Development of a Transient, High-level Expression Platform for Protein Production in Plants

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Abstract

Agroinfiltration is a simple and effective method of delivering transgenes into plant cells for the rapid production of recombinant proteins. In combination with virus-based vectors, Agroinfiltration has become the preferred transient expression platform for both researchers and industry to manufacture biologics in plants, particularly “rapid response” vaccine candidates and antibodies. Despite its popularity, few studies have sought to improve the efficiency of Agroinfiltration in order to further increase protein yields. This study aimed to increase Agroinfiltration-based transient gene expression in *Nicotiana benthamiana* by improving all levels of transgenesis. Using the benchmark pEAQ-HT non-replicating, deconstructed virus vector system and the GUS reporter enzyme, physical, chemical and genetic features were independently assessed for their ability to enhance *Agrobacterium*-mediated transformation frequencies, elevate transgene expression levels and improve protein yield.

Physical characteristics of the Agroinfiltration process, including bacterial strain, culture density and time to harvest all influenced GUS expression levels. The greatest effects were observed using Agrobacterium strain Agl1 at high density (between OD_{600} = 1.0-3.0), four days post infiltration. The addition of chemical additives such as acetosyringone, α-Lipoic acid and Pluronic F-68 in the infiltration media were shown to increase GUS expression in a dose dependent manner. Co-expression of the *Tomato bushy stunt virus* (TBSV) p19 and *Cucumber mosaic virus* (CMV) 2b suppressors of post-transcriptional gene silencing (PTGS), individually and in combination, strongly increased transient expression about 6-fold, as did the *Arabidopsis* BAG4 stress tolerance gene product which enhanced GUS expression 2-fold. While the co-expression of plant-derived heat shock proteins had no effect, a simple 37 °C heat shock treatment to the whole plant two days post infiltration significantly increased GUS levels, 5-fold.

Histones play an important role in stabilising transfer DNA (T-DNA) during *Agrobacterium* transformation and the co-expression of some plant histones can improve transient gene expression. In this study, the gene encoding *Arabidopsis* histone H2A was isolated, cloned into an expression cassette and its effects on transient GUS reporter expression evaluated from either transient co-delivery or from an integrated copy, in *N. benthamiana.* The H2A protein increased GUS levels about 2-fold when co-expressed transiently but failed to augment GUS levels when stably expressed in transgenic plants.

In order to further amplify transient reporter levels in *N. benthamiana*, a replicating vector system based on the genetic machinery of *Tobacco yellow dwarf mastrevirus* (TYDV), a circular, ssDNA geminivirus that infects dicots was developed. The vector, termed RCR-GUS, contained an expression cassette based on pEAQ-HT encoding the GUS reporter enzyme, flanked by copies of the TYDV large intergenic region and was capable of replicative release and rolling circle based amplification by the TYDV replication associated proteins, Rep and RepA. Using Agroinfiltration, the effects of co-expressing TYDV Rep and RepA with pRCR-GUS were assessed independently and in combination. Addition of Rep and RepA together initiated replicative release of the pRCR-GUS cassette resulting in the formation and accumulation of circular dsDNA episomes, molecular forms indicative of rolling circle replication (RCR). Together these proteins enhanced GUS expression about 5-fold over base levels. Interestingly, co-expression of either Rep or RepA alone also increased base GUS expression from pRCR-GUS about 2-fold. This was likely a consequence of two different mechanisms; replication and/or cell cycle control. Both proteins also enhanced transgene expression from a non-replicating control vector, as did the cell cycle control proteins encoded by three related viruses. The abundance of these replication-associated proteins strongly influenced reporter expression, with low levels of the virus gene products generating highest GUS expression. Mutations in the conserved LXCXE motif of both Rep and RepA, showed both proteins likely interact with a plant Retinoblastoma protein to manipulate the cell cycle and subsequently increase transient GUS expression, perhaps by stimulating cellular proliferation, cellular gene expression machinery and/or improving *Agrobacterium* transformation frequencies.

Using those features proven to enhance Agroinfiltration-mediated transient expression a complete and novel expression platform was assembled termed OPTrans (Optimal Transgenesis). This system was strategically engineered to incorporate elements or include treatments that would enhance gene transcription and translation, improve protein stability and positively influence transgenesis and *Agrobacteria*:host interaction. OPTrans vectors were constructed around the pEAQ-HT “Hyper-Translatable” system and were capable of expressing the TBSV p19, CMV 2b, At_BAG4 and TYDV Rep/RepA enhancer proteins. OPTrans vectors were co-delivered in an optimised infiltration medium, MMA-LP (including 500 µM acetosyringone, 5 mM α-Lipoic acid and 0.002 % Pluronic F-68) and plants were physically heat shocked at 37 °C, two days post infiltration. GUS levels obtained with OPTrans were 6-8 fold higher than that from the pEAQ-HT system, representing some of the highest levels recorded from a non-replicating virus vector and rivalling those

afforded by the replicating virus vectors such as MagnICON. OPTrans is, therefore, an effective hyper-expression system and a convenient platform for biofarming recombinant proteins in plants.

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List of abbreviations

aa	amino acids
APS	ammonium persulfate
BAG	Bcl-2 associated athanogene
BAP	6-benzylaminopurine
BeYDV	*Bean yellow dwarf virus*
bp	basepair(s)
BWYV	*Beet western yellows virus*
°C	degrees celsius
CaMV	*Cauliflower mosaic virus*
cDNA	complimentary DNA
CITE	cap-independent translation enhancers
CMV	*Cucumber mosaic virus*
CP	coat protein
CPMV	*Cowpea mosaic virus*
CTAB	cetyl trimethyl ammonium bromide
C-terminal	carboxyl-terminal
dH_20	distilled water
DIG	digoxygenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
dpi	days post infiltration
dsDNA	double stranded DNA
DTT	dithiothreitol
EAQ-HT	"Easy and Quick – Hyper translatable" vector
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
eIFs	eukaryote initiation factors
ER	endoplasmic reticulum
g	gram(s)
g	relative centrifugal force in units of gravity
GRAB	Geminivirus RepA-binding
GUS	β-glucuronidase
h	hour(s)
HRP	horseradish peroxidase
Hsp	heat shock protein
IAA	iso-amyl-alcohol
IME	intron mediated enhancement
IPTG	iso-propyl- β-D-thiogalactopyranoside
IR	intergenic region
IRES	internal ribosome entry site
kb	kilobasepair(s)
kDa	kilodalton(s)

L	litre(s)
LB	h
LIR	large intergenic region
M	molar
mA	milliamp
MCS	multiple cloning site
min	minute(s)
mg	milligram(s)
mL	millilitre(s)
mM	millimolar
MMA	MES; $MgCl_2$; acetosyringone (infiltration buffer)
MMA-LP	MES; $MgCl_2$; acetosyringone; α-lipoic acid; pluronic F-68
mRNA	messenger RNA
MSV	*Maize streak virus*
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl-β-D-glucuronide trihydrate
NAC	NO APICAL MERISTEM, ATAF and CUP-SHAPED COTYLEDON
ng	nanogram(s)
nm	nanometre
nM	nanomolar
nos	gene encoding nopaline synthase
nt	nucleotide(s)
N-terminal	amino-terminal
NTP	nucleoside triphosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCD	programmed cell death
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
pmol	picomole
PVP	polyvinylpolypyrrolidone
pRB	retinoblastoma protein
PRSV	*Papya ringspot virus*
PTGS	post translational gene silencing
PVX	*Potato virus X*
PVY	*Potato virus Y*
RBR	Retinoblastoma-related protein
RCR	rolling circle replication
RdRp	RNA-dependent RNA polymerase
REn	replication enhancer protein
Rep	replication associated protein
RepA	replication associated protein A
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rpm	revolutions per minute

RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)
SDS	sodium dodecyl sulphate
SE	Standard error
SIR	small intergenic region
siRNA	short interfering RNA
SOB	super optimal broth
SOC	super optimal broth with catabolite repression
sp.	species
SSC	standard saline citrate
ssDNA	single stranded DNA
TAE	tris acetate EDTA
TBSV	*Tomato bushy stunt virus*
TbYDV	*Tobacco yellow dwarf virus*
TCV	*Turnip crinkle virus*
T-DNA	transfer-DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
TLS	3' tRNA-Like Structure
TMV	*Tobacco mosaic virus*
ToLCV	*Tomato leaf curl virus*
TrAP	transcriptional activator protein
tris	tris(hydroxymathyl)aminomethane
TSP	total soluble protein
TTBS	Tween Tris Buffer Saline
TWEEN 20	polyoxyethylene (20) sorbitan monolaurate
U	units
µg	microgram(s)
uidA	reporter gene encoding β-glucuronidase
µL	microlitre(s)
µm	micrometre(s)
µM	micromolar
UTR	untranslated region
V	volts
(v/v)	volume per volume
WDV	*Wheat dwarf virus*
WT	Wildtype
(w/v)	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide-cyclohexylamine salt
YM	yeast mannitol broth

Declaration

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: QUT Verified Signature

Date: November 2014

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Chapter 1: Literature Review

1.1 Biofarming: Plants as Bioreactors for the Production of Recombinant Proteins

There remains a growing demand for the safe and inexpensive production of recombinant proteins to be used in a variety of applications including research, industry and medicine (Ganz *et al.*, 1996; Pen, 1996; Rybicki, 2010; Stoger *et al.,* 2014; Twyman *et al.,* 2013). The isolation of proteins from natural sources is often impractically expensive and laborious and as such, considerable research has focused on cheap and high-yielding alternative protein production platforms. Advances in biotechnology have seen the development of numerous genetically modified organisms designed specifically for recombinant protein production, including bacterial and yeast fermenters, insect and mammalian cell culture and transgenic animals and plants (Streatfield, 2007). These production systems, however, are often limited by low quantity or poor quality protein yields, expensive downstream processing costs, the requirement of significant capital and/or the impracticality of up-scaling production to meet demands due to expense or generation time (Giddings, 2001; Hood *et al*., 1999). Also, bacterial and animal generated products intended for human use can pose inherent safety risks in the form of pyrogens, endotoxins and pathogens such as viruses and prions (Yin *et al*., 2007).

Biofarming specifically refers to the production and cultivation of transgenic plants for their exploitation as recombinant protein factories. Plants as bioreactors represent an attractive alternative to conventional expression systems, in that they are relatively simple and inexpensive to grow and generate large quantities of biomass that can be easily scaled up or down depending on demand (Doran, 2000). In addition, plants are essentially free from human pathogens and plant made proteins are therefore considered “safe” (Ma *et al*., 2003; Streatfield, 2007). As higher eukaryotes, plants are able to correctly process, fold and assemble proteins (Faye *et al.*, 2005; Hiatt *et al.*, 1989; Ma *et al*., 2003). There are, however, inherent differences between plant and mammalian glycosylation patterns that may have immunological consequences if the protein is destined for human use. To overcome this, significant research has been directed towards humanizing plant glycosylation by genetically manipulating the enzyme pathway (Lerouge *et al*., 2000; Sriraman *et al*., 2004).

The commercial viability of biofarming is primarily dependent on the yield of the recombinant protein, which can differ depending on the candidate and the application. For

example, low cost industrial enzymes are generally required in crude but large volumes, whereas human therapeutics are preferred in lower volumes but in a highly pure form (Hood *et al*., 1997; Streatfield *et al*., 2002). As such, production costs for plant made pharmaceuticals destined for human use can be high (De Wilde *et al*., 2000; Pen, 1996) due to complex downstream processing and associated safety requirements. Several novel plant gene expression systems have recently been developed capable of producing quantities of protein far beyond those previously recorded (Gleba *et al*., 2005; Lindbo, 2007a; Sainsbury *et al*., 2009; Sainsbury and Lomonossoff, 2008). These technologies all use *Agrobacterium*-based transformation to deliver deconstructed virus vectors that effectively amplify transient gene expression. While these advances may see biofarming evolve from scientific potential to a commercially viable protein production platform, a number of factors can still negatively affect protein expression levels, including RNA silencing, low translational fidelity, poor translational and post-translational processing and targeted proteolysis. This review discusses the various physical, chemical, molecular and virus based methods reported to influence *Agrobacterium*-mediated transformation efficiency, transgene expression and protein accumulation.

1.2 Modes of transformation

Foreign genes may be introduced into plants by either stable or transient transformation. Stable gene expression is defined as the integration of a coding sequence into the host plant genome and is the favoured strategy for long-term protein production. This system is appealing in that it only requires a single transgenic line that can be multiplied and propagated in the same manner as non-transgenic plants allowing for continuous recombinant protein production. However, stable transformation is generally a long process and often provides relatively low levels of transgene expression (Mason *et al*., 1992; Twyman *et al*., 2003). The latter is primarily due to two factors; a “position effect”, where the site of transgene integration into the plant genome can influence expression levels, and “gene silencing”, a phenomenon that results in transgene mRNA degradation and ultimately a complete ‘shut down’ of its expression (Johansen and Carrington, 2001; Nocarova *et al*., 2010).

Transient expression is a rapid, high-level expression system and as such is often employed to assess features of a plant transformation cassette (e.g. promoter strength, gene codon modification, protein targeting and intron mediated enhancement) prior to stable transformation. As gene expression occurs predominantly from extra-chromosomal copies, transient expression is not prone to the position effect (Kapila *et al*., 1997) and

recombinant protein accumulation usually peaks within 3-7 days post transformation (Janssen and Gardner, 1990). Transient expression is still subject to the phenomenon of gene silencing, however, its effects may be overcome by the co-expression of a silencing suppressor (Johansen and Carrington, 2001; Voinnet *et al*., 2003). The derivation and function of viral suppressors of gene silencing will be discussed in detail in the following chapters.

1.2.1 Transformation techniques

1.2.1.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is an oncogenic soil pathogen responsible for the agronomically important crown gall disease (Nester *et al*., 1984). During the infection process, phenolic compounds released from the plant wound site trigger the conjugative transfer of a DNA segment from the bacterial tumour inducing plasmid (Ti plasmid) to the host plant genome (Stachel *et al*., 1986). This transfer DNA (T-DNA) integrates in a semi random manner and carries genes encoding biosynthetic enzymes and plant hormones. Expression of these genes in the plant alters hormone levels and normal cell cycle progression producing tumour-like growths and an environment conducive to the survival of the bacteria. In order to manipulate the natural ability of *Agrobacterium* to transfer its genes to a plant genome, plant biotechnologists “disarmed” the Ti plasmid by deleting the tumour-inducing genes and stripping the T-DNA down to two short (25 bp) repeats, commonly referred to as the right and left borders (Barker *et al*, 1983; Hoekema *et al*, 1983). By introducing the gene of interest, under the transcriptional control of a suitable promoter, between these borders, the bacteria could now be used as an efficient vehicle for gene transfer into plants without the associated tumour-like symptoms (Fraley *et al*., 1986). Transgene expression can occur from both free and integrated forms of T-DNA thus providing for either short-term or stable expression (Nam *et al*. 1999). Suspensions of transgenic *Agrobacterium* may be delivered into plants by two methods of “Agroinfiltration”; vacuum infiltration based on the method of Bechtold *et al* (1993), where vacuum pressure is used to force suspensions into leaf tissue or the method of Schöb *et al*. (1997) with suspensions of *Agrobacterium* delivered into the underside of leaves plantlets using a blunt tipped plastic syringe and applying gentle pressure (Figure 1.1).

Agrobacterium-mediated transformation generally results in low transgene copy number, minimal transgene rearrangement and is typically more efficient than biolistic transformation (Hansen and Wright, 1999; Hiei *et al*., 1994). The major disadvantage of *Agrobacterium*-mediated transformation is that the bacterium tends to have a narrow host

range (i.e. it does not infect all plant species). As a result, considerable effort has been made to optimise *Agrobacterium* infectivity in a number of plant species (e.g. graminaceous monocots), through the addition of inducer molecules such as acetosyringone, antioxidants and anti-apoptotic compounds (all of which will be discussed in greater detail in the following chapters). In addition, the use of super-binary vectors which harbour additional virulence (*vir*) genes in the Ti plasmid, has also increased the ability of the bacteria to infect recalcitrant crops, although this effect appears to be dependent on the bacterial strain (Hiei *et al*., 1994; Vain *et al*., 2004), and is most evident when used in combination with laboratory strain LBA4404 (Komari *et al*., 2006).

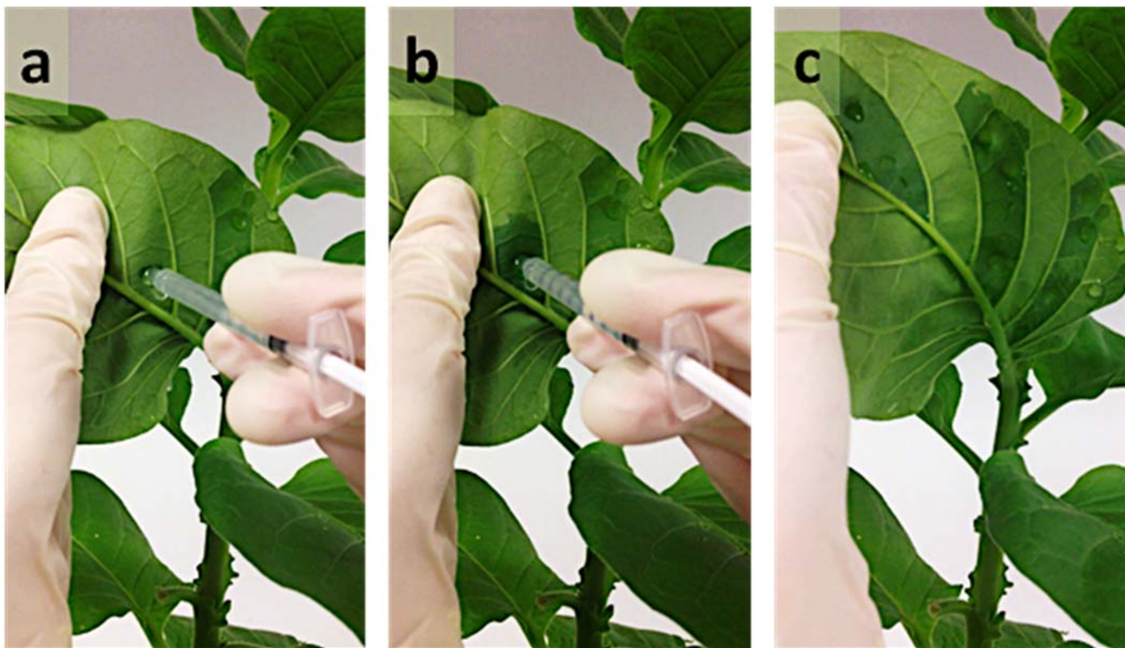

Figure 1. 1 Agroinfiltration of *N. tabacum* leaves for transient transgene expression
Suspensions of transgenic *Agrobacterium* in infiltration medium may be delivered into the underside of leaves using a blunt tipped plastic syringe and applying gentle pressure (Dugdale *et al*., 2014).

1.2.1.2 Biolistic transformation

Biolistic or microprojectile bombardment is also a means for both stable and transient plant transformation and was previously the preferred method for plant species considered recalcitrant to *Agrobacterium*-mediated infection (e.g. monocotyledons including sugarcane). In this case, plasmid DNA is directly coated onto dense, microscopic (~1 μm) inert particles (preferably gold), and bombarded by gas propulsion into the host cell at high speed under pressure (Christou, 1995). This approach often leads to high incidences of transgene rearrangements (Finnegan and McElroy, 1994; Kohli *et al*., 1998; Pawlowski and Somers, 1996) and a high transgene copy number which can result in gene silencing and

genomic instability (Kooter *et al*., 1999). Agroinfiltration has largely superseded microprojectile bombardment as it is a superior technology and improvements in Agrobacterium-mediated transformation have allowed for the transformation of many recalcitrant crops (Shrawat, and Lörz 2006).

1.2.1.3 RNA virus-based vectors

The use of plant viruses as recombinant vectors is appealing as viruses have specifically evolved to utilise a compact, autonomously replicating genome to induce the rapid high titre production of their proteins *in planta* (Mor *et al*., 2003; Porta and Lomonossoff, 1996, 2002; Yusibov *et al.*, 2013). To date, the most effective virus vectors have been adapted from the RNA viruses, (in particular *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) or *Cowpea mosaic virus* (CPMV)), the largest and most widely studied group of plant viruses. Foreign genes may be expressed as read-through fusions with a viral protein or from a viral subgenomic promoter. For read-through fusions, the sequence of interest is introduced immediately downstream of the viral coat protein (CP) coding sequence. In the case of TMV, its CP accumulates to as much as 10% of the dry weight of an infected leaf (Copeman *et al*., 1969). For non-fusions, the gene of interest is usually inserted between a duplicated CP subgenomic promoter and the 3’ untranslated region of the viral genome, downstream of the original CP sequence. As such, instability of the foreign gene in the viral genome can be problematic. The preferred method to transfer the viral genome and transgene into the plant is to use *Agrobacterium tumefaciens* to deliver T-DNA containing cDNA copies. Transcription of T-DNA in the plant nucleus generates RNA that is then capable of self-replication in the cytoplasm.

More recently, a second generation of virus vectors have been developed in which “deconstructed” virus genomes have been streamlined by removing non-essential viral sequences (for example the CP gene). Two such systems based on the genome of TMV, “TRBO” and “Magnifection”, have been adopted by a number of research groups for the manufacture of product prototypes for pharmaceutical, animal health and chemical markets (Daniell *et al.*, 2009; Lindbo, 2007b). Both of these systems are based on a deconstructed TMV genome and are introduced transiently into the host by *Agrobacterium*-mediated infiltration. These virus vectors are capable of self-replication and, in some cases, incapable of systemic movement due to deletion of the coat protein gene. The latter feature alleviates biosafety concerns associated with recombinant virus spread (Gleba *et al*., 2005).

The T-DNA region of the TRBO expression vector is essentially a complete TMV genome under the transcriptional control of a CaMV 35S promoter (Figure 1.2). The CP gene is replaced by the gene of interest (Lindbo, 2007b) and these vectors can reportedly increase expression levels up to ~100-fold over non-virus based expression systems. This increase is similar to that obtained using an unmodified TMV-based vector co-expressed with the TBSV p19 silencing suppressor (Lindbo, 2007a).

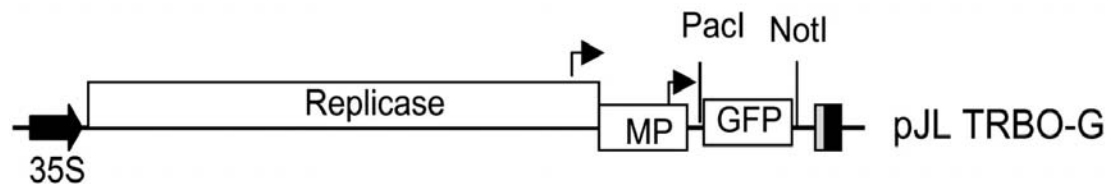

Figure 1. 2 Schematic representation of TRBO TMV-based expression cassette
Block arrow = CaMV duplicated 35S promoter, black box = CaMV polyA signal sequence/terminator, light grey box = ribozyme, bent arrows = sub-genomic promoters, coding regions = white boxes. Note the CP has been replaced by the GFP reporter gene (Lindbo, 2007b).

Unlike TRBO, the replicase and movement protein (MP) coding regions of the MagnICON system were subjected to a series of point mutations (a total of 97 silent nucleotide substitutions) to remove putative cryptic splice sites and increase the G/C content. Additionally, 19 intron sequences were inserted into these coding regions to further increase expression. In this system, plants are systemically transfected by vacuum-infiltration of *Agrobacterium* harbouring the binary plasmid. Protein expression levels generated by MagnICON have been reported to be 1000-fold and 106-fold greater in *N. benthamiana* and *N. tabacum,* respectively, compared to non-optimised TMV vectors (Gleba *et al*., 2005; Marillonnet *et al*., 2005).

Both systems can reportedly yield between 1-5 g of recombinant protein per kilogram of fresh weight (up to 80% total soluble protein), and therefore represent robust biofarming platforms (Gleba *et al*., 2005, Lindbo, 2007b). However, virus-directed replication of RNA has a number of undesirable features, including restrictions on the size of insert that can be accommodated without affecting replication and compromised fidelity of transcripts due to the lack of proofreading by RdRp (Scholthof *et al*., 1996; Steinhauer *et al*., 1992). Further, the biosafety concerns regarding release of a genetically modified *Agrobacterium* would almost certainly preclude this strategy for broad scale biofarming. Further, RNA virus vectors are limited by their cognate virus host range and, as such, the more common systems are generally not suited to plant species outside the *Solanaceae* family.

1.2.1.3.1 CPMV-based pEAQ Vectors

The genome of CPMV has been used effectively as a virus vector for transgene expression in plants for over a decade (Porta and Lomonossoff, 1996). However, it wasn't until 2008 that Sainsbury and Lomonossoff (2008) showed high-level expression from a CPMV-based vector could be achieved in the absence of replication. The researchers identified two elements of the CPMV genome that were essential to recombinant protein accumulation, namely the 5' and 3' UTRs of RNA-2. By placing these sequences immediately up and downstream of a reporter gene, respectively, they were able to increase transgene expression 100-fold (0.3 g/kg), one of the highest expression levels observed for a non-replicating expression platform. How these UTRs effected this increase has yet to be fully determined, however, it is likely these sequences are translation rather than transcription enhancers. As these vectors did not rely on replication, this approach overcame the biosafety and fidelity concerns associated with replicating RNA virus systems.

Based on these findings, Sainsbury *et al*. (2009) later developed a versatile CPMV-based vector for easy and quick expression of heterologous proteins in plants. This vector, referred to as pEAQ-HT (HT an acronym for hyper-translatable) was tailored specifically for transient expression and based on the pBIN-Plus backbone with some modifications; vector size was effectively halved by removing 7 kb of non-essential sequence, a multiple cloning site containing unique restriction enzyme sites was introduced for N- or C-terminal 6XHIS tag fusions with the gene of interest, and a p19 viral silencing suppressor expression cassette was included in the T-DNA (Sainsbury *et al*., 2009). The vector has been subsequently used to produce milligram quantities of a recombinant antibody (IgG 2G12) and human serum albumin (Sun *et al*., 2011) and human gastric lipase, up to 0.5 mg per g of infiltrated *N. benthamiana* leaf tissue (Vardakou *et al*., 2012).

1.2.1.4 DNA virus-based episomal (extrachromosomal) expression

To date, the only DNA viruses to have been successfully exploited as vehicles for high-level gene expression in plants are the *Geminiviridae*, a family of viruses with small, circular, single-stranded DNA genomes. Geminiviruses replicate their genome through a process called "rolling circle replication" (RCR) which is dependent on two genomic *cis*-acting elements (the origin of first strand and second strand synthesis), and the *trans*-acting control of the virally encoded Replication-associated protein (Rep) (Laufs *et al*., 1995). RCR involves two distinct stages; (i) the conversion of the ssDNA genome into a transcriptionally active dsDNA intermediate allowing for the expression of virus encoded proteins (Gutierrez, 1999; Hanley-Bowdoin *et al*. 2000), and (ii) the synthesis of ssDNA from dsDNA, catalysed

by the covalent binding of Rep to a conserved nonanucleotide stem-loop sequence within the origin of plus-strand synthesis. Rep nicks the virion-sense strand in a site-specific manner leading to its displacement and subsequent extension via host DNA polymerases (Gutierrez, 1999, Hanley-Bowdoin *et al*., 2000, Laufs *et al*., 1995). The circular genome allows for the continuous synthesis of DNA, which in turn is nicked and ligated by Rep leading to the accumulation of multiple genomic copies (Campos-Olivas *et al*., 2002).

Geminivirus-based gene amplification vectors are structured such that the gene expression cassette is positioned between tandem repeats of the origin of first strand synthesis. Inside the nucleus, the expression cassette is converted to a transcriptionally active circular dsDNA, extra-chromosomal, plasmid-like molecule or episome that can both express the gene of interest and serve as a template for further replication (Timmermans *et al*., 1994). Rep is generally supplied on the same gene cassette or in *trans*, preferably under the control of an inducible promoter as an accumulation of Rep can negatively impact on plant development (Van Wezel *et al*., 2002). Episomal amplification has some important benefits over RNA virus-based and conventional stable transformation such as (i) the DNA-based system provides for very high transgene copy number, (ii) extra-chromosomal episomal DNA is not subject to the positional effect (Timmermans *et al*., 1992, 1994), and (iii) episomes are not methylated which may preclude them from the negative effects of methylation-dependent transcriptional gene silencing (Mor *et al.,* 2003).

The most effective Geminivirus expression vectors described to date have been based on the single component genomes of three Mastreviruses, *Maize streak virus* (MSV), *Bean yellow dwarf virus* (BeYDV) and *Tobacco yellow dwarf virus* (TYDV). Palmer *et al*, (1999) stably transformed Black Mexican sweet corn cells with a MSV-based system in which virion sense genes were replaced with the bialophos resistance gene (*bar*) under the direction of the CaMV 35S promoter. Between 38-60% of these cell lines contained replicating epsiomes, which were structurally and generationally stable and accumulated to approximately 500 copies per haploid genome. Enhanced *bar* RNA and protein levels were also associated with episomal vector DNA. Deconstructed BeYDV-based vectors have been successfully adapted for both transient and stable expression in a number of plant species including tobacco, potato and lettuce. In tobacco NT1 cells, the BeYDV-based vector increased transient GUS reporter gene expression about 20-40 fold (Mor *et al.*, 2003), while in transgenic potato plants the system enhanced expression of the Norwalk virus capsid protein (NVCP) vaccine candidate up to 10-fold (Zhang and Mason, 2005). In lettuce, the BeYDV system was used for the high-level transient expression of virus-like particles (VLP)

derived from the NVCP and therapeutic monoclonal antibodies against Ebola and West Nile viruses (Lai *et al*., 2012). Recently, a novel Geminivirus vector system based on the genome of TYDV was described that provides both high-level and inducible transgene expression *in planta* (Dugdale *et al*., 2013). The In Plant Activation system or INPACT, was used to over-express three very different recombinant proteins, including a human therapeutic (vitronectin), an industrial enzyme (trypsinogen) and a lethal ribonuclease (barnase). Unlike RNA virus vectors, the INPACT platform does not appear to be host restricted and essentially provides transient expression from a stably transformed plant.

1.3 Increasing *Agrobacterium*-mediated transformation efficiency

In general, the greater the efficiency and frequency of *Agrobacterium*-mediated transformation, the greater the levels of transgene expression (in transient transformation) and the higher the number of transgenic events obtained (for stable transformation). Accordingly, a number of studies have been devoted to optimising factors affecting *Agrobacterium*-mediated transformation, including host plant cultivar, *Agrobacterium* strain, co-cultivation temperature and light conditions and the inclusion of chemical additives during the infection process.

1.3.1 Selection of target species

Over the past decade, the number of plant species that can be routinely transformed by *Agrobacterium* has grown considerably. Some gymnosperms, several forest trees and fruit trees, various legumes, and cereal and non-cereal monocotyledons, once considered recalcitrant to the process, are now in a long list of transformable plants. For biofarming, non-food, high biomass crops that are amenable to *Agrobacterium*-mediated transformation, act as natural hosts to common virus vectors and can support high-level recombinant protein accumulation are preferred. While few target species can fulfill all of these criteria, some members of the *Nicotiana* genus come close, possessing a number of favourable bioreactor characteristics. Readily amenable to genetic manipulation by *Agrobacterium* and with the ability to accumulate significant leaf biomass and relatively high total soluble protein content, tobacco (*N. tabacum*) remains one of the most commonly used plant systems for protein production. Considered to be "biosafe" as it is neither a food nor feed crop (thereby limiting the risk of contamination of the human food chain) it is also a host to most RNA virus vectors and some DNA virus-based expression platforms (Twyman *et al*., 2003). Recombinant protein yield remains a limiting factor of the bioreactor system and in order to overcome this, Streatfield *et al*., (2007) proposed the selection of plant lines suited to high-level protein production and accumulation. Conley *et*

al. (2011) compared the transient expression levels of 52 *Nicotiana tabacum* varieties and found that high-biomass varieties produced a higher amounts of recombinant protein. This is consistent with the findings of Sheludko *et al.,* (2007), who compared biomass and transgene expression levels in six different *Nicotiana* species. They found *N. excelsior* had the highest overall levels of recombinant protein accumulation (3-4 mg compared to 0.5-1 mg in *N. benthamiana)* most likely due to its naturally large biomass. Although *N. benthamiana* was found to support the highest levels of reporter gene expression, this species is physiologically smaller and has a considerably lower biomass. Despite this*, N. benthamiana* is commonly used as a vehicle for transient transgene expression and remains the preferred species for laboratory-based expression studies and small-scale protein production.

1.3.2 Agrobacterium strain

The virulence and host range of *Agrobacterium* is strain-dependent (Wroblewski *et al.*, 2005). Common laboratory *Agrobacterium* strains are categorised according to their opine metabolism, octopine (LBA4404), nopaline (GV3101 and C58C1) and L-succinamopine (Agl1) and members originate from either of the wildtype progenitor isolates C58 (Agl1, GV3101, and C58C1) (Hamilton and Fall, 1971) or Ach5 (LBA4404) (Hoekema *et al.*, 1983). Wroblewski *et al.,* (2005) reported that hypervirulent strains (i.e. Agl1) can cause significant cell damage during the infection process resulting in reduced transgene expression levels. They found laboratory strain C58C1 caused minimal hypersensitive response (HR) in *Arabidopsis* and yielded the highest transient expression. This is in agreement with the findings of Wydro *et al.,* (2006) who showed lower transient GFP expression directed by strain Agl1 than that of strain LBA4404, but directly contradicts studies by Álvarez *et al*., (2004) and Li *et al*., (2010) who reported the invasive nature of strain Agl1 can result in higher transgene expression, perhaps due to a more efficient T-DNA transfer mechanism. The exact reason for these inconsistencies remains unclear, however, differences in plant species, expression vectors and reporter genes between studies may be of relevance. In addition, differences in plant physiology, age and growth conditions or *Agrobacterium* infiltration methodology may contribute.

1.3.3 Physical factors

The effectiveness of the *Agrobacterium* transfer machinery is highly temperature dependent with optimal temperatures for conjugation and tumourigenesis of 19°C and 22°C, respectively (Fullner and Nester, 1996; Riker, 1926). Increasing temperatures above 22°C, during co-cultivation, reportedly decreases transgene expression (Dillen *et al*. 1997).

Light conditions are also important; increasing the typical 16 h photoperiod to continuous light during co-cultivation can increase transgene expression (Zambre *et al*., 2003). If the density of bacteria used during co-cultivation is too high, a decrease in transformation efficiency is often observed due to excessive tissue damage and bacterial overgrowth (Archilletti *et al*., 1995; Howe *et al*., 1994; Mondal *et al*., 2001), whereas a low bacteria to host cell ratio can result in low transformation frequencies. There appears to be no consensus in the literature with respect to the optimal bacterial concentration with variations between plant species and explant type. As such, this parameter often requires optimisation by the individual researcher. Similarly, the optimal time for co-cultivation can vary anywhere between 2-7 days (Mondal *et al*., 2001), with longer co-cultivation times often resulting in a decreased transformation efficiency (De Kathen and Jacobsen, 1990; Dong and McHughen, 1993). The pH of the co-cultivation media is also critical, with pH 5.7 (Rogowsky *et al*., 1987; Stachel *et al*., 1986) or pH 5.6 (Mondal *et al*., 2001) reportedly the most effective.

1.3.4 Chemical additives

The plant-secreted phenolic, acetosyringone, induces *Agrobacterium* virulence gene expression (Hiei *et al*., 1994), and the inclusion of this inducer molecule in the co-cultivation media often improves transformation efficiencies (Jeoung *et al*., 2002; Rogowsky *et al*., 1987; Stachel *et al*., 1986; Wydro *et al*., 2006). Despite conflicting reports, the general consensus appears to be that this enhancing effect increases with increasing concentrations of acetosyringone peaking at ~450 μM (Wroblewski *et al*., 2005; Wydro *et al*., 2006), with higher concentrations sometimes leading to toxicity (Jeoung *et al*., 2002).

α-Lipoic acid and α-lipoic acid analogs are antioxidants that are required for normal cellular growth, cellular respiration and redox regulation (Packer *et al*., 1995, 1997). The addition of α-lipoic acid to the *Agrobacterium* co-cultivation media has been shown to significantly increase transformation and regeneration efficiencies and transgene expression while minimising necrosis and shoot escapes in several plant species (Dan *et al*., 2003, 2009). Similar results were observed using azaserine or acivicin which both inhibit key enzymes in the purine and pyrimidine synthesis pathways of host cells resulting in an increased sensitivity to *Agrobacterium* infection (Cheng *et al*., 1997; Roberts *et al*., 2003; Wu *et al*., 2003; Yang *et al*., 2006).

Use of the surfactant Silwet-L77 has been shown to increase *Agrobacterium*-mediated transformation efficiency in maize, wheat and *Arabidopsis*. Similarly, Break-Thru S 240 (Goldschmidt Chemical, Hopewell VA), a non-ionic surfactant, was used to increase

transient expression in switchgrass (Vandergheynst *et al.*, 2007). Pluronic F-68 and Tween 20 have also been shown to be beneficial in co-culture media (Curtis and Nam, 2001; Khatun *et al.*, 1993). While unproven, it is assumed that surfactants reduce the surface tension of the co-cultivation media assisting in bacterial invasion of plant tissue (Cheng *et al*., 1997; Feng and Ryerse, 1999; Wu *et al*., 2003; A. Yang *et al*., 2006). However, at higher concentrations Silwet-L77 is phytotoxic, killing embryos and/or preventing callus formation (Wu *et al*., 2003; Yang *et al*., 2006).

Chemical chaperones such as sodium phenylbutyrate (NaPBA) and tauroursodeoxycholic acid (TUDCA) stabilise proteins in their natural conformation by improving correct folding in the ER and suppressing ER stress-related cell death. While predominantly used in animal clinical applications for the treatment of neurodegenerative diseases (Ozcan *et al*., 2006; Welch and Brown, 1996), both chemicals have been shown to reduce the sensitivity of *Arabidopsis* seedlings to ER stress (Watanabe and Lam, 2008; Williams *et al.,* 2010). As such, chemical chaperones could theoretically be used to improve *Agrobacterium* infection by minimising cell death brought about by incompatible bacteria:host cell interaction, thereby increasing transformation frequencies and transient transgene expression.

1.4 Molecular Based Methods to Increase Gene Expression

1.4.1 Intron-mediated enhancement (IME)

The inclusion of an intron in the transcribed region of a transgene cassette has been shown to significantly enhance gene expression in a range of eukaryotic organisms (Rose, 2008). When this phenomenon occurs in the absence of enhancer elements or alternative promoters it is termed intron-mediated enhancement (IME) (Mascarenhas *et al*., 1990). Introns that increase expression by IME are predominantly located at the 5’ end of a gene and in their natural orientation (Rose, 2008). The effects of IME coincide with an elevated accumulation of mRNA, however, there is no evidence to suggest they influence mRNA stability (Fong and Zhou, 2001; Furger and Binnie, 2002; Rose and Last, 1997), nor the rate of transcription (Rose, 2008). Deletion analysis of numerous introns failed to elucidate specific elements required for IME but instead revealed that large intron segments have a negligible influence on IME (Clancy *et al*., 1994; Rose and Beliakoff, 2000). This led researchers to hypothesise that intron enhancer elements are non-specific or redundant sequences (Rose and Beliakoff, 2000). Rose (2008) subsequently used a bioinformatics approach to identify two intronic sequences which commonly occurred in highly expressed plant gene introns. Interestingly, the number of copies of these elements directly correlated with the level to which the gene was expressed.

1.4.2 Molecular chaperone proteins and foldases

The *in vivo* process of folding newly synthesised linear polypeptides into functional three-dimensional proteins is an energy dependent process which relies on the presence of two ubiquitous groups of helper molecules (Freedman *et al*., 1994; Hartl and Martin, 1995). Historically referred to as heat-shock or stress-response proteins, because their induction was originally observed under stressful conditions (Georgopoulos and Welch, 1993), molecular chaperones are a conserved set of protein families that participate in protein folding, translocation and assembly (Georgopoulos and Welch, 1993). Molecular chaperones facilitate correct conformational folding by binding to the reactive surfaces of partially folded proteins, effectively sequestering active sites. This limits interactions between partially folded intermediate stages preventing aggregation reactions (Buchner, 1996; Georgopoulos and Welch, 1993; Thomas *et al*., 1997; Xiao *et al*., 2010). Chaperones also participate in the degradation pathway of terminally misfolded proteins (McClellan *et al*., 2005). Heat shock proteins (Hsps) Hsp70 and Hsp90 are two such chaperones with reported anti-apoptotic functions. These proteins are able to directly interact within the programed cell death machinery inhibiting the onset of apoptosis (Joly *et al*., 2010). The activity of Hsps including Hsp70/Hsp90 is not executed in isolation since hundreds of regulators, co-factors and co-chaperone elements have been identified which interact within a network (Hartl *et al*., 2011).

BAG (Bcl-2 associated athanogene) genes belong to an evolutionarily conserved gene family that encode products with a common C-terminal BAG domain (Doukhanina *et al.*, 2006). *Arabidopsis thaliana* contains seven homologs of the BAG family, four of which possess a domain organisation similar to animal BAGs (Kabbage and Dickman, 2008). *BAG* gene products have been shown to inhibit programmed cell death thereby increasing tolerance to biotic and abiotic stresses, and have been referred to as co-chaperones as they interact with and modulate various molecular chaperones (Briknarová *et al*., 2001; Takayama and Reed, 2001). Transgenic tobacco lines over-expressing *AtBAG-4* showed enhanced tolerance to several abiotic stresses through inhibition of programmed cell death pathways (Doukhanina *et al.*, 2006) while *AtBAG-4* knockouts display early senescence (Yabuta *et al.*, 2009). A characteristic of the conserved BAG domain is binding of Hsp70/Hsp90 proteins and modulating their chaperone activity (Briknarová *et al*., 2001; Kabbage and Dickman, 2008).

BAX inhibitor-1 (BI-1) is an anti-apoptotic protein that is evolutionarily conserved and predicted to be a trans-membrane protein that localises to the endoplasmic reticulum (ER)

(Chae *et al*., 2003, 2004; Lee *et al*., 2007; Watanabe and Lam, 2008). Expression of plant BI-1 is enhanced during senescence and under several types of biotic and abiotic stresses (Kawai-Yamada *et al.*, 2004; Kotsafti *et al.*, 2010; Watanabe and Lam, 2006). Over-expression of BI-1 was shown to suppress BAX, pathogen, and abiotic stress-induced cell death in yeast, plants, and mammals (Kawai-Yamada *et al*., 2001; Matsumura *et al*., 2003; Sanchez *et al.*, 2000; Watanabe and Lam, 2009). Analysis of Arabidopsis BI-1 (AtBI1) showed that it is not required for normal plant growth and development, however it is able to ameliorate PCD induced by phytotoxin exposure and heat stress (Watanabe and Lam, 2006). Expression of AtBI1 is up regulated during several stresses including bacterial and fungal pathogens, ozone, norflurazon and salicylic acid (Kawai-Yamada *et al*., 2004; Sanchez *et al*., 2000). Plant BI-1 is thus likely to play an important role as a survival factor under multiple stress conditions (Watanabe and Lam, 2008).

Another class of enzymes, collectively known as foldases or intra-molecular chaperones, are responsible for catalysing the isomerisation of peptidyl-prolyl bonds and forming disulfide bridges (Nagradova, 2008; Shinde and Inouye, 1993; Thomas *et al*., 1997). Foldases are also reported to interact with proteins otherwise incapable of spontaneously achieving their functional conformation (Ikemura *et al*., 1987). The action of foldases differs fundamentally from the passive role of chaperones as they directly interact with transitional stages to facilitate folding (Nagradova, 2008). To date, there have been no reports of using foldases to increase the stability and accumulation of recombinant proteins. The very nature of these proteins however suggests they may be excellent candidates for such applications.

1.4.3 Over-expression of histone genes

Agrobacterium naturally exploits host genes during the infection process (Gelvin, 2003; Tzfira and Citovsky, 2002) including several members of the histone family (Anand *et al*., 2007; Veena *et al.,* 2003). Histones have purported roles in T-DNA integration (Li *et al.*, 2005; Loyter *et al.*, 2005) and have been shown to increase the efficiency of *Agrobacterium*-mediated transformation when induced (Yi *et al.*, 2002) or over-expressed (Tenea *et al*., 2009).

Arabidopsis histone H2A can facilitate the insertion of DNA into the plant genome via interaction with host-encoded VIP1 (VirE2) (Loyter *et al*., 2005) and overexpression of H2A family genes can increase transgene expression 2-fold (Mysore *et al.*, 2000; Tenea *et al*., 2009). Other histone families (H2B, H3 and H4) have also been shown to interact with *Agrobacterium* virulence gene products (Li *et al*., 2005; Loyter *et al*., 2005).

1.4.4 Protein targeting

Recombinant protein stability requires an environment conducive to correct post-translational modifications, protein folding and correct subunit assembly (Faye *et al*., 2005; Goulet *et al*., 2006). In order to achieve high levels of protein accumulation, recombinant polypeptides may be targeted to subcellular locations as opposed to the default cytosolic retention pathway, thereby limiting proteolytic degradation (Biocca *et al*., 1990). Localisation of proteins to a specific organelle, in some cases, can also facilitate the purification process (Torrent *et al*., 2009).

Targeting of plant made proteins to the endoplasmic reticulum (ER) lumen has been used as an effective strategy to increase recombinant protein accumulation and improve overall yield and stability (Gomord *et al*., 2004; Outchkourov and Rogelj, 2003; Schouten *et al*., 1996; Wandelt *et al*., 1992). Retention to the ER is advantageous for a number of reasons including (i) the oxidising environment is suitable for correct folding and disulfide bridge formation (Schillberg *et al*., 1999) and (ii) the ER possesses low levels of proteases and an abundance of stress proteins which function as molecular chaperones. One such example, BiP (binding protein; homologous to human BiP), has been shown to assist in protein folding resulting in an increase in recombinant antibody accumulation (Ma, 1996; Nuttall *et al*., 2002). Further proteins retained in the ER possess high-mannose N-glycans similar to mammalian proteins (Doran, 2006; Triguero *et al*., 2005). Importantly, addition of immunogenic plant specific sugars occurs within the golgi apparatus (Faye *et al*., 2005; Sriraman *et al*., 2004).

The most common method of ER retention is to incorporate a (K/H)DEL tetrapeptide motif into the C-terminus of the protein sequence (Richter *et al.*, 2000; Schouten *et al*., 1996). However, the maize prolamin, γ-zein, has also been used to retain proteins to the ER with great efficiency (Mainieri *et al*., 2004). The resulting fusion leads to the formation of protein bodies (PBs) which are natural ER or vacuole derived protein storage organelles (Torrent *et al*., 2009). ER retention and PBs have been shown to facilitate the stable accumulation of a variety of recombinant antibodies and vaccine candidates (Conrad and Fiedler, 1998; Fischer *et al*., 1999; Nuttall *et al*., 2002; Torrent *et al*., 2009). The ER is not suitable for the accumulation of all recombinant proteins as some may require further post-translational modifications in order to be active or stable. As such, it is sometimes necessary to direct recombinant proteins to compartments or organelles downstream in the secretory pathway by targeting them to the apoplast, chloroplasts, golgi apparatus or in protein storage vacuoles.

In the absence of an ER retention signal, recombinant proteins in the endomembrane system can be secreted to the apoplastic or extra-cellular spaces. Proteins directed to the apoplast often accumulate to levels higher than those retained in the cytosol but are generally 2 to 10-fold lower than those targeted to the ER (Fischer *et al.*, 2004; Gaume *et al.*, 2003; Gils *et al.*, 2005; van Engelen *et al*., 1994). While apoplast targeting may be beneficial for downstream purification and processing of the recombinant protein there is evidence to suggest that proteins in this environment are more prone to degradation by endogenous proteases (Doran, 2006; van Engelen *et al*., 1994).

Plant cell vacuoles are suitable storage sites for recombinant proteins, particularly within seed tissue in which they are abundant (Müntz, 2007; Park *et al.*, 2004; Stoger *et al.*, 2005; Streatfield *et al*., 2003). It is important to note that plants posses more than one type of vacuole. Lytic vacuoles possess a low pH and contain a number of hydrolytic enzymes, while protein storage vacuoles have a neutral to slightly acidic pH (Benchabane *et al*., 2008; Neuhaus and Rogers, 1998; Robinson *et al.*, 2005). Proteins targeted to these storage vacuoles have been shown to accumulate to very high levels, including human epidermal growth factor (hEGF) in tobacco, DP1B (a synthetic analogue of spider silk protein) in *Arabidopsis*, heat-labile enterotoxin B from *E. coli* and thermostable β-glucanase of bacterial origin in barley (Gils *et al.*, 2005; Horvath *et al.,* 2000; Streatfield *et al*., 2003; Wirth *et al.*, 2004; Yang *et al.*, 2006). While the process is not fully understood, vacuolar localisation appears to be governed by small amino acid sequences, which direct nascent proteins to the vacuole (Neuhaus and Rogers, 1998; Vitale and Hinz, 2005; Yang *et al.*, 2005). However, the benefits of vacuole targeting should be considered on a case-by-case basis, as protein stability and yield are often inconsistent. Ultimately, the success of this approach is dependent on both the protein and the tissue type in which it is expressed, as well as the effectiveness of the retention/sorting signal (Vitale and Hinz, 2005; Yang *et al*., 2005).

Very high recombinant protein content, upward of 11% of the total protein, has been reported using chloroplast transformation and retaining the expressed protein in this organelle (Daniell, 2006; Fernández-San Millán *et al*., 2003; Gils *et al*., 2005; Hyunjong *et al*., 2006). Chloroplast-based expression results in a uniform transgene expression rate, multiple copies per cell, minimal gene silencing and low risk of transgene escape into the environment as chloroplasts are inherited maternally (Daniell *et al.*, 2002). Chloroplasts are able to express proteins of both prokaryotic and eukaryotic origin as they allow for some post-translational modifications including multimerisation and disulphide bridge formation

rendering them suitable for the production of proteins which do not require complex modifications (i.e. glycosylation) (Gils *et al*., 2005). Somatotropin, serum albumin, anthrax protective antigen, cholera toxin B subunit and tetanus toxin fragment C, have all been successfully expressed in chloroplasts (Daniell *et al.* 2001, 2005; Tregoning, 2003). However, chloroplasts contain endogenous proteases which can negatively impact on recombinant protein accumulation. This is most problematic in older leaf tissue (Birch-Machin *et al.*, 2004).

1.4.5 Virus based methods to increase expression

1.4.5.1 RNA silencing

RNA-mediated gene silencing or RNA interference (RNAi) is a phenomenon particularly well described in nematodes, and over the past decade has gained considerable interest in plant biotechnology. RNAi is the targeted, systemic, sequence-specific degradation of RNA (Ahlquist, 2002) in response to pathogenic or aberrant RNA (dsRNA) and is considered to be an archaic antiviral defence mechanism (Lindbo *et al*., 1993). The RNA silencing cascade is triggered by dsRNA (Smith *et al*., 2000), the origin of which may be viral genomic dsRNA, or ssRNA that folds into a complex structure such as an inverted repeat (Castel and Martienssen, 2013; Hull, 2002). A large multi-domain enzyme (DICER) cleaves the dsRNA into 21-24 bp short interfering RNAs (siRNA) (Bernstein *et al*., 2001; Kasschau *et al.,* 2007; Mlotshwa *et al*., 2008) which then associate with the RNA-induced silencing complex (RISC) endonuclease, allowing for the targeted degradation of complementary RNA sequences (Ahlquist, 2002; Baulcombe, 2004; Koh *et al.*, 2014; Lu *et al.*, 2009; Voinnet, 2005). The silencing signal is then systemically amplified by host RNA dependent RNA polymerase (RdRp) (Napoli et al., 1990; Voinnet and Baulcombe, 1997; Wassenegger *et al.*, 1994) transcribing additional copies of the target dsRNA which are subsequently processed by DICER into siRNA (Endres *et al*., 2010; Mourrain *et al*., 2000).

RNAi is of considerable importance to biofarming as it can reduce transgene expression to negligible levels resulting in little to no protein accumulation (De Neve *et al*., 1999). In transient transformations, RNAi can be significantly reduced by the co-delivery of a gene cassette capable of expressing a silencing suppressor (Brigneti *et al.,* 1998; Voinnet *et al.,* 2003).

1.4.5.1.1 Suppressors of RNA silencing

In response to the highly efficient PTGS plant host defence, viruses have evolved mechanisms of suppressing gene silencing (Anandalakshmi *et al*., 1998; Burgyán and Havelda, 2011; Kasschau and Carrington, 1998; Voinnet and Baulcombe, 1997). Many of

these suppressors were previously termed pathogenicity determinants and are encoded in both plant and animal virus genomes (Li and Ding, 2006; Scholthof, 2006; Voinnet *et al*., 1999). It is likely that all plant viruses encode a gene product with some form of PTGS suppression activity, however, it seems their exact mode of action differs between plant virus families. The coat protein p38 of *Turnip crinkle virus* (TCV) and the *Cucumber mosaic virus* (CMV) 2b protein interfere with the DICER molecule halting the progression of dsRNA into siRNA (Diaz-Pendon *et al*, 2007; Thomas *et al*., 2003). *Tomato bushy stunt virus* (TBSV) p19 and *Barley yellow dwarf virus* (BYDV) p21 sequester siRNA preventing their association with RISC (Baulcombe and Molnár, 2004; Lakatos *et al*., 2006; Reed *et al*., 2003; Scholthof, 2006). *Beet western yellows virus* (BWYV) p0 suppresses RISC activity (Bortolamiol *et al*. 2007), *Tomato yellow leaf curl virus* (TYLCV) V2 inhibits signal amplification (Glick *et al*., 2008) and the potyvirus HC-Pro affects the DICER molecule (Dunoyer *et al*., 2004) and also sequesters siRNA (Lóránt Lakatos *et al*., 2006). The potexviruses, hordeiviruses, tobraviruses, furoviruses, pecluviruses and carlaviruses all encode gene products with viral pathogenicity and silencing suppressor functions. However, these proteins contain no motifs common to other well characterised silencing suppressors (Alvarado and Scholthof, 2009).

Co-expression of virus-derived silencing suppressors has been used as a means of increasing transient expression levels of heterologous proteins in plants. A potyvirus HC-Pro gene product was shown to increase expression from 2-fold (Wydro *et al*., 2006) to 10-fold (Mallory *et al*., 2002), while Qi *et al* (2004) reported a 2-3-fold increase in expression when the CMV 2b and TBSV p19 were co-delivered in transient assays, even in the presence of dsRNA. In the absence of dsRNA, an 8-fold increase in transgene expression was reported (Reed *et al*. 2003).

Constitutive expression of silencing suppressors can interfere with the normal physiological development of the plant causing phenotypic abnormalities and even plant death (Saxena *et al*., 2011). As such, there have been few examples of their use in stably transformed transgenic plants. One novel approach was recently reported by Alvarez *et al*. (2008). In this study, transgenic tomatoes containing a silenced *Yersinia pestis* vaccine candidate gene were super-transformed with a silencing suppressor gene under the control of ethanol inducible promoter. Upon addition of ethanol, PTGS was reversed and the fruit of these lines accumulated the antigen fusion protein to levels up to 3-times higher than in fruit of non-silenced elite tomato lines. In a different approach, Saxena *et al*. (2011) mutated the TBSV p19 silencing suppressor such that it retained the ability to sequester siRNA but did

not cause a harmful phenotype when over-expressed in *N. benthamiana*. Transgenic plants co-expressing GFP and the p19/R43W mutant showed elevated accumulation of GFP compared with plants not expressing p19/R43W. Further, transgenic expression of P19/R43W caused little to no morphological defects and plants produced normal-looking flowers and fertile seed.

1.4.5.2 Cell cycle control

There is strong evidence for an S phase dependent control mechanism for T-DNA transfer during the *Agrobacterium* transformation process (Villemont *et al*., 1996). During infection, wounding is postulated to stimulate DNA replication and cell proliferation, processes that typically involve recombination and/or repair enzyme activities, which in turn might enhance T-DNA integration. In order to prove this, Villemont *et al*. (1996) synchronised Petunia mesophyll cells into S-G2-M or G0-G1 phase populations using two different phytohormones. Maximum (>95% of explants) transformation efficiency occurred in the S-G2-M phase cell population whereas no transformation was observed in the late G1 phase cells. This suggested an absolute requirement for S phase (DNA duplication) and highlights the pivotal role that the cell cycle plays in *Agrobacterium*-mediated transformation. Based on this requirement it seems likely that the use of chemicals or the expression of gene products that can manipulate the host cell cycle into S phase may significantly increase the efficiency of *Agrobacterium*-mediated gene transfer.

As a consequence of their small genome size, some viruses (e.g. Geminiviruses) rely on host-encoded enzymes and co-factors to replicate their genome and progress the infection. This dependence can be rate limiting, particularly in cells where these replication proteins are not in abundance i.e. non-proliferating terminally differentiated cells (Accotto *et al*., 1993). Accordingly, some viruses encode proteins that are capable of subverting the host cell cycle and transitioning terminally differentiated cells to a more active phase, namely S phase (Aronson *et al*., 2000; Nagar *et al*., 1995; Xie *et al*., 1995).

In addition to catalysing Rolling circle replication (RCR), the Rep protein of begomoviruses induces host synthesis of proliferating cell nuclear antigen (PCNA), a scaffolding protein that tethers DNA and functions to modulate the interactions of other proteins with DNA. This complex interacts with many proteins that are involved in important cellular processes such as replication and repair of DNA, DNA methylation, cell-cycle control and chromatin assembly (Hanley-Bowdoin *et al*., 2000; Castillo *et al*. 2003). The mastrevirus replication associated proteins, Rep and RepA, and the nanovirus cell cycle link (Clink) proteins

function to regulate virus gene expression via protein-protein and protein-DNA interactions (Palmer and Rybicki, 1998) and directly manipulate the host cell cycle (Horváth *et al*., 2000; Xie *et al*., 1995). These proteins contain a LXCXE motif, conserved in animal virus oncoproteins, which specifically binds and sequesters the plant retinoblastoma-like protein RBR. RBR naturally regulates host cell cycle control, thus virus-based sequestration of RBR deregulates the modulation of cell proliferation (Weinberg, 1995) resulting in the induction of S-phase in quiescent cells. This provides an optimal environment for virus replication (Aronson *et al*., 2000; Nagar *et al*., 1995; Xie *et al*., 1995). Clink proteins also contain a F-box that interacts with SKP1 proteins, a family of ubiquitin protein ligases involved in cell cycle regulatory proteolysis (Aronson *et al*., 2000; Deshaies, 1999).

Interactions have also been reported between RepA and two NAC domain containing proteins termed Geminivirus RepA-binding proteins (GRAB1 and GRAB2) (Xie *et al*., 1999). The NAC family are characterised by a conserved N-terminal region (NAC domain) and a highly variable C-terminus. The acronym is derived from the three type members of the family, No Apical Meristem (NAM) (Souer *et al*, 1996), *Arabidopsis* Transcription Activation Factor (ATAF) and the Cup-Shaped Cotyledon (CUC) genes (Aida *et al*, 1997). The NAC domain family proteins are important in plant development and senescence pathways as well as in plant defense response functions (Collinge and Boller, 2001). Importantly, GRAB proteins have been reported to suppress viral replication (Xie *et al*., 1999).

While the begomovirus Replication enhancer protein (REn) is not an essential replication factor, it is responsible for enhancing disease symptoms (Hormuzdi and Bisaro, 1993) and DNA accumulation via several protein-protein interactions (Settlage *et al*, 1996; Sunter *et al*, 1990). Rep-REn interactions are thought to influence the affinity of Rep for the viral origin of replication thus enhancing viral replication (Fontes *et al*, 1994; Gladfelter *et al*, 1997; Hanley-Bowdoin *et al*, 1999; Settlage *et al*., 1996). REn also interacts with host PCNA and RBR (Castillo *et al*., 2003; Settlage *et al*., 2001) and is, therefore, a key regulator of S phase transition (Selth *et al*., 2005; Settlage *et al* , 2001).

1.4.5.3 Translation enhancers

In eukaryotes, translation initiation is dependent on a complex process involving synergistic interactions between eukaryote initiation factors (eIFs), ribosomes, mRNA and tRNA (Prévôt *et al*., 2003). A 5' cap (m7G(5')ppp(5')N) and 3' polyA tail are ubiquitous eukaryotic mRNA features, which in the presence of other eIFs, bind to eiF4E and polyA-binding protein (PABP), respectively. This complex serves as a molecular bridge, forming a closed loop complex necessary for efficient translation (Dreher and Miller, 2006; Hentze, 1997;

Prévôt *et al*., 2003; Thivierge *et al*., 2005). Viruses are intracellular parasites and are almost exclusively reliant on host factors for protein synthesis. As such, viruses have developed novel strategies to ensure preferential translation of their mRNA (Gale *et al*., 2000).

1.4.3.5.3.1 5' UTR Translation enhancers

One of the best-studied 5' enhancer sequences is the 70-nucleotide Ω leader from the genome of *Tobacco mosaic virus* (TMV) (Figure 1. 3). This sequence includes a functional 25 nt polyCAA motif that binds specifically with plant heat shock protein 101 (Hsp101) (Gallie and Walbot, 1992; Sugio *et al*., 2008), and in turn recruits eIF4G (Gallie, 2002). The TMV Ω sequence is functional in *E. coli* and most eukaryotic systems (Gallie *et al*., 1987). Addition of the TMV leader into the 5' untranslated region can reportedly increase transgene expression 2-3-fold (Holtorf *et al*., 1995). Despite reports of limited efficacy in monocot systems (Gallie *et al*., 1989), the TMV Ω sequence has been successfully used in the transformation of recalcitrant monocots including sugarcane (Kinkema *et al*., 2014).

m^7GpppGUAUUUUUACAAUUACCAACAACAACAAACAACAAACAACAUUACAAUUACUAUUUACAAUUACAUG

Figure 1. 3 TMV Ω enhancer sequence

The functional poly(CAA) region of the Ω sequence is underlined and the three direct repeats are indicated with arrows (→).

Members of the Potexvirus family (of which *Potato virus X* (PVX) is the type member) share little nucleotide homology overall, however their 5' leader sequences are generally AC-rich, and include a repeated ACCA motif (Kim and Hemenway, 1996). Deletion/mutation analysis has shown the ACCA motif is essential for replication (Park *et al*., 2008) and computer modeling has identified the presence of stem-loop structures which play a clear role in RNA synthesis (Miller *et al*., 1998). The 5' leader sequences of viruses belonging to the Potyviridae family (of which *Potato virus Y* (PVY) is the type member) have also been reported to increase translation (Carrington and Freed, 1990; Yang *et al*. 1997). Analysis of these sequences revealed they contain a putative stem-loop structure, similar to that of the potexviruses, a CAA repeat similar to the TMV Ω and a UUUCA penta-nucleotide repeat (Yang *et al*. 1997). The leader sequence from the RNA-4 coat protein gene of *Alfalfa mosaic virus* (AMV) (a member of the Bromoviridae), is also able to enhance gene expression by facilitating the formation of a closed-loop structure essential for efficient translation (Jobling and Gehrke, 1987; Krab *et al*., 2005).

1.4.5.3.2 Cap-independent translation

Most (+)ssRNA plant viruses lack a 5' cap or 3' polyA tail with fewer than 20% possessing both structures (Dreher and Miller, 2006). Many viruses employ cap-independent translation, which is advantageous as it avoids cellular-mediated control, permits virus induced shut-off of host cap-dependent translation, minimizes the defensive capabilities of the host and ensures the availability of ribosomes (Kneller *et al*., 2006). As cellular capping is undertaken in the nucleus and RNA virus replication occurs in the cytoplasm, capped RNA viruses must encode their own capping enzymes. This is not necessary for viruses that undergo cap-independent translation (Ahola and Ahlquist, 1999).

1.4.5.3.3 Internal ribosome entry sites (IRES)

Several virus families including Picornaviridae, Potyviridae, Comoviridae and Caliciviridae utilise an internal ribosome entry site (IRES) during translation. Initially observed in Poliovirus (Pelletier and Sonenberg, 1988), the IRES is not subject to regulatory mechanisms that control recruitment of most mRNAs to the translation apparatus (Hellen and Sarnow, 2001). Initiation of cellular translation relies on the two isoforms of eIF4G (eIF4GI and eIF4GII) for capped mRNA/40S ribosome interactions (Pestova *et al*., 2001). The virally encoded cysteine protease 2APro cleaves both isoforms of eIF4G (Barco *et al*., 2000; Etchison *et al*., 1982; Svitkin *et al*., 1999) simultaneously debilitating cap-dependent translation and enhancing internally initiated cap-independent protein synthesis (Bedard and Semler, 2004; Lloyd, 2006; Ohlmann *et al*., 1996). However, even without cleaving eIF4G, IRES sequences function competitively against cellular cap-mediated translation (Mountford and Smith., 1995). The function of IRESs are not dependent on viral gene products but rely on interactions with normal host cellular proteins (Jang and Wimmer, 1990), increasing the affinity of translation initiation factors, ribosomes and mRNA for each other, and allowing ribosome binding without scanning (Kieft *et al*., 2001; Pestova *et al*., 2001; Terenin *et al*., 2005). The mechanism by which ribosomal subunits reach the start codon is not known (Belsham, 1992; Hellen *et al*., 1994; Ohlmann and Jackson, 1999).

IRESs show high sequence variability with no conserved motifs or mechanisms, except in related viruses (Baird *et al*, 2007; Baird *et al*, 2006; Balvay *et al*, 2009; Hellen *et al*., 1994; Mountford and Smith, 1995; Ohlmann and Jackson, 1999). This effectively means these sequences can only be identified based on functionality, rather than sequence homology (Balvay *et al*., 2009; Mountford and Smith, 1995). IRES elements have been used in mammalian cell culture as enhancer elements to facilitate bicistronic expression (Bochkov

and Palmenberg, 2006; Rees *et al*., 1996), however, there are no reports of their use as translation enhancers in plant biotechnology or biofarming.

1.4.5.3.3.1 Virus protein, genome linked (VPg)

Cap-independent translation may also be facilitated by the presence of a small virus encoded protein linked covalently to the 5' end of the RNA, called the Virus Protein genome-linked or VPg (Dreher and Miller, 2006; Gao *et al*., 2004; Thivierge *et al*., 2005). There has been speculation that VPg interactions may inhibit cap dependent translation (Daughenbaugh *et al*., 2003) or facilitate pathogenicity by cell-to-cell trafficking of viral mRNA (Gao *et al*., 2004). The VPg of potyviruses is thought to function as a 5' cap through its interactions with host eiF4E (Grzela *et al*., 2006). Also, it is believed to have a direct role in recruiting translation factors, stimulating translation and competitively inhibiting cap binding (Plante *et al*., 2004).

1.4.5.3.3.2 Cap-independent translation enhancers (CITE)

Viruses which lack both a 5' cap and 3' polyA tail employ a different translation strategy involving a 3' CITE (Kneller *et al*., 2006). 3' CITEs may have a pseudoknot element or an "I" or "T" or "Y" shaped secondary structure, with a terminal stem-loop, and have been implicated in 5'-3' interactions essential for *in vivo* translation (Fabian and White, 2006; Simon and Miller, 2013). The 3' CITE is capable of binding eiF4E and delivering the 43S ribosomal subunit to the 5' UTR (Fabian and White, 2006; Gazo *et al*., 2004; Kneller *et al*., 2006). Interactions between stem-loop structures, the 3' CITE and 5' UTR are often referred to as a "kissing-loop" interaction (Figure 1.4) allowing for long distance RNA-RNA interactions. This association circularises the viral mRNA into a closed-loop complex and facilitates translation (Fabian and White, 2006; Guo *et al*., 2000; Kneller *et al*., 2006; Simon and Miller, 2013).

1.4.3.3.3 3' tRNA-like structure (TLS)

An alternative 3' structure found in viruses that have a 5' cap but lack a polyA tail is a tRNA-like structure (TLS). TLSs generally have a complex secondary and tertiary structure, for example the "pseudoknot" elements that are formed by the loop of an RNA stem-loop base pairing with a distant sequence (Pleij, 1994) (Figure 1.5). Pseudoknots are important for TLS cellular tRNA mimicry, aminoacylation capability (Dreher, 1999), substitution of polyA functions, frame-shifting and enhanced translation (Leathers *et al*., 1993). The full functionality of viral TLSs has yet to be determined, however, they have been shown to bind eIFs, participate in the formation of molecular bridges, assist in the recruitment of host nucleotidyltransferases and are thought to regulate minus strand RNA synthesis (Dreher, 1999). Some reports have demonstrated that 3' TLSs are able to enhance reporter gene expression (Matsuda and Dreher, 2004), with synergistic effects observed in conjunction with a 5' cap.

Figure 1. 4 Putative 3D structure of the PEMV CITE

Possible three-dimensional CITE model of *Pea enation mosaic virus* (PEMV) kissing-loop interaction (Simon and Miller, 2013).

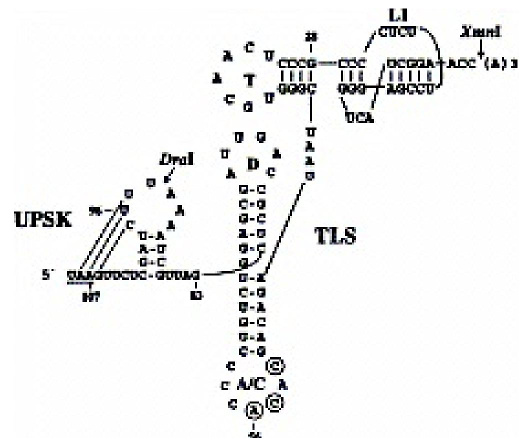

Figure 1. 5 3' tRNA-like structure (TLS) of TYMV

The 3' TLS of *Turnip yellow mosaic virus* (TYMV) and upstream pseudoknot (UPSK), at the 3' end of TYMV RNA (Matsuda and Dreher, 2004).

Chapter 2: General Materials and Methods

This chapter outlines the common methodologies used in the following experimental chapters. Pertinent aspects of the methods and materials will be repeated for the sake of continuity with the results.

2.1 General Materials

2.1.1 Source of general reagents and chemicals

All general laboratory reagents of analytical grade were obtained from Sigma (Aus), Merck Millipore (Aus), Chem-Supply (Aus), unless otherwise stated. Agarose used for gel electrophoresis and bacterial agar were supplied by Roche (Aus) and Oxoid (Aus), respectively. All DNA markers were supplied by Bioline (Aus). Modifying and restriction enzymes were supplied by Roche (Aus) or New England Biolabs (Aus).

2.1.2 Oligodeoxyribonucleotide synthesis

Oligodeoxyribonucleotides were synthesised by GeneWorks (Hindmarsh, South Australia). Primers were diluted to a concentration of 100 µM and working stocks of 10 µM were prepared for PCRs and 3.2 µM stocks for sequencing reactions.

2.1.3 Bacterial strains

Escherichia coli XL1-Blue was used for all general plasmid cloning. *Agrobacterium tumefaciens* strains Agl1, C58 and LBA4404 were readily available within the CTCB. Strain GV3101 was kindly provided by Prof. Peter Waterhouse (University of Sydney).

2.1.4 General media and solutions: abbreviations and composition

1-Naphthaleneacetic acid (NAA):

NAA dissolved in EtOH

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal):

2% (w/v) X-Gal; Prepared in dimethylformamide (DMF) (Stored at -80 °C)

Acetosyringone:

100 mM acetosyringone; Dissolved in DMSO

BAP (6-Benzylaminopurine) (1 mg/mL):

Dissolved in 1 M NaOH

Coomassie Blue solution:

50% ethanol; 10% acetic acid; 0.1% Coomassie Blue G-250 stain (BioRad)

CTAB buffer (DNA):

2% hexadecyltrimethylammonium bromide (CTAB); 2 M NaCl; 25 mM EDTA pH 8.0; 100 mM Tris-HCl (pH to 8.2); 2% polyvinylpyrrolidone (PVP 40 000)

Depurination solution:

0.25 M HCl

Denaturation solution:

0.5 M NaOH; 1.5 M NaCl

Isopropyl β-D-1-thiogalactopyranoside (IPTG):

Isopropyl β-D-1-thiogalactopyranoside; prepared as 0.1 M in sterile dH2O

GUS extraction buffer:

50 mM Na_2HPO_4 pH 7.0; 10 mM Na_2EDTA; 0.1% Sodium Lauryl Sarcosine; 0.1; Triton X-100; 10 mM DTT (Dithiothreitol)

High stringency buffer

20 x SSC 100 mL; 10% SDS 10mL

Low stringency buffer

20 x SSC 5 mL; 10% SDS 10mL

Luria Bertani (LB) agar:

10 g tryptone; 5 g yeast extract; 10 g NaCl (pH to 7.0) (add 1.5% (w/v) agar). Autoclaved.

Luria Bertani (LB) liquid media:

10 g tryptone; 5 g yeast extract; 10 g NaCl; (pH to 7.0. autoclaved)

Maleic acid buffer:

0.1 M maleic acid; 0.15 M NaCl (pH 7.5)

MMA:

10 mM MES; 10 mM $MgCl_2$; 200 µM acetosyringone

MUG Stop buffer:

200 mM Na_2CO_3; (pH 7.5 dissolved in ddH_2O)

MUG substrate:

2 mM 4-methylumbelliferyl-β-D-glucuronidase (4-MU) in GUS extraction buffer

Neutralisation buffer:

0.5 M Tris-HCl (pH 7.0); 1.5 M NaCl; 1 mM EDTA

SDS-PAGE destaining solution (1L):

15% ethanol; 10% acetic acid;

SDS-PAGE Loading Buffer:

50 mM Tris (pH 6.8); 1% SDS; 10% glycerol; 10 mM DTT; 0.025% bromophenol blue

SDS-PAGE Running Buffer:

0.25 M Tris; 0.25 M glycine; 0.1% (w/v) SDS

SOB:

2% (w/v) bacto-tryptone; 0.5% (w/v) yeast extract; 10 mM NaCl, 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$ (pH to 7.0. Autoclaved.)

SOC:

2% (w/v) bacto-tryptone; 0.5% (w/v) yeast extract; 10 mM NaCl, 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$ (pH to 7.0. Autoclaved.)

Prior to used add: 20 mL 1 M filter sterilised glucose and 5 mL of filter sterilised 2 M $MgCl_2$

Solution 1:

25 mM tris-base; 10 mM EDTA; 50 mM glucose. (Autoclaved)

Solution 2:

0.2 M NaOH: 1% sodium dodecyl sulphate (SDS)

Solution 3:

29.4 g potassium acetate: 11.5 mL glacial acetic acid

SSC (x20):

300 mM sodium citrate; 3 M NaCl (pH 7.5)

Transformation buffer (TB):

2.5 g/L Hepes 15 mM $CaCl_2$ (pH 6.7 with KOH) then add: 250 mM KCl 55 mM $MnCl_2$ (Filter sterilised).

TTBS:

100 mM Tris (pH 7.5) 150 mM NaCl; 0.1% Tween-20

Washing buffer

0.1 M maleic acid; 0.15 M NaCl (pH 7.5); 0.3% (v/v) Tween 20

Western transfer buffer

1M Tris; 0.25 M glycine; 20% methanol

Yeast mannitol (YM)

0.4 g/L yeast extract; 55 mM mannitol; 2.8 mM K_2HPO_4; 800 µM $MgSO_4$; 0.1 g NaCl (pH to 7.0. Autoclaved)

2.1.5 Antibiotics

Ampicillin (100 mg/mL)

Dissolved in deionised H_2O, filter sterilised.

Kanamycin (100 mg/mL)

Dissolved in deionised H_2O, filter sterilised.

Rifampicin (25 mg/mL)

Dissolved in DMF.

Timentin (200 mg/mL)

Dissolved in deionised H_2O, filter sterilised.

Tetracyline (25 mg/mL)

Dissolved in 70% Ethanol.

2.2 General Methods

2.2.1 General methods in nucleic acid amplification, cloning and sequencing

2.2.1.1 Extraction of total DNA

Total DNA was isolated from plant tissue (*Arabidopsis thaliana, Nicotiana benthamiana* and *Nicotiana tabacum*) using a standard CTAB method (Porebski *et al*, 1997). Approximately 0.4 g of fresh or frozen leaf tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle and 3 mL of pre-warmed CTAB extraction buffer (65°C) added and allowed to thaw. The homogenate was then transferred to 2 x 2 mL microfuge tubes and incubated at 65°C for 15 min, periodically mixed by inversion. Tubes were centrifuged at 14 000 g for 5 min and ~ 750 µL of the supernatant was transferred to 1.5 mL microfuge tubes. Proteins were denatured by the addition of an equal volume of $CHCl_3$:IAA (24:1) and mixed by vortexing. The tubes were centrifuged at 14 000 g for 5 min and the upper aqueous layer transferred to a new tube and this step repeated. Following the $CHCl_3$:IAA extraction, contaminating RNA was removed by digestion in RNaseA at a final concentration of 1 µg/mL at 37°C for 1 h. Total DNA was precipitated by the addition of an equal volume of isopropanol, mixing by inversion and centrifugation at 14 000 g for 5 min. The DNA pellet was washed in 70% ethanol and resuspended in 50-100 µL of sterile dH_2O. DNA purity and concentration was estimated by agarose gel electrophoresis and spectrophotometry absorbance readings at 260 and 280 nm wavelengths.

2.2.1.2 PCR amplification

PCRs were carried out in a Peltier Thermal Cycler-200 (MJ Research) using GoTaq® Green Master Mix (Promega). All PCRs were performed in a final volume of 20 µL containing 10 µL 2x GoTaq® Green Master Mix and 5 pmol of each primer. PCR cycle conditions were as follows: initial denaturation at 94°C for 2 min prior to 30 cycles of 94°C for 30 s, 55°C (depending on primer set) for 30 s and 72°C for 1 min per kbp of expected product and a final extension at 72°C for 5 min was included.

2.2.1.3 Agarose gel electrophoresis

Agarose gels of 1.5% (w/v) were prepared using agarose (Roche) dissolved in 1 X TAE buffer containing 0.5 X SYBR Safe DNA gel stain (Invitrogen). Gels were run in either mini or midi -multi sub electrophoresis systems (Bio-Rad). A molecular weight marker (either Easy Ladder or Hyper Ladder – Bioline) was added to one lane to determine the approximate size and quantity of the nucleic acid, restriction digest and PCR products. Electrophoresis was carried out at 80-120 V and in 1 X TAE buffer, for approximately 40 min. Agarose gels were visualised using a short wavelength UV transilluminator (Pharmacia) and photographed using the SYNGENE Geldoc system (Syngene).

2.2.1.4 Southern analysis

2.2.1.4.1 Southern transfer of DNA from agarose to nylon membrane

Gels were prepared at 0.8% (w/v) agarose in Tris-Acetate-EDTA (TAE) containing 0.5 X SYBR and electrophoresed at 80 V for 3 h. Agarose gels were prepared for transfer by incubation for 10 min in depurination solution followed by 30 min in denaturation solution and then 2 x 30 min in neutralisation buffer. Gels were rinsed in double distilled water between each treatment. Transfer of DNA to nylon membranes was performed overnight in 20 X SSC using the capillary method of Southern (1975). Membranes were then washed twice for 2 min each in 2 X SSC and then baked for 2 h at 80°C under 800 kPa vacuum pressure prior to hybridisation.

2.2.1.4.2 Synthesis of digoxigenin (DIG)-labelled nucleic acid probes

Probes for hybridisation analysis were synthesised by PCR to incorporate DIG- 11-dUTP (digoxygenin-11-2’-deoxy-uridine-5’-triphosphate; Roche) at a ratio of 3:1 (dTTP:dUTP). The 10X DIG mix (3:1) (2 mM) contained 0.7 mM DIG-11-dUTP, 1.3 mM dTTP and 2 mM of each dATP, dCTP and dGTP (Roche). DIG-labelled PCR products were electrophoresed through agarose gels and purified using a High Pure PCR Product Purification Kit (Roche). All probes were eluted twice in a final volume of 50 µL, of which 10 µL was used for hybridisation. The 10 µL of probe was diluted to 50 µL with dH_2O, denatured by heating to 95°C for 5 min and quenched on ice for 2 min, prior to hybridisation.

2.2.1.4.3 Pre-hybridisation, hybridisation and signal detection nylon

Nylon membranes were pre-hybridised for 1 h at 42°C in 14 mL of Dig-easy Hybe Solution (Roche) using a “Shake and Stack” Rotisserie Oven (Hybaid). The DIG-labelled PCR probe was then added and allowed to hybridise overnight with rotation at temperatures calculated to minimise unspecific binding. The following day, the membrane was washed twice at room temperature with high stringency buffer and twice in low stringency buffer at 68°C. The membrane was rinsed briefly in maleic acid buffer and blocked in maleic acid buffer containing 3% skim milk powder. After blocking, the membrane was incubated in a solution containing 1:20,000 diluted mouse-derived anti-DIG antibody (Roche) in maleic acid buffer with 3% skim milk powder for 30 min at room temperature. Unbound antibody was removed by two washes in washing buffer. Prior to detection, all membranes were equilibrated in detection buffer. Detection of DIG-labelled DNA was achieved using CDP-star (Roche), as per manufacturer’s instructions. X-ray films (AGFA) were exposed for up to 30 min depending on signal intensity. X-ray films were developed manually by dipping in developer solution for 2 min, fixer solution for 5 min, rinsing with water after each step then air-dried.

2.2.1.5 Extraction of total RNA

Total RNA was isolated from plant tissue (*N. benthamiana* and *N. tabacum*) using a RNeasy Kit (QIAGEN) following the manufacturer’s instructions. All tissue samples were snap frozen in liquid nitrogen immediately after harvesting and stored at -80°C prior to processing.

2.2.1.6 Quantification of nucleic acid

The quantity and quality of extracted gDNA and RNA were estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Absorbance was measured at wavelengths of 230, 260 and 280 nm. The purity of the gDNA and total RNA was assessed using the ratio of absorbance at 260 and 280 nm and 260 and 230 nm, respsectively.

2.2.1.7 Reverse transcription PCR (RT-PCR)

Total RNA was transcribed into complementary DNA (cDNA) using M-MLV Reverse transcriptase (Promega, Aus.) following the manufacturer’s instructions. Briefly, two separate Master Mixes (MM) were prepared. MM1 contained 20 ρmol of reverse primer, 1 µg RNA extract template (~ 2 µL) and dH_20 to a final volume of 12 µL. MM1 was then incubated at 70°C for 5 min then quenched on ice before the addition of MM2. MM2 contained 1x RT-PCR buffer, 2 mM dNTPs (Roche), 2 µL RNasin® (Promega), 1 µL M-MLV RT (to a final volume of 25 µL). RT-PCR conditions were as follows: incubation at 42°C for 1 h

and reverse transcriptase was then deactivated by heating to 70°C for 15 min. Second strand synthesis was achieved using GoTaq® Green Master Mix (Promega, Aus.) in a reaction containing 1 μL of cDNA as the template.

2.2.1.8 Restriction enzyme digestion of DNA

Plasmid DNA (up to 1 μg) was incubated with the appropriate restriction endonuclease (between 5-10 U) and specific buffer at the appropriate temperature for at least 1 h. For double digests with non-compatible buffers, sequential digests were performed.

2.2.1.9 DNA ligation

PCR products were purified from agarose using Freeze 'N Squeeze Gel Extraction Spin Columns (Bio-Rad) and ligated with 50 ng of the T-tailed vectors pGEM®-T easy (Promega). For all other ligations, backbone plasmid DNA was prepared by restriction endonuclease digestion followed by alkaline phosphatase treatment (Roche) and agarose gel electrophoresis Freeze 'N Squeeze gel purification. The respective insert DNA was prepared by excision from the pGEM®-T easy vector by endonuclease digestion prior to agarose gel electrophoresis and Freeze 'N Squeeze gel purification. Purified backbone and insert DNA sequences were ligated together in a final volume of 10 μL using the Promega Rapid Ligation buffer (2x) in an insert:vector ratio of 3:1. Ligations all contained 1 U of T4 DNA ligase (Promega) and incubated for at 4°C for at least 16 h.

2.2.1.10 Vector construction

pEAQ-HT (Figure 2.1 a) was a generous gift from G. Sainsbury and G. Lomonossoff, John Innes Centre, UK (Sainsbury *et al.*,2009). pEAQ-HT contains genes important for vector functionality; TrfA which promotes replication, OriV (viral origin of replication), ColEI (*E. coli* origin of replication, for high copy number in *E. coli*), and the NPTII gene for selection of recombinant bacteria with kanamycin. For quantitative and qualitative analysis of transgene expression, the *uid*A reporter gene encoding GUS was selected. Two pEAQ vectors containing the *uid*A reporter were kindly supplied by B. Dugdale; pEAQ-GUS contains an unmodified *uid*A gene in the MCS of pEAQ-HT, while pEAQ-GSN contains the same *uid*A gene but with a small (~100 nt) synthetic intron in the coding region. The *uid*A gene encoding GUS was excised from p35S-GUS as a BamHI (blunt-ended) and SalI fragment and ligated into AgeI (blunt-ended) and XhoI digested pEAQ-HT. The resulting construct was called pEAQ-GUS. The *uid*A gene containing a small synthetic intron was derived from p35S-GSN (Dugdale *et al.*, 2013) and cloned into pEAQ-HT as above. The resulting construct was called pEAQ-GSN (Figure 2.1 b).

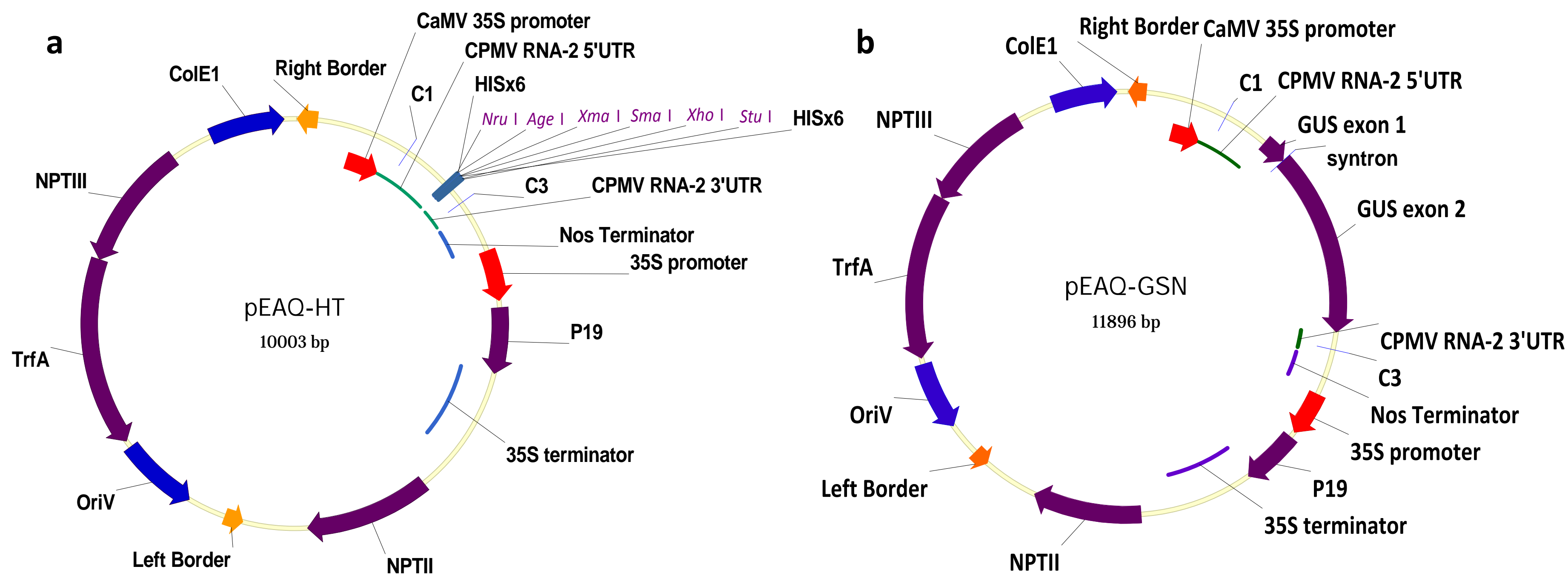

Figure 2.1 Schematic representation of EAQ "Hyper translatable" binary vector maps

(a) pEAQ-HT comprises TrfA which promotes replication, OriV (viral origin of replication), ColEI (*E. coli* origin of replication, for high copy number in *E. coli*), and the NPTII gene to confer kanamycin resistance (Sainsbury *et al.*,2009). (b) pEAQ-GSN contains a *uid*A gene encoding GUS including a synthetic intron (syntron) within the coding region (Dugdale *et al.*, 2013).

2.2.3 Transformation and culture of bacteria

2.2.3.1 Preparation of heat-shock competent *Escherichia coli*

Chemically competent *E. coli* XL1-Blue cells were prepared essentially as described by Inoue *et al*. (1990). Streak plates from a glycerol stock of *E. coli* strain XL1-Blue were incubated overnight at 37°C on LB agar plates containing 25 µg/mL tetracycline. A single colony was grown overnight in 3 mL of LB liquid culture containing 25 µg/mL tetracycline in a shaking incubator (250 rpm). A 1 mL aliquot of the starter culture was inoculated into 1 L conical flasks containing 250 mL of SOB media and incubated at 18°C for a further 40 h with shaking (250 rpm). Cultures were grown to mid-log phase growth (OD_{600} = 0.5-0.6), chilled on ice for 10 min, and cells pelleted by centrifugation at 2 500 g for 10 min. Cells were washed with 80 mL pre-chilled Transformation Buffer (TB) and incubated on ice for 10 min. This was repeated twice and the final pellet was resuspended in 20 mL of ice cold TB (containing dimethyl sulfoxide (DMSO) at a final concentration of 7%) and cells were incubated on ice for a further 10 min. The competent cells were then dispensed in 50 µL aliquots and snap frozen in liquid nitrogen. Cells were stored at -80°C.

2.2.3.2 Transformation of heat-shock competent bacteria with recombinant plasmids

Chemically competent *E. coli* were transformed by heat-shock as described by Inoue *et al*. (1990). Approximately 1 ng of plasmid DNA or 20 µL of ligation reaction was mixed with 50 µL of *E. coli* (thawed on ice) in a 1.5 mL microfuge tube and incubated on ice for 15 min. Cells were heat-shocked at 42°C for 90 s and returned to ice for 1-2 min. Cells were incubated in 1 mL of SOC for 1 h with agitation (250 rpm) and then plated onto LB agar plates containing 100 µg/mL ampicillin. Blue/white screening was performed as required by supplementing plates with 40 µL of 1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 40 µL of 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) prior to plating out cells. Plates were inverted and incubated overnight at 37°C.

2.2.3.3 Purification of plasmid DNA mini-preparation

Plasmid DNA was isolated from *E. coli* cultures using a standard alkaline lysis protocol (Sambrook *et al.*, 1989). A single colony was inoculated into ~ 3-4 mL of LB liquid containing the appropriate antibiotic and incubated with shaking (250 rpm) at 37°C overnight. A 2 mL aliquot of the culture was centrifuged at 14 000 g for 1 min at room temperature and the resulting pellet was resuspended in 100 µL of Solution 1. Bacteria were lysed in 200 µL Solution 2 and mixed by inversion. Bacterial proteins and chromosomal DNA were then precipitated by the addition of 150 µL of Solution 3 and cellular components were separated by centrifugation at 14 000 g for 5 min at room temperature. Plasmid DNA was

then precipitated by transferring 400 µL of the supernatant to 1 mL of ice-cold 100% ethanol and centrifugation at 14 000 g for 5 min at room temperature. The pellet was washed with 70% ethanol, re-centrifuged at 14 000 g for 5 min at room temperature, allowed to air dry and finally resuspended in 50 µL H_2O with 10 ng/mL of RNaseA (Roche).

2.2.3.4 Preparation of electro-competent *Agrobacterium tumefaciens* cells

Preparation of *A. tumefaciens* electro-competent cells was essentially as described by Dower *et al*. (1988). *A. tumefaciens* strains Agl1, C58, GV3101 and LBA4404 (from glycerol) were streaked, under sterile conditions, onto LB agar plates containing 25 µg/mL rifampicin and incubated overnight at 28°C. A single colony of each *Agrobacterium* strain was inoculated into 5 mL of liquid LB containing 25 µg/mL rifampicin and incubated overnight at 28°C with shaking (200 rpm). Aliquots of 2 mL were inoculated into 2x 250 mL of liquid LB in 1 L conical flasks and incubated at 28°C with shaking (200 rpm). Cultures were grown to mid-log phase (OD_{600} = 0.6) for 24 h. Cultures were chilled on ice for 10 min and centrifuged at 4000 g for 15 min at 4°C. Cells were washed in 250 mL of chilled, filter sterilised 1 M HEPES (pH 7.0). This was repeated twice then cells were resuspended in 10 mL of chilled 10% glycerol and centrifuged at 4 000 g at 4°C for 15 min. Final cell pellets were resuspended in 1 mL of 10% glycerol, transferred as 50 µL aliquots into 1.5 mL tubes. Cells were snap frozen in liquid nitrogen and stored at - 80°C.

2.2.3.5 Electroporation of competent *Agrobacterium*

Electro-competent cells were transformed by electroporation using an EC100 electroporator (Thermo EC) based on the method of Dower *et al*. (1988). Approximately 1 µg of plasmid DNA was mixed with 50 µL of *A. tumefaciens* cells and transferred to a pre-chilled electroporation cuvette (path length = 2 mm) (BioRad). Cells were pulsed at 2800 V for 5 msec, immediately resuspended in 1 mL of LB liquid media and incubated for 2 h at 28°C with shaking (250 rpm). Cultures were spread onto LB agar plates containing the appropriate antibiotics and incubated at 28°C for up to 72 h.

2.2.3.6 Preparation of bacterial glycerol stocks

Cultures of *E. coli* or *A. tumefaciens* were preserved by mixing 150 µL of log stage culture with 850 µL of sterile 100% glycerol in 2 mL cryovials. Glycerol stocks were snap frozen in liquid nitrogen and stored at -80°C.

2.2.3.7 DNA sequencing

All sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit^{TM} (version 3.1) (Applied Biosystems) and contained 300 ng of purified plasmid DNA

template, 20 pmol primer and 3.5 µL BDTv3.1 ready mix in a final volume of 20 µL. All sequencing PCRs used an initial denaturation at 96°C for 1 min prior to 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4_min. After cycling, extension products were precipitated by the addition of two volumes of ice cold 100% ethanol, 2 µL of 3 M sodium acetate (pH 5.2), 2 µL of 125 mM EDTA (pH 8.0), incubation at room temperature for 30 min, and centrifugation at 14 000 g for 20 min. DNA pellets were washed with 500 µL of ice cold 70% ethanol and centrifuged for a further 5 min at 14 000 g. DNA pellets were air dried and capillary sequenced at the Molecular Genetics Research Facility (QUT, Brisbane).

2.2.4 Transient *Agrobacterium*-mediated transformation of plants

2.2.4.1 Plant material and growth conditions

N. benthamiana seed was kindly supplied by the Department of Primary Industries, (Indooroopilly, Australia). Seed was sown on a fortnightly basis in seed trays containing Searles Seed Raising mix and plantlets grown in Searles Potting mix in growth cabinets at 27°C with a 16 h photoperiod.

2.2.4.2 Agroinfiltration

A. tumefaciens cells were infiltrated into *N. benthamiana* leaves using the method of Sainsbury and Lomonossoff (2008). In short, a single colony of recombinant bacteria was inoculated into liquid LB media (Agl1 and GV3101) or YM (C58 and LBA4404) containing the appropriate antibiotics and cultures were incubated overnight at 28°C with shaking (200 rpm). Bacteria were pelleted by centrifugation (14,000 g for 5 min) and resuspended to an OD_{600} = 1.0 in MMA (unless otherwise specified). Cultures were then incubated for 2-4 h at room temperature with gentle rocking. Bacteria were delivered into the underside of leaves of 1-2 month old plantlets using a blunt tipped plastic syringe and applying gentle pressure.

2.2.5 General methods for protein analysis

2.2.5.1 Extraction of total soluble protein

N. benthamiana leaf samples were collected between 0 – 8 days post Agroinfiltration and snap frozen in liquid nitrogen. Total soluble protein (TSP) was extracted by homogenising the samples in three volumes (w/v) of GUS extraction buffer using a micropestle. The crude lysate was clarified by centrifugation (14,000 g for 15 min) and protein content estimated using the Bradford assay (Bradford, 1976).

2.2.5.2 GUS fluorometric quantification

GUS expression levels were quantified by fluorometric analysis and repeated in triplicate over an enzymatic time course (T0, 10 min and 20 min). TSP (5 µL) was added to 25 µL of

MUG substrate in a microtitre plate and incubated at 37°C. Reactions were stopped by the addition of 270 μL stop buffer and fluorescence measured using a Perkin Elmer LS50B fluorescence spectrometer (excitation 365 nm, emission 455 nm). Enzyme activities were expressed as nmol 4-MU/mg protein/min.

2.2.5.3 SDS-polyacrylamide (SDS-PAGE) gels

Protein samples were electrophoresed through a 12.5% polyacrylamide gel unless otherwise stated. Protein extracts were mixed with an equal volume of 2 x loading buffer and heated at 95°C for 5 min, immediately quenched on ice then electrophoresed in running buffer on a Protean II Minigel Apparatus (BioRad) at 4°C. A pre-stained MW protein marker (low range, BioRad) was used to estimate the size of the resulting bands. SDS-polyacrylamide gels were usually run at 10 mA for 2 h and then either visualised using Coomassie Blue solution or used for Western blot analysis.

2.2.5.4 Western analysis

Proteins separated by SDS-PAGE were transferred to a NT nitrocellulose transfer membrane (Roche) using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) in Western transfer buffer overnight at 4°C using a constant voltage of 15 V. The membrane was blocked by incubation in blocking solution comprising 5% (w/v) skim milk powder diluted in TTBS buffer for 1 h on an orbital shaker at room temperature. The primary antibody was then diluted 1:3000 in blocking solution and incubated with the membrane on an orbital shaker at room temperature for 1 h. The membrane was washed 4 times for 15 min with TTBS buffer, then incubated with the secondary antibody (horseradish peroxidase (HRP)-conjugated) diluted 1:3000 in blocking solution on an orbital shaker at room temperature for 60 min. Following secondary antibody incubation and washing in TTBS (4 x 15 min), a 1:10 dilution of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was applied directly to the membrane and the signal was detected using X-ray film (AGFA). X-ray films were developed manually by dipping in developer solution for 2 min, fixer solution for 5 min, rinsing with water after each step then air-dried. If signal was not detected after 30 min exposure, membrane was rinsed with TTBS, incubated with a 1:4 dilution of the substrate and exposure to X-ray film was repeated.

2.3 Statistical Analysis

Fluorometric GUS measurements from three leaves on three biological replicates over three separate occasions were pooled and the mean calculated. Graphs and basic statistical analysis were generated in Excel; data were expressed as means ± Standard error (SE). Significant differences from the respective controls were calculated using an unpaired T-test. $P < 0.05$ was considered significant (Townend, 2002).

Chapter 3: Improving Agroinfiltration-based Transient Expression in *Nicotiana benthamiana*

3.1 Introduction

Using plants as bioreactors for the production of bioproducts represents an attractive alternative to conventional expression systems. Plant bioreactors are relatively simple and inexpensive to grow, generating large quantities of biomass that can be easily scaled up or down depending on demand (Doran, 2000). Also, the risk of human pathogens or pathogen-derived contaminants from plant derived proteins is negligible (Ma *et al*., 2003; Streatfield, 2007; Yin *et al*., 2007). The commercial viability of plant-made proteins is strongly yield dependent and considerable effort has been made to increase recombinant protein expression levels.

A transgene can be expressed long-term from an integrated copy, in the case of transgenic plants, or transiently for short-term protein accumulation. While both systems have inherent benefits and disadvantages, the generation of transgenic plants is particularly slow and laborious and typically yields significantly lower expression levels than the latter method (Mason *et al*., 1992; Twyman *et al*., 2003). Transient transformation provides for high-level and very rapid transgene expression as it does not require the production of transgenic plants and protein accumulation usually peaks 2-7 days post transformation (Janssen and Gardner 1990). In many cases, researchers have relied on a single host target species namely, *Nicotiana benthamiana*, for transient expression due its ease of transformation and innate ability to support high levels of recombinant protein accumulation (Sheludko *et al*., 2007).

Commonly, the expression cassette encoding the protein of interest is delivered using disarmed forms of the phytopathogenic bacteria *Agrobacterium tumefaciens. Agrobacterium*-mediated transformation for transient expression involves the physical introduction of recombinant bacteria into leaves or whole plants using a syringe or a vacuum infiltration tank, a process known as Agroinfiltration. During the infection process, transfer DNA (T-DNA) containing the expression cassette is translocated to the plant cell nucleus as a ssDNA molecule complexed to bacteria-encoded virulence gene products. Within the nucleus the T-DNA is integrated into the host cell genome as a dsDNA intermediate via an illegitimate recombination process (Offringa *et al*., 1990). It is now understood transgene expression can occur from both the free and integrated forms of the

T-DNA thus providing for either short-term or stable expression (Nam *et al*. 1999). As such, *Agrobacterium*-mediated transformation is a complex and evolved process that is dependent on both host and pathogen determinants.

Numerous factors can influence the efficacy of *Agrobacterium*-mediated transformation. Physical attributes including temperature, pH, osmotic conditions, explant type, bacterial strain (Wroblewski *et al*., 2005) and density, and co-cultivation time (Mondal *et al*. 2001; Dong and McHughen 1993), have all been shown to affect the frequency of transformation. The design of a suitable artificial environment to promote the interaction of bacteria and explants is also of considerable importance. The plant-secreted phenolic, acetosyringone, induces virulence gene expression in the *Agrobacterium* (Hiei *et al*. 1994), and the inclusion of this inducer molecule to the co-cultivation media often improves transformation efficiencies (Jeoung *et al*., 2002; Rogowsky *et al*., 1987; Stachel *et al*., 1986; Wydro *et al*., 2006). In plant cells, the accumulation of reactive oxygen species (ROS) produced during the oxidative burst response to abiotic stresses or pathogen attack (e.g. *Agrobacterium* infection) can lead to cell damage and necrosis (Welch and Brown 1996; Olhoft *et al*. 2003; Qiusheng *et al*. 2005). The addition of antioxidant or anti-necrotic compounds such as α-Lipoic acid, ascorbic acid and PVP have proven to delay or inhibit the effects of ROS (Dan *et al*. 2009; Khanna and Daggard 2003; Halliwell and Gutteridge 1990; Tang *et al*. 2005).

A number of molecular factors can also significantly influence transgene expression. At the level of transcription, strong constitutive promoters are often desired and the inclusion of an intron in the 5' transcribed region of the transgene has been shown to enhance gene expression, a process commonly known as intron mediated enhancement or IME (Mascarenhas *et al*., 1990; Rose, 2008). However, mRNA can be rapidly degraded in a targeted, systemic and sequence-specific manner through a process known as post-transcriptional gene silencing (PTGS) (Ahlquist, 2002). This natural plant response to pathogenic or aberrant RNA (dsRNA) can drastically reduce transgene expression levels. To overcome this, virus-derived suppressors of PTGS, for example the Tomato bushy stunt virus (TBSV) p19 and the cucumber mosaic virus (CMV) 2b proteins are co-expressed with the gene of interest (Johansen and Carrington 2001; Voinnet *et al*. 2003). At the level of translation, codon optimisation of the transgene to favour host codon usage and the inclusion of virus-derived translation enhancer sequences (e.g. Tobacco mosaic virus Ω leader) in the coding region often improves expression levels (Gallie 2002). Post-translational processing and folding of the nascent protein is critical to maintain polypeptide stability and accumulation. The *in vivo* process of folding newly synthesised

linear polypeptides into functional three-dimensional proteins is an energy-dependent process which relies on the presence of ubiquitous helper molecules referred to as heat-shock proteins or molecular chaperones (Freedman *et al*. 1994; Georgopoulos and Welch 1993; Hartl and Martin 1995).

BAG (Bcl-2 associated athanogene) genes are an evolutionarily conserved family of multifunction co-chaperone proteins (Williams *et al*., 2010). BAG homologues are found in diverse species, and whose products are known to promote cell survival. *At_BAG4* is one of seven *BAG* family homologues identified in *Arabidopsis thaliana.* Transgenic *Arabidopsis BAG4* knockouts display early senescence and unique phenotypes suggesting this gene's product influences plant growth and development. Further, over-expression of *At_BAG4* in tobacco, tomato and banana has been shown to increase tolerance to various biotic and abiotic stresses (Doukhanina *et al*., 2006).

Modern plant expression cassettes have been engineered to include virus-derived genetic elements to enhance transcription and translation, amplify gene copy number and suppress PTGS. One recent example of this is the Hyper-translatable (HT) vector system described by Sainsbury and Lomonossoff (2008). In this vector (pEAQ-HT), transgene expression was controlled by the strong CaMV 35S promoter and the transgene mRNA was engineered to include translation enhancer sequences derived from Cowpea mosaic virus (CPMV) RNA-2. In addition, the cassette co-expressed the TBSV p19 protein, a potent suppressor of PTGS. Very high recombinant protein yields of up to 1.5 g/kg corresponding to about 25-30% total soluble protein (TSP) were obtained in *N. benthamiana* using the HT system (Sainsbury *et al*., 2009). In this chapter a number of parameters were assessed to improve the efficiency of Agroinfiltration of *N. benthamiana* and increase transgene expression levels. Using the HT vector system to express the GUS reporter gene, various physical factors, including *Agrobacterium* strain, concentration, and sampling time post transformation were compared and the effects of assorted chemical additives in the infiltration media were optimised. Further, this chapter demonstrated the positive effects of co-expressing virus-derived silencing suppressors and molecular chaperones on recombinant protein accumulation, and that a whole plant heat treatment can significantly increase transient expression levels.

3.2 Materials and methods

3.2.1 Vector construction

pEAQ-HT was a generous gift from G. Sainsbury and G. Lomonossoff, John Innes Centre, UK (Sainsbury *et al*., 2009). p35S-GSN is a pBIN-Plus vector backbone containing the *uid*A gene (with a small synthetic intron) encoding the GUS reporter enzyme under the transcriptional control of the CaMV 35S promoter and nos terminator (Dugdale *et al*., in press). The *uid*A gene containing the synthetic intron was excised from p35S-GSN as a BamHI (blunt-ended) and SalI fragment and ligated into AgeI (blunt-ended) and XhoI-digested pEAQ-HT. The resulting construct was called pEAQ-GSN (Chapter 2.2.1.10).

To isolate the tobacco *Hsp70* and *Hsp101* genes encoding heat shock proteins, one month old *N. tabacum* cv. Samsun NN plants were heat shocked at 37°C for 90 min and allowed to recover for 1 h at room temperature. Total RNA was extracted from leaf material using a QIAGEN RNeasy kit and *Hsp70* and *Hsp101* cDNA was synthesised using the ImProm-II™ Reverse Transcriptase System (Promega). In short, reaction mixtures containing 1 µg of total RNA and 10 µM of either primer Hsp70_R 5'-CTAGACATTGACAACGCAAAATCGATGACACT-3' or Hsp101_R 5'-GAGCTCTTAATCTTCCATTTCGTCATCTTCAATTTCTTC-3', were incubated at 99°C for 5 min then chilled on ice for 5 min. First strand synthesis was performed at 42°C for 1 h, while second strand synthesis was achieved using GoTaq® Green Master Mix (Promega) in a reaction containing 1 µL of cDNA and 10 µM of the following primer pairs, *Hsp70*: Hsp70-F_AsiSI 5'-GCGCGATCGCATGGCCGGAAAAGGAGAAGGTCC-3' and Hsp70-R_KpnI 5'-CGGGTACCTTAGTCGACCTCCTCAATCTTGG-3' and *Hsp101*: Hsp101-F_AsiSI and Hsp101-R 5'-GCGATCGCATGAATCCTGAAAAATTCACCCACAAGACTAACGAG-3'. The mixtures were cycled using the following conditions: 5 min at 94°C followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min.

To isolate the *At_BAG*4 gene, genomic DNA was isolated from *Arabidopsis thaliana* (cv. Landsberg) using the CTAB method (Porebski *et al*., 1997). The *At_BAG*4 gene was amplified by PCR using GoTaq® Green Master Mix, 0.1–1 µg genomic DNA and 10 µM of the following primers At_BAG4-F_AsiS1 5'-CGGCGATCGCATGATGCATAATTCAACCGAAG-3' and At_BAG4-R_Sac1 5'-GCGAGCTCTCAGTCAAATTTCTCCCAATCTTG-3' using the above cycling parameters.

All silencing suppressor genes were amplified by PCR from cloned viral components. The TBSV p19 gene was amplified from pEAQ-HT using primers p19_AsiSI-F 5'-GCGCGATCGCCATGGAAAGGGCTATTCAGGGAAATGATGC -3' and p19_SacI-R 5'-

GCGCGATCGCCATGGAAAGGGCTATTCAGGGAAATGATGC -3'. Plasmids containing full-length clones of CMV, *Papaya ring spot virus* (PRSV) and *Tomato leaf curl virus* (TOLCV) were kindly provided by Dr Anthony James, Dr Marion Bateson and Dr Ali Rezaian respectively. The CMV 2b gene was PCR amplified using primers CMV_2b_AsiSI-F 5'-GCGCGATCGCATGGAATTGAACGTAGGTGC-3' and CMV_2b_SacI-R 5'-GCGAGCTCTCAGAAAGCACCTTCCGCCCATTCG-3', the PRSV HC-pro gene was amplified using primers Hc-Pro_AsiSI-F 5'-CGGCGATCGCGCAGTACAATGACGTGGCTGAAAAATTCTGGC-3' and Hc-Pro_SacI-R 5'-CGGAGCTCCCGATGTAGTGCTTCATTTCACTATCG-3' and the ToLCV TrAP gene amplified using primers TrAP_AsiSI-F 5'-GCGCGATCGCATGCAGAATTCATCACCCTC -3' and TrAP_SacI-R 5- GCGAGCTCTTAAATACCCTCAAGAAACGCC -3'. A 90 amino acid CMV 2b truncation was amplified using primers CMV_2b_AsiSI-F and CMV_2b(90)-SacI. PCRs used GoTaq® Green Master Mix and contained 10 µM of each primer and either 0.1–1 µg gDNA or 100 ng of plasmid DNA as template. PCR conditions for amplification of these genes was essentially as described above adjusting the extension time based on 1 kb per minute as appropriate. All genes were cloned into pGEM®-T-Easy (Promega) and confirmed by sequencing using the Big Dye™ Terminator system.

All plant and virus-derived genes were excised from pGEM-T Easy as AsiSI and SacI fragments (except *Hsp*70 which was excised as an AsiSI and KpnI fragment) and ligated between the CaMV 35S promoter and nos terminator in a similarly digested pBIN-Plus binary vector. These vectors were called p35S-Hsp70, p35S-Hsp101, p35S-At_BAG4, p35S-TSBV_P19, p35S-CMV_2b, p35S-CMV_2b(trunc), p35S-PRSV_HcPro and p35S-ToLCV_TrAP, respectively.

3.2.2 Agroinfiltration of *N. benthamiana*

Plasmids were mobilised into *Agrobacterium tumefaciens* strains Agl1, C58, LBA4404 and GV301 via electroporation (Dower *et al*., 1988).. Recombinant *Agrobacteria* were prepared for infiltration using a modified protocol of Sainsbury and Lomonossoff (2008), as per Chapter 2.2.4.2. For co-transformations, recombinant bacteria containing different plasmids were mixed at a 1:1 ratio immediately prior to infiltration. The top three leaves of three independent plantlets (approximately 6-8 weeks in old) were infiltrated per plasmid or plasmid combination. This process was repeated on three separate occasions. Plants were germinated from seed, propagated in growth cabinets at 25°C with a photoperiod of 16 h and fertilised with Aquasol™ (1 g L^{-1}) two weeks prior to infiltration.

3.2.3 Chemical additives

Chemicals including α-Lipoic acid (0-100 mM), ascorbic acid (0-100 mM), Pluronic F-68 (0-0.2%) and polyvinylpolypyrrolidone 4 000 (PVP) (0-1 mM) were filter sterilised and added to the MMA:bacteria mix immediately prior to infiltration. For acetosyringone, MMA was prepared containing a final concentration ranging from 0-600 μM.

3.2.4 Protein extraction and GUS fluorometric assays

Leaf samples were sampled and total soluble protein (TSP) was extracted and quantified as described in Chapter 2.2.5.1 (Bradford, 1976). GUS expression levels were quantified by fluorometric analysis and repeated in triplicate over an enzymatic time course, as per Chapter 2.2.5.2. The data obtained and analysed as described in Chapter 2.3.

3.3 Results

3.3.1 *Agrobacterium* strains and time course

Agrobacterium tumefaciens strains Agl1, C58, GV3101 and LBA4404 were selected to compare bacterial virulence and transformation efficiencies between commonly used laboratory bacterial strains. The four strains of recombinant *Agrobacteria* harbouring pEAQ-GSN were infiltrated into the top three leaves of three *N. benthamiana* plantlets and leaves were sampled every 0, 2, 4, 6 and 8 days post infiltration (dpi). GUS activity was measured fluorometrically and data from three separate experiments pooled, statistically analysed and graphed (Figure 3.1). On day 0, negligible levels of GUS expression were observed from all *Agrobacterium* strains suggesting no bacteria-derived GUS expression or endogenous plant-derived GUS activity. For all strains except C58C1, GUS expression increased to their highest levels at 4 dpi and then decreased to 8 dpi where expression levels where undetectable. For strain C58C1, highest GUS expression was observed at 6 dpi. The highest GUS expression was observed using strain Agl1 at 4 dpi. This expression level was approximately 3 to 4-fold higher than the highest expression afforded by strains LBA4404 or GV3101 (4 dpi) and about 2-fold higher than the best expression levels from strain C58C1 (6 dpi).

3.3.2 *Agrobacterium* concentration

To examine the effect of *Agrobacteria* concentration on transgene expression, strain Agl1 harbouring pEAQ-GSN was grown to optical densities ranging from 0.001 to 3.0 and these were independently infiltrated into the top three leaves of three *N. benthamiana* plantlets. Leaves were sampled at 4 dpi and GUS activity data from three separate experiments was pooled and statistically analysed (Figure 3.2). When compared to the GUS expression levels

obtained using recombinant bacteria infiltrated at the control density of OD_{600}=1.0, significantly lower GUS expression was observed in leaves infiltrated with Agl1 at OD_{600} of 0.001 and 0.01, while no significant difference in GUS expression was seen in leaves infiltrated with Agl1 at OD_{600}=0.1, 0.5, 1.5 and 3. However, in leaves infiltrated with recombinant Agl1 at OD_{600}=2.0 and 2.5, GUS levels were significantly higher (3 to 4-fold) than the OD_{600}=1.0 control.

3.3.3 Effects of chemical additives

The effects of five chemical additives on transgene expression were tested by including varying concentrations of these compounds in the MMA co-cultivation media (Figure 3.3 a-e). When compared to GUS expression levels using MMA alone, the addition of α-Lipoic acid at 100 mM resulted in significantly lower expression levels, while a concentration of 50 mM resulted in no significant difference (i.e. $p \geq 0.05$). In contrast, the addition of α-Lipoic acid at concentrations of 5 and 10 mM resulted in significant increases in GUS expression (i.e. $p < 0.05$), with a concentration of 5 mM increasing expression approximately 2-fold over that using MMA alone. The inclusion of acetosyringone to MMA at a final concentration of 100 μM resulted in no significant difference in GUS expression levels. However, at concentrations of 200, 300, 400, 500 and 600 μM, GUS expression was found to significantly increase with increasing acetosyringone concentration, up to a concentration of 500 μM. At this concentration, GUS expression levels were approximately 5-fold higher than those obtained using MMA media alone. The addition of ascorbic acid had no significant effect on GUS expression levels, while the use of PVP at high concentrations (0.5 and 1.0 g/L) resulted in significantly decreased GUS expression levels compared to MMA alone. The addition of a low concentration of the surfactant Pluronic F-68 (0.002%) to MMA was found to significantly (2-fold) increase GUS expression, whereas higher concentrations resulted in either no significant effect (0.02%) or a significant reduction (0.2%) in GUS levels as compared to the MMA control. Based on these observations, leaves were infiltrated with *Agrobacteria* in MMA containing α-Lipoic acid (5 μM), acetosyringone (500 μM) and Pluronic F-68 (0.002%). When compared to the expression levels using MMA alone, GUS expression was found to be significantly higher (approx. 5-fold) (Figure 3.3 f). However the difference between the combined treatments was not significantly higher than that of MMA plus the optimal concentration of the individual chemical treatments

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3.3.3.1 Effects of physical heat-shock and expression of chaperone proteins

To determine the effects of physical heat shock on transient expression, whole plants were subjected to 37°C for 30 min at various time points post-Agroinfiltration (Figure 3.4 a). Although GUS expression levels in plants subjected to a heat shock either immediately following infiltration (Day 0) or at 3 dpi were higher than those in the non-heat shocked controls, the increases were not statistically significant. In contrast, heat shock of plants at both 1 or 2 dpi resulted in significantly increased expression to levels that were 4 to 5-fold higher than the controls.

To examine the effects of the chaperone proteins, tobacco Hsp70, tobacco Hsp101 and At_BAG4 on GUS expression, leaves were co-infiltrated with Agl1 harbouring pEAQ-GSN in combination with Agl1 containing either p35S-Hsp70, p35S-Hsp101, p35S-At_BAG4 or an empty control. Assessment of GUS levels at 4 dpi showed no significant increases in expression levels from the HT expression platform in the presence of either Hsp70 or Hsp101 (Figure 3.4 b) In contrast, the presence of the At_BAG4 gene product was found to significantly increase GUS levels by 2-fold (Figure 3.4 c). Combining At_BAG4 co-expression with a 2 Day post-infiltration heat shock treatment significantly increased GUS transient expression by about 5-fold (Figure 3.4 d), however, this level was not significantly different to GUS levels generated by a 2 dpi heat shock treatment alone.

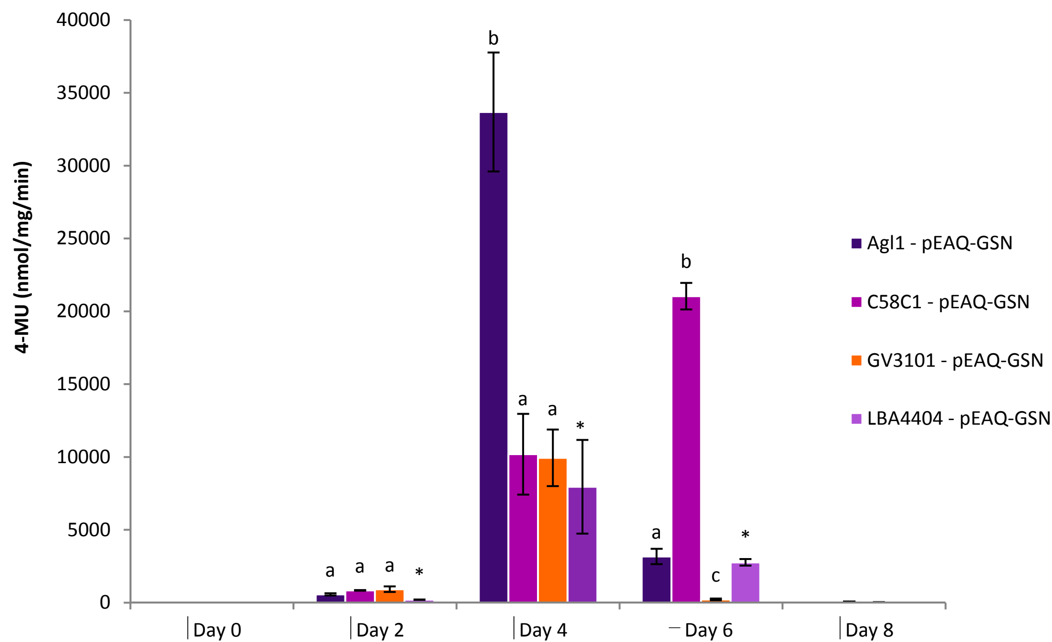

Figure 3.1 Comparison of transient GUS expression using four different strains of *Agrobacteria* over time.

Agrobacterium strains Agl1, C58, GV3101 and LBA4404 harboring pEAQ-GSN were inflitrated into *N. benthamiana* leaves. Leaves were sampled at 0, 2, 4, 6 and 8 days post infiltration (dpi) and TSP was extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment at each time point is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$), b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control as determined using an unpaired T-test ($p < 0.05$).

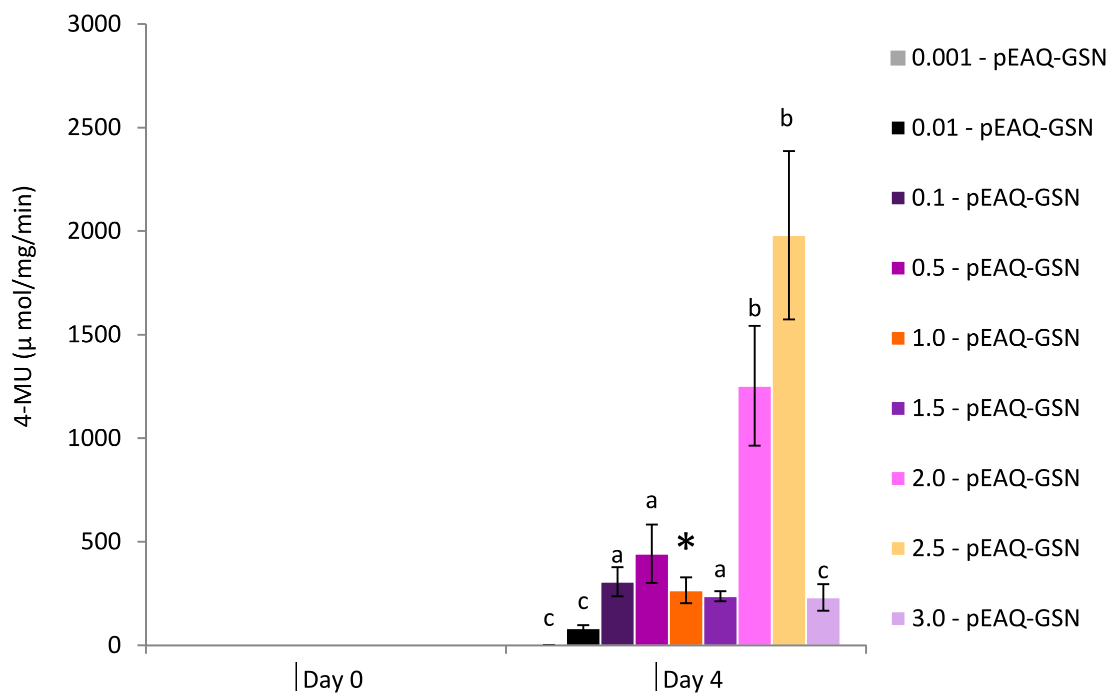

Figure 3.2 Effects of culture density on transient GUS expression.

Agrobacterium strain Agl1 harboring pEAQ-GSN was inflitrated into *N. benthamiana* leaves at increasing concentrations OD_{600} = 0.001, 0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0. Leaves were sampled at 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$), b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control ($p < 0.05$).

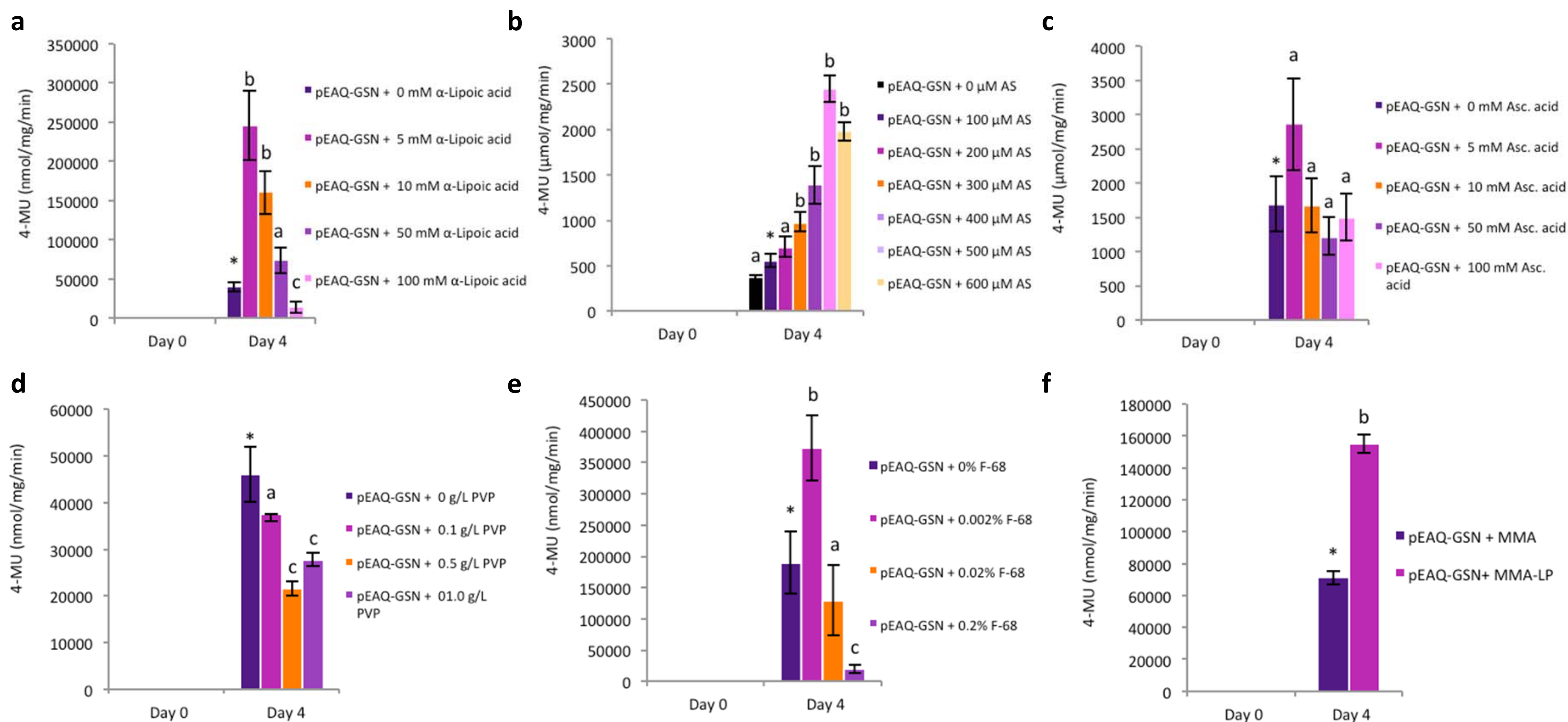

Figure 3.3 Effects of chemical additives on transient GUS expression.

Agrobacterium strain Agl1 harboring pEAQ-GSN was infiltrated in MMA media containing varying concentrations of chemical additives, (a) α-Lipoic acid, (b) acetosyringone, (c) ascorbic acid, (d) PVP, (e) Pluronic F-68, and (f) an optimised media (MMA-LP) containing α-lipoic acid (5 mM), acetosyringone (500 µM) and Pluronic F-68 (0.002%), into *N. benthamiana* leaves. Leaves were sampled 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$), b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control ($p < 0.05$).

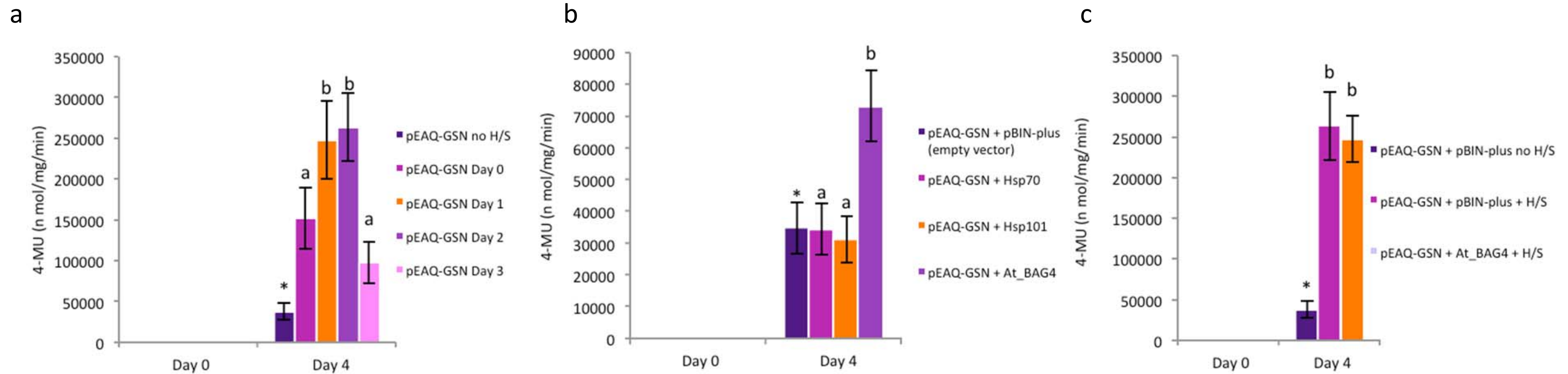

Figure 3.4 Effects of heat shock treatment and co-expressing chaperones on transient GUS expression.

(a) *Agrobacterium* strain Agl1 harboring pEAQ-GSN was infiltrated into *N. benthamiana* leaves and the whole plants heat shocked (H/S) (37°C for 30 min), at either 0, 1, 2, or 3 dpi. (b) Co-transformation with vectors capable of expressing either tobacco Hsp90, Hsp101 or At_BAG4. (c) Co-transformation with or without a vector capable of expressing At_BAG4 and the whole plant heat-treated 2 dpi. Leaves were sampled 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$) and b = data is significantly higher than the control ($p < 0.05$).

3.3.3.2 Effects of virus-derived suppressors of post transcriptional gene silencing

The effect of suppressors of PTGS on transient GUS expression was examined by co-infiltrating leaves with Agl1 harbouring pBIN-GSN (EAQ_GSN expression cassette without TBSV p19 on T-DNA) in combination with Agl1 containing either p35S-TSBV_P19, p35S-CMV_2b, p35S-PRSV_HcPro, p35S-ToLCV_TrAP (and the truncated 2b) or an empty vector control. When GUS expression was assessed at 4 dpi, (Figure 3.5 a), a significant increase in expression was observed in the presence of TBSV p19, CMV 2b and HC-Pro (Figure 3.5 a). The presence of TBSV p19 and CMV 2b resulted in 2 and 3-fold increases in GUS expression, respectively, while the PRSV HC-Pro increased reporter levels by 1.5-fold. The presence of ToLCV TrAP resulted in a significant decrease (2-fold) in GUS expression levels. A truncated CMV 2b protein was a strong suppressor of PTGS, significantly increasing GUS levels 3-fold compared to the empty vector control and about 1.5-fold over the unmodified CMV 2b protein (Figure 3.5 b), however a non-coding CMV construct (CMV 2b (NC)) resulted in a decrease in transgene expression, suggesting that CMV 2b does not operate on an RNA level. Co-expression of the TBSV p19 and the CMV 2b proteins in combination significantly increased GUS levels approximately 6-fold over the empty vector control.

3.3.4 Effect of developmental stage on total soluble protein levels

During this study it was observed that high transient GUS expression often appeared to correlate with high plant TSP levels. To determine whether a direct correlation exists, TSP was measured throughout the *N. benthamiana* lifecycle by sampling leaves from 5 plants weekly and pooling the leaves at each time point. The TSP was estimated by Bradford assay, levels were found to peak (2 µg/µL) at two weeks post-germination, after which these levels declined to below 0.3 µg/µL (Figure 3.6). Application of liquid-soluble fertilizer (Aquasol™) at a concentration of 1 g L^{-1} restored high TSP levels (>2 µg/µL) within two weeks of application. Flowering was initiated 2 months post germination and this developmental change triggered a significant reduction in TSP over the following 6 weeks to negligible levels.

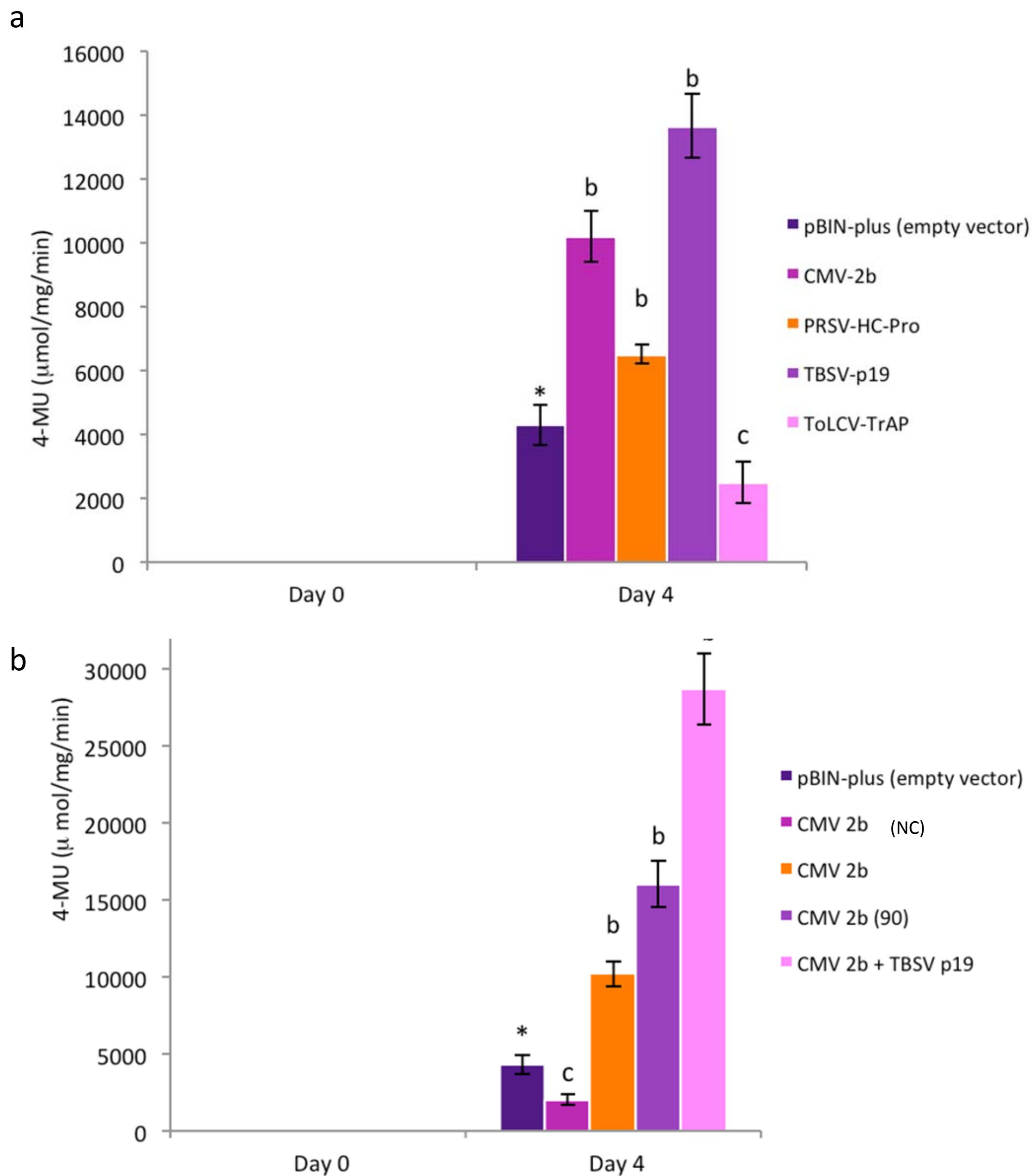

Figure 3.5 Effects of co-expressing suppressors of gene silencing on transient GUS expression.

Agrobacterium strain Agl1 harboring pEAQ-BIN-GSN) was co-infiltrated into *N. benthamiana* leaves with various suppressor of gene silencing. (a) Co-transformation with vectors capable of expressing either CMV 2b, PRSV HCPro, TBSV p19 or ToLCV TrAP. (b) Co-transformation with vectors capable of expressing a truncated CMV 2b(90) or both CMV 2b and TBSV p19. Leaves were sampled 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$), b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control ($p < 0.05$).

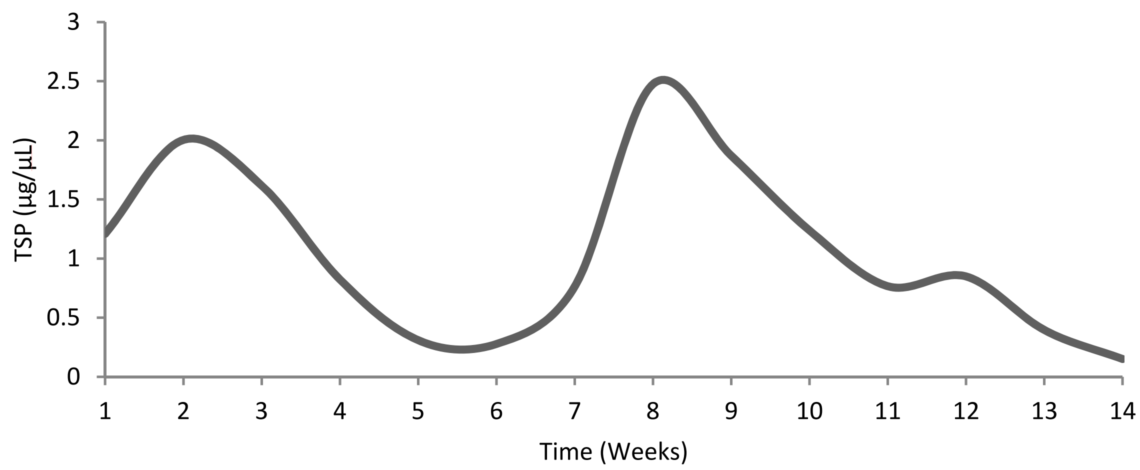

Figure 3.6 Total soluble protein content of *N. benthamiana* over time.

The top 3 leaves of 3 independent *N. benthamiana* plants, grown from seed, were harvested each week for 14 weeks. Leaves were immediately snap frozen and freeze dried for each time point and TSP content measured using the Bradford method. At week 6, plants were treated with Aquasol^{TM} liquid fertiliser and at week 8, flower initiation had commenced

3.3 Discussion

Agroinfiltration has become the preferred method to rapidly express recombinant biologics such as therapeutics and vaccine candidates and to study gene function, gene silencing or gene-for-gene interactions *in planta*. In order to maximise expression levels, researchers have sought to optimise the process at many levels, for example by increasing bacterial transformation rates, tailoring the vector to contain virus-derived elements that increase transgene transcription/translation and minimise PTGS, and by utilising host species that are highly amenable to the infection process and support high levels of recombinant protein accumulation. While these advances have contributed to developing Agroinfiltration as a cost effective platform for the production of plant made proteins, key aspects of the transformation methodology have yet to be fully optimised. In this study, Agroinfiltration of *N. benthamiana* with the HT vector system constructed by Sainsbury and colleagues (2008 and 2009) was used to define factors that contribute to rapid, high-level, transient gene expression.

Genetic background of the *Agrobacterium* can greatly influence the ability of the phytopathogen to act as a vehicle for T-DNA transfer. As such, four common laboratory strains were tested for their ability to support transient GUS expression in *N. benthamiana*. These strains represented three of the four opine utilising types; octopine (LBA4404), nopaline (GV3101 and C58C1) and succinamopine (Agl1) and members originated from either of the wildtype progenitor isolates C58 (Agl1, GV3101, and C58C1) and Ach5 (LBA4404). Of note, GUS levels varied greatly between strains with the hypervirulent strain Agl1 yielding the highest GUS activity and peaking 4 dpi. This may suggest Agl1 has a more aggressive disposition for infection or perhaps a more effective bacteria-encoded T-DNA transfer mechanism in comparison to the other strains (Álvarez *et al*., 2004; Li *et al*., 2010). These findings, however, contrast those of Wydro *et al*. (2006) who reported transient expression levels using strain Agl1 were lower than LBA4404 in *N. benthamiana* leaves Agroinfiltrated with a construct capable of expressing the green fluorescent protein (GFP). While the exact reason for this is unclear, perhaps the use of different reporter genes between studies and the methods of quantification may be of relevance considering the GUS protein is considered more stable than GFP. Alternatively, physiological condition of the host plants, number of assay replicates, and additional leaf wounding in the case of Wydro *et al*. (2006), may have all contributed to these findings. Strain C58C1 provided the second highest level of GUS expression that peaked 6 dpi. High levels of transient gene expression using this strain were also documented by Wroblewski *et al*. (2005) in plant

species that did not elicit a necrotic response to the bacteria, namely *N. benthamiana*, *Arabidopsis* and lettuce.

Bacterial density during the infection process can affect transformation efficiencies; too dilute a culture may result in a low bacteria to target cell ratio thereby decreasing transformation frequencies, whereas concentrated bacterial cultures can lead to bacterial overgrowth causing excessive tissue damage (Archilletti *et al*., 1995; Howe *et al*., 1994; Mondal *et al*., 2001). In this study, it was found that increasing the concentration of *Agrobacteria* generally increased GUS activities. Greatest GUS expression was obtained at OD_{600} = 2.0 and 2.5. However, bacterial cultures at this concentration were more difficult to physically deliver by syringe and GUS activity standard errors ranged considerably. Cultures delivered at concentrations between OD_{600} = 0.01 and 1.5 were markedly easier to infiltrate and provided transient GUS levels that were high and not significantly different. Accordingly, an OD_{600} = 1.0 for bacterial suspensions was selected as the standard concentration for all further comparisons. These findings are in agreement with Wydro *et al*. (2006) who found no differences in the levels of transient *gfp* gene expression from bacterial suspensions delivered at concentrations between OD_{600} = 0.1 and 1.5 into *N. benthamiana*. Similarly, Wroblewski *et al*. (2005) reported bacterial density was critical for optimal transient expression in lettuce, with best results observed between OD_{600} = 0.1 and 1.0.

It is well understood that the wound-induced, plant phenolic signal chemical acetosyringone plays an important role in both chemotaxis and the induction of *Agrobacterium* virulence (*vir*) genes. Incorporation of acetosyringone in the co-cultivation media during bacterial infection has reportedly enhanced the transformation rates of many plant species including those previously considered recalcitrant to transformation and, in some cases, broadened the host range of the *Agrobacterium* strain itself (Godwin *et al*., 1991). Agroinfiltration is a relatively non-pervasive procedure with cell damage often limited to the site of injection. As such, conditioning of the bacteria with acetosyringone prior to delivery most likely up-regulates *vir* gene expression in the absence of this wound response. This study showed that increasing acetosyringone concentrations in the infiltration media resulted in a proportional increase in reporter gene expression, peaking at a concentration of 500 μM. These results are in agreement with Wydro *et al* (2006) who reported a similar increase in *Agrobacterium*-based transient expression of *gfp*.

Innate plant defence mechanisms in response to pathogen invasion often generate an oxidative burst and the induction of pathogenesis-related genes, resulting in necrosis and

cell death at the point of infection. In order to prevent this during the *Agrobacterium*:plant interaction, many chemical additives have been tested for their ability to suppress oxidative stress, minimise necrosis and therefore increase the efficiency of transformation e.g. PVP, DTT, glutathione, ascorbic acid, cysteine, sodium thiosulfate, sodium selenite, and DL-α-tocopherol (Dan *et al*. 2009; Liu *et al.* 2008; Olhoft *et al*. 2003). In this study the effects of including three antioxidant compounds in the infiltration media used to deliver *Agrobacteria*; α-Lipoic acid, ascorbic acid, and the wetting agent PVP were compared. Of these, α-Lipoic acid had the greatest effect increasing transient GUS expression levels in a concentration dependent manner. The greatest GUS activity was obtained with 5 mM α-Lipoic acid, which increased GUS expression 5-fold. α-Lipoic acid is a sulphur-containing compound that exists in nature as a metabolic antioxidant capable of scavenging reactive oxygen species and recycling other antioxidants (Navari-Izzo *et al*., 2002). The compound has successfully been used to increase the frequency of *Agrobacterium*-mediated transformation of a number of crops including soybean, tomato, wheat and cotton (Dan *et al*., 2009). In tomato, the compound was shown to markedly reduce browning in plant tissues following infection and increase the percentage of explants displaying transient reporter expression by 3-fold. Ascorbic acid and PVP have been shown to minimise the secretion of wound-induced phenolics and prevent oxidative stress in rice and peanut transformation (Enríquez-Obregón 1999; Østergaard and Yanofsky 2010; Khanna and Daggard 2003). These results suggest that neither antioxidant increased *Agrobacterium*-mediated transient GUS expression in *N. benthamiana*. In fact, high concentrations of PVP reduced GUS expression levels. Considering this, it was assumed that the effects of these chemicals are dependent on bacteria and host plant compatibility factors and their effectiveness will most likely vary between studies.

The addition of surfactants such as Silwet-L77, Tween 20 and Pluronic F-68 during co-cultivation has been shown to increase *Agrobacterium*-mediated transformation efficiencies in various crops including wheat (Cheng *et al*., 1997; Wu *et al*., 2003), *Arabidopsis* (Clough and Bent 1998), radish (Curtis and Nam 2001), soybean (Liu *et al*. 2008) and switchgrass (Chen *et al*. 2010). While it is unclear exactly how these compounds function, it is presumed they reduce the surface tension of the co-cultivation media and perhaps eliminate certain substances that inhibit cell attachment to improve bacterial invasion and ultimately T-DNA delivery (Cheng *et al*., 1997; Wu *et al*., 2003; Yang *et al*., 2006). Various concentrations of the surfactant Pluronic F-68 were tested for their enhancing effects on transient expression via Agroinfiltration. Similar to other studies, very

low concentrations (0.002%) of the surfactant in the infiltration media were found to greatly influence *Agrobacterium*-mediated transformation efficiency, significantly increasing GUS expression levels two-fold over base levels. Concentrations greater than this had no positive effect, in fact, Pluronic F-68 at a concentration of 0.2% was most likely toxic causing a two-fold reduction in GUS expression compared to base levels. Phytotoxicity associated with the use of surfactants at high concentrations was also observed during *Agrobacterium* transformation of several wheat varieties, causing embryo death and/or preventing callus formation (Wu *et al*., 2003; Yang *et al*., 2006).

A physical heat shock to the entire *N. benthamiana* plant 1-2 days following Agroinfiltration generated a significant (4 to 5-fold) increase in transient GUS expression. It is well known that heat shock proteins (Hsps) and chaperones are up regulated in response to extreme heats and other abiotic stresses (Wang *et al*. 2004) in order to maintain cellular homeostasis. These proteins facilitate the correct conformational folding of native proteins by binding to the reactive surfaces of partially folded proteins, effectively sequestering their active sites. This limits interactions between partially folded intermediate stages preventing aggregation reactions and assisting in degradation of terminally misfolded proteins (Buchner 1996; Georgopoulos and Welch 1993; McClellan *et al*. 2005; Thomas *et al*. 1997; Xiao *et al*. 2010) and provide protection from oxidative stress. Therefore, it was assumed that the increase in transient GUS expression observed in this study is the direct result of these stress related proteins protecting the infiltrated leaf cells from incompatible *Agrobacterium* interactions and increasing cell survival and transformation frequencies. The fact that co-expression of heat shock proteins (either Hsp70 or Hsp101) failed to increase GUS levels suggests this chaperone effect is likely a complex process involving more than one Hsp and/or stress-tolerated protein. While heat treatments of explants (e.g. seedlings) have been shown to increase *Agrobacterium*-mediated transformation frequencies in crops including switchgrass (Chen *et al*. 2010), banana (Khanna *et al*. 2007), rice and maize (Hiei *et al*. 2006), it is believed that this is the first report of its enhancing effects on transient expression via Agroinfiltration in whole plants.

It was proposed that the stress tolerance activity of *At_BAG4* would act to moderate programmed cell death resulting from incompatible *Agrobacterium*:host cell interactions and thereby increase the efficiency of transformation and transgene expression levels. In turn, co-expression of the *At_BAG4* gene significantly increased GUS levels (approximately 2-fold) from the HT expression system. Importantly, BAG4 levels in *Arabidopsis* plants are not up-regulated by heat shock treatment (Doukhanina *et al*., 2006). Hence, *At_BAG4* was

co-expressed in conjunction with a heat treatment in order to determine if this combination would further increase GUS levels. At_BAG4 failed to augment GUS levels above those of heat treatment alone. The reason for this remains unclear, however, it may suggest the enhancing effects brought about by the interaction of *At_BAG4* and *Hsp70* (or other factor(s)) is concentration dependent, and the balance of the two molecules is critical to their chaperone activity.

It is well accepted that low transient *Agrobacterium*-mediated transgene expression is often the result of PTGS and this bottleneck can be overcome by the co-expression of suppressors of gene silencing (Voinnet *et al*., 2003). Many plant viruses encode gene products that are capable of suppressing PTGS, however, their mode of action and potency can vary between virus families. To determine the most effective virus-derived silencing suppressor for our purposes, genes were compared from members of four different virus families, including the Bromoviridae (CMV 2b), Potyviridae (PRSV HC-Pro), Tombusviridae (TBSV p19), and the Geminiviridae (ToLCV TrAP). Geminivirus TRaP mediated silencing suppression operates by inhibiting the systemic amplification of the silencing signal (Glick, 2008), therefore the localised effects on silencing suppression are minimal, accounting for the lower reporter gene expression levels. Of these, the CMV 2b and TBSV p19 gene products were the most effective at suppressing PTGS, both significantly increasing GUS levels by approximately 3-fold. TBSV p19 has long been considered a potent suppressor of gene silencing and has been shown to increase transient expression levels in various plant species (Voinnet *et al*., 2003) by sequestering siRNA and preventing their association with the RISC complex (Baulcombe and Molnár 2004; Lakatos *et al*. 2006; Reed *et al*. 2003; Scholthof 2006). In contrast, CMV 2b is able to directly interact with both the RNA and protein components of the silencing pathway (Diaz-Pendon *et al*. 2007; Thomas *et al*. 2003). A truncation of the 2b protein (1-94 amino acids) effectively suppressed PTGS, increasing GUS levels 1.5-fold higher than the complete 2b protein. Importantly, this truncated protein comprised both nuclear localisation signals (NLS1 and NLS2) a putative phosphorylation site (39-43) and an α-helical domain (62-65) which has been shown to down regulate AGO4 (Duan *et al*., 2012; González *et al*., 2012; Lucy *et al*., 2000; Wang *et al*., 2004). Co-infiltration of both CMV 2b with TBSV p19 resulted in a doubling of GUS expression levels compared to using either silencing suppressor alone. This may indicate that by combining the diverse functions of both proteins i.e. binding and sequestering of siRNAs, preventing siRNA duplex assembly into the RISC, and direct interference with the AGO containing RISC, serves to collectively enhance PTGS suppression.

It has been suggested that the efficiency of transient gene expression in *N. benthamiana* via Agroinfiltration is greatly affected by the developmental stage of the plant, with highest expression obtained using plants shortly before they flower (8-10 weeks old) in comparison to young plants (4-6 weeks old) or intensively flowering plants (>10-12 weeks old) (Sheludko *et al*., 2007). Further, the age of the infiltrated leaf can influence protein accumulation with the top 6 fully expanded upper leaves often providing the highest levels. While the majority of the infiltrations performed in this study used plants and leaves within these optimal age parameters some differences were noted in transient expression between experiments. It was hypothesised that recombinant protein levels may correlate with the plants' natural TSP content at the time of Agroinfiltration, i.e. leaves with naturally high TSP content would accumulate high levels of recombinant protein. Therefore, the TSP content in the top leaves of *N. benthamiana* was examined over a 15-week period of development, including fertiliser treatment and flowering. While TSP profiles changed dramatically over the life cycle of the plant, no significant positive correlation between GUS expression data and TSP content at the time of infiltration, were determined (data not shown). This suggests TSP content alone does not govern the effectiveness of Agroinfiltration based transient expression in this species, rather a combination of host characteristics such as ontogenic factors (plant age and developmental stage), ease of infiltration and protein content may act together to influence the process.

This study sought to increase expression levels from the HT vector system of Sainsbury *et al* (2009) in *N. benthamiana* using Agroinfiltration, by optimising physical and chemical conditions associated with *Agrobacteria*:host interactions and co-expressing products known to assist in protein accumulation or prevent PTGS. In addition to testing parameters previously shown to influence *Agrobacterium*-mediated transformation rates, a number of novel improvements to the system were identified, including the addition of α-Lipoic acid or Pluronic F-68 in the Agroinfiltration media, co-expression of the stress tolerance *At_BAG4* gene, and a simple heat shock treatment to the host plant that significantly increased transient reporter gene expression. Used in combination these factors may contribute to the further development and deployment of this expression platform for the rapid and high-level production of plant-made proteins.

Chapter 4: Effects of the *Arabidopsis* histone H2A protein on Agroinfiltration-based transgene expression

4.1 Introduction

Belonging to an evolutionarily conserved group of proteins, histones are the major constituent of chromatin and function in “spooling” DNA in order to compact and organise it into structural units known as nucleosomes (Luger *et al.*, 1997; Olins and Olins, 1974). Histones have been identified in all eukaryotes except for dinoflagellates, and are also present in Euryarchaea (Archaea) but not other protozoa. Histone proteins belong to 5 distinct classes; core histones (H2A, H2B, H3 and H4) and less highly conserved linker histones (H1/5). The four core histones possess a “helix-turn-helix-turn-helix” motif referred to as the histone fold which allows for the formation of an octameric structure comprising two H2A:H2B dimers and an H3:H4 tetramer by polar and hydrophobic interaction. DNA is wrapped around the octamer and anchored in place by H1 linker histones (Luger *et al.*, 1997; Sullivan *et al.*, 2002) in order to facilitate higher order, supranucleosomal structures which control DNA accessibility. Histone proteins have an overall positive charge and are alkaline in order to facilitate DNA interactions and H_2O solubility (Ehmann *et al.*, 1998).

The nucleosome is of particular importance during meiosis and mitosis and histones are primarily synthesised during S phase. Histones are responsible for packaging eukaryotic DNA and play a role in DNA replication, gene expression and in preventing DNA degradation (Kornberg and Lorch, 1999; Robbins and Borun, 1967). Chromatin can be broadly categorised into two distinct types based on its state of gene expression: transcriptionally active chromatin or euchromatin, and transcriptionally inactive chromatin or heterochromatin. Heterochromatin is more prevalent and tightly bound in regions of the genome that are expressed less often, and has been associated with the “position effect”, a phenomenon in which the context of neighbouring sequences can negatively affect the expression of nearby genes (Eissenberg and Wallrath, 2003). The nucleosome is much more tightly packed in heterochromatin and has more bound histones. In contrast, euchromatin has a relaxed structure that allows its DNA to bind host cellular factors and polymerases required for gene transcription. It is the transitions between heterochromatin and euchromatin states that are thought to regulate cellular gene transcription and genome replication.

Agrobacterium-mediated transformation is used in plant biotechnology to transfer foreign DNA into plant cells for either transient gene expression or for stable DNA integration and the production of transgenic plants. The process is complex involving both host and bacteria-encoded factors for bacterial attachment to the plant cell, transfer-DNA (T-DNA) and virulence protein transfer through the plant cell wall and membrane into the cytoplasm, nuclear targeting of the T-complex, and integration of the T-DNA into the plant genome (Gelvin, 2003; Tzfira and Citovsky, 2002). Several members of the histone family (H2A, H2B, H3 and H4) are known to interact with *Agrobacterium* virulence gene products and are involved in T-DNA integration (Anand *et al.*, 2007; Li *et al.* , 2005; Loyter *et al.*, 2005; Mysore *et al.*, 2000). Further, expression of the *H2A-1* gene is both a marker for, and a predictor of, plant cells most susceptible to *Agrobacterium* transformation (Yi *et al*., 2002). Interestingly, *Arabidopsis thaliana* histone *H2A* gene mutants are resistant to *Agrobacterium* transformation (Mysore *et al*., 2000) suggesting the product of this gene plays a critical role in *Agrobacterium*-mediated transformation.

Stable, over-expression of the *Arabidopsis* H2A, H4, and H3-11 histones (but not H2B and H3) has been shown to increase *Agrobacterium* transformation susceptibility of the plant host (Tenea *et al*., 2009) and in tobacco protoplast transient assays, co-delivery of these genes increased reporter gene expression approximately 2-fold. However, no such transient, histone-mediated increase in reporter gene expression was observed when the reporter gene cassette was stably integrated in the host genome. This would suggest some histones can enhance transient transgene expression perhaps by protecting the incoming T-DNA during the initial stages of transformation, thereby increasing *Agrobacterium*-mediated transformation frequencies. This enhancing effect can be reproduced using phyto-hormones known to induce histone gene expression, in particular those chemicals that up-regulate histone H2A (Yi *et al.*, 2002).

This chapter was aimed at determining whether over-expression of the *Arabidopsis* H2A gene product could enhance transient transgene expression in *Nicotiana benthamiana*. Using Agroinfiltration of the EAQ-HT hyper-translatable vector system encoding the GUS reporter enzyme (Chapter 2.2.1.10), the effects of expressing H2A histone either via transient co-infiltration or from a stably integrated cassette were compared.

4.2 Materials and methods

4.2.1 Vector construction

In order to isolate the *Arabidopsis* histone *H2A-1* gene (*At_H2A-1*), genomic DNA was isolated from *Arabidopsis thaliana* (cv. Landsberg) using the CTAB method (Chapter 2.2.1.1)

and quantified spectrophotometrically. The *At_H2A-1* gene was amplified by PCR using GoTaq® Green Master Mix, 0.1–1 µg genomic DNA and 10 µM of the following primers *At_H2A-1*-F_AsiSI 5'- GCGCGATCGCATGGCTGGTCGTGGAAAAACTCTTGG-3' and *At_H2A-1*-R_Sac1 5'-GCGAGCTCTAATCTTCCTGAGGCTTTGAAGCACC-3'. The *At_H2A-1* gene was cloned into pGEM-T-Easy®® (Chapter 2.2.2.2 – 2.2.2.3) and sequenced using the Big Dye™ Terminator system (Chapter 2.2.2.7). The confirmed *At_H2A-1* gene was excised from pGEM-T Easy (Chapter 2.2.1.5) as an AsiSI-SacI fragment and ligated between the CaMV 35S promoter and nos terminator in a similarly digested pBIN-Plus binary vector as per Chapter 2.2.1.6. The resulting construct was designated vector pBIN-35S-*At_H2A-1* (Figure 4.1).

For GUS reporter gene analysis, the vector pEAQ-GSN (Chapter 2.2.1.7) was used in subsequent experiments. As a negative control, the empty vector pBIN-35S-nos was used.

4.2.2 Transient co-expression of the *At_H2A-1* gene

Wildtype *N. benthamiana* were germinated and maintained as previously described in Chapter 2.2.3.1. The top three leaves of wildtype *N. benthamiana* plants were infiltrated in triplicate over three independent experiments (Chapter 2.2.3.2). All vectors used in this study were mobilised into *Agrobacterium tumefaciens* strain Agl1 as described in Chapter 2.2.2.5. Recombinant bacteria harbouring pEAQ-GSN were co-infiltrated with either the empty vector control (pBIN-35S-nos) or the *At_H2A-1* over-expression vector pBIN-35S-At_H2A.

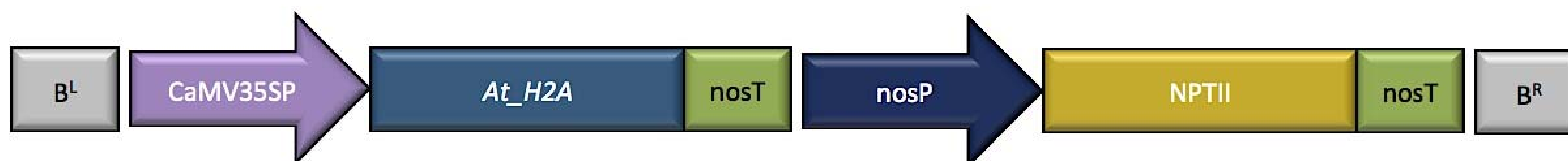

Figure 4.1 Schematic diagram of pBIN-35S-At_H2A vector T-DNA

Vector pBIN-35S-At_H2A was used for both transient co-expression of *Arabidopsis* H2A protein and for stable transformation of *N. benthamiana*.
B^L = left T-DNA border; CaMV35SP = Cauliflower mosaic virus 35S promoter; At_H2A = *Arabidopsis thaliana* H2A histone gene; nosT = nopaline synthase terminator from *Agrobacterium*; nosP = nopaline synthase promoter from *Agrobacterium*; NPTII = selection gene encoding neomycin phosphotransferase II; B^R = right T-DNA border

4.3 Stable transformation of *N. benthamiana*

Vectors pBIN-35S-nos and pBIN-35S-At_H2A were mobilised into *Agrobacterium tumefaciens* strain C58 as described in Chapter 2.2.2.5. *N. benthamiana* plants were transformed by the leaf disc method (Horsch and Klee, 1986) using recombinant *Agrobacteria* harbouring either the empty vector control or pBIN-35S-At_H2A (Chapter 2.2.2.4). Leaf discs were sub-cultured every two weeks on MS104 media containing timentin (200 mg/L) and kanamycin (100 mg/L). After five weeks of culture, shoots of a suitable size were transferred to MSO media containing timentin (200 mg/L) and kanamycin (100 mg/L). Plant replicates were generated by taking nodal cuttings and culturing these on MSO media containing timentin (200 mg/L) and kanamycin (100 mg/L). Plants were PCR screened for the transgene using the following primers: 5'-TCCGGCCAGCCACACCCAGCC-3' and 5'-TTCTTGCGCAGCTGTGCTCG-3' in a reaction containing 5 pmol of each primer, 0.1–1 µg genomic DNA using GoTaq® Green Master Mix and the cycle conditions were as follows: initial denaturation at 95°C for 2 min prior to 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 40 s extension of expected product and a final extension at 72°C for 5 min was included. Expression of the transgene was confirmed by RT-PCR. RNA was extracted from transgenic lines using an RNeasy Kit (QIAGEN) following the manufacturer's instructions and cDNA synthesised as described in Chapter 2.2.1.4 using the At_H2A-R_Sac1 reverse primer. PCR was performed on 1 µL of cDNA using At_H2A-F_AsiSI and nested reverse primers: nested_H2A_1 5'-GTTTCTGACCGCAAGCTGAATGTGACGAGG-3' and nested_H2A_2 5'-GCATGTCAAATTCGGTTCGTGTTTGAATTG-3' in a reaction containing a final volume of 20 µL; 10 µL 2x GoTaq® Green Master Mix, 5 pmol of each primer. PCR cycle conditions were as follows: initial denaturation at 95°C for 2 min prior to 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s extension of expected product and a final extension at 72°C for 5 min was included.

Five independent transgenic *N. benthamiana* lines containing the empty vector control and three independent lines containing the *At_H2A-1* expression cassette were transferred from tissue culture to soil. These plants (T_0 generation) were grown to seed and the T_1 generation seeds germinated in trays containing Searles Seed Raising mix. Plantlets at the four to five leaf stage were transferred to pots containing Searles Potting mix and grown in cabinets at 25°C with a 16 h photoperiod. An outline of the transformation and regeneration process, through to Agroinfiltration of the H2A mutants is shown in Figure 4.2.

Transient GUS expression in transgenic *N. benthamiana* expressing *At_H2A-1*

Ten transgenic T_1 generation *N. benthamiana* plants (confirmed to be expressing the *At_H2A-1* gene by RT-PCR) were selected for transient assays. As controls, five independent T_0 transgenic lines containing the empty vector were also included. The top four leaves of each plant were infiltrated with *Agrobacterium tumefaciens* strain Agl1 harbouring the vector pEAQ-GSN. One leaf from each plant was sampled immediately post infiltration, to serve as a Day 0 control sample for future GUS assays.

4.2.3 GUS fluorometric quantification

Leaf samples, collected at 0 and 4 days post Agroinfiltration, were snap frozen in liquid nitrogen and stored at -80°C. Total soluble protein (TSP) was extracted as described in Chapter 2.2.5.1 and protein content estimated using the Bradford assay (Bradford, 1976). GUS expression levels were quantified by fluorometric analysis and repeated in triplicate over an enzymatic time course (T0 and 10 min), as per Chapter 2.2.5.2. The data obtained and analysed as described in Chapter 2.3.

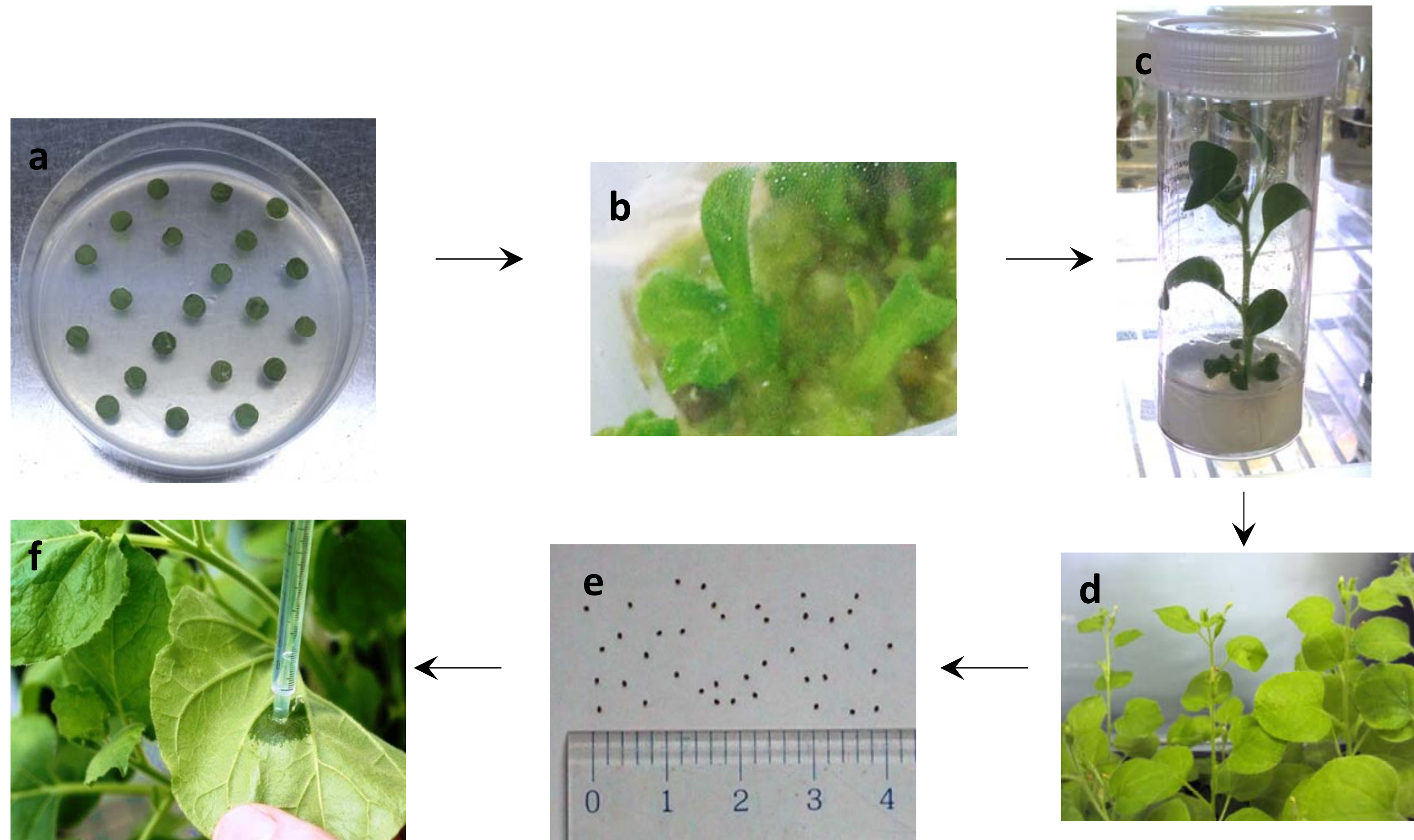

Figure 4.2 Steps involved in the stable transformation of *N. benthamiana* and transient Agroinfiltration of T_1 transgenic lines.

a. Leaf discs from wildtype *N. benthamiana* were co-cultivated with recombinant C58 *Agrobacteria* harbouring either pBIN-Plus (empty vector) or pBIN-35S-At_H2A.
b. Leaf discs were transferred to MS104 media containing kanamycin (100 mg) and timentin (200 mg) and sub-cultured fortnightly until plantlets formed.
c. Plantlets were transferred to MSO media for root formation.
d. Plants were acclimatised from tissue culture to soil and grown in environmental cabinets with a 16 h photoperiod at 25°C. Plants were confirmed to be expressing the *Arabidopsis* H2A gene by RT-PCR (see Figure 4.3).
e. T_0 transgenic lines were grown to the flowering stage and allowed to self-pollinate for seed production. Seeds were sown on Searles Seed Raising mix, and the resulting T_1 transgenic plants confirmed to be expressing the *Arabidopsis* H2A gene by RT-PCR.
f. RT-PCR positive T_1 transgenic plants were infiltrated with recombinant *Agrobacterium* (AGL1) harbouring pEAQ-GSN.

4.3 Results

4.3.1 Effects of transient *At_H2A-1* co-expression on transient reporter gene expression in *N. benthamiana*

In order to determine whether transient over-expression of the histone *H2A-1* gene had a positive effect on the expression of another transgene, recombinant *Agrobacteria* harbouring pEAQ-GSN and pBIN-35S-At_H2A were co-infiltrated into *N. benthamiana* leaves. As a control, *Agrobacteria* harbouring pEAQ-GSN and the empty vector, pBIN-35S-nos, were also co-infiltrated. After 4 days transient expression, leaves were assayed for GUS activity using fluorometric assays. Data from three independent experiments performed in triplicate were pooled, statistically analysed and graphed (Figure 4.3). Negligible GUS expression was detected at Day 0 from both treatments, suggesting the absence of endogenous GUS activity from either the bacteria or plant host. Four days post infiltration, GUS activity from pEAQ-GSN was significantly higher (approximately 2-fold) when co-infiltrated with pBIN-35S-At_H2A compared to when co-delivered with the empty vector control.

4.3.2 Generation of transgenic *N. benthamiana* plants constitutively expressing *At_H2A-1*

Using *Agrobacterium*-mediated transformation, *N. benthamiana* leaf disks were transformed with the vectors pBIN-35S-At_H2A and the empty vector control, pBIN-35S-nos. Ten independent transgenic events containing the *At_H2A-1* expression cassette and five containing the empty vector were regenerated. In order to identify lines that were constitutively expressing *At_H2A-1*, RNA extracted from each plant was used in a RT-PCR with primers specific to the transgene. An amplicon of the expected size (approximately 250 bp) was present in lines #1, 2, 3, 5, 6, 8 and 9 but not lines # 4, 7, and 10, following electrophoresis through a 1% agarose gel (Figure 4.4). The six lines expressing *At_H2A-1* were acclimatised from tissue culture to soil, however, only three of these (lines #1, 3 and 8) survived the transition. These lines were grown through to the flowering stage where they were allowed to self-pollinate and set seed. Similarly, the five transgenic *N. benthamiana* control lines containing the empty vector were also soil acclimatised and grown to seed. Lines expressing *At_H2A-1* showed no obvious phenotypic abnormalities but both the T_0 and T_1 generation were noticeably slower to set seed, in comparison to the empty vector control lines. The subsequent T_1 generation plants from lines # 1, 3 and 8 seed were again screened by RT-PCR to identify those progeny expressing the *At_H2A-1* transgene (see Figure 4.5 for a representative RT-PCR result).

4.3.3 Effects of stable *At_H2A-1* expression on transient reporter gene expression in *N. benthamiana*

Ten T_1 generation plants, confirmed positive by RT-PCR, were selected representing each of the three independent transgenic parent lines (expressing *At_H2A-1*) and five empty vector control lines were used for comparative controls. At the four to five leaf stage, these plants were infiltrated with recombinant *Agrobacteria* (Agl1) harbouring pEAQ-GSN. GUS expression in leaves was measured using fluorometric enzyme assays four days post infiltration. Data from the ten independent plants representing transgenic lines #1, 3, and 8, and a total of fifteen independent plants representing the empty vector control were pooled and statistically analysed using an unpaired T-test and graphed (Figure 4.6). Pooled GUS data suggested there was no significant difference in GUS expression levels between transgenic *N. benthamiana* over-expressing *At_H2A-1* compared to plants not expressing the transgene, following Agroinfiltration with pEAQ-GSN.

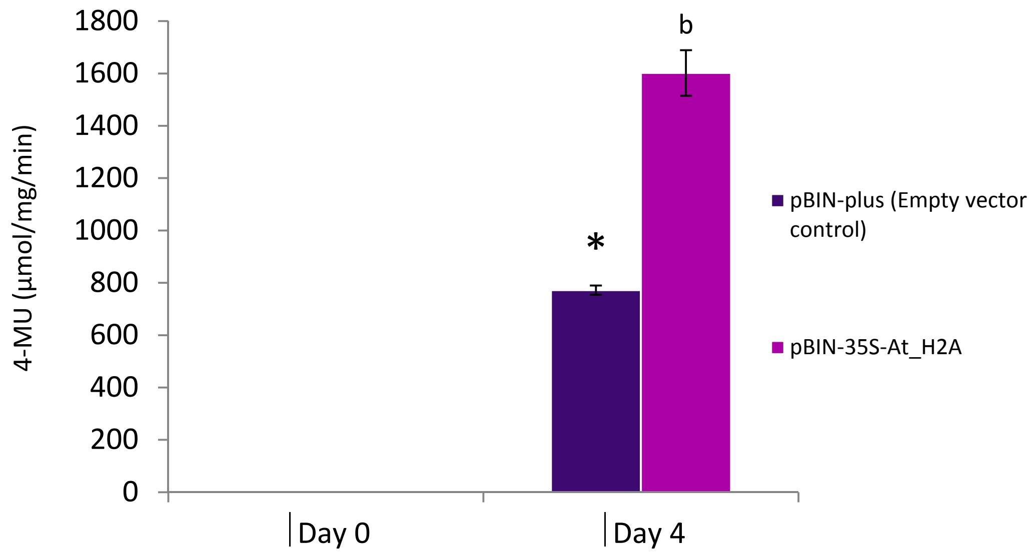

Figure 4.3 Effects of transient expression of the *Arabidopsis H2A-1* gene on transient GUS expression from pEAQ-GSN

Agrobacterium strain Agl1 harbouring pEAQ-GSN were co-infiltrated into *N. benthamiana* leaves with either pBIN-Plus (empty vector) or pBIN-35S-At_H2A. Leaves were sampled at 0 and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Bars represent the standard error of the mean. The control treatment is marked with an asterisk (*); b = data is significantly higher than the control ($p < 0.05$).

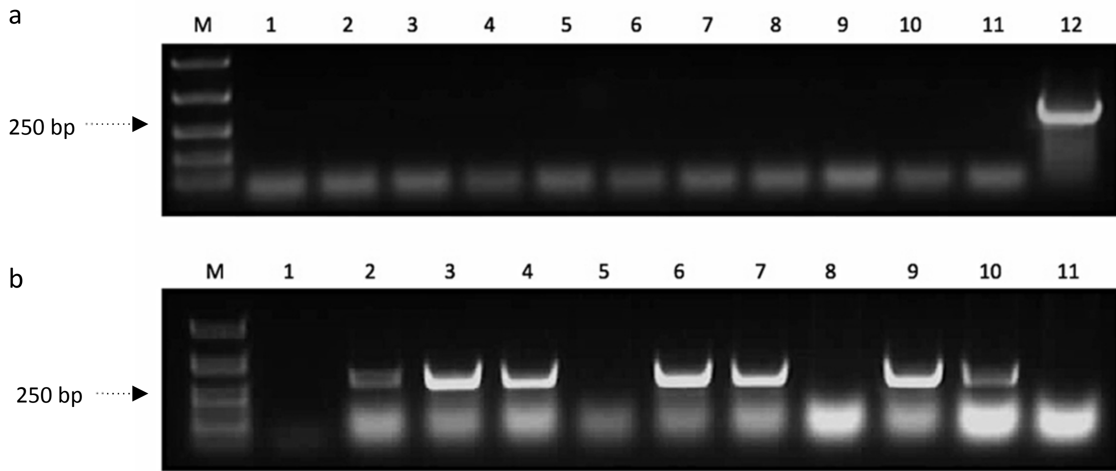

Figure 4.4 RT-PCR to confirm expression of the *Arabidopsis H2A* gene in T_0 Transgenic *N. benthamiana* lines

RNA was extracted from the leaves of independent T_0 generation *N. benthamiana* lines (#1-10) transformed with pBIN-35S-At_H2A. RNA was subjected to RT-PCR using primers specific for the *Arabidopsis* H2A gene and PCR products electrophoresed through agarose.
Panel A = no reverse transcriptase negative control to detect contaminating gDNA.
Panel B = plus reverse transcriptase
M = Easy Ladder (Roche); 1 = no template negative PCR control; 2-11 = transgenic *N. benthamiana* lines (#1-10); 12 = plasmid positive control (10 ng of pBIN-35S-At_H2A)

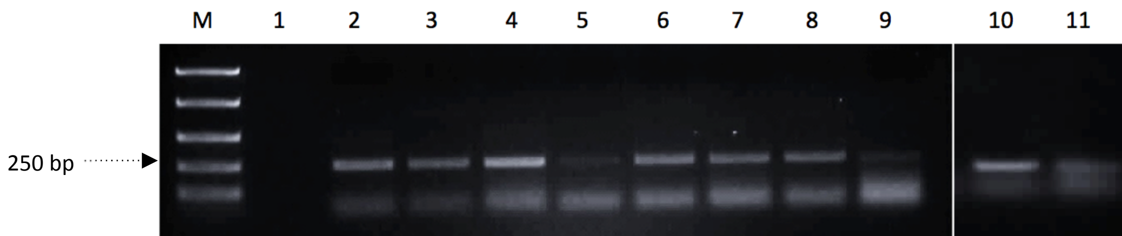

Figure 4.5 Representative RT-PCR to confirm expression of the *Arabidopsis H2A* gene in T_1 transgenic *N. benthamiana* plants

RNA was extracted from the leaves of ten independent T_1 generation *N. benthamiana* plants derived from the seed of transgenic line (#3) transformed with pBIN-35S-At_H2A. RNA was subjected to RT-PCR using primers specific for the *Arabidopsis* H2A gene and PCR products electrophoresed through agarose. No reverse transcriptase negative control to detect contaminating gDNA, data not shown.
M = Easy Ladder (Roche); 1 = no template negative PCR control; 2-11 = 10 individual T_1 generation plants derived from *N. benthamiana* transgenic line #3

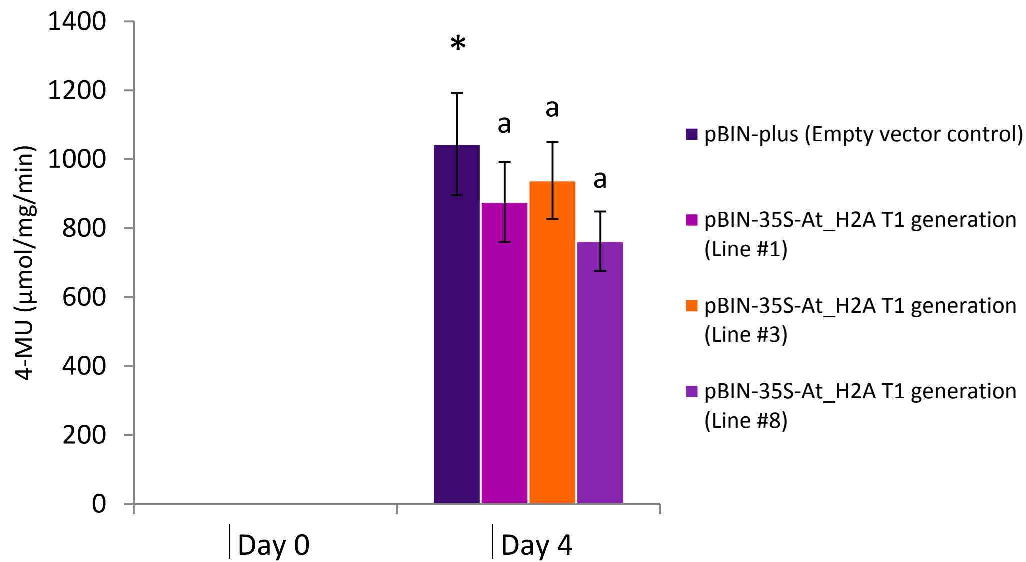

Figure 4.6 Effects of stable *Arabidopsis* H2A gene expression on transient GUS expression from pEAQ-GSN

Agrobacterium strain Agl1 harbouring pEAQ-GSN were co-infiltrated into the leaves of T_1 generation *N. benthamiana* plants transformed with either pBIN-Plus (empty vector) or pBIN-35S-At_H2A. Leaves were sampled at 0 and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Bars represent the standard error of the mean The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$).

4.5 Discussion

Histones comprise one of the most ubiquitous and highly conserved protein families across the domain *Eukarya.* The majority of their functions fall within the purview of complex DNA organisation/structure and the regulation of cellular transcription. Despite the fact that histones are limited to 5 distinct classes, numerous subtype variants of the core histones, H1, H2.A, H2.B and H3, have been identified in numerous species and ascribed specialised functions. One such function is the ability to condense and/or open chromatin, a process that can result in the complete shutdown or up-regulation of cellular gene expression. There is also strong evidence to suggest histones have an involvement in biotic and abiotic stress responses (Ascenzi and Gantt, 1997; Casati *et al*., 2006; Kumar and Wigge, 2010).

Several reports have found that histones and their variants have functional roles in the protection and processing of T-DNA during *Agrobacterium*-mediated transformation, including protection from endonuclease attack, T-DNA translocation from the cytoplasm to the nucleus, and the location-independent integration of T-DNA into the host chromosome (Gelvin, 2010; Li et al., 2005; Loyter et al., 2005; Mysore et al., 2000). The *HTA1* gene encoding the histone *H2A-1* protein, in particular, appears to play an important role in *Agrobacterium*-mediated transformation as a mutation in this gene results in decreased T-DNA integration into the genome (Mysore *et al*., 2000). Further, *H2A* expression is high in the elongation zone of the root, the region that is most susceptible to *Agrobacterium* infection, and studies suggest a role for H2A in directing T-DNA to the site of integration (Li *et al*., 2005a; Loyter *et al*., 2005) possibly by altering host chromatin structure (Mysore *et al*., 2000; Yi *et al*., 2002). Integration of T-DNA into the nucleus is mediated by the *Agrobacterium* virulence protein, *VirE2*. The *VirE2* protein is essential for nuclear transport of T-DNA where it binds the ssDNA, coating the T-strand to form a T-complex (Citovsky *et al.*, 1992; Citovsky *et al.*, 1995; Gelvin, 2010; Zupan *et al.*, 1996). Vir2E in turn is modulated by host VIP1 (Vir2E interacting protein) in conjunction with H2A (Li *et al*., 2005; Loyter *et al*., 2005; Mysore *et al*., 2000).

Previous studies have shown expression of *HTA-1* and a number of other *Arabidopsis* histone genes, can increase the transformation susceptibility of both *Arabidopsis* cells and *Nicotiana tabacum* (tobacco) protoplasts (Tenea *et al*., 2009). These histones can be expressed *in trans* by transient co-transfection or from integrated copies within the host genome and catalyse the accumulation of input DNA with a resulting increase in transformation efficiency and/or transgene expression. They do not, however, increase expression levels from integrated transgenes. This would suggest these proteins protect the

incoming T-DNA from nuclease degradation to stabilise the T-DNA and thereby enhance both transformation frequencies and transgene expression. To practically exploit this and to utilise the *Arabidopsis* H2A protein to further increase transient expression levels afforded by the pEAQ-HT hyper-translatable vector system in *N. benthamiana* using Agroinfiltration*.* The *At_H2A* gene was placed under the transcriptional control of the CaMV 35S promoter and nos terminator and co-delivered with a pEAQ-HT vector containing the *uid*A reporter gene encoding GUS. Co-expression of *Arabidopsis* H2A increased GUS expression levels 2-fold in comparison to the empty vector control as measured four days post Agroinfiltration. This would suggest the *Arabidopsis* H2A protein can function as an enhancer in *Nicotiana benthamiana* and, similar to other studies, acts to increase transient transgene expression most likely by protecting and stabilising the T-DNA. The 2-fold increase in transgene expression mediated by H2A is comparable to that observed by Tenea *et al*. (2009) who reported a 2 to 4-fold increase in expression when histone cDNAs encoding seven different HTAs, one HTR (HTR11), and one HFO were co-transfected with the GUS reporter into tobacco protoplasts. In their study, H2A enhancer activity was localized to the N-terminal 39 amino acids as this region alone was sufficient to increase GUS transgene expression in tobacco cells. This moiety contains motifs for both histone-histone and histone-DNA interaction, and structural analysis suggest it can form two short α-helices (residues 17 to 21 and 27 to 37) interrupted by a short loop. This form allows the two helices to anchor three adjacent phosphates along one strand of the DNA. This region also contains signals associated with nuclear localisation and mutations to basic amino acids within it negatively affect its ability to enhance transgene expression. Together this would suggest the N terminus of H2A plays a critical role in the protein's ability to bind incoming T-DNA and stabilise it.

Having demonstrated that transient co-delivery of the *Arabidopsis H2A* gene product increased transient gene expression in *N. benthamiana*, the effect of *H2A-1* expression on transient protein expression levels from an integrated genomic copy was examined. If increased protein levels were observed, the transgenic plants over-expressing the H2A histone could serve as elite, "hyper-expressing" lines for high-level, transient recombinant protein production and replace conventional wildtype plants. Transgenic *N. benthamiana* plants were regenerated containing the *Arabidopsis H2A* gene under the transcriptional control of the CaMV 35S promoter and nos terminator. Due to external factors, only three independent transgenic events survived acclimatisation from tissue culture to soil and were confirmed to be expressing the transgene by RT-PCR. Since *N. benthamiana* is an

allotetraploid, the introduced T-DNA containing the *H2A* expression cassette, will segregate in the selfed progeny of the T_0 transgenic lines. As such, T_1 plants derived from the seed of each transgenic line were screened by RT-PCR to confirm the presence of the H2A transgene and transcript. These plants and control lines were Agroinfiltrated with the pEAQ-HT vector encoding the GUS reporter and expression levels compared. Transient GUS expression in transgenic *N. benthamiana* lines constitutively expressing the *H2A* gene did not significantly differ to that from control plants suggesting H2A did not stimulate transient gene expression. This finding is in conflict with that of Tenea *et al*. (2009) who showed stable expression of the H2A gene in *Arabidopsis* caused a hyper-susceptibility to *Agrobacterium*-mediated transformation and a resulting increase in the number of transgenic events obtained. Based on transient assays, it appears that an increase in the susceptibility of plant cells to transformation would directly correlate with an increase in transient transgene expression. However, in the case of Tenea *et al*. (1990) this enhancer effect on transient expression was not measured. It is unclear why, in the present study, stable expression of the H2A gene in *N. benthamiana* failed to augment GUS reporter expression following Agroinfiltration. It is possible that the levels of *At_H2A* transgene expression in these transgenic lines were low and H2A abundance was insufficient to promote T-DNA protection and/or stability. It is likely that H2A levels in these plants were considerably lower than those generated in previous Agroinfiltration transient assays as (i) transient expression is generally considered higher than that from stable transgenics and (ii) co-expression of the TBSV P19 silencing suppressor from the pEAQ-GSN vector T-DNA would greatly enhance H2A transient expression levels from pBIN-35S-At_H2A when co-delivered by Agroinfiltration. While RT-PCR did confirm expression of the At_H2A transgene in the T_1 progeny, the exact abundance of H2A in these lines can only be determined by western immunoblot analysis. At the time of this study there were no commercially available antibodies specific for *Arabidopsis* H2A and despite numerous attempts at western analysis with a monoclonal antibody specific for a related *Arabidopsis* histone, cross hybridisation of the antibody with endogenous *N. benthamiana* histones hindered *H2A-1* detection and measurement (results not shown). The generation of high-expressing stably transformed plants often requires the generation of a large population of transgenic events as transgene expression can be greatly affected by a number of factors, such as copy number and “the position effect”. Considering only three independent events were generated and assessed in this study, it is possible that production and characterisation of a larger population of stably expressing transgenic events may yield lines of varying H2A

expression levels and assist in determining whether H2A levels are critical to enhancing transient reporter expression.

This study aimed to increase transient Agroinfiltration-based expression in *N. benthamiana* by over-expressing the *Arabidopsis H2A* gene product from either co-transfected or stably integrated copies. Transient co-expression of the *H2A* gene increased GUS reporter levels directed by the EAQ-HT platform 2-fold, while transgenic lines expressing H2A failed to elevate transient GUS levels. While it remains unclear why H2A expressed from an integrated copy did not increase transient GUS levels, perhaps further studies using a larger population of transgenic lines may assist in answering this question. Transient co-expression of H2A acts to protect and stabilise incoming T-DNA and is a simple approach to substantially increasing transgene expression. The combination of this enhancer activity with other expression platforms may be beneficial for the high-level production of plant made proteins.

Chapter 5: Development of a Geminivirus-based replicating vector and investigation into Replication-associated protein mediated transgene expression

5.1 Introduction

Geminivirus-based vectors have been successfully exploited for the expression of a number of recombinant proteins, including vaccine antigens and monoclonal antibodies, in a wide range of plant cell types (Gleba *et al*., 2007; Hayes *et al*., 1988; Mor *et al*., 2003; Zhang and Mason, 2006). Geminiviruses have a genome of circular, single-stranded DNA that is multiplied via rolling circle replication (RCR), a process dependent on two genomic *cis*-acting elements (the origin of first strand and second strand synthesis), and the *trans*-acting control of the virus-encoded Replication-associated protein (Rep) (Laufs *et al*., 1995). Geminivirus-based vectors are generally structured such that the transgene expression cassette is positioned between tandem repeats of the origin of first strand synthesis and Rep is supplied in *trans* under the transcriptional control of its cognate promoter or a heterologous promoter (Kammann *et al*., 1991; Mor *et al*., 2003; van Wezel *et al*., 2002). RCR allows for the continuous synthesis of DNA, resulting in the accumulation of multiple episomal copies in the plant cell nucleus and amplification of transgene expression (Campos-Olivas *et al.*, 2002). In maize cells, a *Maize streak virus* (MSV) replicating vector was estimated to reach over 500 copies of the replicating episome (replisome) per haploid genome and shown to be structurally stable over multiple generations (Palmer *et al.*, 1999). Using a vector based on the genome of *Bean yellow dwarf virus* (BeYDV), Rep-mediated amplification increased transgene mRNA levels up to 80-fold and protein levels up to 10-fold in tobacco and potato cells (Zhang and Mason, 2006). Similarly, in *Nicotiana benthamiana* leaves, Agroinfiltration of Rep with a modified BeYDV vector increased expression of two subunit vaccine antigens by 2 to 7-fold (Regnard *et al.*, 2010).

While Rep is considered the only virus-derived gene product required for RCR, the process itself is strongly dependent on host cell polymerases and co-factors for effective genome replication. As a direct result of this, geminiviruses and the related nanoviruses have developed a means of overcoming cellular quiescence by subverting the cell cycle control mechanism and synchronising cells to S (synthesis)-phase, a phase in which host cellular DNA synthesis machinery is most abundant. Virus encoded gene products are thought to achieve this by specifically binding the Retinoblastoma-related protein (RBR), a key regulator of the cell cycle, and disrupting the RBR-E2F complex thereby causing premature

entry into S phase. In mastreviruses and nanoviruses this interaction occurs through a conserved canonical LXCXE motif in the RepA and Cell cycle link (Clink) proteins, respectively. In begomoviruses and curtoviruses, Rep and REn bind RBR via a different novel motif (Arguello-Astorga *et al*., 2004).

Agroinfiltration is a convenient and rapid platform for the delivery of virus-based expression cassettes and the rapid production of recombinant proteins in plants (Doran, 2000; Faye *et al.*, 2005; Giddings, 2001; Hiatt *et al.*, 1989; Hood *et al.*, 1999; Ma *et al.*, 2003; Streatfield, 2007; Yin *et al.*, 2007). Like RCR, the effectiveness of *Agrobacterium*-mediated transformation is impacted by cell cycle progression. *Agrobacterium* T-DNA delivery reportedly requires a transition through S phase (Villemont *et al*., 1997) and the early expression of the wheat dwarf geminivirus RepA protein during *Agrobacterium*-mediated transformation has been shown to stimulate cell division and increase transformation frequencies in tobacco and wheat cells (Gordon-Kamm *et al*., 2002). Similar to a virus infection, Agroinfiltration of intact leaves effectively delivers the transgene cassette into fully differentiated leaf and vascular associated cell types; cells that have exited the cell cycle and a cellular environment that is conducive to neither RCR nor *Agrobacterium* transformation.

A TYDV-based vector system for high-level transgene expression was investigate in order to better understand the roles TYDV Rep and RepA play in gene amplification and expression. Further, the effects of replication associated proteins derived from related viruses, namely *Tomato leaf curl virus* (REn), *Banana bunchy top virus* (Clink) and *Maize streak virus* (RepA), on transgene expression were also investigated.

5.2 Materials and methods

5.2.1 Vector construction

pEAQ-HT was a generous gift from G. Sainsbury and G. Lomonossoff, John Innes Centre, UK (Sainsbury *et al.*, 2009). p35S-GSN is a pBIN-Plus vector backbone containing the *uid*A gene (with a small synthetic intron (syntron)) encoding the GUS reporter enzyme under the transcriptional control of the CaMV 35S promoter and nos terminator (Dugdale *et al*., 2013) (Chapter 2.2.1.10). The *uid*A gene containing the syntron was excised from p35S-GSN as a BamHI (blunt-ended) and SalI fragment and ligated into AgeI (blunt-ended) and XhoI digested pEAQ-HT. The resulting construct was called pEAQ-GSN (Figure 5.1 a).

The GUS reporter gene, with syntron, was introduced into p1300-EAQ-HT-SLS (a pCambia vector comprising the EAQ-HT cassette, TYDV SIR and flanked by the LIR of TYDV [GenBank

accession M81103.1 Morris *et al.,* 1992]) using a four-fragment ligation. Fragment 1 consisted of vector backbone following digestion of p1300-EAQ-HT-SLS with AscI and PacI. Fragment 2 was the CaMV 35S promoter, CPMV 5' UTR and part of GUS exon 1 released from pEAQ-GSN (Dugdale *et al*., 2013) using PacI and MfeI. Fragment 3 was the part of GUS exon 1, syntron and part of GUS exon 2 released from pEAQ-GSN using MfeI and SnaBI. Fragment 4 was part of GUS exon 2, CPMV 3' UTR and nos terminator released from pEAQ-GSN using SnaBI and AscI. The four resulting fragments were isolated and ligated to generate the vector pRCR-GUS (Figure 5.1 b)

An empty replicating control vector was constructed using a three-fragment ligation. Fragment 1 was the pCAMBIA1300 vector backbone and the TYDV LIRs from pINPACT-GUS (Dugdale *et al*., 2013) released by digestion with SwaI and PacI. Fragment 2 was the TYDV SIR from pGEM-SIR released by digestion with XhoI (blunt-ended using Klenow polymerase) (Roche) and AscI. Fragment 3 contained the CaMV 35S promoter, CPMV 5' UTR, multiple cloning site, CPMV 3' UTR and nos terminator from pEAQ-HT (Sainsbury *et al*., 2009), released by digestion of pGEM-HT with PacI and AscI. The three resulting fragments were isolated and ligated to generate the vector pRCR-MCS (Figure 5.1 c).

A vector capable of constitutive expression of the TYDV Rep/RepA genes was constructed by excising the CaMV 35S promoter, TYDV Rep/RepA genes, and CaMV 35S terminator from pDH51-Rep/RepA (Dugdale *et al*., 2013) as an EcoRI fragment. The resulting fragment was isolated and ligated into EcoRI digested and alkaline phosphorylase treated pBIN-Plus. The resulting vector was called pBIN-Rep/RepA.

The TYDV Rep gene, in which the small intron was removed, was prepared by overlapping PCR using GoTaq® Green Master Mix, 1 µL of plasmid DNA (pDH51-Rep/RepA) and 10 µM of each of the following primer pairs Rep-Ex1-F (5'-GAATTCATGCCTTCAGCCCCCCAGAAAACCAAATCCTTC-3') and Rep-Ex1-R (5'-GTTCCAGCCCTTCTAGGTTCGTGGTATAGATGCTGGTCTC-3'), and Rep-Ex2-F (5'-GAGACCAGCATCTATACCACGAACCTAGAAGGGCTGGAAC-3') and Rep-Ex2-R (5'-AGAGTCGAGTCAGTGACTCGACGATTCAGGAGCAAA-3'). All PCRs were performed in a final volume of 20 µL; containing 10 µL 2x GoTaq® Green Master Mix, 5 pmol of each primer. PCRs were cycled using the following conditions: 5 min at 95°C followed by 12 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s, with a final extension step of 72°C for 2 min. The resulting two PCR products were electrophoresed through agarose, excised and purified using the High Pure PCR Purification kit (Roche) as per the manufacturer's instructions. These products were then used as a template for a second round of PCR with

the primer pair Rep-Ex1-F and Rep-Ex2-R using standard cycling conditions. The PCR product was purified and ligated into pGEM®-T Easy, cloned and sequenced. The resulting vector was called pGEM.Rep.

The TYDV RepA gene was prepared by PCR amplification with primers Rep-Ex1-F and RepA-R (5'-TCTAGAGTCGAGTTAATTGCTTCCAGAGTGGGACGAATTTGAAG-3') using pDH51-Rep/RepA plasmid template and standard cycling conditions. The PCR product was purified and ligated into pGEM®-T Easy, cloned and sequenced. The resulting vector was called pGEM.RepA.

Vectors capable of expressing either TYDV Rep or RepA were constructed by both genes from pGEM.Rep and pGEM.RepA, respectively, using EcoRI and XbaI. The resulting fragments were ligated between the CaMV 35S promoter and nos terminator in similarly digested pBIN-35S-nos. The resulting vectors were called pBIN-35S-Rep and pBIN-35S-RepA, respectively.

The BBTV Clink gene (GenBank Accession L41578.1) and upstream CaMV 35S promoter were PCR amplified from p35S-BBTV.ORF5 using primers 35S-F (5'-GGCGCGCCCATGGAGTCAAAGATTCAAATAGAGGA-3') and BBTV5-R (5'-TCTAGAGTCGAGTTAGAGTAATGTTACATCATAGTCTGATATAAC-3') using standard cycling conditions. The PCR product was purified and ligated into pGEM®-T Easy, cloned and sequenced. The resulting vector was called pGEM.35S-Clink. A vector capable of strong constitutive expression of the BBTV Clink gene was constructed by excising the CaMV 35S promoter and Clink gene from pGEM.35S-Clink as an AscI and XbaI fragment and ligating it into similarly digested pBIN-35S-nos. The resulting vector was called pBIN-35S-BBTV.Clink

The MSV RepA gene (GenBank Accession AY138520) was codon modified for human codon bias and chemically synthesised by GeneArt® Gene Synthesis (Life Technologies). A vector capable of strong constitutive expression of the MSV RepA gene was constructed in a three fragment ligation containing (i) a CaMV 35S promoter prepared as an AscI and NcoI fragment, (ii) the MSV RepA gene prepared as an NcoI and SacI fragment, and (iii) pBIN-35S-nos prepared as an AscI and SacI fragment. The three fragments were ligated and cloned to generate the vector pBIN-35S-MSV.RepA.

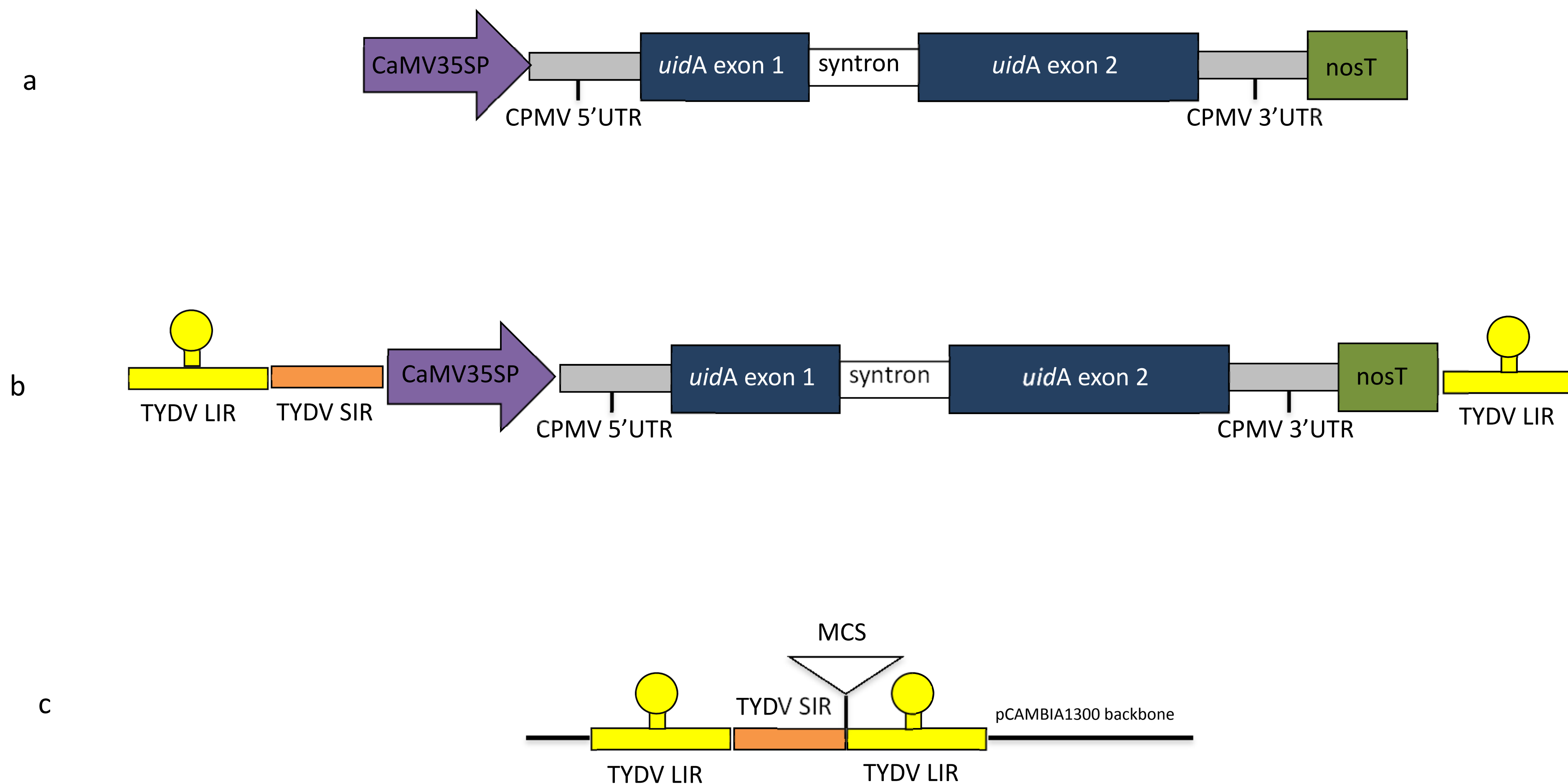

Figure 5.1 Schematic diagram of replicating and non-replicating vectors used in this study

(a) pEAQ-GSN was constructed by inserting the *uid*A reporter gene encoding GUS, and synthetic intron, into the pEAQ-HT hyper-translation binary vector (Sainsbury *et al.*, 2009). (b) The replicating vector, RCR-GUS, contains the GUS expression cassette from pEAQ-GSN with the TYDV SIR located downstream and copies of the TYDV LIR flanking it, in the binary vector pCAMBIA1300. (c)An empty vector control (pRCR-MCS) contains the TYDV *cis* replication machinery without the GUS expression cassette in pCAMBIA1300. CaMV35SP = Cauliflower mosaic virus 35S promoter; CPMV 5' UTR=*Cowpea mosaic virus* RNA-2 5'UTR; *uid*A=gene encoding GUS; syntron=synthetic intron; CPMV 3' UTR=*Cowpea mosaic virus* RNA-2 3'UTR; nosT=nopaline synthase terminator from *Agrobacterium*; TYDV SIR=*Tobacco yellow dwarf virus* small intergenic region; TYDV LIR=*Tobacco yellow dwarf virus* large intergenic region.

The ToLCV REn gene (GenBank Accession S53251) and upstream CaMV 35S promoter were PCR amplified from a pTEST-35S-REn (Williams, 2007) using primers 35S-F and ToLCV.REn-R (5'- TCTAGAGTCGAGTTAATAAAAATTAAATTTTATATCATGATC-3') using standard cycling conditions. The PCR product was purified and ligated into pGEM-T-Easy, cloned and sequenced. The resulting vector was called pGEM.35S-REn. A vector capable of strong constitutive expression of the ToLCV REn gene was constructed by excising the CaMV 35S promoter and REn gene from pGEM.35S-REn as an AscI and XbaI fragment and ligating it into similarly digested pBIN-35S-nos. The resulting vector was called pBIN-35S-ToLCV.REn.

Vectors capable of expressing the virus-derived cell cycle gene products at low levels were constructed by truncating the CaMV 35S promoter at the –(90) position using the unique EcoRV restriction site. Vectors pBIN-TYDV-35S-Rep (Figure 5.2 a), pBIN-TYDV-35S-RepA (Figure 5.2 b), pBIN-TYDV-35S-Rep/RepA (Figure 5.2 c), pBIN-ToLCV-35S-REn (Figure 5.2 d) and pBIN-BBTV-35S-Clink (Figure 5.2 e) were all digested with EcoRV (blunt-ended) and PacI excising a cassette containing the truncated 35S promoter (∆35S), the gene of interest and the nos terminator. This cassette was then ligated into a pBIN-plus digested with SmaI (blunt-ended) and PacI. These constructs were named as follows; pBIN-TYDV-∆35S-Rep (Figure 5.2 f), pBIN-TYDV-∆35S-RepA (Figure 5.2 h), pBIN-TYDV-∆35S-Rep/RepA (Figure 5.2 g), pBIN-MSV-∆35S-RepA (Figure 5.2 i), pBIN-ToLCV-∆35S-REn (Figure 5.2 j) and pBIN-BBTV-∆35S-Clink (Figure 5.2 k).

A point mutation was inserted into the LxCxE motif of Rep and RepA by overlapping PCR and the primer pairs: TYDV AscI_f 5'-GCTATGACCATGATTACG-3' and TYDV^{LxCxK}mut_r 5'-GGTCTCTCCATAACTGCAGATCTTTATGACATTGGAG-3'; TYDV^{LxCxK}mut_f 5'-CATTCACTGAGGAAGATCTCCAATGTCATAAAGATCTGC-3' and TYDV SacI_r 5'-TGTTTGAACGGAGCTCGG-3' using standard cycling conditions. The resulting two PCR products were electrophoresed through agarose, excised and purified using the High Pure PCR Purification kit (Roche) as per the manufacturer's instructions. These purified products were then used in a second round of PCR with the primers TYDV AscI_f and TYDV SacI_r using standard cycling conditions. The PCR products were purified and ligated into pGEM®-T Easy, cloned and sequenced, then the confirmed TYDV-Rep^{LxCxK} and TYDV-RepA^{LxCxK} mutants were digested with AscI/SacI and cloned into a similarly digested pBIN-∆35S backbone. The resulting constructs were called pBIN-TYDV-∆35S-Rep^{LxCxK} (Figure 5.2 l) and pBIN-TYDV-∆35S-RepA^{LxCxK} (Figure 5.2 m).

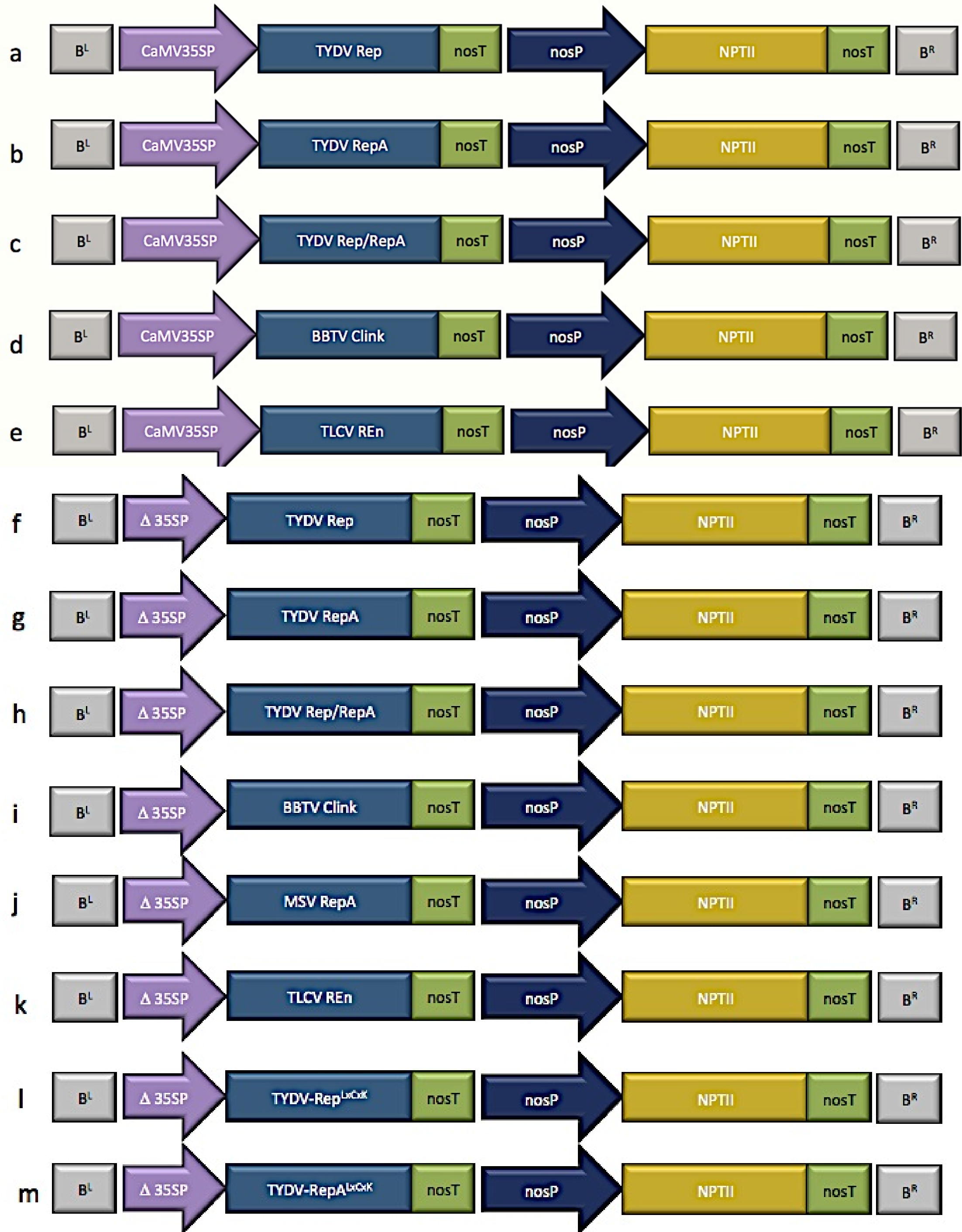

Figure 5.2 Schematic representation of virally derived cell cycle gene cassettes

Cassettes were all inserted into pBIN-plus binary vectors for transient expression in N. *benthamiana* B^L = left T-DNA border; CaMV35SP = Cauliflower mosaic virus 35S promoter; Δ35SP = Cauliflower mosaic virus 35S (-90) promoter; nosT = nopaline synthase terminator from *Agrobacterium*; nosP = nopaline synthase promoter from *Agrobacterium*; NPTII = selection gene encoding neomycin phosphotransferase II; B^R = right T-DNA border. Cassettes a-e contain TYDV Rep, RepA and Rep/RepA, ToLCV REn and BBTV Clink respectively under the control of CaMV35SP. Cassettes f-m comprise TYDV Rep, RepA and Rep/RepA, MSV RepA, TOLCV REn, BBTV Clink TYDV Rep^{LXCXK} and $RepA^{LXCXK}$ mutants all under the control of Δ35SP.

5.2.2 *Agrobacterium* mediated infiltration of *N. benthamiana*

Wildtype *N. benthamiana* were required for transient expression analysis, these plants were germinated and maintained as previously described in Chapter 2.2.3.1.

As described in Chapter 2.2.3.2, the top three leaves of wildtype *N. benthamiana* plants were infiltrated in triplicate over three independent experiments.

5.2.3 Protein extraction and measurement of GUS activity

Leaf samples, collected at 0 and 4 days post Agroinfiltration, were snap frozen in liquid nitrogen and stored at -80°C. Total soluble protein (TSP) was extracted as described in Chapter 2.2.5.1 and protein content estimated using the Bradford assay (Bradford, 1976). GUS expression levels were quantified by fluorometric analysis and repeated in triplicate over an enzymatic time course (T0 and 10 min), as per Chapter 2.2.5.2. The data obtained and analysed as described in Chapter 2.3.

5.2.4 Detection of episomes using outwardly extending PCR

Outwardly extending primers designed to the GUS coding region were used to confirm the presence of circular episomal molecules following Rep mediated replicative release and RCR. Total nucleic acid was extracted from leaf material at 0 and 4 dpi following Agroinfiltration using the CTAB method (Porebski *et al.*, 1997). Episomes were amplified by PCR using GoTaq® Green Master Mix, 0.1–1 µg gDNA and 10 µM of primers GUS-out_F 5’-AACGCTGGACTGGCATGAACTTCGG-3’ and GUS-out_R 5’-TTTTCGCGATCCAGACTGAATGCCC-3’ using the following cycling conditions: 5 min at 94°C followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 90 s, with a final extension step of 72°C for 5 min.

5.2.5 Southern blot analysis

Total DNA was isolated from the leaves of *N. benthamiana* plants at 0 and 4 dpi using the CTAB method. Total DNA (5 µg) was electrophoresed through a 0.8% agarose gel, capillary blotted onto a positively charged nylon transfer membrane (Roche) and hybridised using a Digoxigenin (DIG)-labelled GUS-specific probe. The DIG labelled probe was synthesised by PCR using primers GUS_2 f 5’-AACGCTGGACTGGCATGAACTTCGG-3’ and GUS_2 r 5’-TTTTCGGCATCCAGACTGAATGCCC-3’ and DIG-11-dUTP (9:1) as per the manufacturer‘s protocol (Roche). Membranes were pre-hybridised in DIG Easy Hybe (Roche) for 1 h, and the denatured probe added to the pre-hybridisation solution and hybridised overnight at 42°C. Membranes were then subjected to high stringency washes (0.1 x SSC, 0.1% SDS) at 68°C prior to development as per the manufacturer‘s instructions (Roche).

5.3 Results

5.3.1 Construction of a Geminivirus-based replicating vector

A Geminivirus-based vector, pRCR-GUS (Figure 5.1b), was assembled around a standard expression cassette containing the *uid*A gene encoding the GUS reporter under the transcriptional control of the CaMV 35S promoter and nos terminator. Translational enhancers derived from the 5' and 3' untranslated regions of Cowpea mosaic virus RNA-2 were inserted immediately upstream and downstream of the *uid*A reporter gene. Flanking the cassette were copies of the TYDV large intergenic region (LIR), which contain the origin of first strand synthesis for Rep-mediated RCR. Also, located between the LIRs was inserted the TYDV small intergenic region (SIR) which serves as the origin of second strand synthesis.

The activator Rep and/or RepA genes were placed under the transcriptional control of either the strong, constitutive CaMV 35S promoter or a weaker, truncated version, CaMV 35S(-90) promoter, and similarly delivered *in trans*. In the absence of Rep, normal transcription and translation of the reporter gene occurs. Upon supply of Rep, *in trans*, the protein nicks the stem loop, displaces the sequence between the LIRs and ligates it together to form a circular single stranded DNA molecule. This sequence is converted into a double stranded DNA form via the SIR, which is both transcriptionally active and can serve as the template for further replication. Theoretically, this should result in a dramatic amplification of transgene copy number and an increase in transgene expression levels (Figure 5.3).

To further elevate transient expression levels, recombinant *Agrobacteria* harbouring an expression cassette encoding the *Tomato bushy stunt virus* (TBSV) P19 silencing suppressor were co-infiltrated with the virus vector (Chapter 3.5.3.2) as the p19 cassette is absent from the replicating EAQ-based vector, pRCR-GUS.

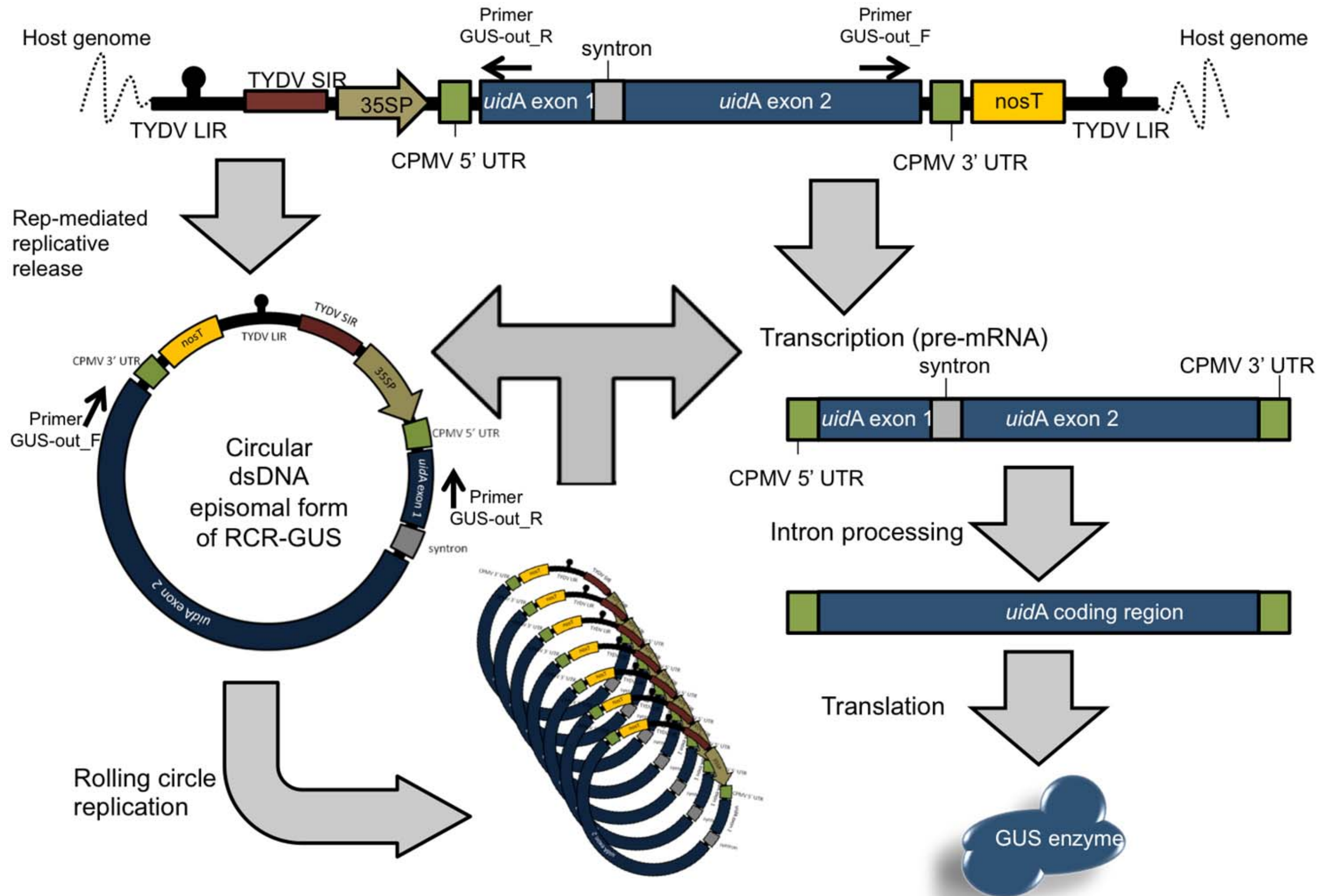

Figure 5.3 Schematic representation of geminivirus-based replicating vector

In the absence of Rep GUS expression is directed by the 35SP and nosT from the pRCR-Gus cassette and is able to carry out normal transcription and translation. Rep supplied *in trans* catalyses RCR allowing for the generation of transcriptionally active circular dsDNA episomes with resulting amplification of the RCR cassette and an increase in GUS expression.

5.3.2 Effects of Rep and/or RepA on GUS expression levels from pRCR-GUS

To determine whether transgene copy number and transgene expression levels from pRCR-GUS could be amplified in the presence of Rep and/or RepA, vectors expressing Rep, RepA or Rep/RepA at high and low levels were co-delivered with pRCR-GUS into *N. benthamiana* leaves by Agroinfiltration and the leaves assayed for GUS activity four days post infiltration (Figure 5.4). No GUS expression was observed in any treatments immediately following Agroinfiltration (Day 0), indicating the absence of bacteria-derived or endogenous plant GUS activity. Four days post infiltration, GUS expression was detected in total soluble protein extracts from leaves infiltrated with the pRCR-GUS vector alone (about 10 nmol 4-MU/mg TSP /min). When the GUS expression levels were compared to those in leaves co-infiltrated with pRCR-GUS and vectors expressing either Rep, RepA or Rep/RepA under the control of the strong CaMV 35S promoter, no significant differences in GUS expression were observed. In contrast, co-delivery of pRCR-GUS with vectors expressing either Rep, RepA or Rep/RepA under the transcriptional control of the weaker, truncated CaMV 35S(-90) promoter resulted in significantly increased GUS levels compared to those with pRCR-GUS alone; Rep increased GUS levels about three-fold, RepA about 4-fold and Rep/RepA together about 5-fold. It was also observed that the infiltrated zones of leaves using vectors expressing either Rep, RepA or Rep/RepA under the control of the strong CaMV 35S promoter were browner than those seen in leaves infiltrated with vectors expressing either Rep, RepA or Rep/RepA under the control of the weaker, truncated CaMV 35S(-90) promoter. Further, TSP extracted from the brown zones was found to be degraded following electrophoresis through polyacrylamide gels and staining with Coomassie Blue, suggesting the occurrence of cellular death.

5.3.3 Detection of pRCR-GUS derived episomes

In order to confirm the formation of circular RCR episomes from pRCR-GUS in the presence of Rep and RepA, a PCR-based strategy was used in which outwardly extending primers were designed to amplify an approx. 2 kb fragment of the uidA coding region of the circular, episomal DNA. *N. benthamiana* leaves were co-infiltrated with pRCR-GUS and pBIN-TYDV-Δ35S-Rep/RepA while, as negative controls, leaves were co-infiltrated with (i) pRCR-GUS and *Agrobacteria* harbouring an empty vector and (ii) a vector similar to pRCR-GUS but without the GUS expression cassette (pRCR-MCS; Fig 5.1c) and pBIN-TYDV-Δ35S-Rep/RepA. Total DNA was extracted at Days 0 and 4 post infiltration was used as a template in a PCR. If the pRCR-GUS cassette (between LIRs) had been effectively cleaved and ligated into circular episomes by Rep/RepA, amplicons of approximately 2 kb were anticipated, whereas no

product would be amplified in the absence of episomes. Using this strategy, a 2 kb amplicon was only detected in DNA extracted from leaves 4 days post co-infiltration with pRCR-GUS and pBIN-TYDV-Δ35S-Rep/RepA (Figure 5.5). This suggested the formation of circular episomes from pRCR-GUS is strongly reliant on Rep/RepA and this primer set is specific for the pRCR-GUS vector only.

5.3.4 Rep/RepA mediated episome formation and transgene amplification of pRCR-GUS via Rolling Circle Replication

To determine whether the pRCR-GUS circular episomes were being replicated to high levels in the presence of Rep/RepA, Southern analysis was used to detect the presence of the hallmark episomal replicative DNA forms including closed, double-stranded circular DNA and open-circular, double-stranded circular DNA. The DNA extracts from above were electrophoresed through an agarose gel, transferred to a nylon membrane and hybridised with a *uid*A-specific probe. Two bands of high-molecular weight (approx. 13 kbp) were present in all lanes at both time points (Figure 5.6) and most likely represent non-specific binding of the GUS probe to the pEAQ-HT input vector backbone, as this vector was common to all treatments. Lower molecular weight hybridisation signals (approx. 3.5 kb) were only detected in the lane containing DNA extracted from leaves containing both pRCR-GUS and Rep/RepA, four days post infiltration. These bands most likely represent the closed, double-stranded circular DNA and open-circular, double-stranded circular DNA forms of the pRCR-GUS episomes. Treatment of these episomal forms with methylation sensitive enzymes (DpnI; cleaves DNA adenine methylase (*Dam*)-methylated DNA at the sequence GA/TC) and DpnII (cleaves unmethylated DNA at the same site)) confirmed them to be non-methylated (results not shown).

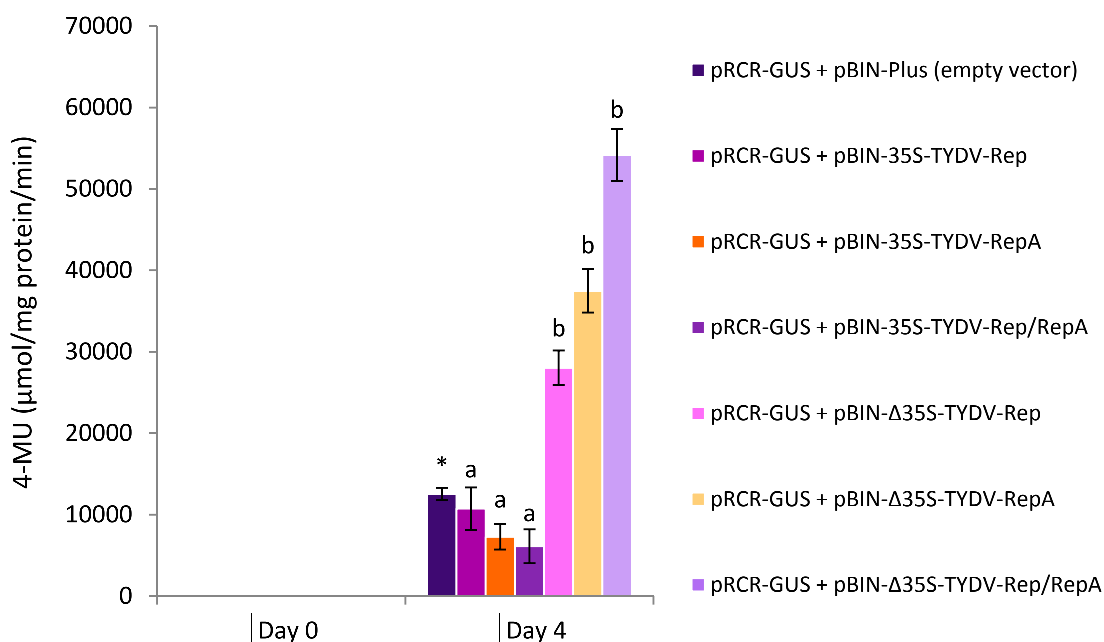

Figure 5.4 Effects of co-expressing TYDV replication associated proteins with pRCR-GUS

Agrobacterium strain Agl1 harbouring RCR-GUS was co-infiltrated into *N. benthamiana* leaves with the TYDV Rep, RepA or Rep/RepA genes under the transcriptional control of either full CaMV 35S or truncated 35S(-90) promoter (Δ35S). Leaves were sampled at 0, and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent ±SE. The control treatment (pRCR-GUS with empty vector) at each time point is marked with an asterisk The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$) and b = data is significantly higher than the control ($p < 0.05$) .

Figure 5.5 Outward extending PCR to detect circular episomal forms of the pRCR-GUS cassette

Vector combinations were Agroinfiltrated into *N. benthamiana* leaves and total DNA extracted 0 and 4 days post infiltration. DNA extracts were subjected to a PCR using primers designed to the *uid*A-coding region and outwardly extend. PCR products were electrophoresed through a 1% agarose gel and photographed.

M = Hyperladder (Roche)
1 = no-template PCR control,
2 = Day 0 pRCR-HT + pBIN-Δ35S-TYDV-Rep/RepA
3 = Day 0 pRCR-GUS + pBIN-plus
4 = Day 0 pRCR-GUS + pBIN-Δ35S-TYDV-Rep/RepA
5 = Day 4 pRCR-HT + pBIN-Δ35S-TYDV-Rep/RepA
6 = Day 4 pRCR-GUS + pBIN-plus
7 = Day 4 pRCR-GUS + pBIN-Δ35S-TYDV-Rep/RepA

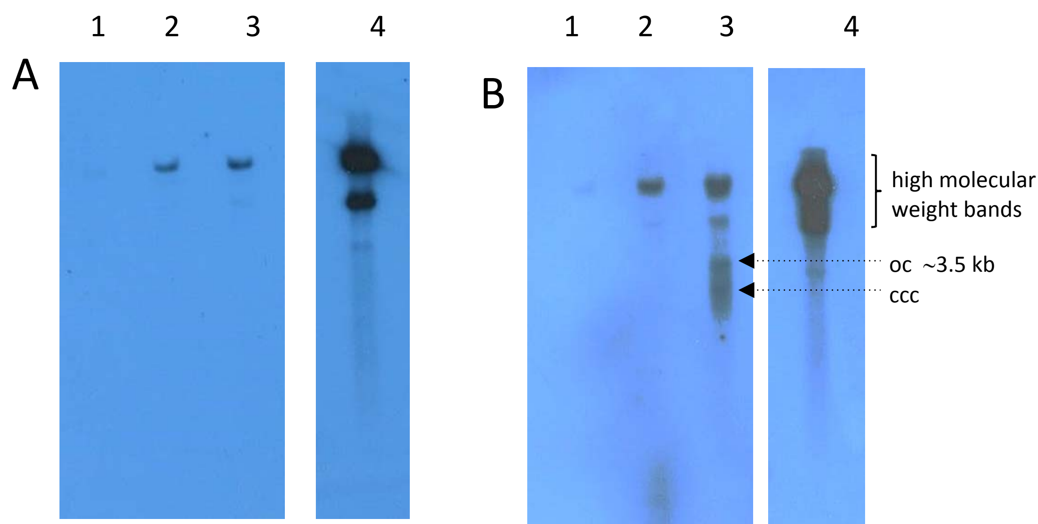

Figure 5.6 Southern analysis to detect Rolling Circle Replication intermediates from pRCR-GUS

Vector combinations were Agroinfiltrated into *N. benthamiana* leaves and total DNA extracted 0 and 4 days post infiltration. DNA extracts were electrophoresed through a 1% agarose gel, transferred to a nylon membrane and hybridised with a DIG-labelled probe specific for the *uid*A-coding region. Hybridisation signal was detected using chemiluminescence. Episomal DNA forms indicative of RCR include (i) double-stranded, open-circular DNA (oc) (ii) double-stranded, closed-circularDNA (ccc).

(A) Day 0 and (B) Day 4

1= pRCR-HT + pBIN-Δ35S-TYDV-Rep/RepA

2 = pRCR-GUS + pBIN-plus

3 = pRCR-GUS + pBIN-Δ35S-TYDV-Rep/RepA

4= pRCR-GUS plasmid DNA (10 ng)

5.3.5 Determining the effects of TYDV Rep and RepA on reporter gene expression from a non-replicating vector

To determine whether the TYDV Rep and/or RepA affected reporter gene expression from a non-RCR based vector, *Agrobacterium* harbouring the EAQ-HT vector encoding GUS (pEAQ-GSN) were co-infiltrated with Rep, RepA or Rep/RepA under the transcriptional control of either the strong CaMV 35S promoter or the weaker truncated CaMV(-90) (Figure 5.2) version. GUS expression levels from leaves infiltrated with each vector combination were measured at 0 and 4 days post infiltration (Figure 5.7). Four days post infiltration, low levels of GUS expression were observed in extracts from leaves infiltrated with the non-replicating EAQ-HT vector alone. These levels of GUS expression were not significantly different from those seen in extracts from leaves co-infiltrated with pEAQ-GSN and vectors expressing either Rep, RepA or Rep/RepA under the control of the strong CaMV 35S promoter. In contrast, co-delivery of pEAQ_GSN with vectors expressing Rep, RepA or Rep/RepA under the transcriptional control of the weaker CaMV 35S(-90) promoter significantly increased base GUS levels from the EAQ-HT vector; Rep increased GUS levels about 2-fold, RepA about 2-fold and Rep/RepA together about 3-fold.

5.3.6 Investigating a point mutation in the LXCXE motif of both TYDV Rep and RepA to determine its role in the enhancement of reporter gene expression from a non-replicating vector

To determine whether the LXCXE RBR-binding motif plays a role in Rep and RepA enhancer activity with non-RCR vectors, an E to K mutation was made in both replication-associated proteins. *Agrobacterium* harbouring pEAQ-GSN were co-infiltrated with Rep, RepA, Rep$^{(LXCXK)}$ or RepA$^{(LXCXK)}$ under the transcriptional control of the weak CaMV(-90) promoter, and GUS expression levels from leaves infiltrated with each vector combination were measured at 0 and 4 days post infiltration (Figure 5.8). No GUS activity was observed in extracts from any treatments immediately following Agroinfiltration (Day 0). Four days post infiltration, co-expression of Rep or RepA significantly increased base GUS levels from the EAQ-HT vector about 3 to 4-fold. In contrast, co-expression of either Rep$^{(LXCXK)}$ or RepA$^{(LXCXK)}$ had no significant effect on base GUS levels from the non-RCR vector, suggesting the LXCXE motif plays an important role in enhancer activity of these proteins.

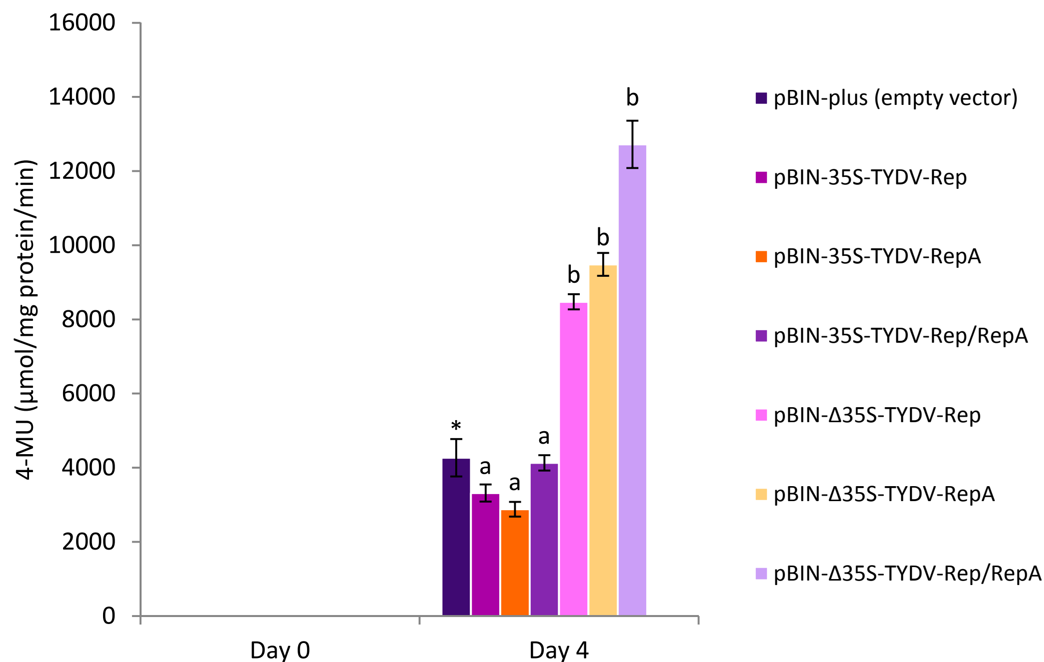

Figure 5.7 Effects of co-expressing virus-derived replication associated proteins with a non-replicating expression vector

Agrobacterium strain Agl1 harbouring pEAQ-GSN was co-infiltrated into *N. benthamiana* leaves with TYDV Rep, RepA or Rep/RepA genes under the transcriptional control of either full CaMV 35S or truncated 35S(-90) promoter (Δ35S). Leaves were sampled at 0, and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent ±SE. The control treatment (pEAQ-GSN + empty vector) at each time point is marked with an asterisk The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$), b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control ($p < 0.05$).

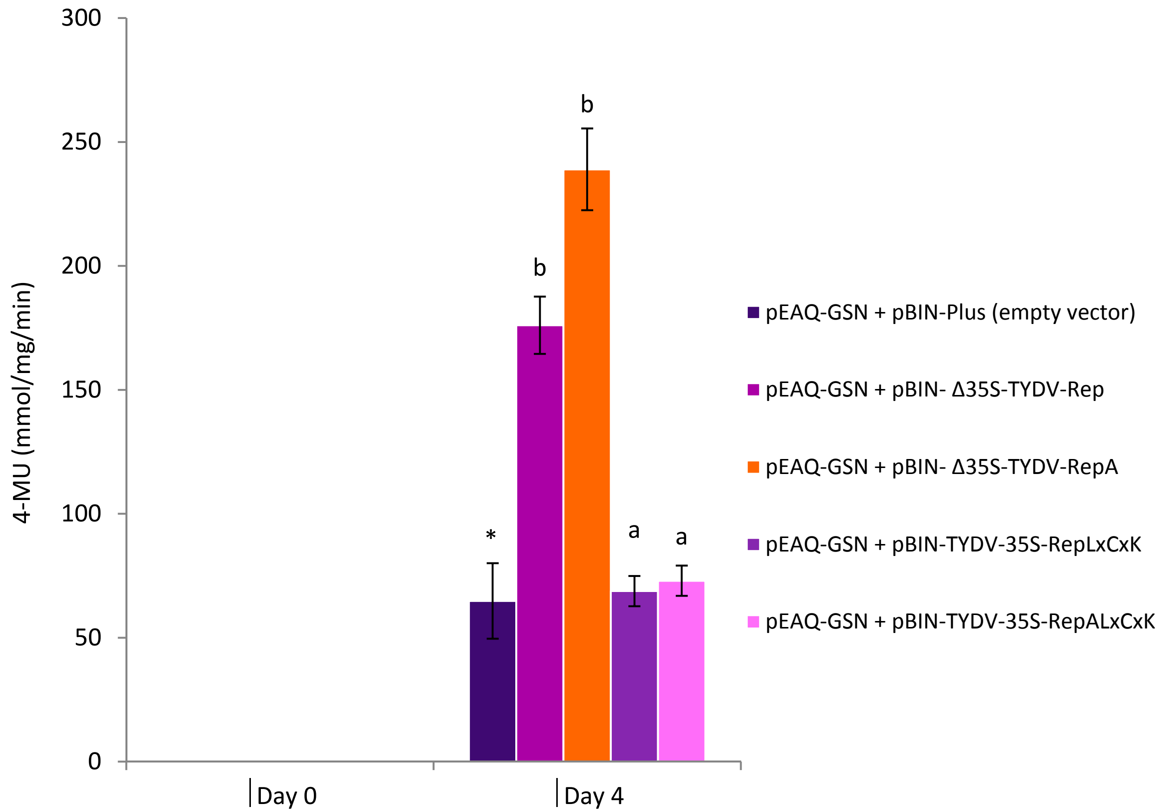

Figure 5.8 Mutation of LXCXE motif in both TYDV Rep and RepA reduces their ability to enhance reporter gene expression from a non-replicating vector

Agrobacterium strain Agl1 harbouring pEAQ-GSN was co-infiltrated into *N. benthamiana* leaves with pBIN-TYDV-Δ35S-Rep, pBIN-TYDV-Δ35S-RepA pBIN-TYDV-Δ35S-Rep^{LxCxK} and pBIN-TYDV-Δ35S-RepA^{LxCxK}. Leaves were sampled at 0, and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent ±SE. The control treatment (pEAQ-GSN + empty vector) at each time point is marked with an asterisk The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$) and b = data is significantly higher than the control ($p < 0.05$).

5.3.7 Investigation of related, virus-derived cell cycle control proteins and their enhancing effects on reporter gene expression from non-replicating vectors

To investigate whether cell cycle control gene products encoded by other related viruses could also enhance gene expression from a non-replicating vector, genes encoding the geminivirus MSV RepA, ToLCV REn and the nanovirus BBTV Clink were each cloned under the transcriptional control of either the strong CaMV 35S promoter (all except MSV RepA) or the weaker truncated CaMV(-90) version (Figure 5.2). *Agrobacteria* harbouring each vector were co-infiltrated with the non-replicating pEAQ-GSN, and reporter gene expression measured at 0 and 4 days post infiltration (Figure 5.9). Again, no GUS activity was observed in any treatments immediately following Agroinfiltration (Day 0). Four days post infiltration, no significant differences were seen in the GUS expression levels in extracts derived from leaves infiltrated with pEAQ-GSN and those co-infiltrated with pEAQ-GSN and vectors expressing ToLCV REn or BBTV Clink using the strong CaMV 35S promoter. In contrast, co-delivery of MSV RepA, ToLCV REn or BBTV Clink under the transcriptional control of the weaker CaMV 35S(-90) promoter significantly increased base GUS levels from the EAQ-HT vector by about 2 to 3-fold.

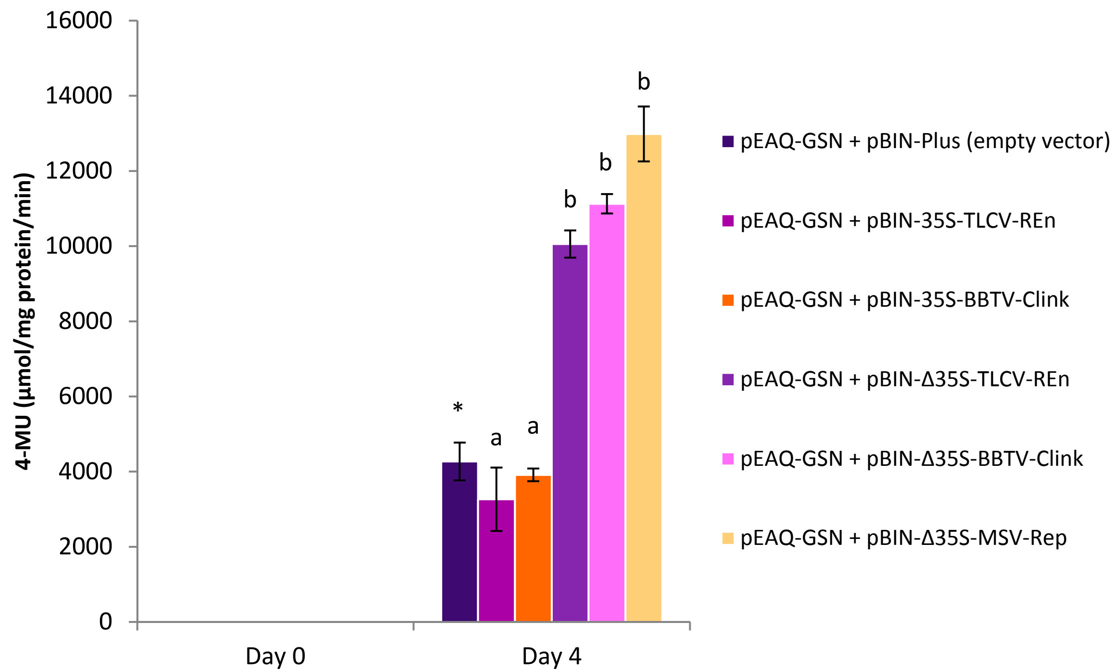

Figure 5.9 Effects of co-expressing Cell cycle control proteins encoded by related viruses with a non-replicating expression vector

Agrobacterium strain Agl1 harbouring pEAQ-GSN was co-infiltrated into *N. benthamiana* leaves with cell cycle control genes from related ssDNA viruses under the transcriptional control of either full CaMV 35S or truncated 35S(-90) promoter (Δ35S). Leaves were sampled at 0, and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent ±SE. The control treatment (pEAQ-GSN + empty vector) at each time point is marked with an asterisk The control treatment is marked with an asterisk (*). a = no significant difference from the control ($p \geq 0.05$) and b = data is significantly higher than the control ($p < 0.05$.

5.4 Discussion

Geminiviruses can replicate their genomes to very high numbers and, as a result, some have been modified or deconstructed into expression vehicles for recombinant protein production in plants. Generally, Rep (and RepA in the case of mastrevirus vectors) is/are provided *in trans* in order to activate RCR and amplify transgene expression. The preferred method of supplying virus-based vectors into plants is via Agroinfiltration, a process that physically delivers the recombinant bacteria harbouring the vector into the extracellular spaces of leaves. Considering this environment is not conducive to either geminivirus RCR or *Agrobacterium*-mediated transformation, the development and use of geminiviruses as protein expression vectors requires a better understanding of how geminivirus replication associated proteins act to increase transgene expression from the virus vector and the roles they play in both RCR and host cell interaction. In order to achieve this, the model geminivirus, TYDV, a monopartite mastrevirus that infects dicots, was used in this study.

A TYDV RCR-based expression cassette was assembled capable of expressing the GUS reporter (pRCR-GUS) and co-delivered with TYDV Rep and/or RepA into *N. benthamiana* leaves via Agroinfiltration. When the Rep and/or RepA genes were placed under the transcriptional control of a truncated CaMV 35S(-90) promoter and co-expressed with pRCR-GUS, a 2 to 5-fold amplification of GUS expression was observed. In comparison, Rep and/or RepA expression directed by the full CaMV 35S promoter sequence appeared to decrease GUS expression from pRCR-GUS and caused visible necrosis within the infiltrated zone of the leaf. This would suggest the two CaMV 35S promoters differ in strength in leaf cells, and that the relative abundance or Rep and/or RepA is critical for the continuation of cellular homeostasis and amplification of transgene expression from pRCR-GUS. The CaMV promoter has a modular organisation and contains two Domains (A and B) based primarily on their roles in promoter tissue specificity and strength. The CaMV 35S(-90) promoter contains only Domain A, comprising a TATA box and a *cis* element termed activation sequence (as)-1. This element consists of a tandem repeat of the sequence TGACG and binds ASF-1 transcription factor that is primarily responsible for strong promoter activity in roots. Upstream of the -90 sequence is located Domain B (-343 to -90) which is able to confer expression in most cell types of leaf and stem as well as the vasculature of roots (Lam and Chua, 1989). Considering Domain B is absent in the CaMV(-90) promoter, it is likely that the level of Rep and/or RepA expression directed by this truncated promoter is considerably lower than that directed by the full CaMV 35S promoter sequence in *N. benthamiana* leaf cells. Also, there is increasing anecdotal evidence to suggest that high

levels of some geminivirus Reps can be toxic to the plant cell and there are few reports of transgenic plants constitutively expressing these gene products (Boulton, 2002; Selth *et al*., 2004; Zhang and Mason, 2006). Whilst the exact cause of this toxicity is unclear, it seems plausible that prolonged interaction of Rep or RepA with host cell cycle regulators and abnormal cell phase transition may trigger a programmed cell death-like event. In this study, Rep and/or RepA expression under the control of the full CaMV 35S promoter caused considerable necrosis in *N. benthamiana* leaves and total soluble protein extracts from these leaves were markedly degraded compared to those in which their expression was placed under the control of the CaMV 35S(-90) promoter. As such, low levels of the replication associated proteins appear to be preferred for maximum transgene expression from the pRCR-GUS vector as high Rep and/or RepA levels may be toxic to the cell causing leaf necrosis. Low level expression of Rep and RepA with the pRCR-GUS vector significantly elevated GUS expression levels about 5-fold which is comparable to that observed for a BeYDV RCR-based vector using a similar plant model and transformation system (Regnard *et al.,* 2010).

Outwardly extending PCR confirmed that the pRCR-GUS cassette was only released and ligated into a double stranded, circular DNA episome upon addition of Rep and RepA. Further, Southern hybridisation confirmed that these episomes were effectively replicated by a rolling circle mechanism as both closed, circular double stranded and open circular double stranded forms indicative of RCR were evident. Although not confirmed, it is unlikely that any of the bands comprised circular, single stranded DNA as these DNA forms only accumulate during virus infection in the presence of the coat protein. Using methylation sensitive enzymes, the extra-chromosomal pRCR-GUS episomes were shown to be non-methylated. This may suggest the transcriptionally active epsiomal forms may be less prone to the negative effects of methylation-associated transcriptional gene silencing, however, it does not preclude them from posttranscriptional gene silencing (PTGS). The initiation and termination of RCR is purportedly under the control of Rep alone, however the possibility that RepA may also share a similar role in RCR cannot be discounted. RepA shares 75% of its primary sequence with Rep and as such contains a number of RCR-related motifs (RCR1: FLTYPXC, RCR2: HLHXXQ, RCR3: VXDYXXK) and DNA binding and replication initiation domains in its N-terminal half. RepA is also thought to form oligomers with Rep as a function of RCR and *in vitro* studies suggest an *E. coli* synthesised RepA has nicking and joining activity. Despite this, there is no reported evidence available to suggest that RepA alone is capable of initiating RCR *in vivo*, independent of Rep. To prove a functional role in

RCR, RepA alone would have to supplied *in trans* with pRCR-GUS and DNA extracts analysed for RCR intermediates by Southern hybridisation.

Mastrevirus RepA proteins have long been considered to directly interact with a plant RBR in a manner similar to that of the oncovirus gene products via a LeuXCysXGlu (LXCXE) motif. Yeast two hybrid studies with the RepA proteins from *Wheat dwarf virus*, *Maize streak virus* and *Bean yellow dwarf virus* have shown these proteins can bind either human or plant RBR and detailed mutational analysis has confirmed the importance of the conserved L, C and E residues for this interaction (Horváth *et al.*, 1998; Liu *et al.*, 1999; Suárez-López and Gutiérrez, 1997). The RepA:RBR interaction is thought to sequester RBR from its normal role in the cell cycle control pathway thereby releasing RBR bound E2F transcription factors and activating a cascade of events leading to a transition from G1 to S phase in the host cell. Using the WDV RepA protein, this function has been practically exploited to synchronise both tobacco and wheat cells for *Agrobacterium*-mediated transformation (Gordon-Kamm *et al*., 2002). In this case, RepA was found to enhance *Agrobacterium* transformation frequencies to such a degree that chemical selection of transformed calli was unnecessary, suggesting S phase is conducive to T-DNA transfer. In the current study, TYDV RepA increased transient GUS expression about 2 to 4-fold from both RCR and non-RCR based expression vectors. This enhancing activity was strongly linked to the LXCXE motif as an E to K mutation in this motif negated its enhancer activity. A similar mutation in the LXCXE motif of WDV RepA reduced RBR binding approximately 95% (Xie *et al*., 1995). Three cell cycle control proteins from related viruses, namely BBTV (Clink), MSV (RepA) and TYLCV (REn) also stimulated GUS expression about 2 to 3-fold from a non-RCR based vector. Together these findings suggest RepA-mediated RBR binding and cell cycle progression positively influences transient transgene expression when delivered into whole leaves via Agroinfiltration. This appears to be the first report of its kind and highlights the fact that these virus derived gene products may be useful tools for further increasing transient expression levels obtained with other Agroinfiltration-based biofarming platforms.

Although all mastrevirus-encoded Rep proteins contain an LXCXE binding motif, their interaction with RBR remains uncertain. Using yeast two hybrid protein assays, an early study (Collin and Fernández-Lobato 1996) showed WDV Rep can directly interact with human RBR. Since then, more stringent two hybrid studies and *in vitro* binding assays have indicated the Reps from MSV, WDV and BeYDV do not interact with a plant RBR (Horváth *et al*., 1998; Liu *et al*., 1999; Suárez-López and Gutiérrez, 1997). It has been suggested the Rep LXCXE motif may be hidden (or not accessible) to RBR as secondary structure predictions

show Rep and RepA protein folding differs greatly around this region due to their unique C-terminal moieties. In the current study, Rep alone was able to significantly increase GUS expression from pRCR-GUS and also from a non-replicating vector (pEAQ-GSN). This would indicate the enhancing effects of Rep are not solely the result of transgene amplification by RCR but perhaps another feature, for example DNA binding, RBR binding and cell cycle control, or a previously unknown function such as PTGS suppression. Of these, RBR binding activity seems the most likely as an E to K mutation in the LXCXE binding motif effectively abolished its enhancer activity when co-delivered with the non-replicating expression vector. Considering Rep's recalcitrance to bind RBR in other studies, this finding was unexpected. Perhaps *in vitro* binding studies in yeast are not a true representation of the normal functional interaction between these proteins in their natural plant host or TYDV Rep simply differs from other mastrevirus Reps with respect to its RBR binding activity.

This study was primarily aimed at developing a replicating virus vector system to amplify transient transgene expression levels and increase recombinant protein production in plants. The resulting TYDV-based vector, pRCR-GUS, was effectively replicated by its cognate Rep and RepA proteins, which increased base reporter expression about 5-fold. RCR-based amplification is therefore a useful method for increasing transient gene expression in order to farm plant-made proteins. This study also showed that virus encoded proteins which regulate the cell cycle can positively influence transgene expression levels from a non-replicating vector when delivered by Agroinfiltration into leaves. Enhancer activity is assumed to arise from the innate ability of these proteins to stimulate cell cycle transition from G1 to S phase, a phase conducive to both *Agrobacterium*-mediated transformation and cellular gene expression. This enhancer function may be practically useful in the construction of novel hyper-expression platforms or used to further increase protein production with existing transient *Agrobacterium*-based expression systems.

Chapter 6: OPTrans: an Agroinfiltration-based platform for extreme transgene expression in *Nicotiana benthamiana*

6.1 Introduction

Transient gene expression by means of Agroinfiltration is a well-established practice that has been demonstrated to yield rapid and high levels of recombinant plant made proteins. Over recent years, improvements in vector design, particularly the use of deconstructed virus vectors, have seen a concomitant improvement in gene expression levels and protein yield to the point where Agroinfiltration is now a commercially viable means of producing functional protein equivalents. Two virus-based vector systems are commonly used today, the replicating virus vectors such as 'MagnICON' based on the genome of TMV and the non-replicating virus vectors such as the hyper-translation (HT) vector, pEAQ-HT, based on the genome of Cowpea mosaic virus (Marillonnet *et al*., 2004; Sainsbury and Lomonossoff, 2008). Both of these platforms reportedly generate very high recombinant protein yields of up to 5 g/kg of fresh biomass (80% total soluble protein (TSP)) and 1.5 g/kg of fresh biomass (30% TSP), respectively. While vector improvements have been of great benefit to biofarming, there have been few studies dedicated to improving other biological and molecular parameters associated with the Agroinfiltration process.

In previous chapters, a number of factors positively affecting Agroinfiltration-based transgene expression levels were identified. The design and make-up of an artificial environment capable of promoting the interaction of *Agrobacteria* with the target explant is an essential part of the Agroinfiltration process. MMA is a medium frequently used to prime and deliver the *Agrobacteria* (Lin and Ratna, 2014; Pang *et al*., 2013; Sainsbury *et al.,* 2009; Sainsbury and Lomonossoff, 2008; Tran *et al.*, 2014), but despite its common use, there have been limited attempts to improve upon its core ingredients. In Chapter 3, a number of chemical additives in the MMA media, including acetosyringone, lipoic acid, and Pluronic F-68, were evaluated and optimised and were found, independently and combined, to significantly increase *Agrobacterium*-mediated transgene expression levels.

Another factor influencing transient expression is post transcriptional gene silencing (PTGS), a natural plant response to pathogenic or aberrant RNA (dsRNA) that can drastically reduce transgene expression levels. Plant viruses have evolved mechanisms to overcome this phenomenon in the form of gene products that can target and disrupt distinct stages of the

PTGS pathway in order to suppress gene silencing in plants (Ahlquist, 2002; Johansen and Carrington, 2001; Reed *et al*., 2003; Voinnet *et al*, 2003). As such, these virus gene products have been exploited in biofarming and are often co-expressed during Agroinfiltration in order to amplify transgene expression. For example, pEAQ-HT contains a cassette in its T-DNA capable of expressing the tomato bushy stunt virus (TBSV) P19 protein. In Chapter 3, it was shown that TBSV P19 and a truncated version of the cucumber mosaic virus 2b protein were strong suppressors of PTGS and co-delivery of these had a combinatorial effect on their action.

Three completely novel approaches to enhancing transgene expression via Agroinfiltration were also described in previous chapters. Co-expression of the Arabidopsis BAG4 gene product (a protein known to confer stress tolerance), co-expression of the TYDV Rep and RepA cell cycle control proteins and a simple heat treatment to the whole plant 1-2 days following Agroinfiltration, were shown to independently increase reporter expression levels 2 to 5-fold. While treatments similar to this have been shown to augment *Agrobacterium* transformation frequencies (Chen *et al*. 2010; Khanna *et al*. 2007; Hiei *et al*. 2006; Gordon-Kamm *et al*., 2002), they have not yet been practically exploited in order to increase the level of Agroinfiltration-based transgene expression.

In the previous chapters a systematic approach was taken to investigate various aspects of the Agroinfiltration technology, including delivery vector, infiltration media, abiotic treatments and the co-delivery of enhancer gene products in order to further increase transient expression levels. This chapter describes the development of a complete system, termed OPTrans (Optimal Transgenesis) that combines these features and maximises gene expression in *N. benthamiana* using Agroinfiltration. The platform uses a dual vector delivery system containing gene cassettes for silencing suppression, cell cycle regulation and stress tolerance, in combination with a modified MMA infiltration medium and heat shock treatment to the plants.

6.2 Materials and Methods

6.2.1 Vector construction

Construction of the vector pEAQ-GSN was described in Chapter 2.2.1.10. The 35S-CMV-2b-nos (94 aa truncation) gene cassette (Chapter 3.3.1) was PCR amplified in order to insert unique restriction sites for future cloning. The cassette was amplified from 35S-CMV-2b plasmid template using 5 pmol of primers CMV_2b_FseI-F 5'-GCGGCCGGCCTCATGGAGTCAAAGATTCAAATAGAGGACC-3' and CMV_2b_FseI-R 5'-CGGGCCGGCCCCGATCTAGTAACATAGATGACACCGCGCGCG-3'. All PCRs were performed in

a final volume of 20 µL, containing 10 µL 2x GoTaq® Green Master Mix and using the following standard cycling conditions: 5 min at 94°C followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension step of 72°C for 5 min (optimised for 1 kb/ 50 s extension). The resulting PCR product was ligated into cloned and sequenced using the Big Dye™ Terminator system (Chapter 2.2.2.7). The expression cassette was excised from pGEM-T-Easy® using restriction enzyme FseI and ligated into FseI digested and dephosphorylated pEAQ-GSN. The resulting construct was called pSPECIAL (Figure 6.1).

The vector pBIN-Δ35S-Rep/RepA contains the TYDV Rep/RepA gene under the transcriptional control of the weak CaMV 35S (-90) promoter and nos terminator. Construction of this vector was previously described in Chapter 5.3.1. The CaMV 35S (-90) promoter, Rep/RepA genes and nos terminator cassette was excised from pBIN-Δ35S-Rep/RepA as an EcoRI fragment and ligated into EcoRI digested and dephosphorylated pBIN-At_BAG4 (described in Chapter 3.3.31). The resulting vector was called pBIN-At_BAG4.Δ35S-Rep/RepA. In order to replace the CaMV 35S promoter driving expression of the At_BAG4 gene, a nos promoter sequence was PCR amplified with primers nos-F_NheI 5'-CCGCGATCGCAGATCCGGTGCAGATTATTTGGATT-3' and nos-R_AsiSI 5'-CAGCTAGCAAATATTTCTTGTCAAAAATGCTCCA-3' from pBIN-At_BAG4 template using the cycling conditions described above. The resulting PCR product was ligated into pGEM-T-Easy®, cloned and sequenced using the Big Dye™ Terminator system (Chapter 2.2.2.7). The nos promoter was excised from pGEM-T-Easy® by restriction digestion with NheI and AsiSI and ligated into similarly digested pBIN-At_BAG4.Δ35S-Rep/RepA. The resulting construct was called pNEEDS (Figure 6.2).

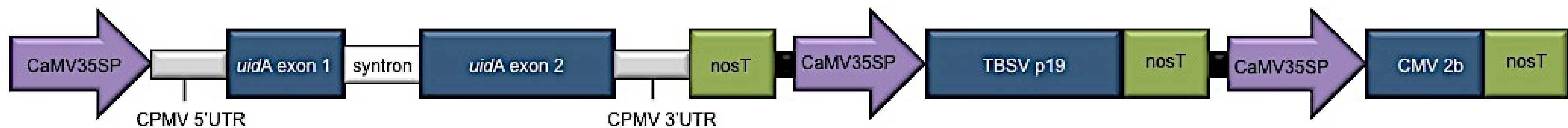

Figure 6.1 Schematic representation of pSPECIAL

pSPECIAL is a binary vector comprising three expression cassettes encoding the GUS reporter enzyme and the TBSV P19 and truncated CMV 2b silencing suppressor proteins CaMV35SP = Cauliflower mosaic virus 35S promoter; CPMV 5' UTR=Cowpea mosaic virus RNA-2 5'UTR; *uid*A=gene encoding GUS; syntron=synthetic intron; CPMV 3' UTR=Cowpea mosaic virus RNA-2 3'UTR; nosT=nopaline synthase terminator from *Agrobacterium*; TBSV P19=Tomato bushy stunt virus P19 silencing suppressor gene; CMV 2b=cucumber mosaic virus truncated 2b silencing suppressor gene.

Figure 6.2 Schematic representation of pNEEDS

pNEEDS is a binary vector comprising two expression cassettes encoding the At_BAG4 stress tolerance protein and TYDV Rep/RepA cell cycle control gene products.
nosP=nopaline synthase promoter from *Agrobacterium*; At_BAG4=Arabidopsis BAG4 gene; nosT=nopaline synthase terminator from *Agrobacterium*; Δ35S P=CaMV 35S (-90) promoter; Rep/RepA=TYDV Rep/RepA bicistronic gene.

6.2.2 Infiltration media

Two infiltration media were prepared, standard MMA (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 100 μM acetosyringone) and an optimised MMA media called MMA-LP (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 500 μM acetosyringone, 5 mM α-Lipoic acid and 0.002% Pluronic F-68).

6.2.3 Agroinfiltration of *N. benthamiana*

Wildtype *N. benthamiana* plants were germinated and maintained as previously described in Chapter 2.2.3.1. The top three leaves of 2-3 month old wildtype *N. benthamiana* plants were infiltrated in triplicate over three independent experiments (as described in Chapter 2.2.3.2). All vectors were mobilised in *Agrobacterium* strain Agl1 by electroporation as previously described (Chapter 2.2.2.5). Recombinant *Agrobacteria* harbouring pEAQ-GSN were co-cultivated and infiltrated in standard MMA media and infiltrated as previously described (Chapter 2.2.3.2). Recombinant *Agrobacteria* harbouring pSPECIAL and pNEEDS were co-infiltrated in the optimised MMA-LP media at a ratio of 1:1. Plants infiltrated with the dual vectors were also physically heat shocked at 37°C for 1 h, 2 days post infiltration (dpi). Leaves were sampled and snap frozen in liquid nitrogen at 0 and 4 dpi and stored at –80°C prior to analysis.

6.2.4 Protein extraction and GUS fluorometric assays

Leaf samples, collected at 0 and 4 days post Agroinfiltration, were snap frozen in liquid nitrogen and stored at -80°C. Total soluble protein (TSP) was extracted as described in Chapter 2.2.5.1 and protein content estimated using the Bradford assay (Bradford, 1976). GUS expression levels were quantified by fluorometric analysis and repeated in triplicate over an enzymatic time course (T0 and 10 min), as per Chapter 2.2.5.2. The was data obtained and analysed as described in Chapter 2.3.

6.2.5 SDS-PAGE and densitometry analysis

TSP extracts were separated using the Nu-PAGE mini-gel system. TSP (20 µg) was loaded onto a NuPAGE® Novex® 4-12% Bis-Tris Protein Gel (Life Technologies) and electrophoresed at a constant voltage (200 V) for 55 min in NuPAGE® MOPS SDS Running Buffer with NuPAGE® Antioxidant (Life Technologies) according to manufacturer's specifications. As a control, 3 µg of purified GUS protein (GUS Type VII-A; Sigma-Aldrich G7646) was loaded. Protein sizes were estimated using the Novex® Sharp Pre-stained Protein Standard (Life Technologies). Following electrophoresis, the acrylamide gel was stained in Coomassie Brilliant Blue dye overnight (approximately 16 h) and destained in SDS-PAGE destaining solution.

Protein bands were analysed with ImageJ software to calculate relative band intensities. Images were first converted to an 8-bit greyscale image using the 'image type' function and background subtracted using the image 'process' function. The parameters of Area, Mean Grey Value and Integrated Density were selected for measurement and the scale set to pixels using the 'Analyze' function. The colour of the image was then inverted, so that an increase in the measured parameters equated to an increase in protein expression. Protein bands were highlighted using the 'Freehand Selection' tool and the parameters defined and measured using the 'Measure' function. Measurements were exported to and analysed with Microsoft Excel.

6.2.6 GUS ELISA

In order to measure GUS levels in TSP, an ELISA protocol described by Dugdale *et al*. (2013) was used. In short, three leaf punches (approx. 10 mg) of fresh leaf tissue (sampled at 0 and 4 dpi) were homogenised and TSP content estimated via the Bradford Assay. High binding 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with 100 µL/well of 0.5 mg/mL rabbit anti-GUS IgG (Sigma-Aldrich G5545) in BBS (25 mM borate and 75 mM NaCl, pH 8.5), then blocked in Blocking buffer (1% BSA in BBS) for 1 h at room temperature. TSP (100 µL) was bound for 1 h at room temperature, then incubated with 100 µL of horseradish peroxidase–labelled rabbit anti-GUS IgG (Invitrogen A5790 conjugated to horseradish peroxidase) diluted to 0.5 mg/mL in blocking buffer for a further 1 h at room temperature. TMB (Sigma-Aldrich) substrate was added for colour development and optical densities read at 650 nm, using 620 nm as a reference, with an absorbency plate reader. Purified GUS (GUS Type VII-A; Sigma-Aldrich G7646) was used to develop a standard curve of reactivity.

6.3 Results

6.3.1 Comparison of the OPTrans and HT Expression systems

To compare expression levels afforded by the OPTrans and HT expression platforms, *N. benthamiana* plants were infiltrated with *Agrobacteria* harbouring pEAQ-GSN in standard MMA as described by Sainsbury *et al.* (2009) or the OPTrans vectors (pSPECIAL and pNEEDS) in the optimised infiltration media MMA-LP. In the latter case, *Agrobacteria* harbouring the OPTrans vectors were co-infiltrated at a ratio of 1:1 and plants were heat shocked at 37°C, two dpi. Four dpi, GUS activity was measured using fluorometric assays. Data, in triplicate, from three independent experiments were pooled, statistically analysed and graphed (Figure 6.3). Negligible GUS expression was detected at Day 0, suggesting that measured GUS activity was not derived from *Agrobacteria* or endogenous plant enzyme

activity. Four dpi, the level of GUS expression afforded by the OPTrans platform was significantly higher (approximately 6 to 8-fold) than that directed by the HT system.

6.3.2 PAGE analysis and densitometry

TSP extracts from leaves infiltrated with *Agrobacteria* harbouring (i) empty vector, (ii) pEAQ-GSN and (iii) pSPECIAL and pNEEDS were electrophoresed through acrylamide and stained with Coomassie Brilliant Blue dye (Figure 6.4). A dense band of approximately 70 kDa, was present in both TSP extracts from pEAQ-GSN and pOPTrans vectors, but not in TSP isolated from the leaves infiltrated with the empty vector. Based on size (GUS is approximately 68.28 kDa in mass) and the co-migration of the bands with the GUS standard, these bands were assumed to represent the GUS enzyme. Using quantitative densitometry on the protein bands, the amount of recombinant GUS produced by the OPTrans system was estimated to be approximately 5.5-fold greater than the HT system.

6.3.3 GUS ELISA

ELISA was used to quantify the amount of recombinant GUS generated by both expression platforms. *N. benthamiana* leaves were infiltrated with *Agrobacteria* harbouring (i) empty vector, (ii) pEAQ-GSN in MMA, and (iii) pSPECIAL and pNEEDS in MMA-LP (heat shocked 2 dpi) and sampled at both 0 and 4 dpi. Leaf samples were taken from the top three leaves of three plants and assayed in triplicate. No GUS was detected by ELISA in the day 0 samples suggesting no bacteria-derived or endogenous plant enzyme background. Four days post infiltration, extracts from pEAQ-GSN yielded an average of approximately 37.8 ng GUS / µg TSP compared to 132 ng GUS / µg TSP from the pOPTrans vectors (Figure 6.5). Based on these ELISA readings, the amount of recombinant GUS generated by the OPTrans platform was significantly greater (about 3.5 fold) than the HT system and represents approximately 13% of the TSP.

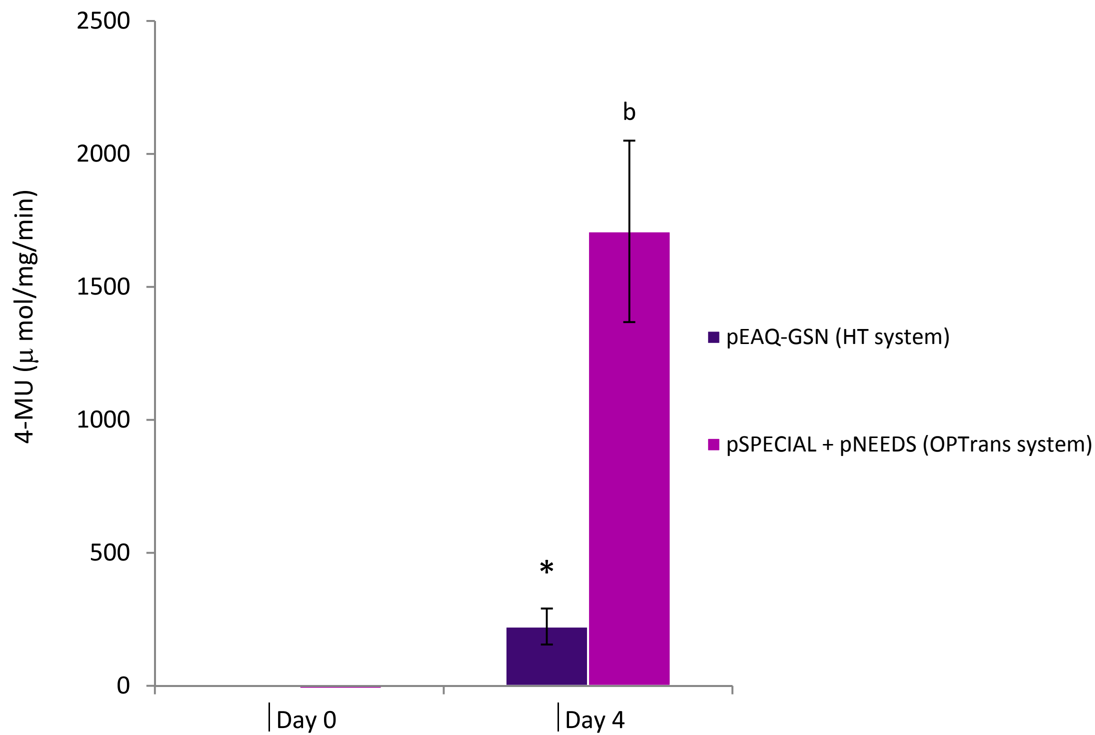

Figure 6.3 Comparison of transient GUS expression afforded by the OPTrans and HT platforms in *N. benthamiana* using GUS fluorometric enzyme assays

Agrobacterium strain Agl1 harbouring either pEAQ-GSN or the OPTrans vectors (pSPECIAL and pNEEDS) were infiltrated into *N. benthamiana* using MMA or optimised MMA-LP media, respectively. Plants infiltrated with OPTrans vectors were also heat shocked at 37°C, two dpi. Leaves were sampled 0 and 4 dpi and TSP extracted for GUS fluorometric enzyme assays. Columns represent mean GUS enzyme activities and bars represent standard error. The control treatment is marked with an asterisk (*), b = data is significantly higher than the control ($p < 0.05$).

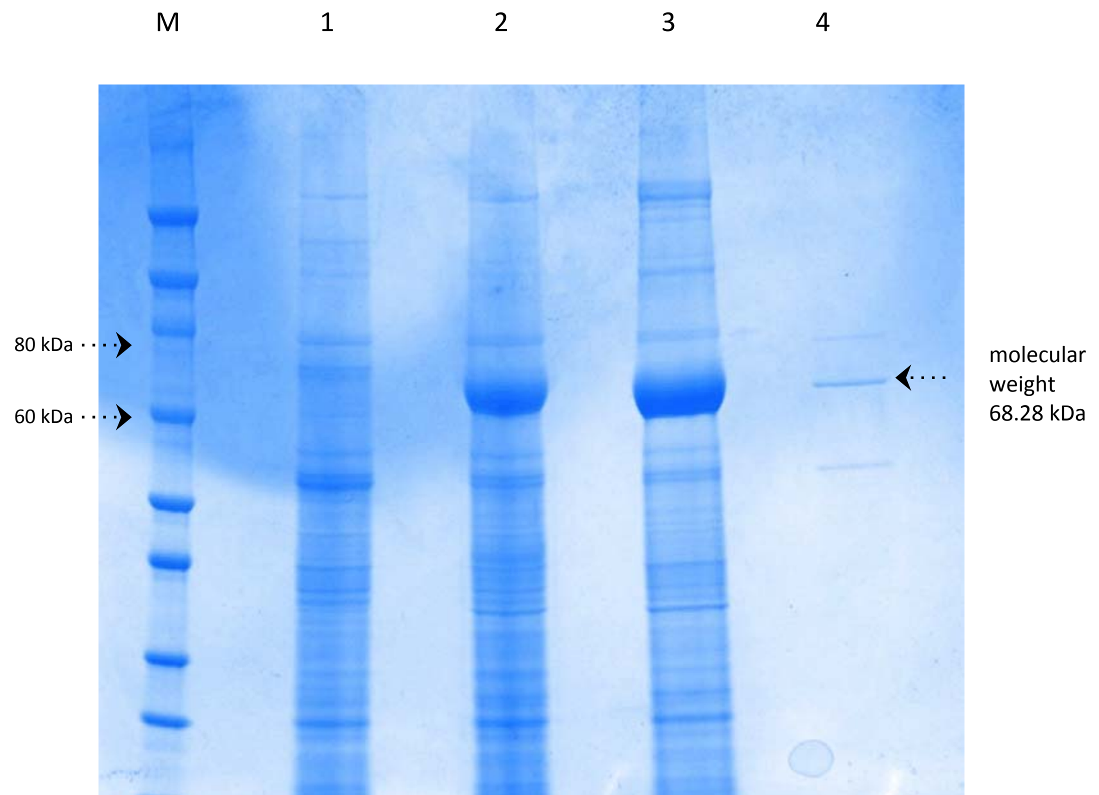

Figure 6.4 SDS-PAGE comparing GUS levels afforded by the HT and OPTrans expression systems

Vector combinations were Agroinfiltrated into *N. benthamiana* leaves and total soluble protein extracted 4 days post infiltration. Protein extracts were separated via SDS-PAGE and visualised using Coomassie Brilliant Blue stain.

M	=	Novex® Sharp Pre-stained Protein Standard
1	=	pBIN-plus in MMA
2	=	pEAQ-GSN in MMA
3	=	pSPECIAL+pNEEDS in MMA-LP with heat shock (2 dpi)
4	=	Purified GUS standard (3 µg) (GUS Type VII-A; Sigma-Aldrich G7646)

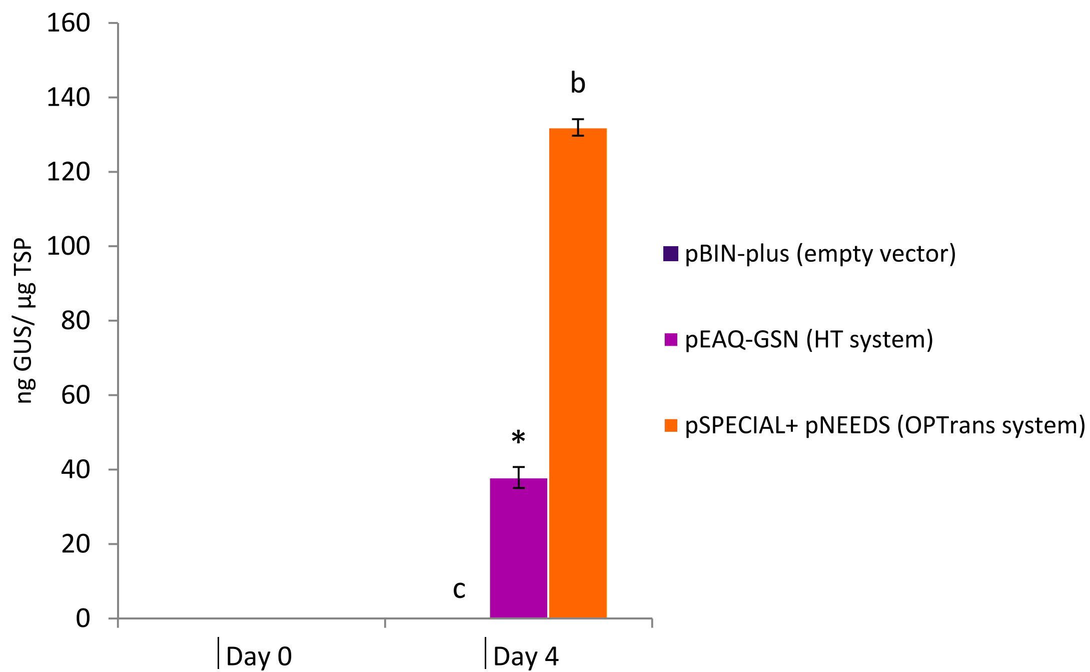

Figure 6.5 Quantification of recombinant GUS levels afforded by the HT and OPTrans systems using ELISA

Agrobacterium strain Agl1 harbouring pBIN-plus (empty vector), pEAQ-GSN (HT system) or pSPECIAL + pNEEDS vectors (OPTrans system) were infiltrated into *N. benthamiana.* OPTrans vectors were delivered in optimised MMA-LP media and plants were heat shocked at 37°C, two dpi. Leaf discs were sampled at 0 and 4 dpi and TSP extracted for GUS ELISA. Columns represent ng GUS per µg of TSP with bars representing ± SEM. The control treatment is marked with an asterisk (*)The control treatment is marked with an asterisk (*). b = data is significantly higher than the control ($p < 0.05$), and c = data is significantly lower than the control ($p < 0.05$).

6.4 Discussion

This chapter aimed to develop a new platform for high-level, transient Agroinfiltration-based expression and recombinant protein production in *N. benthamiana*. In previous chapters, a number of physical, chemical and molecular parameters were investigated in order to maximise *Agrobacteria*:host interactions and enhance transgene expression levels. Of these, several features were shown to significantly influence transgene expression, including *Agrobacterium* strain and density, infiltration medium and chemical additives, translation enhancers, intron-mediated enhancement, suppression of posttranscriptional gene silencing, host cell cycle, and stress tolerance. By combining the best of these features into a single expression platform, it was hoped to increase base expression levels afforded by the non-replicating HT vector system described by Sainsbury and colleagues (2008 and 2009). The resulting system, termed OPTrans (an acronym for Optimal Transgenesis), was a dual vector co-delivery system that incorporated an optimised infiltration medium and heat shock treatment to the whole plant following Agroinfiltration.

The OPTrans vectors, pSPECIAL and pNEEDS, were tailored for two distinct purposes. pSPECIAL is based on the pEAQ-HT vector, in which the gene of interest (in this case the *uid*A reporter gene encoding GUS) was flanked by the CPMV RNA-2 5’ and 3’ translation enhancers and transcription was directed by the CaMV 35S promoter and nos terminator. As the inclusion of an intron into the coding region of some genes has been shown to positively affect their expression, a process known as intron mediated enhancement, the *uid*A gene was engineered to contain a small synthetic intron in its 5’ end. Downstream of this module was inserted two cassettes capable of expressing the TBSV P19 and truncated CMV 2b suppressors of PTGS. As previously discussed in Chapter 3, these virus derived gene products act to suppress gene silencing via different strategies; P19 acts as a head to tail dimer that directly binds to 21 nucleotide siRNA duplexes and quantitatively sequesters these forms from AGO-1 containing RISCs while 2b both binds siRNA and physically interacts with AGO-1 to inhibit its slicing activity. The combinatorial effect of co-expressing both P19 and truncated 2b was previously shown to increase transgene expression about 6-fold.

The second OPTrans vector, pNEEDS, contained two expression cassettes, one encoding the TYDV Rep/RepA gene products and the other, the *Arabidopsis* BAG4 protein. In Chapter 5 co-expression of the TYDV Rep/RepA cell cycle proteins was shown to enhanced transgene expression from a non-replicating vector, most likely by binding plant RBR and creating a cellular phase (S phase) conducive to both *Agrobacterium*-mediated T-DNA transfer and

cellular gene expression. This activity was strongly dependent on Rep/RepA abundance as high expression caused local necrosis whereas low levels were non-toxic and elevated transgene expression. As such, expression of the Rep/RepA bicistronic gene in this vector was placed under the transcriptional control of the weaker truncated CaMV 35S (-90) promoter and the nos terminator. Upstream of this was inserted a cassette capable of expressing *Arabidopsis* BAG4, a protein known to promote cellular tolerance to biotic and abiotic stresses. Previously, co-expression of this product increased transgene expression levels about 2-fold, most likely by moderating programmed cell death resulting from incompatible *Agrobacterium*:host cell interactions. In pNEEDS, expression of the At_BAG4 gene was placed under the transcriptional control of the nos promoter and terminator. The nos promoter was selected in place of the strong CaMV 35S promoter as the latter promoter sequence contains enhancer elements capable of up-regulating neighbouring promoters in a distance and orientation independent manner. This was an important consideration as expression of Rep/RepA from the downstream cassette must remain at a low steady state level for optimal activity.

For maximum effect, the OPTrans system relies on the T-DNA of both vectors to be integrated into the same cell following Agroinfiltration, a process known as co-transformation. It is estimated co-transformation frequencies can vary between 50-80% when T-DNAs are delivered via two different *Agrobacteria* or when two T-DNAs are delivered from the same *Agrobacterium* (Ishida *et al*., 2004; Komari *et al.*, 1996). This would suggest between 20-50% of the cells infected following Agroinfiltration may not contain T-DNAs from both pSPECIAL and pNEEDS vectors, which would likely reduce reporter gene expression levels. To overcome this, perhaps all five expression cassettes could be integrated onto a single T-DNA and delivered using a single strain of *Agrobacterium*. While this approach would overcome the need for co-transformation, the repetition of some sequences (e.g. CaMV 35S promoter and nos terminator) may increase T-DNA instability, as iterated sequences in vectors can be targets for bacteria-mediated recombination. These events are thought to occur via conventional homologous recombination or other causes such as slipped mispairing during DNA replication (Last *et al.*, 1991).

Two additional features of the OPTrans system likely contribute to the high expression levels driven by the platform. The optimised infiltration medium, MMA-LP, was essentially MMA with elevated acetosyringone levels (500 µM) to improve the induction of *Agrobacterium* virulence (*vir*) genes (Wydro *et al.,* 2006), 5 mM α-lipoic acid to minimise *Agrobacterium*-mediated necrosis (Dan *et al.*, 2009) and 0.002% Pluronic F-68, a surfactant

that acts to decrease the surface tension of the infiltration media (Curtis and Nam, 2001). Using this modified infiltration medium, GUS expression from an HT vector was previously shown to be 2.5-fold greater than that from MMA alone (Chapter 3). Plants were also heat shocked at 37°C for 1 h, 2 days post infiltration (dpi). This treatment was previously shown to elevate transgene expression levels about 4-5-fold, most likely by stimulating the production of host-encoded heat shock and chaperone proteins that act to protect the cell from incompatible interactions with the bacteria and maintain normal cellular homeostasis.

Using fluorometric enzyme assays, GUS expression afforded by the OPTrans system was shown to be between 6 to 8-fold higher than that directed by the HT system in *N. benthamiana* leaves, 4 days post Agroinfiltration. When TSP extracts from these leaves were electrophoresed through acrylamide and stained with Coomassie Brilliant Blue dye, a dense GUS protein band of approximately 70 kDa was seen in both OPTrans and HT extracts but not in TSP isolated from the leaves infiltrated with the empty vector. Using quantitative densitometry, the protein expression generated by OPTrans system was estimated to be about 5.5-fold greater than the HT system, which is comparable to that estimated by GUS enzyme assays. Considering protein yields afforded by the TMV-based MagnICON replicating vector are reportedly 3 to 4-fold higher than that of the HT system, OPTrans represents a highly competitive and efficient non-replicating vector platform for protein production in plants. Using ELISA, GUS produced by the OPTrans system was estimated to constitute about 13% of TSP.

It is anticipated that expression levels afforded by OPTrans could be further enhanced with additional refinements to the system. While the system as a whole generated up to an 8-fold increase in reporter gene expression over that of the basic HT system, this increase does not reflect the sum benefits of individual enhancer elements or treatments when tested independently. It is possible that some features, when provided in combination, may negatively impact on transgene expression, for example, an increase in overall transgene expression may in turn elevate Rep/RepA accumulation causing a genotoxic stress response (Weitzman *et al.,* 2004). Alternatively, hyper-expression of each gene may simply deplete the host cellular transcription and translation machinery, thus compromising reporter gene expression levels.

In conclusion, the OPTrans dual vector platform represents an efficient hyper-expression system for the production of plant-made proteins. The OPTrans vectors were strategically engineered to include features that enhance transcription and translation of the gene of interest and express gene products known to positively influence transgenesis by

suppressing PTGS, manipulating the host cell cycle and conferring stress tolerance. In conjunction with a modified infiltration media and a whole plant heat treatment, the system provides superior expression levels compared to that of the HT platform and may potentially rival protein yields afforded by the TMV-based MagnICON replicating vector. With further refinement, the OPTrans vector system has the potential for even greater protein production capacity.

Chapter 7: General Discussion

With advances in plant genetic engineering and transgenesis have come significant improvements to both food and fibre crops, including nutritional biofortification, yield increases and pest and disease management. Biotechnology has also been utilised for the synthesis of valuable heterologous proteins in plants, a spin-off technology termed "molecular farming" or "biofarming" (Fischer and Emans, 2000; Rybicki, 2010; Twyman *et al.*, 2013). There are many advantages to engineering proteins in plant systems over more conventional organisms, such as bacteria and yeasts, including scalability and lower production costs. Plants can be transformed either stably, where the transgene is integrated into the host genome and transgenic plants are regenerated, or transiently where the transgene is expressed extra-chromosomally. While transgenic plants represent a long term resource for protein production, much recent focus has been on transient expression technologies for short-term protein accumulation. Today, Agroinfiltration is by far the preferred method of transient gene expression in plants and has been adopted by both researchers and industry as an effective and simplistic means of transferring genes into plant cells for rapid protein production.

The most commonly utilised experimental plant host for transient expression studies and recombinant protein production is *Nicotiana benthamiana*, a relative of tobacco and a species native to Australia. *N. benthamiana* is particularly suited to biofarming as it is readily amenable to *Agrobacterium*-mediated transformation, is a non-food crop and has a medium range leaf biomass (in comparison to other *Nicotiana* species, such as tobacco). The plant is highly susceptible to infection by a diverse number of plant viruses and, in 2004, Yang *et al*. attributed this extreme susceptibility to a natural mutation in an RNA-dependent RNA polymerase gene (NbRdRp1m) that was prevalent in laboratory-based research accessions. This enhanced virus susceptibility has been particularly useful for biofarming strategies that use fully deconstructed or partially deconstructed virus genomes to deliver, replicate and express foreign genes *in planta*. Based on their high biomass attributes, *N. excelsior* and *N. tabacum* have also been used as alternative biofarming hosts, however, transient expression levels afforded by either species are generally lower than that of *N. benthamiana* (Conley *et al*., 2011; Sheludko *et al.*, 2007).

Yield is paramount for the cost-effective production of plant-made proteins, such that subtle improvements to transgene expression can significantly elevate protein production capacities and decrease manufacture costs. The most substantial improvements to

Agroinfiltration and transient expression have come in the form of vector design. Today, two vector types dominate the biofarming landscape, the replicating deconstructed virus vectors and the non-replicating deconstructed virus vectors. MagnICON is an RNA virus vector system based on the genome of Tobacco mosaic virus (TMV) that can replicate in host cells and move from cell to cell. The MagnICON system is considered the gold standard for transient protein expression and can reportedly yield up to 1-5 g recombinant protein per kilogram fresh weight (Gleba et al., 2005; J. A. Lindbo, 2007). In contrast, the EAQ-HT Hyper-Trans platform uses enhancer elements from the genome of Cowpea mosaic virus (CMPV) and encodes the Tomato bushy stunt virus (TBSV) P19 silencing suppressor. This vector system cannot replicate or move between cells but can generate protein yields of up to 1.5 g recombinant protein per kg fresh weight (Sainsbury *et al*., 2009). Importantly, the MagnICON vectors have not been made available to researchers due to an exclusive licensing agreement between its creators, ICON Genetics, and Bayer CropScience.

While efforts to improve independent aspects of the Agroinfiltration process have been successful, there has been no concerted attempt to combine these optimised features into a single transient expression platform technology. The core objective of this thesis, therefore, was to optimise features of the Agroinfiltration process and develop methods of enhanced transgene expression in order to create a high-level, transient expression platform for recombinant protein production in plants. This would be achieved using the preferred host *N. benthamiana* in conjunction with the readily available EAQ-HT hyper-expression platform delivered via Agroinfiltration. Previously studied and novel improvements were independently assessed with the intent of selecting and combining the most effective elements into a novel platform that would potentially provide transgene expression levels far greater than those currently available.

Effective Agroinfiltration requires complex interaction and compatibility between the bacteria and host cells, induction of bacteria-encoded virulence genes, T-DNA packaging and movement between cells and efficient transgene expression once inside the host cell nucleus. As such, the Agroinfiltration process can be influenced by numerous physical, chemical and genetic factors. For instance, hypervirulent strains of *Agrobacteria* often have a wider host range and are more efficient at transferring their T-DNA into host cells (Álvarez et al., 2004; Li et al., 2010). However, the effectiveness of these strains can vary between plant species as hypervirulence often coincides with host cell necrosis. In this study four common laboratory strains of *Agrobacteria*, with varying levels of virulence, were compared. In order to determine which strain could generate the highest transgene

expression levels with minimal necrosis, reporter gene expression was monitored over time. Hypervirulent strain Agl1 consistently generated the highest expression levels, 4 days post infiltration (dpi), compared to LBA4404, GV3081 and C58. This activity positively correlated with increasing bacterial densities. As such, recombinant *Agrobacteria* strain Agl1, delivered at a convenient concentration (OD_{600} = 1.0), were used for all subsequent assay development and transgene expression measured within 4 dpi.

A number of chemical additives have been reported to enhance *Agrobacterium*-mediated transformation frequencies when incorporated in the co-cultivation media used to promote bacteria and plant cell interaction. In this study, chemicals were selected based on their effectiveness in the literature and added to the co-cultivation/infiltration media at incremental concentrations in order to determine their dose effect on transgene expression. The most significant improvements were observed using 500 μM acetosyringone, 5 mM α-Lipoic acid, 0.002 % of the surfactant Pluronic F-68. These chemicals work differently to augment transient expression levels; acetosyringone acts to stimulate bacteria associated *vir* gene expression, α-Lipoic acid reduces the effects of oxidative stress and Pluronic F-68 is a detergent that reduces the surface tension of the co-cultivation/infiltration media (Wydro *et al*., 2006; Dan *et al*., 2009; Curtis and Nam, 2001). Together these additives improved reporter gene expression 2.5-fold over conventional MMA infiltration media alone. However, it is likely the optimal concentration of these chemicals in other plant hosts may require independent evaluation. For example, in cucumber, highest transient expression was obtained using acetosyringone at a concentration of 200 μM (Nanasato *et al.*, 2013) while in Mexican lime, 50 mM α-Lipoic acid was most effective (Dutt et al., 2011).

Other chemicals, such as chemical chaperones, may also improve Agroinfiltration-based transgene expression levels by minimising cell death associated with incompatible bacteria:host interactions. Two such examples are sodium phenylbutyrate (NaPBA) and tauroursodeoxycholic acid (TUDCA) that serve to stabilise proteins in their natural conformation by improving folding in the ER and suppressing ER stress-related cell death. Predominantly used in animal clinical applications for the treatment of neurodegenerative diseases (Özcan *et al*., 2006; Welch and Brown, 1996), both chemicals were shown to reduce the sensitivity of *Arabidopsis* seedlings to ER stress (Watanabe and Lam, 2008). Azaserine and acivicin are compounds that inhibit key enzymes in the purine or pyrimidine synthesis pathways and have been reported to increase the competency of plant cells to *Agrobacterium*-mediated transformation (Cheng *et al*., 1997, Roberts *et al*., 2003, Wu *et al*.,

2003, Yang *et al*., 2006). Both chemicals may therefore be useful additives for promoting cellular longevity and increasing transformation frequencies.

Heat shock proteins (HSPs) and chaperones are up-regulated in response to extremes in temperature and other abiotic stresses in order to maintain cellular homeostasis. In this study, induction of HSPs using a physical heat shock (37°C) to the entire *N. benthamiana* plant, 2 days following Agroinfiltration, proved to be a simple means of dramatically increasing (4 to 5-fold) transient reporter expression. This increase was likely attributed to the up-regulation of heat stress proteins that in turn protected infiltrated leaf cells from incompatible *Agrobacterium* interactions, thereby increasing transformation frequencies. Co-expression of plant derived heat shock proteins Hsp70 and Hsp101 failed to elicit a similar response, suggesting the process is complex and involves the up-regulation and interaction of many host-encoded factors. *Arabidopsis* Bcl-2-associated athanogene 4 (*At_BAG4*) has a stress tolerance activity that serves to inhibit programmed cell death pathways. In this study, co-delivery of the *At_BAG4* gene product stimulated reporter expression 2-fold. Other stress tolerance genes such as Bcl-xL and CED-9, and the non-coding Bcl-2 3'UTR, have also been shown to enhance the efficiency of *Agrobacterium*-mediated transformation and embryo recovery of both banana and sugar cane (Khanna et al, 2007). These gene products are thought to enhance plant resistance and prevent cell death through the maintenance of organelle homeostasis (Qiao *et al*., 2002) while the transcript of Bcl-2 3' UTR is thought form RNA-level interactions with other pro-apoptotic proteins. The use of these or related stress tolerance products may also serve to further improve *Agrobacterium*-mediated transient reporter gene expression levels in *N. benthamiana.*

The protective effect of co-expressing histone proteins during transient expression was also investigated. Histones naturally interact with DNA and some are known to play a critical role in *Agrobacterium*-mediated transformation, by stabilising incoming T-DNA and increasing integration frequencies (Gelvin, 2010; Li *et al*., 2005; Loyter *et al*., 2005; Mysore *et al*., 2000). Transient co-expression of the *Arabidopsis* H2A gene product effectively increased transient reporter expression about 2-fold, however, expression of the H2A protein from a stably integrated copy did not. It remains unclear why transgenic expression failed to increase reporter levels. Perhaps H2A abundance is critical for its enhancing role, and the levels attained in the small number of lines studied were low. Alternatively, the H2A protein expressed by these lines may be functionally compromised, as certain core residues of this protein are prone to methylation and acetylation. In future studies, At_H2A

and other histone homologues could be isolated from *N. benthamiana* and tested for their enhancing effects in transient assays. Candidate genes could be used to transform *N. benthamiana* and a large transgenic population screened for high-expressing lines in order to determine whether histone abundance is important.

Low transient transgene expression can often be attributed to post transcriptional gene silencing (PTGS), an RNA-based plant defence mechanism primarily evolved to resist invading pathogens (Vaucheret *et al*., 1998). Traditionally, the effects of PTGS are minimised by the co-expression of virus-derived suppressors of PTGS (Voinnet *et al*., 2003) which serve to block or interfere with parts of the PTGS pathway in order to maximise transgene expression. Plant virus families have evolved different strategies in order to achieve this and, as such, these virus-derived PTGS suppressors can differ greatly both in efficiency and mode of function. Of the candidates tested in this study, the Cucumber mosaic virus (CMV) 2b and TBSV p19 were the most effective at suppressing PTGS, increasing reporter gene expression levels by approximately 3-fold. This increase was consistent with the results of Del Toro *et al*., (2014) who showed equivalent reporter gene expression in the presence of either TBSV p19 or CMV 2b. TBSV p19 serves to sequester siRNA, preventing their incorporation into the RISC complex, thus allowing for an increase in transgene accumulation (Baulcombe and Molnár 2004; Lakatos *et al*. 2006; Reed *et al*. 2003; Scholthof 2006). In contrast, CMV 2b interacts at both the RNA and protein level to suppress PTGS (Diaz-Pendon *et al*. 2007; Thomas *et al*. 2003). Interestingly, co-delivery of both CMV 2b and TBSV p19 had a cumulative effect increasing reporter expression almost 6-fold. To our knowledge this is the first report of its kind and suggests combining PTGS suppressors, that work in different ways, may be a useful way of minimising the effects of PTGS and maximising transient expression.

In order to further increase expression levels, a replicating vector based on the genome of Tobacco yellow dwarf mastrevirus (TYDV) was assembled. The vector included the TYDV *cis* replication elements (the large and small intergenic regions) and was designed to be amplified by rolling circle replication when the TYDV Rep/RepA proteins were delivered *in trans*. This TYDV-based system yielded a 2 to 5-fold increase in reporter expression over a non-replicating vector, which was comparable to Regnard *et al*., (2010) who reported a 7-fold increase with a deconstructed *Bean yellow dwarf virus* (BeYDV) vector in *N. benthamiana.* Importantly, the abundance of Rep/RepA activator proteins was critical to the success of the system, with low Rep/RepA levels generating highest transgene expression. An unexpected finding from this study was that both Rep and RepA can

independently elevate Agroinfiltration-mediated transient expression levels directed by a non-replicating vector. This activity was proven to be directly linked to a functional LXCXE motif present in both proteins. Other proteins with this motif are known to directly bind Retinoblastoma protein, sequestering it from its natural role in cell cycle control. This interaction results in the unnatural progression of the host cell into S (Synthesis) phase, a phase in which cellular replication and gene expression machinery is in abundance. Importantly, S phase is also the phase in which *Agrobacterium*-mediated transformation is most effective. We assume the switch from cellular quiescence to active S phase in *N. benthamiana* leaf cells expressing Rep or RepA is preferred for both optimal *Agrobacterium*-mediated transformation and transgene expression. While another Geminivirus replication associated protein has been used to synchronise wheat cells in order to enhance *Agrobacterium* transformation frequencies (Gordon-Kamm *et al*., 2002), this is the first report of utilising these cell cycle control proteins to increase Agroinfiltration-based transient gene expression.

Having investigated all of these parameters, the final step was to combine the elements that provided the most significant improvements into a novel expression platform. To do this, two vectors were carefully planned and generated incorporating the preferred plant or virus-derived enhancer elements and genes. In addition, recombinant *Agrobacteria* harbouring the vectors were infiltrated in an optimised co-cultivation/infiltration medium (termed MMA-LP) and plants were heat shocked 2 days post infiltration. The combined system, termed OPTrans (Optimal Transgenesis), was compared to the EAQ-HT hyper-expression platform delivered using the conditions described by Sainsbury *et al*. (2009). Reporter expression directed by the OPTrans system was about 7-fold higher than the EAQ-HT platform and the amount of recombinant protein produced was estimated to constitute about 13 % total soluble protein. This represents some of the highest transient expression directed by a non-replicating vector system. However, this level is considerably lower than the predicted cumulative effect of each optimised element or feature. Perhaps co-expression of some of these genes together or the implementation of physical and/or chemical treatments in tandem may negate their individual effects. Alternatively, there may be a threshold effect, whereby maximum transgene expression has occurred in the cells that are amenable to and transformed by Agroinfiltration. Importantly, MagnICON vectors are capable of cell-to-cell movement, and as such this vector system can enter almost all leaf cells following Agroinfiltration. It is likely this ability to move and replicate to high levels

in untransformed cell types contributes greatly to its capacity to generate very high protein yields and why it is considered the benchmark platform for biofarming plant made proteins.

While not investigated in this study, a number of other considerations with respect to vector design and the Agroinfiltration process may greatly influence the protein production capacity of OPTrans or other expression systems. The optimal temperature for transient *Agrobacterium*-mediated gene expression is reportedly 25 °C $\pm$ 0.5 °C (Chen *et al*., 2013; Liu *et al*., 2012), and cooler growth temperatures can correlate with a dramatic decrease in siRNA accumulation (Qu *et al.*, 2005; Szittya *et al.,* 2003). This would suggest temperature may play a critical role in the efficiency of *Agrobacterium*-mediated transformation and transgene expression. Therefore, to practically exploit this for Agroinfiltration, target plants should be grown at or below 24 °C in order to increase transformation frequencies and reduce the effects of PTGS.

Modern *Agrobacterium* binary vectors have evolved to include extra virulence (*vir*) genes, such as *vir*B, *vir*E and *vir*G, whose products effectively increase the frequency of T-DNA transfer from the bacteria to the host cell in a dose responsive manner. *Agrobacterium* strains harbouring these so-called “super-binary vectors” also have a wider host range and as such these vectors have been particularly useful for the transformation of crop species considered recalcitrant to *Agrobacterium*-mediated transformation (Hiei *et al*., 1994; Vain *et al*., 2004). While the effectiveness of some super binary vectors reportedly differs between *Agrobacterium* strains (Hiei *et al*., 1997; Vain *et al*., 2004), in general, these vectors can enhance transformation frequencies in many plant species and therefore represent a simple means of increasing Agroinfiltration-based transient gene expression.

Viruses have evolved strategies to divert the eukaryotic translation machinery to preferentially express their gene products in favour of host-encoded genes. While a number of these mechanisms have been well studied, few have been utilised as a means of enhancing transgene expression in plant cells. A few exceptions are the 5’ virus enhancer sequences from the genomes of TMV (the 70 nucleotide Ω leader) (Holtorf *et al*., 1995), members of the potyvirus group, and even the small satellite RNA of tobacco necrosis virus (V Alvarado and Scholthof, 2009; Turner and Foster, 1995). Some viruses employ cap independent translation, using cap independent translation enhancers (CITEs) or internal ribosome entry sites (IRES) to assist in their *in vivo* translation functions (Fabian and White, 2006; Guo *et al*., 2000; Kneller *et al*., 2006; Simon and Miller, 2013; Bedard and Semler, 2004; Lloyd, 2006; Ohlmann *et al*., 1996). Viruses that possess a 5’ cap but lack 3’ poly A

tails utilise a sequence known as a tRNA-like structure (TLS) (Matsuda and Dreher, 2004). Incorporating virus-derived elements such as these into a transgene expression cassette may serve to further improve expression levels, particularly at the level of mRNA translation.

More recently, there has been a shift in research to understand what elements of a traditional transgene expression cassette trigger PTGS and whether the design of this cassette to more closely resemble native plant genes can overcome this. This premise is based on the fact that some endogenous genes are highly expressed throughout the lifecycle of a plant but are not targets for PTGS. In order to mimic these genes, the promoter, terminator and sometimes introns of the endogene are incorporated into the transgene cassette. In transient experiments, a cisgene-like green fluorescent protein (*gfp*) expression cassette based on the regulatory regions of the highly expressed Rubisco gene, maintained high level *gfp* expression with reduced siRNA compared to a *gfp* transgene under the transcriptional control of the CaMV 35S promoter and nos terminator (Dadami *et al.*, 2013). On a similar note, the inclusion of an intron in the transcribed region of a transgene cassette has been shown to significantly enhance gene expression in a range of eukaryotic organisms in a phenomenon known as intron-mediated enhancement (IME) (Rose, 2008). While the exact mechanism of IME remains unclear, this phenomenon may too reflect a cisgene-like effect, as many highly expressed endogenes contain introns in their immediate 5' regions (Christie *et al.,* 2011; Zhang *et al.*, 2013). With this in mind, design of the transgene expression cassette to incorporate regulatory control features and introns from highly expressed endogenes may greatly increase transient expression levels and perhaps obviate the need to co-express suppressors of PTGS. The latter is of particular importance for the production of transgenic plants, as the constitutive expression of PTGS suppressors can cause severe phenotypic abnormalities and inhibit normal plant development (Saxena *et al.*, 2011).

In summary, this thesis was aimed at developing a rapid, high-level, Agroinfiltration-based platform for protein production in *N. benthamiana.* Using both published and novel approaches to optimise the Agroinfiltration process, and plant and virus-derived features to increase transcription, translation and protein stability an efficient platform technology was developed, termed OPTrans. The optimised expression system generated very high levels of protein accumulation, about 7-fold greater than the benchmark EAQ-HT non-replicating vector platform and approached those levels attainable with the gold standard MagnICON replicating vector system. With further refinement, the OPTrans hyper-expression platform

has the potential for greater protein production capacity and may be particularly useful for the cost-effective production of plant-made proteins in the future.

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