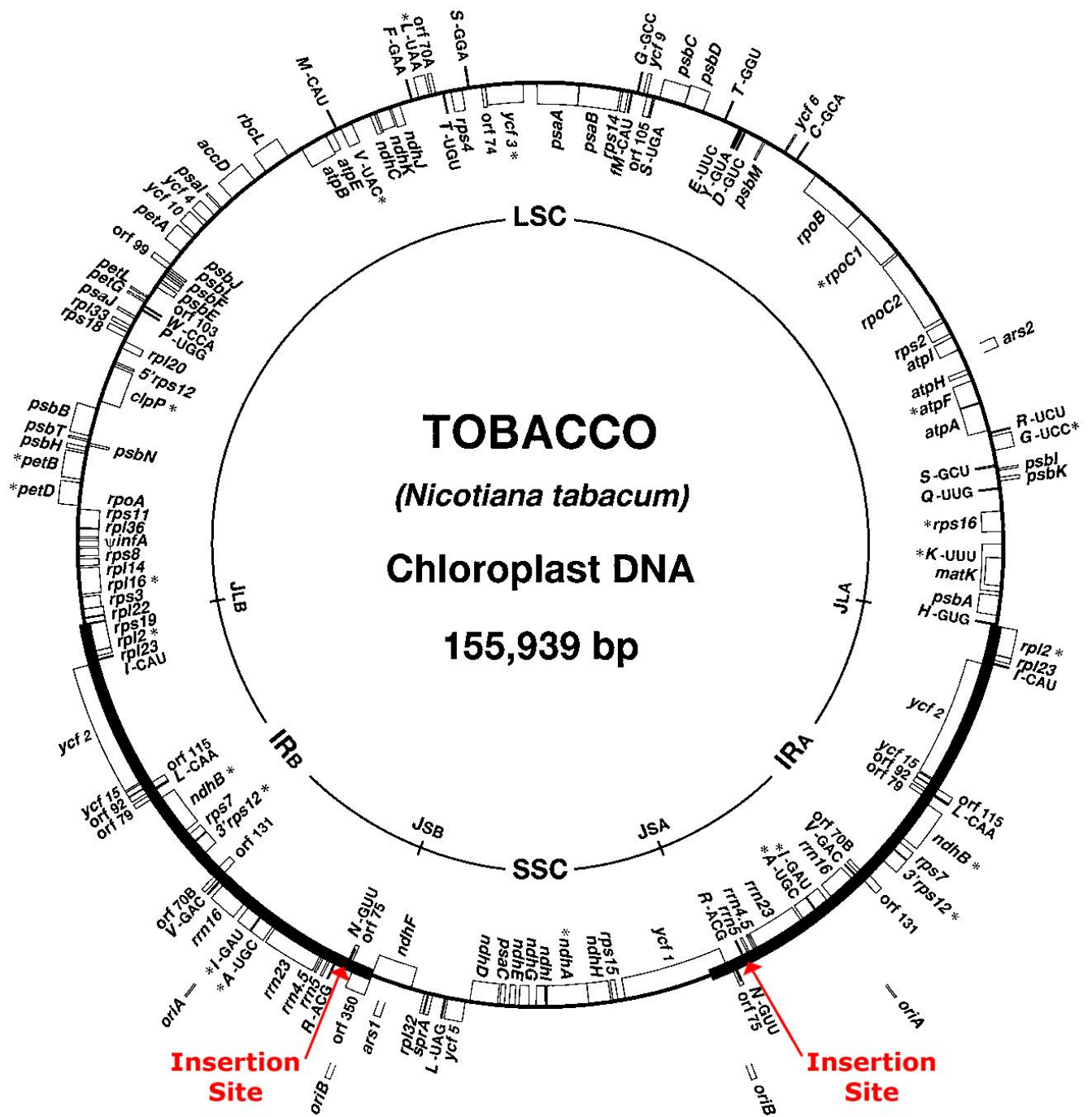


Figure 2.1: Schematic diagram of the chloroplast genome of *Nicotiana tabacum* with the chosen homologous recombination sites marked by arrows (adapted from Wakasugi *et al.*, 1998).

Foreign DNA can be introduced into the chloroplast by means of direct delivery via particle bombardment (Daniell *et al.*, 1990; Sanford *et al.*, 1993) or via PEG permeation (Golds *et al.*, 1993; O'Neill *et al.*, 1993). The foreign DNA is then integrated into the chloroplast genome via two site-directed homologous recombination events between the homologous flanking sequences on the

transformation vector and endogenous sequences on the genome. This process is facilitated by a RecA-type mechanism similar to that of *E. coli* DNA repair mechanisms (Cerutti *et al.*, 1992). Thus, the flanking homologous recombination regions on the chloroplast transformation vector have to be designed specifically according to the genome sequence of the site chosen for transgene insertion (figure 2.1). There are no special requirements for the sequences used for homologous recombination; however, sequences of approximately 1kb are often chosen to provide sufficient specificity and to prevent spurious incorporation of transgenes at non-specific sites (Verma and Daniell, 2007). However care should be taken to ensure that the selected homologous recombination sequence does not provide any extra autonomous functions to the chloroplast transformation vector. Transformation is achieved when transgenes are successfully incorporated into a few copies of the chloroplast genome, followed by 25 to 30 cell division cycles under antibiotic selection pressure to eliminate untransformed chloroplasts. As a result, a homogenous population of chloroplasts with transformed genomes are attained, referred to as a homoplastomic population. The IR region of the chloroplast genome contains a copy correction mechanism which ensures that both IR regions remain identical to each other (Shinozaki *et al.*, 1986). As a result, transgenes that are integrated into the IR region are expected to either undergo transgene deletion or duplication into both IR regions. Thus, effectively doubling the maximum number of active transgene expression cassettes in each chloroplast if successful (Verma and Daniell, 2007). Transgenes have been integrated at multiple sites of the tobacco genome over the past two decades. In initial chloroplast transformation experiments, transgenes were targeted to the transcriptionally silent spacer regions to prevent any possibility for the disruption of endogenous chloroplast genes (Svab and Maliga, 1993). Although transgene expression was successfully achieved, integration of transgenes into transcriptionally active spacer regions proved to be more effective and offered a number of unique advantages such as reduced

dependency on 5'- and 3'-untranslated regulatory elements (UTRs) or promoters for the regulation of transgene expression (Verma and Daniell, 2007). Thus far, the transcriptionally active spacer region between the *trnI* and *trnA* genes within the *rrn* operon which are located at both ends of the IR region closest to the SSC of the chloroplast genome are the most commonly used sites for the integration of transgenes. Transgenes which are integrated and expressed from this site have recorded the highest expression levels as compared to other integration sites on the tobacco chloroplast genome (De Cosa *et al.*, 2001). This would suggest that the integration of transgenes into the spacer regions of transcriptionally active regions may prove to be highly beneficial for high transgene expression levels. However, caution must be exercised as any disruption of surrounding endogenous genes may cause a lethal mutation which would ultimately result in the failure of regenerating homoplastomic plants. It has also been suggested that the inclusion of a chloroplast-specific origin of replication in the chloroplast transformation vector such as the *oriA* may facilitate the rapid recovery homoplastomic plants (Lugo *et al.*, 2004). This was justified with the hypothesis that replication of the chloroplast transformation vector within the chloroplasts would effectively increase the number of transformation vectors within each chloroplast, thus, increasing the template copy number for homologous recombination (Daniell *et al.*, 1990). As a result, the probability of homologous recombination would be enhanced and a state of homoplastomy can be achieved even during the first round of selective regeneration (Guda *et al.*, 2000). Nevertheless, inclusion of an origin of replication in the chloroplast transformation vector is a risky decision as it allows the autonomous maintenance of transformation vectors within the chloroplast. Thus, the antibiotic resistance gene can be expressed indefinitely at high levels even without integration into the chloroplast genome; further complicating the selection and regeneration process.

2.1.3. Regulation of chloroplast gene expression

Chloroplast genes are highly regulated both by external environmental factors such as ambient light intensity, temperature and humidity as well as internal biological factors such as pH levels and the internal oxidative state especially in relation to photosynthetic genes. In addition, regulation of gene expression in the chloroplast is also dependent upon many internal (*cis*-acting) and external (*trans*-acting) factors from the nuclear genome. Although all basic components for gene expression exist within the chloroplast, the nuclear genome plays a major role for the expression of regulatory factors and structural proteins required by the chloroplast. A highly sophisticated signalling pathway has evolved between the chloroplast and nuclear genome allowing them to communicate and coordinate many of the essential functions of the chloroplast which includes the process of photosynthesis. The fundamental aspects of chloroplast gene expression including gene transcription, post-transcriptional mRNA processing, modulation of mRNA stability, and mRNA translation have been extensively reviewed (Gillham *et al.*, 1994; Mayfield *et al.*, 1995; Rochaix, 1996; Sugita and Sugiura, 1996; Barkan and Goldschmidt-Clermont, 2000; Monde *et al.*, 2000; Zerges, 2000). This section attempts to summarise the current understandings regarding the transcription and translation of genes within the chloroplast in relation to transgene expression.

2.1.3.1. Chloroplastic transcription

Evaluation of mRNA abundance in a number of plant species revealed that mRNA abundance fluctuates in response to environmental and developmental stimuli. The observed fluctuation has been argued to be determined by differences in the rate of transcription and mRNA stability. However, a global change in the rate of transcription cannot account for the fact that mRNA abundance is differentially regulated for different genes. The transcription of chloroplast genes have been found to be directed by at least two distinct types of

RNA polymerases; which are, the plastid-encoded RNA polymerase (PEP) and the nuclear-encoded RNA polymerase (NEP). The eubacterial PEP of the chloroplast highly resemble bacterial RNA polymerases and is able to recognise prokaryotic-like promoters which contain the conserved -10 (TATAAT) and -35 (TTGACA) promoter elements. In contrast to *E. coli*, the -35 element has been demonstrated to play only a minor role in regulating promoter activity and was not identified in the promoter sequence of several promoters by both *in-vitro* and *in-vivo* experiments (Gruissem and Zurawski, 1985; Klein et al., 1992). These results suggest that only the -10 element is required for the transcription of genes and the -35 element may have been replaced by another mechanism. In contrast to PEPs, NEPs more closely resemble that of bacteriophage polymerases (Lerbs-Mache, 1993). The NEP promoters mainly regulate the transcription of many 'housekeeping' genes which are required for basic chloroplast metabolism, as well as, the transcription of chloroplast specific regulatory genes such as the ribosomal proteins and the rRNAs. However, NEP promoters do not appear to regulate the transcription of photosynthesis-related genes (Hajdukiewicz *et al.*, 1997). Interestingly, there is also evidence for another distinct type of light-responsive promoter which is known to regulate the transcription of the chloroplast *psbD/C* gene cluster of photosystem II (PSII). Differential transcription by this promoter is known to be mediated by their upstream light-regulated elements by a distinct species of RNA polymerase in synergy with *trans*-acting nuclear-encoded factors (Allison and Maliga, 1995). Further studies revealed that in addition to light, the relative activity of this group RNA polymerases are also influenced by developmental cues (Allison, 2000). The transcription of certain gene clusters have also been shown to be transcribed by both PEPs and NEPs. In general, the genes for the chloroplast genetic system appear to be transcribed by the NEPs during the initial stages of chloroplast development. However, the subsequent transcription of these photosynthesis-related genes is performed by PEPs in the matured chloroplasts (Mullet, 1993;

Kapoor *et al.*, 1997). Fluctuations in the transcription rates of chloroplast genes appear to have an overall effect across all chloroplast species. That is, a particular chloroplast gene that is transcribed at a high rate in one chloroplast is also found to be transcribed at a high rate in other chloroplast species. In addition, the differential transcription rates of chloroplast genes are also found to be independent of its location on the chloroplast genome. Instead, differential transcription is most probably directed by sequence elements that are located downstream of the transcription initiation site, as suggested by the results of experiments using chimeric constructs of chloroplast promoters (Gruissem and Zurawski, 1985). Overall, differential transcription of chloroplast genes can be attributed to plastid type, developmental stage, environmental factors and cell cycle; however, many plastid genes also have a relatively constant rate of transcription throughout development despite having diverse patterns of protein expression. Hence, suggesting that regulation of chloroplast gene transcription is not the limiting step in chloroplast gene expression and post-transcriptional events would likely play a more important role in regulating RNA stability, RNA translation and protein turnover ultimately resulting in the differential expression of chloroplast genes (Mayfield *et al.*, 1995).

2.1.3.2. Chloroplastic translation

The most significant change in chloroplast gene expression is observed during the light induced greening process where an increase in ambient light rapidly affects the expression of genes which are related to photosynthesis. It is believed that the increased expression is due to upregulated rates of protein translation from a pre-existing pool of chloroplast mRNAs. Hence, the regulation of translation appears to be the primary mode of action for light-induced gene expression in the chloroplast. Regulation of *psbA* gene expression has been extensively studied in higher plants and algae and has been regarded as a model for the study of light-induced gene expression. The *psbA* gene encodes for the

D1 protein of PSII which is a key component for the process of photosynthesis. Paradoxically, the D1 protein is itself susceptible to photo-damage; thus, light-induced upregulation of *psbA* gene expression is essential to compensate for photo-damaged D1 proteins in order for the progression of photosynthesis (Klein and Mullet, 1990). Light-induced upregulation of chimeric *uidA* gene fused to the *psbA* 5'-UTR leader region was observed in transplastomic tobacco plants. Therefore, it can be suggested that translational regulation of the *psbA* gene occurs at the initiation phase and is regulated by various elements of the 5'-UTR in response to light (Eibl *et al.*, 1999; Staub and Maliga, 1994). Studies in *Chlamydomonas* indicated that a distinct 47kDa RNA binding protein (RB47) is directly involved in light-induced expression of *psbA*. RB47 was shown to bind specifically to the *psbA* 5'-UTR with high affinity together with three other proteins (RB38, RB55 and RB60) under light, forming a complex (Danon and Mayfield, 1991). Light induced translation, as well as, ribosome binding to mRNA transcripts of the *psbA* gene correlated with the binding activity of the protein complex to *psbA* 5'-UTR (Danon and Mayfield, 1991). Hence, RB47 is likely required for the initiation of *psbA* mRNA translation by the ribosomes and binding of RB47 to *psbA* 5'-UTR is itself modulated by light (Danon and Mayfield, 1991). The RB47 gene has been sequenced and identified as the chloroplast homologue of polyA-binding protein (Yohn *et al.*, 1998). Overall, the *psbA* 5'-UTR appears to play a significant role in modulating the upregulation of D1 protein in response to light and may prove beneficial for the regulation of transgene expression in chloroplasts.

2.2. Transformation strategies

All transformation methods mainly aim to achieve one common objective; that is, the transfer of deoxyribonucleic acid (DNA) molecules into plant cells. Genetically stable transformed plants were first achieved in the 1980s using *Agrobacterium tumefaciens* (Zupan and Zambryski, 1997; Chilton *et al.*, 1977; Zambryski *et al.*, 1983). To date, transformation has been successfully reported in a variety of crop species including maize, tobacco, petunia, tomato, rice, celery, rapeseed, wheat, grape, cassava, millets, and chrysanthemum (Rivera *et al.*, 2012). The first commercial transgenic crop approved by the Food and Drug Administration (FDA) for distribution and consumption in USA was the tomato plant (Herrera-Estrella *et al.*, 2005). Today, transgenic crops account for more than 10% of worldwide crop production and constitute one of the main sources of income for several countries (Schlegel, 2007). In general, an efficient transformation method should be cost effectiveness, not require the use of hazardous or carcinogenic chemicals, have straightforward procedures, allow the precise introduction of transgenes into the intended host, allow the integration of transgenes at a low copy number; and allow convenient regeneration of plants post-transformation. Transformation methods are divided into either direct or indirect categories depending on their mode of action. Indirect methods usually require a biological agent such as bacteria for the delivery of transgenes into the host plant whereas direct methods are usually physical, involving the direct penetration of DNA into the cells of the intended host.

2.2.1. Indirect transformation methods

DNA is usually introduced into the host in the form of a plasmid, which are defined as independent circular forms of DNA usually found in prokaryotes separate from the circular genome of the bacteria. A transformation plasmid typically requires several unique features for the efficient integration and

expression of transgenes and is several orders of magnitude smaller than the bacterial chromosome. The most established method of indirect transformation involves the use of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* for the transfer of transgenes in the form of plasmids into the nuclear genome of plants. Both *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are naturally occurring soil-borne bacteria which are associated with the neoplastic crown gall and hairy root disease, respectively in malus species (Gelvin, 1990). The size of the plasmid used for agrobacterium transformation is generally between 5 and 12kb including the plasmid backbone which is mainly important for plasmid replication (similar to the bacterial genome) and maintenance within the bacteria. Plasmids for agrobacterium transformation are mainly based on the tumour-inducing (Ti) plasmids which cause tumour formation and opine synthesis via the transfer of genes involved in auxin and cytokinin synthesis and nopaline synthase into the nuclear genome of the infected plants (Gelvin, 1990). This unique feature of the Ti plasmid has been exploited for use as a biological vector for the delivery and expression of transgenes in the nuclear genome of plants. However, in order to minimise collateral damage to the infected plant, the non-necessary genes that cause the development of crown gall and production of opines have been removed in current vectors. *Agrobacterium* mediated transformation was first achieved in the 1980s (Zambryski *et al.*, 1983) and has been a popular method for transgene delivery in the industry ever since despite its application mainly only in dicotyledonous plants. However, in recent years the effect of this limitation has been minimised by the used of optimised protocols and the use of acetosyringone as a potent elicitor for *Agrobacterium* infection (Kumar *et al.*, 2006). Several "hybrid" methods have also developed over the past decade composed of a combination of both *Agrobacterium* and a direct approach. Prominent examples that have been used in industrially include agroinfiltration, magniffection and viral vectors (Renier *et al.*, 2000; Gleba *et al.*, 2005).

2.2.1.1. *Agrobacterium tumefaciens* mediated transformation

Evidence pertaining to the ability of *A. tumefaciens* to stably incorporate a region (transfer region or T-DNA) of its tumour-inducing plasmid (Ti-plasmid) into the host's genome has been well established (Galun and Breiman, 1997; Newell, 2000). The T-DNA of the Ti-plasmid is viable for manipulation and *Agrobacterium tumefaciens* can be used to stably incorporate transgenes defined by the right (RB) and left borders (LB) of the T-DNA into the host genome. *A. tumefaciens* has been successfully used in improving crops such as rice to produce β -carotene in the endosperm (Ye *et al.*, 2000). *Agrobacterium* mediated gene delivery is commonly associated with the advantage of a low copy number and stable integration of transgenes into the host's nuclear genome (Newell, 2000). Common disadvantages include low transformation efficiencies, random integration of transgenes, species dependency and the requirement of rigorous optimisation for transformation protocols.

2.2.1.2. Plant viral vector mediated manipulation

This method involves the modification of plant viruses to carry the transgene within the viral capsid while still maintaining virulence (Gleba *et al.*, 2007). The recombinant virus acts as a vector and promotes the propagation of the transgene in the host upon infection. Systemic infection of the host plant has been documented (Gleba *et al.*, 2005). This method has also been used for the experimental production of biopharmaceuticals such as simple vaccines (Gleba *et al.*, 2007). A common approach also involves the linkage of the antigenic polypeptide of interest to the coat protein of plant viruses, a process termed as coat protein fusion or epitope presentation (Gleba *et al.*, 2005; Gleba *et al.*, 2007). The vaccine antigen can then be purified from the recombinant protein. Advantages of this method include a rapid production of recombinant proteins and the potential for high levels of transgene expression. Common disadvantages include a low rate of infection, the risk of post transcriptional gene

silencing, high risk of contamination and failure to establish stable transgenic lines (Gleba *et al.*, 2007).

2.2.1.3. Agroinfiltration and magnifection mediated manipulation

Agroinfiltration and magnifection relies upon an improved method of transgene delivery involving *Agrobacterium* vectors expressing viral replicons linked with the transgene of interest (Gleba *et al.*, 2005). The recombinant *Agrobacterium* vectors are systemically introduced into the plant host using a direct infiltration approach such as by using a force or pressure (Gleba *et al.*, 2005). For example, a syringe can be used to force the *Agrobacterium* suspension through the stomata or an induced wound site (agroinfiltration) or using a vacuum to induce the systemic uptake of an *Agrobacterium* culture through the vascular system into the intercellular space of plant leaves (magnifection) (Gleba *et al.*, 2005; Gleba *et al.*, 2007). This method overcomes the limitations of transgene size and the low infectivity of *Agrobacterium* and the plant viral vectors (Gleba *et al.*, 2005).

2.2.2. Direct transformation methods

Direct transformation methods originated in the 1980s after the advent of *Agrobacterium* mediated transformation as alternative methods for the transformation of recalcitrant plants that were irresponsive to indirect *Agrobacterium* transformation (Neumann *et al.*, 1982; Potter, 1988; Paszkowski *et al.*, 1984). The cellular wall of plant cells acts as a natural barrier and has to be penetrated for the direct delivery of DNA into the host cells. In contrast to *Agrobacterium* mediated transformation, direct methods usually results in the integration of multiple copies of the desired gene into multiple sites on the target genome with minimal cellular toxicity (Smith *et al.*, 2001). However, direct transformation methods are usually associated with disadvantages such as low

transient and stable transgene expression rates, post-transcriptional gene silencing and low post-transformation regeneration rates. Examples of common direct transformation methods include electroporation, particle bombardment, silicon carbide fibres and microinjection.

2.2.2.1. Particle bombardment mediated transformation

Particle bombardment or biolistics is the most widely used method of direct plant transformation. The DNA material which can either be naked DNA, in plasmid form or in a viral or bacterial vector is bound to inert micron to sub-micron sized microcarriers of gold or tungsten and bombarded into the plant tissue (Stanford, 1988; Klein *et al.*, 1988). Initial attempts at particle bombardment involved the use of an explosive charge to propel the microcarriers which caused significant collateral damage to the transgenes and sample tissue (Stanford *et al.*, 1988). In current designs, microcarriers are accelerated using other means such as gas pressure or an electric field (Maliga, 2004). The efficiency of particle bombardment is highly dependent on experimental conditions and optimised protocols based on the available parameters (Hansen and Wright, 1999). Particle bombardment was successfully used to introduce the *Bacillus thuringiensis* (BT)-toxin gene in maize (Sharma *et al.*, 2002). Advantages of gene delivery via particle bombardment include species independency, the ability to regenerate stable transgenic lines, a rapid transformation procedure and the possibility of transforming both the nuclear and plastid genome. Disadvantages of this method include low transformation efficiencies, a tendency of generating high copy numbers, superfluous physical damage and a relatively high cost as compared to *Agrobacterium* mediated transformation.

2.2.2.2. Polyethylene glycol (PEG) mediated transformation

Transformation via PEG-mediated transformation involves the generation of protoplasts. The cells in the tissue of plants have to be enzymatically treated to remove the cell wall and to release the protoplasts (O'Neill *et al.*, 1993). The protoplasts are highly sensitive to osmotic fluctuations and the process of generating protoplasts is often problematic and requires high levels of optimisation. The protoplast then has to be exposed to purified transgenic DNA in the presence of PEG (O'Neill *et al.*, 1993). In the presence of PEG, the protoplast will shrink due to the formation of pores in the cell membrane. Prolonged exposure of PEG will cause the protoplast to lyse due to the disintegration of the cell membrane (O'Neill *et al.*, 1993). The shrinking of the protoplast in the presence of PEG is reversible at any point prior to cell lysis upon which the damage becomes irreversible. The purified DNA is allowed to enter the cell via the pores created by the PEG treatment. PEG treatment is often problematic because of the complication of generating protoplasts as plant cells often reform their cell walls quickly following enzymatic digestion (O'Neill *et al.*, 1993). In addition, PEG treatment only permeates the entry of the transgenic DNA through the cell membrane. For the stable transformation of chloroplasts, the DNA has to pass through the double-membrane bound chloroplast and integrate into the chloroplast genome via homologous recombination.

2.2.2.3. Electroporation mediated transformation

Electroporation is commonly applied for the transport of biological substrates such as proteins, lipids, ribonucleic acid (RNA) and DNA across the cellular membrane. This is achieved via the application of an electric field across the cells which cause the formation of pores in the cellular membrane due to polarity alteration on the membrane (Hofmann and Evans, 1986). The plant cell wall represents a significant barrier which prevents the entry of DNA into the cell. Hence, transformation via electroporation is mainly applied for the

transformation of protoplasts; that is, plant cells which have had their cell walls removed commonly via biochemical digestion of cellulose (Rivera *et al.*, 2012). The success rate of electroporation is fairly variable with recorded rates of 6.2% in maize to 0.3% in wheat (Laursen *et al.*, 1994; Halluin *et al.*, 1992). Electroporation occurs when an alternating or pulsed electric field induces a dipolar moment inside the cells and a potential difference through the cellular membrane which then leads to the reversible permeabilisation of the cellular membrane (Zimmermann and Vienken, 1982). The temporary permeabilisation effects are known to be reversible when the electric pulse lasts for less than 100 μ s after which permanent degradation of the cellular membrane begins to occur (Turgut-Kara and Ari, 2010). A major drawback of this transformation method is that it can only be applied for the transformation of protoplast which is often laborious to produce, as well as, to regenerate post-transformation.

2.2.2.4. Silicon carbide fibres

The physical hardness and the tough crystalline structure of silicon carbide (SiC) fibres allow them to puncture plant cell walls without causing any significant permanent damage. Transformation of plants using SiC was first proposed in 1990 for the transformation of maize and tobacco (Kaepler *et al.*, 1990). The direct method of transformation via SiC is relatively straightforward, cost effective and quick without the requirement of any specialised equipment other than the SiC fibres. SiC mediated transformation is also relatively species-independent and can be implemented for a wide variety of species (Rivera *et al.*, 2012). The general procedure involves the addition of SiC fibres together with a suspension of DNA which is then mechanically introduced into plant tissue via vortexing or abrasion. Alternatively, small plant tissue samples such as cell clusters, immature embryos or callus can also be added together with the SiC-DNA mixture and vortexed in a tube. The DNA coated fibres then pierce the cell wall and cellular membranes allowing the entry of the DNA into the cells via

small pores created by the SiC fibres (Kaeppler *et al.*, 1990). The efficiency of SiC mediated transformation depends on a range of parameters which include the species of plant being transformed and the physical properties of the cell wall of tissue sample, size of the SiC fibres, shape of the tube, and vortexing parameters (speed and duration). Thorough optimisation of these parameters is required in order to improve the efficiency of SiC mediated transformation which can often prove to be difficult. Inadequate optimisation would result in low transformation efficiencies, and may cause irreversible damage to the plant tissue limiting their regeneration capability. Moreover, extreme caution should be taken when working with SiC fibres to prevent laboratory staff from inhaling the fibres as SiC fibres are known to be a respiratory hazard. For the same reason, waste material should also be treated as hazardous waste. The exact mechanism of which SiC fibres introduce DNA into the cell is unknown. However, it has been hypothesised that the SiC fibres act as microscopic needles that perforate the surface of the cell wall and membrane allowing the entry of the DNA suspension into the cell (Wang *et al.*, 1995). Similar results were also observed when other materials with similar properties with SiC fibres were used such as glass, carborundum and silicon nitrate, albeit at lower transformation efficiencies, further supporting the hypothesis (Dalton *et al.*, 1997).

2.2.2.5. Microinjection

Microinjection is regarded as the most precise method of for the genetic transformation of animal cells. The technique of microinjection can also be applied for the transformation of plants and several plant species have been successfully transformed using this method (Davey *et al.*, 1989). Although technically very straight forward, this method does require very high precision instruments for accurate delivery of DNA or even whole chromosomes into the plant cell through a glass microcapillary-injection pipette (Griesbach, 1987). The major disadvantages of this method is that it is a very slow process involving the

transformation of only one cell at a time and the requirement of a specialised micromanipulator system to immobilise the cell and to perform the microinjection. Microinjection results in a transformation efficiency that is approximately 10-times lower than particle bombardment (Rivera *et al.*, 2012). However, the precision of this method is still a valuable asset for the transformation of recalcitrant plants that are irresponsive to all other transformation methods.

2.2.3. Methods for the transformation of the chloroplast genome

A general conclusion that can be drawn from the evaluation of all common methods for plant genetic manipulation is that particle bombardment offers the best chance of success for the transformation of the chloroplast genome. Although the first attempts of transforming the chloroplast genome was made using *Agrobacterium*, success rates were very limited due to the almost exclusive nature of the sophisticated nuclear targeting mechanism of *Agrobacterium*-delivered DNA (De Block *et al.*, 1985; Maliga, 2003). Other methods such as viral vector mediated transformation only allowed the transient expression of transgenes in the cytoplasm of plants (Gleba *et al.*, 2005). Transformation of the chloroplast genome via microinjection has not yet been documented; however, it can be assumed that transforming chloroplasts one at a time would not be a particularly enjoyable experience. The use of SiC fibres for the transformation of microalgae has been successfully reported by Dunahay *et al.* (1997); thus, suggesting that SiC fibres may also be used for the transformation of chloroplasts. However, in addition to having relative low success rates, SiC mediated transformation would also pose an additional health hazard to the experimenter. Other membrane permeabilisation methods such as electroporation and PEG mediated transformation would be highly challenging as the introduced DNA material has to pass through three phospholipid bilayers

before entry into the chloroplasts. Nevertheless, PEG mediated transformation of the chloroplast genome has been successfully reported (Golds *et al.*, 1993; O'Neill *et al.*, 1993). Unfortunately, this method requires the generation of protoplasts which can be technically complicated and difficult to regenerate post transformation (O'Neill *et al.*, 1993). Hence, particle bombardment would offer the highest chance of success for the transformation of the chloroplast genome (Maliga, 2003). Although the process of particle bombardment causes the random delivery of transgenes into plant tissue, the presence of a sufficiently high number of chloroplasts per cell as well as an efficient antibiotic selection procedure would allow the recovery of transplastomic plants.

2.3. Influenza

Influenza is an infectious disease caused by RNA viruses of the family Orthomyxoviridae. Influenza viruses are capable of infecting a wide variety of hosts which include birds and mammals. The main sites of infection of this virus are the nose, throat, bronchi and occasionally the lungs of the host. The symptoms of an influenza infection include extreme fever or coldness, sore throat, runny nose, fatigue, headaches, aching muscles or joints and non-productive (dry) cough. The virus can be transmitted easily from host to host via aerosols and small particles which are produced when the infected host coughs or sneezes. Typical infections of influenza usually persist for about a week before the infected host recovers, often without requiring medical attention. However, mutated or highly pathogenic strains of the influenza virus may result in death if the hosts' immune system is not competent or does not respond quickly enough to establish an immunity to contain the infection (Ng *et al.*, 2006). The most recent outbreak of influenza is caused by the H1N1 strain of swine flu in Mexico which was documented by the World Health Organisation. Based on genetic studies carried out by the Centres for Disease Control and Prevention in the

United States, it was found that this new strain of influenza virus contained genes from four different origins; North American swine influenza, North American avian influenza, human influenza, and swine influenza virus which are typically found in Asia and Europe.

2.3.1. Avian influenza

Avian influenza infections occur naturally among birds and are often spread across the world by infected migratory waterfowl. Wild migratory waterfowl are often non-susceptible to the virus and can carry the virus in their intestines without any visible symptoms, functioning as effective vectors and reservoirs for the virus. Avian influenza is, however, highly contagious among birds and is easily spread from wild migratory fowl to domesticated poultry such as chickens, ducks and turkeys. The virus is shed from an infected bird in its saliva, nasal secretion and faeces and can potentially infect any susceptible host which comes into contact with it or through direct contact with the infected bird itself. Avian influenza infections in domestic poultry can roughly be distinguished into two main categories based on the level of virulence. Low pathogenic infections often go undetected and only cause mild symptoms such as a drop in egg production and ruffled feathers. Highly pathogenic infections, on the other hand, spread more rapidly through flocks of poultry and may establish infections in multiple internal organs. The mortality rate of highly pathogenic infections often reaches 90-100% within 48 hours of an infection. The general structure of the viral particle of influenza virus A is roughly spherical with a genome that is encoded on eight single-stranded-negative-sense RNA segments which encode 1 or 2 proteins each. Each RNA segment is associated with several other proteins such as the RNA polymerase (PB1, PB2 and PA) and nucleoprotein proteins (NP) forming ribonucleoprotein complexes which are enveloped within the viral capsid (Ng *et al.*, 2006). The viral capsid is composed of a complex of matrix protein

(M1) subunits forming a rigid shell which serves to provide structural integrity to the viral particle. A lipid bilayer surrounds the entire viral capsid in which the HA, NA and M2 proteins are embedded. The lipid bilayer is host-derived and serves as a means to avoid detection and to facilitate interactions with the cellular membranes of new host cells. The main function of the RNA polymerase enzyme is for the replication of the viral genome. The RNA polymerase enzyme of influenza virus A is an RNA-dependant RNA polymerase which consists of the viral proteins PB1, PB2 and PA and is found in every virion. The RNA-dependent RNA polymerase is a primer-dependent enzyme. Viral RNA-dependent RNA polymerase enzymes are known to possess the ability to generate their own primers by cleaving existing mRNA molecules of host cells near their 5'-ends. The cleaved portions then act as primers for the replication of the viral genome. The nuclear export protein or NEP (previously named NS2) is assumed to play a role in the export of the viral ribonucleoprotein complex from the nucleoplasm to the cytoplasm by acting as a bridge between nucleoporins of the nuclear pore complex (O'Neill *et al.*, 1998). The ion channel (M2) protein functions as a proton-selective ion channel and plays a role in the uncoating process following receptor-mediated endocytosis of the virus into the host cell (Takeda *et al.*, 2002). The M2 protein is activated by acidic pH and conducts protons across the viral lipid membrane. The drop in pH levels within the virion promotes the dissociation of the matrix protein (M1) and ribonucleoprotein complexes (Takeda *et al.*, 2002). The hemagglutinin (HA) surface glycoproteins found on the viral capsid are found to play a vital role in binding the virus particles to host cells and the subsequent release of the viral genome into the host's system (Wiley and Skehel, 1987; Carr *et al.*, 1997). Neuraminidase (NA) surface glycoproteins possess enzymatic activity and facilitate the release of mature virus particles from the host cells (Sylte and Suarez, 2009; Russell *et al.*, 2006; Huang *et al.*, 2008).

Three significant worldwide (pandemic) outbreaks of avian influenza have been documented over the past century (Kilbourne, 2006). Each outbreak caused significant economic losses and severe cases of morbidity and mortalities. These three historically notable pandemics were the Spanish Flu caused by the influenza virus A, serotype H1N1, which occurred in 1918 causing a death toll of around 40-50 million people (Neumann *et al.*, 2005; Taubenberger and Morens, 2006); the Asian Flu caused by the influenza virus A, serotype H2N2, which occurred in 1957 causing a death toll of around 2 million people and the Hong Kong Flu caused by the influenza virus, serotype H3N2, which occurred in 1968 causing a death toll of around 1 million people. The most recent threat of a potential pandemic of avian influenza is caused by the highly pathogenic H5N1 strain of influenza virus A. This strain of influenza virus A has killed millions of domesticated poultry in a number of countries throughout Asia, Europe and Africa. The WHO has documented 840 cases of human infection by the H5N1 strain of avian influenza from years 2003 to early May 2015. Out of the 840 cases reported, 447 cases resulted in death. Currently, the H5N1 strain of avian influenza, although highly virulent among domestic poultry, only affects humans at a very low rate. However, if the virus gains the ability for sustained human to human transmission, death rates would be expected to increase dramatically. In addition, an unusually high rate of human infection was observed early this year (2015) in Egypt where 132 cases of H5N1 infection were reported with 37 fatal cases. This was a staggering increase over the annual average of only 63 cases per year between 2003 and 2014. Serotypes of avian influenza virus A are characterised according to the two specific glycoproteins found on the surface membrane of the viral particle, namely, hemagglutinin (HA) and neuraminidase (NA) (Qi *et al.*, 2009).

2.3.2. Vaccine development for avian influenza

The concept of vaccines was first established in the year 1796 by Edward Jenner (Huygelen, 1996). Today, vaccines have been developed for a plethora of illnesses (Berg *et al.*, 2008; Joensuu *et al.*, 2008). Vaccines work by triggering an artificial active immune response within a potential host. The majority of conventional vaccines are produced within an animal system (Kang *et al.*, 2009) and are either live-attenuated or inactivated (killed) viruses (Berg *et al.*, 2008). Immunogenic responses triggered by live-attenuated vaccines are higher compared to inactivated vaccines which often have low levels of immunogenicity. However, there is a possibility that live-attenuated vaccines may revert their virulence *in-vivo* (Joensuu *et al.*, 2008). In addition, the processing and delivery of these vaccines are also often problematic and associated with high costs, cumbersome storage requirements and high vulnerability to contamination risks. A more convenient approach involves the exploitation of subunit vaccines. Recent advances in the field of biotechnology have led to the development of DNA and plant-based subunit vaccines. Vaccination using DNA vaccines involves the direct introduction of a gene of an immunogenic component of a pathogen, such as a coat protein gene of a virus into the subject (Chen *et al.*, 1998; Chen *et al.*, 2005; Chen *et al.*, 2009). The gene is transcribed and translated within the vaccinated host to produce the antigenic protein that triggers the immune response. Although a novel approach for vaccine production and delivery, the risk of genetic reassortment and the associated high costs limit the usefulness of this method. Plant-based systems on the other hand, offer many advantages for the production of subunit vaccines. Plants have been used for the production of a wide variety of vaccines (Ma *et al.*, 2003). Notable examples include the hepatitis B surface antigen against the hepatitis B virus (Richter *et al.*, 2000); tuberculosis vaccine (Rigano *et al.*, 2006); recombinant HIV-1/HBV virus-like particles (VLPs) against human immunodeficiency virus (HIV-1) and hepatitis B virus (HBV) (Greco *et al.*, 2007); and hemagglutinin (HA) coat protein against

seasonal influenza (Shoji *et al.*, 2008). All of which have been proven to be effective, at least in functionality, on mice.

Avian influenza is a major threat both clinically and economically. Hence, it is vital that an efficient strategy is developed to contain and control the disease. A common strategy that has been employed to control the spread of the avian influenza infection is through vaccination (Berg *et al.*, 2008). Vaccination involves the pre-exposure of attenuated or inactivated viral particles or components (subunits) of viral capsids collectively referred to as antigens to a potential host before an actual infection takes place (Berg *et al.*, 2008). The host's immune system responds to the antigens by producing specific antibodies that bind to them and disrupts their function. The initial production of antibodies following the first exposure to the antigen is relatively slow. However, antibodies are produced rapidly in subsequent infections due to the fact that memory cells have learnt to recognise the antigens of that particular virus (Czerkinsky *et al.*, 1987; Berg *et al.*, 2008). This allows the host to generate a quick response when the actual infection occurs. Over the past seven decades, influenza vaccines are produced in embryonated chicken eggs. However, this method of production is associated with allergenic factors towards the egg albumin and problems associated with culturing viruses of avian origin. Recently, a new mammalian cell-based method has been developed for the production of avian influenza vaccines. This method of vaccine production employs the use of laboratory cultured mammalian cells (often kidney cells) for the production of vaccines and is proposed to overcome many of the limitations of egg-based vaccines. The first vaccine for humans against avian influenza strain H5N1 was approved by the FDA in 2007 and was targeted for the immunisation of persons between the ages of 18 to 64. The immunisation procedure involved two intramuscular injections given approximately one month apart. Recently, a new single-dose vaccine was also developed against the H5N1 infection (Vajo *et al.*, 2010). Both vaccines

were shown to be efficient in initiating an immunogenic response towards the infective particle in humans. Vaccines produced against the H5N1 avian influenza virus thus far are based on inactivated whole-virions and are highly dependent on large-scale industrial production. Subunit vaccines offer a safer and more cost effective approach for immunisation against the H5N1 infection. Two antigen candidates of the class A influenza virus which are known to effectively induce an immune response are the HA and NA glycoproteins found on the surface of the viral capsids (Chen *et al.*, 1998; Chen *et al.*, 2009; Berg *et al.*, 2008). The HA and NA glycoproteins are essential for the infectivity and reproduction of all strains of class A avian influenza virus.

2.4. Transgenes of interest in this study

Several genes were of particular interest in this study including the green fluorescent protein (GFP) gene and the genes for the hemagglutinin (HA) and neuraminidase (NA) surface antigens of avian influenza A/chicken/Malaysia/5744/2004(H5N1). The GFP gene was identified to be an ideal candidate for the preliminary evaluation of newly constructed chloroplast transformation vectors allowing convenient phenotypic detection of expression under ultraviolet (UV) light. The HA and NA surface antigens are known to be highly immunogenic, hence, they can be considered to be perfect candidate vaccines against the H5N1 infection.

2.4.1. Green Fluorescent Protein

The green fluorescent protein (GFP) has widespread applications in the field of molecular biology and has been used as an efficient marker gene in plants for monitoring gene expression, genetic screening, real-time study of protein expression and to highlight distinct structures for visualisation through

fluorescence confocal microscopy (Tsien, 1988). Native GFP was originally identified in *Aequorea victoria*, a marine jellyfish by Shimomura *et al.* (1962) as a companion protein to aequorin, a protein that produces a blue glow in the presence of Ca^{2+} ions. GFP absorbs blue light and emits green light of a longer wavelength. GFP has a β -barrel structure which is composed of a β -sheet and α -helices that form a compartment around the chromophore which is the active component that fluoresces (Tsien, 1988). A significant advantage of GFP as a marker gene over the commonly used β -galacturonidase (GUS) gene is that it can be viewed *in-situ* in the living tissue of hosts or whole organisms. In contrast, GUS assay requires destructive histochemical treatments for visualisation. Hence, GUS cannot provide accurate temporal data in relation to gene expression patterns *in-vivo* making GFP a better candidate for the identification of primary transformants and real-time tracking of gene expression in living tissue. However, native GFP was unsuited for modern biotechnological applications displaying defective transcription in plants, low levels of fluorescence, toxicity, temperature instability and inefficient protein folding (Tsien, 1988; Haseloff *et al.*, 1997). Major modifications have been made to enable the modern use of GFP as an efficient marker in plants which include the removal of cryptic introns sequences, sequence mutations for the improvement of spectral properties (S65T) and codon optimisation (Tsien, 1988).

2.4.2. Hemagglutinin

In the infected hosts, the HA mRNA is translated into a poly peptide chain of 550 amino acids termed as HA_0 . In the mature form, HA_0 is proteolytically cleaved into the two chains HA_1 and HA_2 which are bound covalently by the removal of arginine at position 329 (Wiley and Skehel, 1987, Guo *et al.*, 2007). The HA monomers then associate non-covalently to form a trimer complex on the surface membrane of the virus (Wiley and Skehel, 1987, Guo *et al.*, 2007).

The HA molecule is known to be heavily glycosylated with 6 oligosaccharide chains extending from asparagine residues at positions 8, 22, 38, 81, 165 and 285 on the HA₁ chain and one from the asparagine residue at position 154 on the HA₂ chain (Wiley and Skehel, 1987). It is proposed that glycosylation events allow the stabilisation of the protein molecule in its 3-dimensional state (Wiley and Skehel, 1987). In addition, glycosylation of the distal tip of the HA molecule is also thought to affect virulence (Perdue and Suarez, 2000). HA facilitates the binding and uptake of viral particles via receptor-mediated endocytosis (Wiley and Skehel, 1987; Carr *et al.*, 1997; Wagner *et al.*, 2000; Wagner *et al.*, 2002). HA binds to terminal sialic acid (*N*-acetyl neuraminic acid) residues found in the cellular receptors of host cells (Wiley and Skehel 1987) and initiates endocytosis. The HA glycoprotein trimer complex is trapped in a metastable conformation at neutral pH (Carr *et al.*, 1997). Upon endocytosis, the host cell engulfs the viral particle within an endosome and attempts to hydrolyse its contents by acidifying the interior, in effect, turning the endosome into a liposome (Carr *et al.*, 1997). At an acidic pH of about 5.0, the native structure of the HA molecule is destabilised and a number of conformational changes are induced (Carr *et al.*, 1997). This results in the dissociation of the HA₁ subunits and the extension of the HA₂ and the fusion-peptide domain into the lysosomal membrane (Carr *et al.*, 1997). Further folding of the rest of the HA peptide results in the 'pulling' of the lysosomal membrane towards the viral membrane ultimately resulting in the fusion of the two membranes (protein-mediated membrane fusion) and the release of the viral nucleocapsid into the host cytoplasm (Carr *et al.*, 1997).

2.4.3. Neuraminidase

The NA tetramer complex of influenza virus has a molecular weight of about 240kDa (Taylor and von Itzstein, 1994). In contrast with HA, NA is shown to possess enzymatic activity to cleave the α -ketosidic linkage between the

terminal sialic acid and an adjacent sugar residue from both viral and host proteins (Liu *et al.*, 1995; Sylte and Suarez, 2009). The catalytic cleavage of sialic acid from viral and host proteins by NA prevents progeny viruses from aggregating on the host cellular membrane or with one another as a result of HA-receptor binding activity. Hence, it is clear that a functional balance between HA and NA activity is a crucial determinant of viral infectivity. Inhibition of NA by antiviral drugs such as oseltamivir (Tamiflu®) and zanamivir (Ralenza®) which act as competitive inhibitors of sialic acid have proven to be effective, at least in functionality, in impeding the spread of the virus. However, there are growing concerns regarding antiviral resistance and adverse drug reactions (ADR) which are often associated with antiviral treatments (Yongkiettrakul *et al.*, 2009). A recent study also suggests that NA may play a role in limiting viral superinfection (Huang *et al.*, 2008). It is proposed that cleavage of terminal sialic acid residues from the receptors of host cells by NA imposes a limit to the number of viral particles that can infect that cell (Huang *et al.*, 2008). This potentially limits viral superinfection which may in turn limit reassortment events between viruses of different subtypes.

Chapter 3: Construction of a versatile 2-part chloroplast transformation vector system for the transformation of *N. benthamiana*

3.1. INTRODUCTION

Chloroplasts are a sub-class of semi-autonomous organelles derived from plastids containing their own self-replicating highly polyploidy genome and transcription-translation machinery. Chloroplasts possess a *recA* DNA repair mechanism similar to *E. coli* which can be potentially manipulated for the integration of transgenes into the chloroplast genome via homologous recombination (Cerutti *et al.*, 1992; Inouye *et al.*, 2008). Successful transformation of plastids in higher plants was first achieved via particle bombardment in 1990 by Svab *et al.* (1990). To date, chloroplast transformation has been widely employed for the improvement of agronomic traits of crops, as well as, for the production of recombinant proteins including industrial enzymes, plantibodies (plant-based antibodies) and vaccines (Kusnadi *et al.*, 1977; Giddings *et al.*, 2000; Ma, 2000; Stoger *et al.*, 2002). In all instances, the construction of efficient chloroplast transformation vectors was the preliminary step for the successful delivery and expression of transgenes in chloroplasts.

3.1.1. The pKCZ chloroplast transformation vector

The pKCZ chloroplast transformation vector is a versatile transformation vector for the tobacco chloroplast genome (Zou *et al.*, 2003). The pKCZ chloroplast transformation vector is composed of eight key components: pUC18 as the backbone, *aadA* antibiotic resistance gene, INSL and INSR homologous sequences, *Prrn* promoter, *rbcl* 3'-UTR (Chl), *rbcl* 3'-UTR (Tob) and the RBS. The INSL and INSR regions of pKCZ are derived from the chloroplast genomic

sequence of *Nicotiana tabacum* and mediate the integration of transgenes onto the chloroplast genome at the *MunI* between the two tRNA genes, *trnR*-ACG and *trnN*-GUU, respectively (Staub and Maliga, 1992; Zou *et al.*, 2003). The bacterial *aadA* gene in pKCZ encodes for aminoglycoside 3'-adenyl transferase which confers resistance to both spectinomycin and streptomycin allowing the efficient selection of transplastomic plants on antibiotic selection media (Eibl *et al.*, 1999; Zou *et al.*, 2003). The vector design allows the removal of each component via RE digestion with a pair of unique enzymes such as *SphI*, *KpnI*, *NcoI*, *PstI*, *BglII*, *EcoRV*, *XbaI*, *NheI*, *Eco47III*, *XhoI* and *SacII*.

In addition, RE digestion of the transformation vector with *NotI* releases the fragment between the *INSL* and *INSR* regions allowing the expression cassette to be potentially transferred into another transformation vector with different insertion sites specific to an alternate location on the genome or for the transformation of another host species. The remaining tobacco *rbcL* 3'-UTR can potentially serve as a universal terminator/stabiliser for transcripts of another transgene expressed in the opposite orientation of the *aadA* gene. As a minor feature, the multiple cloning site (MCS) can also function as a short exogenous spacer region to spatially separate the *aadA* gene from another transgene in the event where both genes are placed under the control of the same *Prrn* promoter in the construct.

3.1.2. Limitations of the original vector

Despite its versatility, the original pKCZ chloroplast transformation vector has several disadvantages. The critical disadvantage of the original pKCZ chloroplast transformation vector in relation to this study was genomic instability that was observed in plants transformed with pKCZ. Zou *et al.* (2003) reported that a segment of the genome of the transformed plants including the *rrn*

operon, involved in the coding of rRNAs for the translation of genes, was lost after a period of cultivation following transformation. Deletion of the chloroplast *rrn* operon is considered a lethal mutation and hence, transformed plants can only survive in a heteroplasmic state. This particular drawback conflicts with the objective of this study as the stable production of transgenic products in plants requires the stable incorporation and expression of transgenes in the chloroplast genome which can only be achieved in a homoplasmic cellular environment. In addition, heteroplasmic plants are also prone to reversion to wild-type once the active selection agent is removed.

Although the transformation vector is designed to allow easy customisation and modification via RE digestion procedures, it still relies heavily on the traditional approach of a step-by-step vector construction method which can be time consuming and highly problematic. This is particularly apparent when many components need to be altered or when the size of the recombinant plasmid becomes too large to work with which limits the high throughput usage of this transformation vector. Hence, the modification of the pKCZ chloroplast transformation vector was imperative in order to accomplish the objectives of this study.

3.1.3. Approaches for improvement

In this study, a highly versatile chloroplast transformation vector system based on a two-part binary design was developed where the antibiotic resistance backbone (carrier plasmid) and transgene expression cassette (expression plasmid) can be customised independently in separate plasmids and finally combined to produce the complete transformation vector. The *aadA* expression cassette in pKCZ was deemed as a suitable candidate for the construction of the carrier plasmid as the *aadA* gene product confers resistance to both

streptomycin and spectinomycin which allow the non-destructive phenotypic screening of leaf explants post-bombardment. The expression plasmid on the other hand employs the use of the endogenous chloroplast *Prrn* promoter and the 5'- and 3'- untranslated regulatory elements (UTR) to facilitate transgene expression.

By using the green fluorescent protein (GFP) as an exemplar cassette to evaluate expression efficiency, a simple yet highly versatile two-part chloroplast transformation vector system was developed for the transformation of *Nicotiana benthamiana*. The success of this system potentially suggests the feasibility of developing a wide range of transformation vectors which can be customised for different host plants and transgenes by substitution of their respective cassettes. This may lead to a large-scale production of highly valuable biopharmaceutical products in plants in future.

3.1.4. Specific objectives

Construction of the carrier and expression plasmids involves the incorporation of various endogenous regulatory elements from the chloroplast genome of *N. benthamiana* such as the promoters and untranslated regulatory elements. The specific objectives of this chapter are: 1) cloning of the *Prrn* and *PtrnFM* promoters as well as the *psbA* 5'-untranslated leader region from the chloroplast genome of *N. benthamiana*, 2) modification of the pKCZ chloroplast transformation vector into a versatile carrier plasmid, 3) construction of an efficient expression plasmid for the expression of transgenes in the chloroplasts of *N. benthamiana*; and 4) development of a highly versatile chloroplast transformation vector system using the carrier and expression plasmids suitable for delivery via particle bombardment.

3.2. METHODS

3.2.1. Plasmid DNA isolation and purification

In this study, plasmid DNA was propagated in *E. coli* for a number of applications. Plasmid DNA was subsequently extracted from overnight cultures of *E. coli* bacteria using commercial kits of via alkaline lysis.

3.2.1.1. Plasmid propagation

An overnight suspension of bacteria carrying the plasmid of interest was prepared by inoculating a single colony of transformed *E. coli* from a pure culture into 10ml of sterile liquid LB medium supplemented with 10 μ l of ampicillin (100mg/ml) in a 50ml Falcon tube. Incubation was performed overnight (16h) at 37°C in an incubator shaker (Innova 42, Eppendorf, Germany) at 200rpm.

3.2.1.2. GeneAll® Exprep™ Plasmid Quick kit

Overnight bacterial cultures carrying the plasmid of interest prepared as described in section (3.2.1.1.) were centrifuged at 6500rpm, 10°C for 5 minutes in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) to pellet bacterial cells. The supernatant was discarded and the tube was inverted on a paper towel to remove residual culture broth. The bacterial pellet was resuspended with 250 μ l buffer S1 from the Exprep™ Plasmid Quick purification kit (GeneAll Biotechnology, Korea) and the mixture was transferred to a new 1.5ml tube. Subsequent extraction and purification procedures were performed as described in the user's manual. The final product was eluted from the GeneAll® column using EB buffer. The volume of EB buffer used for the final elution step could be decreased to obtain more concentrated samples when required. The concentration of the eluted plasmid DNA was determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) as described in section (3.2.4.1.).

3.2.1.3. Alkaline lysis method (mini-preparations)

Overnight cultures of transformed bacteria were prepared as described in section (3.2.1.1.). The bacterial pellet was resuspended with 150µl of pre-chilled solution 1 and transferred to a new 1.5ml tube before 250µl of solution 2 was added to the suspension and mixed by gentle inversion. The mixture was allowed to incubate at room temperature for 1-2 minutes (until clear and viscous) before 250µl of pre-chilled solution 3 was added to the lysate and mixed by gentle inversion for 1 minute, followed by vigorous shaking for another 1 minute. The resultant product was centrifuged at 13 000rpm for 5 minutes to pellet cellular debris (and genomic DNA) before the supernatant was transferred to a new 1.5ml tube. The centrifugation step was repeated to remove any residual cellular debris and the supernatant was again transferred to a new 1.5ml tube. Plasmid DNA was precipitated by adding 750µl of pre-chilled isopropanol (99.7%) and mixing by gentle inversion. Precipitated plasmid DNA was recovered by centrifuging the mixture at 13 000rpm for 5 minutes and discarding the supernatant. The pellet was washed using 500µl of pre-chilled ethanol (95% HPLC grade) and spun for 10 seconds in a microcentrifuge before removing the supernatant. The pellet was allowed to dry in a vacuum desiccator (Concentrator plus, Eppendorf, Germany) for 2-3 minutes before resuspension in 100µl EB buffer. Residual RNA was removed by adding 5µl of RNase A (20mg/ml) and incubating in a 70°C for 10 minutes, followed by room temperature for at least 30 minutes. Plasmid DNA was recovered by adding 10µl of 3M sodium acetate (NaAc) and 100µl of phenol: chloroform: isoamyl alcohol (25:24:1). The mixture was vigorously vortexed for 20 seconds and centrifuged at 13 000rpm for 5 minutes to allow separation into 3 layers: a bottom organic layer, a middle interphase and a top aqueous layer. The top aqueous layer was transferred to a new 1.5ml tube without disturbing the other layers and plasmid DNA was precipitated by adding 100µl of isopropanol, followed by mixing by

gentle inversion. The mixture was centrifuged at 13 000rpm for 5 minutes to pellet plasmid DNA and the supernatant was discarded. The plasmid pellet was washed using 500µl of ethanol (100% HPLC grade) as described earlier and dried in a vacuum desiccator (Concentrator plus, Eppendorf, Germany) for 2-3 minutes before the it was resuspended in 30µl EB buffer. The concentration of the eluted product was determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) as described in section (3.2.4.1.).

3.2.2. Isolation of chloroplast genomic DNA

In order to obtain chloroplast genomic DNA, chloroplast were first isolated from the leaves of *N. benthamiana*. The genomic DNA was subsequently extracted using a commercially available bacterial genome extraction kit with a modified protocol.

3.2.2.1. Chloroplast isolation

All operations were performed at 4°C on ice unless specified otherwise. Chloroplasts were extracted from newly expanded leaves of *N. benthamiana* plants using 2 to 5g of fresh tissue. Leaves were harvested and placed in a pre-chilled mortar together with 30ml of sodium-potassium phosphate (SPP) buffer and homogenised thoroughly. The homogenised sample was filtered through 4 to 5 layers of cheesecloth into a 50 ml conical tube and centrifuged at 1500rcf for 10 minutes at 4°C. The supernatant was discarded leaving the chloroplast pellet which was subsequently resuspended with 35ml of fresh ice-cold SPP buffer and centrifuged again at 1500rcf for 10 minutes at 4°C. The supernatant was discarded and the tube was inverted on a paper towel to drain off residual SSP buffer. Isolated chloroplasts were processed immediately to prevent any degradation of material.

3.2.2.2. Extraction of genomic DNA

Due to the eubacterial nature of chloroplasts, chloroplast genomic DNA can be extracted rapidly and conveniently using any commercial bacteria genomic DNA extraction kit with just a few minor protocol alterations. In this study, genomic DNA was extracted from chloroplasts using the innuPREP Bacteria DNA Kit (Analytik Jena, Germany). Chloroplasts were harvested as described in section (3.2.2.1.). The pellet was completely resuspended in 200µl of TE buffer and mixed by pulse vortexing for 5 seconds. The addition of lysozyme was not required as chloroplasts do not possess a peptidoglycan cell wall. The mixture was transferred to a new 1.5ml tube and 200µl of TLS lysis solution and 25µl of proteinase K were added to the tube, followed by pulse vortexing for 5 seconds and incubation at 50°C for 20 minutes with intermittent agitation. The tube was centrifuged at 12 000rpm for 2 minutes at room temperature to pellet unlysed material and the supernatant containing the genomic DNA was transferred to a new 1.5 ml tube. Genomic DNA was prepared for binding by adding 400µl of TBS binding solution to the tube and mixed thoroughly by vortexing before transferring to a spin filter seated in a 2ml receiver tube. The tube stack was centrifuged at 12 000rpm for 2 minutes at room temperature and the receiver tube containing the filtrate was discarded. The spin filter was placed in a new 2ml receiver tube 750µl of MS washing solution was added to the spin filter. The filter stack was centrifuged at 12 000rpm for 1 minute at room temperature and the filtrate was discarded. Residual wash buffer was removed by centrifuging at 14 500rpm for another 2 minutes at room temperature. The spin filter was removed from the receiver tube and placed in a new 1.5 ml tube. Chloroplast genomic DNA was eluted from the spin filter by dispensing 50-100µl of elution buffer (or dH₂O) directly onto the filter using a pipette. The tube was incubated for 3 minutes at room temperature and centrifuged at 10 000rpm to collect the genomic DNA. The volume of elution

buffer used for the final elution step could be decreased to obtain more concentrated samples. The concentration of the extracted samples of chloroplast genomic DNA was determined using the Nanodrop apparatus as described in section (3.2.4.1.). All genomic DNA samples were stored at -20°C until further processing.

3.2.3. Purification of DNA

DNA purification was important in order to facilitate a number of important steps in the construction process. DNA was purified either using commercially available kits or via ethanol precipitation.

3.2.3.1. Purification of DNA from PCR and restriction enzyme digests

DNA was purified following PCRs and restriction enzyme digestions where needed using the Expin™ PCR SV kit (GeneAll Biotechnology, Korea) according to the user's manual. The volume of EB buffer used for the elution of the purified product could be decreased to obtain more concentrated samples. For the purification of DNA from agarose gels, the bands of interest were purified following gel electrophoresis using the Expin™ GEL SV kit (GeneAll Biotechnology, Korea) according to the user's manual. Again, the volume of EB buffer used for the elution of DNA in the final step could be decreased as described earlier. In most reactions, the final product was eluted from the filter column using 20µl of EB buffer. The concentration of the recovered DNA was determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA).

3.2.3.2. Purification of DNA fragments via ethanol precipitation

This method of DNA purification was designed for the purification and recovery of double stranded DNA fragments (such as the c-Myc tag and RE digested DNA) and produces products that are sufficiently pure for standard PCR amplification, RE digestion and ligation reactions. Ice-cold absolute ethanol was added to the DNA sample at a ratio of 3x DNA sample volume. The mixture was incubated at -80°C for at least 1 hour to allow precipitation of DNA before it was centrifuged at 14 000rpm for 30 minutes at 0°C in a refrigerated centrifuge. The supernatant was carefully removed and the remaining contents of the tube was washed with 750-1000µl of room-temperature 95% ethanol by gentle inversion for 1 minute. The tube was centrifuged at 14 000rpm for 10 minutes at 4°C in a refrigerated centrifuge before the supernatant was removed and the DNA pellet was dried in a vacuum desiccators for 1 minute. The pellet was resuspended in 20-30µl of dH₂O and the concentration was determined using the Nanodrop apparatus.

3.2.4. Concentration determination using a spectrophotometer

Accurate measurements of nucleic acids and optical densities of bacterial cultures were measured using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA) and the Biophotometer plus spectrophotometer (Eppendorf, Germany), respectively.

3.2.4.1. Nucleic acid concentrations

The concentrations of all DNA and RNA products were determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA). The apparatus was initialised using 1µl of distilled water (dH₂O) according to instructions before each session. The spectrophotometer was zeroed accordingly for each sample type with their respective buffer before measurement. For each sample, 1µl was

used for the measurement of concentration. Output measurements of absorbance were recorded at 230nm, 260nm and 280nm wavelengths which correspond to the content of organic constituents, nucleic acids and proteins, respectively. The quantity of nucleic acids (DNA and RNA) is represented by the absorbance at 260nm and the quality of samples with respect to organic and protein contaminations are represented by the 260/230nm and 260/280nm ratios, respectively. DNA samples with 260/230nm and 260/280nm ratios above 1.8 are generally considered to be pure while RNA samples with 260/230nm and 260/280nm ratios above 2.0 are considered to be adequately pure for most biochemical and RT-PCRs.

3.2.4.2. Optical densities of bacterial cultures

The optical densities (OD) of bacterial cultures were measured using the Biophotometer plus spectrophotometer (Eppendorf, Germany). The spectrophotometer was blanked with the appropriate culture media prior to OD measurement of each sample (LB liquid medium as blank for bacterial broth cultures). For each reading, 50 μ l of bacterial broth culture was dispensed into a cuvette (UVette, Eppendorf, Germany) and the OD at 600nm (OD_{600nm}) was measured. The typical OD_{600nm} value of *E. coli* broth cultures in mid logarithmic phase is approximately 0.5.

3.2.5. Polymerase chain reactions (PCRs)

PCRs were carried out routinely throughout the course of this study for various experiments which included the verification and amplification of genes, identification of recombinant plasmids and PCR screening of transformed samples. PCRs generally differed in certain aspects such as the composition of reagents and their programming parameters in relation to their applications. The reaction mixtures for all PCRs were prepared according to appendix (A) and

could be scaled up or down accordingly. Putative *E. coli* transformants carrying the recombinant plasmid constructs were also screened via PCR amplification using a single colony of transformed bacteria as template.

3.2.5.1. Optimisation of primer annealing temperatures and programs for PCRs

Primer annealing temperatures were optimised using the gradient temperature function of the PCR thermal cycler (GS2 Thermal Cycler, G-Storm, UK). The optimum primer annealing temperature was selected based on the highest annealing temperature possible while still maintaining appreciable levels of gene amplification. In cases where the optimum annealing temperature obtained in a single optimisation step was considered to be too low, the optimisation procedure was repeated with 5 initial cycles at the prior optimised temperature, followed by another gradient temperature function for a further 25 cycles. As a result, a 2-temperature PCR program was obtained where the initial 5 cycles were performed at a lower annealing temperature, followed by 25 cycles at a higher annealing temperature according to the second optimisation procedure. The nucleotide sequence and information of all primers are summarised in appendix (B). PCR programs were determined according to the optimised annealing temperature(s) of each primer pair and all PCRs were performed using a standard 25-35 cycle run with either a single or two different annealing temperatures(s) depending on the optimisation results for the respective primer pairs.

3.2.5.2. Assembly of the c-Myc tag fragment via PCR

The c-Myc epitope tag fragment was assembled using 2 perfect complementing strands of synthetic oligonucleotides encoding for the 10-amino acid c-Myc epitope tag sequence (EQKLISEEDL) (appendix B). The c-Myc tag sequence was flanked by the RE sites *Bsi*WI and *Pst*I at the 5'- and 3'-end,

respectively. The TAA nucleotides added at the 3'-end of the c-Myc tag fragment to act as the translational stop codon for the GOI cassette. A calculated amount of each oligonucleotide was mixed and denatured at 95°C for 5 minutes and allowed to cool gradually at 5°C steps in 30 second intervals to allow annealing to occur (appendix C). The short double stranded fragments were then recovered via ethanol precipitation according to section (3.2.3.2.) before further processing.

3.2.6. Restriction enzyme digestion and other enzymatic reactions

RE digestion, CIP treatment and ligation reactions were routinely performed throughout the vector construction process and were achieved as described in the following sections.

3.2.6.1. Restriction enzyme (RE) digestions

RE digestion reactions were performed extensively throughout this study for the construction of recombinant chloroplast transformation vectors. All RE digests were performed in 20µl reaction volumes in 200µl thin-walled PCR tubes. RE digestion assays were performed using the recommended incubation temperatures and duration as specified by the manufacturer. All REs were purchased from either Fermentas (USA) or New England Biolabs (USA) and performed using 1u of RE for every 1000ng DNA material unless specified otherwise.

3.2.6.2. Phosphatase treatment with calf intestinal phosphatase (CIP)

Phosphatase treatments were performed on RE digested products prior to ligation for a number of experiments using the CIP enzyme (New England Biolabs, USA) in order to reduce the formation of empty plasmid vectors. CIP dephosphorylates the overhanging regions of RE digested DNA and prevents the

recircularisation of empty plasmid vectors. Reaction mixtures were set up as described in the user's manual. The mixtures were incubated at 37°C for 1 hour to allow the reaction to proceed.

3.2.6.3. DNA ligation

All ligation reactions were carried out using the T4-DNA ligase (New England Biolabs, USA) for 16 hours at 16°C. The molar ratio of insert to vector was adjusted to 3:1 for each standard ligation reaction. For the ligation of c-Myc to GFP, a molar ratio of 10:1 (c-Myc to GFP) was used instead.

3.2.7. Transformation of competent cells

Competent *E. coli* cells were used routinely throughout this study for the propagation of plasmids as well as for the evaluation of transgene expression (Chapter 3 and 4). Preparation of competent *E. coli* cells was mainly based on the method of Nishimura *et al.* (1990) with minor modifications.

3.2.7.1. Preparation of competent cells by the CaCl₂ method

Top-10 *E. coli* competent cells were prepared according to the method of Nishimura *et al.* (1990). A frozen stock culture of Top-10 *E. coli* competent cells are allowed to thaw on ice for 1-2 minutes. A 5-10µl sample of the stock culture was transferred using a pipette onto a Petri dish containing 20ml of antibiotic-free LB solid medium. The competent cell sample was streaked to single colony and incubated at 37°C for 16 hours. A single bacterial colony was selected from the plate and pure cultured. *E. coli* colonies displaying normal morphologies were selected for broth culture in a sterile Falcon tube containing 10ml of antibiotic-free LB liquid medium. The bacterial broth culture was incubated 37°C with constant agitation at 200rpm for 16 hours in an incubator shaker (Innova 42, Eppendorf, Germany). A 500µl sample from the competent cell broth culture was

transferred by pipetting into a sterile 250ml conical flask containing 50ml of SOC medium (section M2) and placed in an incubator shaker at 37°C with constant agitation at 200rpm for approximately 2 hours. The OD_{600nm} was measured at every subsequent 15-minute intervals using the biophotometer (section 3.2.4.2.). The bacterial culture was chilled on ice immediately when the bacterial growth is in the mid-logarithmic phase (OD_{600nm} of approximately 0.5). The bacterial culture was transferred to a sterile 50ml Falcon tube and incubated on ice for 10 minutes. The bacterial culture was centrifuged (Allegra™ X-22R, Beckman Coulter, USA) at 6000rpm, 4°C for 6 minutes to pellet the bacterial cells. The supernatant was discarded and the following steps were carried out on ice. The pellet was resuspended in 500µl of pre-chilled SOC media. For cryopreservation, 2.5ml of storage solution (section M2) was added to the resuspended bacteria and mixed by gentle pipetting. Aliquots of competent cells were prepared by transferring 100-120µl of the culture into pre-chilled sterile 1.5ml tubes. Samples were stored at -80°C.

3.2.7.2. Transformation of competent cells via heat shock method

An aliquot of competent cells (100µl) was thawed on ice for 1-2 minutes. A 5µl sample (≤ 5% of competent cell volume) of plasmid DNA or ligation reaction mixture was transferred into the competent cell suspension by pipetting. The mixture was mixed briefly by tapping and incubated on ice for 30 minutes. The competent cell mixture was subjected to heat shock by placing the mixture in a 42°C water bath for 45-90 seconds. The mixture was then transferred immediately on ice for 1-2 minutes. Nine hundred microliters of SOC medium was added to the mixture and the mixture was incubated with continuous agitation at 200rpm, 37°C for 1 hour. The culture was centrifuged at 13000 rpm for 1 minute to pellet bacterial cells. Excess supernatant was discarded. The bacterial pellet was resuspended in the remaining supernatant and transferred to a Petri dish containing 20ml of solid LB media supplemented with 20µl of

ampicillin (100mg/ml). The bacterial suspension was streaked evenly to single colony. For the propagation of plasmids, only 10µl of the bacterial suspension was transferred to each plate. The plates were incubated at 37°C for 16 hours.

3.2.8. Construction of the carrier plasmid, pCART

Construction of the carrier plasmid was based on the design of the pKCZ chloroplast transformation vector (Zou *et al.* 2003). Two new restriction enzyme (RE) sites were introduced to facilitate the cloning of expression cassettes for the gene of interest (GOI). The endogenous *Prrn* promoter was also substituted with the constitutive *PtrnFM* promoter from the tobacco plastome to facilitate the stable expression of transgenes.

3.2.8.1. Introduction of new RE site

Based on the overall design of the new chloroplast transformation vector, two unique RE sites were introduced to facilitate the cloning of the expression cassettes for the GOIs. Primers PFMCS and PRMCS were synthesised for the introduction of the *Mlu*I and *Afl*II RE sites, respectively. The primers were designed to flank and amplify a spacer sequence (*Npt*I kanamycin resistance gene; accession no: AY297842). Both the amplified PCR product and the pKCZ original chloroplast transformation vector were completely digested with *Bg*/II and *Nhe*I and purified accordingly. The digested PCR fragment was subsequently ligated into the MCS of the pKCZ backbone as a *Bg*/II-*Nhe*I fragment. The resultant plasmid was subsequently termed as pKCZMCS (section 3.3.1.1.).

3.2.8.2. Amplification of the *PtrnfM* promoter from the chloroplast genome

Primers PF:*NheI-PtrnfM* and PR:*EcoRV-PtrnfM* were designed for the amplification of the promoter region of the initiator tRNA-fMet gene, termed as *PtrnfM*, directly from the chloroplast genome of tobacco (*Nicotiana tabacum* chloroplast genome NC_001879.2; nt.38497-38585). The forward primer PF:*NheI-PtrnfM* was designed to introduce the *NheI* RE site at the 5'-end of the *PtrnfM* amplicon. The reverse primer PR:*EcoRV-PtrnfM* was designed to introduce the blunt *EcoRV* RE site at the 3'-end of the *PtrnfM* promoter. The final *PtrnfM* PCR product was flanked at the 5'-end and the 3'-end by the *NheI* and *EcoRV* RE sites, respectively which were subsequently used for the cloning of the *PtrnfM* promoter into plasmid pKCZMCS.

3.2.8.3. Substitution of the *Prrn* promoter in the *aadA* expression cassette with the *PtrnfM* promoter

The *aadA* antibiotic resistance gene expression cassette in the original pKCZ chloroplast transformation vector and its derivative pKCZMCS is driven by the 16S rRNA promoter (*Prrn*). In regard to the stable regeneration of transplastomic plants, the *Prrn* promoter that drives the *aadA* antibiotic selection gene was replaced with the constitutive *PtrnfM* promoter. The *PtrnfM* promoter was cloned from chloroplast genomic DNA as described in section (3.2.5.). The native *Prrn* promoter of pKCZMCS was removed by the combined digestion of *NheI* and *EcoRV*. The backbone of pKCZMCS was subsequently recovered and ligated with the purified *PtrnfM* PCR product following double digestion with *NheI* and *EcoRV*. The resulting recombinant plasmid pKCZMCS*PtrnfM* was subsequently named as pCART section (3.2.8.).

3.2.9. Construction of the expression plasmid, pGENE

The *psbA* 5'-UTR leader sequence was cloned downstream of the endogenous *Prrn* promoter of the *aadA* expression cassette of a separate pKCZ transformation vector. The GFP:c-Myc fragment was cloned downstream of the *psbA* 5'UTR leader sequence at the previous position of *aadA*.

3.2.9.1. Amplification of the *psbA* 5'-UTR leader region from the tobacco chloroplast genome

A pair of primers was synthesised for the amplification of the 5'-UTR leader region of the *psbA* gene (*psbA* 5'-UTR) directly from the tobacco chloroplast genome (*Nicotiana tabacum* chloroplast genome NC_001879.2; nt.1595-1682). The forward primer PF:*EcoRV-psbA5'* was designed to introduce the *EcoRV* RE site at the 5'-end of the *psbA* 5'-UTR fragment while the reverse primer PR:*NcoI-psbA5'* was designed to introduce the *NcoI* RE site at the 3'-end of the *psbA* 5'-UTR fragment. The resultant *psbA* 5'-UTR PCR product was flanked with the *EcoRV* and *NcoI* RE sites, respectively which could be subsequently used for the cloning of the *psbA* 5'-UTR leader region into pKCZ.

3.2.9.2. Cloning of the *psbA* 5'-UTR leader region into pKCZ

The *psbA* 5'-UTR leader region was introduced downstream of the *Prrn* promoter of the *aadA* antibiotic resistance gene cassette of the pKCZ chloroplast transformation vector. The pKCZ vector was completely digested with *EcoRV* and *NcoI* and a small 27bp nucleotide fragment was removed from the backbone during the subsequent purification process (3.3.2.1.). The *psbA* 5'-UTR leader region was cloned from the genomic DNA of tobacco chloroplasts as described in section (3.2.5.) and digested with *EcoRV* and *NcoI*. The digested product was then purified prior to ligation with the digested pKCZ backbone. The resultant recombinant plasmid was designated as pKCZ*psbA5'UTR*.

3.2.9.3. Preparation of the GFP:c-Myc fragment

A set of primers were designed based on sequence information that was available for the amplification of GFP from control vector HBT95::sGFP(S65T)-NOS (vector accession no: EF090408). The forward primer PF1:GFP-*Nco*I was designed to introduce the *Nco*I RE site at the 5'-end of the GFP amplicon as well as the ATG start codon for the translation of the GFP gene. The reverse primer PR2:GFP-*Bsi*WI was designed to incorporate the *Bsi*WI RE site at the 3'-end of the GFP amplicon. In addition, the reverse primer was also designed to incorporate the factor Xa protease recognition sequence (Thr-Thr-Phe-Asp) between the GFP gene fragment and the c-Myc tag. The c-Myc tag was assembled as described in section (3.2.5.2.). Both the purified GFP PCR product and the c-Myc tag were RE digested with *Bsi*WI and purified accordingly (GFP: section 3.2.3.1.; c-Myc: section 3.2.3.2.) prior to ligation to produce the GFP:c-Myc fragment. The resulting GFP:c-Myc fragment was flanked by the *Nco*I RE site at the 5'-end of the GFP gene and the *Pst*I RE site at the 3'-end of the c-Myc tag fragment which could be used for subsequent cloning steps (section 3.2.9.4.).

3.2.9.4. Construction of the GFP gene expression cassettes

In order to facilitate the construction of expression cassettes for the other GOIs, the GFP:c-Myc fragment was first incorporated into the pKCZ transformation vector as a holding vector. The pKCZ transformation vector was double digested to completion with *Nco*I and *Pst*I and separated from the *aadA* gene via gel electrophoresis. The pKCZ vector backbone was then purified from the gel. The GFP:c-Myc fragment was assembled according to section (3.2.9.3.) and subjected to double digestion to completion with *Nco*I and *Pst*I. Following purification, the digested pKCZ and GFP:c-Myc fragments were then ligated together to produce the recombinant plasmid pKCZGFP:c-Myc which was subsequently named as pROTO.

Once the GFP:c-Myc fragment was stably incorporated in plasmid form, the GFP:c-Myc fragment could easily be reproduced via PCR amplification from pROTO using the PF1:GFP-*Nco*I primer as the forward primer and the reverse complement oligonucleotide used for the assembly of the c-Myc fragment (appendix B) as the reverse primer. In order to produce an expression cassette for the GFP gene with the *psbA* 5'-UTR leader region, the GFP:c-Myc fragment was PCR amplified from pROTO and cloned into plasmid pKCZ*psbA*5'UTR using the same RE digestion and ligation approach. The resultant recombinant plasmid pKCZ*psbA*5'UTRGFP:c-Myc was subsequently named as pGENE.

3.2.10. Assembly of the final pEXPR chloroplast transformation vectors

A pair of universal primers was designed for the cloning of all gene expression cassettes. The forward primer, OMNI:F-*Afl*II was an exact complement to the *Prrn* promoter region and was designed to incorporate the *Afl*II RE site at the 5'-end of the *Prrn* promoter. The reverse primer OMNI:R-*Mlu*I was a reverse complement to the c-Myc tag region and was designed to incorporate the *Mlu*I RE site at the 3'-end of the c-Myc tag. In addition, OMNI:R-*Mlu*I also incorporated 2 consecutive stop codons (TAA TGA) at the end of the c-Myc tag region that act as putative translational terminators. The gene-independent nature of the OMNI primers would allow the same pair of primers to be used for the cloning of all expression cassettes which are flanked by the *Prrn* promoter and the c-Myc tag.

In order for the assembly of the final transformation vectors, the *Npt*I spacer region was first deleted from the MCS of pCART. The carrier plasmid, pCART was double digested to completion with *Afl*II and *Mlu*I and the plasmid backbone was separated from the *Npt*I spacer via gel electrophoresis and recovered accordingly. The gene expression cassettes for the GOIs were

amplified via PCR from their respective (pROTO or pGENE) plasmids using the OMNI primers. The amplified expression cassettes were then subjected to double digestion using REs *AflII* and *MluI*. The digested products were subsequently purified and ligated into the MCS of pCART to produce the final pEXPR chloroplast transformation vectors. The chloroplast transformation vector for the expression of GFP without the *psbA* 5'-UTR was designated as pEXPR-G and the chloroplast transformation vector for the expression of GFP with the *psbA* 5'-UTR region was designated as pEXPR-UG.

Information on all primers used for the construction process are summarised in appendix (B). A schematic diagram of the construction process is outlined in figure 3.1. The pGENE and pEXPR nomenclatures were subsequently used to describe all recombinant plasmids for the expression of different genes of interest. For example, the expression cassette for a particular gene of interest (GOI) was named as pGENE-GOI and the chloroplast transformation vector for said gene was named as pEXPR-GOI. In this study, all intermediate forms of circular double stranded DNA in the construction process are termed as "plasmids" and the term "vector" is only used to describe the original pKCZ and final pEXPR chloroplast transformation vectors.

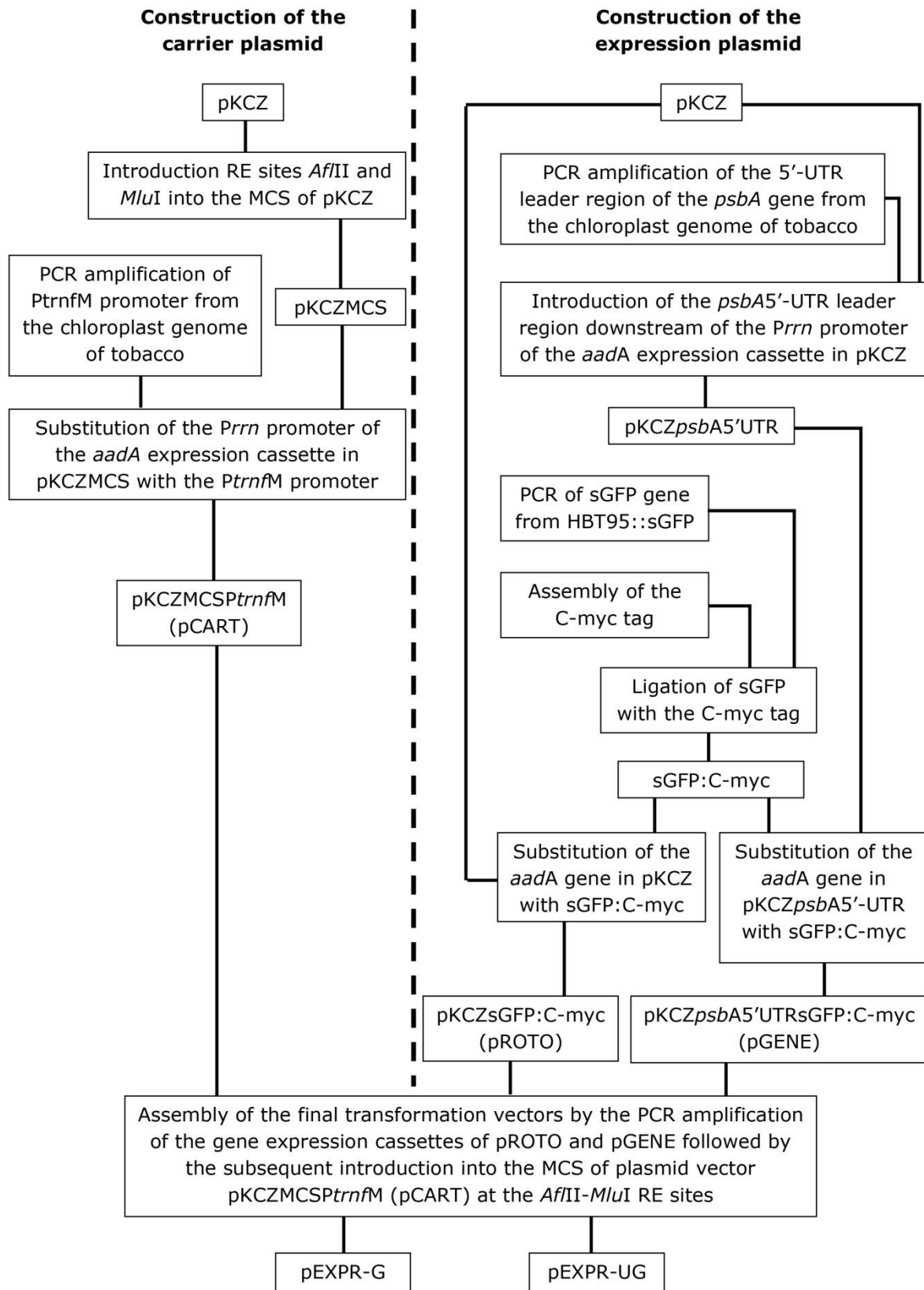


Figure 3.1: Schematic diagram to summarise the construction of recombinant chloroplast transformation vectors, pEXPR-G and pEXPR-UG for the delivery of GFP via modification of chloroplast transformation vector pKCZ.

3.2.11. Construction of chloroplast transformation vectors for other GOIs

Customisation of the chloroplast transformation vectors for the delivery and expression of other GOIs can be easily achieved using the developed 2-part vector system. The GOI was subjected to PCR amplification using gene specific forward and reverse primers to introduce RE sites *NcoI* and *BsiWI* at the 5'- and 3'- ends, respectively. The amplified GOI sequence was then cloned into pGENE after the removal of GFP via double RE digestion with *NcoI* and *BsiWI* to produce recombinant plasmid pGENE-GOI. The entire GOI expression cassette was then amplified from pGENE-GOI and flanked with RE sites *AflII* and *MluI* at the 5'- and 3'- ends, respectively using the universal forward and reverse primers, OMNI:F-*AflII* and OMNI:R-*MluI*. The amplified fragment was subsequently cloned into pCART after the removal of the *NptI* spacer region following double RE digestion with *AflII* and *MluI* to produce the completed chloroplast transformation vector pEXPR-GOI. A schematic diagram for the entire process is outlined in figure 3.2.

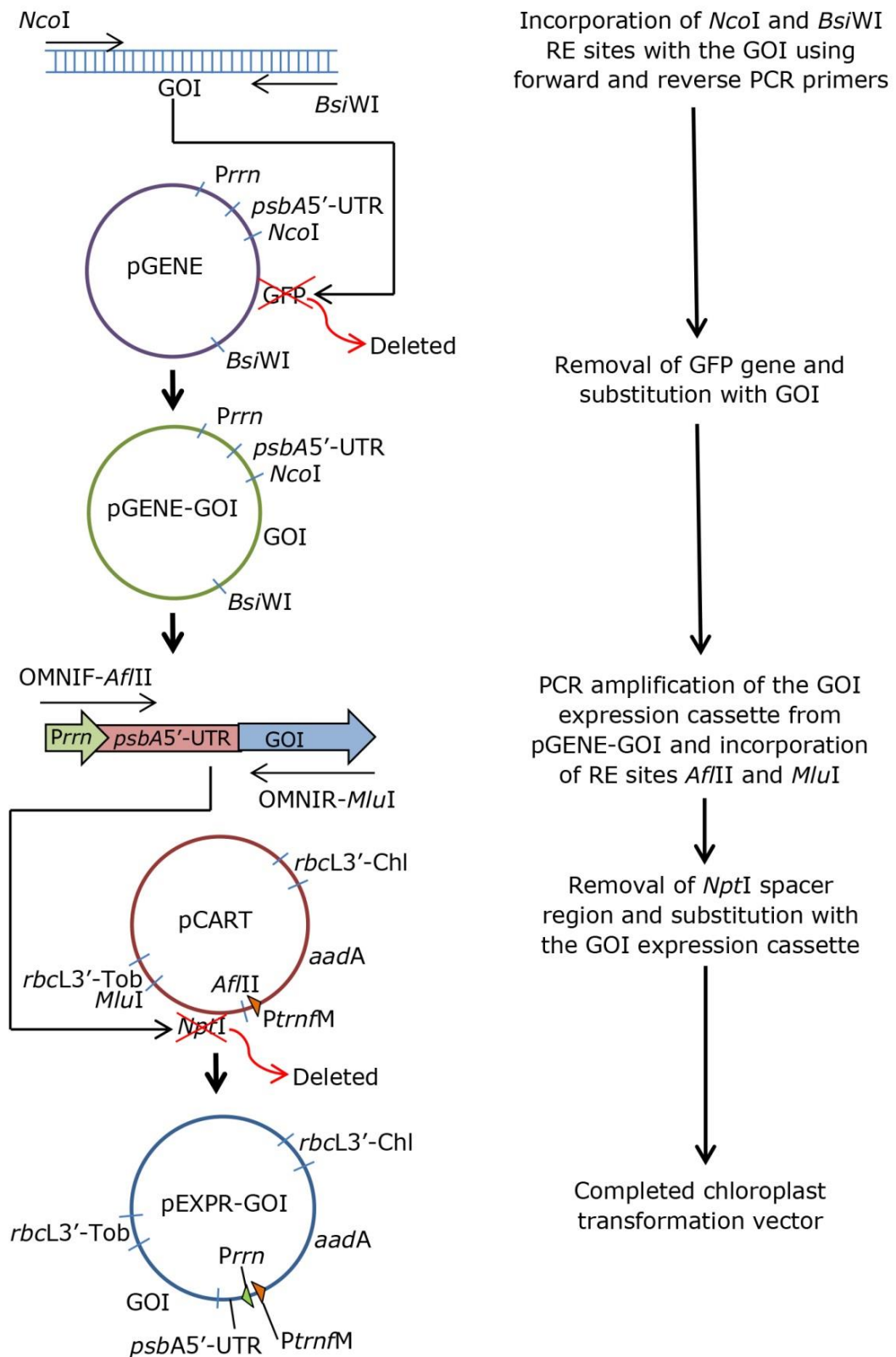


Figure 3.2: Schematic diagram to summarise the methodology for the construction of new chloroplast transformation vectors for the expression of other gene of interest (GOI) using the established 2-part vector system.

3.3 RESULTS

3.3.1. Construction of the Carrier Plasmid (pCART)

3.3.1.1. Introduction of New RE Sites *Afl*II and *Mlu*I Using a Spacer Region

A band of ~800bp in molecular size was obtained in the PCR amplification using primers PFMCS and PRMCS (appendix B) from pGR107 plasmid templates. This corresponded to the size of the *Npt*I gene (816bp). A schematic diagram of the cloning results for the introduction of RE sites *Afl*II and *Mlu*I into the MCS of pKCZ is illustrated in figure 3.3.

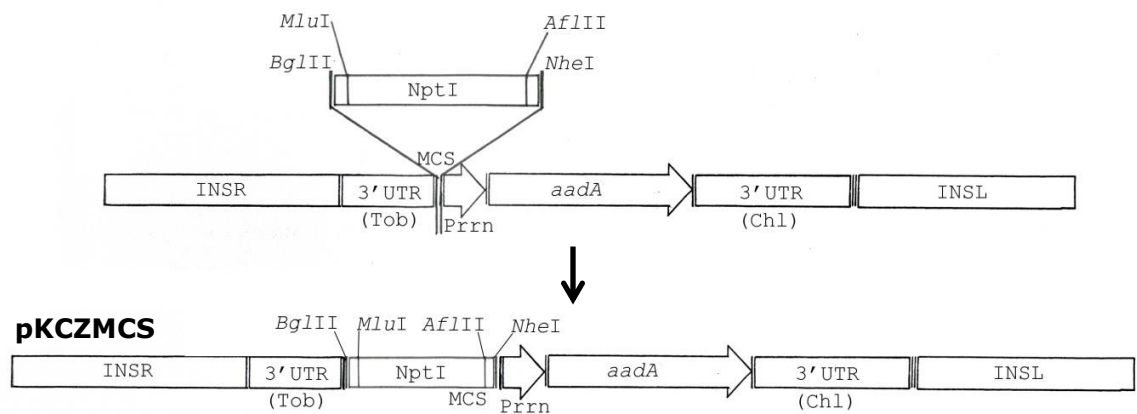


Figure 3.3: Schematic diagram for the construction results of pKCZMCS. Primers, PFMCS and PRMCS were used for the introduction of the 2 new RE sites, *Afl*II and *Mlu*I. The *Npt*I gene was used as a spacer for the incorporation of the 2 new RE sites which can be subsequently removed via double digestion with *Afl*II and *Mlu*I.

The identity of the recombinant plasmid product was characterised via RE digestion with *Afl*II and *Mlu*I by both single and double digestion reactions (figure 3.4). Single RE digestion reactions with either *Afl*II or *Mlu*I produced a single defined band (~7.3kb) which corresponded to the combined sizes of the pKCZ vector backbone (6.5kb) and the *Npt*I gene of pGR107 (~800bp). The double RE digestion reaction with *Afl*II and *Mlu*I produced 2 bands with

molecular sizes of ~6.5kb and ~800bp corresponding to the pKCZ vector backbone and the *NptI* gene, respectively. The sequence identity of the recombinant plasmid product, pKCZMCS, was also conclusively confirmed via sequencing reactions (appendix Fvi).

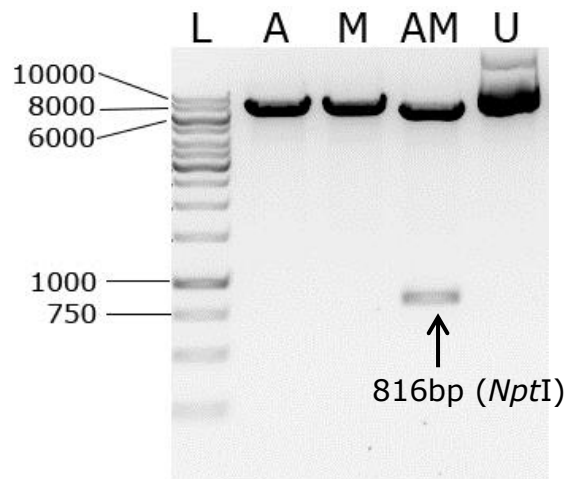


Figure 3.4: RE profiles for the verification of pKCZMCS. The removal of the 816bp *NptI* spacer region is achieved with the double digestion of pKCZMCS with *AflII* and *MluI* (AM) showing that all RE sites were intact. L: Fermentas 1kb DNA ladder, A: *AflII* RE single digest resulting in the linearization of pKCZMCS, M: *MluI* RE single digest resulting in the linearization of pKCZMCS, AM: *AflII* and *MluI* RE double digest causing the removal of the *NptI* spacer region (816bp), U: Undigested control.

3.3.1.2. Substitution of the *Prrn* Promoter of pKCZMCS with the *PtrnfM* Promoter

A band of ~100bp was obtained in the PCR amplification using primers, PF:*NheI*-*PtrnfM* and PR:*EcoRV*-*PtrnfM* (appendix B) from the genomic DNA of tobacco chloroplasts (figure 3.5). This corresponded to the size of the *PtrnfM* promoter (~92bp) of *Nicotiana tabacum*. A schematic diagram showing the results for the substitution of the *Prrn* promoter of the *aadA* gene expression cassette in pKCZMCS with the *PtrnfM* promoter is illustrated below (figure 3.6). The identity of the recombinant plasmid product was evaluated via PCR, RE

digestion with RE *BsrGI* and via sequencing. RE verification with *BsrGI* produced a single band of ~6.7kb corresponding to a single digest at the unique *BsrGI* site located only within the *PtrnfM* promoter region. The sequencing results conclusively confirmed that the expected results were achieved for the construction of recombinant plasmid pKCZMCS*PtrnfM*. The completed carrier plasmid, pKCZMCS*PtrnfM*, was designated as pCART.

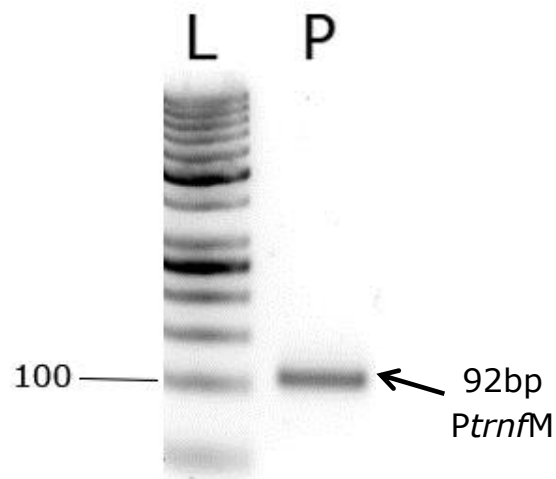


Figure 3.5: PCR profile for the amplification of the 92bp *PtrnfM* promoter of initiator tRNA from the chloroplast genomic DNA of *Nicotiana benthamiana*. L: Fermentas 50bp DNA ladder, P: *PtrnfM* PCR product.

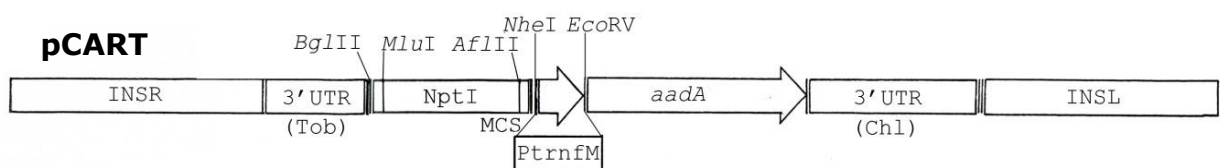


Figure 3.6: Schematic diagram for the results of the construction of pCART (pKCZMCS*PtrnfM*). The endogenous *Prrn* promoter was removed from pKCZMCS via double digestion with *NheI* and *EcoRV*. The *PtrnfM* promoter was then ligated into the previous position of *Prrn* to produce the recombinant plasmid, pCART.

3.3.2. Construction of the Expression Plasmid (pGENE)

3.3.2.1. Introduction of the *psbA* 5'UTR Leader Sequence

Primers, PF:*EcoRV-psbA5'* and PR:*NcoI-psbA5'* (appendix B) were used for the amplification of the *psbA* 5'-UTR leader region of the *psbA* photosynthetic gene from the genomic DNA of tobacco chloroplasts. A band of ~100bp was obtained which corresponded to the size of the *psbA* 5'-UTR (~88bp) of *Nicotiana tabacum* (figure 3.7). The results for the cloning of the *psbA* 5'-UTR leader region downstream of the endogenous *Prrn* promoter of the *aadA* gene expression cassette in pKCZ are shown in figure 3.8. The identity of the recombinant plasmid product was evaluated via PCR and sequencing. PCR detection of the *psbA* 5'-UTR leader region from the recombinant plasmid product produced a band of ~100bp confirming the presence of the *psbA* 5'-UTR leader region. The sequencing results conclusively confirmed that the *psbA* 5'-UTR was integrated in the correct orientation downstream of the endogenous *Prrn* promoter to produce recombinant plasmid, pKCZ*psbA5'UTR*.

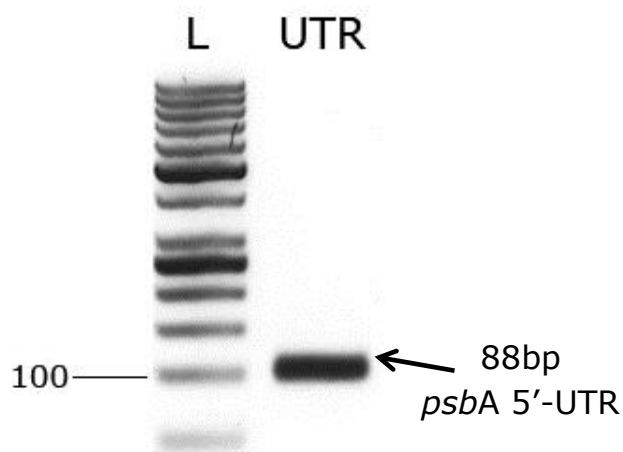


Figure 3.7: PCR profile for the amplification of the 88bp *psbA* 5'-UTR leader region from the chloroplast genomic DNA of *Nicotiana benthamiana*. L: Fermentas 50bp DNA ladder, UTR: *psbA* 5'-UTR PCR product (88bp).

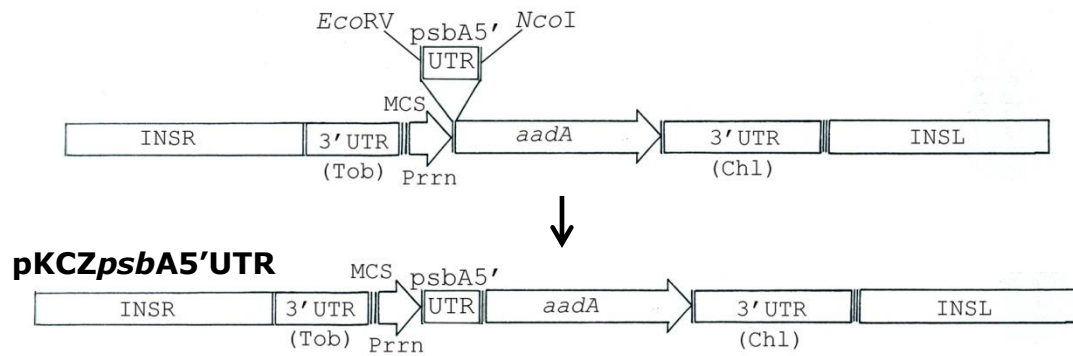


Figure 3.8: Schematic diagram for the results of the construction of pKCZ*psbA5'UTR*. The *psbA5'-UTR* leader region was cloned downstream of the endogenous *Prrn* promoter sequence for the *aadA* expression cassette in pKCZ at the *EcoRV-NcoI* site to produce pKCZ*psbA5'UTR*.

3.3.2.2. Construction and Cloning of the GFP:c-Myc fragment

The successful ligation of the GFP gene with the c-Myc tag was evaluated via PCR using primers, PF:GFP-*NcoI* and c-Myc (-) (appendix B). A defined ~700bp band was obtained which indicated the successful ligation of the GFP gene fragment with the c-Myc epitope tag. A schematic diagram for the results of the cloning of the GFP:c-Myc fragment into vector pKCZ is shown in figure 3.9. The recombinant plasmid product was evaluated via PCR, RE digestion with *BsiWI* and sequencing. PCR screening of the recombinant plasmid product with primers, PF:GFP-*NcoI* and c-Myc (-) (appendix B) produced a defined band of ~700bp corresponding to the size of the GFP:c-Myc fragment. RE verification with *BsiWI* caused the linearization of the plasmid (~7.2kb band) due to a single cleavage at the *BsiWI* RE recognition site between the GFP gene and the c-Myc tag. Sequencing results conclusively confirmed the orientation of GFP:c-Myc within pKCZ and that the cloning of the GFP:c-Myc fragment into pKCZ proceeded as expected for the construction of recombinant plasmid pKCZGFP:c-Myc. The resulting recombinant plasmid, pKCZGFP:c-Myc, served as a prototype expression plasmid and was subsequently named as pROTO.

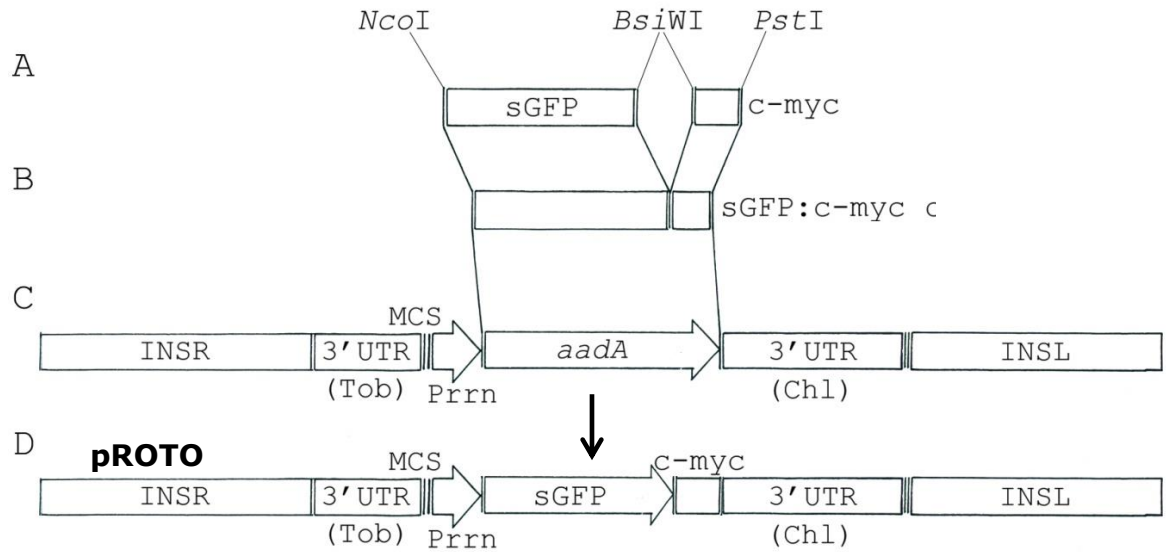


Figure 3.9: Schematic diagram for the results of the construction of recombinant plasmid pKZGFP:c-Myc (pROTO). A: PCR of GFP and the assembly of c-Myc, B: Ligation of the GFP gene and the c-Myc tag to produce GFP:c-Myc, C: Substitution of the endogenous *aadA* gene with the GFP:c-Myc fragment, D: The completed construction of the preliminary GFP expression cassette within the pKZ vector backbone.

3.3.2.3. Cloning of GFP:c-Myc into pKZ*psbA5'UTR*

Following the construction of recombinant plasmid pROTO, primers, PF:GFP-*NcoI* and c-Myc (-) (appendix B) were used for the cloning of the GFP:c-Myc fragment from pROTO into plasmid pKZ*psbA5'UTR*. A schematic diagram for the results of the construction of recombinant plasmid, pKZ*psbA5'UTR*::GFP:c-Myc is shown in figure 3.10. The identity of the recombinant plasmid product was evaluated via PCR analysis and sequencing. PCR analysis of pKZ*psbA5'UTR*::GFP:c-Myc using primers, PF:*EcoRV-psbA5'* and c-Myc (-) (appendix B) produced a ~800bp band which corresponded approximately to the sizes of *psbA5'-UTR*, GFP and c-Myc. Sequencing results for recombinant plasmid, pKZ*psbA5'UTR*::GFP:c-Myc confirmed the correct assimilation of the GFP:c-Myc fragment into the pKZ*psbA5'UTR* plasmid

backbone. The recombinant plasmid, pKCZ*psbA5'*UTR::GFP:c-Myc, was subsequently nominated as pGENE.

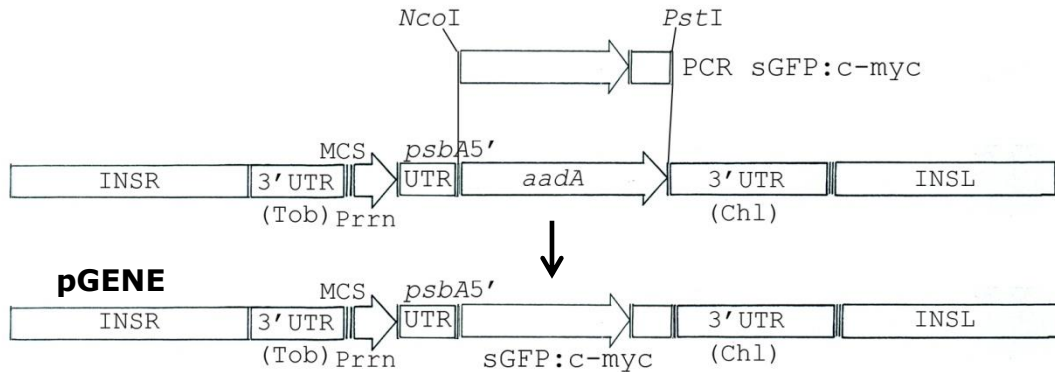


Figure 3.10: Schematic diagram for the results of the construction of recombinant plasmid pKCZ*psbA5'*UTRGFP:c-Myc (pGENE). The *aadA* gene of pKCZ*psbA5'*UTR was removed via double digestion with *NcoI* and *PstI*. The GFP:c-Myc fragment was amplified from pROTO via PCR using primers, PF:GFP-*NcoI* and c-Myc (-) and cloned into the previous position of the *aadA* gene of pKCZ*psbA5'*UTR producing the completed GFP expression cassette in pGENE.

3.3.3. Assembly of the final transformation vectors

Primers, OMNI:F-*AflII* and OMNI:R-*MluI* (appendix B) were used for the cloning of the gene expression cassettes from recombinant plasmids, pROTO, and pGENE, respectively. A single defined band of ~900bp was observed for the PCR amplification of pROTO and pGENE using primers, OMNI:F-*AflII* and OMNI:R-*MluI* (figure 3.11). The bands correspond approximately to the sizes of the GFP gene expression cassettes of pROTO (*Prrn*:GFP:c-Myc; 896bp) and pGENE (*Prrn*:*psbA5'*-UTR:GFP:c-Myc; 984bp) (appendix Fiii), respectively.

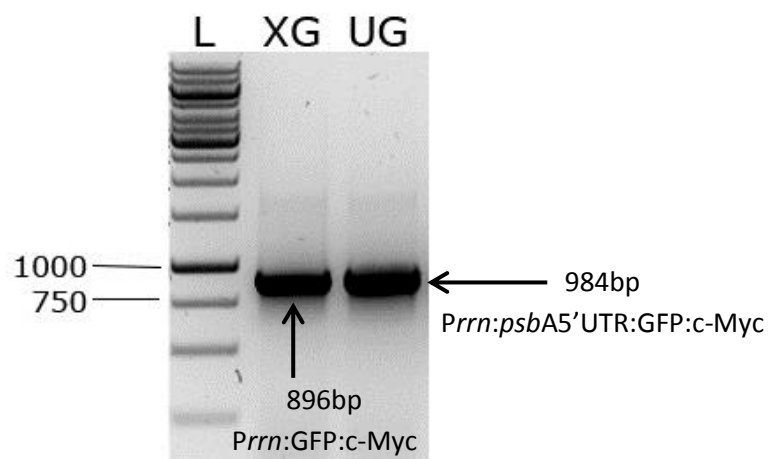


Figure 3.11: PCR amplification profile of the GFP expression cassettes from pROTO and pGENE using primers, OMNI:F-*Afl*II and OMNI:R-*Mlu*I resulting in the amplification of the 896 and 984bp GFP expression cassettes, respectively. L: Fermentas 1kb DNA ladder, XG: GFP expression cassette of pROTO (*Prrn*:GFP:c-Myc), UG: GFP expression cassette of pGENE (*Prrn*:*psbA5'*-UTR:GFP:c-Myc).

3.3.4. Verification of the identity of the final chloroplast transformation vectors

A schematic diagram for the results of the cloning of the gene expression cassettes of recombinant plasmids, pROTO and pGENE into pCART is illustrated in figure 3.12. The final assimilation of the gene expression cassettes of pROTO and pGENE into pCART produced the two chloroplast transformation vectors, pEXPR-G and pEXPR-UG, respectively. The identity of chloroplast transformation vectors, pEXPR-G and pEXPR-UG were evaluated via RE verification and sequencing. The final vectors, pEXPR-G and pEXPR-UG were double digested with REs *Afl*II and *Mlu*I which caused the release of the respective gene expression cassettes from the pCART vector backbone (figure 3.13). Both double RE digestion reactions produced two distinct bands. A band of ~6.5kb was observed in all four samples which correspond to the size of the backbone of pCART. Bands of ~900bp and ~1000bp were also observed corresponding to the sizes of the cloned gene expression cassettes of pROTO (896bp) and pGENE

(984bp), respectively. Further sequencing results conclusively confirmed the correct assimilation of the gene expression cassettes into the backbone of pCART and the identity of both chloroplast transformation vectors. A complete summary of the results of the modification and construction of the recombinant chloroplast transformation vectors is shown in figure 3.14.

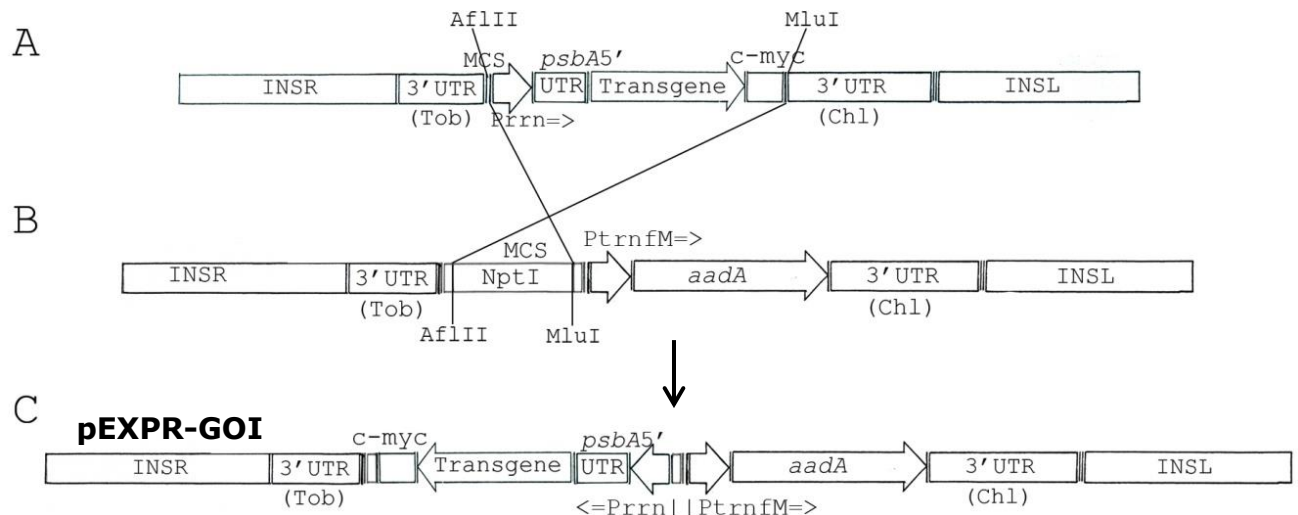


Figure 3.12: Schematic diagram for the results of the construction of the final chloroplast transformation vectors pEXPR-G (transformation vector for GFP without the *psbA* 5'-UTR) and pEXPR-UG (transformation vector for GFP with the *psbA* 5'-UTR). A: The gene expression cassettes were amplified from their respective vectors as an *AflII*-*MluI* fragment, B: The *NptI* spacer fragment was removed from pCART via double RE digestion with *AflII* and *MluI*. The amplified gene expression cassettes were subsequently cloned into the backbone of pCART as an *AflII*-*MluI* fragment, C: The final chloroplast transformation vectors contained two expression cassettes for the expression of *aadA* and the GOI in opposite orientations.

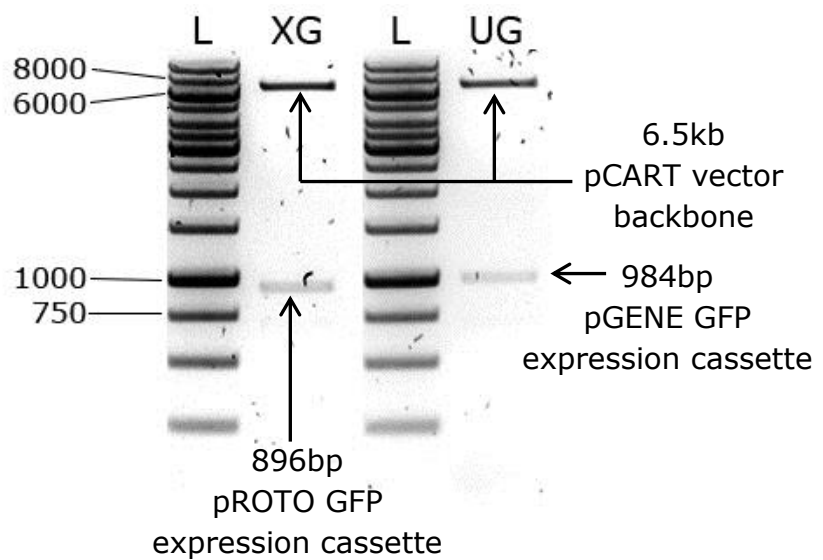


Figure 3.13: RE profile for the verification of pEXPR-G and pEXPR-UG via double digest with REs *AflII* and *MluI*. The double digestion of the final recombinant chloroplast transformation vectors with *AflII* and *MluI* will result in the excision of the GFP expression cassettes of pROTO (896bp) and pGENE (984bp) from the pCART backbone (6.5kb). L: Fermentas 1kb DNA ladder, XG: pEXPR-G, UG: pEXPR-UG.

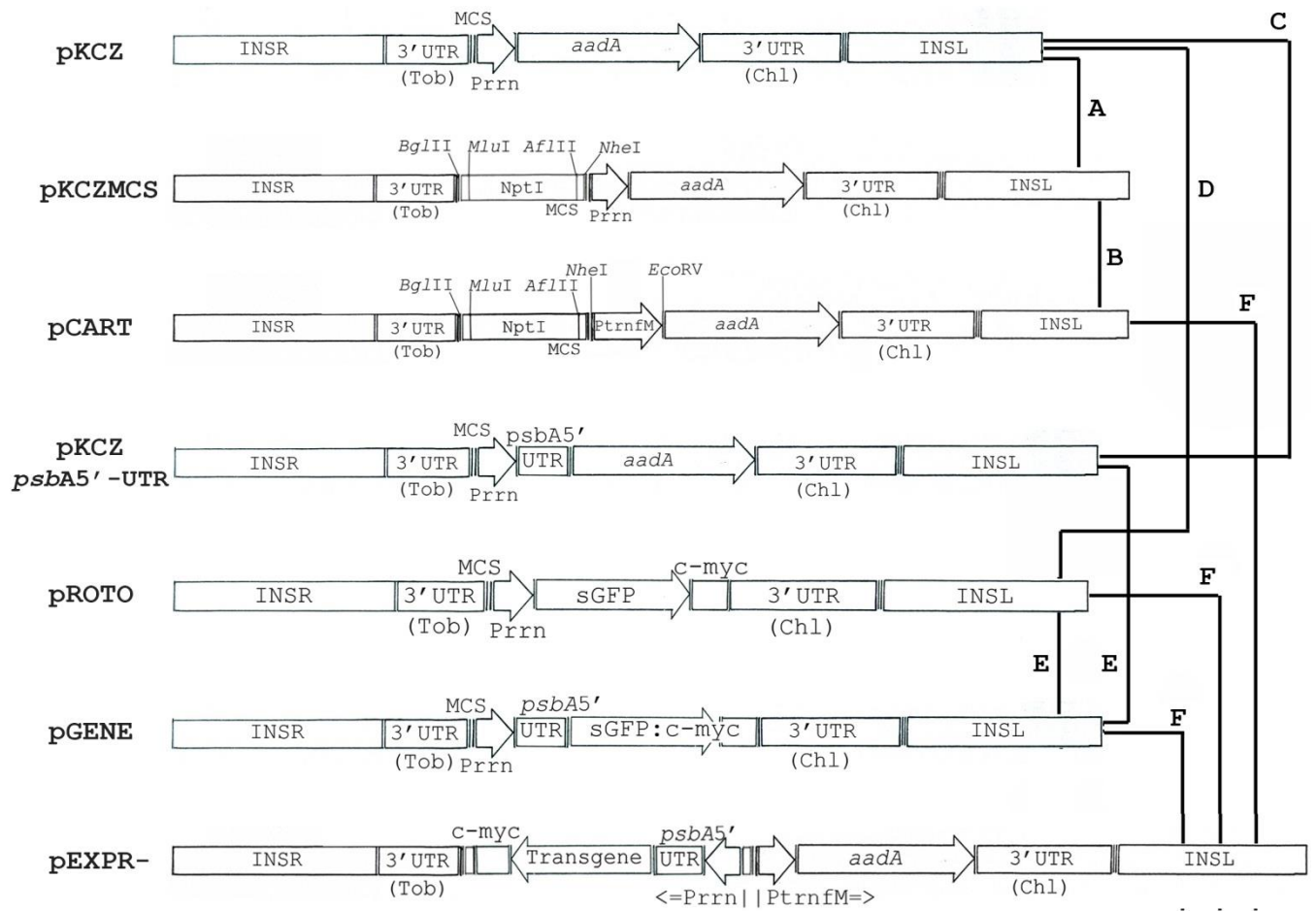


Figure 3.14: Schematic diagram to summarise the construction of the completed chloroplast transformation vector, pEXPR. A: Introduction of the new *AflII* and *MluI* RE sites into the MCZ of pKCZ, B: Substitution of the endogenous *Prrn* promoter of the *aadA* gene expression cassette with the *PtrnfM* promoter, C: Introduction of *psbA* 5'-UTR into pKCZ downstream of the endogenous *Prrn* promoter for the *aadA* gene, D: Substitution of the *aadA* gene in the gene expression cassette of pKCZ with the GFP:c-Myc fragment, E: Cloning of the GFP:c-Myc fragment from recombinant plasmid, pPROTO into the plasmid backbone of pKCZ*psbA5'UTR*, F: Assembly of the final chloroplast transformation vectors, pEXPR-G and pEXPR-UG. The expression cassettes for the GOIs were cloned from plasmids pPROTO and pGENE, respectively into the MCS of recombinant plasmid pCART at the new *AflII*-*MluI* RE sites.

3.4. DISCUSSION

This study was commenced by generating an improved chloroplast transformation vector system. The design concept of the original pKCZ chloroplast transformation vector focused on high versatility. However the usefulness of the original vector was overshadowed by several disadvantages such as the tendency to cause aberrant intermolecular recombination, inefficient gene expression cassettes, complicated customisation procedures, and most unfavourably, the inability to generate stable homoplastomic plants. Nevertheless, the original idea of a versatile chloroplast transformation vector was an intriguing concept and the original pKCZ vector was used as a template for improvement. In this study, a 2-part system was developed in order to improve on the concept of 'versatility', where the "host-specific" elements of the vector was encompassed in one plasmid (pCART) and the "transgene-specific" elements were encompassed in another (pGENE). Hence, this allowed easy access to each individual component for easy customisation and the flexibility of mixing and matching several different pCART and pGENE plasmid constructs for maximum expression. In addition, improvements were also made to eliminate aberrant intermolecular recombination events which would potentially allow the regeneration of stable homoplastomic plants, a critical aspect of chloroplast transformation. A schematic diagram summarising the key modifications made to plasmid vector pKCZ to produce carrier plasmid pCART and expression plasmid pGENE is shown in figure 3.15 and the function of each component of pCART, pGENE and pEXPR-GOI is outlined in table 3.1.

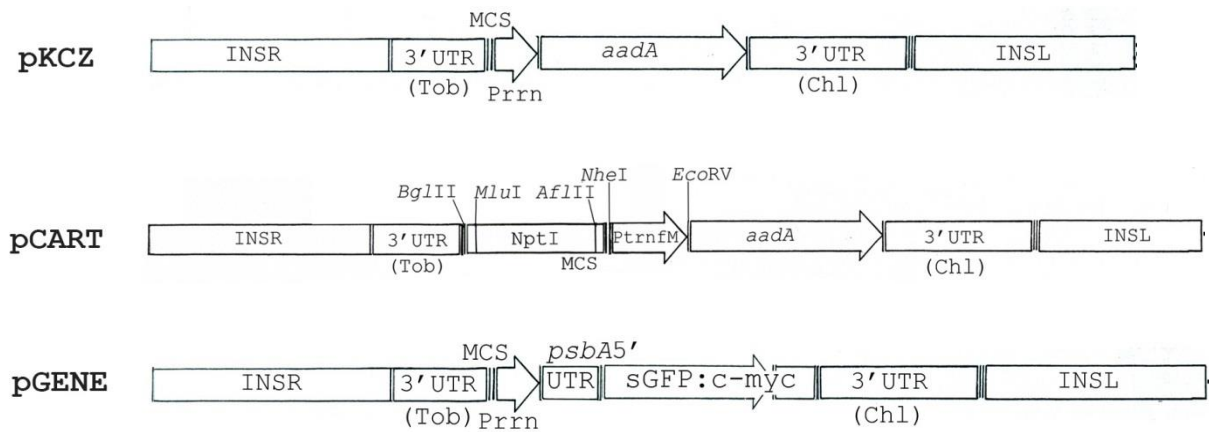


Figure 3.15: Schematic diagram of the original pKCZ chloroplast transformation vector, carrier plasmid pCART and expression plasmid pGENE. The *aadA* antibiotic resistance gene in carrier plasmid pGENE is regulated by the newly introduced *PtrnFM* which prevents aberrant homologous recombination events between direct *Prrn* repeats. The *psbA* 5'UTR leader sequence is introduced downstream of the *Prrn* promoter in expression plasmid pGENE to facilitate post-transcriptional processing of mRNAs and protein synthesis in the chloroplast of tobacco.

Table 3.1: Summary of modifications made during the construction of carrier plasmid pCART, expression plasmid pGENE and the final chloroplast transformation vector pEXPR-GOI together with their respective functions.

Carrier plasmid pCART	
1) Introduction of RE sites <i>Afl</i> II and <i>Mlu</i> I	<ul style="list-style-type: none"> New non-cutting RE sites to facilitate the construction of the final chloroplast transformation vectors
2) Introduction of the <i>Npt</i> I spacer region	<ul style="list-style-type: none"> Allows confirmation of double digestion and as a spacer to allow the incorporation of the expression cassette from pGENE
3) Substitution of the <i>Prrn</i> promoter with <i>Ptrnf</i> M	<ul style="list-style-type: none"> Prevents aberrant intermolecular recombination with endogenous <i>Prrn</i> promoter sequences located close to the intended integration site
Expression plasmid pGENE	
1) Introduction of the <i>psbA</i> 5'UTR leader sequence	<ul style="list-style-type: none"> Facilitates post transcriptional processing of mRNAs and the translation of proteins in the chloroplast of tobacco
2) Introduction of the c-Myc epitope tag	<ul style="list-style-type: none"> Facilitates the detection, recovery and purification of transgenic proteins
3) Introduction of the factor-Xa protease cleavage site	<ul style="list-style-type: none"> Allows removal of the c-Myc epitope tag post-purification
Chloroplast transformation vector pEXPR-GOI	
1) Inversion of the expression cassette from pGENE	<ul style="list-style-type: none"> Prevents overlapping expression of genes and aberrant intermolecular recombination events between the <i>Prrn</i> promoter of pEXPR-GOI and endogenous <i>Prrn</i> promoter sequences. Potentially allows the subsequent removal of the antibiotic resistance gene

3.4.1. Modification of the pKCZ chloroplast transformation vector and construction of carrier plasmid, pCART

In a previous study, Zou *et al.* (2003) reported that the main reason for the genomic instability of the transformed chloroplast genome was due to internal recombination events between the *Prrn* promoter of the *aadA* gene expression cassette of pKCZ and the endogenous *Prrn* promoter of the *rrn* operon which was located approximately 10kb from the transgene integration

site. Intermolecular recombination events between the two direct repeats of *Prrn* caused the excision of the flanked region of the chloroplast genome leading to the deletion of the transformed expression cassette for the GOI and the endogenous *rrn* operon. In this study, the constitutive *Prrn* promoter for the *aadA* cassette was replaced with another constitutive *PtrnFM* promoter for the initiator tRNA (tRNA^{fMet}). This modification would potentially prevent any spurious intermolecular recombination events as the endogenous *PtrnFM* promoter is located at the long single copy (LSC) region which is spaced sufficiently distant from the transgene insertion site. The *Prrn* promoter which is used to drive the expression cassette for the GOI in pKCZ is oriented as an inverted repeat to the *Prrn* promoter of the endogenous *rrn* operon. The inverted repeats of *Prrn* should not be affected by intermolecular recombination as intermolecular recombination events only occur between direct repeat regions (Zou *et al.*, 2003). In addition to the modification of the *aadA* gene expression cassette, the modification of the MCS region of pKCZ was also essential to facilitate the construction of the final chloroplast transformation vectors. The RE sites on the MCS of pKCZ were limited and all RE sites that were available in the MCS were also found on at least one of the GOIs of this study. In order to simplify the construction process of the final chloroplast transformation vector, the expression cassettes for the GOI was developed in a separate expression plasmid, pGENE. The expression cassettes for the GOIs could then be cloned from pGENE into the MCS of the carrier plasmid, pCART. Hence, an approach could be taken where only two unique RE sites were required for the cloning of all GOI expression cassettes into the carrier plasmid, pCART to produce the final chloroplast transformation vectors. Following the analysis of the sequencing data of pKCZ, all GOIs and the overall approach of the construction process, *AflII* and *MluI* RE sites were found to be the most suitable candidates as the recognition sites for both *AflII* and *MluI* were not found on pKCZ and all GOIs of this study. The introduction of the *AflII* and *MluI* RE sites into the MCS allowed the convenient cloning of the completed

expression cassettes for all GOIs into the MCS using the OMNI primers. In summary, the carrier plasmid, pCART was constructed based on the original pKCZ chloroplast transformation vector. The *Prrn* promoter which was deduced to be the main cause of intermolecular recombination was removed and substituted with the *PtrnFM* promoter. In addition, new *Afl*II and *Mlu*I RE sites were also introduced into the MCS to allow the convenient cloning of GOI expression cassettes from pGENE into pCART.

3.4.2. Construction of the expression plasmid. pGENE

With respect to high transgene expression rates, the expression cassette had to contain several essential components such as an efficient promoter, compatible 5'- and 3'-untranslated regions, an optimised gene sequence with familiar codon usage and a suitable epitope tag for the detection and purification of transgenic protein. The *Prrn* promoter which was chosen to drive the expression cassettes is a plastid-encoded RNA polymerase (PEP) type promoter which resembles the σ^{70} promoters of prokaryotes which recognise typical -10 and -35 elements (Suzuki *et al.*, 2003; Klein *et al.*, 1992). The *Prrn* promoter was first described and sequenced by Tohdoh *et al.* (1981). It is a constitutive promoter and is known to modulate the transcription of transgenes at relatively high levels (Newell *et al.*, 2003). The *psbA* 5'-UTR was selected as a leader sequence for transgenes in the expression cassette. Translation is known to be heavily modulated by the 5'-UTR leader region of mRNAs. More than 90% of chloroplast mRNA species contain sequences upstream of their respective initiator codon which modulates translation by facilitating base pairing with the 3'-terminal of the small subunit rRNA (Ruf and Kössel, 1988). The 5'-leader region of the *psbA* gene which encodes for the 32kDa D1 polypeptide of photosystem II was found to be highly efficient in modulating the translation of genes (Staub and Maliga, 1994; Zou *et al.*, 2003). The stem-loop region of the

psbA 5'-UTR is known to be a crucial determinant for mRNA stability and translation efficiency of *psbA* mRNA transcripts (Zou *et al.*, 2003). In addition, the *psbA* 5'-UTR is also known to mediate light-induced activation of *psbA* mRNA transcripts in the chloroplast of tobacco which may be an interesting aspect in this study (Staub and Maliga, 1994; Eibl *et al.*, 1999). The light-induced activation of the *psbA* 5'-UTR is mainly attributed to the AU-box (UAAAUAAA) of the *psbA* 5'-UTR which interact with *cis*- and *trans*-activating factors to stabilise the mRNA transcript of genes under light (Hirose and Sugiura, 1996; Agrawal *et al.*, 2001). This light-induced activation can be potentially manipulated for the post-transcriptional control of the expression of transgenes driven by the constitutive *Prrn* promoter. The Shine-Dalgarno sequence (SD-sequence) is known to be an important determinant for the initiation of translation in prokaryotes. The SD-sequence interacts with the 3'-end of the of the 16S rRNA to initiate the translation of the genes downstream of the 5'-leader region (Shine and Dalgarno, 1975). The gene expression machinery of chloroplasts is highly similar to that of prokaryotes and a homolog of the SD-sequence is also known to mediate the initiation of the translation of genes in the chloroplasts (Tanaka *et al.*, 1986). The endogenous SD-sequence of *Prrn* was deleted during the cloning of the *psbA* 5'-UTR into pKCZ. The endogenous SD-sequence of pKCZ was not required as the *psbA* 5'-UTR also contains a putative SD-like ribosome binding site (RBS) towards the 3'-end (Zou *et al.*, 2003). Codon usage is known to vary greatly even within closely related organisms (Campbell and Gowri, 1990). It is highly recommended that the codons of the GOIs are optimised according to the codon usage frequency of the intended host. However, in this experiment, the *aadA* gene has been shown to be efficiently expressed in the chloroplast of tobacco in previous experiments, thus, was not optimised. The codon sequence of the GFP gene was compared to the codon usage frequency for the chloroplasts of *N. benthamiana* (<http://www.kazusa.or.jp/codon/>) and was found to contain no significant usage of rare codons; hence, codon optimisation was predicted to

be unnecessary. The c-Myc tag sequence was linked in frame at the 3'-end of the GOI expression cassette to facilitate the detection and extraction of transgenic proteins. Incorporation of c-Myc at the 3'-end of the gene sequence would ensure that only fully translated protein chains are detected during screening and purification assays. The c-Myc tag is a small 10 amino acid (EQKLISEEDL) residue of approximately 1.2kDa in size. The c-Myc tag is relatively small in comparison to the tagged transgenic proteins and should not interfere with the immunogenicity or the folding of the tagged protein (Terpe, 2003). However, as a precautionary measure, a factor-Xa protease cleavage site was also incorporated between the GOI and the c-Myc epitope tag. Two consecutive stop codons (UAA UGA) were placed at the 3'-end of the c-Myc tag sequence to halt the translation of genes. The expression cassettes of the GOIs were cloned into the MCS of the modified chloroplast transformation vector in the opposite orientation to the endogenous *aadA* expression cassette to prevent the overlapping of gene expression. The endogenous *rbcL* 3'-UTR located downstream of the cloned transgene expression cassette serves as the translational terminator for the expression cassette. In addition, the *rbcL* 3'-UTR is also proposed to confer additional mRNA stabilising effects to the mRNA transcripts of the GOIs (Eibl *et al.*, 1999). In brief, the GOI expression cassette of pGENE contains the *Prrn* promoter to drive the transcription of genes and the *psbA* 5'-UTR to modulate the translation of mRNAs. In addition, the expression cassette also incorporates a c-Myc epitope tag at the 3'-end of the introduced GOI sequence which is separated by a factor-Xa protease cleavage site to facilitate its removal if necessary. Gene-independent amplification of the entire GOI expression cassette from pGENE can be easily achieved via the use of primers, OMNI:F-*Afl*II and OMNI:R-*Mlu*I. Integration of the gene expression cassette of pGENE into the MCS of pCART would result in the final chloroplast transformation vector, pEXPR.

3.5. CONCLUSION

In conclusion, a prototype for the proposed 2-part chloroplast transformation system has been successfully assembled. Two separate chloroplast transformation vectors, pEXPR-G and pEXPR-UG have been constructed using the 2-part system for the expression of GFP as an exemplar cassette. Transformation vector pEXPR-G does not contain a *psbA* 5'-UTR sequence and was intended as a control to evaluate the function of *psbA* 5'-UTR in regulating chloroplast gene expression. All things considered, improved chloroplast transformation vectors have been successfully constructed and are ready for preliminary testing in *E. coli* prior to actual transformation of *N. benthamiana*.

Chapter 4: Evaluation of the pEXPR-G and pEXPR-UG chloroplast transformation vectors in *E. coli*

4.1. INTRODUCTION

In this chapter, the pEXPR-G and pEXPR-UG chloroplast transformation vectors that were constructed in the previous chapter were evaluated in the prokaryotic model organism, *E. coli*. The eubacterial chloroplasts and *E. coli* share similar elements for the regulation of gene expression, thus allowing *E. coli* to be used as a suitable surrogate for the preliminary evaluation of the two transgene expression cassettes of the developed transformation vectors. Preliminary evaluation of the transformation vectors in *E. coli* is critical to ensure that the constructed vectors are primarily functional prior to chloroplast transformation.

4.1.1. *E. coli* as a suitable surrogate for preliminary evaluation studies

It is widely accepted that chloroplasts arose from the endosymbiotic relationship between an ancient cyanobacterium and an ancient non-plastid-bearing cell (Martin and Kowallik, 1999; Mereschkowsky, 1905). Today, the biosynthetic machinery of chloroplasts still retains their prokaryotic-like nature and highly resembles that of free-living algae. The transcriptional regulation of many photosynthetic genes of the chloroplasts is controlled by P1 plastid-encoded polymerase (PEP) promoters which are highly similar to the prokaryotic σ^{70} type promoters of *E. coli* (Tanaka *et al.*, 1986; Newell *et al.*, 2003). Hence, in this study, the efficiency of the recombinant chloroplast transformation vectors were evaluated in *E. coli* at both the RNA (transcription) and protein (translation) levels. Preliminary evaluation in *E. coli* is crucial to ensure the functionality of the

recombinant transformation vectors prior to commencement of particle bombardment. In this study, testing of the recombinant chloroplast transformation vectors in *E. coli* offers a number of unique advantages, such as 1) rapid screening procedures due to the short life cycle of *E. coli* bacteria, 2) comparative analysis between the *Prrn* and *PtrnfM* promoters due to structural homology with prokaryotic σ^{70} type promoters, 3) troubleshooting of problematic expression cassettes prior to actual transformation, 4) straightforward transformation procedures, 5) transgene expression can be evaluated without integration into the bacterial genome, and 6) convenient detection of antibiotic resistance via growth on antibiotic media. These advantages make *E. coli* an ideal candidate for the preliminary evaluation recombinant vectors.

4.1.2. Similarities between the expression machinery of plastids and *E. coli*

Chloroplast genes are mostly regulated by promoters which are recognised by the plastid-encoded RNA polymerase (PEP) and consist of important conserved regions at positions -35 and -10 from the start site of transcription. This promoter structure is highly similar to the sequences of promoters in *E. coli* (Hawley and McClure, 1983). Given that a sequence similarity exists between the promoter elements of chloroplasts and *E. coli*, it is expected that chloroplast promoters in recombinant chloroplast transformation vectors are potentially active and capable of directing the heterologous expression of transgenes in *E. coli*. Similarly, promoter elements derived from *E. coli* and also chimeric promoters have also been demonstrated to be active in the chloroplasts. For example, Hibberd *et al.* (1998) demonstrated the use of the chimeric *trc* (a portmanteau of *trp* and *lac*) promoter which combines the -35 region from the promoter of the *trp* (tryptophan) operon of *E. coli* with the -10 region from the promoter of the *lac* (lactose utilisation) operon (Brosius *et al.*,

1985) for the transient expression of GFP in plastids following particle bombardment. The same chimeric promoter was also used for the expression of *aadA* antibiotic resistance gene in the chloroplasts of tobacco (Mäenpää *et al.*, 2000). These studies indicate that σ^{70} type prokaryotic promoters were functional in both chloroplasts and *E. coli* bacteria and the plausibility of evaluating the expression cassettes of chloroplast specific transgene expression cassettes in *E. coli*.

4.1.3. Approaches for evaluation

In this study, the expression of *aadA* under several different chloroplast-specific expression elements was evaluated in *E. coli*. Evaluation was performed using a minimal inhibitory concentration assay (MIC) to determine promoter strength. Within the context of this study, the MIC was defined as the minimum concentration of antibiotics required for 100% inhibition of *E. coli* growth in a defined culture media within a defined period of time. Recombinant plasmid constructs with different elements for the expression of *aadA* gene were transformed into *E. coli* via heat shock and promoter strength was determined based on their growth rates in culture media containing different concentrations of antibiotics. The GFP expression cassettes of chloroplast transformation vectors pEXPR-G and pEXPR-UG were also evaluated based on the phenotypic detection of GFP expression in *E. coli*.

4.1.4. Specific objectives

Two key functions of the newly constructed chloroplast transformation vectors pEXPR-G and pEXPR-UG can be evaluated in *E. coli*. The *aadA* gene expression cassette is a fundamental part of the chloroplast transformation vector and has to be functional in order to allow the selective regeneration of

antibiotic resistant plants. The transgene expression cassettes can also be evaluated in *E. coli* to ensure that the transgenes were incorporated in frame with the regulatory elements. The specific objectives of this chapter are: 1) to evaluate the expression of transgenes in *E. coli* under the regulation of heterologous chloroplast promoters, 2) to evaluate the efficiency of the *P_{trnFM}* promoter as a suitable replacement of the *P_{rrn}*, 3) to evaluate the effects of the *psbA* 5'-UTR leader sequence in modulating the expression of transgenes, 4) to evaluate the transcription and translation of GFP in pEXPR-G and pEXPR-UG transformed *E. coli*.

4.2. METHODS

4.2.1. Determination of minimal inhibitory concentration (MIC) values for transformed *E. coli*

Competent *E. coli* cells were transformed with recombinant plasmids using the heat shock method as described in section (3.2.7.2.) and plated onto LB agar supplemented with 100mg/l ampicillin. Antibiotic resistant colonies were pure cultured onto LB agar containing 100mg/l ampicillin and incubated at 37°C for 16 hours. A single colony was selected and inoculated into 50ml LB broth supplemented with 100mg/l ampicillin using a sterile inoculation loop and allowed to incubate at 37°C for 16 hours at 200rpm according to section (3.2.1.1.). The optical density of the bacterial culture was measured at 600nm (OD_{600}) using a spectrophotometer as described in section (3.2.4.2.) and diluted to OD_{600} of 0.1 in LB broth without any antibiotics. MIC screening was performed by inoculating 10 μ l ($OD_{600}=0.1$) of the bacterial suspension into 50ml of LB broth supplemented with spectinomycin at concentrations 0, 25, 50, 100, 150, 200, 300, 400, 500, 750 and 1000mg/l, respectively. The bacterial cultures were allowed to incubate at 37°C for 20 hours at 200rpm according to section (3.2.1.1.) and quenched in an ice bath before the results were measured using a spectrophotometer as described in section (3.2.4.2.).

4.2.2. Qualitative evaluation of transgenic mRNA expression in *E. coli*

The transcription of both the *aadA* antibiotic resistance and the green fluorescent protein (GFP) genes were evaluated in a prokaryotic expression background (*E. coli*) for each of the newly developed chloroplast transformation vectors. Competent *E. coli* cells were transformed with the recombinant chloroplast transformation vectors and allowed to propagate overnight as

described in section (3.2.1.1.). Total RNA samples were extracted from overnight cultures of *E. coli* bacteria and subjected to gene-specific reverse transcription PCR (RT-PCR) analysis for the detection of mRNA transcripts of the respective genes.

4.2.2.1. RNA extraction from bacterial samples using TRIzol

Bacteria carrying the recombinant plasmids were cultured overnight in LB broth according to section (3.2.1.1.). The overnight culture was centrifuged at 6500rpm, 10°C for 5 minutes in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) to pellet bacterial cells. The supernatant was discarded and the tube was inverted on a paper towel to drain off residual culture media before 1ml of TRIzol (Invitrogen, USA) was added into the tube containing the bacterial pellet. The pellet was resuspended and vortexed with the TRIzol for 5 minutes or until complete homogenisation is achieved. The homogenised mixture was transferred to a new sterile RNase-free 1.5ml tube using a pipette and incubated at 60°C for 5 minutes with constant agitation. The tube was centrifuged at room temperature for 5 minutes at 13 500rpm to pellet cell debris and the supernatant was transferred to a new 1.5ml tube without disturbing the debris pellet. In order to recover the RNA, 200µl of chloroform was added to the supernatant and the mixture was vortexed for 10 seconds, followed by vigorous shaking by hand for 30 seconds and incubation for 5 minutes at room temperature with intermittent agitation. The sample was allowed to stand for a further 5 minutes at room temperature without agitation and centrifuged at 12 000rpm for 15 minutes at 4°C in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) to separate the aqueous and organic phases. The upper aqueous phase was transferred to a new 1.5ml tube without disturbing the other phases (RNA is contained exclusively in the upper aqueous phase, DNA in the middle phase and proteins in the lower organic phase). Approximately 1 volume (500µl for every 1ml TRIzol used) of 4M LiCl solution was added to the

supernatant and mixed by gentle inversion. Samples were incubated overnight at 4°C to allow the precipitation of RNA and spun the following day at 13 000rpm for 10 minutes at 4°C to pellet the precipitated RNA. The supernatant was discarded without disturbing the pellet and the pellet was resuspended in 250µl ice-cold Tris-SDS solution, 25µl of ice-cold 3M NaAc solution and 275µl of ice-cold isopropanol and mixed by gentle inversion. The mixture was spun at 13 000rpm for 10 minutes at 4°C to pellet the RNA and the supernatant was removed. The RNA pellet was rinsed twice with 500µl of ice-cold 95% ethanol (HPLC grade), followed by 500µl of ice-cold 100% ethanol (HPLC grade). The tube was vortexed gently and centrifuged at 13 000rpm for 5 minutes at 4°C to pellet the RNA. The supernatant was removed and the RNA pellet was dried in a vacuum desiccator (Eppendorf concentrator plus, Eppendorf, Germany) for 2-3 minutes and resuspended in 20µl of EB buffer. The concentration of the recovered RNA product was determined using the Nanodrop-1000 spectrophotometer as described in section (3.2.4.1.).

4.2.2.2. DNase treatment of RNA samples

DNase treatment of extracted RNA samples were performed by resuspending 20µg of RNA in 1x DNaseI reaction buffer to a final volume of 100µl, followed by the addition of 6 units of DNaseI to the RNA sample. The mixture was mixed thoroughly by brief vortexing for a few seconds, followed by incubation at 37°C for 1 hour to allow DNA digestion to proceed. The DNase digestion reaction was quenched by the addition of 25µl Proteinase K (100mg/ml) to the sample mixture, followed by thorough vortexing for a few seconds and incubation at 37°C for 30 minutes. RNA was subsequently recovered by the addition of 1ml of TRIzol, followed by thorough vortexing for 20-30 seconds. The mixture was incubated at 65°C with continuous vortexing for 5 minutes in a heat block. The samples were chilled on ice and 200µl of chloroform (100%) was added, followed by vigorous vortexing for 20 seconds.

The phases were separated by centrifugation at 12 000rpm for 15 minutes at 4°C in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA). The upper aqueous layer was transferred to a new RNase-free 1.5ml tube containing 500µl of isopropanol (100%) and mixed by gentle inversion. The sample was incubated at room temperature for 5 minutes to allow the precipitation of RNA and centrifuged at 13 000rpm for 10 minutes at 4°C to pellet the precipitated RNA. The supernatant was discarded and the RNA pellet was rinsed twice with 500µl of ice-cold ethanol (95-100%). The RNA pellet was recovered by centrifuging the mixture at 13 000rpm for 5 minutes at room temperature. The supernatant was discarded and the RNA pellet was allowed to dry for 1 minute in a vacuum desiccator, followed by resuspension in a suitable volume of RNase-free dH₂O or EB. The concentration of the recovered RNA samples was determined using a spectrophotometer according to section (3.2.4.1.) and were stored at -20°C until use.

4.2.3. Reverse Transcription (RT)-PCR: evaluation of transgene expression

RT reactions were carried out using the Qiagen Omniscript® Reverse Transcription kit (Qiagen, Netherlands). Reverse transcription of mRNA was performed via the 2-step method according to the user's manual in 20µl reactions using 1.0µM of the respective reverse primer and 2µg of template RNA. Reaction mixtures were incubated at 37°C for 2 hours to allow reverse transcription to proceed. PCR was performed using 5µl of the product of reverse transcription in 50µl PCR reactions using standard PCR reagents and amplification protocols according to appendix (A). Products of the RT-PCR reaction were subjected to gel electrophoresis and visualised (M4).

4.2.4. Qualitative evaluation of transgenic protein expression in *E. coli*

The qualitative analysis of protein expression of the *aadA* and GFP gene for each of the recombinant chloroplast transformation vectors constructs were evaluated in *E. coli* bacteria. Competent *E. coli* cells were transformed with the recombinant chloroplast transformation vectors and allowed to propagate over a period of 16 hours. Total cellular protein samples were extracted from the overnight cultures of *E. coli* bacteria and subjected to both GFP-specific and c-Myc specific Western hybridisation analysis to qualitatively determine the expression of the gene of interest (GOI).

4.2.4.1. Extraction of protein from bacterial samples

Two different methods were used for the extraction of total cellular protein from overnight cultures of *E. coli* bacteria. The boiling method involved a single boiling step and allowed for the rapid and convenient preparation of crude protein extracts (section 4.2.4.2.). The soni-thaw method of protein extraction was more laborious and required multiple snap-freezing, sonication and thawing cycles (section 4.2.4.3.). However, higher levels of homogenisation and total protein yield (up to 10x) could be achieved using the soni-thaw method. By replacing the respective protein extraction buffers (section M3), the extraction of both soluble and insoluble proteins can be achieved using the same extraction method.

4.2.4.2. Boil Method

An overnight suspension of bacteria carrying the plasmid of interest was prepared as described in section (3.2.1.1.) and 1ml of the bacterial suspension was transferred to a new 1.5ml tube. The tube was centrifuged at 12 000rpm for 1 minutes at 10°C in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) to pellet the bacterial cells and the supernatant was discarded.

The bacterial pellet was resuspended in 100µl of NP40 protein extraction buffer (section M3) and the mixture was boiled at 100°C for 5 minutes. The boiled samples were centrifuged at 13 000rpm for 5 minutes at room temperature to pellet the cell debris. The supernatant containing the extracted proteins was transferred carefully to a new tube without disturbing the pellet and a 5µl aliquot was taken from each protein sample for concentration measurements. The remainder of the protein extract was stored at -80°C until further use.

4.2.4.3. Soni-thaw method

An overnight suspension of bacteria carrying the plasmid of interest was prepared as described in section (3.2.1.1.) and 1ml of the bacterial suspension was transferred to a new 1.5ml tube. The tube was centrifuged at 12 000rpm for 1 minutes at 10°C in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) to pellet the bacterial cells and the supernatant was discarded. The bacterial pellet was resuspended in 100µl of NP40 protein extraction buffer (section M3) and the mixture was snap-frozen in liquid nitrogen. The frozen mixture was sonicated for 30-40 seconds in an ice bath and allowed to thaw to a 'slush' stage. The thawed mixture was snap-frozen again in liquid nitrogen and sonicated again for 30-40 seconds. The freeze-sonicate-thaw cycle was repeated for 2-3 times for each sample. The mixture was allowed to thaw completely after the last sonication cycle and centrifuged at 13 000rpm for 5 minutes at room temperature to pellet the cell debris. The supernatant containing the extracted proteins was transferred carefully to a new tube without disturbing the pellet and a 5µl aliquot was taken from each extracted protein sample for concentration measurements. The remainder of the protein extract was stored at -80°C until further use.

4.2.4.4. Determination of protein concentrations

The concentration of extracted protein samples was determined using the Biophotometer (Eppendorf, Germany). A 5µl aliquot was taken from each extracted protein sample and mixed with 75µl of Pierce 660 nm protein assay reagent (Thermo Scientific, USA). The mixture was allowed to incubate at room temperature for at least 5 minutes before the absorbance readings were measured using the Biophotometer (Eppendorf, Germany). Absorbance readings at the 650nm wavelength were recorded for each sample and the concentration of each protein extract was determined from the equation of a standard curve, $y=0.0007x+0.0058$, y =absorbance at OD_{650} and x =protein concentration (µg/ml) (appendix D) generated using defined concentrations of bovine serum albumin (BSA) protein which was used as a protein concentration standard.

4.2.5. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used in order to determine the quality of the extracted protein samples. In addition, SDS-PAGE also allowed the separation of proteins according to their molecular weight and is the first step for Western detection of protein expression.

4.2.5.1. Casting of polyacrylamide gels for protein products

All polyacrylamide gels for SDS-PAGE were prepared according to section (M4). Polyacrylamide gels were casted in either pre-sealed Novex[®] (Invitrogen, USA) Midi Cassettes or resealed cassettes. The sealed cassettes were checked for leaks by filling them up with dH₂O before each cast. The cassette was placed in a vertical position on a stable rack. A 12% polyacrylamide separating gel mixture was prepared according to section (M4). The mixture was transferred carefully into the sealed cast using a 5ml pipette and 1ml of 100% isopropanol

was overlaid onto the separating gel mixture to remove any trapped air bubbles. The mixture was allowed to polymerise at room temperature for 1 hour. After gel polymerisation, the isopropanol was decanted away and the polymerised separating gel was rinsed with ddH₂O. The 4% stacking gel mixture was then prepared according to section (M4) and transferred on top of the separating gel using a pipette. An appropriate comb was inserted carefully into the cast without introducing any air bubbles and the stacking gel was allowed to polymerise at room temperature for 30 minutes to 1 hour. The comb was removed gently and the wells of the gel were rinsed with 1x SDS-PAGE running buffer before use. Alternatively, polymerised gels can be stored for up to a week in 1x SDS-PAGE running buffer in a sealed plastic bag in the fridge at 4°C.

4.2.5.2. SDS-PAGE setup

Protein extracts were subjected to denaturing and reducing SDS-PAGE for analysis. Protein samples were prepared by adding 10µl of 2x SDS-PAGE sample loading buffer (Invitrogen, USA) to 10µl of protein sample in a 0.2ml PCR tube. The samples were heated at 70°C for 10 minutes to allow complete denaturation of proteins, followed by brief centrifugation to pool the liquid at the bottom of the tube. A prepared polyacrylamide gel cassette (section 4.2.5.1.) was placed in the XCell *SureLock*[™] Mini-Cell SDS-PAGE module (Invitrogen, USA) and was assembled according to manufacturer's instructions. The upper buffer chamber was filled with 200ml of ice-cold SDS-PAGE running buffer (M3) and the SDS-PAGE running assembly was checked for leakage. Once no leakage was confirmed, the lower buffer chamber was filled with 600ml of ice-cold SDS-PAGE running buffer. SDS-PAGE analysis was performed using 20µl of the prepared protein samples which were loaded into the wells of the polyacrylamide gel together with a suitable volume of protein ladder/marker (Nacalai, USA) according to manufacturer's instructions. Gel electrophoresis was allowed to proceed at a constant voltage of 125V for 90 minutes.

4.2.5.3. Gel staining

The polyacrylamide gel cassette was retrieved from the XCell *SureLock*[™] Mini-Cell SDS-PAGE module (Invitrogen, USA) and rinsed with ddH₂O. The cassette was cracked carefully along the 3 sealed edges of the cast and the polyacrylamide gel was carefully removed. The gel was rinsed by adding enough ddH₂O to cover the gel and rocking gently on an orbital shaker for 5 minutes and repeated for 3-4 times. The gel was immersed completely in fresh coomassie blue staining solution (BioRad, USA) and placed on an orbital shaker for 1 hour. The staining solution was discarded and the stained gel was washed by adding enough ddH₂O to cover the gel completely and rocking gently on an orbital shaker for 30 minutes. The washing step was repeated with fresh ddH₂O every hour until the desired resolution is achieved. The gel image was captured using the GS800 Densitometer (BioRad, USA) according to manufacturer's instructions.

4.2.6. Western Blot

In this study, all Western blot procedures were performed using polyclonal primary antibodies for the specific detection of the protein of interest and a secondary antibody conjugated to horseradish peroxidase (HRP) enzyme which binds to the primary antibody. In most experiments, results were visualised by adding an HRP substrate which allows the colorimetric visualisation of results.

4.2.6.1. Western transfers

Blotting pads and sheets of blotting paper were fully saturated with sufficient transfer buffer. The polyacrylamide gel cassette was retrieved from the XCell *SureLock*[™] Mini-Cell SDS-PAGE module (Invitrogen, USA) following SDS-PAGE (section 4.2.5.2.) and rinsed with ddH₂O. The two halves of the cassette were separated carefully along the 3 sealed edges and the polyacrylamide gel

was carefully removed. The wells and the 'foot' of the polyacrylamide gel were removed using a suitable cutting implement. A 10 x 10cm sheet of nitrocellulose membrane was prepared and soaked briefly in transfer buffer and carefully overlaid on the back-side of the polyacrylamide gel without introducing any air bubbles to allow the transfer of a carbon-copy of the SDS-PAGE results onto the membrane. The gel, nitrocellulose membrane, blotting paper and blotting pads were arranged into a sandwich stack as shown in figure 4.1. The sandwich stack was carefully placed in the XCell II™ Blot Module (Invitrogen, USA) while ensuring the correct orientation with the positive and negative terminals (figure 4.1). The blotting module was filled completely with pre-chilled transfer buffer. Ice-cold dH₂O was filled into the gel tank to dissipate heat from the blotting module. The blotting procedure was allowed to proceed at a constant voltage of 25V for 90 minutes. After 90 minutes, the blotting assembly was carefully dismantled and any remaining gel fragments were removed from the nitrocellulose membrane which now contains the blotted proteins. The nitrocellulose membrane was then rinsed with ddH₂O and subjected to immunodetection immediately or placed between 2 sheets of filter paper and allowed to dry in a vacuum desiccator for 5 minutes before storing in a fridge at 4°C in a sealed plastic bag.

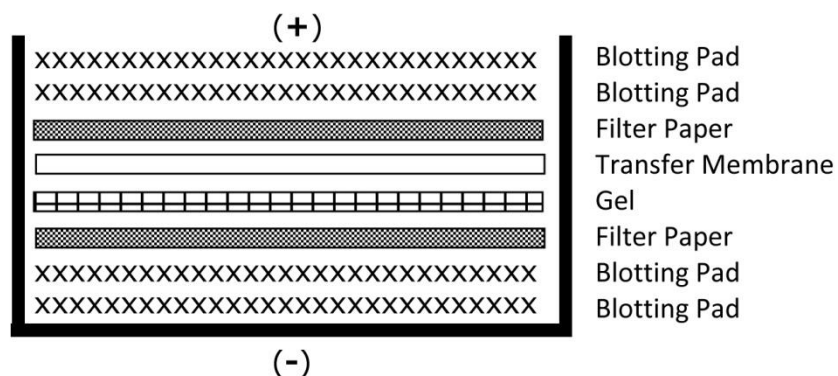


Figure 4.1: Schematic representation of the arrangement of blotting pads, filter papers, transfer membrane, and protein gel in an electroblotting sandwich stack.

4.2.6.2. Western hybridisation and immunodetection

The nitrocellulose membrane was immersed in enough blocking buffer (section M3) to cover the entire membrane and allowed to incubate at room temperature on an orbital shaker at 60rpm for 1 hour. The blocking buffer was removed after the blocking step. A primary antibody solution was prepared using either rabbit polyclonal antibody against GFP (Abcam, UK) or rabbit polyclonal antibody against c-Myc-tag (Abcam, UK), diluted at 1:25 000 in blocking buffer for the specific detection of GFP and c-Myc tagged proteins, respectively. The nitrocellulose membrane was incubated in the primary antibody solution at room temperature on an orbital shaker at 60rpm for 2 hours. The membrane was removed from the primary antibody solution and placed in a new container, followed by the addition of 20ml of TBST washing buffer (section M3). The container was placed on an orbital shaker and allowed to shake at 60rpm for 5 minutes. The washing procedure was repeated 4-5 times. The secondary antibody solution was prepared using horseradish peroxidase (HRP) conjugated goat polyclonal antibody against rabbit IgG (Abcam, UK), diluted at 1:10 000 in TBST. The nitrocellulose membrane was incubated in the secondary antibody solution at room temperature on an orbital shaker at 60rpm for 1 hour. The membrane was removed from the secondary antibody solution and placed in a new container, followed by the addition of 20ml of fresh TBST washing buffer (section M3). The container was placed on an orbital shaker and allowed to shake at 60rpm for 5 minutes. The washing procedure was repeated 4-5 times, followed by a final rinse with ddH₂O after the last wash cycle. Results were visualised by the addition of 1ml of 1 Component TMB Membrane Peroxidase Substrate (KPL, USA). The substrate was pipetted continually onto the membrane, cycling repeatedly, until protein bands were observed at the desired intensity and the reaction was quenched in ddH₂O before excessive background noise began to appear.

4.3. RESULTS

4.3.1. Phenotypic evaluation of *aadA* expression under various regulatory elements

The *aadA* gene was used as an indicator gene in order to investigate the effects of various regulatory elements of gene expression, such as the *Prrn* and *PtrnFM* promoters, as well as, the *psbA* 5'-UTR leader sequence. Data was recorded based on how these elements affect the expression of *aadA* in transformed *E. coli* grown on culture media supplemented with antibiotics.

4.3.1.1. Confirmation of *aadA* as the source of spectinomycin resistance

Competent *E. coli* bacteria transformed with recombinant plasmid pKCZ Δ *aadA* (original pKCZ chloroplast transformation vector where *aadA* has been deleted) was observed to be able to propagate on LB agar supplemented with 100mg/l ampicillin following incubation at 37°C for 16 hours (figure 4.2: A). The results were as expected as an *ampR* ampicillin resistance gene expression cassette is located on the pUC18 backbone of pKCZ which should be still active in pKCZ Δ *aadA*. In contrast, *E. coli* bacteria transformed with the same recombinant plasmid was unable to propagate on LB agar supplemented with 100mg/l of spectinomycin and no colonies were observed after incubation at 37°C for 16 hours (figure 4.2: B). No visible growth was recorded in all non-transformed *E. coli* bacteria on antibiotic media following incubation at 37°C for 16 hours (negative control data not shown).

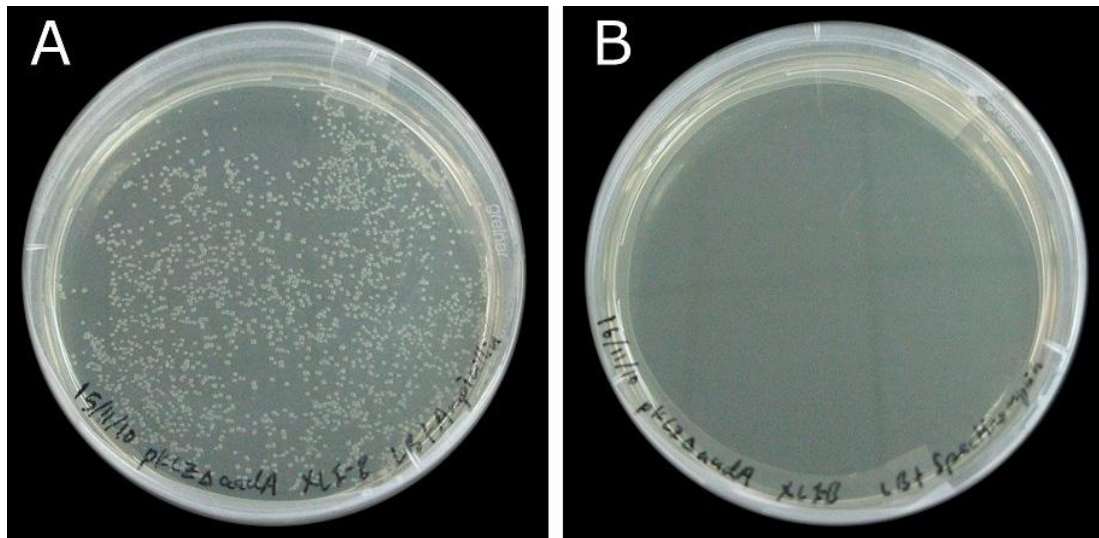


Figure 4.2: Growth profiles of *E. coli* bacteria transformed with recombinant plasmid pKCZΔ*aadA* on LB agar plates supplemented with 100mg/l of ampicillin. A: Competent *E. coli* bacteria transformed with pKCZΔ*aadA* showing growth on ampicillin, B: Competent *E. coli* bacteria transformed with pKCZΔ*aadA* failed to form colonies on spectinomycin.

4.3.1.2. Preliminary evaluation of *aadA* expression in pKCZ, pCART, pKCZ*psbA*5'-UTR and pKCZΔ*Prrn*

The unmodified pKCZ chloroplast transformation vector represents *aadA* expression under the regulation of the *Prrn* promoter alone, the carrier plasmid pCART represents *aadA* expression under the regulation of the *P_{trnFM}* promoter alone, the recombinant plasmid pKCZ*psbA*5'-UTR represents *aadA* expression under the regulation of the *Prrn* promoter and the *psbA* 5'-UTR leader sequence, and the recombinant plasmid pKCZΔ*Prrn* represents *aadA* expression without a promoter with only the ribosome binding site (RBS) present. Preliminary evaluations were based on growth on LB agar supplemented with spectinomycin at increasing concentrations of 0, 25, 50, 75, 100, 125 and 150mg/l, respectively. Competent *E. coli* bacteria transformed with pKCZ was observed to be able to propagate without any adverse effects on LB agar supplemented with spectinomycin up to a concentration of 150mg/l (figure 4.3: Ai, Bi). Similar

results were also observed for the growth of competent *E. coli* bacteria transformed with the carrier plasmid pCART (figure 4.3: Aii, Bii) and the recombinant plasmid pKCZ $psbA5'$ -UTR (figure 4.3: Aiii, Biii) on LB agar supplemented with spectinomycin at a concentration of 150mg/l. In contrast, growth of competent *E. coli* bacteria transformed with pKCZ ΔPrn was completely suppressed when propagated on LB agar supplemented with 150mg/l of spectinomycin (figure 4.3: Biv). However, despite the absence of a promoter to drive the transcription of the *aadA* gene, pKCZ ΔPrn transformed *E. coli* still managed to survive to a certain extent on LB agar supplemented with spectinomycin up to a concentration of 125mg/l (figure 4.3: Aiv).

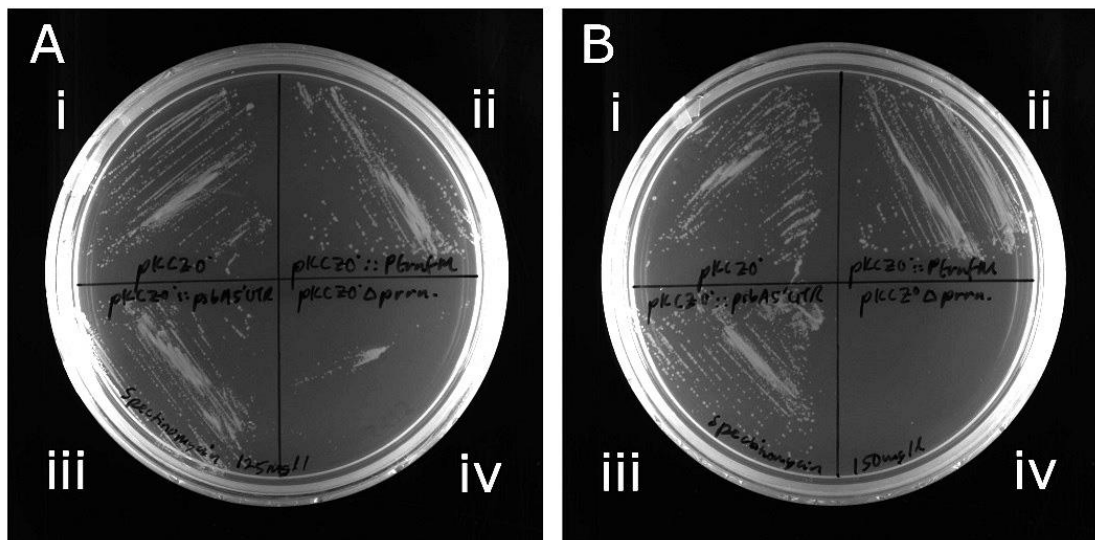


Figure 4.3: Preliminary evaluation of the regulatory elements for gene expression using *aadA* as an indicator gene. The *aadA* gene under the regulation of both *Prn* (Ai, Bi) and *PtrnFM* (Aii, Bii) promoters, as well as, the *Prn* promoter and the *psbA* 5'-UTR element (Aiii, Biii) allowed growth of transformed *E. coli* bacteria on LB agar supplemented with spectinomycin up to a concentration of 150mg/l. Expression of *aadA* without a promoter region with only the RBS allowed weak growth of transformed *E. coli* on LB agar supplemented with 125mg/l spectinomycin (Aiv). A: LB agar supplemented with 125mg/l spectinomycin, B: LB agar supplemented with 150mg/l spectinomycin, i: *E. coli* bacteria transformed with pKCZ, ii: *E. coli* bacteria transformed with pCART, iii: *E. coli* bacteria transformed with pKCZ $psbA5'$ -UTR, iv: *E. coli* bacteria transformed with pKCZ Δprn .

4.3.1.3. MIC screening: evaluation of regulatory elements for gene expression

The relative effects of *Prrn*, *PtrnfM* and *psbA* 5'-UTR were further evaluated in *E. coli* via MIC assays (section 4.2.1.) and compared based on their ability to modulate the expression of the *aadA* gene. Data was recorded based on the optical density (OD_{600}) of *E. coli* bacteria transformed with recombinant plasmids pKCZ, pCART, pKCZ*psbA*5'-UTR and pKCZ Δ *Prrn* as described in section (3.2.4.2.), respectively. The growth patterns of *E. coli* transformed with recombinant plasmids pKCZ (*Prrn* promoter) and pCART (*PtrnfM* promoter) were relatively identical (within the standard deviation values). A significant decline in the growth rates of both bacterial cultures were observed at a spectinomycin concentration of 200mg/l and growth was found to be 100% inhibited at a spectinomycin concentration of 500mg/l (figure 4.4: ● and ■). In comparison, the growth rates of *E. coli* transformed with recombinant plasmid pKCZ*psbA*5'-UTR (*Prrn* promoter with the *psbA* 5'-UTR leader sequence) was observed to display a significant decline at a spectinomycin concentration of 150mg/l (figure 4.4: ▲). Nevertheless, growth of pKCZ*psbA*5'-UTR transformed bacteria was found to be 100% inhibited only at a spectinomycin concentration of 500mg/l, similar to pKCZ and pCART transformed bacteria. The growth pattern of pKCZ Δ *Prrn* transformed *E. coli* was noticeably different as compared to pKCZ, pCART and pKCZ*psbA*5'-UTR transformed *E. coli* and displayed a significant decline in growth rates at a spectinomycin concentration of only 50mg/l (figure 4.4: ▼). Interestingly, despite having no promoter to direct the transcription of the *aadA* gene, growth of *E. coli* transformed with pKCZ Δ *Prrn* could only be 100% inhibited at a spectinomycin concentration of 150mg/l. No growth was observed for all cultures at spectinomycin concentration above 500mg/l.

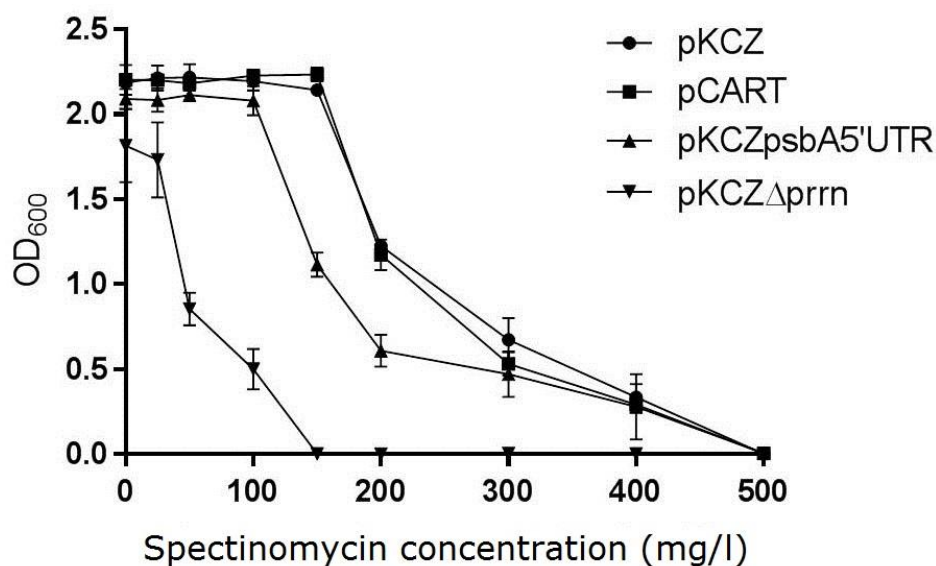


Figure 4.4: Effects of different regulatory elements on the expression of *aadA* in *E. coli* based on minimal inhibitory concentration (MIC) assays. pKCZ: *aadA* regulated by *Prrn* promoter; pCART: *aadA* regulated by *Ptrn*^{fM} promoter; pKCZ*psbA*5'-UTR: *aadA* regulated by *Prrn* promoter with the *psbA* 5'-UTR leader sequence; pKCZΔ*Prrn*: *aadA* with no promoter and only the ribosome binding site (RBS). (N=3).

4.3.2. Molecular evaluation of transgene expression in *E. coli*

In this study, the GFP expression cassettes of chloroplast transformation vectors pEXPR-G and pEXPR-UG were evaluated in *E. coli* at both the transcriptional and translational levels. The presence of GFP mRNA transcripts in pEXPR-G and pEXPR-UG transformed *E. coli* was evaluated via RT-PCR and the presence of c-Myc-tagged GFP proteins were detected using GFP-specific and c-Myc-specific antibodies via Western blot.

4.3.2.1. RT-PCR: qualitative evaluation for the transcription of GFP in *E. coli*

The RT-PCR profiles for the detection of GFP mRNAs in total RNA samples extracted from pEXPR-G and pEXPR-UG transformed *E. coli* are shown in figures 4.5 and 4.6, respectively. Single defined bands with an estimated molecular size

of 700bp were produced in the RT-PCR reactions, specific for the detection of GFP mRNA transcripts in total RNA samples of both pEXPR-G (figure 4.5: lanes X1, X2,X3) and pEXPR-UG (figure 4.6: lanes G1, G2,G3) transformed *E. coli* using primers, PF1:GFP-*Nco*I and PR2:GFP-*Bsi*WI. These bands corresponded with the PCR positive control for the amplification of the GFP gene from the pEXPR-G (figure 4.5: lane X+) and pEXPR-UG (figure 4.6: lane G+) chloroplast transformation vectors, respectively using the same primers. Additionally, the observed bands also corresponded with the expected molecular size of the GFP gene of 720bp. The internal positive control for the RT-PCR reaction targeting the amplification of *aadA* mRNA transcripts from the same total RNA samples produced bands with an estimated molecular size of 780bp using forward and reverse primers, PF*aadA* and PR*aadA* (appendix B), respectively (figures 4.5 and 4.6: lane A). These bands also corresponded with the positive control for the PCR amplification of the *aadA* gene from pKCZ (figures 4.5 and 4.6: lane a+) as well as the expected molecular size of the *aadA* gene of 798bp. All negative controls; non-RT controls for the amplification of GFP (figure 4.5, lane -RTx; figure 4.6, lane -RTg), untransformed negative controls (figures 4.5 and 4.6: lane -C), non-RT controls for the amplification of *aadA* (figures 4.5 and 4.6: lane -RTa), and the non-template controls (figures 4.5 and 4.6: lane NTC) did not produce any detectable bands of any size, thus, validating the RT-PCR results.

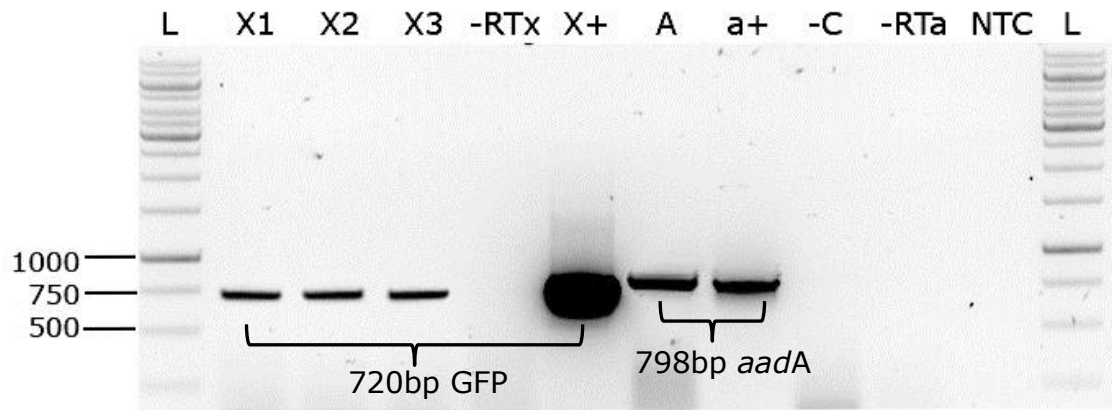


Figure 4.5: RT-PCR results for the qualitative detection of mRNA transcripts of GFP from total RNA samples extracted from overnight cultures of *E. coli* bacteria transformed with recombinant chloroplast transformation vector pEXPR-G. L: Fermentas 1kb DNA ladder, X1-X3: RT-PCR products of GFP from total RNA isolated from transformed *E. coli*, -RTx: Non-RT control for the PCR amplification of GFP gene, X+: positive control for the amplification of the GFP gene, A: positive control for the RT-PCR of antibiotic resistance gene (*aadA*), a+: *aadA* PCR positive control, -C: Untransformed bacteria negative control, -RTa: Non-RT control for the amplification of the *aadA* gene, NTC: Non-template control.

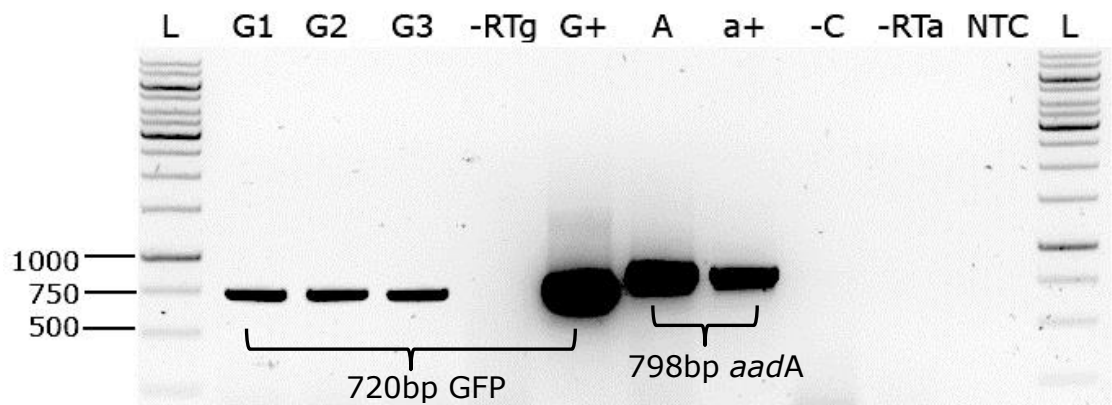


Figure 4.6: RT-PCR results for the qualitative detection of mRNA transcripts of GFP from total RNA samples extracted from overnight cultures of *E. coli* bacteria transformed with recombinant chloroplast transformation vector pEXPR-UG. L: Fermentas 1kb DNA ladder, G1-G3: RT-PCR products of GFP from total RNA isolated from transformed *E. coli*, -RTg: Non-RT control for the PCR amplification of GFP gene, G+: positive control for the amplification of the GFP gene, A: positive control for the RT-PCR of antibiotic resistance gene (*aadA*), a+: *aadA* PCR positive control, -C: Untransformed bacteria negative control, -RTa: Non-RT control for the amplification of the *aadA* gene, NTC: Non-template control.

4.3.2.2. SDS-PAGE: the protein expression profile of transformed *E. coli*

The results for the SDS-PAGE analysis of total cellular protein extracted from competent *E. coli* bacteria transformed with recombinant plasmids pEXPR-G and pEXPR-UG are shown in figure 4.7. The protein profiles of competent *E. coli* bacteria transformed with recombinant plasmids pEXPR-G (figure 4.7: lanes X1 and X2) and pEXPR-UG (figure 4.7; lanes U1, U2) were generally identical without any noticeable difference which can be observed in the stained SDS-PAGE gel. The total protein profiles of untransformed competent *E. coli* cells which served as negative controls (figure 4.7; C1 and C2) were also generally similar to the protein profiles of pEXPR-G and pEXPR-UG transformed bacteria with only minor discrepancies in expression patterns with one exception. A distinct band with an estimated molecular size of 28kDa was observed only in the SDS-PAGE profiles of pEXPR-G and pEXPR-UG transformed *E. coli* bacteria and was not present in both the negative controls (figure 4.7). The observed band of approximately 28kDa corresponded approximately to the expected sizes of the GFP and the *aadA* proteins which are predicted to be 28kDa and 30.0kDa respectively.

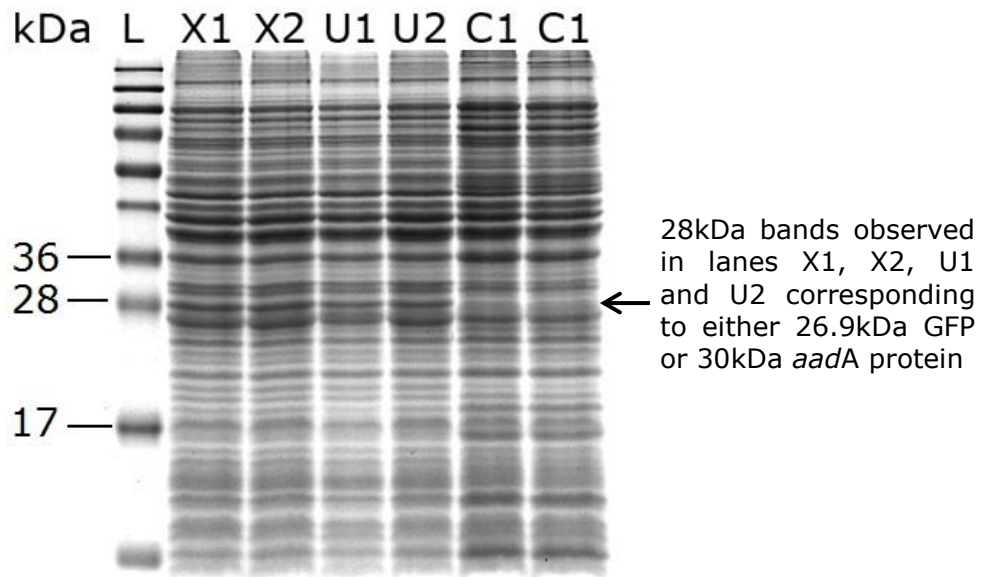


Figure 4.7: SDS-PAGE profiles of total cellular protein samples extracted from pEXPR-G and pEXPR-UG transformed *E. coli* bacteria. Distinct bands of approximately 28kDa could be observed in the protein profiles of the transformed bacteria (lanes X1, X2, U1 and U2) which were not present in the protein profiles of the untransformed controls. The 28kDa GFP or the 30.0kDa *aadA* protein may be responsible for the observed 28kDa band in the protein profiles of pEXPR-G and pEXPR-UG transformed bacteria. L: Protein marker (Nacalai, USA), X1 and X2: Total cellular protein from pEXPR-G transformed *E. coli* bacteria, U1 and U2: Total cellular protein from pEXPR-UG transformed *E. coli* bacteria, C1 and C2: Total cellular protein from untransformed *E. coli* bacteria (negative control).

4.3.2.3. Western Blot: Qualitative detection of expressed transgenic proteins (GFP)

Results for the Western detection of GFP in samples of total cellular protein extracted from pEXPR-G (X1, X2) and pEXPR-UG (U1, U2) transformed *E. coli* using GFP-specific and c-Myc-specific primary antibodies are shown in figures 4.8 and 4.9, respectively. Unique distinct bands estimated at about 28kDa were observed in lanes X1, X2, U1 and U2 in both Western blot profiles at identical positions for both GFP-specific (figure 4.8: lanes X1, X2, U1 and U2) and c-Myc-specific (figure 4.9: lanes X1, X2, U1 and U2) immunodetection assays. The bands which were observed in figure 4.8; lanes X1, X2, U1 and U2 corresponded to the size of the positive control for GFP (figure 4.8: +C). The +C GFP positive control sample was extracted from a separate hyper-expressive strain of *E. coli* that had been confirmed to be expressing the 28kDa GFP protein. The positive control band (C+) was aligned accordingly using the protein ladders to allow accurate comparison of molecular sizes. The negative control lanes are composed of samples of total cellular protein extracted from non-transformed *E. coli* bacteria. No distinct bands were detected in the Western blot profiles of the negative control samples (figures 4.8 and 4.9: C1 and C2). Although no positive controls were available for the Western detection of c-Myc-tagged GFP (the GFP protein expressed in the hyper-expressive strain of *E. coli* did not contain a c-Myc tag), bands that were detected using c-Myc-specific antibodies corresponded to the 28kDa band of the protein marker (figure 4.9: lanes X1, X2, U1 and U2).

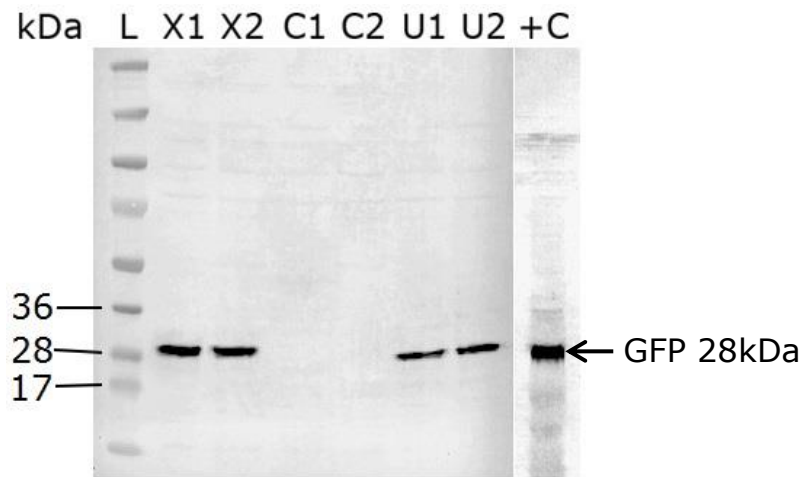


Figure 4.8: Western detection profiles for GFP in total cellular protein extracts from transformed *E. coli* bacteria using a GFP-specific primary antibody. L: Protein marker (Nacalai, USA), X1 and X2: Total cellular protein extracted from pEXPR-G transformed *E. coli* bacteria, U1 and U2: Total cellular protein extracted from pEXPR-UG transformed *E. coli* bacteria, C1 and C2: Total cellular protein extracted from untransformed *E. coli* bacteria (negative control), +C: Total cellular protein extracted from transformed *E. coli* bacteria carrying the pRSET::GFP plasmid (GFP positive control from a separate Western blot aligned according to the protein marker).

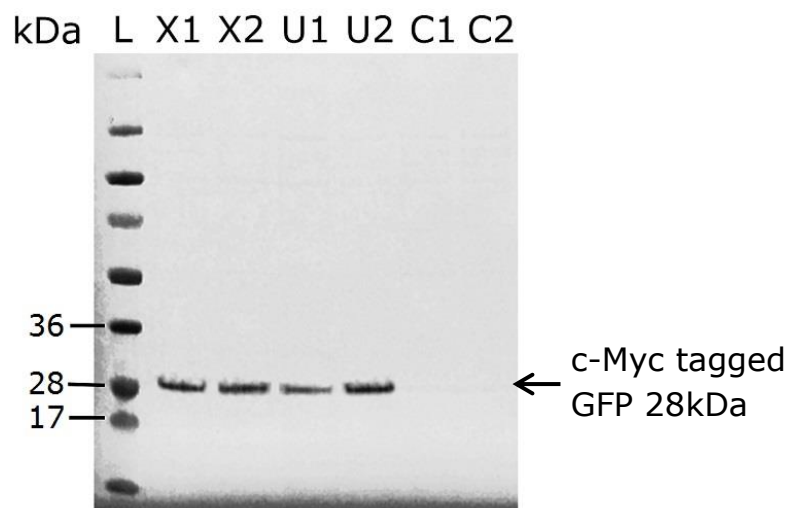


Figure 4.9: Western detection profiles for c-Myc tagged proteins in total cellular protein extracts from transformed *E. coli* bacteria using a c-Myc-specific primary antibody. L: Protein marker (Nacalai, USA), X1 and X2: Total cellular protein extracted from pEXPR-G transformed *E. coli* bacteria, U1 and U2: Total cellular protein extracted from pEXPR-UG transformed *E. coli* bacteria, C1 and C2: Total cellular protein extracted from untransformed *E. coli* bacteria (negative control).

4.3.2.4. Phenotypic detection of GFP in transformed *E.coli*

The results for the phenotypic detection of GFP in competent *E. coli* bacteria transformed with recombinant chloroplast transformation vectors pEXPR-G and pEXPR-UG are shown in figure 4.10. GFP fluorescence could not be detected with the naked eye in both pEXPR-G and pEXPR-UG transformed samples of bacteria even under UV-light. The green fluorescence of the GFP protein could only be detected when the transformed bacterial pellet was viewed under UV with the assistance of a digital camera with a green pass filter and an extended exposure time of 2 minutes. No fluorescence was observed in the negative control which contains a sample of untransformed *E. coli* bacteria under UV light (figure 4.10: Aii) even with an extended exposure time of 5 minutes. With regard to a standardised incubation period and sample optical density (OD_{600}), the green fluorescence was stronger in pEXPR-G transformed bacterial samples (figure 4.10; Bii) as compared to pEXPR-UG transformed bacterial samples (figure 4.10; Cii).

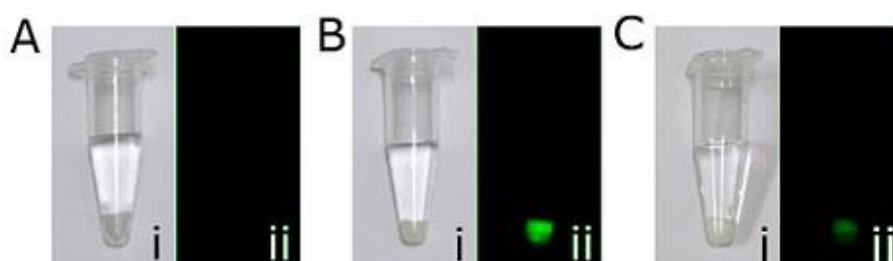


Figure 4.10: Visualisation of pEXPR-G and pEXPR-UG transformed *E. coli* bacterial pellet under (i) white light and (ii) UV-light (350nm). Fluorescence signals were detected only in transformed bacteria (B and C) but not in the untransformed negative control (A). A: Non-transformed negative control, B: pEXPR-G transformed *E. coli* bacterial pellet, C: pEXPR-UG transformed *E. coli* bacterial pellet, i: Visualisation under white light, ii: Visualisation under UV-light (350nm). Image captured through a digital camera equipped with a green pass filter with an exposure time of 2 minutes.

4.4. DISCUSSION

It is essential that the two basic expression cassettes of the newly constructed chloroplast transformation vectors, pEXPR-G and pEXPR-UG are proven to be functional prior to the actual transformation of *N. benthamiana*. The *aadA* antibiotic resistance gene is crucial for the selective regeneration of transplastomic plants post-bombardment. Hence, a defective *aadA* gene expression cassette would inevitably result in the failure of all transformation events. The transgene expression cassette is responsible for modulating the transcription and translation of GOIs in the transformed host. Thus, its proper function is also of considerable importance. The basic gene expression mechanism of the prokaryotic bacteria, *E. coli* bears striking homology with eubacterial chloroplasts. Thus, preliminary evaluation of the rudimentary elements of the newly constructed chloroplast transformation vectors was evaluated in *E. coli*. In this study, the efficiency of the *P_{trnFM}* promoter was evaluated to determine if it was a compatible replacement for the *P_{rrn}* promoter of the *aadA* gene expression cassette. The efficiency of the GOI expression cassette was also evaluated to ensure that all regulatory elements that were incorporated in the previous chapter were in-frame and primarily functional to direct the expression of GFP.

4.4.1. Phenotypic evaluation of *aadA* expression

An evaluation of the *aadA* antibiotic resistance gene expression cassette based on phenotypic expression indicated that *aadA* was fundamentally expressed in *E. coli* transformed with the pKCZ chloroplast transformation vector. The results of section (4.3.1.1.) confirmed that antibiotic resistance towards spectinomycin was conferred solely by the *aadA* gene and the deletion of *aadA* from pKCZ resulted in 100% growth inhibition on LB agar supplemented with 100mg/l spectinomycin (Svab and Maliga, 1993) (figure 4.2). The *aadA*

gene encodes for (aminoglycoside 3'-adenyltransferase) and confers resistance to aminoglycoside antibiotics such as spectinomycin and streptomycin. The mode of action of aminoglycoside antibiotics and how *aadA* confers antibiotic resistance to these antibiotics are discussed in Chapter 6, section (6.4.3.). Inactivation of aminoglycosides by *aadA* is an enzymatic reaction. Thus, higher expression levels of *aadA* are expected to correlate with higher levels of tolerance towards aminoglycosides and vice versa. Both the *Prrn* promoter (which directs the transcription of the *rrn* operon) and the *Ptrn^{fM}* promoter (which directs the transcription of the initiator tRNA^{fMet}) were found to be active in *E. coli* and could initiate the expression of the *aadA* gene. The results of section (4.3.1.3.) indicated that both the *Prrn* and *Ptrn^{fM}* promoters had very similar efficiencies and allowed the accumulation of *aadA* protein at similar levels in transformed *E. coli* bacteria as indicated by their growth patterns in the MIC assay (figure 4.4). Hence, the *Ptrn^{fM}* promoter can be considered as an equivalent substitute for the *Prrn* promoter for the expression of *aadA* in the carrier plasmid, pCART. Incorporation of the *psbA* 5'-UTR leader sequence downstream of the *Prrn* promoter did not appear to significantly affect the expression of *aadA*. The *psbA* gene encodes for the D1 protein in the chloroplasts of tobacco which is an important protein involved in the process of photosynthesis. The expression of D1 is light-responsive and is known to be upregulated in response to light by the *psbA* 5'-UTR (Eibl *et al.*, 1999). However, no significant difference was observed in the expression of *aadA* in *E. coli* which was cultured in the dark or in the presence of light. The mechanism for light-induced upregulation of *psbA* is unlikely to be present in *E. coli* as it is most probably a mechanism which evolved in line with the process of photosynthesis and is known to involve additional nuclear-encoded *trans*-acting factors as well (Sriraman *et al.*, 1998). If anything, the *psbA* 5'-UTR was observed to have a marginally negative influence on *aadA* expression where an earlier decline in growth rate was observed in pKCZ*psbA*5'-UTR transformed *E. coli* (figure 4.4). The internal RBS

of *Prrn* was deleted during the cloning of *psbA* 5'-UTR. Although the *psbA* 5'-UTR also contains an RBS, there would be a larger distance between the RBS and the -35 and -10 promoter elements of *Prrn* which is atypical for most σ^{70} -type prokaryotic promoters (Harley and McClure, 1983). This difference may account for the earlier decline in *aadA* expression. Nevertheless, displacement of the RBS was not a rate-limiting factor as the final MIC value was similar to that of *E. coli* transformed with pKCZ and pCART. Thus, regulation of *E. coli* gene expression is mainly promoter dependent. This is in agreement with the results of Chuang *et al.* (1993) who suggested that regulation of gene expression in *E. coli* occurs at the transcriptional level. The MIC screening results of pKCZ Δ *Prrn* transformed *E. coli* also demonstrated that the expression of the *aadA* gene can occur without the regulation of an upstream promoter element to a certain extent albeit weaker than promoter regulated expression (section 4.3.1.3.; figure 4.4). Interestingly, in a separate experiment; *E. coli* bacteria transformed with recombinant plasmid pCART was also observed to propagate at low frequencies on LB agar plates supplemented with 100mg/l of kanamycin. The only possible source of kanamycin resistance was the *NptI* spacer region that was used for the introduction of new RE sites into the MCS of pCART (Chapter 3; section 3.2.8.1.). However, the *NptI* gene did not contain a promoter region or an active RBS and was incorporated in the reverse orientation to the *aadA* gene expression cassette as a precautionary measure to prevent overlapping expression. It is currently unknown if *NptI* was expressed while still in the plasmid or if it was incorporated into the bacterial genome in close proximity to an endogenous gene allowing the overlapping expression of genes. Nevertheless, this anomaly is unlikely to affect the integrity of the experimental results as no growth was ever observed in non-transformed *E-coli* bacteria cultured on antibiotic media, ruling out the possibility of antibiotic resistance gained through spontaneous mutations. In addition, the *NptI* spacer region would also be removed during the construction of the final chloroplast transformation vectors.

4.4.2. Evaluation of transgene expression at RNA level

The RT-PCR results of total RNA samples extracted from *E. coli* bacteria transformed with recombinant chloroplast transformation vectors pEXPR-G and pEXPR-UG indicate that the GFP and *aadA* genes were expressed at the mRNA level (section 4.3.2.1.). The pEXPR-G vector contains an expression cassette for the expression of GFP under the regulation of the *Prrn* promoter alone while GFP expression is regulated by both the *Prrn* promoter as well as the *psbA* 5'-UTR region in pEXPR-UG. Results of the RT-PCR reactions indicate that mRNA transcripts of GFP were present in the total RNA samples of pEXPR-G and pEXPR-UG transformed *E. coli* bacteria (section 4.3.2.1.). These results were further corroborated by the absence of bands in all negative control lanes proving that the observed bands did not arise due to DNA contamination from either a genomic or plasmid source. The *aadA* internal control also serves as an important control as the expression of the *aadA* gene can be phenotypically characterised via growth on culture media supplemented with spectinomycin. The reliability of the RT-PCR reaction can be gauged as a defined band is expected to be produced for the detection of *aadA* mRNA transcripts if the *aadA* gene has been determined to be phenotypically expressed in advance. The other negative control reactions were also necessary to ensure the reliability of the obtained results as RT-PCR is a very sensitive assay that can often lead to many false positives or negatives. The application of RT-PCR for the detection mRNA transcripts in total RNA samples extracted from *E. coli* was a particularly challenging process. The expression of genes in prokaryotes is often coupled where the translation of the mRNA transcript occurs simultaneously as the gene is being transcribed. As a result, the half-life of the majority of mRNA transcripts in prokaryotes is extremely short. Gel electrophoresis results indicate that the quality of mRNA in total RNA samples extracted from *E. coli* degraded rapidly during and after extraction even with careful handling. This observation can be attributed the findings of Bernstein et al. (2002) who reported that

approximately 80% of mRNA transcripts of *E. coli* have half-lives between 3 to 8 minutes. The high turnover rate coupled with the degradation of mRNA via endonucleolytic cleavage imposes a challenge for the evaluation of gene expression in *E. coli* and stresses the importance of proper sample preparation during the RNA extraction and RT-PCR process. Nevertheless, GFP mRNA transcripts were successfully detected in pEXPR-G and pEXPR-UG transformed *E. coli* which suggests that the *Prrn* promoter is able to drive the transcription of GFP in the GOI expression cassettes of the newly constructed chloroplast transformation vectors. Similar to the results of *aadA* expression (section 4.3.1.3.), the *psbA* 5'-UTR did not appear to significantly alter the transcription rates of the GFP gene in *E. coli*. Any discrepancies between the band intensities of the RT-PCR results were minor (figure 4.5 and 4.6) and can be attributed to different levels of exposure during the imaging process as indicated by the relative intensities of the DNA ladder. The expression of transgenes under the control of chloroplast-specific heterologous regulatory elements in *E. coli* is discussed further in Chapter 8, section (8.2.).

4.4.3. Evaluation of transgene expression at protein level

Gene expression in *E. coli* is mainly regulated at the transcriptional level (Chuang *et al.*, 1993), however mRNA stability and turnover is also known to contribute to final protein expression levels. The stability of mRNA transcripts of *E. coli* is known to be affected by determinants which are localised to specific mRNA segments which mediate mRNA decay leading to a decline in protein expression (Belasco *et al.*, 1986). There are several different mechanisms which modulate the decay of mRNA in *E. coli*, one of which is initiated by the endonucleolytic cleavage within the mRNA transcript resulting in the gradual segmented decay of the mRNA (Schmeissner *et al.*, 1984). Another method involves the rate-limiting endonucleolytic cleavage at the 5'-end of the mRNA

transcript within the RBS region. The targeted disruption of the RBS prevents further association of ribosomes and effectively strips the mRNA transcript of all ribosomes and renders it susceptible to rapid and completed degradation by RNases (Belasco *et al.*, 1986). The major difference between the two methods of mRNA decay is that the former method allows the translation process to continue on attenuated mRNA transcripts resulting in truncated proteins whereas the latter method ensures that all translated proteins are complete. The addition of rifampicin to a final concentration of 100mg/l prior to protein extraction ensured that transcription of genes by *E. coli* RNA polymerase is arrested. The absence of shorter non-specific bands in the Western blot profiles for the detection of GFP proteins (figure 4.8) suggests that the stability of mRNA transcripts of GFP in pEXPR-G and pEXPR-UG transformed *E. coli* is most probably modulated by the second method of mRNA decay. The 28kDa bands which were observed in the Western blot profiles using GFP specific primary antibodies indicate the successful detection of GFP expression at the protein level (figure 4.8). In contrast to the results of section (4.3.1.3.), which was based on the expression of the *aadA* antibiotic resistance gene, the Western blot profiles for the detection of GFP in pEXPR-UG transformed *E.coli* showed lower levels of final GFP protein accumulation as compared to pEXPR-G transformed *E.coli* bacteria (figure 4.8). There is evidence where the *psbA* 5'-UTR was shown to have destabilising effects on the mRNA transcripts of *psbA* in the chloroplasts of plants which were kept in the dark (Staub and Maliga, 1994; Hirose and Sugiura, 1996). Nevertheless, it is highly implausible that the destabilising effect of the *psbA* 5'-UTR is to be blamed for the discrepancies in GFP protein levels as the chloroplast specific *cis*- and *trans*- acting factors that interact with the *psbA* 5'-UTR is unlikely to be present in *E. coli* as discussed previously in section (4.4.1.). Instead, the *psbA* 5'-UTR will more than likely be recognised as foreign by the translation machinery of the *E. coli* resulting in the cessation of translation or recycling of the mRNA strand. The extended distance of the RBS from the -35 and -10 conserved elements of

the *Prrn* promoter may also have an effect in GFP expression as discussed earlier in section (4.4.1.). Taken together, the results suggest that discrepancies that were observed between the Western blot profiles for the detection of GFP in both pEXPR-G and pEXPR-UG transformed *E. coli* (figure 4.8) is more likely related to variations in the binding affinity of bacterial ribosomes to the mRNA transcripts. Samples of total cellular protein extracted from pEXPR-G and pEXPR-UG transformed *E. coli* bacteria probed with the c-Myc-specific primary antibody also produced the expected band of approximately 28kDa corresponding to the expected size of the GFP protein (figure 4.9). Hence, the c-Myc tag can be used for the efficient detection of transgenic proteins, as well as, for the immunopurification of tagged proteins from total protein extracts of transformed plants. Bands that were relatively similar in intensity were observed in the Western blot profiles probed with c-Myc specific primary antibodies (figure 4.9). This was in contrast to the results obtained using GFP-specific antibodies. The reason for this observation may be due to that fact that the c-Myc tag only recognises a short 10 amino acid sequence (EQKLISEEDL) which may produce a less quantitative result as compared to GFP specific antibodies. The Western blot profile produced using GFP specific primary antibodies was assumed to be a more accurate representation of actual GFP expression levels. This was further corroborated by the results for the phenotypic detection of GFP fluorescence under UV light which also seems to suggest that GFP expression was higher in pEXPR-G transformed *E. coli* bacteria (section 4.3.2.4.).

4.5. CONCLUSIONS

In summary, the chloroplast-specific *Prrn* and *PtrnFM* promoters were found to be primarily functional and can be used for heterologous expression of *aadA* and GFP genes in *E. coli*. The *PtrnFM* promoter has also been established as a compatible replacement for the *Prrn* promoter and displayed similar transcription efficiencies for the expression of the *aadA* gene in the recombinant plasmid, pCART. The *psbA* 5'-UTR leader sequence was shown to have a marginally negative effect on the expression of both the *aadA* and GFP genes in *E. coli*. However, the *psbA* 5'-UTR leader sequence is non-native to *E. coli* and, hence, cannot be concluded to be unfavourable for gene expression in chloroplasts. Nevertheless, phenotypic expression of GFP was detected in pEXPR-UG transformed *E. coli* which indicated that the *psbA* 5'-UTR was integrated in-frame on the transgene expression cassette. Phenotypic expression of GFP in *E. coli* also indicated that the transgene expression cassettes of pEXPR-G and pEXPR-UG were functional and all elements in the chloroplast transformation vectors were in proper order. All in all, the newly constructed chloroplast transformation vectors pEXPR-G and pEXPR-UG are fundamentally functional and can be potentially used for the transformation of the chloroplast genome of *N. benthamiana*.

Chapter 5: Optimisations of Particle Bombardment Parameters and Transformation of Leaf Explants of *Nicotiana benthamiana*

5.1. INTRODUCTION

Optimisation of particle bombardment is an absolute requirement for the effective transformation of plants. Conventional optimisation of particle bombardment parameters are usually performed by monitoring the transient expression of a marker gene such as β -glucuronidase (GUS) or green fluorescent protein (GFP). Nevertheless, this method of optimisation usually results in parameters for the maximum delivery of DNA-coated gold particles into the bombarded sample which may lead to excessive tissue damage and may be problematical for long-term stable chloroplast transformation. Hence, in this chapter, particle bombardment parameters were optimised in the interest of facilitating the maximum recovery of transformed leaf explants of *N. benthamiana* during the regeneration process.

5.1.1. Particle bombardment: a species independent approach

Particle bombardment is a highly versatile transformation technique which, once established, can be applied to a wide array of tissues and cells across the species barrier. The process of particle bombardment generally follows a relatively simple and efficient methodology which is virtually identical for the transformation of all samples regardless of the type of DNA used or the nature of the host species. The process of particle bombardment also has its uses beyond the scope of genetic transformation, for example, for the generation of expression mutants for the study of gene expression and regulation (Yang and Christou, 1990) and for the investigation of various *cis*- and *trans*- regulatory

elements in promoters and untranslated leader regions (UTR) (Zou *et al.*, 2003; Hayashi *et al.*, 2003). In addition, particle bombardment can be used for the transformation of cells from a wide variety of sources which include callus (Christou *et al.*, 1988), coleoptiles (Reggiardo *et al.*, 1991), protocorms (Kuehnle and Sugii, 1992), immature embryos (Christou *et al.*, 1991), cell suspensions (Fromm *et al.*, 1990), meristematic tissues (McCabe and Martinell, 1993), and pollen (Twell *et al.*, 1989). In contrast, other transformation methods are generally restricted to a limited range of explant types. For example, polyethylene glycol (PEG)-mediated transformation can only be used for the delivery of transgenes into protoplast suspension (Potrykus *et al.*, 1985) and *Agrobacterium* which is almost exclusively used for the transformation of dicotyledonous species (Pua *et al.*, 1987). In addition, particle bombardment also allows the transformation of sub-cellular organelles such as the chloroplasts. The transient expression of chloramphenicol acetyltransferase (*cat*) gene in chloroplasts under the regulation of a chloroplast specific promoter was achieved using particle bombardment (Daniell *et al.*, 1990).

5.1.2. Particle bombardment: history and concept

The concept of directly delivering microparticles coated with nucleic acids into a host was initially conceived by Sanford and co-workers in 1984. The first results were published three years later (Sanford *et al.*, 1988) and were obtained using a gunpowder-driven device to deliver tungsten microcarriers coated with viral RNA into the epidermal cells of onion. In the same year, the introduction of plasmid DNA carrying a foreign gene into onion was also documented using the same technique (Klein *et al.*, 1987). The particle bombardment apparatus was developed as a method of host-independent direct gene transfer. DNA would be precipitated onto microcarriers which are often made of an inert material such as gold or tungsten using a precipitation solution

containing calcium chloride (CaCl_2) and spermidine together with the DNA of interest. The DNA-coated microcarriers would then be loaded onto a macrocarrier film which is then accelerated towards a stopping screen. The stopping screen halts the linear acceleration of the macrocarrier but allows the microcarriers to pass through and continue until it penetrates the sample below (Maliga, 2004). Early models of the particle bombardment device utilises a gunpowder charge as a method of macrocarrier acceleration (Sanford *et al.*, 1988). This traditional method was often risky and posed a major threat of an accidental or uncontrolled explosion. In addition, gunpowder charges were also highly unpredictable at small scales and were hard to calibrate especially between different production batches. Biological samples which were bombarded using the gunpowder charges were also subjected to collateral damage from the explosion. Today, the modern design of the particle bombardment apparatus has eliminated the use of gunpowder and instead relies on inert pressurised helium (He) gas as a means of macrocarrier acceleration. In addition to being a lot safer to operate, the burst pressure which determines the accelerative force of the macrocarrier film can be carefully controlled. This is achieved by halting the flow of He using a rupture disk which has been calibrated to fail at a specific burst pressure. This improvement has significantly increased the reliability of particle bombardment as a convenient and reproducible means of genetic transformation across species (Verma and Daniell, 2007). An example of such a device is the particle delivery system (PDS)-1000/He marketed by BioRad (USA). A schematic diagram of the PDS-1000/He device is shown in figure 5.1.

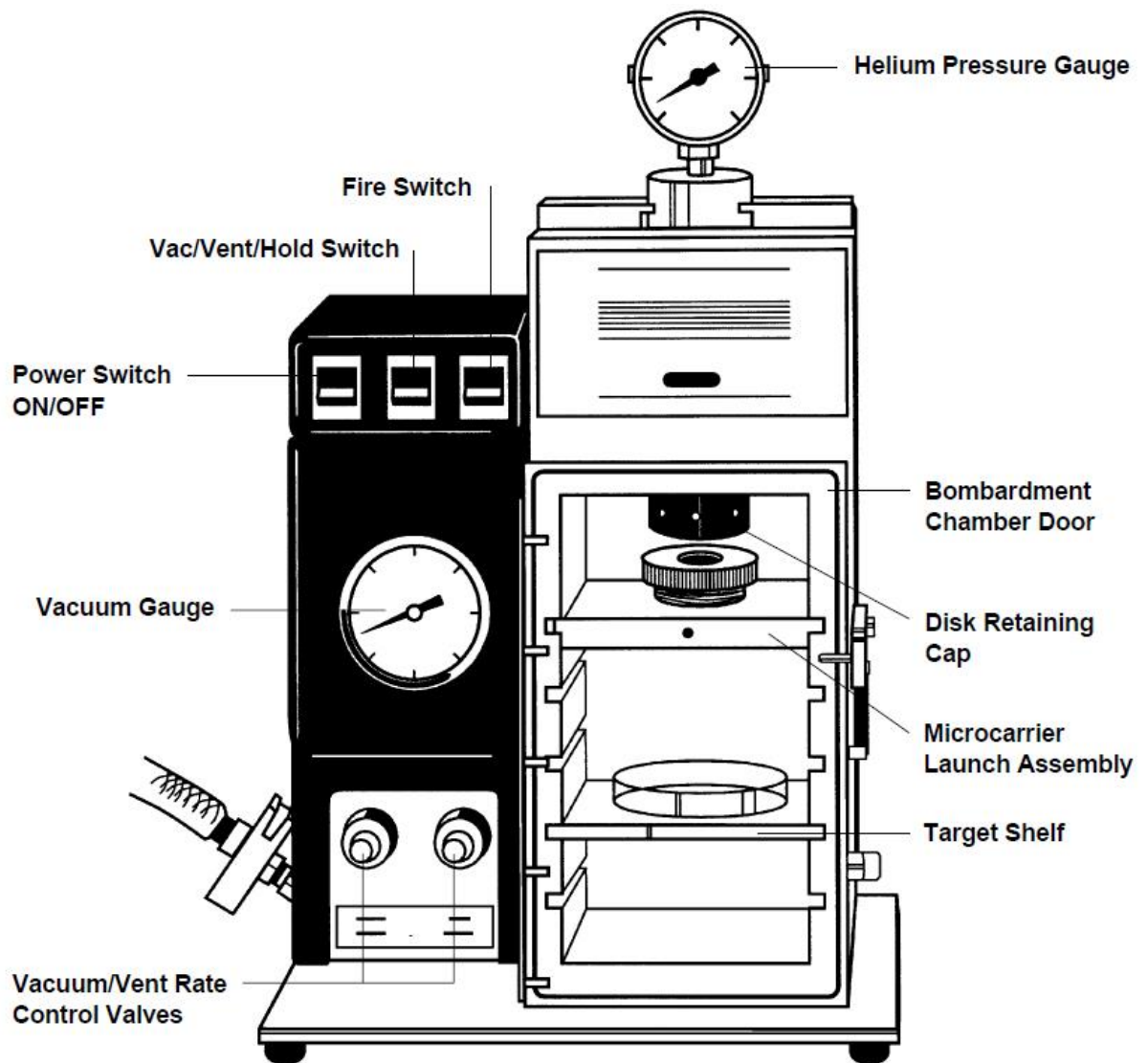


Figure 5.1: Schematic diagram of the PDS-1000/He helium powered particle bombardment apparatus marketed by BioRad. All major components are annotated in the diagram (adapted from the Biolistic® PDS-1000/He Particle Delivery System user manual; BioRad, USA)

5.1.3. Parameters of optimisation

The particle delivery system (PDS)-1000/He (BioRad, USA) allows the operator to manipulate a wide range of variables to optimise DNA delivery into the target cells. These parameters include the burst pressure, distance of the macrocarrier from the rupture disc, distance between the macrocarrier and stopping screen, distance between the stopping screen and target stage and the

vacuum pressure. Some of parameters may produce a more pronounced effect on the success rate of particle bombardment than others. Parameters that are known to play a more important role require careful optimisation whereas parameters that only affect the outcome of the particle bombardment in a minor way requires little to no optimisation at all. A schematic diagram of the PDS-1000/He bombardment chamber is shown in figure 5.2 and a summary of all optimisable parameters are shown in table 5.1.

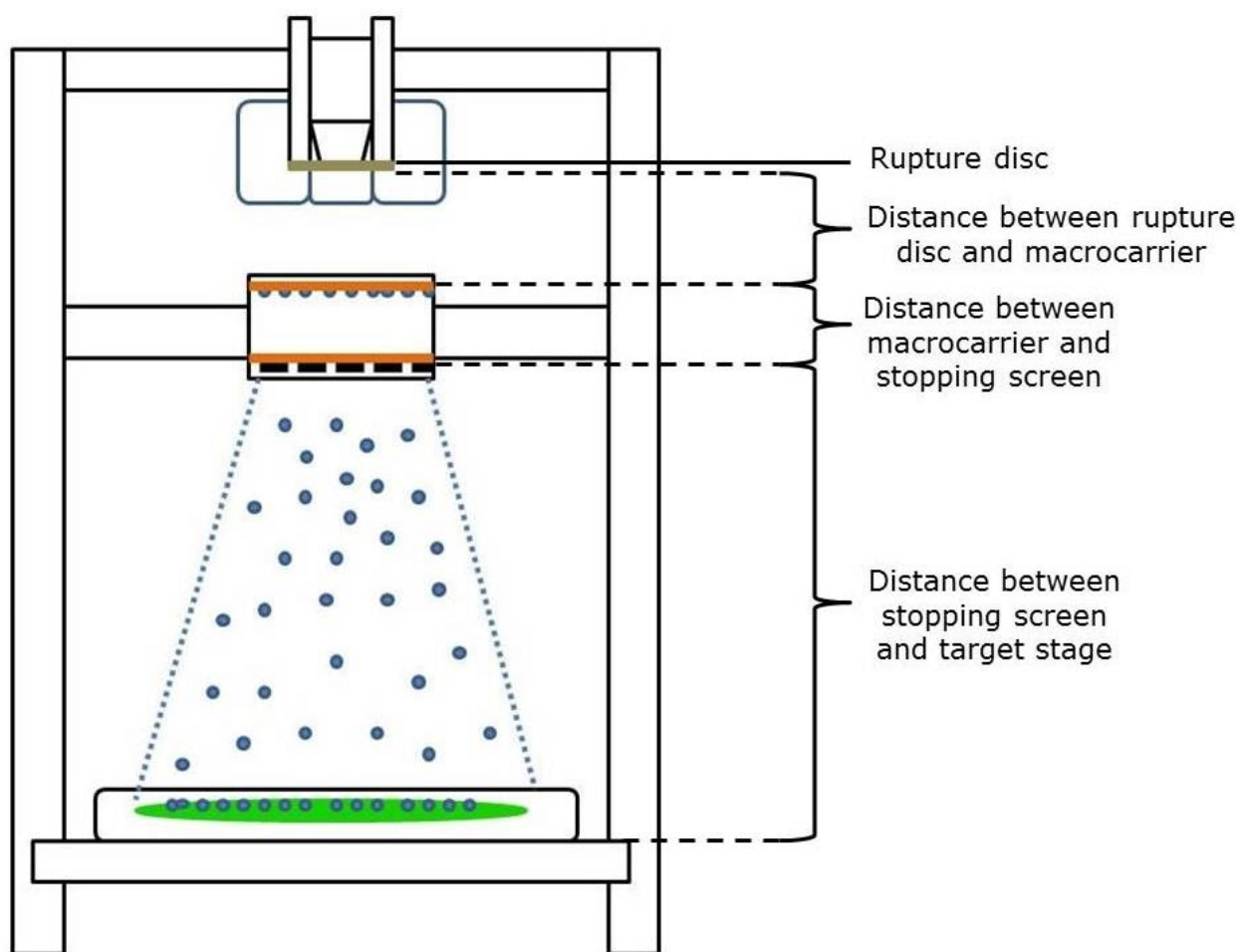


Figure 5.2: Schematic diagram of the bombardment chamber of the PDS-1000/He particle bombardment device. The physical variables which are commonly optimised for particle bombardment are annotated in the diagram.

Table 5.1: Summary of the adjustable parameters of the BioRad (USA) PDS-1000/He particle bombardment device and the respective parameters that are affected.

Variable	Affected parameters
Rupture disc	<ul style="list-style-type: none"> • Acceleration of macrocarrier • Force of bombardment
Distance between rupture disc and macrocarrier	<ul style="list-style-type: none"> • Acceleration of the macrocarrier • Force of bombardment
Distance between the macrocarrier and stopping screen	<ul style="list-style-type: none"> • Accuracy of bombardment • Force of bombardment • Speed of microcarriers
Distance between stopping screen and target stage	<ul style="list-style-type: none"> • Penetrative force of microcarriers • Scattering of the microcarriers • Density of bombardment • Area of bombardment
Vacuum pressure	<ul style="list-style-type: none"> • Accuracy of bombardment • Efficiency of microcarrier migration
Helium regulator pressure	<ul style="list-style-type: none"> • Affects the speed of helium pressure build-up • Affects the speed of bombardment

*Adapted from Klein *et al.*, 1988.

5.1.4. Approaches for optimisation

In this study, the particle bombardment process was optimised for optimal delivery of DNA-coated gold microcarriers in to the chloroplast of *N. benthamiana* with particular emphasis on two key areas; the DNA coating process, and the particle bombardment parameters. Careful optimisation of the DNA coating process would theoretically allow the maximum amount of vector DNA to adhere to the gold microcarriers which is expected to correlate with higher transformation efficiencies. Optimisation of the DNA-coating process was primarily based on obtaining a set of parameters which allowed the maximum amount of plasmid DNA to be precipitated onto the gold microcarriers while minimising the amount of wasted DNA. On the other hand, optimisation of particle bombardment parameters were primarily based on the viability of the

leaf explants of *N. benthamiana* post bombardment. Establishment of a set of parameters which allow the maximum delivery of DNA-coated microcarriers; while, at the same time, minimising excessive damage to the plant tissue is vital for the regeneration of stable transplastomic plants.

5.1.5. Specific objectives

The first part of this chapter focuses on optimising the concentration of reagents required for the DNA-coating process and the second part of this chapter evaluates the effects of various particle bombardment parameters on the viability of *N. benthamiana* leaf explants post-bombardment. The specific objectives of this chapter are: 1) to optimise the DNA-coating process of gold microcarriers by evaluating the effects of varying concentrations of plasmid DNA, CaCl₂ and spermidine on the binding efficiency of DNA to the gold microcarriers; and 2) to evaluate the effects of various parameters of particle bombardment; such as, the amount of microcarriers delivered per bombardment, the number of bombardments (per transformation), distance between stopping screen and target stage, and rupture disk burst pressure on the viability of *N. benthamiana* leaf explants post-bombardment.

5.2. METHODS

5.2.1. Preparation of leaf explants for particle bombardment

Seeds of *N. benthamiana* were sterilised and germinated as described in Chapter 6, section (6.2.1.1.). Plants with leaves of approximately 2cm in diameter were transferred to lower light conditions for incubation 3 days prior to bombardment. Tobacco leaves were harvested and prepared no longer than 1 hour prior to commencement of particle bombardment. Sterile tobacco leaves with an approximate diameter of 2cm were excised from healthy young plants and transferred adaxial side up into a Petri dish containing 25ml of solid Murashige and Skoog (MS) media without any additional components. Petri dishes containing the leaf explants were wrapped in aluminium foil and kept in the dark until commencement of particle bombardment.

5.2.2. Coating of Ø0.6µm microcarriers with plasmid DNA

A stock solution of Ø0.6µm gold microcarriers (BioRad, USA) with a concentration of 60mg/ml was prepared to facilitate the coating process. Plasmid DNA (HBT95::sGFP(S65T)-NOS; accession no: EF090408) with absorbance ratios of $260/230 \geq 1.8$ and $260/280 \geq 2.0$ was prepared and precipitated onto the gold microcarriers with the assistance of spermidine and Ca^{2+} ions supplied by a solution of calcium chloride, followed by immersion in 100% ethanol. The DNA-coated microcarriers were then washed prior to loading onto the macrocarrier film.

5.2.2.1. Washing and preparation of the gold microcarrier stock solution

The gold microcarrier stock solution was prepared by weighing out 30mg of Ø0.6µm gold microcarriers into a 1.5ml tube containing 1ml of 70% (v/v) molecular grade ethanol. The tube was vortexed vigorously for 3-5 minutes until the microcarriers were thoroughly resuspended before they were allowed to settle for a further 15 minutes. The mixture was spun briefly in a microcentrifuge for 5 seconds to pellet the microcarriers and the supernatant was discarded. The gold microcarriers were washed by adding 1ml of sterile dH₂O, followed by vigorous vortexing for 1 minute. The microcarriers were allowed to settle for 1 minute before the tube was spun in a microcentrifuge to pellet the microcarriers. The supernatant was discarded and the wash procedure was repeated for another two cycles. After the third wash cycle, the microcarrier pellet was resuspended with 500µl of 50% glycerol which brought the concentration of the stock solution to 60mg/ml. The stock solution was stored at 4°C and used within 1 month of preparation.

5.2.2.2. Coating of plasmid DNA onto the microcarriers

The microcarrier stock solution was vortexed and sonicated for 5 minutes to resuspend the mixture. A 50µl aliquot (3mg gold particles) of the microcarrier stock solution was transferred by pipetting into a new 1.5ml tube. While the tube was being vortexed, 10µl of plasmid DNA (1µg/µl), 50µl of 2.5M calcium chloride (CaCl₂) solution and 20µl of 100mM spermidine solution was added to the microcarrier suspension in the stated order. The mixture was vortexed for another 3 minutes before allowing the microcarriers to incubate for 1 minute. After vortexing, the tube was spun briefly in a microcentrifuge for 5 seconds to pellet the microcarriers. The supernatant (residual binding solution, RB) was removed and the microcarrier pellet was immersed with 150µl of 100% ethanol (HPLC grade) and resuspended by gentle vortexing. Microcarriers were recovered by spinning the mixture in a microcentrifuge for 5 seconds before

removing the supernatant (waste solution A). The wash procedure was repeated for a second time and the supernatant was removed (waste solution B). The microcarrier pellet was resuspended in 50µl 100% ethanol (HPLC grade). The DNA-coated microcarrier mixture was kept on ice to minimise evaporation.

5.2.3. Particle bombardment procedure

Prior to commencement, all autoclavable parts of the PDS-1000/He (BioRad, USA) particle bombardment device (rupture disc retaining cap, macrocarrier launch assembly and macrocarrier holder) were sterilised by autoclaving. All other non-autoclavable parts were sterilised by exposure to UV-light and by wiping with a 70% ethanol solution in a sterile laminar flow hood. Macrocarrier films and the stopping screens were sterilised by soaking in 100% molecular grade ethanol for 1 minute, followed by air drying in the laminar flow hood. Rupture discs were sterilised by dipping briefly into 99.7% (laboratory grade) isopropanol prior to insertion into the rupture disc retaining cap. This process also allowed the formation of an airtight liquid gasket between the components. All consumables used for the particle bombardment process were purchased from BioRad Laboratories Inc. (USA).

5.2.3.1. Loading of DNA-coated microcarriers onto the macrocarrier film

The microcarrier suspension was resuspended by gently tapping the tube and the contents of the tube were divided equally between 6 sterilised macrocarriers. The suspension was allowed to spread evenly around the central area of each macrocarrier film with a pipette and allowed to air dry for 10 minutes in a laminar flow hood.

5.2.3.2. Transformation of *N. benthamiana* leaf explants

Leaf explants of *N. benthamiana* were prepared as described in section (5.2.1.). Following verification by sequencing, the recombinant plasmid constructs were delivered into *N. benthamiana* leaf explants via particle bombardment using the PDS-1000/He particle delivery system (BioRad, USA) according to the user's manual. Modifications for the optimisation of the standard bombardment procedure are summarised in table 5.2, with the standard bombardment parameters (Klein *et al.*, 1988) indicated with (^a). Each parameter under investigation was evaluated independently while keeping all other parameters constant according to the bombardment protocol of Klein *et al.* (1988). DNA delivery was performed using a completely randomised order across each treatment with a minimum of three replicates per treatment. For example, evaluation of the effects of gold particle load, rupture disk burst pressure, distance between stopping screen and target stage, and the number of bombardments per transformation were performed sequentially using a randomised selection of *N. benthamiana* leaf explants before the cycle was repeated for the second and third replicates, within the same day.

Table 5.2: Summary of particle bombardment parameters evaluated independently for the transformation of leaf explants of *in-vitro* cultivated *N. benthamiana*. Parameters which are denoted by the superscript (^a) are standard particle bombardment parameters as described by Klein *et al.* (1988); whereas, parameters denoted by the superscript (^b) are parameters that have been determined to be optimal according to this study.

Parameters	Conditions tested
A. Preparation of microcarriers	(for 6 bombardments)
Gold particle load	3mg ^{ab}
Plasmid amount	1, 5, 10 ^b , 15 ^a , 20 µg
Final concentration of CaCl ₂	0, 0.5, 1.0 ^b , 1.5, 2.0 ^a M
Final concentration of Spermidine	0, 8 ^a , 16 ^b , 24, 32 mM
B. Particle Bombardment	(for each bombardment)
Gold particle load	100 ^a , 300, 500 ^b , 700, 900 µg
Rupture disk burst pressure	450, 900 ^a , 1100 ^b , 1350, 1800 psi
Distances:	
Rupture disk – macrocarrier	6mm ^{ab}
Macrocarrier – stopping screen	10mm ^{ab}
Stopping screen – target stage	3.0, 6.0 ^{ab} , 9.0, 12.0 cm
Bombardment times	1x ^{ab} , 2x, 3x
Vacuum pressure	-711.20mmHg ^{ab}

^a Parameters used in a standardised bombardment procedures.

^b Parameters used in the optimised bombardment procedures.

5.2.4. Optimisation of DNA coating parameters

The amount of DNA bound to the gold microcarriers (section 5.2.2.2.), remaining in the residual binding solution (section 5.2.2.2.) and the waste solutions (section 5.2.2.2.) were evaluated to determine the effects of varying concentrations of plasmid DNA, CaCl₂ and spermidine on the efficiency of DNA precipitation onto the gold microcarriers.

5.2.4.1. Recovery of DNA from gold particles

In order to determine the amount of plasmid DNA precipitated onto the gold microcarriers under varying conditions, 30µl of TE buffer (10mM Tris, 1mM EDTA at pH8.0) was added to the microcarrier pellet instead of 150µl of 100% ethanol as described in section (5.2.2.2.). The pellet was then resuspended and incubated at 60°C for 5 minutes with continuous vortexing. Bound DNA was recovered in the supernatant after centrifuging the mixture at room temperature, 12 000rpm, for 10 minutes. The concentration of plasmid DNA in the recovered sample was measured using the Nanodrop device as described in section (3.2.4.1.) and the total DNA recovered was determined.

5.2.4.2. Recovery of DNA from residual binding solution and waste solutions

Plasmid DNA (wasted) was recovered from the residual binding solution (RB) (section 5.2.2.2.) to determine the efficiency of the DNA binding process under different parameters. DNA recovery was performed by adding 0.1 volumes of 3M sodium acetate solution (pH5.2) and 2 volumes of 100% ethanol to RB. The mixture was vortexed briefly and incubated at -20°C for 30 minutes. To recover the DNA pellet, the mixture was centrifuged at 4°C in a refrigerated centrifuge (Allegra™ X-22R, Beckman Coulter, USA) at 13 000rpm for 25 minutes. The DNA pellet was rinsed with 70% ethanol and allowed to dry in a vacuum desiccator for 2 minutes, followed by resuspension in 30µl of TE buffer.

The concentration of the recovered sample was measured using the Nanodrop device as described in section (3.2.4.1.). For the recovery of DNA from waste solutions (section 5.2.2.2.), both waste solutions A and B were pooled together in a 1.5ml tube and recovered using the same method as described for solution (RB).

5.2.5. Optimisation of particle bombardment parameters

Particle bombardment parameters were optimised to achieve maximum particle delivery while minimising damage to the target tissue. Leaf explants of *N. benthamiana* were subjected to particle bombardment with HBT95::sGFP(S65T)-NOS coated gold particles and results were scored based on the viability of the bombarded explants during regeneration over a period of 6 weeks. The transient expression of the GFP gene was also used as a means of determining the efficiency of particle bombardment with different burst pressures after all other parameters have been optimised.

5.2.5.1. Evaluation of viability: determining levels of necrosis

Transformed leaf explants were regenerated as described in Chapter 6, section (6.2.6.). The percentage of leaf explant segments which entered a necrotic state was evaluated using the Munsell notation (Munsell, 1912; Nickerson, 1946). Explant samples were categorised based on their colour under a 'cool white' fluorescent light with pale white (Munsell hue value/chroma of 5Y 7/2) indicating chlorosis, pale brown (5YR3/6) indicating 50% necrosis and dark brown (5YR1/2) indicating full necrosis (figure 5.3). Data were analysed by one-way analysis of variance (ANOVA) and the differences between treatments were contrasted with a multiple range test (MRT). All statistical analyses were performed at the 5% level ($p=0.05$) using Microsoft Excel (Microsoft Corp. USA.) with a minimum average of 3 replicates per treatment.

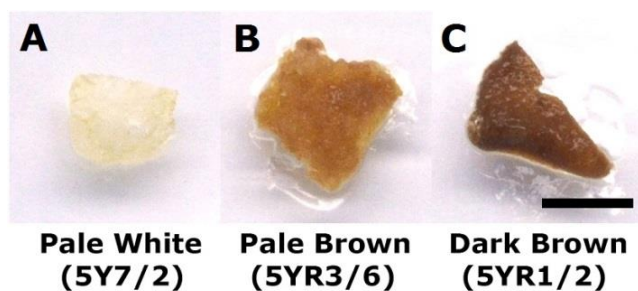


Figure 5.3: Munsell notation of colours representing regenerated explants undergoing chlorosis (A: pale white, 5Y7/2), 50% necrosis (B: pale brown, 5YR3/6) and 100% necrosis (C: dark brown, 5YR1/2). All evaluation procedures were performed under cool-white fluorescent lighting. In this experiment, the 50% necrosis level (pale brown, 5YR3/6) is known as the 'point of no return'; at which, no further growth or regeneration of shoots have been recorded. Bar= 0.5cm.

5.2.5.2. Evaluation of transient GFP expression

Transient expression of GFP in leaf explants bombarded with control vector HBT95::sGFP(S65T)-NOS were evaluated at the phenotypic level three days post-bombardment by observation under a stereoscopic microscope (SMZ-1500, Nikon, Japan) equipped with a GFP illuminator (C-HGFI Intensilight, Nikon, Japan) with an excitation wavelength range of 380-400 nm. A total of 3 replicates were evaluated for each treatment and the average number of observable GFP spots in a rendered monochromatic image of the bombarded leaves of *N. benthamiana* was manually recorded. The obtained data was evaluated as described in section (5.2.5.1.).

5.3. RESULTS

5.3.1. Evaluation of parameters affecting DNA precipitation onto microcarriers

Data on plasmid DNA content was gathered from three critical sources during the precipitation process in order to determine the optimum parameters for the reaction. Recovery of precipitated DNA from the gold microcarriers provides valuable information on the amount of DNA that was precipitated. The recovery of plasmid DNA from the residual precipitation solution and the wash solutions, on the other hand, provides information on the amounts of plasmid DNA wasted in the precipitation and washing process, respectively. The efficiency of the precipitation process under varying parameters can then be determined based on the gathered data.

5.3.1.1. Amount of plasmid DNA loaded

The statistical results on the effects of varying plasmid DNA concentrations on the efficiency of DNA precipitation are summarised in figure 5.4. The highest amount of plasmid DNA precipitated ($7.7\mu\text{g}$) was recorded when $20\mu\text{g}$ of DNA was used. However, only 38.4% of total amount was precipitated onto the microcarriers (figure 5.4: panel A). The amount of DNA remaining in the residual precipitation solution was calculated at 27.5% and DNA loss in subsequent washing steps were determined to be 6.9% of the total precipitated (figure 5.4: panel B and C). The maximum efficiency was achieved when $10\mu\text{g}$ of plasmid DNA was precipitated onto the microcarriers which allowed the precipitation of 55.5% of plasmid DNA with 16.1% loss in the residual precipitation solution and 4.6% loss in subsequent washing steps (figure 5.4: panel A, B and C). Although a respectable percentage for DNA precipitation (54.6%) was achieved when $1\mu\text{g}$ of DNA was used, the total amount of precipitated DNA was; however, 10 times lower as compared to when $10\mu\text{g}$ of

DNA was used. Furthermore, precipitation using 1 μ g DNA also recorded the highest percentage of DNA loss during subsequent washing steps (recorded at 28% of precipitated DNA).

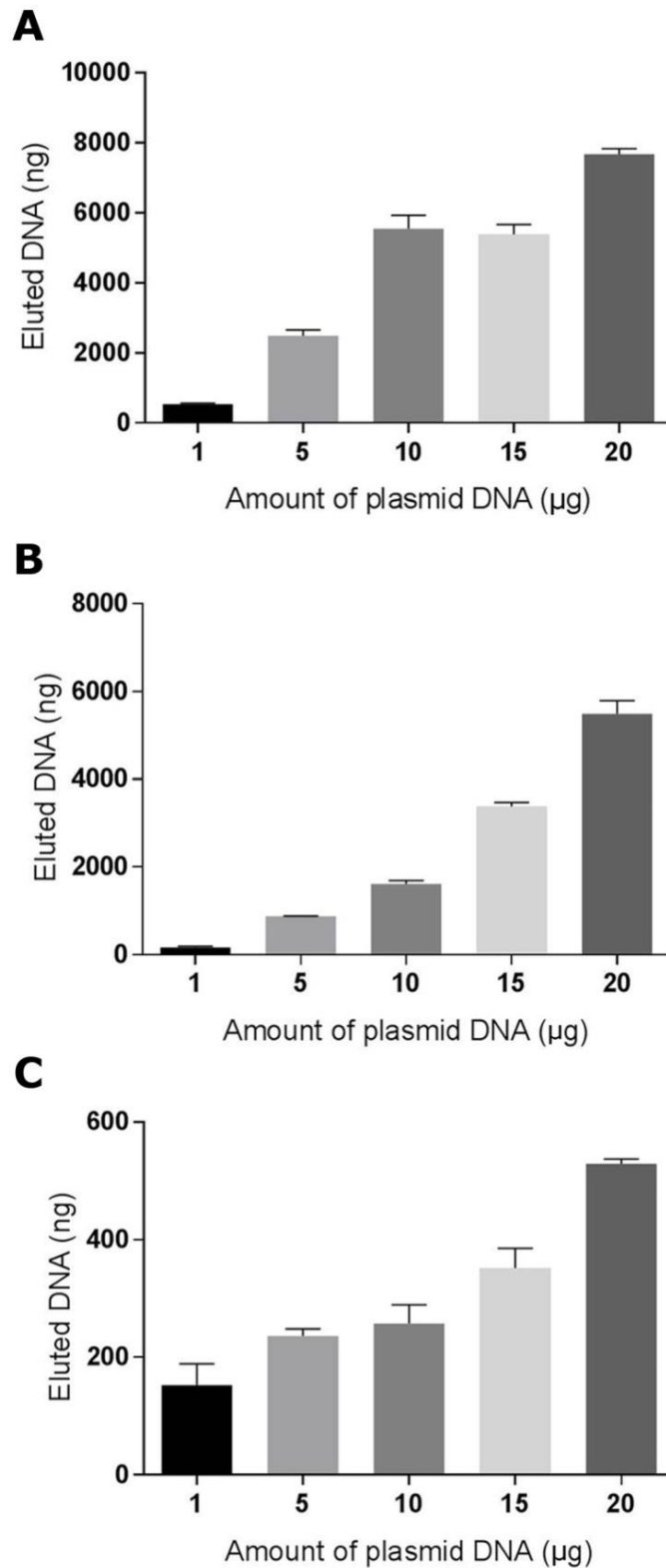


Figure 5.4: Effects of varying plasmid DNA concentrations on the efficiency of precipitation onto gold microcarriers. Precipitated plasmid DNA was recovered from (A) gold microcarriers (section 5.2.4.1.); (B): residual binding solution (section 5.2.4.2.); (C): waste solution (section 5.2.4.2.). Bars represent the mean values and error bars correspond to standard deviation (n=3).

5.3.1.2. CaCl₂ Concentration

The statistical analysis on how different concentrations of CaCl₂ affect the precipitation of plasmid DNA onto the gold microcarriers is summarised in figure 5.5. In the absence of CaCl₂, only a negligible amount (<0.3%) of DNA was recovered from the gold particles even when 15µg of plasmid DNA was used (figure 5.5: panel A). A significant improvement in the efficiency of precipitated DNA was observed when CaCl₂ was introduced to the precipitation solution. The amount of plasmid DNA precipitated in reactions containing CaCl₂ typically ranged from 24.8% to 36.6% (figure 5.5: panel A) of total DNA used. The highest efficiency of DNA precipitation was recorded when a final concentration of 1M CaCl₂ was used (36.6%). Interestingly, precipitation reactions performed at this concentration also recorded the lowest amount of DNA loss in the residual precipitation solution at only 2.0% (figure 5.5: panel B). The amount of DNA loss during subsequent washing steps did not appear to be affected by the concentration of CaCl₂ used in the precipitation reaction (figure 5.5: panel C).

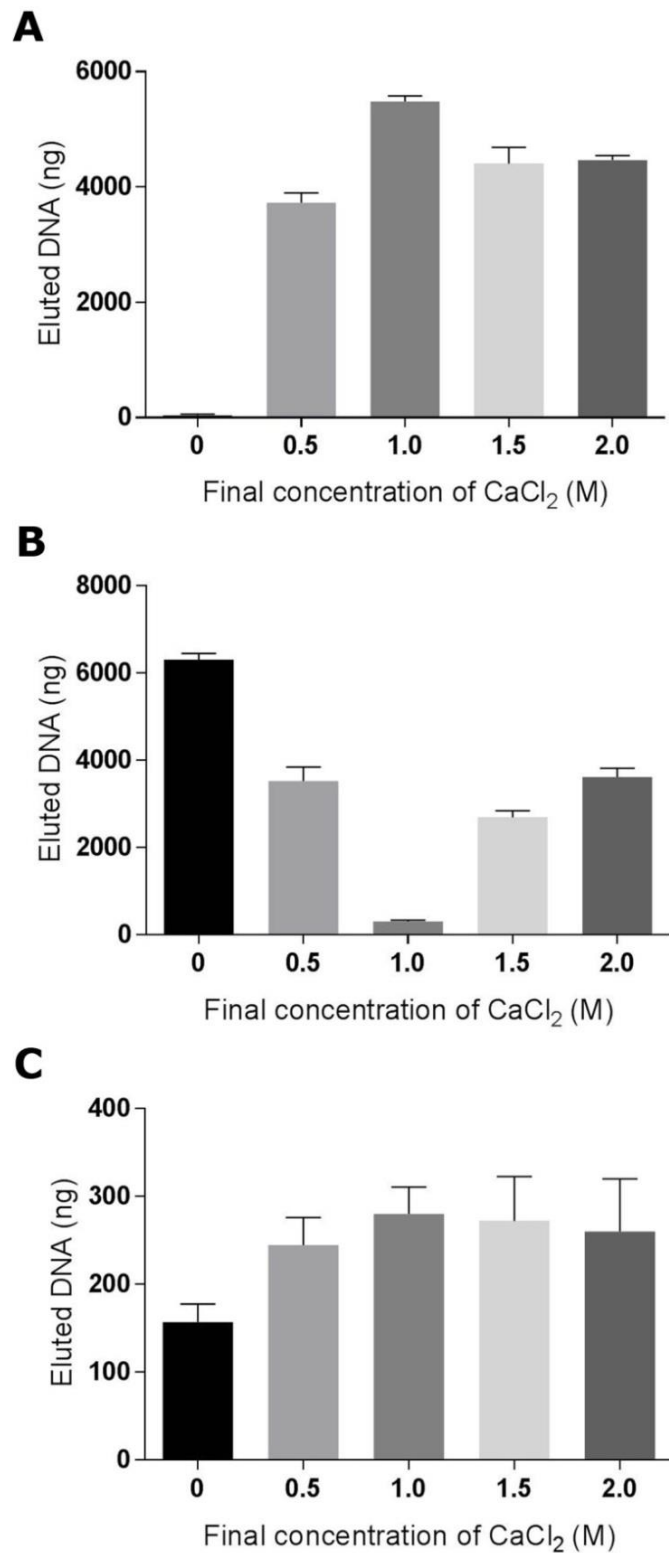


Figure 5.5: Effects of varying CaCl₂ concentrations on the efficiency of plasmid DNA precipitation onto gold microcarriers. Precipitated plasmid DNA was recovered from (A): gold microcarriers (section 5.2.4.1.); (B): residual binding solution (section 5.2.4.2.); (C): waste solution (section 5.2.4.2.). Bars represent the mean values and error bars correspond to standard deviation (n=3).

5.3.1.3. Spermidine concentration

The results for the statistical analysis on how the concentration of spermidine affects the efficiency of plasmid DNA precipitation onto gold microcarriers are summarised in figure 5.6. DNA precipitation was highly inefficient in the absence of spermidine and was almost untraceable with only 0.3% recovered from the gold microcarriers (figure 5.6: panel A). Most of the DNA was unbound and remained in the residual binding solution (figure 5.6: panel B). DNA precipitation was significantly improved when spermidine was added to the mixture. The data also seemed to suggest that DNA precipitation was not permanent at low spermidine concentrations. At a concentration of 8mM, the precipitation efficiency was recorded at 17.0% of the total DNA (~2.5µg DNA). However, 58% of the precipitated DNA was lost in subsequent washing steps (figure 5.6: panel C, 8mM). A loss of this magnitude was not reflected in other reactions where higher concentrations of spermidine were used (figure 5.6: panel C, 16-32mM). The amount of DNA loss in the other reactions with higher spermidine concentrations typically ranged between 5 and 6% of the total amount of precipitated DNA. The lowest concentration of spermidine required to achieve optimum binding was recorded at a final concentration of 16mM where the precipitation efficiency was 36.8% of total plasmid DNA with only a 1.7% loss in the residual precipitation solution and 5.5% loss of precipitated DNA in subsequent washing steps.

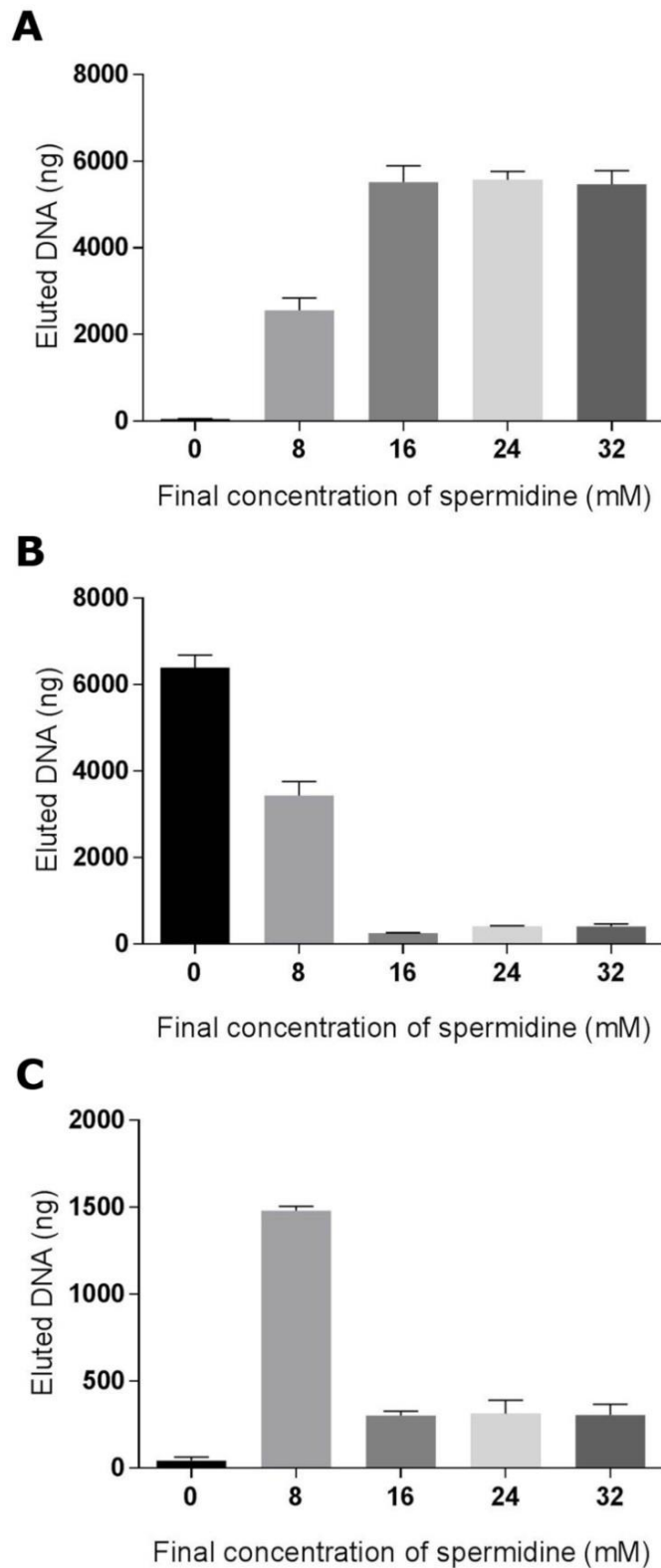


Figure 5.6: Effects of varying spermidine concentrations on the efficiency of plasmid DNA precipitation onto gold microcarriers. Precipitated plasmid DNA was recovered from (A): gold microcarriers (section 5.2.4.1.); (B): residual binding solution (section 5.2.4.2.); (C): waste solution (section 5.2.4.2.). Bars represent the mean values and error bars correspond to standard deviation (n=3).

5.3.2. Evaluation of particle bombardment parameters

In this experiment, empty gold microcarriers were used to assess the effects of different bombardment parameters on the amount of damage caused to the bombarded tissue. Optimisation of bombardment parameters was performed to achieve maximum delivery of microcarriers into *N. benthamiana* leaf explants while minimising unnecessary collateral damage. The main parameters under investigation were; the gold particle load, the number of bombardments per transformation, the distance between the stopping screen and target stage and rupture disk burst pressure. Data was recorded over a period of 6 weeks based on the viability of *N. benthamiana* leaf explants which was determined by observing the percentage of leaf specimens undergoing necrosis (browning of leaf tissue). The severity of necrosis was evaluated based on Munsell notation. Examples of regenerated explants displaying chlorosis (0% necrosis), 50% necrosis and 100% necrosis are shown in figure 5.3. Leaf explants that displayed $\geq 50\%$ necrosis were categorised as "in the state of necrosis" as no further growth or regeneration of shoots have been recorded from the leaf explants in this category. A summary of the statistical analyses of the data on how these parameters affected the viability of leaf explants post-bombardment is shown in figure 5.7.

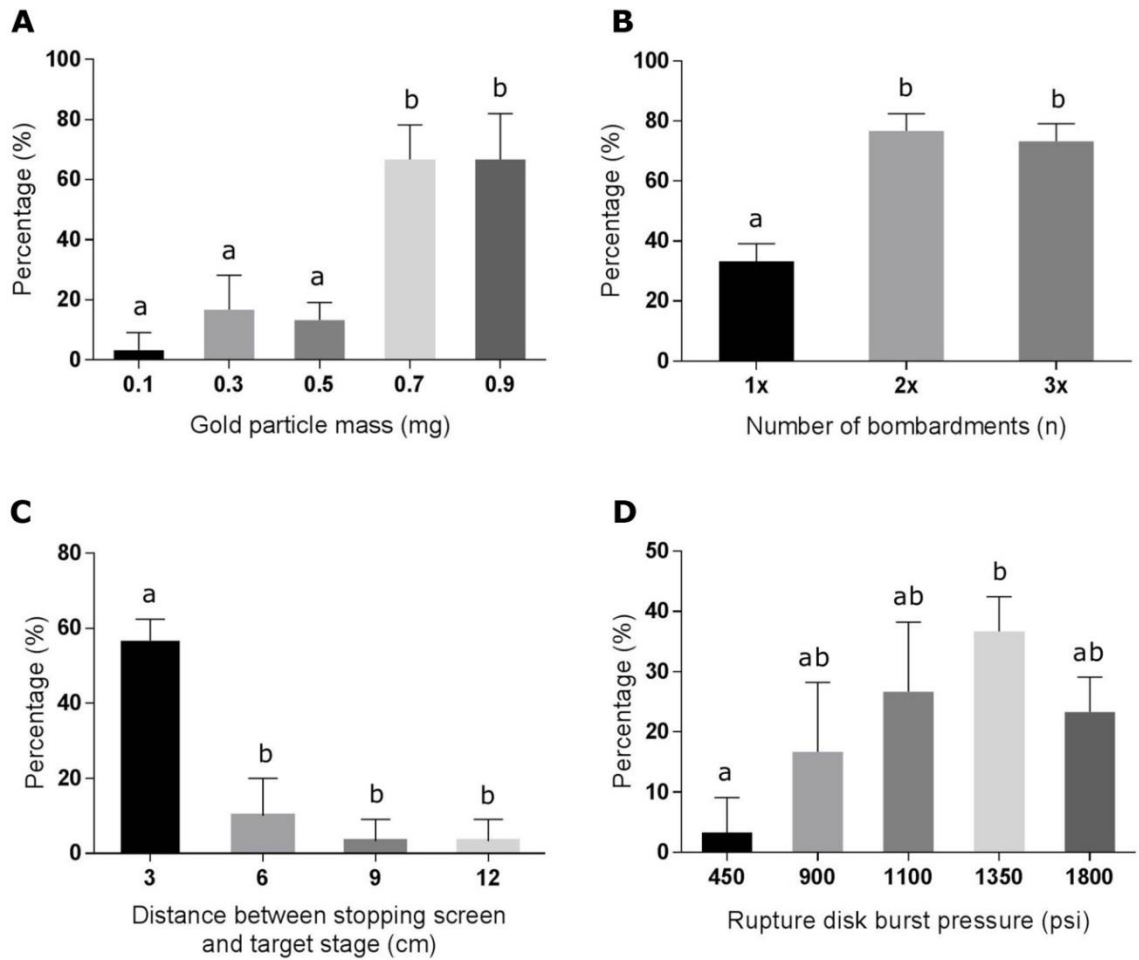


Figure 5.7: Severity of collateral damage sustained by the leaf explants of *N. benthamiana* during the particle bombardment process under varying conditions. The data represents the percentage of leaf explants that have undergone necrosis. (A): Gold particle load per bombardment; (B): Number of bombardments; (C): Distance between the stopping screen and the target stage; (D): Rupture disk burst pressure. Bars represent the mean values and error bars correspond to standard deviation (n=3); Bars marked with different letter are significantly different at p<0.05.

5.3.2.1. Gold particle load

The optimal amount of gold particles per bombardment was generally found within the range of 0.1 to 0.5mg where emergence of necrosis was low at 3.3 to 13.3% (figure 5.7: panel A). A significant increase in the emergence of necrosis was observed in bombarded samples when higher amounts of gold particles were used. Leaf explants bombarded with 0.7 and 0.9mg of gold particles per bombardment resulted in the emergence of necrosis in 66.7% of the samples (figure 5.7: panel A). A gold particle load of 0.5mg per bombardment was assumed to be optimum; allowing both the delivery of high gold particle loads with minimal collateral damage to the plant tissue.

5.3.2.2. Number of bombardments

Emergence of necrosis was lowest (at 33.3%) when leaf explants were subjected to a single bombardment per transformation event (figure 5.7: panel B). The emergence of necrosis was significantly higher when leaf explants were subjected to 2 or 3 bombardments per transformation with necrotic rates of 76.7 and 73.3%, respectively (figure 5.7: panel B).

5.3.2.3. Distance between stopping screen and target stage

Emergence of necrosis was highest when the closest possible distance of 3cm was used for particle bombardment with a rate of 56.7% (figure 5.7: panel C). A significant decline in the emergence of necrosis ranging from 10.0 to 3.3% was observed when the distance between the stopping screen and the sample stage was increased within the range of 6 to 12cm. However, standard deviation values indicated that the data recorded for bombardment distances of 9.0 and 12.0cm were highly variable with respect to the actual mean value (figure 5.7: panel C).

5.3.2.4. Rupture disk burst pressure

In general, the increase in the emergence of necrosis appeared to correlate with increasing burst pressures within the range of 450 to 1350psi (figure 5.7: panel D). However, an anomaly was observed in the trend when the burst pressure of 1800psi was used for bombardment (figure 5.7: panel D). The emergence of necrosis in *N. benthamiana* leaf explants bombarded at 1800psi showed a uniform decline, observed within all three sample replicates. These results were further corroborated by the standard deviation value of the mean which strongly indicate that the results were not merely the result of outlying data points.

5.3.3. Evaluation of optimised particle bombardment parameters via transient GFP expression

The optimised particle bombardment parameters were subsequently tested based on transient GFP expression using control vector HBT95::sGFP(S65T)-NOS (figure 5.8) and data was recorded according to section (5.2.5.2.). However, prior optimisation results of rupture disk burst pressure based on necrosis were inconclusive. Hence, in this experiment, the effects of different rupture disk burst pressures were re-evaluated based on transient GFP expression. Transient expression of GFP was highest with a burst pressure of 1100psi with a recorded mean of 49.3 for the number of observed spots per bombardment (figure 5.9). Particle bombardment at burst pressures 900, 1350 and 1800psi generally produced similar results with mean values of 26.7, 25.3 and 31.3, respectively for the number of observed GFP spots. However, it should be noted that at a burst pressure of 1800psi, there was a high degree of variance between replicate samples causing the standard deviation of the mean to be remarkably high at 18.8 (59.9% of the mean value).

Transformation with a burst pressure of 450psi produced the lowest results with a mean value of only 0.7 for the number of GFP spots observed (figure 5.9).

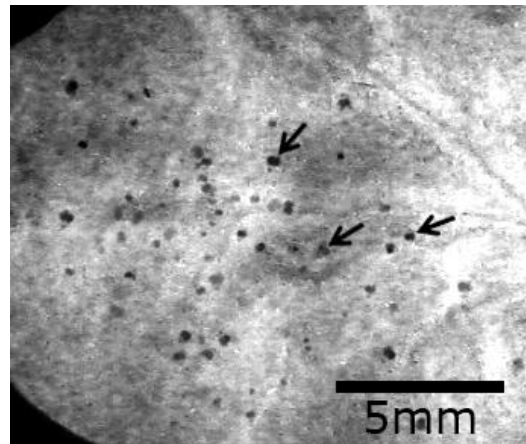


Figure 5.8: Inverted monochromatic image of HBT95::sGFP(S65T)-NOS bombarded leaf of *N. benthamiana* displaying transient expression of GFP (dark spots indicated by arrows) 3 days post bombardment. Bar= 5mm.

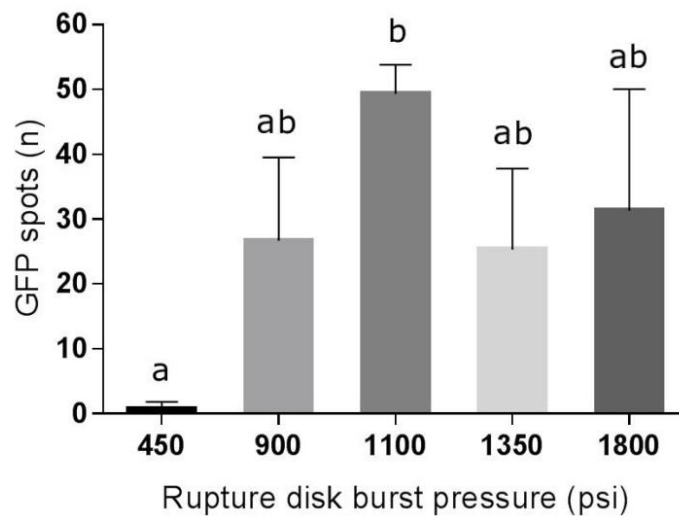


Figure 5.9: Transient expression of GFP in leaf explants of *N. benthamiana* under different particle bombardment burst pressures. Bars represent the mean values and error bars correspond to standard deviation (n=3). The average number of GFP spots is recorded per bombardment. Bars marked with different letters are significantly different at $p < 0.05$.

5.4 DISCUSSION

Optimisation of the particle bombardment process was typically focused around the two key areas; which are DNA precipitation onto the gold microcarriers, and delivery of the gold microcarriers into the plant tissue. The effects of different concentrations of plasmid DNA, CaCl₂ and spermidine on the DNA precipitation process was evaluated by the recovery of plasmid DNA from the gold microcarriers, precipitation mixture and wash solutions. Based on the obtained results, an optimised protocol was developed to allow the maximum precipitation of plasmid DNA onto the gold microcarriers while avoiding excess wastage of materials. Optimisation of particle bombardment parameters for the delivery of gold particles into the leaf explants of *N. benthamiana* was evaluated based on the viability of the post-bombarded tissue. Empty microcarriers were used for the optimisation process as the mass of the precipitated DNA is, on average, only 0.001% of the mass of the gold microcarriers and can be assumed to be negligible. Within the context of this experiment, the optimum bombardment parameters would allow the maximum delivery of gold microcarriers into the leaf explants of *N. benthamiana* with minimal detrimental effects to its regenerative potential. Thus, the effects of different bombardment parameters; such as, gold particle load, number of bombardments, distance between stopping screen and target stage, as well as, rupture disk burst pressures were evaluated based on the regenerative potential of *N. benthamiana*. Nevertheless, data obtain from experiments pertaining to the optimisation of rupture disk burst pressures were inconclusive when evaluated based on necrosis. Hence, the effects of rupture disk burst pressures were re-evaluated based transient GFP expression in conjunction with other optimised parameters of particle bombardment.

5.4.1. Preparation of explants for particle bombardment

The incubation conditions of *N. benthamiana* plants prior to particle bombardment are known to affect the distribution of chloroplasts within the leaves. It is known that chloroplasts of plants possess the ability to avoid light in order to minimise photodamage as well as to accumulate towards low light as a response to maximise photosynthesis (Kasahara *et al.*, 2002). For all experiments, *in-vitro* cultured *N. benthamiana* plants were kept in low light levels 3 days prior to particle bombardment. This period of incubation under low light is important to allow the chloroplasts of the palisade mesophyll cells to move and accumulate towards the periclinal walls to allow more direct exposure to particle bombardment. This phenomenon was also observed in *A. thaliana* where chloroplasts in plants grown under weak light remain close to the periclinal walls where they can then respond quickly to any added light (Trojan and Gabrys, 1996). It is proposed that phototropins play an important role as photo-receptors for the detection of varying light conditions (Kagawa *et al.*, 2001) which then mediate the movement of chloroplasts in the cytosol via the cytoskeletal network along the actin filaments (Kandasamy and Meagher, 1999). It is important that leaves are excised no longer than 1 hour prior to bombardment as continued exposure to intense fluorescent illumination may encourage the movement of chloroplast towards the antipericlinal walls which would greatly reduce the exposure of chloroplasts to the microcarriers during bombardment.

5.4.2. Particle Bombardment: coating of DNA onto microcarriers

In recent years, gold microcarriers are often preferred over tungsten as gold microcarriers are known to have better surface and size uniformity as compared to tungsten (Maliga, 2004). In addition, tungsten was also found to be toxic in certain cell types and has been shown to reduce the efficiency of particle

bombardment (Russell *et al.*, 1992). The precipitation of plasmid DNA onto the gold microcarriers is a fundamental step which predetermines the maximum amount of DNA that can be delivered into the target tissue. The effects of several key components in the precipitation solution were evaluated in order to determine the most efficient combination to maximise precipitation of DNA on the microcarriers while minimising DNA wastage. In general, the amount of DNA which is precipitated onto the gold microcarriers increased in relation to increasing plasmid DNA loads (section 5.3.1.1.). This is expected as an increase in the amount of loaded DNA would theoretically provide a higher concentration of DNA for binding to the gold microcarriers until the point of saturation is attained. This has been clearly demonstrated in the data which showed an increasing trend in the percentage of unbound DNA with increasing amounts of loaded DNA. The optimum amount of plasmid DNA for 3mg of gold particles in relation to this study was deduced to be 10 μ g which corresponds to a concentration of about 3.3 μ g DNA/mg microcarriers as it allowed maximum precipitation with minimum wastage. Although the amount of precipitated DNA was 38.3% higher when 20 μ g of DNA was used, the amount of DNA leftover in the precipitation solution was substantial at 27.5% of the total amount loaded. Furthermore, excessive levels of DNA have been known to cause the aggregation of microcarriers which reduce the efficiency of particle bombardment (Oard, 1991). The aggregation of gold particles has been linked to the uneven distribution of microcarriers during bombardment which may result in heavy damage to a localised point (Humara *et al.*, 1999). The aggregation of gold microcarriers was also known to happen over time when gold particles are left in an aqueous solution (Sanford *et al.*, 1993). Hence, it is highly advisable that the gold microcarrier stock solution is sonicated each time before use in order to dissociate the microcarriers and to allow more even coverage of DNA. DNA-coated microcarriers should also be used immediately and should not be stored

for extended periods of time to prevent the aggregation of microcarriers which is often the cause for the lack of homogeneity between bombardments.

Calcium chloride (CaCl_2) was found to be an absolute requirement in the precipitation solution for the efficient precipitation of plasmid DNA onto the gold microcarriers. In the absence of CaCl_2 , most of the plasmid DNA remained in the residual precipitation solution and any remaining DNA that was associated with the microcarriers was lost in subsequent washing steps. It was interesting to note that the amount of wasted DNA left in the residual precipitation solution was lowest at a final concentration of 1.0M and increased at higher final concentration levels of CaCl_2 (1.5M and 2.0M). The increase in the amount of plasmid DNA remaining in the residual precipitation solution may be caused by the negative effects of high salt concentrations on the precipitation of DNA (Rasco-Gaunt *et al.*, 1999). Hence, from the results, it can be deduced that the optimal final concentration of CaCl_2 which produced the most efficient DNA precipitation results was at 1.0M. No significant difference between the precipitation efficiencies of CaCl_2 solutions at pH5 and pH10 were observed in this experiment. In contrast, Perl *et al.* (1992) reported that sources of calcium with high pH levels (e.g. pH10) effectively doubled expression by improving the precipitation of DNA and found calcium nitrate to be superior to calcium chloride at both pH5 and pH10 producing up to a four-fold increase in expression. However, work done by Rasco-Gaunt *et al.* (1999) also indicated that no significant difference was observed in the amount of precipitated DNA when different sources of calcium ions were used as well as for CaCl_2 solutions at both pH5 and pH10.

Similar to CaCl_2 , the data also suggest that spermidine was a crucial element for the efficient precipitation of plasmid DNA onto gold microcarriers. Almost the entire amount of introduced DNA was found wasted in the residual precipitation solution in the absence of spermidine. Even at low concentrations of

spermidine (8mM), precipitation of DNA was generally poor and most of the precipitated DNA was subsequently lost in downstream washing procedures. This suggests that a certain threshold concentration of spermidine is required for the proper precipitation and adsorption of DNA onto the microcarriers which has been experimentally deduced to be at a final concentration of $\geq 16\text{mM}$. These results were not in agreement with those of Perl *et al.* (1992) who stated that spermidine was not a necessary component for the precipitation of DNA and reported that the maximum expression of transgenes were observed when spermidine was completely omitted from the commonly used procedure of Klein *et al.* (1988). However, it is worth mentioning that current results were based on the amount of bound DNA recovered from the microcarriers while the results of Perl *et al.* (1992) was based on the transient expression of the GUS gene in the scutellar calli of wheat. The delivery of excessive amounts of plasmid DNA or residual spermidine into the host tissue may have negative effects on the expression of transgenes; however, these effects were not tested in this study. Interestingly, the age of the spermidine stock solution also appeared to have a significant effect the efficiency of DNA precipitation. In preliminary experiments, the efficiency of DNA precipitation onto gold microcarriers was severely compensated when stock solutions of spermidine were stored at -20°C and subjected to multiple freeze-thaw cycles. This was in agreement with the report of Sanford *et al.* (1993) who also established that older spermidine stock solutions were generally ineffective after repeated freeze-thaw cycles. Southgate *et al.* (1995) also reported that even when spermidine aliquots were stored at -20°C , transient gene expression in several plant species declined as the stock solution aged. Hence, it is imperative that the spermidine solution should only be prepared when it is required and the spermidine stock should be kept frozen indefinitely at -80°C . In this study, the optimal amount of plasmid DNA has been determined to be at $10\mu\text{g}$, and the optimal final concentrations of CaCl_2 and

spermidine were deduced to be 1.0M and 16mM, respectively; for every 3mg of gold particles used.

5.4.3. Particle bombardment: delivery of microcarriers

Optimisation of particle bombardment for the delivery of microcarriers was conventionally based on parameters which allow maximum transient expression of either the GFP or GUS marker genes in the bombarded plant tissue. However, it was found that maximum transient expression of GFP did not always correlate with the stable transformation of *N. benthamiana* leaf explants. In theory, increasing the proportion of target cells which are bombarded with DNA coated microcarriers should increase the likelihood of a stable transformation event. Unfortunately, a larger proportion of plant cells would also be subjected to damage which correlated with a decline in viability during the selection and regeneration process. As a result, a large percentage of putatively stable transformants would be lost during selection. Hence, in an effort to improve the recovery of stable transplastomic plants, optimisation of microcarrier delivery parameters were based on the viability of *N. benthamiana* leaf explants post-bombardment. In this study, bombarded leaf explants of *N. benthamiana* have been found to display a characteristic process of necrotic browning in response to heavy damage sustained during particle bombardment (section 5.3.2.). This observation indicated the onset of necrosis and can be potentially used as a measurement of viability. In order to obtain empirical data for the extent of necrosis within leaf explants of *N. benthamiana*, the colour of leaf samples were evaluated using Munsell notation (Munsell, 1912). The Munsell Book of Colour (Munsell Colour Company, USA.) was used instead of the colour charts from the Royal Horticultural Society because it allows the potential interpolation of data for future work; a feature which is not possible using the colour charts from the Royal Horticultural Society (Griesbach and Austin, 2005).

The Munsell notation describes a very specific colour based on three colour dimensions of hue, value and chroma in the stated order (Munsell, 1912). The Munsell notation has also been comprehensively compared with the Royal Horticultural Society's colour chart (Voss, 1992) and used routinely for describing flower colours (Griesbach and Austin, 2005) as well as in the field of soil science. Current results have identified the colour of (5YR3/6) has identified to represent the level of 50% necrosis (figure 5.3). All leaf explant samples of *N. benthamiana* which displayed a level of necrosis $\geq 50\%$ failed to regenerate viable shoots. Hence, in the context of this experiment, the level of 50% necrosis (5YR3/6) was identified as 'the-point-of-no-return' beyond which leaf explants undergo progressive necrosis with a 0% chance of survival.

Mature chloroplast of tobacco typically have an average diameter of 5-8 μm (Hayashi *et al.*, 2003). In order to minimise damage to the chloroplast of *N. benthamiana* leaf explants, all experiments were performed using gold microcarriers of $\text{\O}0.6\mu\text{m}$. In addition to minimising damage to the chloroplast, the smaller microcarriers also provide a larger total surface area per unit mass for the precipitation of plasmid DNA. In all optimisation experiments, the gold microcarrier load was standardised at the lowest tested concentration of 100 μg per bombardment to ensure that all results for the evaluation of necrosis were mainly influenced by the independent variable under investigation. In theory, a higher microcarrier load would allow higher amounts of DNA to be bound and delivered into the target tissue corresponding to better transformation efficiencies. However, in practice, excessive microcarrier load is often associated with high levels of tissue damage. This was clearly reflected in the current results where no significant difference was observed in the development of necrosis in bombarded leaf explants of *N. benthamiana* at lower microcarrier loads of 0.1 to 0.5mg per bombardment (figure 5.7: panel A). In contrast, when higher microcarrier loads of 0.7 and 0.9mg per bombardment were used, a

significant increase in the development of necrotic tissue was observed within bombarded leaf explant (figure 5.7: panel A). Similar results were also obtained by Klein *et al.* (1988) and Aragao *et al.* (1993) who found that increasing the microcarrier load beyond a specific threshold point caused an increase in cell damage as well as the decline in transient gene expression. Rasco-Gaunt *et al.* (1999) also observed better shoot regeneration in plant tissues bombarded with lower microcarrier loads as compared to that of higher microcarrier loads. In contrast, McCown *et al.* (1991) demonstrated that although viability of bombarded plants declined when higher microcarrier loads were used, transformation frequencies in *Populus* remained unaffected. Based on current results, it is found that a microcarrier load of 0.5mg per bombardment was ideal for chloroplast transformation. A microcarrier load of 0.5mg per bombardment would allow the maximum amount of DNA to be transferred into the leaf explants of *N. benthamiana* while still maintaining low levels of collateral damage to the plant tissue.

The final velocity of the microcarrier is the main factor that affects the penetrative force and is determined by the acceleration (burst pressure) and the distance of the target tissue from the stopping screen (referred to as "microcarrier travel distance" hereafter) (Southgate *et al.*, 1995). In theory, the penetrative force of the microcarriers is enhanced by increasing the burst pressure, reducing the microcarrier travel distance; or both. This can be particularly useful when transforming plant samples with thick cuticles or epidermal layers which require higher microcarrier velocities for effective penetration but this may be detrimental to delicate tissue samples.

The results indicated that increasing the burst pressure from 450psi to 1350 psi at a microcarrier travel distance of 6cm caused a noticeable increase in the percentage of necrotic leaf explants (figure 5.7: panel D). This suggests that high burst pressures were unfavourable for the transformation of *N.*

benthamiana leaf explants which was in agreement with the results of Russell *et al.* (1992). Interestingly, a noticeable decline in the percentage of necrotic leaf samples was observed at a burst pressure of 1800psi. It can be hypothesised that the distribution of microcarriers is affected by high burst pressures which may have skewed or caused a high density of microcarriers to be deposited over a narrow area. This would cause a smaller proportion of leaf explants to be damaged by bombardment and hence undergo necrosis. This hypothesis was further substantiated by the data obtained from the detection of transient GFP expression under varying burst pressures (figure 5.9). A smaller number of GFP spots were observed when leaf explants of *N. benthamiana* were transformed at a burst pressure of 1800psi as compared to 1100psi. These results were in agreement with those obtained by Rasco-Gaunt *et al.* (1999) who observed a similar reduction in GUS expression at high bombardment pressures of 1300 to 1550psi and noted, based on the distribution of blue spots, that high bombardment pressures caused a smaller area of the tissue to be very heavily targeted and more likely to be damaged. Based on the data obtained in this experiment, the optimum burst pressure was determined to be at 1100psi at a microcarrier travel distance of 6cm. A shorter microcarrier travel distance is undesirable as even at a lower bombardment pressure of 900psi, a significantly higher occurrence of necrosis was recorded in bombarded leaf explants of *N. benthamiana* (figure 5.7: panel C). The longer microcarrier travel distance of 9 and 12cm were also undesirable as statistical analysis of the data showed that results were highly variable at both 9 and 12cm indicated by the high standard deviation value under the conditions tested. Klein *et al.* (1988) and Aragao *et al.* (1993) also found that increasing the microcarrier travel distance led to a decrease in the frequency of transformation. This clearly demonstrates that burst pressures have to be increased to maintain impact velocity when the microcarrier travel distance is increased.

With respect to the number of bombardments performed per transformation, the results indicated that the lowest amount of damage was sustained when leaf explants of *N. benthamiana* were subjected to a single bombardment per transformation. The sustained damage was significantly higher when subjected to 2 and 3 bombardments per transformation (figure 5.7: panel B). Accumulation of damage due to multiple bombardments is expected. However, the data showed no significant difference in the damage sustained by leaf explants that were bombarded twice per transformation and three times per transformation. It can be hypothesised that during the third bombardment, the microcarriers were penetrating cells which have already been previously damaged during the first and second bombardments. Hence, no significant increase in necrosis was observed between samples that were subjected to 2 and 3 bombardments per transformation. Multiple bombardments are mainly useful only if the primary delivery of microcarriers to the target tissue is inefficient (Lonsdale *et al.*, 1990). However, Rasco-Gaunt *et al.* (1999) found no significant difference between single and multiple bombardments in the transient expression of the GUS gene in wheat tissue. Multiple bombardments are not recommended for the transformation of *N. benthamiana* due to the significant decrease in viability. In addition, multiple bombardments using the PDS-1000/He gun would require subjecting the plant tissue to multiple vacuum and bombardment cycles which would cause excessive damage to the cell walls and result in cytoplasm leakage. Nevertheless, a vacuum condition was crucial to reduce or eliminate any effects of air drag which may affect the displacement of the microcarriers. In addition, elimination of air from the bombardment chamber also reduces any risk of explosion or shockwave effects which may cause damage to the plant tissue. In all experiments, particle bombardment was performed with 711.20mmHg of vacuum. Particle bombardment performed at vacuum pressures weaker than 685.80mmHg did not produce any result which was in agreement with the reports of other researchers. Klein *et al.* (1988)

reported that transformation efficiency improved as the vacuum pressure increased. Rasco-Gaunt *et al.* (1999) found that transformation did not occur at vacuum pressures lower than 685.80mmHg and that transformation at 711.20mmHg was significantly better than 685.80mmHg. Genga *et al.* (1991) also reported a three-fold increase in the transient expression of the GUS gene in tobacco when the vacuum pressure was increased from 609.60mmHg to 711.20mmHg. Southgate *et al.* (1995) noted that high vacuum pressures allowed a more uniform distribution of microcarriers over the target area. A more uniform distribution of microcarriers would allow better resolution of individual transient gene expression events allowing previously grouped spots to appear as individual spots. This may, in part, account for the apparent increase in expression levels observed by other research groups. In this study, it was essential that particle bombardment was performed under high vacuum pressures as small Ø0.6µm microcarriers were used for the transformation of the chloroplast genome and air drag may be a significant factor (Sanford *et al.*, 1993). It is best to perform the bombardment and release the vacuum as soon as possible to minimise vacuum damage to the plant tissue. Exposure of plant tissue to excessive levels of vacuum over an extended period of time may lead to the expansion of soluble gases within the cytoplasm which may potentially rupture the plant cells. Excessive vacuum exposure may also induce the removal of water from the plant material and induce plant tissue damage (Sanford *et al.*, 1993).

5.4.4. Other factors that affect particle bombardment

Several other technical factors can also cause a significant effect on the outcome of particle bombardment. In this study, it was discovered that rupture disks manufactured between different production lots can have an uncertainty of up to ±150psi. The hydrophobic/hydrophilic properties of the macrocarrier films

are also highly variable between production lots and were found to be the major factor affecting the distribution of DNA-coated gold microcarriers during the loading process (figure 5.10). In this study, both strongly hydrophobic and hydrophilic macrocarriers caused a high degree of variability in the results of particle bombardment. Reproducible results could only be obtained from 'neutral' macrocarriers. It can be hypothesised that pre-wetting of the macrocarrier film with a 0.1% solution of Triton X-100 followed by thorough rinsing with dH₂O may improve the distribution of the microcarriers on the macrocarrier film leading to more reliable particle bombardment results.

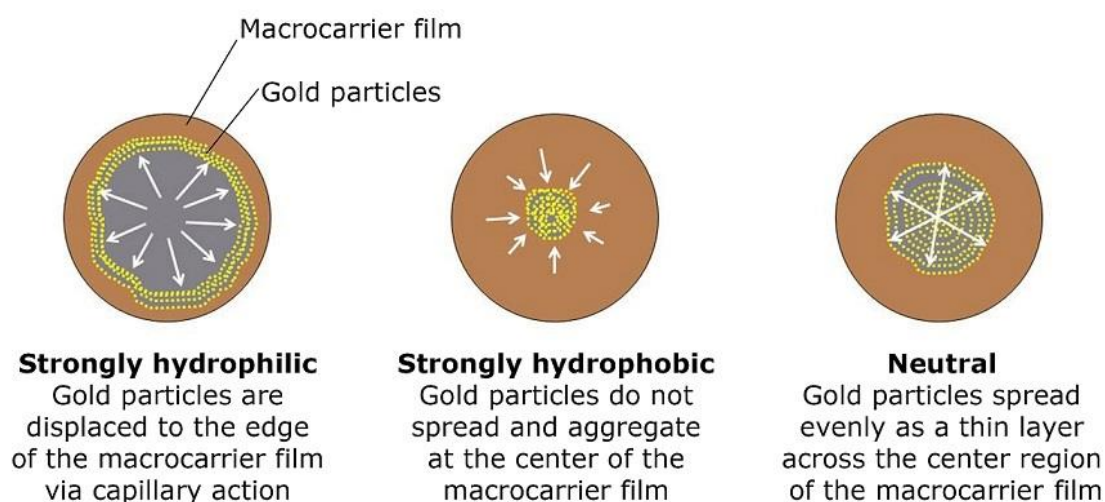


Figure 5.10: Profiles for the distribution pattern of the DNA-coated gold microcarriers on strongly hydrophilic, strongly hydrophobic and neutral macrocarrier films. Strongly hydrophilic and strongly hydrophobic macrocarrier films caused the uneven distribution of gold microcarriers during the loading process. Reproducible results could only be obtained from evenly distributed gold microcarriers on 'neutral' macrocarriers.

5.5. CONCLUSION

In summary, the optimum combination of parameters for the efficient precipitation of plasmid DNA onto gold microcarriers has been obtained and is deduced to comprise 10µg of plasmid DNA with 1.0M CaCl₂; 16mM spermidine and 3mg of Ø0.6µm gold microcarriers. Optimum particle bombardment parameters for the delivery of DNA coated gold microcarriers into the leaf explants of *N. benthamiana* were deduced to be a single bombardment using 500µg of DNA coated gold particles performed at a burst pressure of 1100psi with a distance of 6cm between the stopping screen and target tissue. The combination of optimised parameters obtained in this chapter was subsequently used for the transformation of *N. benthamiana* leaf explants with the newly constructed chloroplast transformation vectors aiming at significantly improving the likelihood of a successful stable transformation event (discussed further in Chapter 7).

Chapter 6: Post-Bombardment Selection and Regeneration of Transformed Plants

6.1. INTRODUCTION

Plant tissue culture is essential in order to provide sterile plant material for the particle bombardment process as well as for the recovery of explants post-bombardment. In this chapter, various aspects of plant tissue culture were optimised in the interest of maximising the recovery and regeneration of leaf explants of *N. benthamiana* post-bombardment.

6.1.1. Tissue culture

Plant tissue culture is the general term used to describe the practice of growing plant cells, tissues or organs in an artificially prepared nutrient medium of known composition, solid or liquid, in an aseptic environment (Purohit, 2012). It has many applications across the field of agriculture, forestry, plant breeding, horticulture, phytopathology, somatic cell hybridisation, modern biotechnology, botany and industrial production of plant metabolites. Tissue culture was first used in large scale for the propagation of virus-free orchids (Morel and Martin, 1952). It later became apparent that the technique of tissue culture could be applied to any plant as long as the right media formulation and the right processes were optimised for its culture. Hence, in theory, the totipotent nature of plants would allow them to be regenerated indefinitely in tissue culture. The propagation of any plant *in-vitro* involves 4 stages of culture growth. Namely, 1) explant establishment or initiation, 2) propagation or multiplication, 3) rooting; and 4) acclimatisation or hardening off for growth on soil (Purohit, 2012).

6.1.2. Regeneration of post-bombardment

The tissue culture process is a fundamental part of transformation via particle bombardment and is crucial in order to supply healthy explants for the transformation procedure as well as to maximise the recovery of putative transformants post bombardment. Establishment of stress tolerant plants through *in-vitro* selection may prove essential in providing healthy resilient explants of *N. benthamiana* which are capable of withstanding the extreme stress from the transformation and the selective regeneration procedure (Murashige, 1974; Rai *et al.*, 2011). The proper optimisation of plant growth regulator levels is also an essential part of the regeneration process as it will determine the pathway of regeneration (Gaspar *et al.*, 1996). In addition, unbalanced plant growth regulator levels are also known to cause detrimental effects during regeneration. Other factors that relate to the success of regeneration include the use of suitable media types for the recovery of damaged explants as well as the proper minimal inhibitory concentration (MIC) of the selective agent in order to provide a clear distinction between transformed and non-transformed explants.

6.1.3. Somaclonal variation

Plant cells are known to spontaneously develop genetic variability during tissue culture. This phenomenon is known as "somaclonal variation" and was first introduced by Larkin and Scowcroft (1981) to describe the differences observed between plant (somaclone) variants in tissue culture. The term "gametoclonal variation" was also introduced by Evans *et al.* (1984) to describe the variations observed between variant clones raised specifically from gametic or gametophytic cells. The occurrence of somaclonal variation in plants is now considered to be a rule rather than an exception (Purohit, 2012). Initially, tissue culture was viewed mainly as a means for the rapid cloning of explants and the

occurrence of somaclonal variation was viewed only as a burden to the process. However, in recent years, somaclonal variation is hailed as a novel way of introducing new traits into tissue cultured plants without directly resorting to genetic manipulation particularly for the introduction of traits related to stress resistance (Rai *et al.*, 2011).

6.1.4. Approaches for investigation

The focus of this chapter was to detail the aspects that were required for the efficient recovery and regeneration of post-transformed leaf explants of *N. benthamiana*. Several key areas of the regeneration process were investigated and optimisation experiments were performed in order to improve regeneration efficiency. These areas include the establishment of *N. benthamiana* explants *in-vitro*, optimisation of auxin and cytokinin concentrations, evaluation of MIC levels and evaluation of media types for regeneration.

6.1.5. Specific objectives

In order to assist in the successful recovery of stable transplastomic lines of *N. benthamiana*; the specific objectives of this chapter are: 1) to establish stress tolerant plants of *N. benthamiana* via cycling of *in-vitro* selection, 2) to evaluate the effects of auxin and cytokinin on the growth and development of *N. benthamiana* leaf explants, 3) to determine the best combination of plant growth regulators for the regeneration of calli and shoots, 4) to determine the minimum level of antibiotics required to distinguish between transformed and non-transformed explants; and 5) Determine the media type best suited for the recovery of damaged leaf explants of *N. benthamiana* post-bombardment.

6.2. METHODS

6.2.1. *In-vitro* propagation of *N. benthamiana*

Establishment of sterile cultures of *N. benthamiana in-vitro* was fundamental to the process of particle bombardment. Wild-type plants generally do not cope well with the stress of tissue culture and a period of acclimatisation may potentially prove beneficial for the development of stress tolerance over time.

6.2.1.1. Establishment of sterile cultures of *N. benthamiana*

Approximately 100 seeds of *N. benthamiana* were transferred into a sterile 1.5ml tube and 500µl of Triton solution (2-3 drops of Triton X-100 in 400µl dH₂O) was added to the tube which was shaken vigorously for 1 minute to thoroughly rinse the seeds. Seeds were allowed to settle for 30 seconds and the wash solution was removed using a pipette before adding 500µl of 20% bleach solution. The tube was inverted to disperse the seeds and vortexed continuously for 10 minutes before another 500µl of Triton solution was added to the tube. The tube was vortexed again and seeds were allowed to settle for 1 minute before removing the wash solution. The washing procedure was repeated again using 500µl of Triton solution. The seeds were then immersed with 500µl of 70% ethanol (HPLC grade) and resuspended by inversion and allowed to settle for 30 seconds. The ethanol solution was discarded and the seeds were rinsed with 1ml of dH₂O. Another 1ml of dH₂O was added to the tube and the seeds were imbibed for 1 hour before they were spread evenly onto moist filter paper saturated with dH₂O in a sterile Petri dish and allowed to germinate under white fluorescent light for 7-10 days. The seedlings were transferred aseptically onto solid Murashige and Skoog (MS) media after 2 weeks and were cultivated under a photoperiod of

16 hours light and 8 hours darkness at 25°C. Routine subculturing of plantlets onto fresh solid MS media was performed every 3-4 weeks.

6.2.1.2. Establishing stress tolerant plants via *in-vitro* selection

Within the context of this experiment, “stress tolerant” plants are defined as plants which display a normal phenotype during *in-vitro* propagation despite being subjected to unavoidable tissue culture related abiotic stress factors, such as, unfavourable temperature and humidity conditions. Cultured *N. benthamiana* plants were subjected to *in-vitro* selection for abiotic stress tolerance 2 months post germination. Shoots (comprising the apical meristem together with 2 or 3 young leaves) were excised and subcultured aseptically onto fresh solid MS media. Explants were cultivated under a photoperiod of 16 hours light and 8 hours darkness at 25°C and growth performance was monitored after 4 weeks. Regenerated explants were sorted based on their susceptibility towards *in-vitro* related abiotic stress which was denoted by the onset of aberrant morphological traits such as decreased leaf diameter, decreased internodal length, increase in leaf number, and elongated petioles (figure 6.9: panel B). Internodal segments were excised from plants in the top 80-percentile (least affected by abiotic stress) and subcultured onto fresh solid MS media. Cycling of shoot regeneration was repeated every 4 weeks using the internodal segments from the top 80-percentile of regenerated plants, for a minimum of 6 months in order to establish stress tolerant plants. Within the context of this experiment, stress tolerant plants are defined as plants that do not display (or only display minimal) aberrant morphological traits when subjected to abiotic stresses of *in-vitro* culture. Changes in the morphology of wild-type, stress tolerant and stress susceptible plants were recorded using a sample size of 10 plants (n=10). The morphology of regenerated plants was compared with wild-type plants of the same age and the data was evaluated according to section (6.2.5.).

6.2.2. Optimisation of plant growth regulators for regeneration

In preliminary studies, leaf explants of *N. benthamiana* were subjected to varying concentrations of 1-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) to determine the effects of the plant growth regulators on growth and development during regeneration. Subsequent optimisation experiments were performed in order to determine the optimum combination of NAA and BAP for the regeneration of callus and shoots, respectively from leaf explants of *N. benthamiana*.

6.2.2.1. Preparation of stock solutions of NAA and BAP

Stock solutions of NAA and BAP (Duchefa Biochemie, Netherlands) were prepared by dissolving 50mg of the respective compound with 1ml of 0.1M NaOH in a 1.5ml tube. The mixture was vortexed until all contents were completely dissolved before transferring to a 50ml conical tube. Distilled water was added to the solution to a final volume of 50ml (1mg/ml). The solution was sterilised by filtration through a 0.15µm pore filter into a sterile 50ml conical tube and kept at 4°C until use. Stock solutions were stored for no longer than 2 months.

6.2.2.2. Evaluating the effects of NAA and BAP on plant growth and development

Solid MS media supplemented with varying concentrations of NAA and BAP was prepared by mixing 50ml of molten MS media (~60°C) with the required volumes of NAA and BAP in a sterile 50ml conical tube before pouring into sterile disposable petri dishes. Leaf explants of *N. benthamiana* with an average diameter of 2cm were harvested and dissected aseptically into squares of 0.5 X 0.5cm. Experiments were performed using 20 random leaf explants in each plate with 3 replicates for each treatment cultivated under a photoperiod of 16 hours light and 8 hours darkness at 25°C for 4 weeks. Data on callus induction was recorded based on the number of leaf explants that had a callus

mass of more than 50% of the total mass of the regenerated tissue. Data on shoot induction was recorded based on the number of shoots that were observed in each plate. All experiments for the optimisation of plant growth regulators were performed using 3 replicates for each treatment (n=3) and the data was evaluated as described in section (6.2.5.). A summary of the treatments that were performed is shown in table 6.1.

Table 6.1: Combinations of plant growth regulators used in the optimisation process to determine the optimum concentration of NAA and BAP for callus and shoot regeneration, respectively.

Preliminary evaluation	NAA (mg/l)	0.10	0.50	1.00	1.50	2.00
	BAP (mg/l)	2.00	1.50	1.00	0.50	0.10
Optimisation of NAA Conc.	NAA (mg/l)	0.10	0.25	0.50	0.75	1.00
	BAP (mg/l)	1.00				
Optimisation of BAP Conc.	NAA (mg/l)	1.00				
	BAP (mg/l)	0.10	0.25	0.50	0.75	1.00

6.2.3. Minimal inhibitory concentration for selective regeneration

Stock solutions of spectinomycin and streptomycin (Duchefa Biochemie, Netherlands) were prepared by dissolving the antibiotics in dH₂O to a final concentration of 100mg/ml in a sterile 50ml conical tube. Selective MS media containing either spectinomycin or streptomycin were prepared using the molten agar method similar to section (6.2.2.2). Experiments were performed using dissected leaf explants of *N. benthamiana* as described in section (6.2.2.2) with 20 explants in each Petri dish and 3 replicates per treatment cultivated under a photoperiod of 16 hours light and 8 hours darkness at 25°C. Data was recorded over a period of 4 weeks based on the colour change observed in the regenerated leaf samples. Explant samples were only described as chlorotic

when a pale white phenotype (Munsell notation of 5Y7/2) or lighter is displayed. All experiments for the optimisation of MIC were performed using 3 replicates for each treatment (n=3) and any necrotic (discussed in Chapter 5, section 5.2.5.1.) samples were omitted from the data for statistical analysis. The recorded data was evaluated according to section (6.2.5.).

6.2.4. Effects of different plant tissue culture media on regeneration

The efficiency of explant regeneration was evaluated on three different types of regeneration media. Solid agar plates of MS media including vitamins (MS+Vit), Gamborg B5 media including vitamins (GB+B5) and MS media including B5 vitamins (MS+B5) were prepared according to manufacturer's instructions with the addition of 0.5mg/l of NAA and 1.0mg/l of BAP (section 6.2.2.2.). Experiments were performed using a random selection of *N. benthamiana* leaf explants as described in section (6.2.2.2) with 20 explants in each Petri dish and 5 replicates for each media type cultivated under a photoperiod of 16 hours light and 8 hours darkness at 25°C. Leaf explants were allowed to regenerate over a period of 4 weeks and the data was recorded based on the mean weight of the regenerate callus tissue per sample per plate. All experiments were performed using 5 replicates for each treatment (n=5) and the recorded data was evaluated as described in section (6.2.5.).

6.2.5. Statistical analysis of optimised parameters

All experiments for the establishment stress tolerant plants (section 6.2.1.2.); optimisation of plant growth regulators (section 6.2.2.2.), MIC optimisation (section 6.2.3.), and evaluation of regenerative media (section 6.2.4.) were performed with the specified number of replicates for each

treatment as indicated in each section. The relative states of chlorosis or necrosis were evaluated using Munsell notation as described in Chapter 5, section (5.2.5.1.), where necessary. All collected data were analysed by one-way analysis of variance (ANOVA) and the differences between treatments were contrasted using a multiple range test (MRT) as described previously in Chapter 5, section (5.2.5.1.).

6.2.6. Regeneration of plant samples post-bombardment

Transformed leaf explants of *N. benthamiana* were allowed to incubate in the dark at 25°C for 48 hours post-bombardment. The bombarded leaf explants were then dissected into 0.5 x 0.5cm segments and transferred onto selective regeneration media containing 500mg/l spectinomycin. Regeneration was performed using two different combinations of plant growth regulators for the induction of callus (0.5mg/l NAA, 1.0mg/l BAP) and shoots (0.1mg/l NAA and 1.0mg/l BAP), respectively. Plates containing leaf explants were cultivated under a photoperiod of 16 hours light and 8 hours darkness at 25°C. Antibiotic selection pressure was maintained by subculturing the regenerated explants onto fresh selective media containing the respective plant growth regulators and antibiotics; alternating between spectinomycin and streptomycin every 2 weeks to minimise the recovery of spontaneous mutants. Swollen leaf explants were also dissected into 0.5 x 0.5cm segments to maintain contact with the selective media. Any antibiotic resistant shoots or calli that emerged during the selective regeneration process were subcultured onto fresh MS media containing 500mg/l spectinomycin.

6.3. RESULTS

6.3.1. Establishment of stress tolerant *N. benthamiana in-vitro*

The morphological differences observed between the wild-type, stress tolerant and stress susceptible plants are summarised in table 6.2. In brief, stress tolerant plants had a smaller leaf diameter and a shorter internode length but had a higher number of leaves, a higher average height and slightly longer petiole lengths as compared to the wild-type plants. However, these discrepancies were not statistically significant at $p > 0.05$. On the other hand, the stress susceptible plants had a much smaller leaf diameter, shorter internodes and a shorter average height but higher number of leaves and longer petiole lengths when compared to both wild-type and stress tolerant plants. The differences in the data for leaf diameter, internode length and number of leaves were statistically significant at $p < 0.05$ between stress susceptible and wild-type plants. However, the data obtained for the height and petiole lengths were not statistically significant at $p > 0.05$. Stress susceptible plants were also observed to display several abnormal morphological traits as compare to wild-type plants. Among them were irregular or deformed vascular structures and elongated light green translucent leaves as opposed to the usual round, opaque, dark green leaves observed in both wild-type and stress tolerant plants (figure 6.9).

Table 6.2: Summary of measurements and observations made between wild-type, stress tolerant and stress susceptible plants cultivated *in-vitro*. All data represents the mean and standard deviation values (n=10).

Trait	Wild-type	Stress tolerant	Stress susceptible
Average leaf diameter (cm)	2.05 ±0.31 ^a	1.85 ±0.28 ^a	0.75 ±0.41 ^b
Average internode length (cm)	1.05 ±0.24 ^c	0.93 ±0.21 ^c	0.35 ±0.13 ^d
Average height (cm)	4.52 ±0.48 ^e	5.07 ±0.53 ^e	3.52 ±0.87 ^e
Average petiole length (cm)	0.71 ±0.17 ^f	0.74 ±0.13 ^f	1.45 ±0.57 ^f
Number of leaves (n)	7.3 ±1.2 ^g	8.0 ±1.5 ^g	13.9 ±2.2 ^h
Leaf colour	Dark green	Dark green	Light green
Leaf shape	Round	Round	Elongated
Vascular structure	Regular	Regular	Deformed

*Values marked with different superscript letters are significantly different at p<0.05

6.3.2. Optimisation of plant growth regulators for regeneration

Results from the preliminary experiments using varying combinations of NAA and BAP were highly variable giving rise to the regeneration of shoots, callus and a combination of both (figure 6.1). A combination of 0.1mg/l NAA and 2.0mg/l BAP mainly produced shoots (2.3 ±1.2 calli and 39.7 ±4.2 shoots per plate) 14 to 21 days post culture while the inverse combination of 2.0mg/l NAA and 0.1mg/l BAP mainly produced calli (16.3 ±1.5 calli and 1.7 ±1.2 shoots per plate) 21 to 28 days post culture. The induction of both shoots and calli were observed in samples treated with 0.5mg/l NAA, 1.5mg/l BAP; 1.0mg/l NAA, 1.0mg/l BAP and 1.5mg/l NAA, 0.5mg/l BAP, respectively (figure 6.1). Calli induction showed an increasing trend (6.7 ±1.2, 15.6 ±1.5, 16.0 ±2.0 calli per plate) with increasing concentrations of NAA and decreasing concentrations of

BAP (figure 6.1: panel A) while shoot induction showed a decreasing trend (40.3 ± 2.5 , 16.0 ± 2.7 , 2.3 ± 0.6 shoots per plate) with decreasing concentrations of BAP and increasing concentrations of NAA (figure 6.1: panel B). Shoots recovered from treatments using high BAP concentrations (2.0 and 1.5mg/l) were observed to be stressed and had deformed translucent yellowing leaves (discussed in section 6.4.2.). Interestingly, callus samples recovered from treatments using high concentrations of NAA (1.5 and 2.0mg/l) also showed similar characteristics where the callus structure was friable and composed of translucent yellowing cells. The best results for the regeneration of calli and shoots were obtained with a combination of 1.0mg/l NAA and 1.0mg/l BAP.

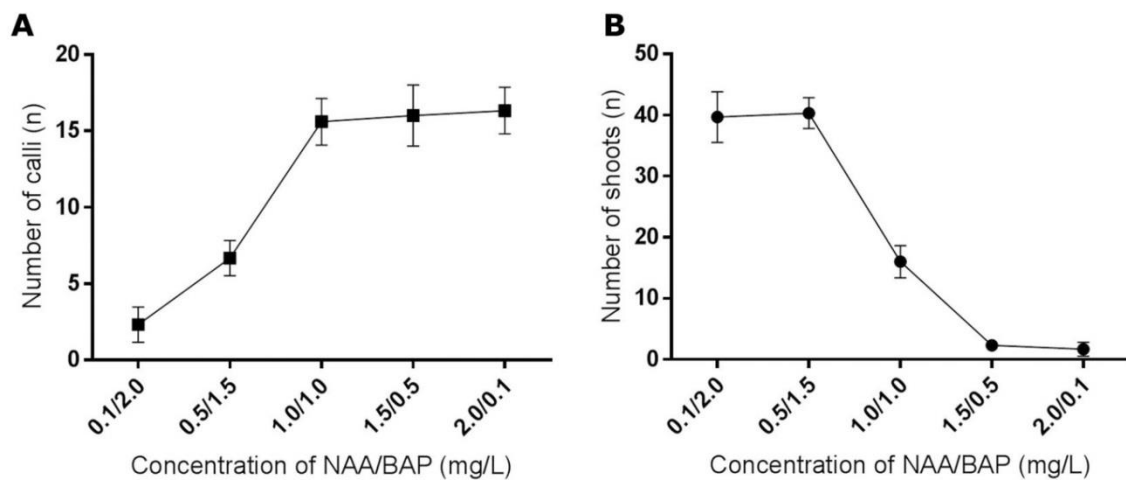


Figure 6.1: Effects of combined treatments using different concentrations of NAA and BAP with respect to (A) calli and (B) shoot induction from regenerated leaf explants of *N. benthamiana* (n=3). (A): Callus induction was observed to increase with increasing NAA/BAP ratios. (B): Shoot induction was observed to decline with increasing NAA/BAP ratios.

6.3.2.1. Effects of BAP on callus and shoot induction

When the concentration of NAA was standardised at 1.00mg/l and the concentration of BAP was varied (0.10 to 1.00mg/l), callus induction remained high (14.3 ± 1.5 , 16.3 ± 2.1 , 17.0 ± 1.7 , 16.0 ± 1.7 , and 17.3 ± 0.6 calli,

respectively per plate) and appear to be unaffected by the concentration of BAP (figure 6.2: panel A). Shoot induction on the other hand showed an increasing trend (2.3 ± 0.6 , 11.7 ± 3.1 , 36.3 ± 6.7 , 37.0 ± 4.4 shoots, respectively per plate) with increasing concentrations of BAP (0.10mg/L to 0.75mg/l in combination with 1.00mg/l NAA) (figure 6.2: panel B). However, a slight decline in shoot induction (18.3 ± 3.1 shoots per plate) was observed when 1.00mg/l BAP and 1.00mg/l NAA was used in combination (figure 6.2: panel B). A combination of 1.00mg/l NAA together with 0.10 or 0.25mg/l BAP allowed the regeneration of calli with relatively few shoots. However, calli produced at that combination of plant growth regulators were friable and had a light green phenotype.

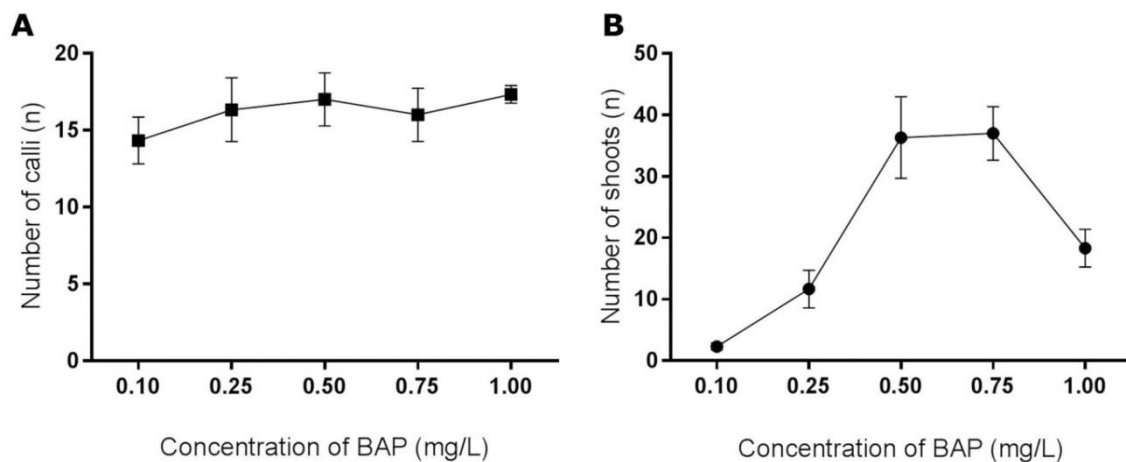


Figure 6.2: Regeneration profiles on (A) callus and (B) shoot induction in leaf explants of *N. benthamiana* following combined treatments of different concentrations of BAP (0.10, 0.25, 0.50, 0.75 and 1.00mg/l) in combination with 1.00mg/l of NAA (n=3). (A): The different concentrations of BAP (0.10 to 1.00mg/l) did not appear to significantly affect the rate of callus induction when used in combination with 1.00mg/l of NAA. (B): BAP concentrations within the range of 0.50 to 0.75mg/l were found to increase the rate of shoot induction when used in combination with 1.00mg/l of NAA.

6.3.2.2. Effects of NAA on callus and shoot induction

When the concentration of BAP was standardised at 1.00mg/l, callus induction showed an increasing trend as the concentration of NAA increased (2.7 \pm 0.6, 9.7 \pm 0.6, 17.3 \pm 1.5 calli, respectively per plate) from 0.10 to 0.50mg/l (figure 6.3: panel A). Callus induction did not show a further increase (15.7 \pm 1.5, 16.3 \pm 0.6, respectively) when higher concentrations of 0.75 and 1.00mg/l NAA were used (figure 6.3: panel A). On the other hand, shoot induction was highest (41.3 \pm 3.5, 41.3 \pm 2.1, respectively) when a concentration of 0.10 and 0.25mg/l NAA was used in combination with 1.00mg/l BAP (figure 6.3: panel B). There was a notable drop in shoot induction (19.3 \pm 1.5, 17.3 \pm 4.9, 19.0 \pm 4.4 shoots, respectively per plate) when higher concentrations of NAA (0.50 to 1.00mg/l) were used in combination with 1.00mg/l BAP (figure 6.3: panel B). A combination of 1.00mg/l BAP and 0.10mg/l NAA allowed the efficient regeneration of healthy green shoots with very few callus induction events. In contrast, callus induction was most efficient when a combination of 1.00mg/l BAP and 0.50mg/l NAA was used which allowed the regeneration of compact green calli from the leaf explants of *N. benthamiana*.

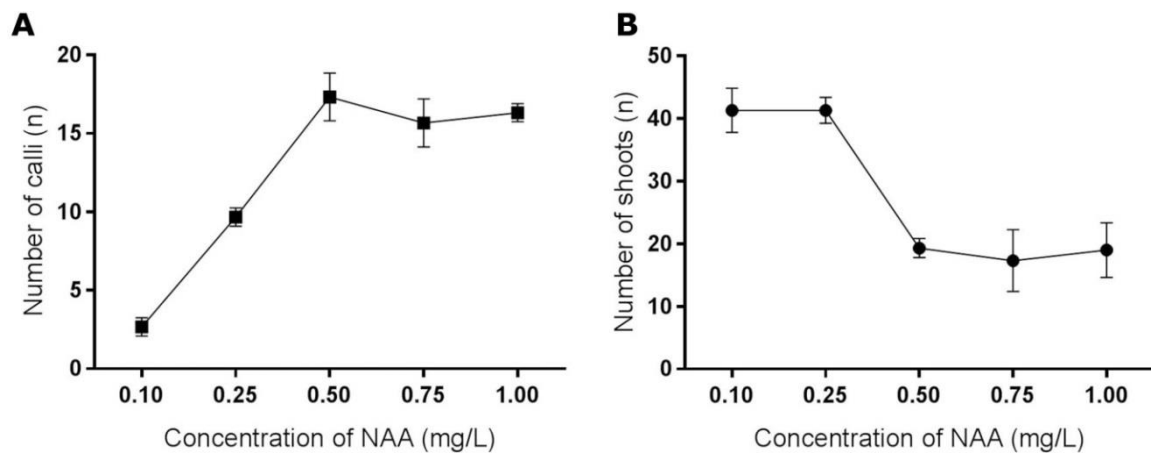


Figure 6.3: Regeneration profiles on (A) callus and (B) shoot induction in leaf explants of *N. benthamiana* following combined treatments of different concentrations of NAA (0.10, 0.25, 0.50, 0.75 and 1.00mg/l) in combination with 1.00mg/l of BAP (n=3). (A): NAA concentrations within the range of 0.50 to 1.00mg/l were found to increase the rate of callus induction when used in combination with 1.00mg/l of BAP. (B): Shoot induction was highest when a concentration of NAA within the range of 0.10 to 0.25mg/l was used in combination with 1.00mg/l BAP. A decline in shoot induction was observed at higher NAA concentrations of 0.50, 0.75 and 1.00mg/l.

6.3.3. Minimal inhibitory concentration: selective regeneration

Within the context of this experiment, the MIC is defined as the minimal concentration of antibiotics required for 100% inhibition of all wild-type chloroplast within the leaf explants of *N. benthamiana* resulting in chlorosis of wild-type leaf samples. No significant difference was observed between the MIC results of spectinomycin and streptomycin (figure 6.4). All leaf explant samples were observed to undergo a certain degree of chlorosis even at the lowest concentration of antibiotics (50mg/l) except for control samples. A gradual decline in the survival rate of leaf explants of *N. benthamiana* was observed in response to increasing antibiotic concentrations in both spectinomycin and streptomycin samples from a concentration of 50 to 250mg/l. However, at an antibiotic concentration of 500mg/l, there was a sudden decline in the survival

rate of leaf explants to essentially 0%. No surviving plants were recovered at higher antibiotic concentrations of 750 and 1000mg/l. In addition, leaf explants cultivated on media containing 750 and 1000mg/l antibiotics were also observed to have undergone chlorosis well before the fourth week.

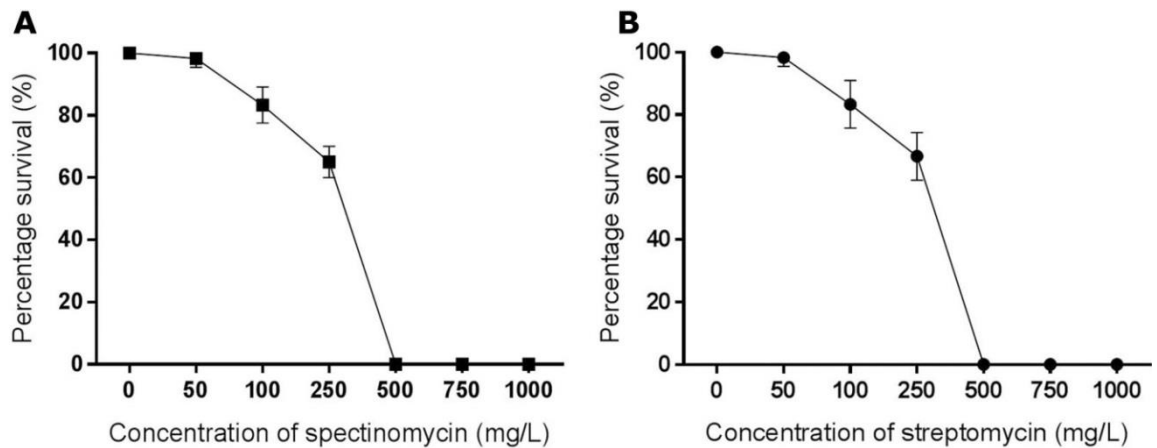


Figure 6.4: Minimal inhibitory concentration profiles for the selective regeneration of *N. benthamiana* leaf explants post-bombardment on MS media supplemented with different concentrations of (A) spectinomycin and (B) streptomycin after 4 weeks (n=3). The minimal inhibitory concentration profiles indicate that an antibiotic concentration of 500mg/l for both spectinomycin and streptomycin was sufficient to cause 100% chlorosis in wild-type *N. benthamiana* leaf explants.

6.3.4. Evaluation of regenerative media

Callus tissue was used in order to evaluate the effects of different media types on the regenerative potential of *N. benthamiana* leaf explants post-bombardment by measuring the increase in biomass. A general decline in callus induction (callus mass) was observed in all bombarded samples as compared to the non-bombarded samples for all treatments. Non-bombarded leaf explants were observed to perform equally well on all three types of regenerative media with mean callus mass values of $2.10 \pm 0.31\text{g}$, $2.03 \pm 0.55\text{g}$ and $2.12 \pm 0.28\text{g}$ for media types MS+Vit, GB+B5 and MS+B5, respectively (figure 6.5). On the other

hand, bombarded leaf explants were observed to regenerate with the highest efficiency on media type MS+B5 followed by MS+Vit and GB+B5 with mean values of callus mass at $1.89 \pm 0.49\text{g}$, $1.59 \pm 0.44\text{g}$ and $1.40 \pm 0.26\text{g}$, respectively (figure 6.5). Although statistically not significant ($p > 0.05$), regeneration of bombarded leaf explants of *N. benthamiana* via callus induction was observed to be most efficient on MS media supplemented with B5 vitamins (MS+B5) (figure 6.5).

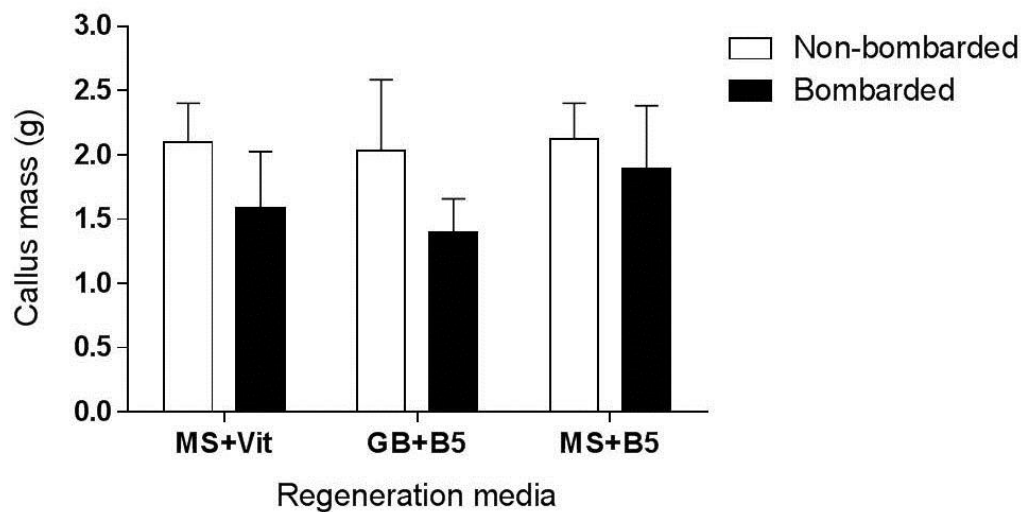


Figure 6.5: Regeneration profiles of non-bombarded and bombarded leaf explants of *N. benthamiana* via callus induction on different types of regenerative media ($n=5$). The three types of media were MS with vitamins (MS+Vit), Gamborg with B5 vitamins (GB+B5) and MS with B5 vitamins (MS+B5).

6.3.5. Regeneration of *N. benthamiana* leaf explants post bombardment

Antibiotic resistant shoots and calli were successfully regenerated from post-bombarded leaf explants of *N. benthamiana* after a cultivation period of 5 and 6 weeks, respectively. A general summary of the regeneration results indicated that calli induction occurred at a higher frequency as compared to

shoot induction. However, results suggest that regeneration of shoots via direct organogenesis was a better option for the recovery of putative transformants.

6.3.5.1. *N. benthamiana* leaf explants pre- and post-bombardment

No immediate significant morphological changes were observed between pre- and post-bombarded leaf explants of stress tolerant *N. benthamiana*. However, a noticeable decline in the structural integrity of the leaf was observed after an incubation period of 48 hours (figure 6.6: panel A and B). The decline in structural integrity was even more pronounced in bombarded leaf explants of stress susceptible plants (figure 6.6: panel C). The viability of bombarded stress tolerant leaf explants did not seem to be noticeably affected by the decline in structural integrity during regeneration. In contrast, stress susceptible leaf explants did not cope well and steadily progress towards a state of necrosis during the regeneration period. Wild-type leaves could not be transformed as they were highly susceptible to abiotic stress and did not survive well during regeneration.

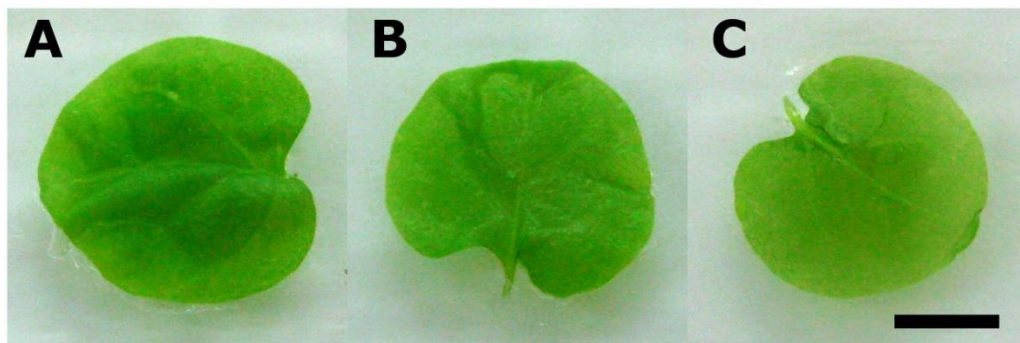


Figure 6.6: Leaf explants from stress tolerant and stress susceptible *N. benthamiana* before and after particle bombardment. All bombarded leaf explants showed a noticeable decline in structural integrity 48 hours post-bombardment; however, the effects were more significant in the leaves of stress susceptible plants. (A): Stress tolerant leaf before bombardment, (B): stress tolerant leaf 48 hours after bombardment, (C): Stress susceptible leaf 48 hours after bombardment. Bar= 1cm.

6.3.5.2. Selective regeneration: callus induction

Callus tissue (referred to as primary calli hereafter) was only recovered from transformed leaf explants of *N. benthamiana* regenerated on MS+B5 media with a plant growth regulator combination of 1.0mg/l BAP and 0.5mg/l NAA after a minimum of 4-5 weeks post-transformation figure 6.7. An average of 3 individual green calli was recovered for each bombardment using the optimised particle bombardment parameters (Chapter 5). No significant difference was observed in the frequency of callus induction between plants transformed with recombinant vectors pEXPR-G and pEXPR-UG. However, two distinct phenotype variations were observed in the recovered calli. One displayed a tightly packed dark green callus structure while the other had a friable light green structure. Both variations did not respond well to shoot induction with 1.0mg/l BAP and 0.1mg/l NAA and healthy shoots could not be recovered from the callus cultures. However, both callus cultures were observed to propagate well on culture media supplemented with 500mg/l of spectinomycin or streptomycin.

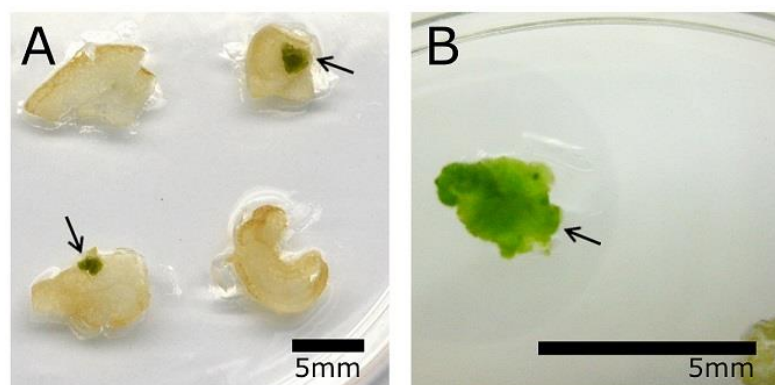


Figure 6.7: Callus initiation (arrows) was observed after 5 weeks in both pEXPR-G and pEXPR-UG transformed *N. benthamiana* leaf explants regenerated on MS media with B5 vitamins supplemented with 1.0mg/l BAP, 0.5mg/l NAA and 500mg/l spectinomycin. (A): Antibiotic resistant calli regenerated from pEXPR-G transformed *N. benthamiana* 5 weeks post-bombardment, (B): Isolated antibiotic resistant callus regenerated from pEXPR-UG transformed *N. benthamiana* 7 weeks post-bombardment. Bar= 5mm.

6.3.5.3. Selective regeneration: shoot induction

Shoots were successfully recovered from transformed leaf explants of *N. benthamiana* cultured on shoot induction media (MS+B5 with 1.0mg/l BAP, 0.1mg/l NAA and 500mg/l spectinomycin or streptomycin) 4-6 weeks post-bombardment (figure 6.8). Recovered shoots had green leaves and were able to survive on tissue culture media supplemented with 500mg/l spectinomycin or streptomycin. However, several prominent morphological variances were observed in the recovered plants as compared to untransformed stress tolerant plants (section 6.3.6.). In contrast to callus induction, shoot induction frequencies were considerably lower with the average recovery of only a single shoot for every 3 bombardments under optimised particle bombardment conditions (Chapter 5). There was no significant difference between the shoot induction frequencies of pEXPR-G and pEXPR-UG transformed leaf explants. Callus induction was successfully achieved when excised leaf explants were cultured on MS+B5 media containing 1.0mg/l BAP, 0.5mg/l NAA and 500mg/l streptomycin for a period of 4 weeks. Calli that were induced using this method (referred to as secondary calli hereafter) also showed resistance towards spectinomycin and streptomycin at a concentration of 500mg/l.

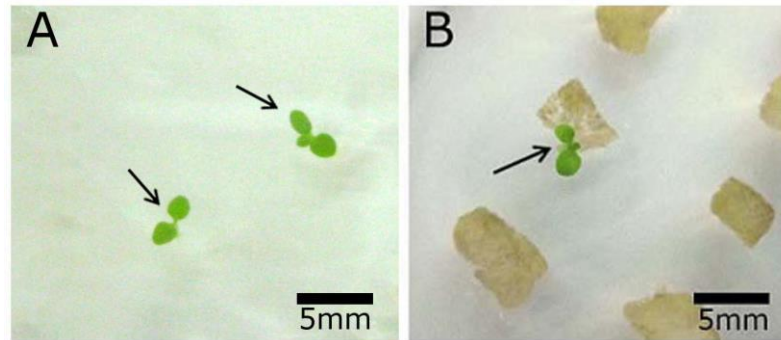


Figure 6.8: Regenerated shoots (arrows) recovered from pEXPR-G (A) and pEXPR-UG (B) transformed leaf explants of *N. benthamiana* cultured on MS media with B5 vitamins supplemented with 1.0mg/l BAP, 0.1mg/l NAA and 500mg/l spectinomycin. (A): Regenerated shoots of pEXPR-G transformed *N. benthamiana* 7 weeks post-bombardment, (B): Regenerated shoots emerging from pEXPR-UG transformed leaf explants of *N. benthamiana* 6 weeks post-bombardment. Bar= 5mm.

6.3.6. General morphology of regenerated whole plants

The recovery of whole plants was only possible from transformed leaf explants regenerated via shoot induction and from secondary calli (induced from the leaf tissue of regenerated shoots). Primary calli failed to generate normal shoots and, hence, could not produce whole plants. The morphological characteristics observed in the majority (94.1%) the regenerated plants closely resemble that of the stress susceptible variety (figure 6.9). Evident variations include a smaller average leaf diameter, shorter internode lengths, shorter average heights, decrease in stem diameter and an increase in the number of leaves (figure 6.9). In addition, many adventitious shoots were also observed to emerge from the lateral buds of the stems. Only the remaining 5.9% of recovered whole plants displayed a normal morphology. The development of roots was observed to be very inefficient across all regenerated plant lines even when cultured on media containing high auxin (1.0mg/l NAA) to cytokinin (0.1mg/l BAP) ratios. Surprisingly, the recovered plants that displayed a normal

morphology were still able to develop flowers *in-vitro* regardless of the type of media they were cultured on and were observed to produce seeds upon maturity (figure 6.10). Similar results were observed for whole plants recovered from both pEXPR-G and pEXPR-UG transformed leaf explants.

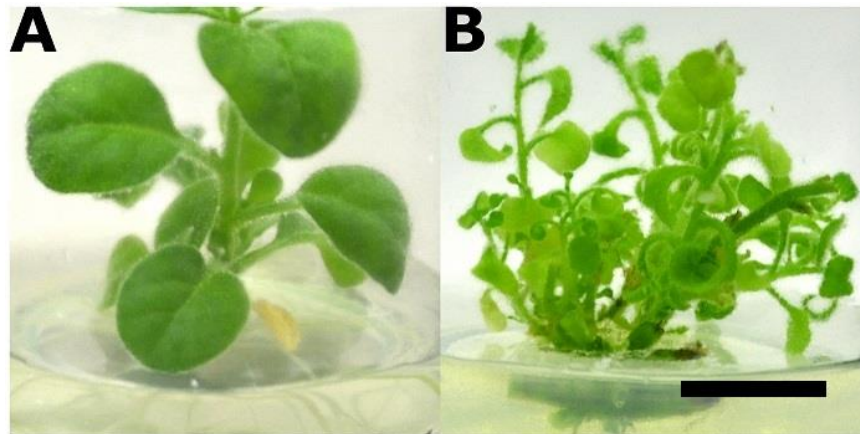


Figure 6.9: Recovery of whole plants from transformed leaf explants of *N. benthamiana* displaying stress tolerance and stress susceptibility towards abiotic stresses of *in-vitro* culture. Only 5.9% of whole plants recovered post-transformation displayed a normal morphology while the remaining 94.1% of recovered plants exhibit morphological characteristics related to stress susceptibility. A: Recovered whole plant displaying the normal morphology of *N. benthamiana in-vitro*, B: Recovered plants displaying stress susceptibility *in-vitro*. Bar= 2cm.



Figure 6.10: Fertilised flowers containing seeds excised from *in-vitro* cultivated *N. benthamiana* regenerated from pEXPR-UG transformed leaf explants. Bar= 0.5cm.

6.4. DISCUSSION

In order to improve the success rate of particle bombardment, stress tolerant lines of *N. benthamiana* were established *in-vitro* to provide resilient explant samples for transformation. The concentration of plant growth regulators were optimised for the recovery of putative transformants via shoots and callus induction, respectively while MIC levels for the selective agents were determined to ensure the efficient selection of putative transformants. Several media types were also evaluated to determine their potency for the regeneration of post-bombarded leaf explants.

6.4.1. Establishment of stress tolerant *N. benthamiana*

The establishment of stress tolerant plants was crucial for the successful regeneration of whole plants from post-bombarded leaf explants of *N. benthamiana*. Based on the results, the most common form of abiotic stress observed to affect *in-vitro* cultivated plants is the waterlogging of plant tissues, commonly known as hyperhidrosis (formerly vitrification) (Kevers *et al.*, 2004). Wild-type plants that were cultivated *in-vitro* began displaying signs of hyperhidrosis 4 weeks post germination and did not respond well to particle bombardment. The onset of hyperhidrosis is caused by a wide array of stress factors which are commonly present during cultivation such as wounding, high humidity in an enclosed vessel, high moisture and ionic solutes in the media, excessive nitrogen levels and certain specific combinations of plant growth regulators are known to induce hyperhidrosis in *in-vitro* cultivated plants (Kevers and Gaspar, 1986). Stress susceptible *N. benthamiana* explants which were in a state of hyperhidrosis displayed several aberrant morphological traits such as a translucent light green appearance, a flaccid structure, smaller leaves, emergence of many adventitious shoots and reduced root formation. Gaspar (1991) reported that hyperhydric tissues show several changes in biochemistry

and homeostasis such as; an increase in water uptake, decrease in lignin and cellulose synthesis, lowered chlorophyll content, increased auxin catabolism and an overall reduction in ethylene production. The results agree with these findings as a decrease in the chlorophyll content together with an increased uptake of water would result in the formation light green translucent shoots while a decrease in lignin and cellulose biosynthesis would result in a flaccid plant structure. An increased rate of auxin catabolism would also explain the decrease in average leaf diameter and root formation (Gaspar *et al.*, 1994) as well as the loss of apical dominance which caused the development of adventitious shoots in response to low auxin levels (Tamas, 1995). In an effort to establish stress tolerant varieties of *N. benthamiana*, explant propagation was only performed using plants (from the top 80-percentile) that were able to survive well *in-vitro*. This ensures that a constant artificial selection pressure is exerted in favour of stress tolerant varieties of *N. benthamiana* resulting in the increase in the population size over time. Similar methods were also used for the establishment of stress tolerance towards heavy metals (copper) in tobacco (Rout and Sahoo, 2007) and drought in potato, carrot and rice (Sabbah and Tal, 1990; Fallon and Phillips, 1989; Adkins *et al.*, 1995) *in-vitro*. However, the morphology of whole plants in the majority of samples regenerated from post-transformed stress resistant leaves of *N. benthamiana* showed a reversion back to the morphology of the stress susceptible variety. This observation indicated that most the stress tolerant *N. benthamiana* plants that were established *in-vitro* only underwent proximal acclimatisation to the stress factors allowing them to grow well *in-vitro* and only a small fraction underwent adaption for survival under stressful *in-vitro* conditions. Consequently, stress tolerance was lost in stress-acclimated plants when whole plants were regenerated at the cellular level especially after the process of cell dedifferentiation and only the small proportion that underwent epigenetic adaptation developed normally. This is in agreement with the proposal of Kevers *et al.* (2004) who suggested that development of abiotic stress

tolerance *in-vitro* could either emerge via plastic avoidance or tolerance of the abiotic stress factor which allows the plant to survive and grow or via epigenetic changes caused by somaclonal variation or epigenetic mutations which allow the plant to permanently adapt to the stress factors. This would explain the loss of stress tolerance in most of the plant samples post-regeneration as acclimatisation to abiotic stress generally occurs at a systemic level (Mettraux *et al.*, 1990). Hence, it can be hypothesised that if the period of the selection process is further lengthened, during the establishment of stress tolerant plants *in-vitro*, the population of somaclonal variants permanently adapted to tolerate abiotic stress may further increase. As a result, a higher percentage of plants recovered post-regeneration would be expected to display normal morphology. However, a more thorough evaluation of somaclonal variation is also required for the characterisation of any genotypic or epigenetic changes that might have provided stress adaptive characteristics to the *N. benthamiana* plants established *in-vitro*, as tissue culture induced somaclonal variation is known to be somewhat random (Robert *et al.*, 1994; Larkin and Scowcroft, 1981).

6.4.2. Optimisation of plant growth regulators for regeneration

Auxins and cytokinins concentrations together with their specific interactions are known to be the most important plant hormones for the regulation of growth and organised development in plant tissue and organ cultures (Evans *et al.*, 1981). Auxins are known to be important determinants of cell expansion and growth, initiation of cell division, acidification of cell walls, delaying senescence in leaves and in fruit ripening (Addicott, 1982; Sabater, 1985; Chandler and Thorpe, 1986). In addition, auxins are also known to exert a strong influence on the organisation of meristematic tissue, the development of defined organs such as roots and in facilitating vascular differentiation (Liu and Reid, 1992; Aloni, 1995). Polar auxins transport is also important in maintaining

apical dominance and in the initiation of tropistic curvatures (Tamas, 1995). In contrast to auxins, cytokinins appear to affect plant growth and development in a very different manner and appear to possess antagonistic effects to auxin (Gaspar *et al.*, 1996). Cytokinins are known to enhance chlorophyll synthesis, promote chloroplast development, promote leaf expansion and lateral bud growth as well as delay leaf senescence in intact plants (Kuhnle *et al.*, 1977). In tissue culture, cytokinins are known to stimulate cell division (usually together with auxins) as well as the formation of lateral adventitious buds and the release of bud dormancy which leads to the formation of adventitious shoots (Fabijan *et al.*, 1981) (section 6.3.2.). This particular property of cytokinin allows the rapid propagation of plant cuttings *in-vitro* as whole plants can be conveniently regenerated from the adventitious shoots. Cell division is a process which is regulated by the synergistic effects of auxin and cytokinin, with each hormone independently influencing different phases of the cell cycle. Auxin is known to affect the DNA replication process, whereas cytokinin appears to be involved in the events leading to mitosis (Vesely *et al.*, 1994) and cytokinesis (Gaspar *et al.*, 1996). Hence, auxin and cytokinin levels need to be carefully optimised for the regeneration of transformed explants *in-vitro* to ensure proper growth and development. The synthetic plant growth regulator 1-naphthaleneacetic acid (NAA) was used in place of auxin and 6-Benzylaminopurine (BAP) was used in place of cytokinin. Naturally occurring auxins (indole-3-acetic acid) and cytokinins (zeatin) were not used as natural plant hormones tend to be unstable and denature easily in media and are metabolised rapidly within plant tissues (Gaspar *et al.*, 1994). During optimisation experiments, the emergence of hyperhydric characteristics was observed in the regenerated explants even though stress tolerant plants were used. This was particularly prevalent at high concentrations of auxin and cytokinin where regenerated shoots were observed to be translucent and flaccid and calli were observed to be friable, translucent and light green in colour. Cytokinins have been known to be involved in the

onset of hyperhydricity and excessive levels of cytokinin may be responsible for the observed response. The cytokinin-like substance thidiazuron is known to cause rapid hyperhydricity on repeated subcultures *in-vitro* (Gaspar *et al.*, 1994). High cytokinin concentrations were also reported to cause hyperhidrosis in lavender, crab apple and grape vine when cultured *in-vitro* (Andrade *et al.*, 1999; Phan, 1991; Gribaudo and Fronda, 1991). However, Kadota and Niimi (2003) reported that hyperhydricity was mainly caused by the type of cytokinin that was used and the concentration had little influence. This may explain the onset of hyperhidrosis even when low concentrations of BAP (0.1mg/l) were used for callus induction. On the other hand, it is also possible that the onset of hyperhidrosis may be caused by excessive levels of NAA; however, no reports have been published so far describing such events. It can be hypothesised that excessive levels of NAA may promote the hyperhydric response by acidification of the cytoplasmic environment which may increase oxidative stress levels or by altering the catabolism of endogenous cytokinin levels. Plant growth regulator levels were optimised for the independent regeneration of callus and shoots. Due to the rapid proliferation rate of tobacco cells, regeneration via callus culture would allow the rapid propagation of transformed cells *in-vitro*. However, precautions should be taken as callus cultures tend to exhibit a higher degree of somaclonal variation which may lead to the emergence of epigenetic mutants resistant to antibiotic selection (Kaepler *et al.*, 2000). Unfortunately, the recovery of shoots from primary callus tissue was not possible as any shoots that were recovered displayed critical levels of hyperhidrosis and progressed rapidly towards necrosis during subculture. Similar results were also observed by Kadota and Niimi (2003) in pear plants. In contrast, regeneration of transformed leaf explants via shoot induction would theoretically allow better recovery of non-chimeric explants as early differentiation of tissue through direct organogenesis would prevent cross-protection of surrounding non-transformed cells. In addition, regeneration via direct organogenesis would also shorten the

regeneration period, during which, plant tissues are most susceptible to abiotic stresses and hyperhidrosis (Joyce *et al.*, 2003; Rojas-Martínez *et al.*, 2010). It was interesting to note that callus induction was possible using the leaf explants of both normal and hyperhydric shoots resulting in the formation of compact and friable calli, respectively. However, redifferentiation into shoots was only possible in the calli of the normal compact variety. These results suggest that plant tissue in the hyperhydric state seem to have a decreased response to exogenously applied plant growth regulators (Kadota and Niimi, 2003; Bairu *et al.*, 2007; Rojas-Martínez *et al.*, 2010). However, this effect is mainly observed to inhibit the differentiation process but not the reverse process of dedifferentiation. This is illustrated in the results where hyperhydric shoots were able to dedifferentiate into callus tissue but hyperhydric calli were unable to differentiate into shoots.

6.4.3. Minimal inhibitory concentration and regeneration

Streptomycin was the first aminoglycoside antibiotic to be discovered in 1944. The mode of action of streptomycin is attributed to four major events of ribosomal blockade, misreading in translation, membrane damage and irreversible uptake of the antibiotic (Davis *et al.*, 1974; Gale *et al.*, 1981; Tai and Davis, 1985; Wallace *et al.*, 1979). The basic bactericidal mode of action of streptomycin involves a multistep approach whereby streptomycin uptake leads first to the disruption of membranes followed by total ribosomal blockade (Davis *et al.*, 1986). Davis (1987) described the sequence of events as an initial entry of the aminoglycoside antibiotics into the cell via an unknown mechanism where it binds to chain-elongating ribosomes causing misreading (Gorini, 1974). The misread proteins are subsequently incorporated into the membrane creating channels that permit the influx of more antibiotics initiating an autocatalytic event of increased influx, misreading and channel formation. This causes a continued increase in the intracellular antibiotic concentration to the point where

all initiating ribosomes are inhibited, thus preventing any further protein synthesis. Lethality ensues from this irreversibility in inhibition, due to irreversible uptake and binding of aminoglycosides to the ribosomes. Homologs of the prokaryotic 16S rRNAs are also found within the gene expression machinery of chloroplasts (Zablen *et al.*, 1975; Bonen and Doolittle, 1975). Thus, in practice, aminoglycoside antibiotics can also be used for the disruption of ribosomes within chloroplasts which would result in eventual disruption of the chloroplast and thylakoid membranes leading to the cessation of chlorophyll synthesis and chlorosis (Svab and Maliga, 1993; Franklin *et al.*, 2003). Caveat the importance of distinguishing between the process of chlorosis which causes the destruction of chlorophyll resulting in the bleaching of plant tissues; and necrosis which results in the browning of plant tissues and death (Culver *et al.*, 1991). Conversely, the *aadA* gene encoding for aminoglycoside 3'-adenyltransferase provides resistance against aminoglycoside antibiotics such as streptomycin and spectinomycin (Chinault *et al.*, 1986). Resistance towards these antibiotics are caused by the adenylation of the 3-hydroxyl group on the amino-hexose III ring of streptomycin or the 9-hydroxyl group on the actinamine ring in spectinomycin (Davies and Benveniste, 1974). It is not entirely clear how these modifications provide resistance against aminoglycosides but some authors suggest that these modifications may inhibit further entry of antibiotics into the cell (Davies and Smith, 1978), while others have suggested the plausibility that the modified antibiotics are inefficient at binding and inhibition of prokaryotic rRNAs (Yamada *et al.*, 1968). Thus, the use of *aadA* as a marker gene coupled with antibiotic selection using aminoglycosides provides a powerful selection tool for the recovery of transplastomic chloroplasts (Svab and Maliga, 1993). However, a limitation to this method of selection is that non-transformed spontaneous 16S rRNA mutants will also display similar characteristics of antibiotic resistance leading to false positives. For example, Pfister *et al.* (2003) described that a U1406C/U1495A double mutation at the A site (aminoacyl-

transfer RNA decoding site) of the bacterial 16S rRNA provided high-level resistance against aminoglycosides in *E. coli*.

Optimisation of MIC was important for the selective recovery of transplastomic chloroplasts; providing a convenient method of distinguishing between transformed and non-transformed tissue via a phenotypic change in colour. However, the inactivation of aminoglycosides by *aadA* is an enzymatic process. Therefore, exposure to excessive levels of aminoglycosides would still cause damage to transformed chloroplasts if the substrate concentration exceeds the maximum rate of reaction of *aadA*. Hence, it was especially important that post-transformed leaf explants were incubated for 48 hours in the dark to minimise photooxidative stress while allowing the recovery of damaged membranes as well as the integration and initiation of transgene expression (Inzé and Montagu, 1995). Nevertheless, transformed explants should be subcultured onto antibiotic media as soon as possible after recovery to ensure that the transgene is not deleted by the endogenous copy correction mechanism of the chloroplasts (Shinozaki *et al.*, 1986). An MIC value of 500mg/l was determined to be optimum for 100% inhibition of non-transformed chloroplasts as it allowed complete chlorosis of wild-type explants over a period of 4 weeks. This is considered to be optimum as callus and shoot initiation was determined to occur after a minimum of 4 weeks post culture (section 6.3.5.2.). This was in agreement with the results of Svab and Maliga (1993) who reported that an antibiotic concentration of 500mg/l spectinomycin was optimum for high-frequency chloroplast transformation of tobacco leaf explants with the *aadA* gene. It was important that resistant cells were selected for before the onset of callus initiation as somaclonal variation is known to occur frequently in callus tissue and may result in an increased recovery of spontaneous mutants (Skirvin *et al.*, 1994). It was also important to maintain maximum exposure between the explants and the selective agent throughout regeneration to ensure that an even

selection pressure was exerted against wild-type chloroplasts. This would ensure a gradual shift in the equilibrium of the population in favour of the transformed chloroplasts (Svab and Maliga, 1993). Extended cycling of shoots on antibiotic media was also essential in order to achieve homoplastomy within the plastome to ensure the persistence of the transformed genome and to prevent wild-type reversion when the selection pressure was removed (Ruiz *et al.*, 2011). The results of this study were in agreement with the results of Davarpanah *et al.* (2009) for the stable chloroplast transformation in *N. benthamiana* with a polycistronic *aadA*-GFP expression cassette.

6.4.4. Evaluation of tissue culture media

The composition of culture media has a significant influence over the success of plant propagation and regeneration as it provides the only source of exogenous nutrition to the plants *in-vitro*. All three media types tested provided the essential micro and macro nutrients required for growth and development *in-vitro* as well as additional vitamins which may prove to be beneficial for recovery and regeneration. Although the MS media with vitamins (MS+Vit) was developed specifically for the optimal growth of tobacco callus in tissue culture, various additional factors have to be taken into account for the regeneration of damaged leaf tissue. Some reports suggest that added vitamins may be beneficial for the regeneration of cultured tissues (Roest and Bokelmann, 1975). This may be plausible as the results for the evaluation of regenerative media indicated that post-bombarded leaf explants of *N. benthamiana* grew best on MS media supplemented with B5 vitamins (MS+B5) which had higher levels of thiamine, pyridoxine and nicotinic acid as a source of vitamins. Thiamine (vitamin B₁ or aneurine) is known to be an important co-factor for carbohydrate metabolism (George *et al.*, 2008) and may facilitate the catabolism of sucrose to support the heavy metabolic burden of cellular recovery. Thiamine requirement has also

been shown to become more apparent with consecutive subculture cycles (Polikarpochkina *et al.*, 1979). Other authors found thiamine to be an essential component for the formation of somatic embryos in *Glycine max*, embryogenic callus induction in *Zoysia japonica* and adventitious rooting in *Taxus* spp. (Barwale *et al.*, 1986; Asano *et al.*, 1996; Chée, 1995). The addition of nicotinic acid (niacin) and pyridoxine (vitamin B₆) was also thought to stimulate growth of plant cells in tissue culture (Ohira *et al.*, 1976) and an increase in nicotinic acid was reported to improve the growth of isolated roots of pea, radish and tomato *in-vitro* (Bonner and Devirian, 1939). However, it cannot be assumed that the improved regeneration results on MS+B5 to be merely due to increased concentrations of thiamine, pyridoxine and nicotinic acid in the B5 vitamins alone. If this was indeed the case, the paradoxical results where regeneration declined on Gamborg media with B5 vitamins (GB+B5) would not be observed. Further evaluation of the compositions of MS and GB media indicated that several micro and macro nutrients were significantly lower in GB compared to MS (appendix E). These include the micro nutrient salts of boron (H₃BO₃), manganese (MnSO₄.H₂O), zinc (ZnSO₄.7H₂O) and the macro nutrient salts of calcium (CaCl₂), potassium and phosphorus (KH₂PO₄), magnesium and sulphur (MgSO₄) and nitrogen (NH₄NO₃). These micro and macro nutrients significantly affect the normal function of plant cells and are especially important for cell damage repair, normal enzymatic activity, photosynthesis, stress response and cell division. Boron is involved in maintaining plasma membrane integrity and influences membrane bound proteins (Blevins and Lukaszewski, 1998) possibly by stabilising metal chelates which are important in the maintenance of membrane structure and function (Clarkson and Hanson, 1980). Manganese, on the other hand, is involved in the definition of metalloproteins which are involved in respiration and photosynthesis and in superoxide dismutase which are commonly involved in the oxidative stress response (Clarkson and Hanson, 1980). Zinc is also known to affect the action of superoxide dismutase as well as

RNA and DNA polymerases in bacteria (George *et al.*, 2008). Calcium is required for the proper function of many enzymes and for the hydrolysis of ATP while potassium is important for phosphate adsorption and the prevention of hyperhydricity (George *et al.*, 2008). Other macronutrients include phosphorus, often found as phosphates, which are essential for many cellular processes such as the synthesis of phospholipids, phosphorylation, ATP synthesis, respiration and photosynthesis. Magnesium is also directly involved in photosynthesis and is the central atom of the porphyrin structure of the chlorophyll molecule (George *et al.*, 2008). Sulphur is important for lipid synthesis and also in maintaining secondary and tertiary protein structures via S-S bonds. Nitrogen is a constituent part of proteins, nucleic acids and chlorophyll as well as being involved in cell division and cell wall formation in plants (George *et al.*, 2008). Hence, it can be concluded that elevated levels of micro and macro nutrients together with increased vitamin concentrations are important to facilitate many of the major events leading to recovery and regeneration of damaged *N. benthamiana* leaf explants *in-vitro*.

6.5. CONCLUSION

In conclusion, the establishment of stress tolerant plants for transformation via *in-vitro* selection was successful, giving rise to explants that were able to survive well in high humidity conditions. Surprisingly, results also suggest the emergence of stress resistant plants which displayed persistent resistance towards abiotic stress. However, a thorough investigation is still required to fully elucidate the underlying mechanisms of stress resistance. The effects of varying concentrations of auxin (NAA) and cytokinin (BAP) on growth and development of *N. benthamiana* leaf explants have also been determined. An optimum concentration of plant growth regulators for the regeneration of callus was deduced to be 1.0mg/l BAP and 0.5mg/l NAA while the regeneration of shoots required 1.0mg/l BAP and 0.1mg/l NAA. A minimum inhibitory concentration of 500mg/l of streptomycin or spectinomycin was required for the effective selection of transformed plants which allowed the complete chlorosis of non-transformed explants over a period of 4 weeks. The regeneration of post-bombarded leaf explants was also found to be most effective in media containing high amounts of micro and macro nutrients together with increased levels of vitamins (MA+B5). Antibiotic resistant calli and shoots have been successfully recovered from pEXPR-G and pEXPR-UG transformed leaf explants of *N. benthamiana* using the optimised parameters detailed in this chapter.

Chapter 7: Evaluation of pEXPR Transformation Vector for Transgene Delivery and Expression in Chloroplasts of *Nicotiana benthamiana*

7.1 INTRODUCTION

In this chapter, transformed plants were evaluated at the genomic, transcriptional and translational levels in order to confirm the expression of transgenes within the chloroplasts. In addition, transformation vectors were also constructed using the developed 2-part system for the delivery and expression of the hemagglutinin (HA) and neuraminidase (NA) surface antigens of influenza A strain H5N1 in the chloroplast of *N. benthamiana* as a preliminary step towards the development of chloroplast-expressed plant-based vaccines.

7.1.1. Transformation of the chloroplast genome of *N. benthamiana*

Transformation of the chloroplast genome provides many distinct advantages over the nuclear genome. Among the advantages of chloroplast transformation include: 1) lack of gene silencing mechanisms within the chloroplasts, 2) absence of positional effects due to site-specific integration of transgenes via homologous recombination, 3) maternal inheritance provides a natural mechanism for the biological containment of transgenes preventing gene flow to other related species, 4) the possibility of expressing multiple heterologous genes in polycistronic operons under a single regulatory sequence, and 5) the potential of integrating transgenes into any part of a sequenced chloroplast genome even into endogenous gene expression cassettes (de Cosa *et al.* 2001; Daniell *et al.* 2002; Lee *et al.* 2003; Jeong *et al.* 2004). In addition, the highly polyploid nature of chloroplasts as well as the existence of up to 100

chloroplasts in a single photosynthetically active cell would potentially allow very high levels of expression of transgenic proteins (Verma and Daniell, 2007). Chloroplasts are also able to sequester transgenic proteins for storage, potentially preventing cytotoxic proteins from harming the eukaryotic cytoplasm (Tran *et al.*, 2013). Additionally, chloroplast transformation has also been extensively used in the field of reverse genetics for the study of unknown genes or sequences of the chloroplast genome via targeted disruption or for the study of cryptic open reading frames (ORFs) (Rochaix, 1997; Goldschmidt-Clermont, 1998; Bock, 2015). The concept of stable chloroplast transformation in higher plants has been around for more than two decades and has been successfully applied for the transformation of major crops; including soybean, tomato and cotton (Svab *et al.*, 1990; Ruf *et al.*, 2001; Kumar *et al.*, 2004; Dufourmantel *et al.*, 2004). However, in order to transform the chloroplast genome of a particular plant, sequence information is required before a suitable chloroplast transformation vector can be customised for transgene delivery. *Nicotiana benthamiana* is a valuable model system for higher land plants and has been used extensively in research at the organellar, nuclear, cellular, organ and whole-plant levels. In addition, the short life cycle of *N. benthamiana* is also highly favourable for the recovery of stable transplastomic plants in chloroplast transformation studies. *N. benthamiana* is amphiploid, with 38 chromosomes, and is thought to be the product resulting from the hybridization of *N. suaveolens* (n=16) and *N. debneyi* (n=24) (Davarpanah *et al.*, 2009). It is a member of the Solanaceae (Nightshade) family which also includes important crop plants such as tomatoes and potatoes. *N. benthamiana* is a close relative of *N. tabacum* (cultivated tobacco) which is also a model plant often used in research. In addition to particle bombardment, *N. benthamiana* is most commonly used as a host for the transient expression of transgenic proteins via viral vectors due to its unique susceptibility to a diverse range of plant viruses (Raquel *et al.*, 2008). It has been used as a model plant for the investigation of

virus-induced gene silencing (VIGS); a useful tool for the functional analysis of genes and investigation of plant disease (Baulcombe, 1999). In addition, *N. benthamiana* is also used for the study of viral protein-mediated and artificial miRNA-mediated cross-protection in plants (Lin *et al.*, 2007). In this study, *N. benthamiana* was used for the expression of transgenic proteins in the chloroplast in order to evaluate the efficiency of the newly developed chloroplast transformation vectors.

7.1.2. Characterisation of transplastomic plants

In order to identify a plant as truly transplastomic, plants which were regenerated post-bombardment had to fulfil a number of requirements: 1) the introduced transgene expression cassette(s) had to be present on all copies of the chloroplast genome, 2) the transgenes had to be transcribed into functional mRNAs, and 3) the mRNA transcripts had to be translated into the correct proteins. Experimental methods of molecular biology such as; Southern blot, reverse transcription polymerase chain reaction (RT-PCR) and Western blot provide the necessary tools for the thorough characterisation of transformed *N. benthamiana* plants. The presence of transgenic DNA in the transformed plants can be conveniently determined via PCR-screening at the tissue level. However, data that is generated via this method of evaluation only provides information on the presence or absence of the transgene and does not provide any information on the location of the gene, site of integration or copy number. The presence of transgenic DNA in the chloroplast genome can be further evaluated via Southern blot. Southern blot was developed as a method to transfer DNA directly from an agarose gel to a nitrocellulose or nylon membrane. This method was first described by Edwin Southern in 1975, hence the name, Southern blot (Southern, 1975). Today, this method is routinely used for the analysis of DNA for a wide array of applications. In most applications, extracted genomic DNA is subjected

to restriction enzyme (RE) digestion and separated based on their size via agarose gel electrophoresis. The double-stranded DNA fragments are then denatured in the gel with an alkaline solution and transferred onto a nylon or nitrocellulose membrane via capillary action. The bound DNA is then immobilised onto the membrane by exposing the membrane to a UV source or by baking which cross-links the single-stranded DNA to the membrane. Specific DNA sequences can be detected by hybridising the membrane with a labelled probe that is complimentary to the sequence of interest. Initially, DNA probes were labelled using radioactive ³²P-labelled deoxynucleotides. However, in recent years a number of non-radioactive detection methods have been developed which include the commonly used biotin-streptavidin and the digoxigenin-antidigoxigenin system in combination with alkaline-phosphatase or horseradish-peroxidase enzyme (Cate *et al.*, 1991; Engler-Blum *et al.*, 1993). Following the detection of transgenes in the chloroplast genome, the presence transgenic mRNA transcripts were detected in the transformed plants via RT-PCR. First strand cDNA synthesis was performed with total RNA samples extracted from the transformed plants using gene-specific primers. This allowed the reverse transcription of transgenic mRNAs into cDNAs which were subsequently amplified via PCR to produce a visible band if transgenic mRNAs were present in the sample. Nevertheless, RT-PCR was a very sensitive process which was prone to many false positives and negatives. Hence, it was imperative that appropriate controls were included in the RT-PCR reaction. Following the detection of transgenic mRNA transcripts, the presence of transgenic proteins was detected in the transformed plants via Western blot using primary antibodies which were specific for the detection of the proteins of interest. The concept of Western blot derived from Southern blot (Southern, 1975) and the name "Western blot" was coined by Burnette (1981) to describe the procedure that was modified by Towbin *et al.* (1979) for the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Similar to Southern blot, Western

blot involves the separation of protein samples in sodium dodecyl sulphate polyacrylamide gels via gel electrophoresis (SDS-PAGE) based on their size followed by transfer to an adsorbent matrix such as nitrocellulose or polyvinylidene fluoride (PVDF). However, in contrast to Southern blot where RE digested genomic DNA was transferred via capillary action; Western blotting employs the use of an electric potential to drive the transfer of proteins from the gel onto the membrane resulting in an exact replica of the protein gel (Towbin *et al.*, 1979). Despite the high resolving ability of SDS-PAGE, its usefulness was limited before the advent of Western blot as separated proteins within the gel matrix were not readily accessible for analysis with molecular probes (Kurien and Scofield, 2006). The development of Western blot for the analysis of proteins was a turning point in the field of immunology as it allowed direct antibody-protein interactions between the separated protein bands. Today, Western blotting together with subsequent process of immuno-detection has found widespread applications in the fields of biochemistry, life sciences and biotechnology.

7.1.3. Avian influenza

The most notable genus of influenza virus is of the class A variety known simply as influenza virus A which mainly affects birds and some mammals. This virus is the main causal agent of outbreaks of highly pathogenic avian influenza (HPAI) which include the highly pathogenic H5N1 strain of infection amongst domestic poultry. Influenza virus A is also known to be the only genera of influenza virus capable of causing infection across the species barrier. Consequently, influenza virus A are prone to antigenic shifts; known to be the main reason for its' high virulence (Qi *et al.*, 2009; Qui *et al.*, 2009). Serotypes of influenza virus A are characterised respectively according to two specific surface antigens, namely, hemagglutinin (HA) and neuraminidase (NA) (Qi *et al.*,

2009). For instance, the H5N1 serotype of avian influenza contains the H5 and N1 subtypes of HA and NA surface antigens, respectively. There are 16 different subtypes of HA and 9 different subtypes of NA glycoproteins which are designated as H1 through H16 and N1 through N9 (Russell *et al.*, 2006), respectively (Qi *et al.*, 2009; Qui *et al.*, 2009). The HA and NA surface antigens are known to be highly antigenic making them prime targets for the development of plant based candidate vaccines (Redkiewicz *et al.*, 2014).

7.1.4. Approaches for investigation

The focus of this chapter involves the identification of transplastomic individuals within the population of antibiotic resistant plants regenerated post-bombardment *N. benthamiana* plants. Experiments were performed for the detection of transgenes, mRNA and proteins in the chloroplasts of transformed *N. benthamiana* plants in order to confirm transgene integration and expression. Following the success of GFP expression, the system would then be implemented for the delivery and expression of HA and NA surface antigens of avian influenza virus A/chicken/Malaysia/5744/2004(H5N1) in the chloroplasts of *N. benthamiana* as a preliminary step towards the production of plant-based vaccines.

7.1.5. Specific objectives

The main focus of this chapter is to evaluate the efficiency of the newly constructed chloroplast transformation vectors for the delivery and expression of transgenes in the chloroplast genome of *N. benthamiana*. The specific objectives of this chapter are to identify transplastomic plants by; 1) detection of transgenes in the genomic DNA of chloroplasts via PCR and Southern blot, 2) qualitative evaluation of transgene expression at the transcription level via the

detection of mRNA transcripts using RT-PCR, 3) evaluation of protein expression via Western detection; and 4) construction of chloroplast transformation vectors for the delivery and expression of HA and NA in the chloroplast of *N. benthamiana*.

7.2 METHODS

7.2.1. Genomic evaluation of transgene insertion

Antibiotic resistant pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants that regenerated post-bombardment were subjected to experiments to determine their transplastomic status at the genomic level. The presence of the green fluorescent protein (GFP) gene was evaluated via polymerase chain reaction (PCR) experiments using both tissue and chloroplast genomic DNA samples from transformed *N. benthamiana* plants.

7.2.1.1. PCR screening for GFP in plant tissue

PCR reactions were performed according to section (3.2.5.) using forward and reverse primers, PF1:sGFP-*Nco*I and PR2:sGFP-*Bs*/WI, respectively. Template DNA for the PCR reactions was substituted with a 1x1mm sample of leaf or callus tissue for the *in-situ* detection of GFP in antibiotic resistant shoots and calli, respectively. PCR products were subsequently subjected to gel electrophoresis (M4) and results were visualised.

7.2.1.2. PCR detection of GFP in chloroplast DNA

Chloroplasts were isolated from antibiotic resistant leaves and calli as described in section (3.2.2.1.) using 1g of starting material and genomic DNA was extracted from isolated chloroplasts using the innuPREP Bacteria genomic DNA Kit (Analytik Jena, Germany) as described in section (3.2.2.2.). PCR reactions for the detection of GFP were performed according to section (3.2.5.) using the same forward and reverse primers with the template DNA substituted with 100ng of extracted chloroplast genomic DNA. Likewise, PCR products were also processed as described in section (7.2.1.1.).

7.2.1.3. Evaluation of transgene integration

Chloroplasts genomic DNA was extracted from antibiotic resistant leaves and calli as described in section (7.2.1.2.) and PCR was performed as described in section (3.2.5.) using INSL and INSR specific forward and reverse primers, INSR-TF and INSL-TR, respectively for the detection of the entire transgene insert. PCR products were processed and visualised as described in section (7.2.1.1.).

7.2.2. Southern blot for the detection of GFP gene

The presence of the GFP gene expression cassette was further evaluated in chloroplast genomic DNA samples of regenerated *N. benthamiana* plants via Southern blotting. Detection of DNA fragments were performed using biotinylated probes prepared specifically for the detection of GFP gene and results were visualised using a chemiluminescent nucleic acid detection method.

7.2.2.1. Restriction digestion of genomic DNA

Genomic DNA was extracted from isolated chloroplasts according to section (7.2.1.2.). Restriction enzyme (RE) digestion of chloroplast genomic DNA was performed according to section (3.2.6.1.) using 5µg of genomic DNA with REs, *AflII* and *MluI*. All digestion reactions were allowed to run at 37°C for 16 hours to completion using sufficient REs for a 10x over digestion assay (generally 1u of each enzyme for every 1µg DNA, unless stated otherwise by the manufacturer) in a 45µl reaction mixture. The digested DNA was purified according to section (3.2.3.2.) and eluted in 15ul of dH₂O. The concentration of the purified DNA was determined according to section (3.2.4.1.) and 2µg of DNA was transferred to a new 200µl tube with loading dye diluted to a working concentration of 1x in dH₂O to a final volume of 10µl. Samples were heated at

65°C for 3 minutes and chilled on ice for 2 minutes before they were spun in a microcentrifuge to pool all contents of the tube.

7.2.2.2. Gel electrophoresis of digested genomic DNA

Agarose gels were prepared according to section (M3) using 1xTAE and 1% agarose without the addition of SYBR Safe DNA gel stain. A bubble leveller was used to ensure that the casted gel had a uniformly even surface area. The prepared gel was submerged with 1xTAE solution in the running tank and the RE digested samples prepared in section (7.2.2.1.) were loaded into the wells of the gel together with a 1kb DNA ladder marked with Ez-vision™ loading dye (Amresco, USA). Gel electrophoresis was performed at 45V for 2 hours or until the dye front reaches three-quarters of the way into the gel. The gel was viewed under UV and a fluorescent ruler was used to gauge the migration distance of the DNA ladder bands from the wells. The wells were removed using a scalpel and the orientation of the gel was marked by cutting a corner off.

7.2.2.3. Preparation of agarose gels for Southern blotting

The gel was rinsed with dH₂O before it was placed into a plastic dish and soaked with enough denaturation solution (0.5M NaOH, 1.0M NaCl) to completely cover the gel. Denaturation was allowed to proceed at room temperature on an orbital shaker set at 50rpm for 30 minutes. The denaturation solution was removed and the gel was allowed to soak for another 20 minutes in neutralisation solution (0.5M Tris-HCl (pH7.5), 3M NaCl) at room temperature at 50rpm. The neutralisation solution was removed and the gel was rinsed briefly with dH₂O before proceeding to the blotting procedure.

7.2.2.4. Southern Blot: Assembly and transfer

The transfer stack was assembled according to figure 7.1. A container (reservoir) was filled to 2/3 full with 20x saline-sodium citrate (SSC: 3M NaCl, 0.3M sodium citrate, pH7.0) transfer buffer and a glass support was placed across the container. A strip of Whatman® 3MM blotting paper with an equal width to the gel was placed across the glass support with both ends immersed in the transfer buffer (bridge). The bridge was saturated with 20x SSC from the reservoir and any air bubbles trapped under the bridge were removed by rolling a glass rod over the bridge. The gel containing digested genomic DNA (section 7.2.2.3.) was placed bottom-side-up on the bridge and any air bubbles were again removed using a glass rod. Four strips of plastic wrap were aligned to overlap 0.5cm of the gel edge to prevent 'short circuit' of transfer buffer. The nylon membrane (BrightStar®-Plus, Ambion, USA) was trimmed to the size of the gel and allowed to soak in 20x SSC buffer for 5 minutes before it was placed on top of the gel (figure 7.1) followed by 4 pieces of Whatman® 3MM blotting paper saturated with 20x SSC from the reservoir. Air bubbles were removed using a glass rod and an 8 cm stack of paper towels was placed on top of the blotting paper followed by a flat rigid support and a weight of approximately 500g. Blotting was allowed to proceed for 16 hours overnight at room temperature. The paper towel stack and blotting paper was removed to expose the nylon membrane and the corner of the membrane corresponding to the cut gel was notched. Lanes were marked using a pencil before the membrane was carefully removed from the surface of the gel and rinsed gently with 2x SSC buffer DNA-side-up in a clean plastic dish without shaking. Crosslinking of bound DNA was performed by exposing the side of the membrane that was facing the gel to ultraviolet light in a UV transilluminator ($7000\mu\text{W}/\text{cm}^2$) for 3 minutes followed by the reverse side for 2 minutes. Blotted membranes were then dried and stored at 4°C or hybridised immediately.

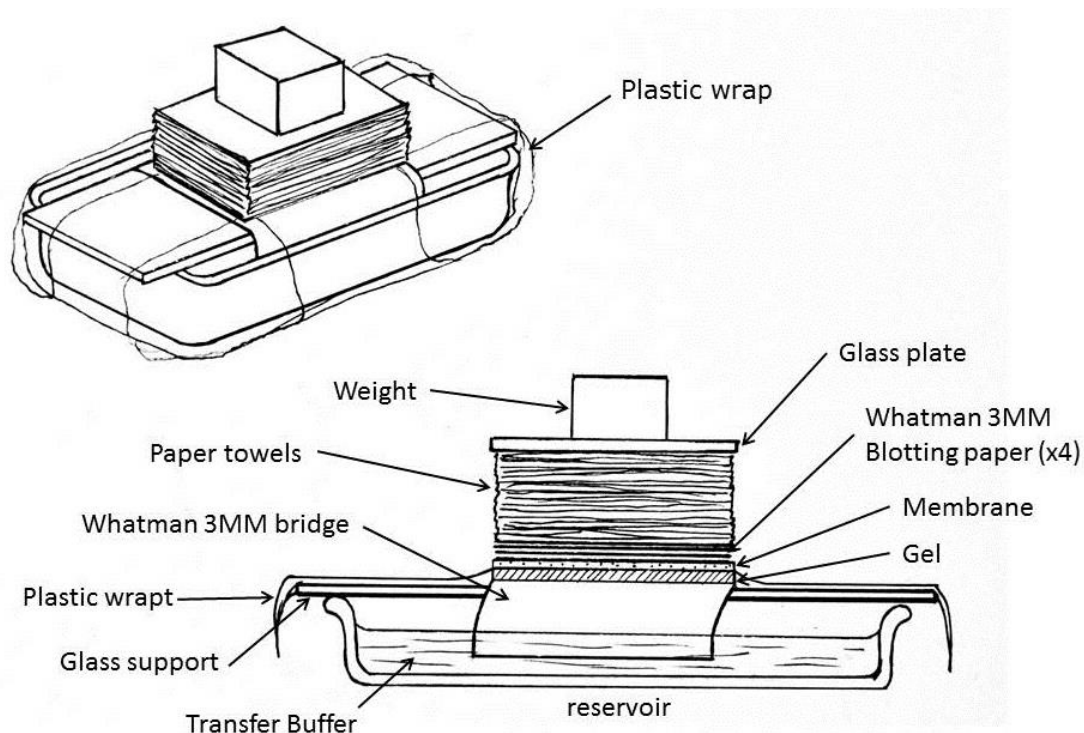


Figure 7.1: Schematic representation of the arrangement of components for the Southern blot of RE digested chloroplast genomic DNA fragments from agarose gel onto nylon membrane via capillary transfer.

7.2.2.5. Preparation of biotinylated probes

Biotinylated DNA probes were prepared using the BioNick™ Labelling System (Invitrogen, USA) according to the user's manual. The gene of interest was amplified by PCR as described in section (3.2.5.) from their respective transformation vectors and the PCR product was gel purified according to section (3.2.3.1.) and eluted in 10µl of dH₂O. The reaction mixture was prepared in a 1.5ml tube on ice with a final concentration of 1x dNTP mix, 1µg of amplified DNA and 1x enzyme mix to a final volume of 50 µl. The mixture was vortexed briefly and spun in a microcentrifuge to pool the liquid before incubating at 16°C for 1 hour. The reaction was halted by the addition of 5µl stop buffer and unincorporated nucleotides from the labelling process were purified away by repeated ethanol precipitation as described in section (5.2.4.2.). The purified

probes were resuspended in 20µl EB buffer and stored at -20°C. Alternatively, labelled DNA was also purified using a standard PCR clean-up kit as described in section (3.2.3.1.).

7.2.2.6. Pre-hybridisation procedure

Hybridization buffer (ULTRAhyb[®], Thermo Fisher Scientific, USA) was preheated in a 68°C water bath to dissolve any precipitated material. Sheared salmon sperm DNA was denatured by preheating in a 95-100°C water bath for 5 minutes and chilled on ice for 2 minutes. Pre-hybridisation buffer was prepared by adding 100µl of 50x Denhardt's solution (Thermo Fisher Scientific, USA) to 5ml of hybridisation buffer together with 10µg of denatured sheared salmon sperm DNA. The nylon membrane was placed into a hybridisation tube with the bound DNA facing inwards the membrane was pre-wet using 20ml of 2x SSC. The SSC solution was carefully discarded without leaving any air bubbles under the membrane and 5ml of pre-hybridisation buffer was introduced into the tube without any direct contact to the membrane. Incubation was performed at 42°C for 1 hour in a hybridisation oven (Thermo Fisher Scientific, USA).

7.2.2.7. Hybridisation procedure

Biotinylated probes prepared in section (7.2.2.5.) were denatured by preheating in a 95°C water bath for 5-10 minutes and cooled immediately on ice for 2 minutes. Hybridisation buffer was prepared by adding 500ng of biotinylated probe DNA to the pre-hybridisation buffer as described in section (7.2.2.6.). The only difference between pre-hybridisation and hybridisation buffer is the addition of probe DNA. Pre-hybridisation buffer was carefully discarded without disturbing the membrane following the pre-hybridisation step and 5ml of hybridisation buffer was added to the tube without any direct contact to the membrane. Hybridisation was allowed to proceed for 16 hours overnight at 65°C in a hybridisation oven.

7.2.2.8. Washing of unbound probes

The hybridisation solution was removed by decanting into a sterile 50ml conical tube (hybridisation buffer can be reused again) and the membrane was washed twice with 10ml of 2X SSC solution at room temperature for 5 minutes, twice with 10ml of 2X SSC; 1% (w/v) SDS at 50°C for 15 minutes and twice again with 10ml of 0.1X SSC solution at 60°C for 15 minutes.

7.2.2.9. Detection of nucleic acids

Nucleic acid detection was performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, USA). The provided blocking buffer and 4x wash buffer was preheated in a 45°C water bath to dissolve any precipitated material. The washed membrane (section 7.2.2.8.) was placed in a clean plastic container and the blocking procedure was performed using 10ml of blocking buffer with gentle shaking on an orbital shaker at 50rpm for 15 minutes at room temperature. Conjugate/blocking buffer solution was prepared by adding 33µl of stabilised streptavidin-horseradish peroxidase conjugate to 10ml of blocking buffer (1:300 dilution). The blocking buffer was discarded and 10ml of conjugate/blocking solution was added without direct contact to the membrane. Binding was allowed to proceed at room temperature with gentle shaking at 50rpm for 15 minutes. The membrane was transferred to a new container and rinsed briefly with 20ml of 1x wash solution (5ml 4X wash buffer; 15ml dH₂O). Washing was performed using 20ml 1x wash solution with gentle shaking at 50rpm for 5 minutes at room temperature for a total of four times. The membrane was transferred to a new container and 15ml of substrate equilibration buffer was added followed by incubation at room temperature with gentle shaking at 50rpm for 5 minutes. The membrane was removed from the substrate equilibration buffer and residual liquid was carefully blotted away by touching a corner of the membrane on a paper towel before it was placed in a clear plastic folder. Chemiluminescent substrate working solution was prepared

by adding 3ml of luminol/enhancer solution to 3ml of stable peroxide solution under reduced light. The substrate working solution was transferred onto the membrane using a pipette and the solution was spread evenly across the entire membrane using the plastic folder cover followed by incubation for 5 minutes at room temperature under reduced light. Excess substrate working solution was removed after incubation by gently rolling a glass rod over the plastic folder. Chemiluminescent signals were detected by photographing the membrane in the dark using the FluorChem[®]HD2 gel dock system (Alpha Innotech, USA) with an exposure time of 2-5 minutes.

7.2.3. Evaluation of GFP transcription in *N. benthamiana*

GFP expression was evaluated at the transcription level for both pEXPR-G and pEXPR-UG transformed plants. A qualitative evaluation for the presence of GFP mRNA transcripts in total RNA samples extracted from the leaves of antibiotic resistant *N. benthamiana* plants was performed via reverse transcription-polymerase chain reaction (RT-PCR).

7.2.3.1. Preparation of RNA samples for RT-PCR

All hardware to be used for RNA extraction such as mortar and pestles were treated overnight with a 0.4M NaOH solution, rinsed with dH₂O and autoclaved prior to usage. RNA extraction was performed by grinding 100mg of plant tissue in liquid nitrogen to a fine powder together with 1ml of TRIzol (Invitrogen, USA). The homogenised plant material was allowed to thaw completely before transferring to a new RNase-free 1.5ml tube using a pipette. The tube was incubated at 60°C for 5 minutes at 400rpm with intermittent vortexing. Subsequent steps for the recovery of RNA were performed as described in section (4.2.2.1.), subjected to DNase treatment and purification as

described in section (4.2.2.2.) to obtain RNA of sufficient purity for RT-PCR (absorbance ratios of 260/230 and 260/280 ≥ 2.0).

7.2.3.2. RT-PCR: qualitative detection of GFP mRNA transcripts

RT-PCR was performed on 2 μ g samples of extracted total RNA (section 7.2.3.1.) using the Omniscript[®] reverse transcription kit (Qiagen, Germany) via the 2-step method according to the user's manual as described in section (4.2.3.). The reverse transcription reaction was achieved using the GFP specific reverse primer, GFPR-Seq. The subsequent PCR amplification was performed using forward and reverse primers, GFPF-Seq and GFPR-Seq, respectively and results of the RT-PCR reaction were evaluated as described in section (7.2.1.1.).

7.2.4. Evaluation of GFP protein expression in *N. benthamiana*

The presence of GFP was detected in the protein extracts of pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants via Western blot. The detection of GFP was performed using GFP specific primary antibodies on both pre- and post-immunopurified samples and contamination of non-specific binding was evaluated using primary antibodies specific to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein.

7.2.4.1. Protein extraction from chloroplasts

Chloroplasts were isolated from the leaves of antibiotic resistant *N. benthamiana* plants as described in section (3.2.2.1.) using 1g of starting material. The chloroplast pellet was resuspended with 200ul working solution of Sample Buffer 1 (ReadyPrep[™] Protein Extraction Kit, Bio-Rad Laboratories, USA) and snap-frozen in liquid nitrogen. Subsequent steps for protein extraction were performed using the soni-thaw method as described in section (4.2.4.3.) and the

concentration of extracted proteins was determined as described in section (4.2.4.4.).

7.2.4.2. Immunoprecipitation of c-Myc tagged protein

Protein was extracted from 100mg samples of leaf and callus tissue using the soni-thaw method as described in section (7.2.4.1.) and the volume of the final product was adjusted to 500ul with ddH₂O in a 1.5ml tube. Immunoprecipitation of c-Myc tagged proteins was performed using monoclonal c-Myc antibodies crosslinked to immobilized protein A agarose beads (Clontech Laboratories, USA). The agarose bead suspension was vortexed briefly and 25µl of the suspension was transferred using a pipette into the tube containing the extracted protein sample. The mixture was incubated overnight at 4°C in the thermomixer (Eppendorf, Germany) with agitation at 200rpm. The mixture was spun in a microcentrifuge to pellet the agarose beads and the supernatant was removed before the pellet was washed using 1ml of TBS solution for 2 minutes for a total of five times. The supernatant was discarded after the final wash and the pellet was resuspended in 25µl of 2x SDS sample buffer. Samples were heated to 95-100°C for 5 minutes to unbind the immunoprecipitated protein and centrifuged at 10 000rpm. The supernatant containing the c-Myc tagged proteins was transferred to a new 1.5ml tube and stored at -80°C.

7.2.4.3. Western detection of transgenic protein

Samples were prepared for denaturing SDS-PAGE by mixing 10µg of total protein (sections 7.2.4.1. and 7.2.4.2.) with 1x NuPAGE[®] LDS sample buffer (Invitrogen, USA) to a final volume of 20µl in ddH₂O. The prepared samples were subjected to SDS-PAGE as described in section (4.2.5.) and evaluated via Western blot as described in section (4.2.6.). Detection of GFP and c-Myc tagged proteins were performed according to section (4.2.6.2.). Detection of RuBisCO was performed using chicken polyclonal primary antibody against RuBisCO

(Sigma, USA) at a 1:20 000 dilution in blocking buffer and HRP conjugated donkey polyclonal secondary antibody against chicken IgG (Antibodies, Germany) at a 1:10 000 dilution in TBST according to section (4.2.6.2.).

7.2.5. Expression of Hemagglutinin (HA) and Neuraminidase (NA) viral antigens of avian influenza A virus strain H5N1 in the chloroplasts of *N. benthamiana*

Chloroplast transformation vectors were constructed for the delivery and expression of HA and NA in the chloroplasts of *N. benthamiana* using the developed 2-part (pCART and pGENE) vector construction system. The completed chloroplast transformation vectors were delivered into leaf explants of *N. benthamiana* via particle bombardment and regenerated using the optimised parameters. The expression of HA and NA was evaluated at the genomic, transcription and translation levels in leaf tissue samples from the regenerate shoots of transformed *N. benthamiana*.

7.2.5.1. Construction of HA and NA transformation vectors

The native hemagglutinin (HA) and neuraminidase (NA) genes of avian influenza virus A/chicken/Malaysia/5744/2004(H5N1) were predicted to contain several rare codons that are expected to severely impede the translation process *in-vivo*. Hence, codon optimised HA (appendix Fiv) and NA (appendix Fv) genes were synthesised based on the chloroplast codon usage frequencies of *N. benthamiana* with reference to source data from NCBI-GenBank (<http://www.kazusa.or.jp/codon/>; date last accessed: 25/05/2015). Transformation vectors for the expression of HA and NA in chloroplasts of *N. benthamiana* were constructed using the developed 2-part vector system as described in (section 3.2.11.) and the identities of the constructed vectors were verified as described in (section 3.3.4.).

7.2.5.2. Transformation of *N. benthamiana* with HA and NA chloroplast transformation vectors

Chloroplast transformation vectors for the expression of HA and NA were delivered into leaf explants of *in-vitro* propagated *N. benthamiana* plants using optimised protocols and bombardment parameters as described in Chapter 5, table 5.2 and regeneration of leaf explants post-bombardment were performed based on the optimised procedures outlined in Chapter 6, section 6.5.

7.2.5.3. Evaluation of HA and NA expression in *N. benthamiana*

Preliminary evaluation of HA and NA insertion into the chloroplast genome of pEXPR-HA and pEXPR-NA transformed *N. benthamiana* was performed via gene specific PCR-screening of leaf tissue using primers, ZUHF and ZUHR (appendix B) for the detection of HA; and primers, ZUNF and ZUNR (appendix B) for the detection of NA, respectively according to section (7.2.1.1.). The chloroplast genome was extracted from PCR-positive plants as described in section (7.2.1.2.) and the presence of the HA and NA gene was detected via PCR amplification using the same PCR screening primers. The presence of HA and NA was further verified via Southern blot detection using HA and NA gene-specific probes that were prepared from the amplified gene transcripts of HA and NA from their respective pEXPR-HA and pEXPR-NA chloroplast transformation vectors as described in section (7.2.2.). The detection of mRNA transcripts of HA and NA was performed via RT-PCR using primers, ZUHF and ZUHR; and primers, ZUNF and ZUNR for the detection of HA and NA, respectively according to section (7.2.3.). Immunoprecipitation of c-Myc-tagged proteins were performed according to section (7.2.4.2.) and Western detection of immunoprecipitated proteins was performed using rabbit polyclonal antibody against c-Myc-tag (Abcam, UK) diluted at 1:25 000 in blocking buffer; together with horseradish peroxidase (HRP) conjugated goat polyclonal antibody against rabbit IgG (Abcam, UK), diluted at 1:10 000 in TBST as described in Chapter 4, section

(4.2.6.2.). Detection of transgenic NA proteins was performed via Western blot using rabbit polyclonal primary antibodies against avian influenza A neuraminidase (Abcam, Cambridge, UK), diluted at 1:15 000 in blocking buffer; together with HRP conjugated goat polyclonal antibody against rabbit IgG (Abcam, Cambridge, UK), diluted at 1:10 000 in TBST as described in Chapter 4, section (4.2.6.2.).

7.3 RESULTS

7.3.1. Detection of GFP gene in chloroplast genomic DNA

In order to facilitate the identification of transplastomic plants, detection of the *aadA* gene was first performed via PCR screening of small tissue samples harvested from antibiotic resistant shoots and calli. Regenerated lines that were found to be positive were further evaluated via PCR detection of GFP in the chloroplast genome. Evaluation of transgene integration was further confirmed via gene-independent PCR and Southern blot experiments.

7.3.1.1. Detection of *aadA* in regenerated shoots and callus tissue

Results for the PCR amplification of the *aadA* gene (795bp) from the tissue samples of antibiotic resistant shoots and calli using primers, *aadA*-SeqF and *aadA*-SeqR indicated that *aadA* was more abundant in shoots as compared to calli. Bands of approximately 795bp were produced by all positive samples which corresponded to the expected size of the *aadA* gene. A total of 83% of antibiotic resistant shoots regenerated from pEXPR-G transformed *N. benthamiana* displayed positive results for the detection of *aadA* (figure 7.2: lanes 1-6). In contrast, only 50% of pEXPR-G transformed calli were found to be positive (figure 7.3: lanes 1-4). Similarly, a higher percentage (100%) of pEXPR-UG transformed shoots were also found to display positive results for the detection of *aadA* (figure 7.2: lanes 7-14) as compared to pEXPR-UG transformed calli (0%) (figure 7.3: lanes 5-11).

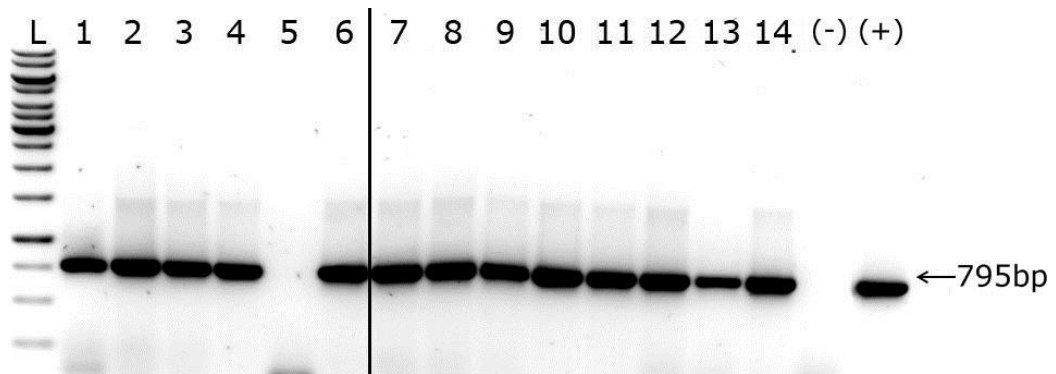


Figure 7.2: PCR profiles for the amplification of *aadA* gene (795bp) from tissue samples of antibiotic resistant shoots regenerated from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants. L: Fermentas 1kb DNA ladder, Lanes 1-6: PCR products of pEXPR-G transformed samples, Lanes 7-14: PCR products of pEXPR-UG transformed samples, (-): Negative control, (+): *aadA* positive control.

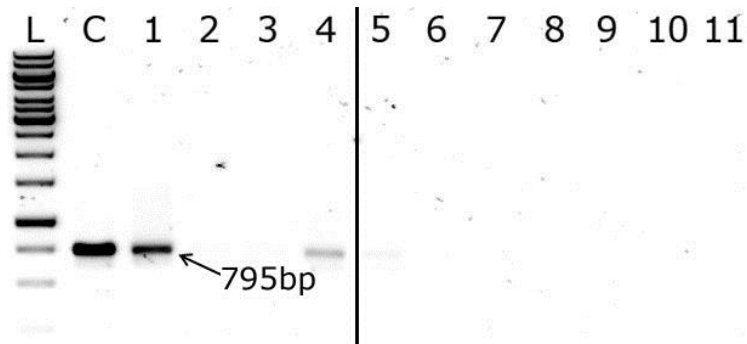


Figure 7.3: PCR profiles for the detection of *aadA* (795bp) in tissue samples of antibiotic resistant calli regenerated from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants. L: Fermentas 1kb DNA ladder, C: *aadA* positive control, Lanes 1-4: PCR products of pEXPR-G transformed samples, Lanes 5-11: PCR products of pEXPR-UG transformed samples.

7.3.1.2. Detection of GFP in genomic DNA of shoots and callus tissue of *N. benthamiana*

The purity of extracted chloroplast genomic DNA was evaluated by PCR detection of contaminating DNA sequences of the 25S rRNA of *N. benthamiana* (accession: KP824745.1). GFP specific primers, PF1:sGFP-*Nco*I and PR2:sGFP-

*Bsi*WI were used for the detection of GFP in the genomic DNA extracted from antibiotic resistant shoots and calli regenerated from pEXPR-G and PEXPR-UG transformed *N. benthamiana*. All positive samples produced a band of approximately 720bp which corresponded to size of the GFP positive control and the expected size of the GFP gene. Results for the detection of GFP in chloroplast genomic DNA samples extracted from antibiotic resistant shoots of pEXPR-G and pEXPR-UG transformed *N. benthamiana* (figure 7.4) were identical to prior PCR screening results (section 7.3.1.1., figure 7.2). However, results for the detection of GFP in chloroplast genome samples extracted from antibiotic resistant calli did not produce any positive results and only produced non-specific bands of approximately 250bp (figure 7.5) which may be the result low primer annealing temperatures or truncated gene inserts caused by spurious recombination events. Interestingly, even callus lines 1 and 4 which were positive in earlier PCR screening experiments (section 7.3.1.1., figure 7.3) did not produce any positive results (figure 7.5).

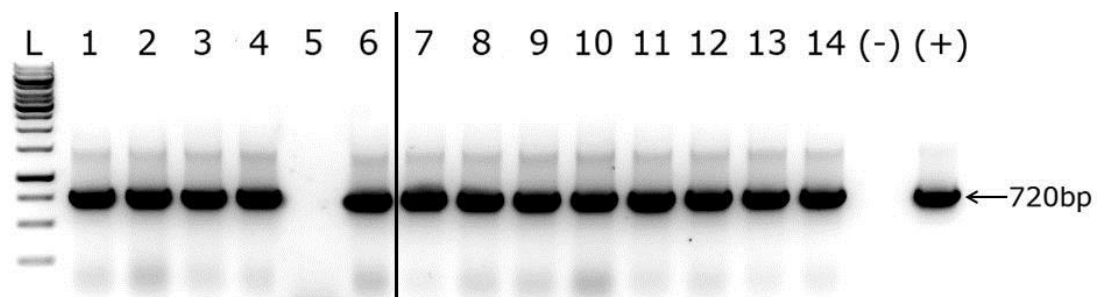


Figure 7.4: PCR profiles for the amplification of GFP gene (720bp) from chloroplast genomic DNA samples extracted from antibiotic resistant shoots of regenerated pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants. L: Fermentas 1kb DNA ladder, Lanes 1-6: PCR products of pEXPR-G transformed samples, Lanes 7-14: PCR products of pEXPR-UG transformed samples, (-): Negative control, (+): GFP positive control.

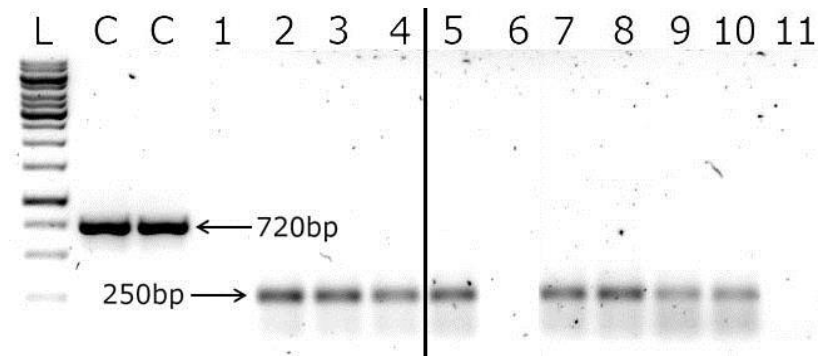


Figure 7.5: PCR profiles for the detection of GFP in chloroplast genomic DNA extracted from antibiotic resistant calli regenerated from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants. L: Fermentas 1kb DNA ladder, C: GFP positive control, Lanes 1-4: PCR products of pEXPR-G transformed samples, Lanes 5-11: PCR products of pEXPR-UG transformed samples.

7.3.1.3. Genotype screening of transformed shoots and calli

Primers INSR-TF and INSL-TR were used for the amplification of the endogenous homologous recombination sequence of INSR and INSL in the inverted repeat (IR) regions of the tobacco chloroplast genome. The forward primer INSR-TF was designed to bind to the INSR homologous recombination site at positions 110 714 and 131 899 of the chloroplast genome sequence while the reverse primer INSL-TR was designed to bind at positions 110 001 and 132 612 of the chloroplast genome sequence. The amplification of bands of approximately 700bp is expected from both the right and left IR regions of wild-type chloroplast genomic DNA when PCR was performed using primers, INSR-TF and INSL-TR (figure 7.6: Wt). In contrast, results for the PCR amplification of chloroplast genomic DNA extracted from all pEXPR-G (figure 7.6: A, lanes 1-6) and pEXPR-UG (figure 7.6: A, lanes 7-14) transformed antibiotic resistant shoots using primers, INSR-TF and INSL-TR produced bands of approximately 3.3kb in addition to the 700bp band (figure 7.6: A). Interestingly, the 700bp wild-type band was observed to be lost in lines 7 and 13 of pEXPR-UG transformed shoots of *N. benthamiana* (figure 7.6: A). In contrast, a larger band was never observed

in all evaluated samples of genomic DNA extracted from transformed antibiotic resistant calli using the same primers. Remarkably, even the 700bp wild-type band was found to be missing in a number of calli samples (figure 7.6: B, lanes 1, 4, 6, 9, 10 and 11).

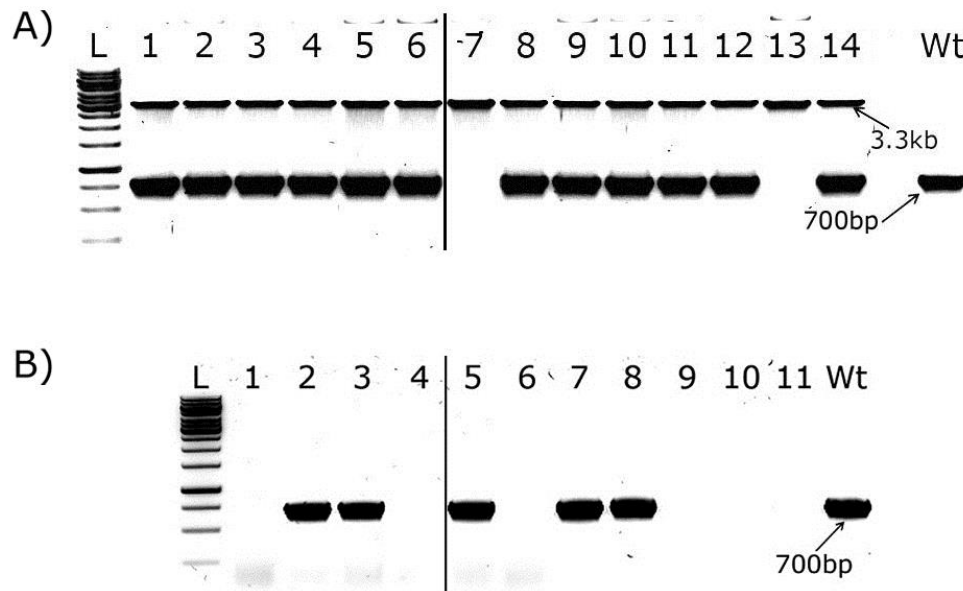


Figure 7.6: PCR profiles for the amplification of extracted chloroplast genomic DNA using primers, INSR-TF and INSL-TR from pEXPR-G and pEXPR-UG transformed shoots (A) and calli (B). The homologous recombination site specific primers allow the detection of transgene integration into the chloroplast genome via a change in the size of the amplified bands. L: Fermentas 1kb DNA ladder, A) Lanes 1-6: PCR products of pEXPR-G transformed shoots, A) Lanes 7-14: PCR products of pEXPR-UG transformed shoots, B) Lanes 1-4: PCR products of pEXPR-G transformed calli, B) Lanes 5-11: PCR products of pEXPR-UG transformed calli, Wt: Wild-type control.

7.3.1.4. Southern blot: detection of GFP in genomic DNA of transformed *N. benthamiana*

Southern blot experiments were performed for the identification of the GFP gene in the chloroplast genome of the transformed explants. Bands of approximately 980bp were observed in the Southern blot profile of pEXPR-UG transformed shoots of *N. benthamiana* (figure 7.7: Lanes 1-8). The bands were

found to be highly similar to the positive control (figure 7.7: C1) which was produced by double digestion of the pEXPR-UG chloroplast transformation vector with REs, *Afl*II and *Mlu*I. The other positive control (figure 7.7: C2) which was produced by the PCR amplification of the GFP gene from pEXPR-UG using PCR primers, GFPF-Seq and GFPR-Seq produced a band with an approximate size of 720bp which corresponded to the expected size of the GFP gene.

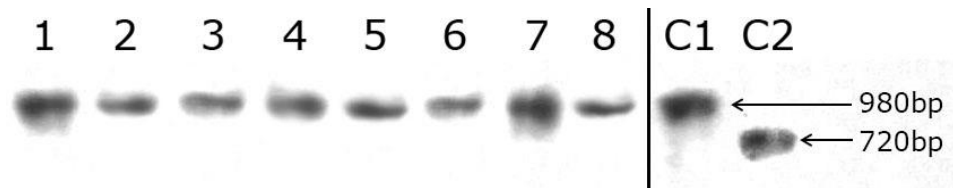


Figure 7.7: Southern blot profiles for the detection of GFP in the chloroplast genome of pEXPR-UG transformed shoots of *N. benthamiana*. Lanes 1-8: Chloroplast genomic DNA from pEXPR-UG transformed shoots double digested with REs *Afl*II and *Mlu*I, C1: pEXPR-UG double digested with REs, *Afl*II and *Mlu*I, C2: GFP PCR product.

7.3.2. Evaluation of GFP transcription in *N. benthamiana*

The expression of GFP was evaluated at the transcription level in pEXPR-G and pEXPR-UG transformed shoots via qualitative detection of GFP mRNA transcripts. Total RNA was extracted from the leaves of transformed *N. benthamiana* plants and subjected to 2-step RT-PCR for the detection of GFP transcription.

7.3.2.1. Total RNA extraction from the leaves of regenerated shoots

No significant differences were observed in the gel electrophoresis results for total RNA samples extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants (figure 7.8). Observed bands were mainly the result of the abundant ribosomal RNAs (rRNAs) of both eukaryotic and prokaryotic origin due to the global nature of the RNA sample. Defined bands of approximately 3.7kb

and 1.9kb were observed which corresponded to the expected sizes of the eukaryotic 25S and 18S rRNAs, as well as, bands of approximately 2.9kb and 1.5kb which corresponded to the expected sizes of the prokaryotic-like 23S and 16S chloroplast rRNAs. The defined rRNA bands also indicate that the extracted samples were not contaminated with RNase and can be used for further evaluation experiments.

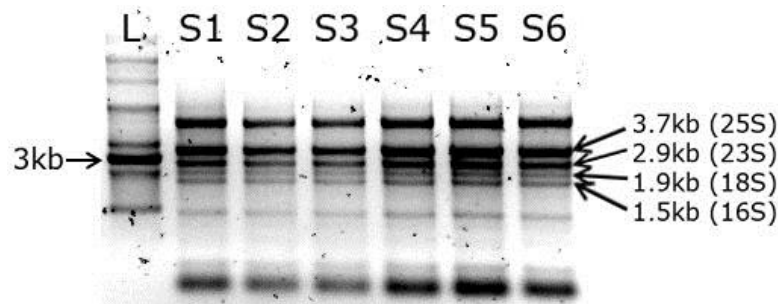


Figure 7.8: RNA profiles for the extraction of total RNA samples from the leaves of pEXPR-G and pEXPR-UG transformed *N. benthamiana*. Clearly defined rRNA bands indicate that the extracted RNA was not contaminated with RNase and can be subjected to RT-PCR experiments. L: NEB ssRNA ladder, Lanes S1-S3: Total RNA extracted from pEXPR-G transformed *N. benthamiana*, Lanes S4-S6: Total RNA extracted from pEXPR-UG transformed *N. benthamiana*.

7.3.2.2. RT-PCR: Qualitative detection of GFP mRNA expression

Bands of approximately 720bp were obtained in the RT-PCR results for the detection of GFP mRNA transcripts in total RNA extracted from pEXPR-G (figure 7.9: lanes X1-3) and pEXPR-UG (figure 7.9: lanes U1-3) transformed *N. benthamiana* plants. The observed bands corresponded to the size of the GFP positive control (figure 7.9: C2) as well as the expected size of the GFP gene. The negative controls C1 (NTC), C3 (*aadA* non-RT), C6 (non-RT pEXPR-G total RNA) and C7 (non-RT pEXPR-UG total RNA) did not produce any detectable bands while positive controls C2 (GFP positive control: 720bp), C4 (*aadA* RT-PCR

internal control: 798bp) and C5 (*aadA* positive control: 798bp) produced bands with the correct expected sizes (figure 7.9).

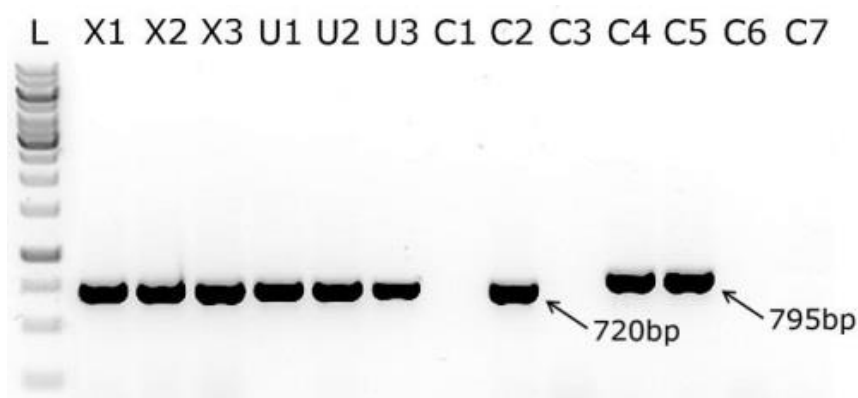


Figure 7.9: RT-PCR profiles for the qualitative detection of mRNA transcripts of GFP from total RNA samples extracted from pEXPR-G and pEXPR-UG transformed shoots of *N. benthamiana*. L: Fermentas 1kb DNA ladder, Lanes X1-3: RT-PCR products of GFP from total RNA extracted from pEXPR-G transformed *N. benthamiana*, Lanes U1-3: RT-PCR products of GFP from total RNA extracted from pEXPR-UG transformed *N. benthamiana*, C1: Non-template control, C2: GFP positive control, C3: Non-RT control for the *aadA* gene, C4: Internal control for the RT-PCR of *aadA*, C5: *aadA* positive control, C6: GFP Non-RT control (total RNA from pEXPR-G transformed plants), C7: GFP Non-RT control (total RNA from pEXPR-UG transformed plants).

7.3.3. Western blot: Detection of GFP protein expression

Expression of GFP protein was detected from total protein samples extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants via Western detection using both GFP-specific and c-Myc specific primary antibodies. However, expression of GFP was detected only in protein samples from pEXPR-UG transformed plants.

7.3.3.1. Detection of GFP: Effects of immunoprecipitation

Results for the Western detection of transgenic GFP proteins in total protein samples extracted from the regenerated shoots of pEXPR-G and pEXPR-UG transformed *N. benthamiana* using GFP-specific primary antibodies are shown in (figure 7.10). A high degree of non-specific binding was observed when GFP-specific polyclonal primary antibodies were used for the detection of GFP in total protein samples extracted from pEXPR-G transformed plants before immunoprecipitation (figure 7.10: lanes X1 and X2). Similarly, Western blot profiles for total protein samples extracted from pEXPR-UG transformed plants also showed a high degree of non-specific binding before immunoprecipitation when probed using the same primary antibodies (figure 7.10: lanes U1 and U2). Interestingly, bands of approximately 28kDa were only observed in the Western blot profiles of protein samples extracted from pEXPR-UG transformed *N. benthamiana* plants after the immunoprecipitation process (figure 7.10: lanes U3 and U4). The observed bands corresponded to the positive control for GFP and the expected molecular mass of the GFP protein. Similar bands were not observed in pEXPR-G transformed samples (figure 7.10: lanes X3 and X4).

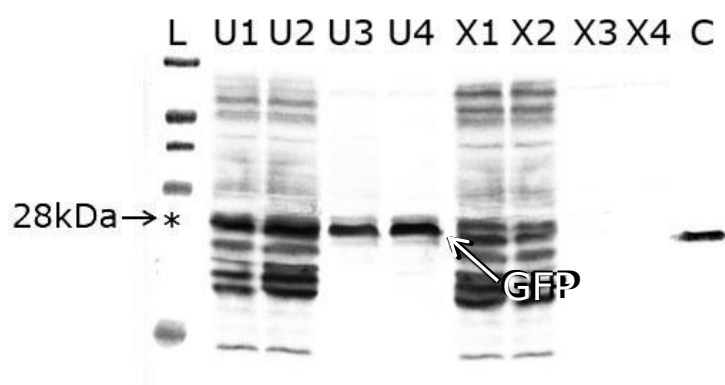


Figure 7.10: Western blot profiles for the detection of GFP protein in total protein samples extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* before and after c-Myc tag immunoprecipitation using GFP-specific primary antibodies. L: Nacalai broad range protein ladder one, triple colour (Nacalai, USA), U1 and U2: protein samples from pEXPR-UG transformed plants before c-Myc tag immunoprecipitation, U3 and U4: protein samples from pEXPR-UG transformed plants after c-Myc tag immunoprecipitation, X1 and X2: protein samples from pEXPR-G transformed plants before c-Myc tag immunoprecipitation, X3 and X4: protein samples from pEXPR-G transformed plants after c-Myc tag immunoprecipitation, C: bacteria expressed GFP positive control, (*): the imaging device automatically applied a blue pass filter for the detection of HRP substrate which made the red/orange ladder bands disappear.

7.3.3.2. Detection of c-Myc tagged proteins post-immunoprecipitation

Immunopurified samples were further verified via Western blot using c-Myc-specific primary antibodies for the detection of c-Myc tagged proteins. As expected, bands with sizes of approximately 28kDa were observed in immunopurified protein samples extracted from pEXPR-UG transformed plants which corresponded to the expected molecular mass of the GFP protein (figure 7.11: U1 and U2). No positive controls were available for c-Myc tagged GFP proteins, however, the observed results highly resemble the results for the Western detection of GFP using GFP-specific primary antibodies (figure 7.10: lanes U3 and U4). Immunopurified protein samples extracted from pEXPR-G

transformed plants did not produce any detectable bands when probed using c-Myc specific primary antibodies (figure 7.11: lanes X1 and X2).

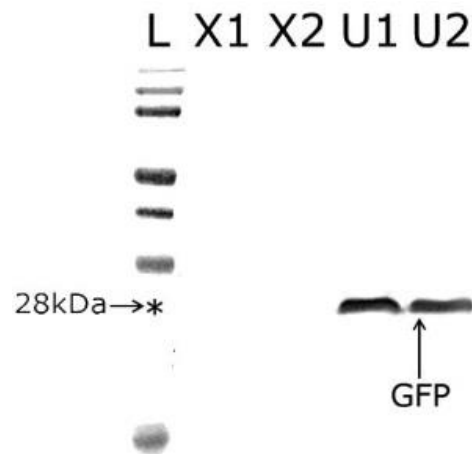


Figure 7.11: Western blot profiles for the detection of c-Myc tagged proteins in total protein samples extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* after immunoprecipitation using c-Myc-specific primary antibodies. L: Broad range protein ladder one, triple colour (Nacalai, USA), X1 and X2: protein samples from pEXPR-G transformed plans, U1 and U2: protein samples from pEXPR-UG transformed plants, *the imaging device automatically applied a blue pass filter for the detection of HRP substrate which made the red/orange ladder bands disappear.

7.3.3.3. Detection of non-specific binding of RuBisCO

The efficiency of immunoprecipitation for the specific isolation of c-Myc tagged proteins was evaluated by the detection of RuBisCO contamination in the immunoprecipitated protein samples. The immunopurified samples of total protein extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* were subjected to Western detection using polyclonal primary antibodies against RuBisCO. All pEXPR-G (figure 7.12: X1 and X2) and pEXPR-UG (figure 7.12: U1 and U2) samples as well as the negative control (figure 7.12: C) did not produce any bands for the detection of RuBisCO.

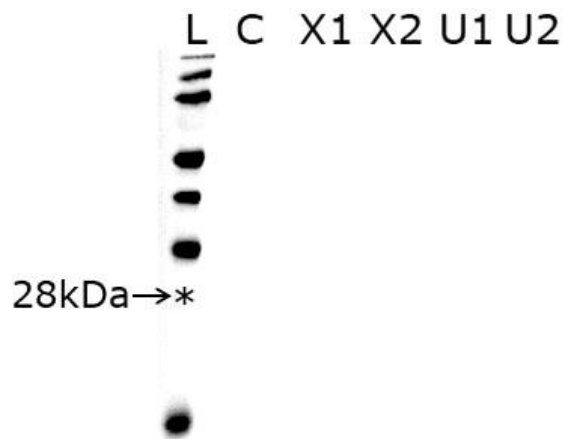


Figure 7.12: Western blot profiles for the detection of plant expressed RuBisCO in the c-Myc tag immunopurified protein samples extracted from pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants using polyclonal primary antibodies against RuBisCO. L: Broad range protein ladder one, triple colour (Nacalai, USA), C: bacteria expressed GFP positive control, X1 and X2: immunopurified protein samples from pEXPR-G transformed plants, U1 and U2: immunopurified protein samples from pEXPR-UG transformed plants, *the imaging device automatically applied a blue pass filter for the detection of HRP substrate which made the red/orange ladder bands disappear.

7.3.3.4. Phenotypic expression of GFP in *N. benthamiana*

Phenotypic GFP expression was evaluated by viewing tissue samples of pEXPR-G and pEXPR-UG transformed *N. benthamiana* plants under a stereoscopic dissecting microscope equipped with a GFP filter (SMZ-1500, Nikon, Japan). GFP expression was only detected in tissue samples harvested from antibiotic resistant shoots of pEXPR-UG transformed samples (figure 7.13: panel B). Tissue samples that were harvested from pEXPR-G transformed shoots and wild type control samples did not display any green fluorescence when viewed under UV light (figure 7.13: panel D and F).

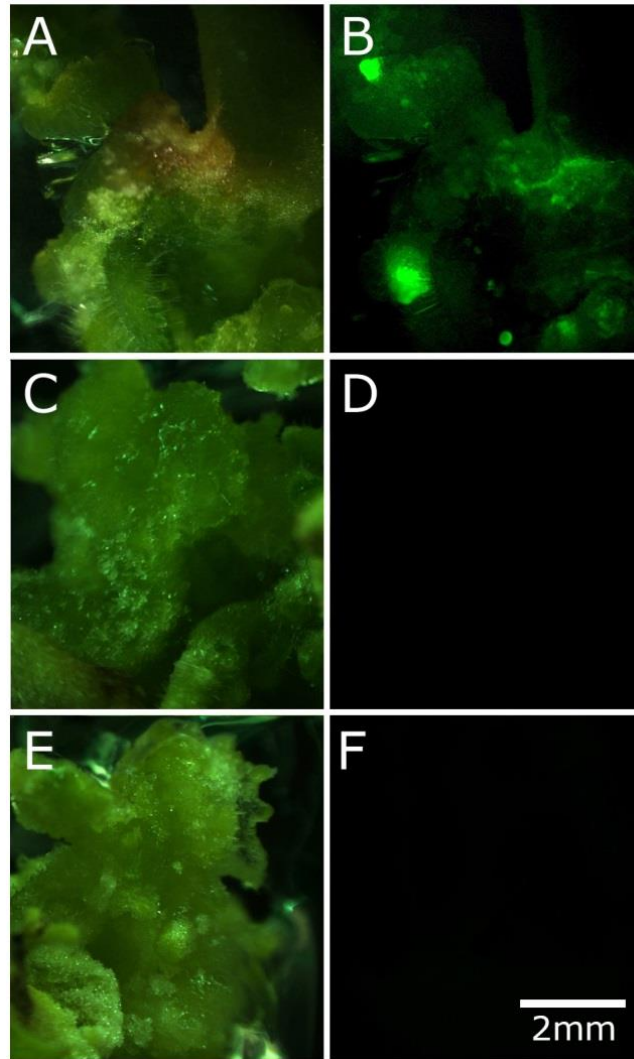


Figure 7.13: Visualisation of pEXPR-G and pEXPR-UG transformed *N. benthamiana* tissue samples under a stereoscopic dissecting microscope equipped with a GFP filter. Images of the same samples were captured under both white light and UV light (379-401 nm) at 20x magnification. Only pEXPR-UG transformed *N. benthamiana* tissue samples displayed systemic expression of green fluorescence under UV light (B). Results for the detection green fluorescence in pEXPR-G transformed *N. benthamiana* tissue samples under UV light were negative (D) and identical to the non-transformed negative control (F). (A): pEXPR-UG transformed plant tissue viewed under white light, (B): pEXPR-UG transformed plant tissue viewed under UV light, (C): pEXPR-G transformed plant tissue viewed under white light, (D): pEXPR-G transformed plant tissue viewed under UV light, (E): Non-transformed plant tissue viewed under white light, (F): Non-transformed plant tissue viewed under UV light. Bar=2mm.

7.3.4. Construction of HA and NA chloroplast transformation vectors

Results for the construction of chloroplast transformation vectors for the delivery and expression of the hemagglutinin (HA) and neuraminidase (NA) genes in the chloroplast of *N. benthamiana* using the developed 2-part vector system were evaluated as described in Chapter 3, section (3.3.4.). PCR amplification of codon optimised HA was successfully achieved using forward and reverse primers, ZUHF and ZUHR (figure 7.14: A) while amplification of codon optimised NA was achieved using primers, ZUNF and ZUNR, respectively (figure 7.14: B). Gel electrophoresis results for the PCR products of the HA and NA gene using their respective forward and reverse primers produced single bands of approximately 1700bp and 1350bp, respectively (figure 7.14). These bands corresponded to the expected length of the synthesised HA and NA gene sequence of 1704bp and 1347bp, respectively.

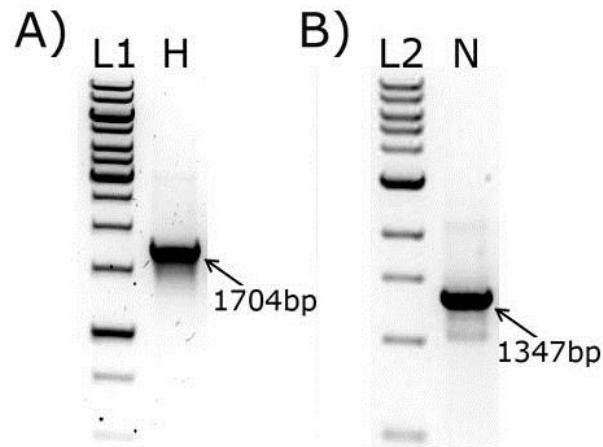


Figure 7.14: PCR amplification profiles of codon optimised HA (A) and NA (B) from their respective pUC57 holding vector. Amplification of the 1704bp HA gene was achieved using primers ZUHF and ZUHR while amplification of the 1347bp NA gene was achieved using primers ZUNF and ZUNR. The forward and reverse primers were also designed to incorporate the *Nco*I and *Bsp*HI RE sites at the 5'- and 3'-ends of the HA and NA genes, respectively. L1: Fermentas 1kb DNA ladder, H: PCR amplified HA, L2: NEB 1kb ladder, N: PCR amplified NA.

The primers that were used for the PCR amplification of HA and NA genes were also designed to incorporate RE sites *NcoI* and *BsiWI* which were subsequently used for the cloning of HA and NA genes into the transgene expression cassette of pGENE. The resultant plasmid products were designated as recombinant plasmids pGENE-HA and pGENE-NA, respectively. PCR amplification of pGENE-HA and pGENE-NA using universal primers, OMNI:F-*AflII* and OMNI:R-*MluI* produced single defined bands of approximately 1950bp and 1600bp, respectively. These bands corresponded to the expected size of the expression cassettes of HA (*Prrn:psbA5'-UTR:HA:c-myc*) and NA (*Prrn:psbA5'-UTR:NA:c-myc*) in pGENE, respectively (figure 7.15).

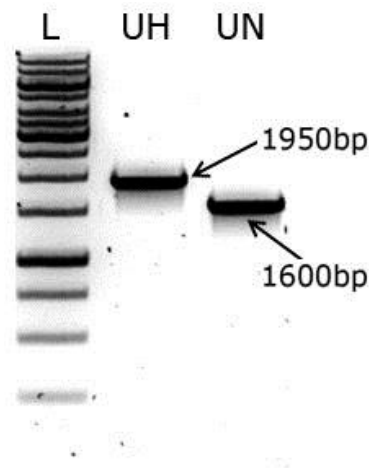


Figure 7.15: PCR profiles for the amplification of the 1950bp HA and 1600bp NA gene expression cassettes from pGENE-HA and pGENE-NA using the universal primers, OMNI:F-*AflII* and OMNI:R-*MluI*. L: Fermentas 1kb DNA ladder, UH: 1950bp HA expression cassette amplified from pGENE-HA (*Prrn:psbA5'-UTR:HA:c-myc*), UN: 1600bp NA expression cassette amplified from pGENE-NA (*Prrn:psbA5'-UTR:NA:c-myc*).

The PCR amplified HA and NA gene expression cassettes were cloned into pCART to produce the complete chloroplast transformation vectors pEXPR-HA and pEXPR-NA, respectively. The identity of pEXPR-HA and pEXPR-NA were evaluated via RE verification and sequencing reactions. Double RE digestion with

*Afl*II and *Mlu*I is expected to cause the disassociation of the transgene expression cassette from the chloroplast transformation vector backbone. This was observed in the RE verification results of pEXPR-HA (figure 7.16: A) and pEXPR-NA (figure 7.16: B) where a band of approximately 6.5kb was produced which corresponded to the expected size of the pCART vector backbone. A band with an approximate size of 1950bp was also present in the RE profile of pEXPR-HA which corresponded to the expected size of the HA expression cassette (figure 7.16: A). Similarly, a band of approximately 1600bp was also observed in the RE profile of pEXPR-NA which corresponded to the expected size of the expression cassette of NA (figure 7.16: B). Sequencing results also confirm the correct integration of HA and NA transgene expression cassettes into the chloroplast transformation vectors.

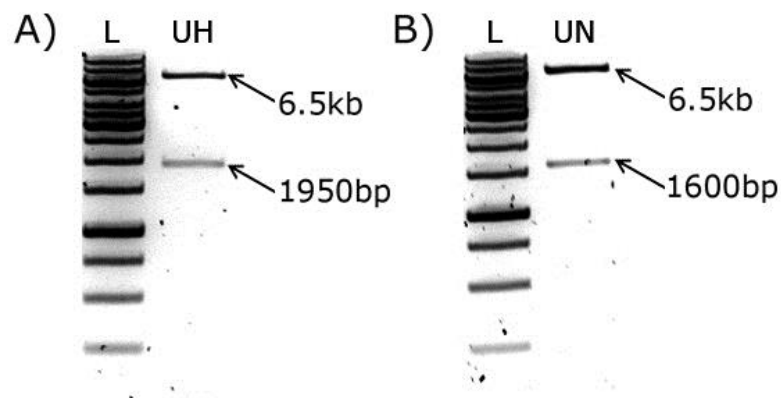


Figure 7.16: RE digestion profiles for the verification of pEXPR-HA and pEXPR-NA via double digestion with REs, *Afl*II and *Mlu*I. Double digestion with *Afl*II and *Mlu*I would cause the dissociation of the HA and NA transgene expression cassettes from the 6.5kb chloroplast transformation vector backbone.

7.3.5. Detection of HA and NA genes in chloroplast genomic DNA

Leaves of antibiotic resistant shoots regenerated from pEXPR-HA and pEXPR-NA transformed *N. benthamiana* plants were subjected to PCR screening for the detection of HA and NA. Plant lines that produced a positive result were subjected to further evaluation via PCR detection of HA and NA in the chloroplast genome and genotype screening experiments.

7.3.5.1. Detection of HA and NA in regenerated shoots of *N. benthamiana*

Leaf explants of *N. benthamiana* that were transformed with chloroplast transformation vectors pEXPR-HA and pEXPR-NA were regenerated via shoot induction as data from previous experiments indicate that regenerated shoot responded better to transformation. PCR screening was performed for the detection of HA and NA genes in antibiotic resistant shoots of post-transformation *N. benthamiana* using their respective forward and reverse primers as described in section (7.2.1.1.). Four out of the eight antibiotic resistant lines of pEXPR-HA transformed *N. benthamiana* plants produced bands of approximately 1700bp when subjected to PCR screening for the detection of HA (figure 7.17: A, lanes 1, 2, 4 and 7) which corresponded to the size of the positive control, as well as, the expected size of the HA gene. Similarly, four out of the eight antibiotic resistant lines of pEXPR-NA transformed *N. benthamiana* plants were also positive (figure 7.17: B, lanes 3, 5, 6 and 8) producing bands with an approximate size of 1350bp which corresponded to NA positive control and the expected size of the NA gene.

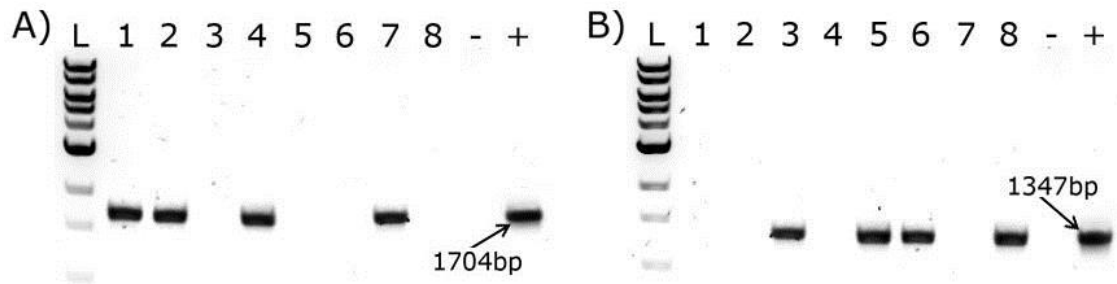


Figure 7.17: PCR amplification profiles for leaf tissue samples of pEXPR-HA (A) and pEXPR-NA (B) transformed shoots. Small segments of leaf tissue were harvested from transformed *N. benthamiana* plants and subjected to PCR screening for the detection of HA and NA genes. (A) L: NEB 1kb DNA ladder, lanes 1-8: PCR products amplified from pEXPR-HA transformed samples, (-): Negative control, (+): HA positive control; (B) L: NEB 1kb DNA ladder, lanes 1-8: PCR products amplified from pEXPR-NA transformed samples, (-): Negative control, (+): NA positive control.

7.3.5.2. Detection of HA and NA in chloroplast genomic DNA

Results for the detection of HA and NA genes in extracted chloroplast genomic DNA samples from pEXPR-HA and pEXPR-NA transformed shoots of *N. benthamiana* are shown in figure 7.18. Positive results were observed for all antibiotic resistant shoots that screened positive in previous PCR screening experiments (section 7.3.5.1.). PCR amplification of chloroplast genomic DNA extracted from pEXPR-HA transformed shoots using HA specific primers produced a single defined band with an approximate size of 1700bp which corresponded with the expected size of the HA gene (figure 7.18: A, lanes 1-4). Similarly, bands of approximately 1350bp which corresponded to the expected size of the NA gene were also produced when chloroplast genomic DNA from pEXPR-NA transformed shoots were subjected to PCR amplification using NA specific primers (figure 7.18: B, lanes 1-4).

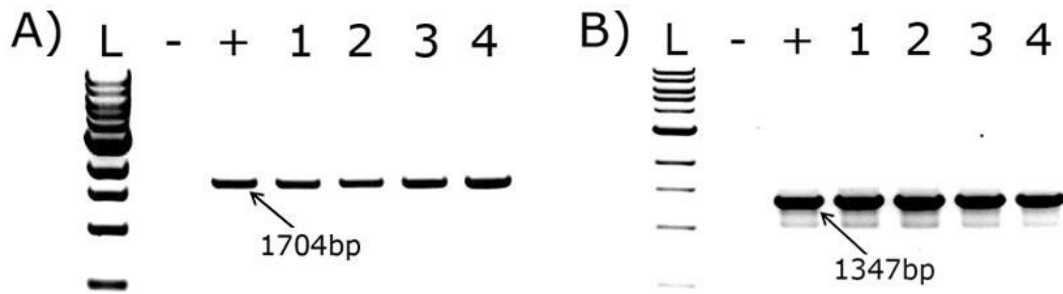


Figure 7.18: PCR profiles for the detection of HA and NA in chloroplast genomic DNA extracted from pEXPR-HA (A) and pEXPR-NA (B) transformed *N. benthamiana*. A) L: NEB 1kb DNA ladder, (-): Negative control, (+): HA positive control, lanes 1-4: PCR products amplified from chloroplast genomic DNA of pEXPR-HA transformed plants; B) L: NEB 1kb DNA ladder, (-): Negative control, (+): NA positive control, lanes 1-4: PCR products amplified from chloroplast genomic DNA of pEXPR-NA transformed plants.

7.3.5.3. Genotype screening of pEXPR-NA transformed shoots

Samples of chloroplast genomic DNA extracted from pEXPR-NA transformed shoots were subjected to genotype screening using forward and reverse primers, INSR-TF and INSL-TR for the amplification of the homologous recombination binding sites as described in section (7.2.1.3.). Three out of the four samples that were subjected to PCR amplification using primers, INSR-TF and INSL-TR produced two distinct bands of approximately 3.9kb and 700bp, respectively (figure 7.19: lanes 1, 2 and 4). The remaining sample (figure 7.19: lane 3) only produced a single band of approximately 3.9kb when subjected to PCR amplification using primers, INSR-TF and INSL-TR. Further evaluation of pEXPR-HA transformed plants was not possible due to inadequate plant material following progressive necrosis of all tissue cultured samples.



Figure 7.19: PCR profiles for the amplification of chloroplast genomic DNA extracted from pEXPR-NA transformed shoots of *N. benthamiana* using primers, INSR-TF and INSL-TR. PCR amplification of flanking homologous recombination sites allow the detection of transgene integration into the chloroplast genome via an increase in the size of the amplified bands. L: Fermentas 1kb DNA ladder, Wt: Wild-type control, Lanes 1-4: PCR products of pEXPR-NA transformed samples.

7.3.5.4. Southern blot: Detection of NA in the chloroplast genome

Chloroplast genomic DNA extracted from pEXPR-NA transformed shoots were subjected to double digestion using REs *Afl*II and *Mlu*I and subjected to Southern blot using NA specific probes for the detection of the NA gene sequence. Bands with an approximate size of 1600bp were detected in the Southern blot profiles of pEXPR-NA transformed shoots of *N. benthamiana* (figure 7.20: lanes 1-4) which corresponded to the expected size of the NA gene expression cassette. A similar band was also observed in the Southern blot profile of *Afl*II and *Mlu*I digested pEXPR-NA (figure 7.20: C1) which is expected to cause the disassociation of the 1600bp NA transgene expression cassette (*Prrn-psbA5'*-UTR-NA-c-Myc) from the chloroplast transformation vector backbone. The NA gene PCR product which served as a positive control for the detection of NA also produced a visible band of approximately 1350bp (figure 7.20: C2) which corresponded to the expected size of the optimised NA gene further validating the results of the Southern blot.

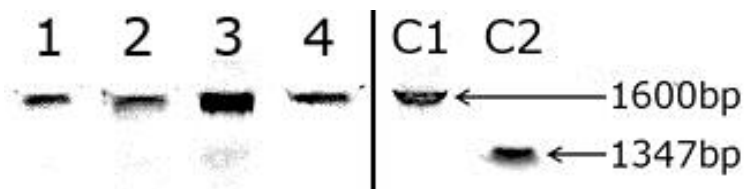


Figure 7.20: Southern blot profiles for the detection of NA in the chloroplast genome of pEXPR-NA transformed shoots of *N. benthamiana*. Lanes 1-4: Chloroplast genomic DNA from pEXPR-NA transformed shoots double digested with REs, *AflII* and *MluI*, C1: pEXPR-NA double digested with REs, *AflII* and *MluI*, C2: NA PCR product.

7.3.6. RT-PCR: Evaluation of NA transcription in *N. benthamiana*

Detection of mRNA transcripts of NA in total RNA samples was achieved by 2-step RT-PCR using primers, ZUNF and ZUNR. Bands of approximately 1350bp were observed in the RT-PCR profiles of total RNA extracted from all four lines of pEXPR-NA transformed shoots of *N. benthamiana*. These bands corresponded with the expected 1347bp size of the codon optimised NA gene (figure 7.21: lanes 1-4) and the NA positive control (figure 7.21: C2). The *aadA* internal control for the RT-PCR reaction also produced a band of approximately 800bp which corresponded with the NA positive control, as well as, the expected size of the NA gene (figure 7.21: C4 and C5). No bands were visible in all negative controls for the RT-PCR reaction (figure 7.21: C1, C3 and C6) which further validates the observed results.

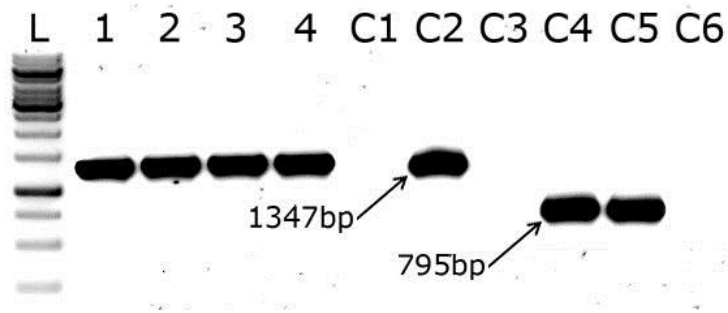


Figure 7.21: RT-PCR profiles for the qualitative detection of mRNA transcripts of NA from total RNA samples extracted from pEXPR-NA transformed shoots of *N. benthamiana*. L: Fermentas 1kb DNA ladder, Lanes 1-4: RT-PCR products from total RNA extracted from pEXPR-NA transformed shoots, C1: Non-template control, C2: NA positive control, C3: *aadA* Non-RT control, C4: *aadA* RT-PCR Internal control, C5: *aadA* positive control, C6: NA Non-RT control.

7.3.7. Western blot: Detection of NA protein expression

The 10 amino acid c-Myc epitope tag (EQKLISEEDL) provides a convenient method for the purification and detection of transgenic proteins in total protein samples. Total protein was extracted from pEXPR-NA transformed shoots and subjected to immunoprecipitation for the isolation of c-Myc tagged proteins. The presence of c-Myc tagged NA in the immunopurified protein samples were subsequently evaluated via Western blot using NA and c-Myc specific primary antibodies. Distinct bands with a molecular mass of approximately 50kDa were detected in both immunopurified protein samples extracted from pEXPR-NA transformed *N. benthamiana* using both antibodies (figure 7.22: A and B). The observed bands corresponded to the expected size of the NA protein which contains 449 amino acid residues with a predicted molecular mass of 49.06kDa (http://www.bioinformatics.org/sms/prot_mw.html, date last accessed: 25/05/2015). The presence of RuBisCO contamination in the immunopurified protein samples of pEXPR-NA transformed shoots were also evaluated via Western blot using RuBisCO-specific primary antibodies as

described in section (7.2.4.3.). Western blot profiles for the detection of RuBisCO did not produce any detectable bands (figure 7.22: C).

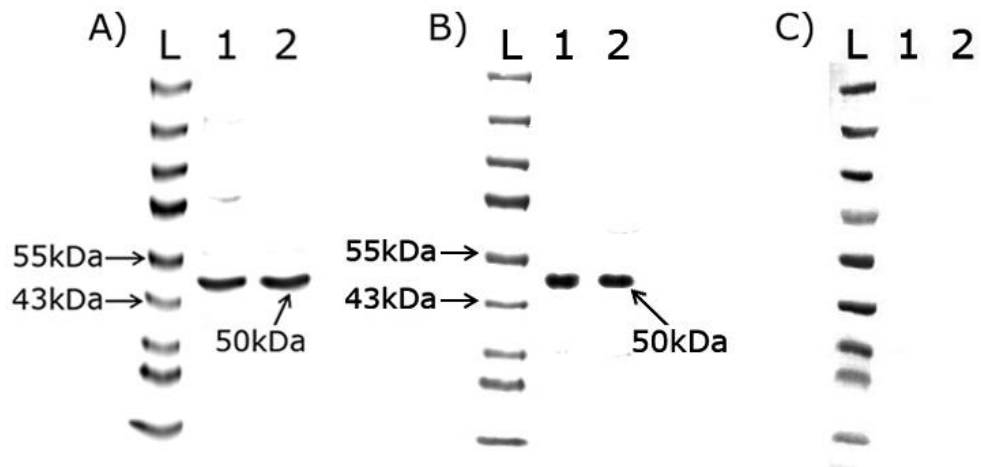


Figure 7.22: Western blot profiles for the detection of c-Myc tagged NA using NA-specific polyclonal primary antibodies (A) and c-Myc-specific polyclonal primary antibodies (B) in c-Myc tag immunopurified protein samples extracted from pEXPR-NA transformed *N. benthamiana*. Detection of RuBisCO protein contamination in the immunopurified samples was also performed using RuBisCO-specific polyclonal primary antibodies (C). L: Broad range protein ladder one, triple colour (Nacalai, USA), 1 and 2: immunopurified protein samples from pEXPR-NA transformed *N. benthamiana* plants.

7.4 DISCUSSION

Transformed plants can only be classified as truly transplastomic if they fulfil three main conditions; 1) transgenes have to be contained within the chloroplast genome, 2) transgenes have to be transcribed within the chloroplasts to produce stable mRNA transcripts; and 3) the mRNA transcripts of transgenes have to be translated into proteins. Efficient integration and expression of transgenes in the transformed chloroplasts is dependent upon many *cis*- and *trans*-acting factors that interact with the components of the chloroplast transformation vector, as well as, the regulatory elements of the expression cassette. Hence, various mechanisms relating to the integration and expression of transgenes in the chloroplast are discussed in relation to the results.

7.4.1. Transgene integration into the chloroplast genome

The direct method of transformation via particle bombardment causes the unbiased delivery of DNA coated gold microcarriers throughout the entire tissue sample resulting in transgenic DNA being delivered into both the chloroplast and the nuclear genome. As a result, the data obtained from PCR screening experiments using tissue samples from post-transformed plants is insufficient to confirm transgene integration into the chloroplast genome as this method of evaluation, although convenient, does not distinguish between nuclear and chloroplast transformed samples. Hence, in order to acquire more accurate data for the detection of transgenes, it was essential that the chloroplast genome was isolated from other genomic sources. The data suggest that regeneration of transformed tissue via shoot regeneration (direct organogenesis) to be more reliable with a 100% rate of transgene detection in all pEXPR-G and pEXPR-UG transformed plants that were positive for PCR screening. In contrast, samples that were regeneration via callus induction (indirect regeneration) failed to produce any transplastomic plants even though antibiotic resistance was

displayed. This was in agreement with the findings of (Ruhlman *et al.*, 2010) who suggested that the rapid recovery of transplastomic plants via direct organogenesis without callus induction was essential to minimise somaclonal variation which may lead to increased spontaneous mutation events. Since the primary site of action for the aminoglycoside antibiotics are the rRNAs responsible for translation of membrane bound proteins, spontaneous mutations in the rRNA sequence may lead to antibiotic resistance. Conversely, non-transplastomic antibiotic resistant mutants may also arise from the nuclear expression of *aadA*. This may be possible if the *aadA* gene was accidentally integrated into the nuclear genome within close proximity to an endogenous promoter following particle bombardment. As a result, the *aadA* gene product may be present in the cytoplasm causing the neutralisation of aminoglycoside antibiotics before they can affect the chloroplasts. Alternatively, *aadA* may also be expressed and translocated into the chloroplasts via various protein import and routing systems of the chloroplasts (Keegstra and Cline, 1999). This may explain the results observed in sections (7.3.1.1.) and (7.3.1.2); where the *aadA* antibiotic resistance gene was detected in transformed plant tissue (figure 7.3: lanes 1 and 4) but the GFP gene was not detected in the extracted chloroplast genome (figure 7.5: lanes 1 and 4).

In order to produce stable transplastomic plants, a homoplastomic state has to be established within the chloroplast population to prevent the possibility of reversion back to wild-type upon the removal of the selective agent (Maliga, 2002). A homoplastomic state is achieved when all copies of the chloroplast genome in all chloroplasts of the transformed plant are of the transformed variety. The sequence of events following particle bombardment leading to the establishment of homoplastomicity begins with the integration of the transgene expression cassettes from the chloroplast transformation vectors into the chloroplast genome. Two recombination events take place between the right

(INSR) and left (INSL) insertion regions of the chloroplast transformation vector and the endogenous sequences of the chloroplast genome. The homologous recombination process is thought to be mediated by a plant homolog of the RecA bacterial protein which is mainly responsible for facilitating DNA recombination/repair mechanisms (Cerutti *et al.*, 1992; Inouye *et al.*, 2008). The selection of proper homologous recombination sequences for the integration of transgenes is crucial for the success of chloroplast transformation. Although sequences with 150-200bp of sequence similarity have been found to be sufficient for the initiation of homologous recombination (Newman *et al.*, 1990; Suzuki *et al.*, 1997), homologous recombination sites of 700bp-1000bp are more commonly used for the transformation of the chloroplasts genome as longer sequences provide a higher degree of specificity (Maliga, 2002). The chloroplast genome has its own genetic machinery of eubacterial origin (Bhattacharya and Medlin, 1998). A high degree of conservation is found within the arrangement of gene transcription units and in the ribosomal RNAs of the chloroplast genome (Palmer, 1985). In contrast, the intergenic and UTR sequences are highly variable in the chloroplast genome (Daniell *et al.*, 2006, Timme *et al.*, 2007). For this reason, it would be more sensible to select the homologous recombination sequences based on coding regions rather than non-coding regions for the efficient integration of transgenes into the chloroplast genome. However, it is crucial that the origin of replication of the chloroplast genome (*oriB*) is not included in any of the recombination sequences as it would likely allow the autonomous maintenance of the transformation vector within chloroplasts. The pMB1-type origin of replication in the pUC18 backbone of the pEXPR chloroplast transformation vector is *E. coli* specific and should be of no concern. Direct repeat regions should also be excluded from homologous recombination sites to prevent intermolecular recombination events which can result in the deletion of the entire region between the direct repeats (Newman *et al.*, 1992). The unusual bands which were observed in the results for the detection of GFP in the

chloroplast genomic DNA extracted from callus samples may be attributed to aberrant or multiple homologous recombination events which might have causing the truncation of transgenes. Alternatively, the truncation of transgenes may also be the result of indiscriminate intermolecular recombination events between short repeating nucleotide sequences (Ogihara *et al.*, 1988, Newman *et al.*, 1992).

Following the insertion of the transgene into the inverted repeat (IR) region of the chloroplast genome, the endogenous copy correction mechanism may either cause the removal or the duplication of the transgene in both IR regions (Shinozaki *et al.*, 1986, Zou *et al.*, 2003). Antibiotic selection pressure causes the preferential selection for the persistence of the transformed genome harbouring the *aadA* antibiotic resistance gene leading to a gradual shift in the equilibrium in favour of the transformed genome until all wild-type genomes have been eliminated. The population of transformed chloroplasts then increase in relation to the wild-type variety under continued antibiotic selection pressure until all chloroplast within the plant are of the transformed variety whereupon a homoplasmic state is achieved. Evaluation of transgene integration into the chloroplast genome in pEXPR-G and pEXPR-UG transformed shoots were further evaluated by PCR using primers that were designed for the amplification of flanking insertion regions. In addition to the detection of transgenes, this method of evaluation also provides some information regarding genotype identity. A band of approximately 700bp was expected for the PCR amplification of the insertion sites of wild-type plants using primers, INSR-TF and INSL-TR. Integration of transgene expression cassettes between the insertion sites via homologous recombination would cause the size of the amplified fragment to increase in relation to the size of the transgene insert. This was observed in the results of sections (7.3.1.3.) and (7.3.5.3.) where pEXPR-G, pEXPR-UG and pEXPR-NA transformed plants produced bands with sizes of approximately 3.3kb,

3.3kb and 3.9kb, respectively. The increase in the band sizes matched the size of the transgene insert of 2475bp, 2570bp and 3197bp, respectively for chloroplast transformation vectors pEXPR-G, pEXPR-UG and pEXPR-NA. Interestingly, the 700bp band was not produced by a number of pEXPR-UG transformed samples (section 7.3.1.3., figure 7.6: A, lane 7 and 13), as well as, pEXPR-NA transformed samples (section 7.3.5.3., figure 7.19: lane 3). The absence of the 700bp wild-type band putatively suggests the possibility that the aforementioned samples were of a homoplastomic nature with transgenes in both IR regions. This hypothesis is further supported by the fact that the observed bands also appear to be slightly thicker, possibly indicating a higher copy number. Similar results were also reflected in Southern blot experiments for the detection of the GFP gene where noticeably thicker bands were observed for the putative homoplastomic pEXPR-UG and pEXPR-NA transformants (figure 7.7: lanes 1 and 7; figure 7.20: lane 3). Samples in figure 7.7: lanes 1-8 correspond to samples in figure 7.6: A, lanes 7-14.

7.4.2. Evaluation of transcription in *N. benthamiana*

Transcription of the genes of interest (GOI) in pEXPR chloroplast transformation vectors is modulated by the constitutive promoter of the chloroplast *rrn* operon (*Prrn*). The *Prrn*P1 promoter is a σ^{70} -type promoter of eubacterial origin reminiscent of *E. coli* promoters containing conserved blocks of hexameric (TATATT) and (TTGACG) sequences at the -35 and -10 regions, respectively (appendix Fi) which are recognised by the plastid-encoded plastid RNA polymerase (PEP) (Igloi and Kössel 1992, Allison *et al.*, 1996). Chloroplast *Prrn* P1 promoters are relatively compact in comparison to similar promoters in *E. coli* (Suzuki *et al.*, 2003) with sequences upstream of nucleotide -83 rarely contributing to promoter function. In contrast, the *E. coli rrnB* P1 promoters contain two distinct sets of regulatory elements located -40 to -60nt and -64 to -

150nt upstream corresponding to the UP element (part of the promoter recognition domain) and the Fis binding sites (Hirvonen *et al.*, 2001). The PEP which recognises the chloroplast *Prrn* P1 promoter is highly similar in relation to the multi-subunit RNA polymerase of *E. coli* and is composed of the plastid encoded core subunits (α , β , β' , β'') and a nuclear-encoded σ (sigma)-like factor (SLF) (Vera and Sugiura, 1995; Maliga, 1998). The SLF is an important determinant for the initiation of mRNA transcription and has been hypothesised to be responsible for the recognition of promoters, as well as, melting of the DNA around the transcription initiation site (Maliga, 1998). Transcription of genes within the chloroplast is regulated at the nuclear level by nuclear-expressed SLFs (Tsunoyama *et al.*, 2002). Various plant SLFs have been shown to exhibit promoter preference both *in-vivo* (Kanamaru *et al.*, 2001) and *in-vitro* (Tiller and Link, 1993; Hakimi *et al.*, 2000). The interaction between nuclear-expressed SLFs and chloroplast promoters are known to be species specific with a suite of SLFs required for the active transcription of photosynthetic genes by the PEPs (Allison *et al.*, 1996; Hess and Börner, 1999). Interestingly, in addition to the nuclear controlled expression of SLFs, the *rpoB* operon which encodes for the core subunits of PEP is also transcribed by a nuclear-encoded plastid RNA polymerase (NEP), a phage-like RNA polymerase (Allison *et al.*, 1996; Hajdukiewicz *et al.*, 1997; Hess and Börner 1999; Liere and Maliga 1999). Moreover, NEPs are also responsible for in the transcription of many housekeeping genes involved in the genetic system and metabolism together with PEPs (Hayashi *et al.*, 2003). Hence, the use of endogenous promoter elements of genes which are involved in photosynthesis (such as the *psbA* promoter) or any other constitutive genes (*rrn* operon promoter) would, in theory, allow elevated transgene expression levels in the chloroplast. However, care should be taken to ensure that the selected promoter elements are not integrated as direct repeats to the endogenous sequence to prevent spurious intermolecular recombination (Ogihara *et al.*, 1988) as in the case of the original

unmodified chloroplast transformation vector, pKCZ. In addition to the -10 and -35 elements of the *Prrn* P1 promoter, the hexameric sequence (GTGGGA) directly upstream of the -35 element has also been identified as an important element for the transcription of genes. This sequence has been described by Suzuki *et al.* (2003) as the rRNA operon upstream activator (RUA) and is assumed to be the binding site of nuclear-expressed SLFs. Hence, the RUA can be hypothesised to be among the key determinants of promoter strength with a role similar to the UP element of the *rrnB* P1 promoter in *E. coli* which directly interacts with the α -subunit C-terminal domains of the bacterial RNA polymerase (Ross *et al.*, 1993). Suzuki *et al.* (2003) also found the -10 element of the *Prrn* P1 promoter to be non-essential for proper promoter function and suggested that it could be replaced in part with direct protein-DNA interactions between PEP and RUA or by protein-protein interactions between PEP and an RUA binding transcription factor. This observation further supports the proposal that nuclear expressed σ -factors binds to the RUA of *Prrn* and modulates the dissociation of DNA and the recruitment of PEP core subunits leading to the initiation of mRNA transcription (figure 7.23).

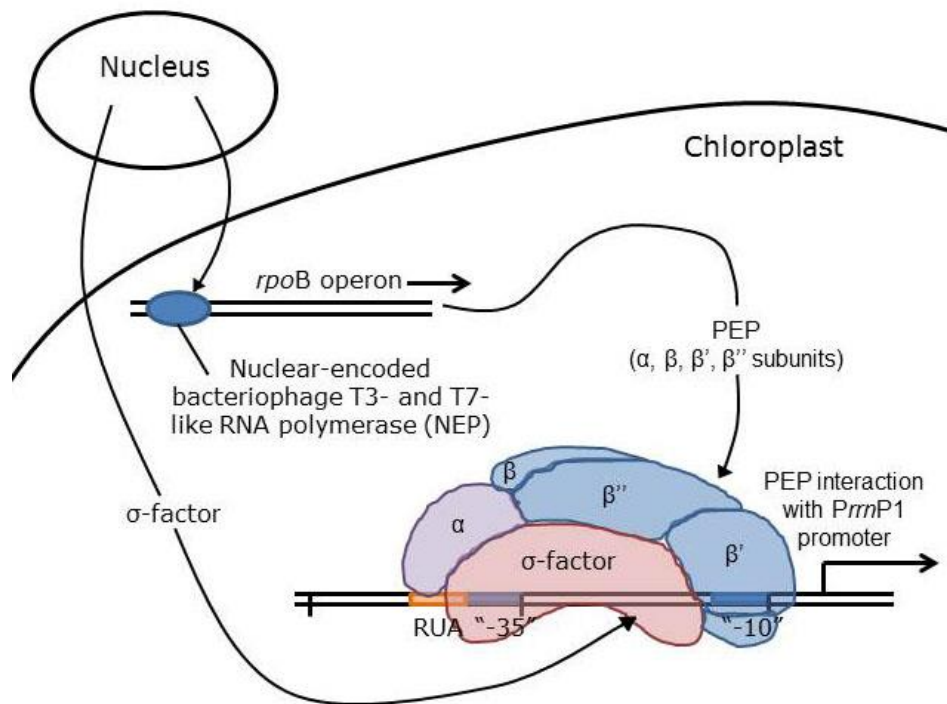


Figure 7.23: Schematic diagram for the initiation of mRNA transcription from the *Prrn* P1 PEP promoter. Nuclear-expressed σ -factors bind to the RUA region of the promoter facilitating the recruitment of core subunits of the PEP complex leading to mRNA transcription.

Results for the detection of mRNA transcripts in total RNA samples of pEXPR-G, pEXPR-UG and pEXPR-NA transformed *N. benthamiana* plants indicated that the GFP and NA transgenes were primarily transcribed at the mRNA level in all tested samples (figures 7.9 and 7.21). Unfortunately, unlike chloroplast genomic DNA samples, total RNA could not be extracted from isolated chloroplasts as the resulting RNA samples were found to be of low quality and purity. This is likely to be due to RNA degradation which occurred during the chloroplast isolation process. It is suggested that the 3'-UTR plays an important role in mediating mRNA stability by forming stem-loop structures that associate with various plastid-localised RNA-binding proteins (Stern and Grussem, 1987; Eibl *et al.*, 1999). It can be hypothesised that RNA degraded rapidly during the course of chloroplast isolation in the absence of nuclear-

expressed mRNA-stabilising elements resulting in poor yield and quality of the extracted RNA samples. Thus, results from the RT-PCR experiments do not conclusively confirm that the transgenes were expressed in the chloroplast. Nevertheless, chloroplast expression would be the most likely scenario given that GFP and NA transgenes were successfully detected in the chloroplast genome (sections 7.3.1. and 7.3.5.) and that chloroplast specific promoter elements were used to drive transcription.

7.4.3. Evaluation of transgenic protein expression in *N. benthamiana*

The majority of mRNAs in the chloroplasts of higher plants are transcribed as polycistronic sequences and are subsequently edited and/or spliced by endonucleolytic enzymes into monocistronic mRNAs (Sugiura, 1992; Hirose *et al.*, 1994; Ruf *et al.*, 1994). The subsequent translation of mRNAs is generally mediated by the 5'-untranslated regulatory elements (UTR) in most chloroplast genes. More than 90% of chloroplast mRNA species contain sequences upstream of their respective initiator codon which modulates translation by facilitating base pairing with the 3'-terminal of the small 16S rRNA (Ruf and Kössel, 1988). The translation of GOIs in the pEXPR-UG and pEXPR-NA chloroplast transformation vector is facilitated by the *psbA* 5'-UTR. The *psbA* gene encodes for the 32kDa D1 polypeptide of photosystem II and the 5'-UTR leader region was found to be highly efficient in modulating the translation of genes (Staub and Maliga, 1994; Eibl *et al.*, 1999; Zou *et al.*, 2003). Sequencing results of the *psbA* 5'-UTR of *N. benthamiana* has revealed several important conserved features which include three putative ribosome binding sites (RBS1, RBS2 and RBS3 named in relation to their positions from the AUG initiation codon and an AU-box consisting of a conserved octameric (UAAAUAAA) sequence located between RBS1 and RBS2 (appendix Fii). Identical results were previously obtained by Hirose and Sugiura

(1996) in the *psbA* 5'-UTR sequence of *N. tabacum* which suggest that these features were highly conserved between species. In addition to nuclear control over the transcription of chloroplast genes via NEPs and SLFs, nuclear control over the translation of mRNA employs the use of numerous plastid-localised RNA-binding proteins (RBPs). Studies have shown that RBP-mRNA interactions are highly specific and RBPs only have a strong affinity towards their cognate sequences in the UTRs of chloroplast mRNAs (Nakamura *et al.*, 2001; Shen *et al.*, 2001; Meierhoff *et al.*, 2003). This "lock and key" mechanism of regulation allows comprehensive nuclear-control over the spatial and temporal expression of chloroplast genes at both transcriptional and translational levels. The use of endogenous *psbA* regulatory elements has also been attributed for the successful expression of variety of soluble (Verma *et al.*, 2008) and membrane-associated (Singh *et al.*, 2008) proteins in tobacco.

A remarkable feature of the *psbA* 5'-UTR involves the presence of *cis*-elements which have been proposed to form secondary stem-loop structures as predicted based on minimum Gibbs free energy (ΔG) (Mathews *et al.*, 2004; Gruber *et al.*, 2008; Zou *et al.*, 2003). The stem-loop structure of the sequenced *psbA* 5'-UTR of *N. benthamiana* is illustrated in figure 7.24. The stem-loop structure causes the looping out of an AU-rich region that has been suggested to facilitate the interaction between nuclear-encoded RBPs (Yang *et al.*, 1995; Hirose and Sugiura, 1996; Zou *et al.*, 2003; Merhige *et al.*, 2005). RBPs are known to be important determinants for an array of functions including RNA editing and maturation, endonucleolytic cleavage of polycistronic transcripts, modulating transcript stability and mRNA turnover, and the recruitment of additional protein factors for the initiation of translation (Alexander *et al.*, 1998; Eibl *et al.*, 1999; Zou *et al.*, 2003; Nickelsen, 2003). In most cases, the stem-loop structure of *psbA* 5'-UTR was found to be essential for the translation of genes and the disruption of the secondary structure led to a decrease in

translation activity (Zou *et al.*, 2003). The presence of an endonucleolytic cleavage site in the stem-loop region which is protected upon the binding of protein factors has also been suggested (Alexander *et al.*, 1998) which may be involved in regulating mRNA translation and/or turnover. In addition, chloroplast ribonucleoproteins (cpRNPs) are also hypothesised to associate with the stem-loop structure to stabilise the mRNA molecule (Nakamura *et al.*, 2001) and might act as molecular chaperones for the delivery of mRNAs to membrane-bound ribosomes for translation (Hauser *et al.*, 1998). Stromal RBPs are also known to associate with the *psbA* 5'-UTR of endogenous genes providing transcript stability (Nakamura *et al.*, 2001; Ruhlman *et al.*, 2010).

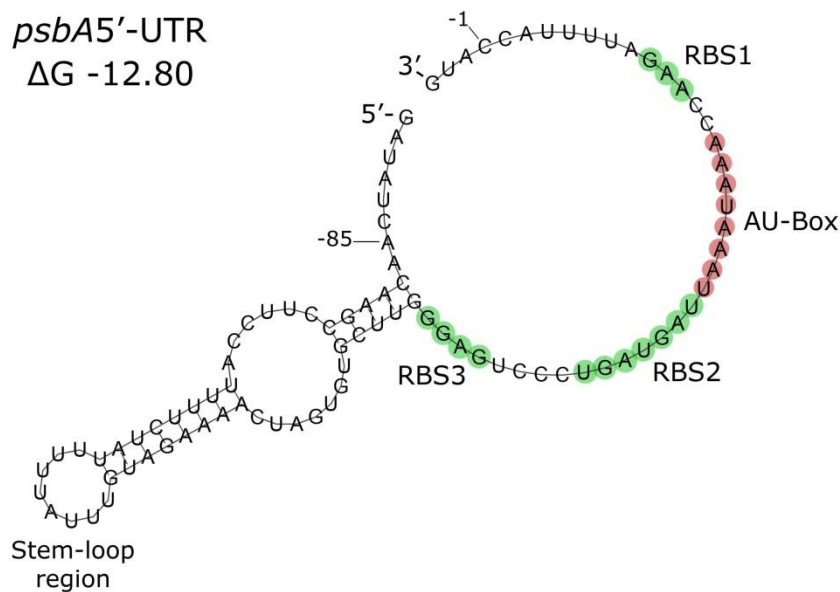


Figure 7.24: Schematic diagram of the theoretical secondary structure within the *psbA* 5'-UTR. The two-dimensional structure was produced using the Vienna RNA Websuite tool (Gruber *et al.*, 2008) for the *psbA* 5'-UTR of *N. benthamiana* based on minimum free energy (ΔG). Unpaired bases upstream and downstream of the stem-loop structure are shown as circular with the 5'- and 3'-ends labelled accordingly. This is an arbitrary software-dependant rendition and is not intended as an indication of the actual two-dimensional structure. Green nucleotide sequences indicate putative ribosome-binding sites (RBS) and the red nucleotide sequence indicate the AU-box.

In contrast to *E.coli*, where the prokaryotic 30S ribosomes usually bind to the Shine-Dalgarno (SD) sequence commonly located 7 ± 2 nt upstream of the initiator codon in the 5'-UTR of prokaryotic mRNA (McCarthy and Brimacombe, 1994; Hirvonen *et al.*, 2001), the binding of 30S ribosomes to chloroplast mRNAs seem to involve a more complex mechanism involving the AU-box. The AU-box has been determined to be an absolute requirement for efficient translation of mRNA transcripts and has been hypothesised to be the primary target sequence for nuclear-expressed *trans*-activating factors with mutation or deletion of the AU-box often resulting in abolished translation (Hirose and Sugiura, 1996). Three main elements of the *psbA* 5'-UTR sequence have been empirically proven to be essential for the binding of 30S ribosomes during translation which includes the two RBS sequences (RBS1 and RBS 2) and the AU-box (Hirose and Sugiura, 1996). Hirose and Sugiura (1996) suggested that RBS1 and RBS2 would bind cooperatively to the 3'-end of the 16S rRNA causing the looping out of the AU-box which facilitates interaction with various *trans*-activating factor(s). Interactions between nuclear-expressed *trans*-activating factors and the AU-box have also been suggested to be affected by ATP/ADP ratios and the/or the redox potential in higher plants (Danon and Mayfield, 1994). This may account for the differences observed in the expression profiles of plants cultivated in *in-vitro* environments under prolonged exposure to abiotic oxidative stress. A hypothetical model for the expression of transgenes under the regulation of the *psbA* 5'-UTR in pEXPR-UG and pEXPR-NA transformed chloroplasts is illustrated in figure 7.25. The stem-loop conformation of *psbA* 5'-UTR autonomously formed based on the minimum energy state leading to binding with cpRNPs which mediated the association of mRNAs with membrane-bound ribosomes on the thylakoid membrane. The 3'-end of the 16S rRNA subunit was then bound transiently to BRS1 and RBS2 causing the AU-box to be looped out allowing nuclear-expressed *trans*-activating factors to associate with

the AU-box which stabilised the 30S ribosome subunit initiating translation (Hirose and Sugiura, 1996).

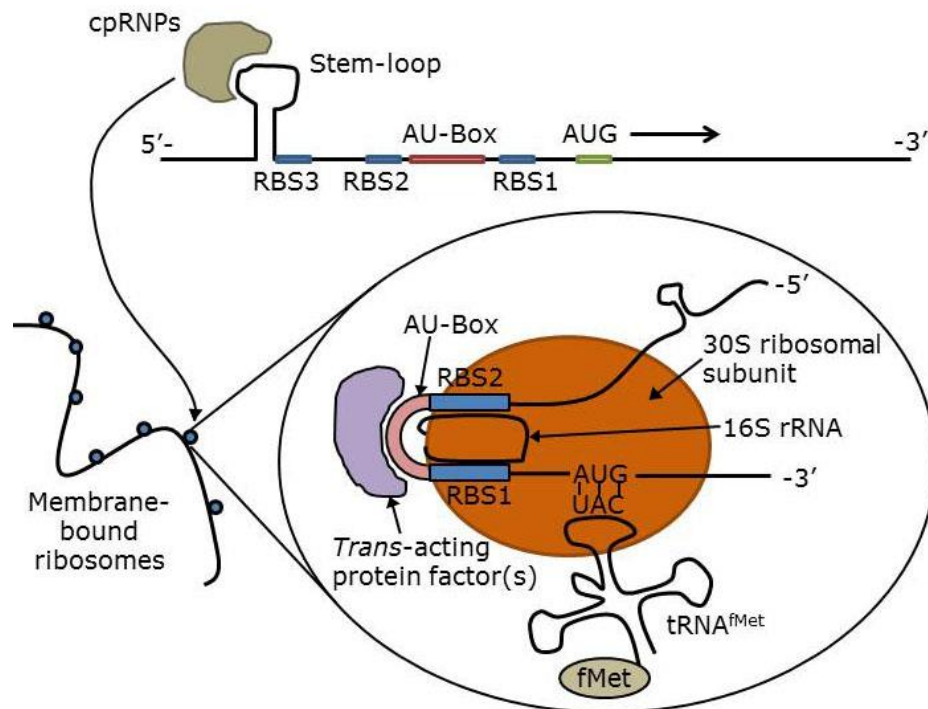


Figure 7.25: Hypothetical model for the translational expression of transgenes under the regulation of the *psbA* 5'-UTR. The chloroplast ribonucleoproteins (cpRNPs) mediate the association of mRNA transcripts with membrane-bound ribosomes which then bind to the two ribosome binding sites (RBS) sequences of *psbA* 5'-UTR causing the looping out of the AU-box allowing interaction with nuclear-expressed *trans*-activating factors which initiate translation.

Taken together, it is clear that the *psbA* 5'-UTR in the GOI expression cassette was an important determinant for the translation of transgenic proteins within the chloroplasts. This is clearly illustrated in the Western blot results for the detection of transgenic proteins of GFP and NA (section 7.3.3. and 7.3.7.) where specific protein bands were only detected for genes under the regulation of the *psbA* 5'-UTR. Phenotypic expression of GFP was only observed in pEXPR-UG (GFP expression vector with the *psbA* 5'-UTR leader sequence) transformed plants (section 7.3.3.4.). The failure of pEXPR-G (GFP expression cassette

without the *psbA* 5'-UTR leader sequence) transformed *N. benthamiana* plants to express GFP proteins despite the successful detection of GFP specific mRNAs (section 7.3.2.2.) strongly suggest that transcribed mRNAs cannot be translated into proteins without appropriate 5'-leader sequences in the chloroplasts despite having an RBS in the *Prrn* promoter region. This was in contrast to prior expression studies in *E. coli* in Chapter 4, Section (4.3.2.4.) where GFP proteins were detected in both pEXPR-G and pEXPR-UG transformed samples suggesting that the endogenous RBS of *Prrn* was sufficient to initiate translation in *E. coli* but not in chloroplasts.

7.4.4. Immunoprecipitation of c-Myc-tagged proteins

The recovery of recombinant proteins of high purity is essential especially when intended for pharmaceutical applications. Purification of a specific plant-derived recombinant protein from crude extracts has always been problematic procedure and is known to be a major task for protein chemists in the pharmaceutical industry. Plant-derived products are often contaminated with other contaminants such as; secondary metabolites, alkaloids, herbicides, insecticides, toxins, and other proteins with allergenic or immunogenic properties which may impact human health (Geada *et al.*, 2007). Hence, in this study, the chloroplast transformation vector construct was designed to incorporate the c-Myc epitope tag at the C-terminal of the expressed transgenic protein to facilitate recovery via immunopurification protocols (section 7.2.4.2.). The c-Myc epitope tag (or affinity-tag) of 10 amino acid residues (EQKLISEEDL) is highly favourable for the recovery of recombinant proteins as it: 1) is relatively small and is expected to have minimal effect on the tertiary structure and biological activity of the recombinant proteins (see GFP expression section 7.3.3.4.), 2) allows one-step adsorption purification (section 7.2.4.2.), 3) allows convenient detection of protein expression (section 7.3.3.2.), and 4) can be applied for the

purification of a wide variety of proteins. Nevertheless, as a precautionary measure; the design of the expression construct also allowed the removal of the c-Myc tag using factor-Xa protease to reproduce the native protein. Removal of the c-Myc tag may also be essential to facilitate protein crystallisation or for antibody production. The murine anti-c-Myc antibody (Clone 9E10) was developed in 1985 and is commonly used for the detection of c-Myc-tagged fusion-proteins (Evan *et al.*, 1985). In this experiment, c-Myc-tagged recombinant proteins expressed in the chloroplasts of *N. benthamiana* were successfully recognised by the 9E10 immunoglobulin (bound to agarose beads) and allowed the subsequent immunopurification of the tagged proteins (section 7.3.3.2. and 7.3.7.). This was in agreement with the report by Munro and Pelham (1986) who stated that the 9E10 immunoglobulin still configures recognition towards c-Myc epitope tags which are expressed in different protein contexts. To date, the c-Myc tag has been extensively used for monitoring the expression of recombinant proteins in bacteria (Dreher *et al.*, 1991; Vaughan *et al.*, 1996), mammalian cells (McKern, 1997; Moorby and Gherardi, 1999), plants (Ferrando *et al.*, 2001), yeast cells (Sequi-Real *et al.*, 1995; Weiss *et al.*, 1998), and insect cells (Schiöth *et al.*, 1996). The c-Myc tag is also used in Western blot, immunopurification and flow cytometry (Kipriyanov, 1996). The c-Myc tag can be placed at either the N- or the C-terminal of the protein chain (Manstein *et al.*, 1995). However, in this study, assignment of the c-Myc tag to the C-terminal ensured that all recombinant proteins that were recovered during the immunopurification process consisted of only full-length polypeptide chains.

In nature, the c-Myc proto-oncogene product (Myc) is a transcription factor that binds to thousands of genomic loci and is related to the process of apoptosis and cell-cycle progression (Eilers and Eisenman, 2008; Sabò *et al.*, 2014). Uncontrolled expression of c-Myc has been known to cause the unregulated expression of many genes, some of which are involved in cell-cycle

regulation, resulting in the formation of cancerous cells (Sabò *et al.*, 2014). In addition, c-Myc has also been shown to be involved in the down-regulation of the NF- κ B and interferon pathways in a dose-dependent manner (Schlee *et al.*, 2007). All things considered; although the c-Myc tag only represents a short chain of 10 amino acid residues of c-Myc, it is highly recommended to remove the tag if the recombinant protein product is intended for application in pharmacological studies. This was particularly important in this study where the c-Myc tag was used for the immunopurification of recombinant NA proteins from pEXPR-NA transformed *N. benthamiana* plants (section 7.3.7.). Association of the c-Myc tag with an antigenic protein such as NA may lead to an immune response towards the c-Myc tag. This may prove to be problematic and may also lead to the eventual disruption of the regulatory control of endogenous c-Myc levels of the host. Nevertheless, the c-Myc tag allowed efficient recovery of immunopurified recombinant NA proteins without any trace of RuBisCO contamination (section 7.3.7.). RuBisCO is known to be the most abundant protein on earth and is potentially the main contaminating agent of any recombinant protein expressed in plants. Although RuBisCO is commonly found in most crop plants and is safe for human consumption, intravenous delivery of RuBisCO contaminated vaccine antigens may cause adverse side effects and allergic reactions. In addition, measurements of dosage would also be inaccurate if vaccine antigens cannot be properly purified. In this study, c-Myc-tagged NA proteins could be recovered efficiently via immunopurification procedures and may be potentially applied for future studies. The potential application of chloroplast expressed recombinant NA is discussed in further detail in Chapter 8 section (8.6.). Unfortunately, the expression of HA in pEXPR-UG transformed *N. benthamiana* plants could not be further evaluated as the antibiotic resistant shoots that were recovered post-bombardment did not display persistent tolerance towards abiotic stress (discussed earlier in Chapter 6, section 6.4.1.). As a result, the recovered shoots underwent progressive

necrosis following several unfavourable temperature spikes which occurred during the regeneration process; thus, emphasising again the importance of establishing resilient lines of *N. benthamiana* for chloroplast transformation. Nevertheless, preliminary results indicated that the HA gene was successfully detected in the chloroplast genome of transformed *N. benthamiana* plants (section 7.3.5.2.). This was highly encouraging as it suggested that the HA gene could also be potentially expressed in the chloroplast genome of *N. benthamiana*.

7.5. CONCLUSION

In summary, results for the evaluation of antibiotic resistant plants regenerated from post-bombardment *N. benthamiana* indicated that recovery via direct organogenesis was crucial for the recovery of true transplastomic plants. The rapid recovery of shoots post-bombardment was necessary to minimise somaclonal variation and to prevent aberrant intermolecular recombination events which was hypothesised to occur extensively in rapidly dividing callus tissue which failed to produce transplastomic plants. The plastid *rrn* promoter (*Prrn*) was found to be highly efficient in modulating the transcription of the GOIs within the chloroplasts of *N. benthamiana* allowing the accumulation of mRNA transcripts in total RNA samples of the transformed plants. Plants that were transformed with GOI expression cassettes without the *psbA* 5'-UTR allowed the accumulation of mRNAs but failed to produce proteins suggesting that the *psbA* 5'-UTR leader sequence was an absolute requirement for the translation of mRNAs. Similar results were also achieved when the developed 2-part chloroplast vector system was employed for the expression of NA viral antigen of avian influenza A (H5N1) in the chloroplasts of *N. benthamiana* demonstrating the versatility of this system. To date, there are still no commercial chloroplast transformation vector systems in the market and the development of a versatile chloroplast transformation vector system would definitely assist the development of this field in the long run.

Chapter 8: General discussion and conclusions

Transgenic plants offer a potent alternative platform for the large-scale production of high-value proteins for a variety of industrial and therapeutic applications. The nuclear and chloroplast genomes are often chosen as the preferred sites for the integration and expression of transgenes with the latter option often leading to higher accumulation of total soluble transgenic proteins (Tregoning *et al.*, 2004). Transplastomic plants are primarily produced via particle bombardment followed by arduous regeneration of homoplastomic plants; the success of which is highly dependent on the nature and efficacy of the chloroplast transformation vector being used. An efficient chloroplast transformation vector is expected to fulfil a minimum of three functions. The chloroplast transformation vector has to 1) facilitate the assimilation of the transgene expression cassette(s) into the chloroplast genome, 2) promote the transcription of mRNAs; and 3) modulate the translation of mRNAs into proteins (Verma and Daniell, 2007). A major bottleneck in this field of study is the absence of versatile chloroplast transformation vectors which can be easily customised to suit a variety of applications. At the time of writing, no commercial vectors are available for the transformation of the chloroplast genome and most constructed chloroplast transformation vectors are highly specific to their function and do not translate well for expression of different genes or in other plant species (Verma and Daniell, 2007). The concept of a binary vector provides some insight for the design of a versatile chloroplast transformation vector system composed of two separate plasmids; individually responsible for transgene integration into the chloroplast genome; and transgene expression, respectively. Thus, this approach was utilised in this work for the development of a highly versatile 2-part chloroplast transformation vector system using green fluorescent protein (GFP) as an exemplar transgene for the evaluation of expression in *N. benthamiana*. Modifications were made to the

original pKCZ chloroplast transformation vector in order to overcome its limitations and two separate plasmids; namely, pCART and pGENE, were constructed for the integration of transgene expression cassettes and the expression of transgenes in the chloroplast genome, respectively. A total of four complete pEXPR chloroplast transformation vector constructs were produced by integrating transgene expression cassettes from pGENE into pCART for the expression of GFP (pEXPR-G and pEXPR-UG) as a proof of concept, followed by hemagglutinin (pEXPR-HA) and neuraminidase (pEXPR-NA) in the chloroplasts of *N. benthamiana*. The HA and NA genes of avian influenza virus A/chicken/Malaysia/5744/2004(H5N1) were chosen as functional gene candidates for the prospective development of plant-based vaccines. The efficiency of the pEXPR chloroplast vectors were evaluated in a prokaryotic model system of *E. coli* and was determined to be primarily functional prior to delivery into *N. benthamiana* leaf explants via particle bombardment under optimised conditions. Streptomycin and spectinomycin resistant shoots and calli were obtained for all constructs and transgenes were successfully detected in isolated chloroplast genomic DNA. The expression of GFP and NA protein was also detected in the regenerated transformants. By analysis of the entire workflow for the establishment of transplastomic plants, the following key results were found: 1) the newly developed pEXPR chloroplast transformation vectors were capable of directing efficient integration and expression of transgenes in the chloroplasts of *N. benthamiana*, 2) the prokaryotic model system of *E. coli* can be used for the evaluation of heterologous chloroplast promoters but is unsuitable for the evaluation of chloroplast-specific 5'-regulatory elements, 3) optimisation of particle bombardment parameters based on the survival of post-bombarded plants in combination with transient expression of a marker gene (such as GFP) allowed efficient recovery of stable transformants, 4) the timely recovery of post-bombarded explants via direct organogenesis is important in order to prevent the loss of transgenes, as well as, to minimise the effects of

abiotic stresses, and 5) the developed 2-part chloroplast transformation vector system is highly versatile and can be easily customised for the expression of different transgenes as initially intended. Prior to this study, the pKCZ chloroplast transformation vector has been evaluated and found to be incapable of producing stable homoplasmic transformants, however, the basic expression construct was found to be effective for the expression of transgenes and was used as a model for the construction of pCART and pGENE.

8.1. The 2-part chloroplast transformation vector system

One of the most important aspects for the design of a chloroplast transformation vectors is the selection of suitable sites for the integration of transgene into the chloroplast genome. In contrast to nuclear transformation, the integration of transgenes into the chloroplast genome is site-directed via homologous recombination (Newman *et al.*, 1990; Svab and Maliga, 1993); meaning that, transgenes can be integrated into the chloroplast genome at a very specific predetermined location. This provides a significant advantage over nuclear transformation where transgenes integrate at random parts of the nuclear genome with different copy numbers resulting in multiple transgenic lines with highly variable patterns of transgene expression (Kindle *et al.*, 1989; Daniell *et al.*, 2005). However, this would also mean that an improperly selected integration site would always result in a failed chloroplast transformation event or cause the disruption of endogenous chloroplast genes resulting in genome instability (Sriraman *et al.*, 1998). Thus, the homologous recombination sites are proposed to be the determining factor for the baseline efficiency of chloroplast transformation and should be carefully evaluated prior to actual application. For this reason, the well characterised chloroplast genome of tobacco, as well as, its gene organisation provided valuable insight for the design of plasmids, pCART and pGENE. The carrier plasmid, pCART was designed to integrate the transgene

expression cassettes into the inverted repeat (IR) regions of the chloroplast genome of tobacco. Since two IR regions are present in each copy of the chloroplast genome, transgenes which are integrated into the IR regions would undoubtedly be duplicated by endogenous copy correction mechanisms. The increase in transgene copy number is expected to correlate with higher expression levels in the absence of post-transcriptional gene silencing (Daniell *et al.*, 2002). This approach had already been successfully demonstrated by Zoubenko *et al.* (1994) and Daniell *et al.* (2001) and was thought to contribute to the highest expression of transgenic protein. The expression plasmid, pCART, on the other hand, employed the use of the constitutive *Prrn* promoter together with the *psbA* 5'-untranslated leader sequence to regulate the transcription and translation of genes. Unfortunately, the complete chloroplast genome sequences are not readily available for many important commercial and crop plants posing a significant bottleneck for chloroplast transformation. However, chloroplast genes in higher plants are often clustered into transcriptional operons separated by non-coding spacer regions. In theory, integration of transgenes into the spacer regions should not disrupt any endogenous genes and are ideal target locations for transformation. Thus, the detection of non-coding spacer regions together with some nucleotide sequence data of the approximate location would be sufficient for the modification of the INSL and INSR regions of pCART, allowing transgene integration into the chloroplast genome of the new host. In addition, the *aadA* expression cassette in pCART is transcriptionally active and can be used independently for the evaluation of the selected transgene insertion site in the new host prior to actual transformation. With the advent of next-generation sequencing (NGS) technologies, limited access to complete chloroplast genome sequences will no longer be a hindrance. This would potentially facilitate the identification and characterisation of potential intergenic regions within the chloroplast genomes of commercially important plants for the integration of transgenes. A library of pCART vectors can then be constructed

based on the obtained information for the transformation of a wide variety of plants, while at the same time, still being compatible to the same pGENE plasmid. Likewise, the expression plasmid pGENE can also operate independently allowing expression studies to be performed for a variety of transgenes prior to final vector construction. The versatility of pCART and pGENE was clearly demonstrated by the successful delivery of HA and NA genes into the chloroplast genome of *N. benthamiana*. In addition, the expression plasmid derivative, pGENE-UG that was developed for the evaluation of GFP expression in *N. benthamiana* using the aforementioned system may also be potentially applied for the expression of other transgenes. Most chloroplast genes are expressed as polycistronic cassettes (Gruissem, 1989) suggesting that a GOI that is cloned upstream of the GFP gene in pGENE-UG will result in the synthesis of GOI-GFP fusion proteins allowing the phenotypic detection of transgenic protein expression under UV light. Taken together, the 2-part chloroplast transformation vector system involving pCART and pGENE have the potential to be applied for the delivery of a wide variety of transgenes into many different host plants.

8.2. Evaluation of transformation vectors

The prokaryotic model system of *E. coli* offers a convenient platform for the preliminary evaluation of chloroplast transformation vectors. The gene expression machinery of *E. coli* is highly similar to that of chloroplasts where genes are transcribed by RNA polymerases which are dependent on σ - or σ -like factors. The initiation of mRNA translation in both *E. coli* and chloroplasts are also mediated with the initial association of the 5'-leader region of the mRNA transcript with the prokaryotic 16S rRNA (Sprengart *et al.*, 1990). This is further encouraged by the advantage of rapid *E. coli* growth rates (doubling time of 25 minutes in LB broth) (Ellwood and Nomura, 1980; Marr, 1991), as well as, the ability to express transgenes without integration into the genome and similar

susceptibility towards antibiotic agents. GFP expression was evaluated in pEXPR-G and pEXPR-UG transformed *E. coli* prior to transformation of *N. benthamiana* and the *aadA* antibiotic resistance gene was observed to be expressed efficiently by both chloroplast *Prrn* and *PtrnfM* promoters in *E. coli*. A conclusion can be drawn that the eubacteria-like chloroplast P1 type PEP promoters are mainly functional in a prokaryotic background due to their structural similarity in comparison with prokaryotic σ^{70} -type promoters (Igloi and Kössel 1992, Allison *et al.*, 1996). The ribosome binding site (RBS) in the *Prrn* and *PtrnfM* chloroplast promoters highly resemble the Shine-Dalgarno (SD) sequence of σ^{70} -type promoters of *E. coli*. Hence, it can be hypothesised that *E. coli* σ -factors and RNA polymerases are able to interact with the -10 and -35 upstream regulatory elements of the chloroplast P1 promoters as well as the rRNA operon upstream activator (RUA) to a certain degree and initiate mRNA transcription (Hirvonen *et al.*, 2001; Suzuki *et al.*, 2003). Both the *Prrn* and *PtrnfM* promoters were also found to be sufficient to drive the translation of proteins from the mRNA transcripts of *aadA* using their internal RBS; allowing the transformed *E. coli* bacteria to be resistant to antibiotic concentrations of up to 500mg/l as observed in the results of the minimal inhibitory concentration (MIC) assays. Similar results were also observed for the expression of GFP in *E. coli* where the *Prrn* promoter was sufficient to drive protein expression. Furthermore, the *psbA* 5'-untranslated leader region (UTR) even appeared to have an inhibitory effect on GFP protein expression in *E. coli* (see section 4.4.1.). Interestingly, the *aadA* antibiotic resistance gene could also be expressed in the chloroplast genome of *N. Benthamiana* under the control of the *PtrnfM* promoter alone without the requirement of additional 5'-untranslated regulatory elements to direct the translation of mRNAs. However, in contrast to *E. coli*, the *psbA* 5'-UTR was determined to be an absolute requirement for efficient GFP protein translation in chloroplasts (Chapter 7, section 7.4.3.). These results suggest that, unlike *E. coli*, chloroplasts of higher plants possess a higher order of post-transcriptional

control over gene expression. As a result, genes under the regulation of similar promoter types can be differentially expressed depending on their nature and function as required by the chloroplasts. The regulation of gene expression in *E. coli* occurs globally at the transcriptional level, where a group of genes involved in response to a particular stimulus consisting of multiple operons are under the control of similar promoter(s) (Chuang *et al.*, 1993). Post-transcriptional regulation of gene expression in *E. coli* is relatively uncommon and is mediated via an antisense mechanism involving small regulatory RNAs which bind to their mRNA targets leading to degradation by RNase E possibly through the assembly of a degradosome (Massé *et al.*, 2003; Udekwu, 2010). In comparison, transcriptional regulation is known to have a limited effect on chloroplast gene expression during growth and development. Most chloroplast genes of higher plants are regulated at the post-transcriptional level (Deng and Gruissem, 1987) via various *cis*- and *trans*-acting factors which interact with the 5'- and 3'-UTRs of chloroplast mRNA transcripts. This is highly counterintuitive as 5'-UTR elements are also common features of *E. coli* mRNA transcripts and are known to mediate the association of the pyrimidine rich region on the 16S rRNA component of the 30S ribosome with the Shine-Dalgarno (SD) sequence (Komarova *et al.*, 2005, Laursen *et al.*, 2005). Hence, the only conclusion that can be drawn is that the organisation and spacing between the elements of the 5'-UTR region is highly specific to their endogenous application and cannot be used effectively for the regulation of genes between *E. coli* and chloroplasts. In prokaryotes, the SD sequence essential for ribosome binding and accurate initiation of translation is commonly located 7 ± 2 nucleotides upstream of the AUG initiation codon in the 5'-UTR of prokaryotic mRNAs. However, spacing of the SD-like sequences on chloroplast 5'-UTRs are much less conserved, ranging from 2 to 29 nucleotides upstream of the AUG initiation codon. Hence, the increased distance between the SD-like sequence from the initiation codon within *psbA* 5'-UTR may account for the observed decrease in protein expression (Ruf

and Kössel, 1988). Alternatively, the secondary stem-loop structure that is proposed to be adopted by the *psbA* 5'-UTR may have formed in the proximity of the SD-like sequence (section 7.4.3.), thus, inhibiting ribosome binding in the absence of chloroplast specific *cis*- and *trans*-acting factors leading to decreased rates of protein expression. In addition, multiple putative-ribosome binding sites are also present on the *psbA* 5'-UTR (figure 7.24) which may have a negative effect on the initiation of translation in prokaryotes. In summary, the prokaryotic *E. coli* system can be conveniently used for the evaluation of eubacterial chloroplast-specific promoters due to their similarity with prokaryotic promoters. However, evaluation of transgene expression cassettes under the regulation of chloroplast-specific 5'-UTRs in *E. coli* did not produce results which reflect actual expression patterns in chloroplasts. Hence, evaluation of 5'-UTRs of the transgene expression cassette must still be performed directly in the intended host prior to actual transformation. Fortunately, the pEXPR-UG expression plasmid that was constructed for the expression of GFP in the chloroplasts of tobacco provides an excellent platform for the evaluation of 5'-UTRs which can be monitored based on GFP expression. It can be assumed that the pEXPR-UG GFP expression plasmid can be used for the identification and characterisation of suitable UTR elements for the regulation of transgene expression in a new host plant.

8.3. Effects of abiotic stress and regeneration

The regeneration and recovery of transplastomic plants is regarded as the bottleneck for the entire chloroplast transformation process. Even if transformation vectors have been successfully delivered into the chloroplasts of *N. benthamiana* and integration of transgenes into the chloroplast genome has been positive, the transformed cells are still required to recover, multiply and regenerate into whole plants. Recovery of antibiotic shoots was noticeably

improved with the used of stress-tolerant lines of *N. benthamiana*. The tissue culture process is an integrated part of the particle bombardment procedure and is required for the preparation of plant material for transformation, as well as, for the recovery of whole plants from post-bombarded tissue samples. Unfortunately, most wild-type plants do not cope well with tissue culture and generally require a period of acclimatisation before they are able to proliferate under *in-vitro* conditions. Preliminary experiments using un-acclimatised plants for particle bombardment resulted in a 100% mortality rate where all bombarded samples underwent progressive necrosis 7 to 9 days post bombardment. The abnormal growth and developmental patterns observed in the majority of the wild-type *N. benthamiana* plants can be attributed to high humidity levels within the culture jars, fluctuations in ambient temperature and photooxidative stress which often leads to hyperhidrosis. Hyperhydric leaves do not respond well to particle bombardment and often resulted in failed transformation events due to a decline in the survival rate of post bombarded plant tissue. Onset of hyperhidrosis in the leaves of *in-vitro* cultured *N. benthamiana* plants is believed to have close correlations with increased humidity levels in the culture vessel (Debergh *et al.*, 1992; Chen, 2004). Uptake of nutrients and water by plants usually occurs via the relatively passive process of transpiration which is highly dependent on relative humidity (%RH) levels. In tissue culture, %RH is affected by the ambient temperature as well as the moisture content in the tissue culture media and can reach ~100%RH under unfavourable conditions. It can be hypothesised that diurnal fluctuations in ambient temperature causes the cycling of evaporation and condensation of water in the culture medium. As a result, the cultured plants are subjected to conditions of ~100%RH during the day and later logging stress during the night when the water vapour condenses back into a liquid. It can be assumed that at ~100%RH, the process of transpiration is impaired and water can no longer exit the leaves via evaporation from the stomata. As a result, water accumulates in the leaves resulting in hyperhidrosis

(Ziv, 1991). This condition is further worsened by the continual uptake of water from the culture media via passive diffusion due to root pressure which eventually leads to the disruption of the compact palisade mesophyll layer to accommodate the excess moisture leading to a vitrified appearance (Ziv, 1990). This also accounts for the observed leakage of moisture from the leaves as excess water is being forced out via the leaf spaces and the stomatal pores by high internal osmotic pressures. Hence, the decline in transformation efficiency observed in hyperhydric leaves can be explained as the majority of chloroplasts are located within the palisade mesophyll layer. In addition, perforation of the cell walls by the gold microcarriers would also result in severe damage due to cytoplasm leakage in turgid cells leading to cellular necrosis. Root initiation was also observed to be impaired in hyperhydric plants. This is expected as the proliferation of roots would likely lead to the worsening of hyperhidrosis. All things considered, the establishment of stress-resistant lines of *N. benthamiana* significantly improved the overall recovery of transplastomic plants as it allowed antibiotic resistant lines to be clearly distinguished from non-transformed plants without the interference of abiotic stress effects. In addition, the stress tolerant lines also allowed the clear distinction between the process of chlorosis and cellular necrosis which was an important aspect for the optimisation of parameters for particle bombardment.

8.4. Variables of particle bombardment

The PDS-1000/He particle delivery system is highly customisable and offers an entire suite of parameters which can be combined to obtain optimal delivery of DNA coated microcarriers into virtually any tissue. These parameters include burst pressure, distance between rupture disk and launch apparatus, distance between macrocarrier and stopping screen, distance of the target sample from the stopping screen and vacuum pressure. Based on preliminary

experiments, it has been determined that all parameters are dependent, that is, the adjustment of one parameter can compensate for another to attain similar results. A summary that can be drawn from the optimisation of particle bombardment (Chapter 5) is that all bombardment parameters ultimately affect only two characteristics of microcarrier delivery: 1) the acceleration of microcarriers which determines the force of impact; and 2) the spread and distribution of microcarriers which determines the homogeneity of the transformation event. Acceleration of microcarriers is mainly affected by the bombardment pressure, distance between the rupture disk and launch apparatus and; the distance between the macrocarrier and the stopping screen. In theory, the bombardment pressure is expected to correlate with the volume of helium that rushes into the head space (area above the launch apparatus) of the bombardment chamber when the rupture disk bursts. A longer distance between the launch apparatus and the rupture disk would increase the head space volume causing a significant decline in the force transferred to the macrocarrier. Another factor that is commonly overlooked in most particle bombardment optimisation procedures is the distance between the macrocarrier and the stopping screen. Acceleration of the macrocarrier effectively only occurs along this distance. Once halted by the stopping screen, acceleration is no longer applied and the initial velocity of the microcarriers is determined by this value. Hence, increasing the distance between macrocarrier and stopping screen would allow acceleration over a longer period of time; thus, increasing the final velocity of the microcarriers. In this study, all bombardments were performed using the minimal distance between rupture disc and launch apparatus; and a constant distance of 10mm between macrocarrier and stopping screen. Hence, these parameters can be treated as a constant variable allowing the acceleration of microcarriers to be controlled solely by burst pressure alone. On the other hand, the spread and distribution of microcarriers is mainly attributed to the distance between stopping screen and target stage. During particle bombardment, the

macrocarrier is stopped by the stopping screen but the microcarriers are allowed to pass through and continue towards the target tissue. As a result, tissue that is placed closer to the stopping screen would be more densely bombarded within a narrower area as compared to targets that are placed further away from the stopping screen. The results of this experiment strongly support this assumption as tissues bombarded with a shorter distance between stopping screen and target stage were found to sustain a higher degree of damage over a narrower region as indicated by the trend of necrosis (Chapter 5, section 5.3.2.3.). In this experiment, optimisation of particle bombardment parameters based on necrosis was vital as conventional optimisation via transient reporter gene expression often resulted in maximum gold particle delivery into the host tissue. Although this is expected to correlate with high levels of transient gene expression, it would also result in heavy damage to the target tissue which would be detrimental for the regeneration of stable transformants. In addition to the optimised parameters, the outcome of particle bombardment can also be affected by inconsistencies in consumable materials such as rupture disks and macrocarriers even when produced by the same manufacturer. Hence, it is vital that optimisation of particle bombardment and actual transformation is performed using consumables manufactured within the same production lot.

8.5. Establishment of homoplastomy

In order to minimise the effects of hyperhidrosis and somaclonal variation, the regenerated shoots have to be subcultured onto soil as soon as a homoplastomic state is achieved. Establishment of homoplastomicity was important to ensure that transformed plants do not revert back to their 'wild-type' genome upon removal from antibiotic selection. Establishment of a homoplastomic state is a complicated process involving the gradual replacement of the wild-type genome with the transformed genome. This replacement

process is generally dependent on the efficiency of transformation; which is, in turn, dependent upon the efficiency of the chloroplast transformation vector. A homoplastomic state is usually achieved after 2-4 cycles of selective regeneration on antibiotic media (Verma and Daniell, 2007). In this study, putative homoplastomic plants began to emerge after the second cycle of selective shoot regeneration on antibiotic media (Chapter 7, section 7.3.5.3.). A homoplastomic state cannot be achieved if the transformed genome is not fully competent to replace the wild-type genome or was damaged following transgene integration. Most commonly, damage to the chloroplast genome is the result of aberrant intermolecular recombination events caused by inefficient transformation vectors or cassettes leading to the deletion of essential genes. The chloroplast genome of tobacco has a highly conserved structure which is maintained by copy correction mechanisms in the IR region (Shinozaki *et al.*, 1986) together with intermolecular recombination which deletes any region between two direct repeat sequences; thus, preventing the accidental duplication of genes (Sriraman *et al.*, 1998). When recombination of the chloroplast genome was activated by multiple direct repeats, recombination events will continue until all local direct repeat sequences have been removed (Iamtham and Day, 2000). Hence, when the integration of transgenes causes damage to endogenous gene sequences, both copies of the transformed and wild-type genomes are maintained in order to thrive during the antibiotic selection process. However, once antibiotic selection stress is removed, replication of the defective transformed genome would be down-regulated and subsequently lost resulting in wild-type reversion. Thus, substitution of the *Prrn* promoter in the *aadA* expression cassette was essential as the endogenous *rrn16* operon was located approximately 10kb away from the selected integration site on both IR regions. In contrast, only one copy of the endogenous *PtrnFM* promoter was present in the large single copy region (LSC) of the chloroplast genome located approximately 95kb from the selected intended site (Shinozaki *et al.*, 1986).

Taken together, the substitution of *Prrn* with *PtrnFM* was a crucial improvement that allowed the regeneration of homoplastomic transformants from pEXPR-UG and pEXPR-NA transformed *N. benthamiana* plants. Thus, this suggests that homologous regulatory elements should be used with caution to prevent intermolecular recombination with endogenous sequences. Nevertheless, intermolecular recombination induced by direct repeat regions could potentially be used for the removal of antibiotic resistance genes from the chloroplast genome post transformation to generate marker free plants. This can be achieved by flanking the antibiotic resistance gene expression cassette with short direct repeat sequences of 100 to 200bp in length leading to its eventual excision by intermolecular recombination mechanisms (Maliga, 2003).

8.6. Chloroplast expression of HA and NA

The hemagglutinin (HA) and neuraminidase (NA) glycoproteins are two main surface antigens located on the outer lipid membrane of the influenza A virus. HA and NA are fundamental glycoproteins required for the infection and spread of the virus within host cells (Wiley and Skehel, 1987; Sylte and Suarez, 2009). The HA and NA molecules have been reported to display the highest level of antigenicity as compared to all other viral proteins making them valuable targets for the construction of candidate vaccines against the infection (Chen *et al.*, 2009). In this study, HA and NA genes of the avian influenza A virus strain H5N1 were successfully delivered into the chloroplast of tobacco using the developed 2-part chloroplast transformation vector system. In brief, codons optimised genes for the HA and NA viral antigens of avian influenza A/chicken/Malaysia/5744/2004(H5N1) were synthesised for expression in the chloroplasts of *N. benthamiana*. Expression plasmids were constructed by inserting the codon-optimised HA and NA genes into pGENE to produce expression plasmids pGENE-HA and pGENE-NA, respectively. The pGENE-HA and

pGENE-NA expression plasmids were subsequently combined with pCART to produce the completed chloroplast transformation vectors, pEXPR-HA and pEXPR-NA, respectively. The recombinant vectors were then delivered into the leaf explants of stress tolerant lines of *N. benthamiana* via particle bombardment under the optimised parameters detailed in Chapter 5 (table 5.2). Antibiotic resistant lines of pEXPR-HA and pEXPR-NA transformed *N. benthamiana* were regenerated on selective media using optimised protocols as described in Chapter 6 and homoplastomic lines were recovered after 2 cycles of selective regeneration. Expression of c-Myc tagged NA proteins was successfully detected using both NA-specific and c-Myc-specific primary antibodies suggesting that the NA proteins could be expressed in the chloroplasts of *N. benthamiana*. The band of approximately 50kDa that was observed in the Western detection for NA (Chapter 7, section 7.3.7.) corresponds to the expected size of the core peptide of the non-glycosylated N1 protein. This is expected as the process of glycosylation does not occur in chloroplasts which lack both the endoplasmic reticulum and the Golgi apparatus. Although heterologous expression of NA in the chloroplasts of *N. benthamiana* results in non-glycosylated NA proteins, the role of glycosylation and its effects on the molecular and kinetic properties of NA are relatively unknown. Nonetheless, glycosylation sites are known to be localised on the head domain of the NA protein (Russell *et al.*, 2006) which may suggest that the non-glycosylated NA expressed in chloroplasts may stimulate a lower immunogenic response. Nevertheless, a study performed by Yang *et al.* (2012) indicated that similar non-glycosylated recombinant NA proteins expressed in *E. coli* were still immunogenic in a dose-dependent manner. In the same study, it was also shown that Balb/c mice immunised three times with 3µg of purified non-glycosylated recombinant NA protein resulted in an antibody titre of (1:3800) (Yang *et al.*, 2012). Alternatively, the lower immunogenic response can also be overcome with the aid of adjuvants which act by stimulating the innate immune system; therefore, priming a stronger response by the adaptive

immune system. A diverse range of compounds have been used as vaccine adjuvants which include oil-in-water emulsions, mineral salts, liposomes, saponin-based adjuvants, cytokines, microparticles and polysaccharides (Aucouturier *et al.*, 2001). Adjuvants mainly function by; 1) assisting in the chemical stabilisation of vaccine antigens, 2) improving the delivery of antigens to antigen presenting cells, 3) improving the processing and presentation of antigens by antigen presenting cells, 4) stimulating the production of desirable immunomodulatory cytokines; thus, 5) permitting a reduction in the required dosage of the administered vaccine antigen (Jalilian *et al.*, 2013). Examples of adjuvants which are commonly used for influenza vaccines include aluminium salts (Hem and Hogenesch, 2007), MF59 (oil-in-water emulsion) (O'Hagan *et al.*, 2013), AS03 (oil-in-water emulsion) (Garcon *et al.*, 2012), and virosomes (liposomes) (de Jonge *et al.*, 2006). Although the HA surface antigen is known to have a greater antigenic effect as compared to NA; there is plenty of evidence to support the usefulness NA as a candidate vaccine and its application for inducing protective immunity against the influenza virus (Jahiel and Kilbourne, 1966; Kilbourne *et al.*, 1968; Schulman *et al.*, 1968; Webster *et al.*, 1988; Johansson *et al.*, 1989; Aymard *et al.*, 1998). In addition, influenza vaccine supplemented with purified viral NA antigens also showed an increase in immune response against a viral challenge (Kilbourne *et al.*, 1968; Schulman *et al.*, 1968). Recombinant NA protein produced in a baculovirus-expression system also managed to induce the production of NA-specific antibodies in mice (Kilbourne *et al.*, 2004). Hence, the immunogenic potential of NA as a protective antigen against avian influenza A (H5N1) is still fascinating and worthy of further investigation. It is interesting to think that the recombinant NA protein produced in the chloroplasts of *N. benthamiana* in this study could potentially have a real application as an influenza vaccine. However, proper precautions should be taken to ensure that the extraneous c-Myc tag does not interfere with the desired immune response. For this reason, a factor Xa protease cleavage site

(Ile-Glu-Gly-Arg) was placed between the C-terminal of NA and the c-Myc epitope tag during vector construction allowing the convenient recovery of pure NA. Antibiotic resistant lines of pEXPR-HA transformed *N. benthamiana* were also successfully recovered post-bombardment. Unfortunately, stably transformed pEXPR-HA plants could not be maintained due to uncontrolled fluctuations in temperature (between 20 and 42°C) which caused the subsequent demise of all antibiotic resistant lines of pEXPR-HA transformed plants during tissue culture. Nevertheless, preliminary results that were obtained from the antibiotic resistant shoots before they underwent progressive necrosis indicated that the HA gene was successfully delivered into the chloroplast genome. This observation suggests that the HA gene can be potentially expressed in the chloroplast of *N. benthamiana* under favourable conditions. Hence, the failure of regeneration was primarily attributed to unfavourable environmental factors rather than vector or protein-toxicity related problems. Various forms of recombinant HA including monomers, trimers, virus-like particles and chimeric proteins have been successfully expressed in plants using both transient and stable expression strategies (Redkiewicz *et al.*, 2014). In this study, the newly developed 2-part chloroplast transformation vector system has been proven to be an effective tool for the delivery and expression of foreign genes in the chloroplast of *N. benthamiana*; as demonstrated by the successful expression of GFP and NA at the protein level. To date, chloroplast expression of HA and NA has not yet been reported and this study signifies the first real attempt for the expression of HA and NA in the chloroplasts of *N. benthamiana* as an attempt towards vaccine development. Further work is still required to thoroughly characterise the full potential of chloroplasts as a platform for the production of plant-based recombinant vaccines. In summary, the 2-part chloroplast transformation vector system is workable and has been successfully used for the heterologous expression of the NA gene from avian influenza virus A/chicken/Malaysia/5744/2004(H5N1) in the chloroplast of *N. benthamiana*. This

expression system would potentially provide a valuable cost effective alternative to animal-based systems for the large scale production of heat-stable vaccines in future.

8.7. General conclusions

An efficient chloroplast transformation vector is essential for the delivery and expression of transgenes in the chloroplast genome. A typical chloroplast transformation vector is required to have chloroplast-specific homologous recombination sites, expression cassette(s) for the expression of the antibiotic resistance marker gene as well as the gene of interest, efficient promoters to direct the transcription of genes, and suitable 5'-untranslated regulatory elements to mediate post-transcriptional regulation of gene expression. The homologous recombination sequences of the chloroplast transformation vector are host-specific and have to be customised accordingly for each new host. On the other hand, the transgene expression cassette generally allows the expression of any gene of interest. The construction of a single chloroplast transformation vector for the delivery and expression of a particular gene is time consuming and highly impractical as each component has to be modified using a step-by step method involving multiple cloning events. To overcome this problem, a 2-part system was designed separating the elements required for transgene integration and antibiotic resistance into a single plasmid (carrier plasmid, pCART) and the elements required for the expression of the gene of interest in another (expression plasmid, pGENE). This allows convenient access for the customisation and evaluation of each individual element prior to final assembly to produce the completed chloroplast transformation vector. Chloroplast transformation vectors constructed using the developed 2-part system with GFP as an exemplar cassette was found to be fundamentally functional in *E. coli*. GFP expression was also successfully detected in *N.*

benthamiana plants that were recovered from particle bombarded leaves. Chloroplast transformation vectors for the delivery and expression of HA and NA was also successfully constructed and delivered into the leaf explants of *N. benthamiana* using the 2-part system. DNA and RNA analysis of the recovered transplastomic plants indicated that the genes integrated successfully into the target site on the chloroplast genome and were also transcribed to produce the correct mRNA transcripts. Immunological detection assays using GFP and NA specific antibodies also indicated the presence of GFP and NA proteins in the chloroplast of transformed *N. benthamiana*. The main conclusions of this study are the following:

- 1) The new chloroplast transformation vectors were competent and allowed the stable incorporation of transgenes into the chloroplast genome of *N. benthamiana*. Modifications that were made in the construction process eliminated aberrant intermolecular recombination events allowing the regeneration of homoplastomic plants. Hence, the pEXPR chloroplast transformation vector can be used for stable chloroplast transformation and is useful for future applications.
- 2) The evaluation of chloroplast transformation vectors can be fundamentally evaluated in *E. coli* with a caveat that complex chloroplast-specific regulatory elements such as the *psbA* 5'-UTR may inhibit the translation process. Remarkably, the *E. coli* system was highly suited for the evaluation of chloroplast P1 promoters such as the *Prrn* promoter of the *rrn* operon, as well as, the *Ptrn^{fM}* promoter of tRNA^{fMet}.
- 3) Optimisation of particle bombardment parameters based on transient expression of a marker gene does not always translate well for stable chloroplast transformation. High rates of transient expression usually correlate with maximum damage to the bombarded tissue, thus, severely limiting its regenerative potential. Successful transformation was successfully

achieved only when bombardment parameters were optimised to allow maximum DNA delivery without causing excessive damage to the plant tissue.

- 4) True transformants were only recovered from antibiotic resistant shoots that were regenerated via direct organogenesis and all antibiotic resistant callus samples that were evaluated did not produce the desired mutant genotype. Callus cells are non-specialised cells that divide rapidly resulting in higher rates of somaclonal variation. In addition, chloroplasts are also found in much lower numbers in calli as compared to leaves. Hence, the regeneration of transformed plants via direct organogenesis is essential for the recovery of true transplastomic plants.
- 5) The gene expression cassette of the chloroplast transformation vector allowed the efficient expression of transgenes in the chloroplasts of *N. benthamiana*. The *Prrn* promoter allowed the efficient transcription of mRNA from the gene sequence of GFP and NA. The *psbA* 5'-UTR was found to be an absolute requirement for the translation of mRNA transcripts as demonstrated in the analysis of GFP expression in transplastomic *N. benthamiana* plants transformed with pEXPR-G and pEXPR-UG.

The versatile 2-part chloroplast transformation vector system can be used for the convenient delivery and expression of different genes in the chloroplast genome of *N. benthamiana*. This was demonstrated by the successful delivery of HA and NA genes. The NA gene was also shown to be fundamentally expressed at both the transcriptional and translational levels in transplastomic tobacco. Interestingly, similar transformation results were observed regardless of the GOI that was being delivered, which suggests that transgene insert length is not a limiting factor in the homologous recombination process (at least between the range of 700-1700bp).

Chapter 9: Limitations of the Study and Future Perspectives

Although all general and specific objectives of this study had been achieved, there were some unavoidable limitations during the process. Despite performing particle bombardment using optimised protocols, a number of random elements still appeared to affect microcarrier delivery. The majority of such elements have been discussed in Chapter 5 such as, premature bursting of rupture disks and irregular hydrophobic/hydrophilic properties of the macrocarrier film and precautionary steps have also been suggested to minimise these effects. However, an element of variability was still apparent in the results of particle bombardment where a successful transformation event was not always guaranteed even under optimised bombardment parameters. Development of an automated platform for particle bombardment may prove useful in reducing such random elements and removing human-induced errors to ensure more reproducible results. Inconsistencies were also observed in the results of the regeneration process. Although the concentration of plant growth regulators have been optimised for the induction of shoots and calli, the expected response was not always observed in all subcultured samples. This was especially apparent in plants which have been subjected to multiple treatments. For example, plant tissue that was subjected to callus induction using 1.0mg/l of BAP and 0.5mg/l of NAA did not produce shoots when cultured on media containing 1.0mg/l BAP and 0.1mg/l NAA. Instead, shoots were only induced when a higher concentration of BAP (2.0mg/l) was used. Thus, this indicates that exogenously applied plant growth regulators may alter the regulation of endogenous levels of plant hormones causing a "carry-over" effect during subsequent subculture cycles. In addition, plants that were cultivated for extended periods on culture media supplemented with an exogenous source of plant growth regulators also underwent progressive necrosis when subcultured

onto culture media without plant growth regulators. This is in agreement with the previous hypothesis suggesting that exogenously applied plant growth regulators may disrupt the biosynthesis of endogenous plant hormones (Tamas, 1995). Hence, for efficient regeneration of transplastomic plants, it is highly recommended that antibiotic resistant shoots are regenerated via direct organogenesis and transferred onto soil as soon as a state of homoplastomy is achieved. In this study, the major problems which limited the regenerative potential of pEXPR-HA transformed *N. benthamiana* plants were determined to be heat damage and hyperhidrosis. Abiotic stress factors are present throughout the entire duration of tissue culture and can severely impair the regeneration capability of the transformed plants. Due to the contained nature of the culture vessel, water vapour which evaporates from the culture media cannot escape leading to an overall increase in humidity. This disrupts the process of transpiration, causing hyperhidrosis, which triggered a vicious cycle of cellular asphyxiation, increased oxidative stress, upregulated expression of stress related genes (causing various morphological anomalies); and ultimately, programmed cell death (Kevers *et al.*, 2004). This problem has been well addressed in this study and a number of pre-emptive measures have been taken to facilitate the recovery of plants post-bombardment; such as, careful control of ambient temperatures, more frequent subculture cycles onto fresh culture media, the use of "cool-white" fluorescent lamps to reduce the emission of heat to minimise temperature fluctuations during light and dark cycles, and the establishment of stress-tolerant lines of *N. benthamiana*. Nevertheless, fluctuations in temperature due to diurnal or weather conditions can neither be predicted nor controlled in a tropical country such as Malaysia. Hence, in future studies, it is imperative that the regeneration of transformed plants is performed in a more readily controlled environment such as a growth chamber. Establishment of stress-tolerant lines of *N. benthamiana* was a defining moment which allowed the subsequent recovery of transplastomic lines of pEXPR-UG (expression of

GFP) and pEXPR-NA (expression of NA) transformed plants. Although this was consistent with the objectives of the study, the decision to work with stress-tolerant lines of *N. benthamiana* also had its disadvantages. Stress-tolerant lines of *N. benthamiana* were selected based on their ability to adapt to the unfavourable conditions of *in-vitro* culture. One of the characteristic traits of stress-tolerant plants was a decline in root development. Reduced root formation is hypothesised to minimise the influx of water into roots via passive diffusion; thus, preventing hyperhidrosis under high humidity conditions. At the later part of this study, it was discovered that the stress-tolerant lines of pEXPR-UG and pEXPR-NA transformed plants did not survive well when transferred onto soil. This was expected, as the main problem during acclimatisation onto soil was dehydration; the direct opposite of hyperhidrosis. Consequently, stress-tolerant plants which have adapted to thrive in an environment of high humidity would now be highly susceptible to dehydration. This problem was worsened by their reduced root formation which impedes the uptake of water from the soil. Acclimatisation of the transformed plants using a hydroponic system may potentially prevent dehydration to a certain extent and is worth consideration in future studies. All things considered, although the objectives of this study have been successfully achieved, the recommended improvements would more than likely improve the results in future studies. A schematic diagram of a prototype culture vessel is also depicted in appendix (G) which may be useful for reducing humidity levels within the culture jars with the aid of a vacuum pump. Such an invention could also be used for the acclimatisation of *in-vitro* propagated plants for growth on soil.

In summary, the recommendations for future studies are:

- 1) To develop an automated system for the controlled loading of DNA-coated microcarriers onto the macrocarrier films.
- 2) To minimise the regeneration period for the recovery of shoots from the transformed leaf explants.
- 3) To perform the regeneration process in a controlled environment to limit the effects of environmental factors.
- 4) To acclimatise the regenerated plants on culture media which promote root development prior to transfer onto soil.

The developed 2-part chloroplast transformation vector system has been proven to be a competent tool for the delivery and expression of transgenes in the chloroplast genome of *N. benthamiana*. Nevertheless, more investigations are required as suggested in the aforementioned points of discussion to further improve the recovery and regeneration of transplastomic plants *in-vivo*. This would potentially allow the accumulation of recombinant proteins, such as NA, to much higher levels. Evaluation of the immunogenicity of the chloroplast-expressed NA in mice would also be an interesting prospect for future studies.

Appendix

A) Composition of PCR reagents for standard gene amplification, cloning of DNA fragments and identification of recombinant plasmids.

Application Reagents		Stock concentration	Concentration required		
			Standard PCR mixture	High fidelity PCR mixtures	PCR screening
10x <i>Taq</i> reaction buffer		10x	1x	-	1x
10x EXT reaction buffer		10x	-	1x	-
<i>Taq</i> DNA polymerase		5u/μl	1u	-	1u
EXT DNA polymerase + 1.5mM MgCl ₂		1u/μl	-	2u	-
MgCl ₂		25mM	1.5mM	-	1.5mM
dNTP mix		10mM	0.3mM	0.3mM	0.3mM
Forward primers		5mM	0.3mM	5.0μl	0.3mM
Reverse primers		5mM	0.3mM	5.0μl	0.3mM
Template	Bacteria	-	-	-	Single colony
	DNA	50ng/μl	50ng	50ng	
	Recombinant plasmid	50ng/μl	-		
	Chloroplast genomic DNA				
Total volume with dH ₂ O		-	20μl	50μl	20μl

B) Primers list

Primer name	Sequence 5'- to 3-'	Target	O
PF1:GFP- <i>Nco</i> I	GATCCATGGTGAGCAAGGGCGAGGAGC	GFP	F
PR2:GFP- <i>Bsi</i> WI	GTTTCGTACGTTACGACCTTCGATTTTCTTGACAGCTCGTCCAT		R
PF <i>aadA</i>	TAGTCTAGAGATGGCTCGTGAAGCGGT	<i>aadA</i>	F
PR <i>aadA</i>	AGCCTCGAGTCATTATTTGCCGACTACCTTGGT		R
PFMCS	GGAAGATCTGCAACGCGTATGAGCCATATTCAA CGGGA	<i>NptI</i> / MCS	F
PRMCS	CTCGCTAGCCTCCTTAAGTTAGAAAACTCATC GAGCA		R
PF: <i>Nhe</i> I- <i>Ptrn</i> fM	GCCGCTAGCAAGGATTAACGCTTCATTTCT	<i>Ptrn</i> fM	F
PR: <i>Eco</i> RV- <i>Ptrn</i> fM	GCCGATATCTGTACAAATAGAATAGCCCAT		R
PF: <i>Eco</i> RV- <i>psbA</i> 5'	GCCGATATCAACAAGCCTTCCATTTTCTA	<i>psbA</i> 5'-UTR	F
PR: <i>Nco</i> I- <i>psbA</i> 5'	CCGCCATGGTAAAATCTTGTTTTATTTAA		R
c-Myc (+)	CAGCGTACGGAACAAAAATTGATTTCTGAAGAA GATTTGTAAGTGCAGAGG	c-Myc	F
c-Myc (-)	CCTCTGCAGTTACAAATCTTCTTCAGAAATCAAT TTTTGTTCCGTACGCTG		R
ZUHF	GCACCATGGAAAAGATTGTATTGTTGT	HA	F
ZUHR	GAGCGTACGACAAATACAAATTCGGCATTGAAG		R
ZUNF	GAGTCATGAATCCCAATAAGAAAATT	NA	F
ZUNR	AGCCGTACGACACTTATCAATAGTAAAGGGAAG		R

OMNI:F- <i>Afl</i> II	GAACCTAAGGATGCAATTCGCCGTCGTTCAATG	OMNI	F
OMNI:R- <i>Mlu</i> I	CCAACGCGTGTGTCATTAGATCAAATCTTCTTC AGAAAT		R
INSL-TF	GAGCACGTGGCTACGAAC	cassette	F
INSR-TR	CTCTATGAGATCGAATCT		R
GFPF-Seq	ATGGTGAGCAAGGGCGAG	GFP	F
GFPR-Seq	TTACTTGTACAGCTCGTC		R
aadA-SeqF	GATGGCTCGTGAAGCGGT	<i>aadA</i>	F
aadA-SeqR	TTTGCCGACTACCTTGGT		R

Orientation (O): Forward (F) or Reverse (R)

C) Assembly of the c-Myc fragment

Synthetic oligonucleotide fragment:



Calculations to determine the mass/ μ l of c-Myc tag

Total M_w of oligonucleotides: 31383 g/mole

Concentration of each oligonucleotide: 5 μ M

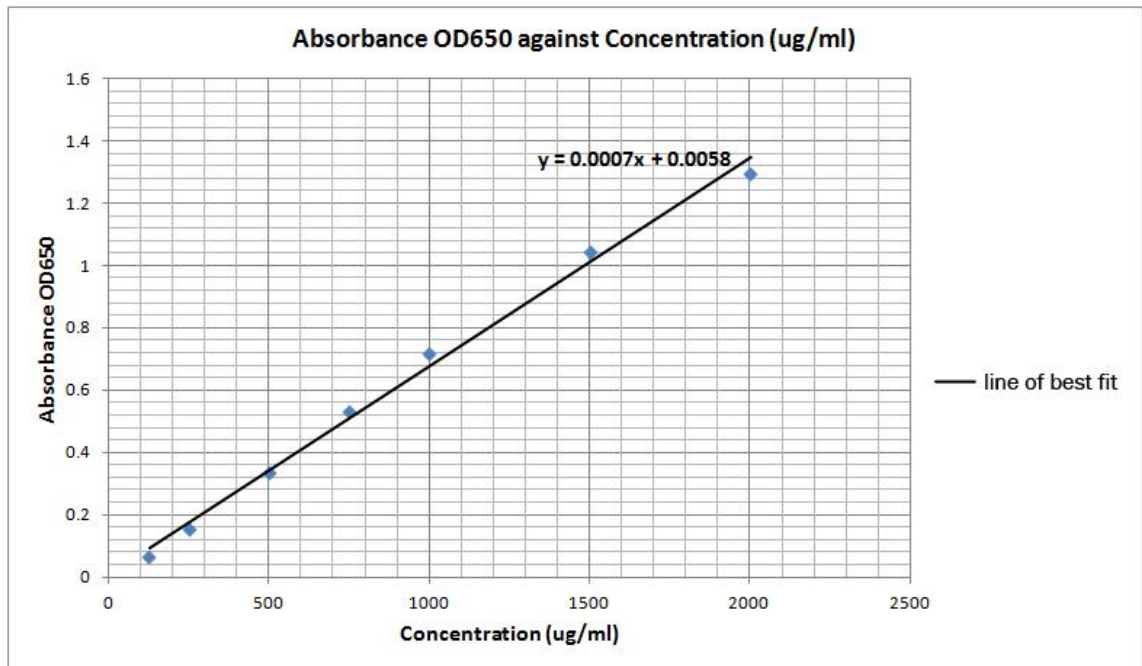
$$\begin{aligned} \text{Mass} &= 5 \mu\text{moles}/\text{dm}^3 \times 31383\text{g}/\text{mole} \\ &= \underline{156915 \mu\text{g}/\text{L}} \end{aligned}$$

$$\begin{aligned} \text{Mass}/\mu\text{l} &= 156915 \times 10^{-6} \\ &= 0.157 \mu\text{g}/\mu\text{l} \\ &= \underline{157\text{ng}/\mu\text{l}} \end{aligned}$$

Hence, 1 μ l of oligonucleotide dimer contains 157ng of DNA (c-myc linker)

The calculations were used to facilitate the synthesis of the c-Myc tag fragment as well as further processing by restriction enzyme (RE) digestion and ligation.

D) Standard curve for the measurement of protein concentration



Standard curve of absorbance at OD650nm against concentration of protein ($\mu\text{g/ml}$) for the determination of protein concentration calibrated using BSA protein concentration standards. ($y=0.0007x + 0.0058$).

E) Composition of various tissue culture media

Composition of micro elements, macro elements and vitamins in Murashige and Skoog media with vitamins (MS+Vit), Gamborg media with B5 vitamins (GB+B5) and Murashige and Skoog media with B5 vitamins (MS+B5). Bold values indicate higher concentrations of the individual components compared among media types.

Micro elements	Concentration (μm)		
	MS+Vit	GB+B5	MS+B5
CoCl ₂ .6H ₂ O	0.11	0.11	0.11
CuSO ₄ .5H ₂ O	0.10	0.10	0.10
FeNaEDTA	100.00	100.00	100.00
H ₃ BO ₃	100.27	48.52	100.27
KI	5.00*	4.52*	5.00*
MnSO ₄ .H ₂ O	100.00	59.16	100.00
Na ₂ MoO ₄ .2H ₂ O	1.03	1.03	1.03
ZnSO ₄ .7H ₂ O	29.91	6.96	29.91
Macro elements	MS+Vit	GB+B5	MS+B5
CaCl ₂	2.99	1.02	2.99
KH ₂ PO ₄	1.25	-	1.25
NaH ₂ PO ₄	-	1.09	-
KNO ₃	18.79	24.73	18.79
MgSO ₄	1.50	1.01	1.50
NH ₄ NO ₃	20.61	-	20.61
(NH ₄) ₂ SO ₄	-	1.01	-
Vitamins	MS+Vit	GB+B5	MS+B5
Glycine	26.64		
myo-Inositol	554.94	554.94	554.94
Nicotinic acid	4.06	8.12	8.12
Pyridoxine HCl	2.43	4.86	4.86
Thiamine HCl	0.30	29.65	29.65

*difference not significant enough to be considered

F) DNA Sequences and Sequencing Results

i) Sequenced *Prrn* promoter sequence:

GCTAGCGGCAATTCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGACG
TGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGATATCACTAGTTG
TAGGGAGGGATCCATG

ii) Sequenced *psbA* 5'-UTR sequence:

GATATCAACAAGCCTTCCATTTTCTATTTTTATTTGTAGAAAAGTGTGCTTGGGAGT
CCCTGATGATTAATAAACCAAGATTTTACCATGG

iii) Sequenced pGENE (Expression cassette + GFP)

CTGCAGTTACAAATCTTCTTCAGAAATCAATTTTTGTTCCGTACGTTACGACCTTCGAT
TTTCTTGTACAGCTCGTCCATGCCGTGAGTGATCCCGGCGGCGGTACGAACTCCAGC
AGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTGTCTAGGGCGGACTGGGTGCTCA
GGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTGCGCGATGGGGGTGTTCTGCTGGT
AGTGGTCGGCGAGCTGCACGCTGCCGTCTCGATGTTGTGGCGGATCTTGAAGTTCAC
CTTGATGCCGTTCTTCTGCTTGTGCGCCATGATATAGACGTTGTGGCTGTTGTAGTTGT
ACTCCAGCTTGTGCCCCAGGATGTTGCCGTCTCCTTGAAGTCGATGCCCTTCAGCTCG
ATGCGGTTACACAGGGTGTGCGCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGC
CGTCGTCTTGAAGAAGATGGTGCCTCCTGGACGTAGCCTTCGGGCATGGCGGACTT
GAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAG
GTGAAGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATG
AACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGACACGCTGA
ACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAA
CAGCTCCTCGCCCTTGTCCACCATGGTAAAATCTTGGTTTATTTAATCATCAGGGACTCC
CAAGCACACTAGTTTTCTACAAATAAAAATAGAAAATGGAAGGCTTGTGATATCGCCC
GGAGTTGCTCCCAGAAATATAGCCATCCCTGCCCCCTCACGTCAATCCCACGAGCCTC
TTATCCATTCTCATTGAACGACGGCGAATTGCCGCTAGC

iv) Codon-optimised hemagglutinin (HA)

ATGGAAAAGATTGTATTGTTGTTTGCTATTGTATCACTTGTAAGTCAGATCAAATTTGT
ATTGGATACCATGCTAACAATTCTACTGAACAAGTTGATACTATTATGGAAAAGAATGTT
ACTGTAACCCATGCTCAAGATATTCTTGAAGAAGACCCATAATGGGAAATTATGTGATTTG
GATGGAGTTAAACCATGATTTTAAGAGATTGTTCTGTAGCTGGATGGTTACTTGAAAT
CCTATGTGCGATGAATTCATTAATGTACCCGAATGGTCTTATATAGTTGAAAAGGCTAAT
CCCGTAAATGATTTGTGTTATCCAGGAGATTTTAATGCTTATGAAGAATTAACATTTG
TTATCTCGAATTAATCATTTTGAAGAAGATTCAAATTATACCTAAGTCTTCATGGTCTTAC
ATGAAGCATCTTATAGGAGTTTCTTACGCTTGCCCTATCAAGGAAAATCTTCTTCTTTC
GAAATGTTGTATGGTTAATTAAGAAAAATTCTACTTATCCCACCATTAAAAGATCATATA
ATAACACTAATCAAGAAGATTTGTTAGTTCTTTGGGGAATTCATCATCAAATGATGCTG
CTGAACAAACCAAATTGTATCAAAATCCTACTACCTATATATCTGTAGGAACTTCAACCT
TAAATCAAAGACTTGTCCACGAATTGCTACTCGATCTAAAGTAAATGGGCAATCTGGA
CGTATGGAATTTTTCTGGACCATTCTTAAACCAATGATGCTATTAATTTTGAATCTAATG
GGAATTTTATAGCTCCAGAATATGCTTATAAGATTGTTAAAAAAGGAGATTCTACTATTA
TGAAGTCAGAATTTGAATATGGAAATTGTAATACTAAGTGCCAAACCCCTATGGGAGCT
ATTAATCTTCAATGCCATTTTATAATATACATCCTTTAGCTATAGGAGAATGCCCAAAT
ATGTTAAGTCTAATCGACTTGTATTGGCTACTGGATTACGAAATTCACCTCAAAGAGAAC
GAAGACGAAAAAGAGAGGACTTTTTGGAGCTATTGCTGGGTTTATTGAAGGAGGATG
GCAAGGAATGGTTGATGGACGATATGGGTATCATCATTCTAACGAACAAGGATCAGGAT
ATGCTGCTGATAAAGAATCTACTCAAAGGCTATTGATGGAGTTACCAATAAGGTTAATT
CAATTATAGATAAGATGAATACTCAATTTGAAGCTGTAGGACGAGAATTAATAACTTAG
AAAGACGAATTGAAAATCTTAATAAGAAGATGGAAGATGGATTTTTAGATGTTTGGACTT
ATAATGCTGAATTAATGACTTATGAAAACGAAAGAACCTTGGATTTTTCATGATTCTA
ATGTTAAAAATTTGTATGATAAGGTACGATTACAACCTTAGAGATAATGCTAAGGAACTTG

GAAATGGGTGTTTTGAATTTTATCATAAGCGAGATAACGAATGCATGGAATCTGTTTCAA
ATGGTACCTATGATTATCCACAATATTCTGAAGAAGCTAGACTTAAACGAGAAGAAATTT
CTGGTGTTAAGTTAGAATCAATAGGAATCTATCAAATTTCTTCTATCTATTCAACTGTAG
CTTCTTCATTGGCTTTAGCTATTATGGTTGCTGGACTTTCTTTGTGGATGTGCTCTAACG
GATCTCTTCAATGCCGAATTTGTATT

v) Codon-optimised neuraminidase (NA)

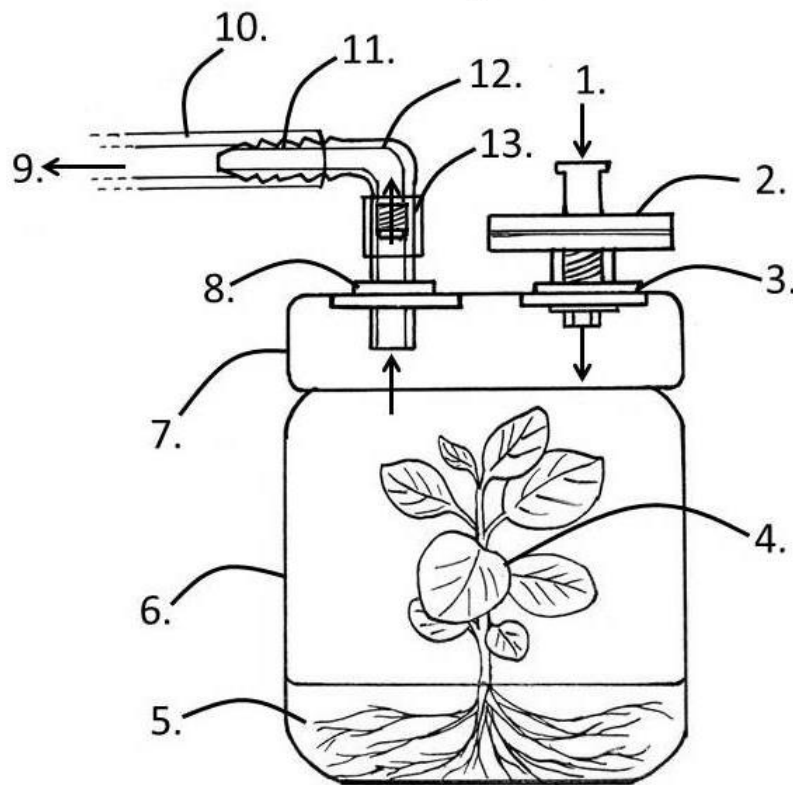
ATGAATCCCAATAAGAAAATTATAACTATAGGATCAATATGTATGGTAACTGGAATGGTA
TCTTTAATGCTTCAAATAGGAAATCTTATATCTATTTGGGTATCTCATTCAATTCATACTG
GAAATCAACATAAGGCTGAACCAATTTCTAATACTAATCTTCTTACCGAAAAAGCTGTTG
CTTCAGTAAAGTTAGCTGGAAATTTCTTCACTTTGTCCAATTAATGGGTGGGCTGTTTATT
CTAAGGATAATTCAATTCGAATAGGATCTAAGGGAGATGTTTTTGTAAATAAGAGAACCTT
TTATTTCTTGTTACATTTGGAAATGCCGAATTTCTTTTTAACCCAAGGAGCTTTGCTTAA
TGATAAGCATTCTAATGGGACTGTTAAGGATCGATCTCCTCATAGAACCTTGATGTCATG
TCCCGTAGGAGAAGCTCCATCTCCTTATAATTCAAGATTTGAATCTGTTGCTTGGTCTGC
TTCAGCTTGCCATGATGGAAATTTCTGGCTTACTATTGGAATATCAGGACCTGATAATGG
GGCTGTTGCTGTATTGAAATATAATGGGATTACTGATACCATTAAGTCTTGGCGAAA
TAACATACTTAGAACTCAAGAATCTGAATGTGCTTGCCTAATGGGTCTTGCTTTACTGT
AATGACCGATGGACCCTCAAATGATCAAGCATCTCATAAAATTTTTAAGATGGAAAAGG
GAAAGGTTGTTAAATCTGTAGAATCTGATGCTCCTAATTATCATTATGAAGAATGTTCTT
GCTATCCCGATGCTGGAGAAATTTGCTTGTGTTGCCGAGATAATTGGCATGGATCTAAT
AGACCTTGGGTTTTCTTTAATCAAATCTTGAATATCAAATTGGATATATATGTTCTGGA
GTATTTGGAGATAATCCCCGACCAATGATGGAGCTGGATCTTGCGGACCAGTTTCTTC
AAATGGGGCTTATGGAGTTAAAGGATTTTCTTTAAGTATGGAAATGGGGTTTGGATTG
GACGAATAAATCAACCAATCCACGATCTGGATTTGAAATGATTTGGGACCCAAATGGG
TGGACTGAAACCGATTCTTCATTTTCTGTTAAACAAGATATTGTAGCTATTACTGATTGG
TCAGGATATTCTGGATCTTTTGTCAACATCCCGAACCTTACCGGATTGGATTGTATTCGA
CCATGCTTTTGGGTTGAATTAATACGAGGAAGACCTAAAGAATCAACTATTTGGACCTCT
GGATCTTCAATATCATTTTGTGGAGTTAATTCTGATACCGTAGGATGGTCTTGGCCCGAT
GGAGCTGAACTTCCCTTACTATTGATAAG

vi) Sequenced *NptI* gene from pKCZMCS

ATGAGCCATATTCAACGGGAAACGTCTTGCTCAAGGCCGCGATTAAATCCAACATGGA
TGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAA
TCTACCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGT
AGCGTTGCCAATGTTGTTACAGATGAGATGGTCAGACTAACTGGCTGACGGAATTTAT
GCCTCTCCGACCATCAAGCATTTTATCCGTAATCTCTGATGATGCATGGTTACTCACCAC
TGCGATCCCAGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTGAGGTGAAA
ATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCTGTTTGAATT
GTCCTTTTAAACAGCGATCGCGTATTTCTGCTCGCTCAGGCGCAATCACGAATGAATAAC
GGTTTGGTTGATGCGAGTGATTTTGTGACGAGCGTAATGGCTGGCCTGTTGAACAAGT
CTGGAAAGAAATGCATAAACTTTTGCCATTCTCACCAGATTGAGTCGTCATCATGGTG
ATTTCTCACTTGATAACCTTATTTTGTGACGAGGGGAAATTAATAGGTTGTATTGATGTTG
GACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACCTGCTCGG
TGAGTTTTCTCCTTCAATACAGAAACGGCTTTTTCAAATATGGTATTGATAATCCTGAT
ATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAA

G) Prototype design of a culture jar with a vented lid

Fig 1.



- | | |
|---------------------------|-------------------------|
| 1. Air inflow | 8. Sealing gasket |
| 2. Disposable filter unit | 9. Air outflow |
| 3. Airtight rubber gasket | 10. Hose to vacuum pump |
| 4. Cultured plant | 11. Hose nozzle |
| 5. Culture media | 12. Elbow fitting |
| 6. Glass culture jar | 13. One-way valve |
| 7. Jar cap | |

The prototype design of the vented lid allows the unidirectional flow of air through the culture jar. When a vacuum is applied (9, 10), air with high humidity (in the jar) is extracted through the one-way valve (13). The decrease in pressure draws air of lower humidity from the outside (1) through a disposable filter unit (2) which sterilises the air. This ensures that the contents of the jar are kept sterile at all times. The overall effect would be a net reduction in humidity within the culture vessel.

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Presented Abstracts

Construction of a Gateway-Like Vector System for Transplastomic Integration of a Transgene in *Nicotiana benthamiana* Chloroplasts

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Chloroplasts are subcellular organelles found in all higher plants. They are a fundamental component in many biochemical reactions including photosynthesis. Here we report the construction of a highly versatile gateway-like chloroplast transformation vector for the selective transformation of *Nicotiana benthamiana* chloroplast genome. The completed chloroplast transformation vector was composed of two independent cassettes responsible for transgene expression and transplastomic integration respectively. The transgene expression cassette used the constitutive *Prrn* plastid promoter to drive the expression of transgene(s) as well as the *psbA* 5'-UTR to stabilise the translation of transgenic products. The transformation vector backbone contained the *aadA* gene conferring resistance against the aminoglycoside antibiotics, spectinomycin and streptomycin to allow the selection of transformed *N. benthamiana* explants on antibiotic media. The completed transformation vector was delivered into the host plant via particle bombardment where it directed the integration of the transgene expression cassette into a specific region of the chloroplast genome via homologous recombination of the left (INSL) and right (INSR) flanking regions. Preliminary evaluation of the transformation vector using green fluorescent protein (GFP) indicated successful expression at the protein level. Expression of more complex transplastomic products such as the neuraminidase (NA) surface antigen of the avian influenza strain H5N1 is currently underway. The successful expression of NA in the chloroplast of plants may prove useful for the development of a putative form of edible vaccines which is safer and more economical in future.

***In planta* chloroplast engineering of the avian influenza vaccine candidates through the gateway cassettes of a reporter gene**

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The possibility to directly manipulate the prokaryotically organised circular genome of the chloroplast has paved the way for the convenient expression and accumulation of high levels of transgenic protein within the chloroplasts. With regard to stable and high levels of transgene expression, new transformation vectors based on the versatile pKCZ chloroplast transformation vector backbone were developed to facilitate the delivery and integration of transgenes into the chloroplast genome (plastome). Some key elements of the chloroplast transformation vector were modified to improve its performance such as the replacement of the canonical *Prrn* plastid promoter with the *PtrnfM* promoter to eliminate unwanted intermolecular recombination events and to enhance the regeneration of homoplastomic plants. The 5'-untranslated leader region (5'-UTR) of the *psbA* gene was also incorporated to confer improved levels of mRNA stability and translation efficiency leading to an increase in the yield of transgenic protein accumulation within the transformed chloroplasts. The improved chloroplast transformation vectors were firstly verified by the expression of the Green Fluorescence Protein (GFP) reporter gene. Subsequently, the codon-optimised hemagglutinin (HA) and neuraminidase (NA) surface antigens of the highly pathogenic avian influenza (HPAI) strain H5N1 were incorporated into the improved vector through the gateway approach by substitution of the GFP cassette with the finalised HA and NA expression cassettes. In order to improve time and cost efficiency, preliminary evaluation of the functionality of the completed chloroplast transformation vectors were first conducted in a model prokaryotic system (*E. coli*) prior to transformation into plants as protein expression systems in plastids share a prokaryotic origin. The chloroplast-specific expression cassettes showed successful expression of GFP in *E. coli* further confirming the endosymbiotic theory for the origin of chloroplasts as well as the conservation of prokaryotic gene expression machinery between the two systems. The expressivity and functional characteristics of the HA and NA in chloroplast are currently underway. The global spread of HPAI of the subtype H5N1 has encouraged the development of human vaccines against potential pandemic outbreaks. The stable expression of immunogenic HA and NA surface antigens within the chloroplasts of plants will provide valuable insight for the potential development of a stable source of edible vaccines against the H5N1 infection.

Towards development of plastid-based candidate vaccines against avian influenza strain H5N1

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The possibility to directly manipulate the prokaryotically organised circular genome of the chloroplast has paved the way for the convenient expression and accumulation of high levels of transgenic protein within the chloroplasts. The expression and accumulation of biopharmaceuticals such as transplastomic vaccines in the form of viral surface antigens in the chloroplasts of plants can be highly beneficial in curbing the infection of highly pathogenic strains of avian influenza. Influenza virus class A is the main causal agent of outbreaks of highly pathogenic avian influenza (HPAI) such as the H5N1 strain of avian influenza infection. The global spread of HPAI of the subtype H5N1 has encouraged the development of human vaccines against potential pandemic outbreaks. However, the current approach to the production of human vaccines remains cumbersome, often requires high investment costs and is subjected to reliability issues due to the rapid mutation of avian influenza viruses within the infected host. In this study, we investigate the feasibility of expressing the hemagglutinin (HA) and neuraminidase (NA) surface antigens of the H5N1 strain of avian influenza virus in the plastids of tobacco plants. Synthetic genes for the HA and NA surface antigens were synthesised to optimise their expression in the chloroplasts of tobacco. Chloroplast-specific gene expression cassettes were then constructed for the expression of the codon-optimised HA and NA transgenes. The completed expression cassettes were subsequently cloned into customised chloroplast transformation vectors for the delivery and integration of the transgene expression cassettes into the chloroplast genome. Preliminary evaluation of the chloroplast-specific gene expression cassettes were performed in the prokaryotic model organism (*E. coli*). Interestingly, the chloroplast-specific expression cassettes showed successful expression in *E. coli* which indicate that the mechanisms for the regulation of gene expression in chloroplasts are highly similar to that of prokaryotes. This observation further enforces the endosymbiotic theory for the origin of chloroplasts. Biolistics-mediated transformation of tobacco leaf explants with the recombinant plasmid constructs is currently underway. The stable expression of immunogenic HA and NA surface antigens within the plastids of plants will provide valuable insight for the potential development of a stable source of edible vaccines against the H5N1 infection. Crop plants expressing the immunogenic HA and NA surface antigens can then be generated and incorporated into poultry feed. The prevention of the spread H5N1 at the primary host level will in turn prevent the spread of the infection across the species barrier to humans.