

# **Plant-based strategies aimed at expressing a functional human adenosine deaminase at high levels**

---

**S. A. D. Sanjeewa Singhabahu**

A thesis submitted in partial fulfilment of the  
requirements of the University of East London  
for the degree of Doctor of Philosophy

**June 2012**

# Abstract

An inherited disorder, ADA deficiency is a form of severe combined immunodeficiency, which is ultimately caused by an absence of adenosine deaminase (ADA), a key enzyme of the purine salvage pathway. The absence of ADA-activity in sufferers eventually results in a dysfunctional immune system due to the build-up of toxic metabolites. To date, this has been treated with mixed success, using PEG-ADA, made from purified bovine ADA coupled to polyethylene glycol. It is likely however, that an enzyme replacement therapy protocol based on recombinant human ADA would be a more effective treatment for this disease. Therefore, as a preliminary step to produce biologically active human ADA in transgenic tobacco plants and tobacco BY-2 cell suspensions a human ADA cDNA has been inserted into a plant expression vector under the control of the CaMV 35S promoter and terminator. In an attempt to maximise the yield various recombined gene constructs containing apoplast targeting sequences were tested along with different translational regulatory sequences such as TMV omega and RUBISCO untranslated regions.

Tobacco plants and BY-2 cells transformed with cytosolic constructs showed levels of recombinant ADA of up to 80 ng mg<sup>-1</sup> TSP. By comparison, transgenic calli expressing constructs containing apoplast-directing signals showed higher levels of recombinant ADA expression of up to 115 ng mg<sup>-1</sup> TSP. The most significant ADA activities were measured in transgenic BY-2 cell suspensions, however. Where, incorporation of a signal for arabinogalactan addition at the C-terminus of the recombinant ADA gene, targeted for secretion, produced a maximum yield of approximately 13 mg L<sup>-1</sup>. Representing a 336-fold increase over ADA activities recorded in a BY-2 suspension transformed with a cytosolic counterpart.

# **Disclaimer**

I hereby declare that the work and results presented in this thesis are of my own investigations except where reference has been made to published literature.

S.A.D.Sanjeewa Singhabahu

# Acknowledgements

I would like to express my sincere gratitude to Dr David Bringloe for his utmost support and advice throughout the course of my PhD. Further, I'm extremely grateful to Dr John George and Dr Joanne Tocher for all their support, advice and encouragement. I'm extremely honoured to be supervised by such an exceptional supervisory team. Thanks also to the academic, technical and support staff at the University of East London. I'm also extremely grateful to the School of health, sports and biosciences for granting the required funding and giving me the opportunity to successfully complete the PhD.

Special thanks to my loving parents Mr. Amarasiri and Mrs. Sriyani for the support and pride throughout my life in felicity and anguish. Very special thanks to my wife Deepthi for standing by my side and supporting me.

# Table of contents

<b>List of figures</b>	<b>IX</b>
<b>List of tables</b>	<b>XII</b>
<b>Abbreviations</b>	<b>XIV</b>
<b>1. Introduction</b>	<b>2</b>
1.1 Adenosine deaminase and ADA deficiency	2
1.2 Management options for ADA-SCID	6
1.3 ADA and cancer treatment	8
1.4 Production of biopharmaceuticals in transgenic plants and cell cultures	8
1.5 Expression of recombinant genes in plant cells and tissues	12
1.6 Suspension cultures	18
1.7 Plant produced human enzymes for enzyme replacement therapy	20
1.8 Production systems for ADA	22
1.9 Aims of study	23
 <b>2. Materials and Methods</b>	 <b>24</b>
<b>2.1 Materials</b>	<b>25</b>
2.1.1 Bacterial strains and plasmids	25
2.1.2 Plant material	28
2.1.3 Sources of reagents and enzymes	29
2.1.4 Solutions and media not described in the text	29
<b>2.2 General DNA techniques</b>	<b>32</b>
2.2.1 Small-scale isolation of plasmid DNA (minipreps)	32
2.2.2 Small-scale isolation of plasmid DNA (minipreps) using Qiagen QIAprep Kit	33
2.2.3 Large-scale isolation of plasmid DNA using QIAGEN Maxi Kit	33

2.2.4 Isolation of total DNA from plant material	34
2.2.5 Isolation of total DNA from plant material using Qiagen DNeasy Plant Mini Kit	34
2.2.6 Digestion of plasmid and PCR amplified DNA with restriction endonucleases	35
2.2.7 Agarose Gel electrophoresis of DNA	35
2.2.8 Recovery of DNA from agarose gels using Qiagen QIAquick Gel Extraction Kit	35
2.2.9 Ligation of DNA restriction fragments with vector DNA	36
2.2.10 Transformation of <i>E. coli</i> with plasmids	36
2.2.11 Amplification of DNA	37
<b>2.2.12 DNA hybridization techniques</b>	<b>37</b>
2.2.12.1 Preparation of Southern blots	37
2.2.12.2 Blotting	38
2.2.12.3 Random primer labelling of DNA with DIG	39
2.2.12.4 Hybridization of membrane-bound nucleic acid with DNA probes and immunological detection using Roche DIG High Prime Detection Kit	40
<b>2.3 RNA methods</b>	<b>41</b>
2.3.1 Isolation of total RNA using a Sigma Plant total RNA Kit	41
2.3.2 Gel electrophoresis of RNA	42
2.3.3 Preparation and hybridization of northern blots	42
2.3.4 Blot analysis	43
<b>2.4 Tobacco plant and tobacco BY-2 cell transformation</b>	<b>43</b>
2.4.1 Transfer of a pGreenI0029 plasmid constructs to <i>Agrobacterium</i> by electroporation	43
2.4.2 Transformation of tobacco BY-2 cell suspensions	44
2.4.3 Transformation of tobacco and regeneration of whole plants	45
<b>2.5 Protein methods</b>	<b>46</b>

2.5.1 Isolation of total soluble proteins from plant material	46
2.5.2 Enzymatic assay for adenosine deaminase	46
2.5.3 Protein Assay	48
2.5.4 SDS-polyacrylamide gel electrophoresis of proteins	49
2.5.5 Western blotting	50
2.5.6 Thrombin cleavage of ADA-hydroxyproline fusion	51
<b>2.6 Statistical analysis</b>	<b>51</b>
<b>3. Design and generation of ADA gene expression constructs</b>	
<b>in pGREEN for tobacco transformation</b>	<b>52</b>
<b>3.1 Overview</b>	<b>53</b>
<b>3.2 Constructs designed to increase cytosolic levels of human ADA</b>	<b>57</b>
3.2.1 Human ADA cDNA (pCDNA_ADA) construct design	57
3.2.2 Minimal 5' UTR (pΔ 5' UTR_ADA) construct design	62
3.2.3 TMV Omega 5' UTR (p5' Omega_ADA) construct design	64
3.2.4 TMV Omega 5' & 3' UTR (p5'_3' Omega_ADA) construct design	66
3.2.5 RUBISCO small subunit 5' UTR (pSS 5'UTR_ADA) construct design	67
<b>3.3 Constructs designed to increase levels of human ADA in tobacco</b>	
<b>by directing recombinant protein to apoplast and ER</b>	<b>69</b>
3.3.1 TMV Omega 5'UTR PR1a and TMV Omega 5'UTR Extensin	
(p5'Omega-PR1a_ADA and p5'Omega-Extensin_ADA) construct design	69
3.3.2 3'Hydroxyproline (p5'Omega-Extensin_ADA-3'Hydroxyproline) construct design	73
<b>4. Cytosolic expression of human adenosine deaminase in transgenic tobacco</b>	
<b>plants and BY-2 calli</b>	<b>77</b>

4.1 Overview	78
<b>4.2 Results</b>	<b>79</b>
<b>4.2.1 Cytosolic expression in transformed plants</b>	<b>79</b>
4.2.1.1 Detection of adenosine deaminase protein in transgenic tobacco plants using western blot analysis	82
4.2.1.2 Southern blot analysis of genomic DNA from plants transformed with the pCDNA_ADA construct	83
4.2.1.3 Northern blot analysis of ADA mRNA from plants transformed with the pCDNA_ADA construct	86
<b>4.2.2 Expression of five ADA cytosolic constructs in tobacco BY-2 calli</b>	<b>88</b>
4.2.2.1 Northern blot analysis of transcribed ADA mRNA from tobacco BY-2 calli transformed with five cytosolic constructs	91
4.3 Discussion	95
 <b>5. Subcellular directed expression of human adenosine deaminase in transgenic tobacco BY-2 calli</b>	 <b>100</b>
5.1 Overview	101
5.2 Results	102
5.2.1 Apoplast-directed expression in transformed BY-2 calli	102
5.2.2 Northern blot analysis of transcribed mRNA from calli transformed with different apoplast-directed constructs	105
5.3 Discussion	109
 <b>6. Expression of human adenosine deaminase in transgenic tobacco BY-2 cell suspension cultures</b>	 <b>113</b>
6.1 Overview	114



6.2 Results	115
6.2.1 Extra-cellular ADA-activities in BY-2 cell suspension transformed with ADA expressing constructs	116
6.2.2 Intra-cellular ADA-activities in BY-2 cell suspension transformed with ADA expressing constructs	118
6.3 Discussion	124
<b>7. Discussion</b>	<b>127</b>
<b>8. References</b>	<b>138</b>
<b>9. Appendices</b>	<b>162</b>
<b>Appendix A- Presentations, posters and abstract publications</b>	<b>163</b>

## List of figures

Figure 1.1: Catalytic conversion of adenosine into inosine	2
Figure 1.2: Purine metabolism pathway	4
Figure 1.3: Summary of the effects of ADA deficiency	5
Figure 1.4: Lymphocyte development and effect of ADA deficiency and other SCID disorders	5
Figure 2.1: A restriction map of pJIT	26
Figure 2.2: Restriction enzyme maps of Plant binary vectors (a) pGreenI0029 plasmid, (b) pSoup plasmid	27
Figure 3.1: Human ADA cDNA	59
Figure 3.2: (a) A human cDNA clone for ADA inserted into pJIT <i>Xba</i> I sites and (b) SafeView <sup>TM</sup> stained 1 % agarose gel of <i>Xba</i> I digested pJITADA	60
Figure 3.3: (a) Human ADA cDNA including 35S CaMV promoter and terminator inserted into <i>Eco</i> RV site in pGreenI0029 and (b) SafeView <sup>TM</sup> stained 1 % agarose gel of <i>Eco</i> RV digested pGreenI002935SADA	61
Figure 3.4: SafeView <sup>TM</sup> stained 1 % agarose gel of <i>Sal</i> I digested pGreenI002935SADA and pSoup	62
Figure 3.5: Schematic diagram depicting minimal 5' UTR configuration	63
Figure 3.6: Schematic diagram of the gene construct pΔ5'UTR_ADA	64
Figure 3.7: Tobacco Mosaic Virus (TMV) 5' Omega UTR DNA sequence	64
Figure 3.8: A PCR-amplified ADA cDNA and 5'Ω TMV UTR DNA inserted into pJIT	65
Figure 3.9: Tobacco Mosaic Virus (TMV) 3' un-translated region sequence	66
Figure 3.10: TMV 3'UTR inserted into p5' Omega_ADA construct	66
Figure 3.11: 5' Tobacco RUBISCO small subunit 5' UTR DNA sequence	67

Figure 3.12: pSS 5'UTR_ADA construct	68
Figure 3.13: Synthesised PR1a-ADA fusion	71
Figure 3.14: Synthesised extensin-ADA fusion	71
Figure 3.15(a): p5'Omega-PR1a_ADA construct and (b): p5'Omega-Extensin_ADA construct	72
Figure 3.16: ADA-3' Hydroxyproline fusion	75
Figure 3.17: p5'Omega-Extensin_ADA-3'Hydroxyproline construct	75
Figure 4.1: ADA specific-activities measured in the leaves of 6 transgenic plant lines transformed with pCDNA_ADA cytosolic construct	80
Figure 4.2: ADA specific-activities measured in tobacco plants transformed with various cytosolic constructs	80
Figure 4.3: Western blot of proteins extracted from the leaves of 3 transgenic tobacco plants transformed with cytosolic construct, pCDNA_ADA	83
Figure 4.4: Southern blot of genomic DNA of transformed (pCDNA_ADA) and non-transformed tobacco plant leaves	85
Figure 4.5: (a) Northern blot of total RNA extracted from pCDNA_ADA transformed and non-transformed tobacco plant leaves and (b) SafeView <sup>TM</sup> stained 2 % agarose gel of rRNA	87
Figure 4.6: ADA specific-activities measured in BY-2 calli transformed with various cytosolic constructs	89
Figure 4.7: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli and (b) SafeView <sup>TM</sup> stained 2 % agarose gel of rRNA	93

Figure 4.8: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli and	
(b) SafeView™ stained 2 % agarose gel of rRNA	94
Figure 5.1: ADA specific-activities measured in BY-2 calli transformed with various cytosolic and apoplast directing constructs	103
Figure 5.2 & 5.3: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli and	
(b) SafeView™ stained 2 % agarose gel of rRNA	107
Figure 5.4: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli and	
(b) SafeView™ stained 2 % agarose gel of rRNA	108
Figure 6.1: Extra-cellular ADA-activities measured in various cytosolic and apoplast-directing constructs in transformed BY-2 cell suspensions	117
Figure 6.2: Intra-cellular ADA-activities measured in various cytosolic and apoplast-directing constructs in transformed BY-2 cell suspensions	119
Figure 6.3: Total soluble proteins ( $\text{g L}^{-1}$ ) measured in Tobacco BY-2 cell suspension	120

## List of tables

Table 1.1: Plant-based vaccines, antibodies and therapeutic proteins in clinical development or on the market	10
Table 1.2: Comparison of recombinant protein production in a range of model systems	11
Table 1.3: Plant produced human enzymes	22
Table 2.1: Dilution series of DIG labelled DNA	40
Table 2.2: Protein assay	49
Table 3.1: Cytosolic constructs	57
Table 3.2: Subcellular targeting constructs	69
Table 4.1: Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco leaves using Mann-Whitney U test (5 % level)	81
Table 4.2: Pair-wise comparisons of median ADA specific-activities measured in transgenic tobacco leaves	81
Table 4.3: Genome T-DNA copy number and ADA specific-activities of transgenic tobacco plants transformed with pCDNA_ADA construct	86
Table 4.4: ADA specific-activities of tobacco plants transformed with pCDNA_ADA alongside ADA mRNA amounts measured in northern blots & T-DNA copy number	87
Table 4.5: Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco BY-2 calli using the Mann-Whitney U test (5% level)	90
Table 4.6: Pair-wise comparisons of median ADA specific activities measured in transgenic tobacco BY-2 calli	90
Table 4.7: ADA specific-activities of BY-2 calli transformed with cytosolic constructs alongside ADA mRNA amounts measured in northern blots	94

Table 5.1: (a) Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco calli using Mann-Whitney U test (5% level)	104
Table 5.1: (b) Pair-wise comparisons of median ADA specific-activities measured in transgenic tobacco BY-2 calli	104
Table 5.2: ADA specific-activities of tobacco BY-2 calli transformed with cytosolic and apoplast-directing constructs alongside ADA mRNA amounts measured in northern blots	108
Table 6.1: Pair-wise comparisons of highest extra-cellular ADA specific-activities measured in transgenic tobacco BY-2 cell suspensions	117
Table 6.2: Pair-wise comparisons of highest intra-cellular ADA-activities measured in transgenic tobacco BY-2 cell suspensions	119
Table 6.3: Highest intra and extra-cellular ADA-activities in transgenic tobacco BY-2 suspensions	120
Table 6.4: High-yield expression of pharmaceutical proteins in plant cell suspension cultures	122
Table 7.1: Summary of the highest ADA levels obtained in plants, BY-2 calli and BY-2 cell suspensions transformed with various constructs	133
Table 7.2: Summary of highest combined intra and extra-cellular ADA levels obtained in BY-2 cell suspensions transformed with various constructs	133

# Abbreviations

4-AA	4-aminoantipyrine
ADA	Adenosine deaminase
AGPs	Arabinogalactan proteins
BiP	Protein binding motifs
BMT	Bone marrow transplant
bp	Base pairs
BSA	Bovine serum albumin
BY-2	Bright yellow-2
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CHO	Chinese hamster ovary
dAdo	Deoxyadenosine
dATP	Deoxyadenosine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
EDTA	Ethylenediaminetetraacetic acid
EHSPT	N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline
eIF4F	Eukaryotic translation initiation factor 4F
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FDA	Food and drug administration
GCD	Glucocerebrosidase
GD	Gaucher's disease

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GT	Gene therapy
GUS	$\beta$ -glucuronidase
HBsAg	Hepatitis B surface antigen
HCl	Hydrochloric acid
HDEL	H-Histidine, D-Aspartic acid, E-Glutamic acid, L-Leucine
hGH	Human growth hormone
hGM-CSF	Human granulocyte-macrophage colony-stimulating factor
HSCs	Haematopoietic stem cells
HRGPs	Hydroxyproline(Hyp)-rich glycoproteins
HRP	Horseradish peroxidase
Hyp	Hydroxyproline
IFN $\alpha$ 2	Interferon $\alpha$ 2
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin-G
IPTG	Isopropylthio-(3-D-galactoside)
kDa	Kilo Dalton
KDEL	K-Lysine, D-Aspartic acid, E-Glutamic acid, L-Leucine
KOAc	Potassium acetate
LAMAN	Lysosomal acid $\alpha$ -mannosidase
LB	Luria broth
MCS	Multiple cloning site
mRNA	Messenger RNA
MS	Murashige Skoog
MW	Molecular weight



NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NDV	Newcastle disease virus
NOS	Nopaline synthetase
NT	Non-transformed
nt	Nucleotide
(ocs)3mas	Octopine synthase 3mas promoter
ORF	Open reading frame
oriV	Virulence origin gene
PEG-ADA	Bovine ADA coupled to polyethylene glycol
PBLs	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNP	Purine nucleoside phosphorylase
POD	Peroxidase
PR1a	Pathogenesis related protein 1a
PVP	Polyvinylpyrrolidone
rAAT	Recombinant human $\alpha$ 1-antitrypsin
RAmy3D	Rice $\alpha$ -amylase 3D
RBCs	Red blood cells
RNA	Ribonucleic acid
RUBISCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
SAH	S-adenosylhomocysteine hydrolase
scFv	Single-chain variable fragment
SCID	Severe combined immune deficiency disorders

SDS -PAGE	Sodium dodecyl sulphate/ polyacrylamide gel electrophoresis
T1, T2 etc	Primary transformed plants
T-DNA	Transfer DNA
TdT	Deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
TMV	Tobacco mosaic virus
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TSP	Total soluble protein
USFDA	United States Food and Drug Administration
UTR	Un-translated region
UV	Ultraviolet
Var:	Variety
WT	Wild type
W/V	Weight/volume
X-gal	5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside
XOD	Xanthine oxidase

#### Nucleic acid abbreviations

A- Adenine.

C- Cytosine.

G- Guanine.

T- Thymine.

### Amino acid abbreviations

#### **Abbreviation   1 letter abbreviation   Amino acid name**

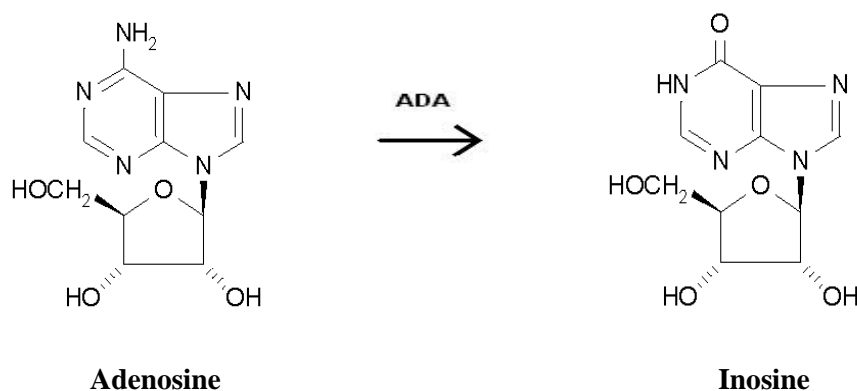
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Pyl	O	Pyrrolysine
Ser	S	Serine
Sec	U	Selenocysteine
Thr	T	Threonine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

# **Chapter 1**

## **Introduction**

## 1.1 Adenosine deaminase and ADA deficiency

Human adenosine Deaminase (ADA; EC 3.5.4.4) is an enzyme, which consists of 363 amino acids and is 41kDa in size. Also referred to as adenosine aminohydrolase, ADA is a ubiquitous enzyme, belonging to the purine metabolism pathway (figure 1.2), it catalyses the conversion of adenosine into inosine (figure 1.1) and deoxyadenosine (dAdo) into deoxyinosine, through hydrolysis of a purine amino group (Benveniste and Cohen, 1995). In addition to catalytic function in the cell cytoplasm, ADA was found to interact with cell surface receptor CD26 and other specific adenosine receptors. ADA appears to co-localise with these adenosine receptors found on dendritic cells and interact with CD26 on T-lymphocytes leading to an increased production of pro-inflammatory cytokines (interferons and interleukins) and T-helper cells (Pacheco *et al.*, 2005).



**Figure 1.1:** Catalytic reaction of adenosine into inosine. ADA also hydrolyses deoxyadenosine. The amino groups are cleaved during hydrolysis, which results in an amide, and an ammonia molecule.

The disease adenosine-deaminase-deficiency is a severe combined immunodeficiency, a fatal inherited disorder caused by mutations in the ADA gene on chromosome 20, which affects the development and function of immune cells (figure 1.4). Although ADA is present in all tissues, there is higher activity in lymphocytes during their development (Tsuboi *et al.*, 1995). The number of individuals diagnosed with deficiency of ADA ranges from 1:200,000 to 1:1,000,000 births, affecting all ethnic groups. However the prevalence is higher in certain

communities where the consanguinity is greater (Hershfield, 2011). The disease is characterized by a purine defect that leads to impaired immune function, recurrent infections and systemic metabolic abnormalities. It is an autosomal-recessive disorder representing 20% of the severe immune deficiency disorders (SCID) (Aiuti, 2004). As shown in figure 1.3, deficiency of ADA causes an increase in adenosine and 2'-deoxyadenosine (dAdo) in the plasma and an increase of nucleotides in lymphoid tissues, red blood cells and organs such as the kidney and the liver. These accumulations lead to an inactivation of S-adenosylhomocysteine hydrolase which in due course inhibit certain lipids, proteins and nucleic acid methylations. When dAdo levels increase there is also a significant inhibition of another enzyme, ribonucleotide reductase, which in turn causes a deoxynucleotide (dNTP) imbalance. The imbalance of dNTP leads to impairment of the T lymphocytes' ability to repair and synthesise DNA. This causes an increase of denatured DNA in the cell nucleus, which in turn activates apoptosis (Benveniste and Cohen, 1995). Improper ADA function, imbalances of dATP, dAdo, and dNTP, as well as S-adenosylhomocysteine hydrolase impairment leads to an active apoptosis of thymocytes, resulting in T-cell death and severe combined immunodeficiency syndrome (Gangi-Peterson *et al.*, 1999). As B-cell activity is controlled by T-cells, a decrease in T-cell function also results in dysfunctional B-cells. Furthermore, during maturation and recombination of the T-cell receptor, thymocytes are increasingly affected by ADA deficiency (Benveniste and Cohen, 1995). At this stage an imbalance of the nucleotide pool, caused by a deficiency of ADA, leads to N-region insertion alterations during T-cell receptor V(D)J recombination. A change in N-region substitutions with A-T nucleosides rather than G-C nucleosides leads to reduced frequency of recombination in T-cell immunoglobulins. In addition to the above, Van de Wiele *et al.*, (2002) have shown that dATP-induced release of cytochrome c in mitochondria, also initiates

apoptosis and this is now thought to be the main mechanism of dATP toxicity in ADA deficient patients.

Since the disorder is genetic, symptoms of ADA deficiency SCID are seen in affected infants at birth. However in some instances, ADA deficiency cannot be detected until later childhood or even adulthood due to mutations which partially impair the activity of ADA. Complete deficiency of ADA causes severe onset syndrome in SCID infants which exhibit lymphopenia, growth failure, opportunistic infections, and impaired function of cellular and humoral immunity, due to severe depression of T-cell function and antibody responses. There are about 40 identified alleles known to cause ADA SCID and some locations on the gene are highly susceptible to mutations (Hirschhorn *et al.*, 1990).

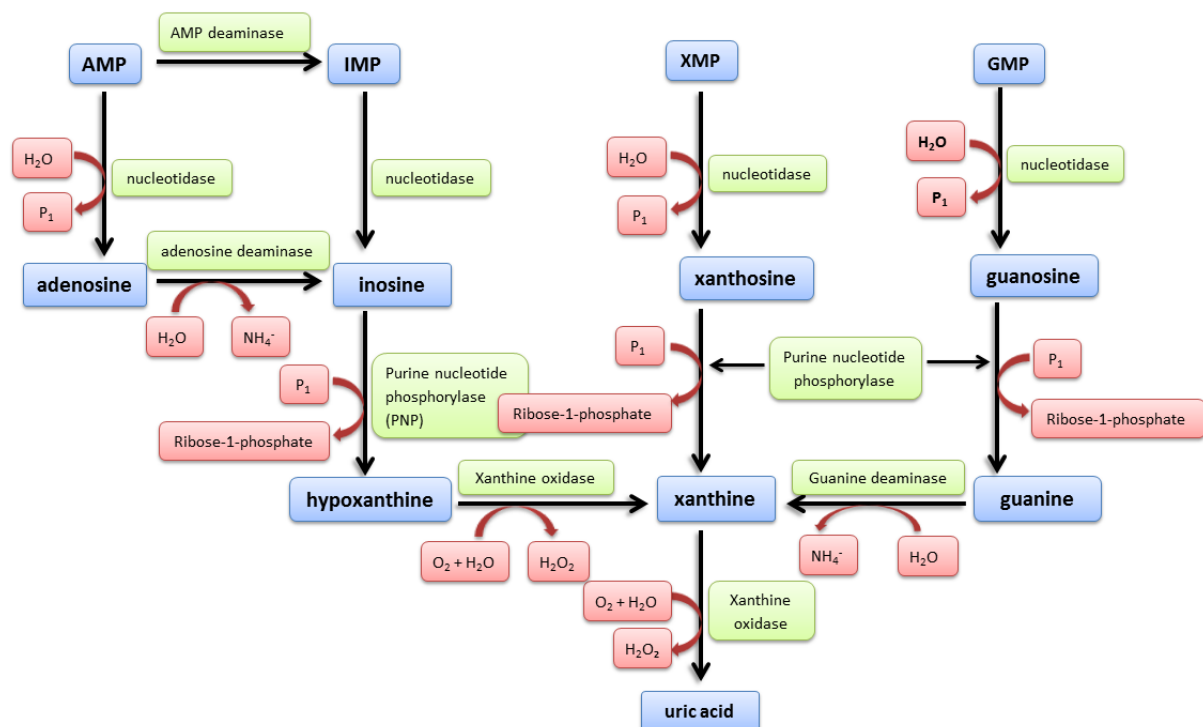


Figure 1.2: Purine metabolism pathway, modified from Kather, (1990).

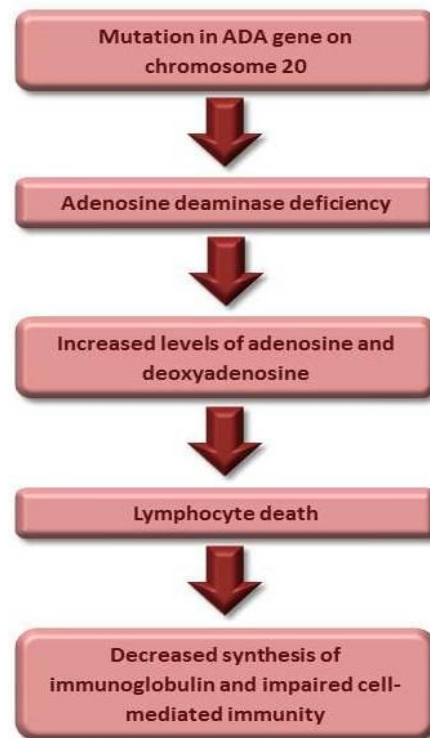


Figure 1.3: Summary of the effects of ADA deficiency.

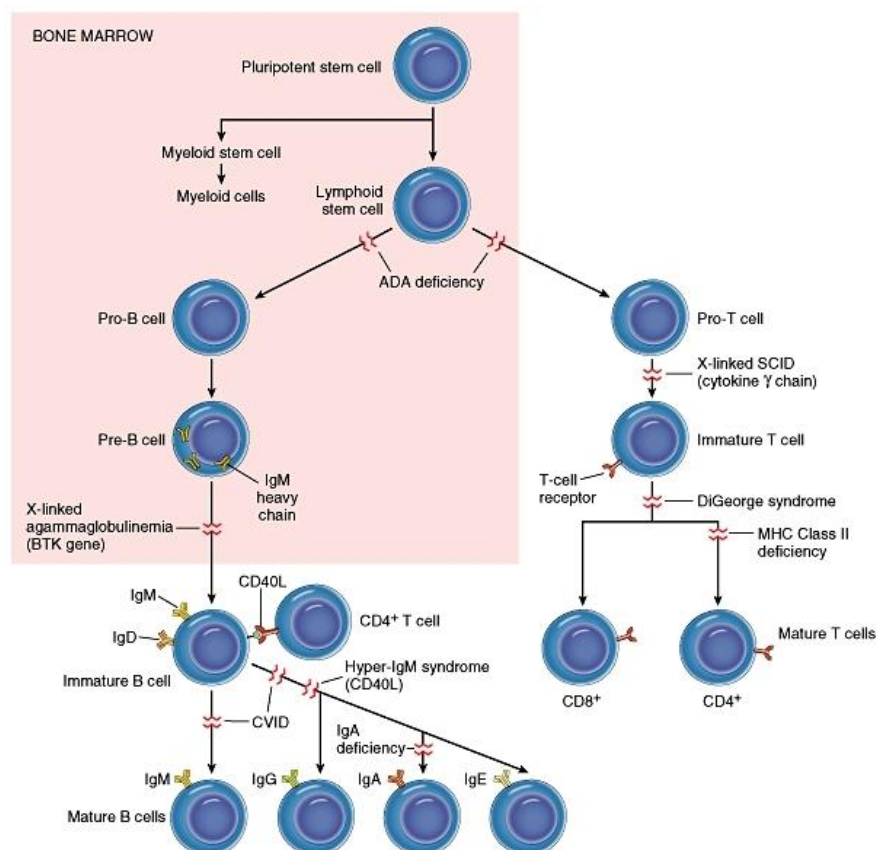


Figure 1.4: Lymphocyte development and effects of ADA deficiency and other SCID disorders, adapted from Kumar *et al.*, (2009).



## 1.2 Management options for ADA-SCID

Management of ADA deficiency is based on restoration of a functional immune system and can be attained by three main management options which include bone marrow transplant (BMT), somatic gene therapy (GT) and enzyme replacement therapy (ERT) (Booth *et al.*, 2007). In the first of these, the bone marrow transplant from a haploidentical donor is a fairly up-front process as the patients are already immunocompromised. It is difficult however to find donors with haploidentical characteristics and who themselves are not ADA deficient (Haddad *et al.*, 1999). Once the bone marrow transplant has been performed, the success rate of recovery is above 90% with fully recovered B-cell and T-cell functions (Hershfield *et al.*, 2001; Rubocki *et al.*, 2001). Buckley *et al.* (1999) reported that 11 ADA deficient patients out of 13 survived after bone marrow transplantation with an improvement of T-cell numbers and function after 3 to 4 months of transplantation.

The second option, somatic gene therapy, represents a promising therapeutic option in general for inherited disorders of the immune system. Treatment of inherited diseases using gene transfer approach was first performed on adenosine deaminase deficiency (Anderson *et al.*, 1990). The early trials of gene therapy showed the possibility and safety of engineering peripheral blood lymphocytes and haematopoietic stem cells using retroviral vectors (Aiuti, 2004). Results of these studies showed that gene therapy with peripheral blood lymphocytes allowed correction of the T-cell defect, but provided insufficient systemic detoxification shown by increased purine metabolites leading to decreased numbers of B-cells. However the gene transfer of bone marrow stem cells demonstrated a full correction of metabolic and immunological defects of adenosine deaminase deficiency with normalisation of peripheral blood lymphocyte counts, thymic activity restoration and improved immunoglobulin levels (Aiuti, 2004).

The third option, enzyme replacement therapy (ERT) with PEG-ADA has been used for more than 20 years to treat ADA deficient SCID patients (Booth *et al.*, 2007). To begin with, ERT was based on the use of frozen irradiated red blood cells (erythrocytes) which can indirectly add the missing supply of ADA. However, Polmar *et al.*, (1976) showed that some patients treated with this therapy showed a similar depressed immune response as that found in untreated ADA deficient patients. As a result it was thought that introduction of ADA, by directly injecting the enzyme, is a far better approach than using irradiated red blood cells. However, non-conjugated ADA enzyme was found to undergo rapid degradation in the serum and in some cases deleterious immune responses resulted. Hence, polyethylene glycol (PEG) conjugated bovine ADA (PEG-ADA) is used by intramuscular injection to treat ADA deficiency. PEG conjugation blocks protease access to ADA in the serum and consequently lengthens the plasma half-life of ADA by more than 100-fold (Bordignon *et al.*, 1995). Weekly administration of PEG-ADA ( $15\text{--}60\text{ U kg}^{-1}$  per week) improves the deleterious effects of ADA deficiency by correcting metabolic abnormalities, as PEG-ADA permits variable improvements in lymphocyte counts and immune function. As expected dAdo and dATP levels are also decreased and improvements in growth, reduced incidents of life-threatening infections resulted from this treatment (Gaspar *et al.*, 2009; Hershfield, 2011). If treatment is continued for longer than a year most individuals develop antibodies against bovine PEG-ADA which leads to inhibition of catalytic activity and enhanced clearance in some patients (Chaffee *et al.*, 1992). Continuing PEG-ADA therapy is also extremely costly and can cost from \$ 200,000 to \$ 300,000 per patient annually (Chan *et al.*, 2005). Hence, ERT with a recombinant human ADA is thought to be a viable and cheaper alternative in the treatment of patients over a life-long period. As plants possess well established foreign protein production mechanisms and human-like post-translational modification systems,

along with many other advantages over mammalian and prokaryotic production systems, plant production systems represent a reasonable choice for the development of a recombinant human ADA production system.

### 1.3 ADA and cancer treatment

High levels of adenosine are known to indirectly stimulate the proliferation of some forms of cancer, inhibiting the activation of cytokine production in killer T-cells which in turn stimulates metastasis (Sitkovsky *et al.*, 2005; Loshkin *et al.*, 2006). Since ADA catalyzes the conversion of adenosine to inosine, it has a potential as a drug in the treatment of adenosine-sensitive tumours. For example, Sitkovsky *et al.*, (2005) have shown that ADA along with adenosine receptor antagonists can enhance the immune reaction against some tumours. In addition, adenosine deaminase in conjugation with intravenous doses of polyalkylene oxide has been shown to substantially reduce the growth and spread of some tumours in mice (Filpula *et al.*, 2009). Hence, there would seem to be another potential use for recombinant human adenosine deaminase in the treatment of some types of cancer.

### 1.4 Production of biopharmaceuticals in transgenic plants and cell cultures

Plants and plant products have been used for centuries for the treatment of human disease. Botanical gardens presented a wealth of *materia medica* for therapeutic use by the latter part of 16<sup>th</sup> century. In the 17<sup>th</sup> century, a scientific approach and the discovery of new pharmacological remedies led to the identification of active principles and purification for therapeutic use (Winslow and Kroll, 1998). At present, about a quarter of prescription drugs also contain medicaments which are of botanical origin. The current advances in biotechnology have led to renewed hopes in producing new medicaments from botanical sources, including fully functional pharmacologically imperative recombinant human

proteins (Fischer and Emans, 2000). Recombinant human insulin produced in bacteria was the first commercially produced biopharmaceutical to be made in 1982 by Lilly, USA; and coincides with the development of a first genetically modified plant (Fraley *et al.*, 1983). The development of genetically modified plant was followed by successful production of biopharmaceuticals in plants, such as expression of human growth hormone fusion protein (Barta *et al.*, 1986), interferon (DeZoeten *et al.*, 1989), monoclonal antibodies (Hiatt *et al.*, 1989), and human serum albumin (Sijmons *et al.*, 1990). Since then, scores of biopharmaceuticals were produced in plant expression systems (table 1.1).

Although bacteria are classed as convenient and cost-effective production systems for producing smaller human proteins (e.g. human insulin), incapacity of assembly steps and post-translational modifications, which are essential for biological activity of most human proteins, hinders their use for production of more complex proteins. In addition, effective removal of contaminating lipopolysaccharides from recombinant protein is extremely expensive in bacterial expression systems. Plants on the other hand possess an efficient protein synthesis mechanism, and with established gene expression systems, plants can be used to produce large amounts of proteins (Fischer and Emans, 2000). Due to the absence of mammalian pathogens, unlike mammalian cell systems, plant expression systems possess some important advantages over mammalian and bacterial expression systems (table 1.2) (Fischer and Emans, 2000). Other advantages of plant-derived biopharmaceuticals also include: higher product safety, higher production scale, lower production costs, easy storage and distribution.

**Table 1.1: Plant-produced therapeutic proteins, antibodies and vaccines in clinical development or on the market.**

Product	Plant host	Indication	Route of administration	Product development stage	Reference
<b>Vaccines</b>					
E.coli LT-B	Potato Maize	Diarrhoea	Oral	Phase 1 Phase 1	Mason <i>et al.</i> , (1998); Tacket <i>et al.</i> , (2004)
Norwalk virus CP	Potato	Diarrhoea	Oral	Phase 1	Mason <i>et al.</i> , (1996)
Rabies virus GP/NP	Spinach	Rabies	Oral	Phase 1	Modelska <i>et al.</i> , (1998)
Newcastle disease virus HN	Tobacco cell suspension	Newcastle disease (poultry)	Subcutaneous	USDA approved (Marketed)	<a href="http://www.dowagro.com">http://www.dowagro.com</a>
Personalized anti-idiotyped scFVs:	<i>Nicotiana benthamiana</i>	Non-Hodgkin's lymphoma	Subcutaneous	Phase 1 (ongoing)	Bendandi <i>et al.</i> , (2010)
H5N1 influenza HA VLP	<i>Nicotiana benthamiana</i>	H5N1 "avian" influenza	Intramuscular	Phase 1 (ongoing) Phase 2 (Health Canada approved and enrolling volunteers)	Yusibov <i>et al.</i> , (2012)
H5N1 influenza HAI1	<i>Nicotiana benthamiana</i>	H5N1 "avian" influenza	Intramuscular	Phase 1 (ongoing)	Yusibov <i>et al.</i> , (2012)
H1N1 influenza HAC1	<i>Nicotiana benthamiana</i>	H1N1 "swine" influenza	Intramuscular	Phase 1 (ongoing)	Shoji <i>et al.</i> , (2012)
<b>Antibodies</b>					
Anti-CD20	Duckweed	Non-Hodgkin's lymphoma, rheumatoid arthritis	Intravenous	Pre-clinical	Cox <i>et al.</i> , (2006)
Anti-Streptococcus surface antigen I/II	Tobacco	Dental caries	Topical	Phase 2; EU approved	<a href="http://www.planetbiotechnology.com">http://www.planetbiotechnology.com</a>
Anti-αCCR5	<i>Nicotiana benthamiana</i>	HIV	Topical	Pre-clinical	Pogue <i>et al.</i> , (2010)
Anti-HIV gp120	Maize <i>Nicotiana benthamiana</i>	HIV	Topical	Pre-clinical	Yusibov <i>et al.</i> , (1997)
Hepatitis B surface antigen	Potato Lettuce	Hepatitis B vaccine purification	Not applicable	Phase 1	Kapusta <i>et al.</i> , (1999); Streatfield, (2006)
<b>Therapeutic and dietary proteins</b>					
Glucocerebrosidase	Carrot cell suspension	Gauchers disease	Intravenous	USFDA approved in 2012 (Marketed)	Shaaltiel <i>et al.</i> , (2007)
Insulin	Safflower	Diabetes	Subcutaneous	Phase 1/2 (marketing expected)	Markley <i>et al.</i> , (2006)
Gastric lipase	Maize	Cystic fibrosis, pancreatitis	Oral	Phase 2 (marketed as analytical reagent)	Zhong <i>et al.</i> , (2006)
Lactoferrin	Maize	Gastro-intestinal infections	Oral	Phase 1 (marketed as analytical reagent)	Samyn-Petit <i>et al.</i> , (2001)
Intrinsic factor	<i>Arabidopsis thaliana</i>	Vitamin B12 deficiency	Oral	Phase 2	Fedosov <i>et al.</i> , (2003)

Table modified from Yusibov *et al.*, (2011); Paul and Ma, (2011).

**Table 1.2: Comparison of recombinant protein production in a range of model systems.**

	Transgenic plants	Plant viruses	Yeast	Bacteria	Mammalian cell cultures	Transgenic animals
Cost/storage	Cheap/RT	Cheap/- 20 °C	Cheap/-20 °C	Cheap/-20 °C	Expensive	Expensive
Distribution	Easy	Easy	Feasible	Feasible	Difficult	Difficult
Gene size	Not limited	Limited	Unknown	Unknown	Limited	Limited
Glycosylation	'Correct'?	'Correct'?	Incorrect	Absent	'Correct'	'Correct'
Multimeric protein assembly (SigA)	Yes	No	No	No	No	Yes
Production cost	Low	Low	Medium	Medium	High	High
Production scale	Worldwide	Worldwide	Limited	Limited	Limited	Limited
Production vehicle	Yes	Yes	Yes	Yes	Yes	Yes
Propagation	Easy	Feasible	Easy	Easy	Hard	Feasible
Protein folding accuracy	High?	High?	Medium	Low	High	High
Protein homogeneity	High?	Medium	Medium	Low	Medium	High
Protein yield	High	Very high	High	Medium	Medium-High	High
Public perception of risk	High	High	Medium	Low	Medium	High
Safety	High	High	Unknown	Low	Medium	High
Scale-up costs	Low	Low	High**	High**	High**	High
Therapeutic risk*	Unknown	Unknown	Unknown	Yes	Yes	Yes
Time required	Medium	Low	Medium	Low	High	High

\*-Residual viral sequences, oncogenes, endotoxins; \*\*-Large, expensive fermenters. Table adapted from Fischer & Emans, (2000).

In the year 2006, Dow AgroSciences acquired the licence for first plant-produced biopharmaceutical product, a poultry vaccine developed against Newcastle disease virus. This achievement of Dow AgroSciences is a significant breakthrough for plant-produced biopharmaceuticals. The Concert™ Plant-cell-produced, bio-contained system utilizes tobacco plant cells as an alternative to an expression of foreign proteins in whole plants (Kaiser, 2008). A recombinant glucocerebrosidase (Taliglucerase alfa), an enzyme produced by Protalix Biotherapeutics which is used to treat Gaucher's disease, was the first plant cell-produced

human enzyme to be approved by the United States Food and Drug Administration which was approved in 2012 (Pfizer Press Releases, 2012).

Table 1.1, shows an array of plant species used in recent years to produce biopharmaceuticals. The choice of plant species varies and may depend on the type of pharmaceutical product, storage of the product and ease of downstream processing. Most of the early attempts to generate transgenic plants were performed in tobacco which remains a model expression system to date. Due to ease of gene transfer, well established expression systems and being a non-food crop, tobacco has an advantage over other plant systems. Furthermore, well-established tobacco suspension culture expression systems provide a better containment opportunity and also evade the undesirable effects of alkaloids which are present in tobacco whole plants. They also allow foreign protein secretion into the medium, which greatly cuts the cost of downstream processing.

### **1.5 Expression of recombinant genes in plant cells and tissues**

Expression of foreign proteins in plant cells and tissues requires a promoter to drive the expression of linked foreign cDNAs. The Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell *et al.*, 1985) is the most commonly used constitutive promoter when foreign genes in plants are expressed. Mason *et al.*, (1992) demonstrated the production of first plant-derived vaccine (hepatitis B surface antigen (HBsAg) in tobacco plants and established the use of the CaMV 35S promoter in expressing biopharmaceuticals. Mason *et al.*, (1992) prepared a construct containing CaMV 35S promoter upstream of the hepatitis B surface antigen (HBsAg) coding region trailed by the downstream nopaline synthetase (NOS) terminator. The CaMV 35S promoter sequence has been modified in recent years to enhance efficiency of expression by approximately 10-fold (He *et al.*, 2008). The *Ubiquitin* promoter used in

expression of scFv antibodies and insulin-like growth factor-1 (IGF-1) in rice cells (Panahi *et al.*, 2004; Torres *et al.*, 1999) and the hybrid (ocs)3mas promoter used in over expression of HBsAg in soybean cell cultures (Smith *et al.*, 2002) are two other constitutive promoters commonly used in foreign gene expression in plant cells, especially monocotyledons.

Another kind of promoter which has been reported extensively in recent years is the inducible promoter, where expression regulation is controlled by external stimuli such as sucrose, salts, steroids, alcohol or environmental stress factors (Huang and McDonald, 2009). As cell growth and protein production are not related to each other, use of inducible expression may be advantageous in some cases, allowing individual optimisation of both processes, to yield maximum protein levels (Sharma and Sharma, 2009). Rice  $\alpha$ -amylase 3D (*RAmy3D*) promoter is a commonly used inducible promoter where sucrose starvation promotes the high-level expression of the foreign genes in rice and has been used to produce a range of human therapeutic proteins in rice cell suspension cultures including  $\alpha$ 1-antitrypsin, serum albumin, lysozyme (Huang and McDonald, 2009), granulocyte-macrophage colony-stimulating factor (Shin *et al.*, 2003), growth hormone (Kim *et al.*, 2008a) and interleukin-12 (Shin *et al.*, 2010b).

Once transformed, transcription of the gene of interest can be affected by different factors such as, the position of gene insertion in the plant genome (Springer *et al.*, 1995) and the organization of the gene integration (Hepburn *et al.*, 1983). *Agrobacterium*-mediated transformation is associated with random integration of T-DNA into the plant genome (DeBeuckeleer *et al.*, 1981). The earliest reports show that transgene silencing by DNA methylation also plays a pivotal role in transcription (Hepburn *et al.*, 1983). Stam *et al.*, (1997) described that transfer of tandem copies of T-DNA at a single locus is frequently associated



with *Agrobacterium*-mediated transformation and it was also found that tandem inverted T-DNA repeats situated closer to the right border are also frequently associated with transgene silencing due to promoter methylation.

Choice of strong translational enhancer/regulation sequences is equally as important as the choice of promoter in increasing the yield of recombinant products. Gallie and Walbot, (1990) reported that the addition of 204-bases of the tobacco mosaic virus (TMV) 3'-untranslated region to foreign mRNA increased the gene expression a further 100-fold, increasing the stability of mRNA and enhancing the translation in a similar manner to polyadenylated mRNA. Furthermore, Gallie, (2002) demonstrated that the RNA 5'-leader sequence (omega- $\Omega$ ) of TMV functions as a translational enhancer through recruiting the ribosomal initiation factor, eIF4F, and significantly enhances translation in both eukaryotes and prokaryotes. Another reported translational regulator used in plant expression constructs is the 5' UTR of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO), an enzyme which catalyzes the primary stage in carbon fixation in plants (Mazur and Chui, 1985). Patel *et al.*, (2004) and (2006) demonstrated that the untranslated region from C4 *Amaranthus* and *Flaveria bidentis* RUBISCO small subunit1 mRNAs causes translational enhancement which led to increased expression of downstream foreign genes.

Although expression of foreign genes in plants can be enhanced using various promoter sequences such as 35S CaMV, 5' and 3'-untranslated regions, in order to achieve commercial success of plant based production systems, improved strategies employed to accumulate foreign proteins are essential (Doran, 2006). Foreign proteins produced in plant systems undergo continuous degradation due to processes such as proteolysis and irreversible surface adsorption (Doran, 2006). Hence, it is of prime importance to employ measures in order to

reduce foreign protein degradation and loss. One such technique to preserve foreign proteins from proteolytic degradation is to target and anchor the recombinant proteins in the endoplasmic reticulum (ER) (Conrad and Fiedler, 1998). The plant cell ER contains less endogenous protease-activity, providing a protective environment for foreign proteins (Nuttall *et al.*, 2002). The incorporation of C-terminal motifs, such as KDEL and HDEL into recombinant proteins directed to the ER causes the retention of proteins within the ER and in turn prevents the protein from proteolytic degradation in the cytoplasm. Hellwig *et al.*, (2004) described that foreign protein retention in the ER resulted in 10-100 times greater yields than those obtained by secretion. Petruccelli *et al.*, (2006) also demonstrated the accumulation of monoclonal antibodies increased by 4-11-fold when retained in the ER as a fusion with the KDEL peptide.

Direction of recombinant proteins to the apoplast through sub-cellular targeting is another approach used to protect proteins from proteolytic degradation in the cytosol and also eject such proteins into the medium of cell suspensions. The apoplast is the non-living part of plant tissue, located out-side the cell membrane and composed of the cell walls, the intercellular spaces, and the lumen of dead structures such as xylem cells. Cell wall proteins which contain appropriate *N*-terminal signal peptide sequences undergo secretion into the apoplastic space. Signal sequences from proteins such as tobacco pathogenesis related protein (PR1a), when used in combination with recombinant proteins can target foreign proteins into the apoplast (Pen *et al.*, 1993a). For instance the incorporation of the PR1a signal peptide at the *N*-terminus of the thermostable eubacterium endo-1, 4- $\beta$ -D-glucanase was found to increase the yield by up to 26% of TSP in *Arabidopsis* leaves (Ziegler *et al.*, 2000). Another *N*-terminal apoplast directing signal is found in plant extensins which are a well-characterized hydroxyproline-rich glycoprotein family, forming a major component of the cell wall

(Showalter, 1993). Like the PR1a signal peptide, incorporation of an extensin signal peptide at the *N*-terminus of recombinant proteins directs secretion into the apoplast. Francisco *et al.*, (1997) expressed bryodin 1, a potent ribosome-inactivating protein, as a soluble recombinant protein using the extensin signal peptide to secrete the protein into the culture medium. The purified bryodin 1 yield was 30 mg L<sup>-1</sup> of culture and showed an identical inhibitory activity to the native protein isolated from the roots of *Bryonia dioica*. Apoplast direction of recombinant therapeutic proteins has also been reported by Xu *et al.*, (2007), where, an extensin signal peptide was used in conjunction with a *C*-terminal hydroxyproline (Hyp)-rich peptide to produce a hydroxyproline (Hyp)-rich glycoprotein. Chimeric expression of such glycoproteins (HRGPs) where a short *C*-terminal Hyp-Ser sequence directs extensive *O*-glycosylation, leads to addition of arabinooligosaccharides. Recombinant interferon- $\alpha$ 2-arabinogalactan peptides (IFN $\alpha$ 2-AGPs) targeted for secretion in this way increased yields by 350–1400-fold, compared to the non-glycosylated IFN $\alpha$ 2 control. Finally, the signal peptide, AP24, from osmotin was used to direct recombinant proteins into the apoplast causing a 10<sup>4</sup>-fold increase in the yield of recombinant epidermal growth factor in tobacco plants (Wirth *et al.*, 2004).

### **Glycosylation of the recombinant proteins**

During protein trafficking in plant cells, proteins undergo several post-translational modifications such as glycosylation, phosphorylation, methylation, ADP-ribosylation, oxidation and glycation (Gomrod and Faye, 2004). Covalently attaching an oligosaccharide side chain to a protein is known as glycosylation. Carbohydrates moieties attached to proteins are of two types. Carbohydrates linked to the amide moiety of asparagine (Asn) are known as *N*-glycans. Whereas carbohydrates linked to the hydroxyl group of threonine (Thr), serine (Ser) or hydroxyproline (Hyp) are known as *O*-glycans. Apart from nucleocytoplasmic *O*-

GlcAcylation, both *N*- and *O*-glycosylation occur in the secretory pathway (Gomrod *et al.*, 2010). Biological activity of many therapeutic glycoproteins depends on the glycosylation structure of the protein. Hence, it is of extreme importance to have production systems which exert complex glycosylation patterns. The use of plant production system over production systems such as bacterial or yeast is beneficial due to the plant's capability to exert complex glycosylation patterns similar to that found in human cells. Although plant expression systems exert complex glycosylation, inability to produce exact human-like glycosylation patterns on recombinant proteins plays a major limitation in using plant-made pharmaceuticals in human therapy especially for parenteral administration (Faye *et al.*, 2005). This is because the existence of immunogenic glycoepitopes in complex plant *N*-glycans has been found to be immunogenic to mammals (Gomrod *et al.*, 2005). However, strategies such as retaining recombinant proteins in the ER, inhibition of host plant glycosyltransferases and expression of heterologous glycosyltransferases for the addition of mammalian-like glycans, have been developed in recent years to re-design the protein *N*-glycan structures in plant expression systems (Saint-Jore-Dupas *et al.*, 2007).

As well as *N*-glycans, plant protein *O*-glycans are also structurally different from human glycol-proteins. The most abundant *O*-glycans in human cells are mucin-type, *O*-GlcNAc and a xylose residue linked to Ser and Thr residues. Some of these may be extremely important in the physicochemical properties of some proteins. The most abundant *O*-glycosylated proteins present in plants are the extensins and various other arabinogalactan proteins. The extensins are *O*-glycosylated with galactose residues linked to most of the Serine (Ser) residues and arabinosyl residues linked to hydroxyproline (Hyp) residues (Showalter, 2001). The AGPs are the largest and the most extensively glycosylated proteins where, Hyp residues are linked with very large arabinogalactan glycomodules or smaller

arabinooligosaccharides (Qi *et al.*, 1991). The use of extensive plant *O*-glycosylation to increase the stability of administered recombinant proteins *in vivo* has been investigated recently by Xu *et al.*, (2007). Human INF $\alpha$ 2 fused to an AGP *O*-glycomodule had an increased serum half-life in mice of 9 hours. Furthermore, these INF $\alpha$ 2 fusions are non-immunogenic when administered intravenously in mice (Xu *et al.*, 2007). Immuno-tolerance of AGPs fused to recombinant proteins was also recently confirmed with recombinant human growth hormone (hGH), which showed no adverse immune reaction when injected into mice (Xu *et al.*, 2010).

## 1.6 Suspension cultures

Production of recombinant proteins of industrial and pharmaceutical importance in plant cell cultures is common because the cell cultures can be scaled up in controlled environments such as fermenters which provide containment. Developed in the 1950's, plant cell suspension culture technology has been used for producing secondary metabolites such as digoxin, paclitaxel, and artemisinin (Georgiev *et al.*, 2009; Huang and McDonald, 2009). With the latest advances in modern plant molecular biology, cell culture systems are used as a feasible alternative production system for therapeutic proteins. Human serum albumin (Sijmons *et al.*, 1990) and chloramphenicol acetyltransferase (Hogue *et al.*, 1990) were the first therapeutic proteins to be produced in plant cell suspension cultures. The first plant cell suspension culture produced therapeutic protein to be approved by the USDA was the poultry vaccine manufactured in transgenic tobacco cell cultures by Dow AgroSciences, against Newcastle disease virus (NDV) (Kaiser, 2008). Although, many recombinant proteins have been produced in plant cell suspension culture systems to date, tobacco BY-2 cell suspensions are the most common in use. Owing to a rapid doubling time, well established genetic transformation, low proteolytic activity, low production of secondary metabolites, the

capability to carryout post-translational modifications and reduced post-translational silencing effects, expression of therapeutic proteins in tobacco BY-2 plant cell cultures possess prominent advantages over whole plants (Lee *et al.*, 2000; Su and Lee, 2007). Furthermore, purification of recombinant proteins from suspension cultures is relatively straightforward when targeted for secretion (Doran, 2000): where secreted proteins can be isolated from the suspension medium by filtration, precipitation and chromatographic techniques (Fischer *et al.*, 1999a)

Efficient downstream processing of recombinant proteins from suspension cultures is extremely vital in order to increase the yield of recombinant proteins from plants. Hence, it is pivotal to adopt techniques to extract entire recombinant protein products preserved from any degradation. Once cells lyse to release intracellular products, the proteases in plant cells begin to degrade the proteins. Hence, choice of a suitable buffer and growth medium containing either protein stabilizing agents or protease inhibitors is imperative to enhance yields. Incorporation of  $0.75 \text{ g L}^{-1}$  polyvinylpyrrolidone (PVP)(MW 360,000), a protein stabilizing agent, into the culture medium was found to enhance the yield of extra-cellular heavy chain monoclonal antibodies by 35-fold due to the protein stabilizing effect of PVP (LaCount *et al.*, 1997). Further to this, Lee *et al.*, (2002) tested two other stabilizing polymers (polyethylene glycol and gelatine), as well as PVP in order to stabilize the secreted protein, hGM-CSF, in the culture medium. Gelatine was found to be the most effective stabilizing agent among three polymers. Addition of 5 % gelatine into the culture medium increased the yield by 4.6-fold over a corresponding control (Lee *et al.*, 2002). However, it was further revealed that the addition of gelatine into the culture medium seemed to be negatively affecting cell growth in suspension after day 5 and in turn reduced overall production of hGM-CSF (Lee *et al.*, 2002).

## 1.7 Plant produced human enzymes for enzyme replacement therapy

Like ADA deficiency, Gaucher's disease (GD) is another autosomal recessive inherited disorder which can be treated by ERT. Gaucher's disease is a condition caused by glucocerebrosidase (GCD) enzyme deficiency which leads to lysosomal dysfunction. Gaucher's disease is of three types. Type I (non-neuropathic type), Type II (acute infantile neuropathic form) and Type III (the chronic neuropathic form). The most common of the three, GD type 1 can be treated by ERT owing to the lack of a central nervous affect (Pastores *et al.*, 2011). The concept of treating GD with ERT was first proposed in the 1960s and with the advent of Alglucerase extracted from human placental tissue (Genzyme) in 1991, ERT for GD became a reality. A recombinant version of glucocerebrosidase, Imiglucerase (Cerezyme), produced in Chinese hamster ovary (CHO) cells was soon after approved by the FDA for patient use in 1994 and it was found that ERT with Cerezyme significantly improved the condition of the patients (Aerts *et al.*, 2010). In 2010 another recombinant version of GCD, Velaglucerase Alfa (Vpriv), produced in cell lines of human fibroblasts through gene-activation technology, was approved by the FDA. A recombinant GCD produced in plant cells is also in production as Shaaltiel *et al.*, (2007) have successfully produced a recombinant human GCD (prGCD) with terminal mannose glycans, in carrot cell suspensions, directed to vacuoles with the use of C-terminal plant-specific sorting signals. Biological uptake of the GCD in humans' suffering from Gaucher's disease, via macrophage mannose receptors is assisted by mannose residues. Hence, the *in vitro* exposure of mannose residues, as in the case for Cerezyme, is not required. Following the scrutiny of clinical, preclinical and phase I trials (Aviezer *et al.*, 2009) prGCD (Taliglucerase alfa) has been approved by the United States Food and Drug Administration (FDA) in 2012 and is the first plant-derived human enzyme product to be approved by FDA (Pfizer Press Releases, 2012).

In another attempt to produce a recombinant enzyme for ERT, De Marchis *et al.*, (2011) have demonstrated the production of recombinant  $\alpha$ -mannosidase (LAMAN) in transgenic tobacco plants for the treatment of a rare lysosomal storage disorder, alpha-mannosidosis: a congenital autosomal recessive disorder that leads to mental and physical deterioration due to accumulation of oligosaccharides in lysosomes. The cause of the disease is a deficiency of the enzyme  $\alpha$ -mannosidase which cleaves  $\alpha$ -linked mannose residues from glycoproteins preventing the accumulation of oligosaccharides in normal individuals.

Fabry's disease is another rare autosomal recessive lysosomal storage disease caused by a deficiency in the lysosomal enzyme  $\alpha$ -galactosidase A (GAL-A). A defect in this enzyme results in accumulation of glycosphingolipids substrates, which fatally affect heart, brain and kidneys and in due course bring about organ failure and death (Breunig and Wanner, 2003). ERT with a once in two weeks administration of recombinant human  $\alpha$ -galactosidase A (rh-GAL A) produced in cultured human fibroblast (Agalsidase  $\alpha$ ) and Chinese hamster ovary (CHO) cell lines (Agalsidase  $\beta$ ) has shown significant improvement in the symptoms of this disease (Breunig and Wanner, 2003). As the production of recombinant  $\alpha$ -galactosidase A in cultured human fibroblast and CHO cell lines are extremely costly, efforts are underway to produce human  $\alpha$ -galactosidase A in plant expression systems (Garger *et al.*, 2005).

The production system for most of these enzymes is based on well-established protocols using Chinese hamster ovary (CHO) cell lines. Alternative production options based on plant cell systems, however, came to forefront after a viral infection disrupted the CHO-based production of glucocerebrosidase at Genzyme (Rybicki, 2010). Moreover the cost of recombinant enzyme production based on CHO cell lines is extremely high which questions the widespread affordability of such a production system. Hence, like GCD, research into the,



production of many other human enzymes used in ERT are currently underway using plant cell based systems (table 1.3).

**Table 1.3: Plant produced human enzymes.**

Disease	Enzyme	Current system	Plants	reference
Gaucher's disease	Human Glucocerebrosidase	Carrot cell suspension	Carrot cell suspension	Shaalitiel <i>et al.</i> , (2007)
$\alpha$ -mannosidosis	Human $\alpha$ -mannosidase	Chinese Hamster Ovary	Tobacco	De Marchis <i>et al.</i> , (2011); Fogh <i>et al.</i> , (2003)
Fabry's disease	Human $\alpha$ -galactosidase A	Chinese Hamster Ovary and human fibroblast	Carrot cell suspension	www.protalix.com
Maroteaux-Lamy syndrome	Human lysosomal enzyme N-acetylgalactosamine-4-sulfatase	Chinese Hamster Ovary	Tobacco	Garger <i>et al.</i> , (2005)
Mucopolysaccharidosis-I	Human $\alpha$ -iduronidase	Chinese Hamster Ovary	Tobacco BY-2 cell suspension	Fu <i>et al.</i> , (2009)
Pompe disease	$\alpha$ -glucosidase	Chinese Hamster Ovary	Tobacco	Garger <i>et al.</i> , (2005)
Organophosphate poisoning (Nerve agents)	Human Acetylcholinesterase-R	Carrot cell suspension	<i>Nicotiana benthamiana</i>	Woods <i>et al.</i> , (2008)
			Carrot cell suspension	www.protalix.com
Diabetes	Human insulin	Bacteria and yeast	<i>Arabidopsis thaliana</i>	Nykiforuk <i>et al.</i> , (2006)
			Safflower	Markley <i>et al.</i> , (2006)

## 1.8 Production systems for ADA

In an attempt to develop a non-antibiotic selection system for transgenic plants and calli, Petolino *et al.*, (1999) demonstrated the expression of a murine ADA cDNA in transgenic maize under the control of the maize *ubi-1* promoter. Transformation was executed using a micro-particle bombardment method in embryonic maize calli along with plasmids comprising, *gusA* and *bar* gene. Transformants expressed  $\beta$ - glucuronidase (GUS) and accumulated a 41 kDa protein which was immunoprecipitated with ADA-specific polyclonal antibodies and

ADA enzyme activities were measured in transgenic calli and regenerated plants (Petolino *et al.*, 1999). Use of ADA as a selection marker for selecting plant transformants has also been investigated in hot pepper plants (Lim *et al.*, 1999). Recombinant ADA has also been produced in insects, where Medin *et al.*, (1990) successfully expressed a human ADA cDNA in baculovirus-infected *Spodoptera frugiperda* and *Trichoplusia ni* insect larvae and obtained ADA enzyme levels up to 70 U mg<sup>-1</sup> TSP. There are also several other reports of the production of human ADA in *E.coli* for the purpose of investigating folding mutations of the enzyme (Santisteban *et al.*, 2003).

## 1.9 Aims of study

The overall aim of the work presented in this thesis was to effectively express a human ADA cDNA in whole tobacco plants and cell suspensions under the control of various cytosolic and sub-cellular targeting signals. Constructs for cytosolic expression, containing different translational regulation sequences, would be investigated for their ability to increase levels of ADA enzyme activity in transformants. These ADA activities would be compared with activities measured in transformants containing corresponding sub-cellular targeting constructs.

# **Chapter 2**

## **Materials and methods**

## 2.1 Materials

### 2.1.1 Bacterial strains and plasmids

#### Bacterial strains

*Escherichia coli*: DH5 $\alpha$ : F<sup>-</sup> endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG  $\Phi$ 80dlacZ $\Delta$ M15  $\Delta$ (lacZYA-argF)U169, hsdR17(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>),  $\lambda$ - (Hanahan, 1985)

*Agrobacterium tumefaciens*: AGL1: AGL0 (C58 pTiBo542) recA::bla, T-region deleted Mop (+) Cb(R) [AGL0 is an EHA101 with the T-region deleted, which also deletes the aph gene] (Lazo *et al.*, 1991).

#### Plasmids

##### a. pCMV6-ADA

The pCMV6-ADA plasmid was used as a source of the human ADA cDNA (1.5 kbp) and was purchased from Origene<sup>TM</sup> (Andersson *et al.*, 1989). The full-length ADA cDNA fragment is unidirectionally inserted downstream from a eukaryotic transcriptional promoter in the pCMV6 vector between *Eco*RI and *Sal*I.

##### b. pUBS

The pUBS plasmid is a small, high-copy number *E. coli* cloning vector containing a pUC18 plasmid backbone and Bluescript plasmid KS+ multiple cloning site (Bringloe *et al.*, 1995).

## c. pJIT

The pJIT vector carries the 35S cauliflower mosaic virus (CaMV) promoter and terminator along with a small MCS (figure 2.1) (Hellens *et al.*, 2000).

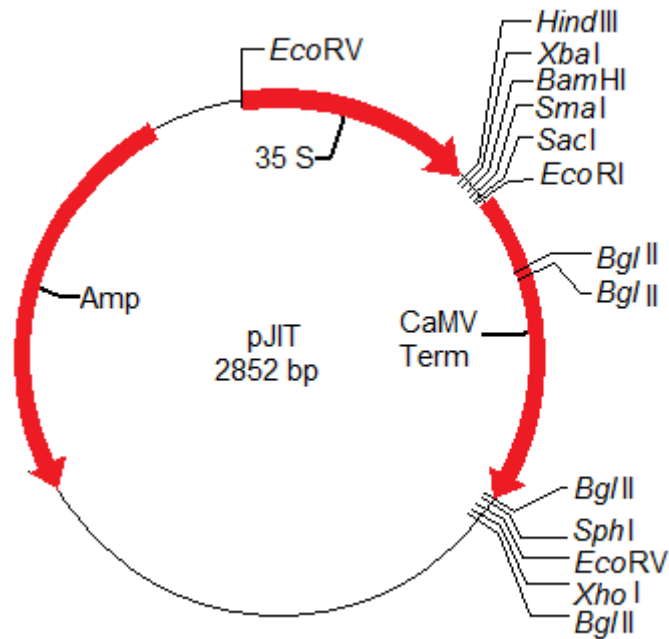
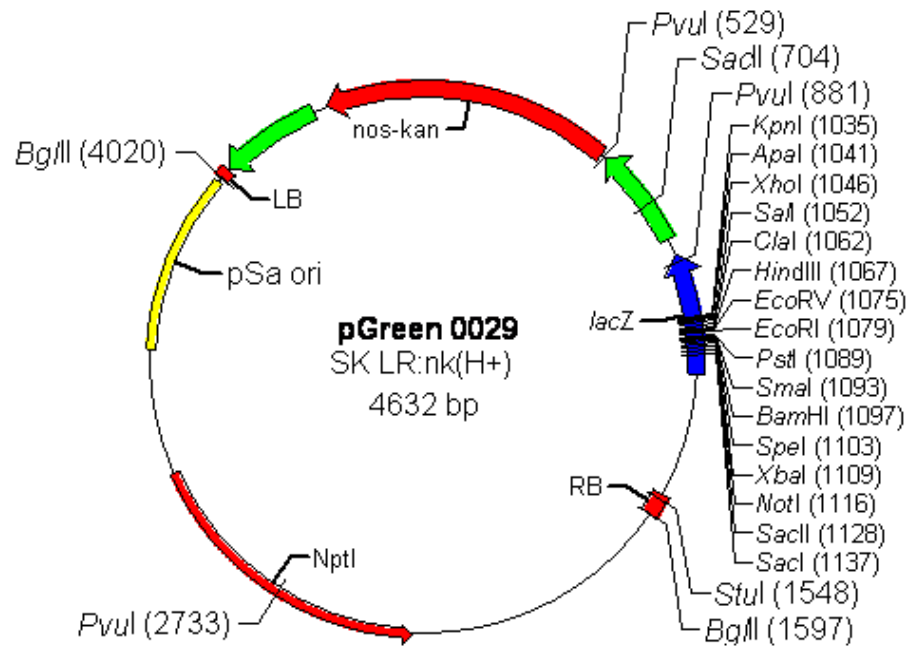


Figure 2.1: A restriction map of pJIT ([www.pgreen.com](http://www.pgreen.com)).

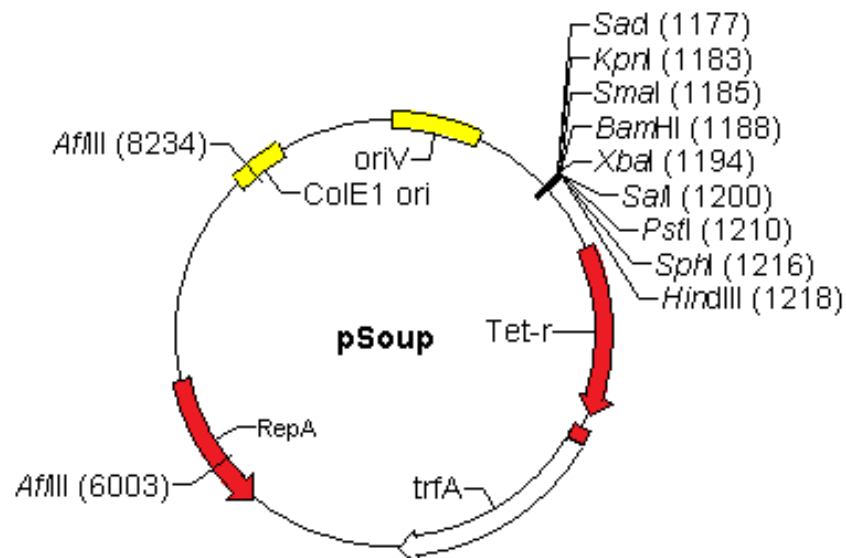
## d. pGreenI0029 and pSoup

The pGreenI0029 and pSoup binary vectors are used for *Agrobacterium*-mediated transformation of plants. The *NptI* gene of pGreenI0029 conveys resistance to kanamycin, and can be used as a selection marker for plasmid transformation. The pGreenI0029 plasmid contains a MCS incorporated in *lacZ'* gene which allows blue/white selection. Constructs can be subcloned from pJIT using the *EcoRV* site.

The pSoup vector contains a virulence origin gene (*oriV*) which acts in *trans* for T-DNA incorporation into the plant genome (Hellens *et al.*, 2000).



(a)



(b)

Figure 2.2: Restriction enzyme maps of Plant binary vectors (a): pGreenI0029 plasmid, (b): pSoup plasmid (Hellens *et al.*, 2000).

### 2.1.2 Plant material

#### BY-2 callus

*Nicotiana tabacum* BY-2 calli obtained from Syngenta, UK were maintained on MS agar medium at 25 °C and subcultured monthly by excising a small part of calli and transferring onto fresh sterile MS Agar medium for callus (section 2.1.4).

#### Plant material

Tobacco plants (*Nicotiana tabacum*: Samsun Var:) grown *in vitro* were initially obtained from Syngenta, UK. They were propagated in an incubator at 25 °C with supplementary artificial lighting providing photosynthetically-active irradiance of 150  $\mu\text{mol photons m}^{-2} \text{S}^{-1}$  on a 24 hour photoperiod and sub-cultured monthly by excision of inter-nodal stems with an auxiliary bud and replanting in sterile MS agar medium (section 2.1.4).

#### BY-2 cell suspension

A small part (2 g) of *Nicotiana tabacum* BY-2 calli initially obtained from Syngenta, UK was cut into small 2 mm<sup>2</sup> pieces in a sterile Petri dish and transferred into 20 ml of fresh sterile culture medium in a 100 ml conical flask, containing MS medium for cell suspension (section 2.1.4). The cultures were shaken at 120 rpm in the dark at 25 °C for 7 days after which the tobacco BY-2 starter suspension was transferred into 100 ml of fresh sterile MS-culture medium in a 250 ml conical flask (section 2.1.4). Tobacco BY-2 cell suspensions were propagated weekly by inoculating 100 ml of modified MS medium with 20 ml of a week-old suspension culture.

### 2.1.3 Sources of reagents and enzymes

Restriction endonucleases were purchased from Sigma Aldrich (UK), Roche (UK) or New England Biolabs (UK). Bradford reagent, Isopropylthio-(3-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-(3-D-galactoside (X-gal), Polyvinylpyrrolidone, MS basal medium, total mRNA isolation kit, ampicillin, kanamycin, carbenicillin, tetracycline, cefotaxime, Rifampicin, acetosyringone, RNase A, proteinase K, *O*-phenanthroline and rabbit antiserum to ADA were purchased from Sigma Aldrich (UK). Calf intestinal alkaline phosphatase, T4 DNA ligase, restriction endonucleases, DNA molecular weight markers and DIG nucleic acid hybridization kit were obtained from Roche Diagnostics. Primers used in PCR and DNA sequence analysis were obtained from Eurofin MWG operon, Germany. Plasmid miniprep, gel-extraction and total plant DNA isolation kit were obtained from Qiagen, UK. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG was obtained from Novus biological, UK. Adenosine deaminase assay kit was obtained from Bioquant, USA. Sodium hypochlorite solution was obtained from Unilever, UK. Stock reagents, solutions and media were sterilised by autoclaving under one atmospheric pressure at 121 °C for 15 min. Materials unsuitable for autoclaving were sterilised where necessary by passage through Millipore disposable filters (0.4 µm). Most reagents were AnalaR grade and obtained from BDH unless otherwise indicated.

### 2.1.4 Solutions and media not described in the text.

#### TE buffer

10 mM Tris-HCl pH 8.0, 1 mM EDTA.Na<sub>2</sub>

#### Solution I

50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA.Na<sub>2</sub>, 100 µg ml<sup>-1</sup> RNase A



**Solution II**

0.2 M NaOH, 1 % (w/v) SDS

**Solution III**

1.32 M potassium acetate pH 5.0

**1X TAE buffer**

0.04 M Tris (BDH AnalaR), 1.43 % (v/v) Glacial Acetic Acid (BDH AnalaR), 0.0125 M EDTA (BDH AnalaR)

**20x SSC**

3.0 M NaCl (Oxoid), 0.3 M Sodium Citrate (Sigma)

**10x SSC**

1.5 M NaCl (Oxoid), 0.15 M Sodium Citrate (Sigma)

**LB broth**

1 % (w/v) Bacto Tryptone (Oxoid), 1 % (w/v) NaCl (Oxoid), 0.5 % (w/v) yeast extract (Oxoid) to pH 7.5 with NaOH

**LB plates**

LB broth, 1.5 % agar (Oxoid)

**MS broth**

0.44 % (w/v) Murashige and Skoog basal medium (Sigma), 30 % (w/v) Sucrose (Oxoid)

**MS for BY-2 cell suspension**

MS broth, 0.02 % (w/v) 2,4-Dichlorophenoxy acetic acid (Sigma)

**MS Agar for Callus**

MS broth, 0.02 % (w/v) 2, 4-Dichlorophenoxy acetic acid (Sigma), 0.8 % (w/v) Plant agar (Sigma)

**MS Agar for plants**

MS broth, 0.8 % (w/v) Plant agar (Sigma)

**NBM Agar**

MS broth, 0.00001 % (w/v)  $\alpha$ -Naphthaleneacetic acid (Sigma), 0.0001 % 6- Benzylaminopurine (Sigma) 0.8 % (w/v) Plant agar (Sigma)

## 2.2 General DNA techniques

### 2.2.1 Small-scale isolation of plasmid DNA (minipreps)

The method was based on that of Birnboim and Doly (1979). A culture of *E. coli* containing the required plasmid was grown overnight at 37 °C in 10 ml of LB broth containing appropriate antibiotic for the plasmid (100 µg ml<sup>-1</sup> ampicillin or 100 µg ml<sup>-1</sup> kanamycin or 25 µg ml<sup>-1</sup> tetracycline). Aliquots of bacteria (1.5 ml) were pelleted in microfuge tubes by centrifugation for 5 min and re-suspended in 200 µl of solution I by vortexing. Solution II (200 µl) was added and the tube gently inverted several times at room temperature. After 5 min, 200 µl of solution III was added and the tubes were left on ice for 5 min to allow precipitation of *E. coli* chromosomal DNA and denatured protein. The insoluble material was pelleted by centrifugation for 10 min in a microfuge. Protein contaminants dissolved in the supernatant were removed by successive extractions with phenol and chloroform as follows. The supernatant containing plasmids from each tube was transferred into a clean microfuge tube and 0.5 ml phenol/chloroform (1:1 v/v) solution was added. The two layers were mixed vigorously using a vortex for 1 min. The layers were separated by centrifugation in a microfuge for 5 min at top speed. The upper aqueous phase was transferred into clean microfuge tube without disturbing the white precipitate at the interface and the extraction was repeated with fresh phenol/chloroform (1:1 v/v). The aqueous layer was again transferred into a new microfuge tube and an equal volume of chloroform was added to remove any residual phenol. The tube was vortexed and layers were separated by centrifugation in a microfuge for 1 min at top speed. The aqueous phase (about 0.4 ml) was transferred into a clean microfuge tube and DNA was precipitated by addition of one tenth volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of ice-cold absolute ethanol. The contents were mixed thoroughly and incubated on ice for 15 min. The ethanol-precipitated DNA was pelleted in a microfuge at top speed for 10 min and the supernatant discarded and the pellet washed carefully with 0.5 ml

70 % (v/v) ethanol to remove excess sodium acetate. The supernatant was discarded carefully and the DNA pellet air-dried for 10 min to remove the last residue of ethanol. The pellet was finally re-suspended in 50  $\mu$ l of TE buffer pH 8.0.

### **2.2.2 Small-scale isolation of plasmid DNA (minipreps) using Qiagen QIAprep Kit**

This alkaline lysis miniprep method is partially based on that of Birnboim and Doly (1979) as described in section 2.2.1. A culture of *E. coli* containing the required plasmid was grown overnight at 37 °C in 10 ml of LB broth containing appropriate antibiotic for the plasmid (100  $\mu$ g ml<sup>-1</sup> ampicillin or 100  $\mu$ g ml<sup>-1</sup> kanamycin or 20  $\mu$ g ml<sup>-1</sup> tetracycline). The plasmid extractions were carried using the Qiagen QIAprep Kit by following the manufacturers' protocol.

### **2.2.3 Large-scale isolation of plasmid DNA using QIAGEN Maxi Kit**

A culture of *E.coli* containing the required plasmid was grown overnight in 100 ml of LB broth, containing the appropriate antibiotic for the plasmid (100  $\mu$ g ml<sup>-1</sup> ampicillin or 100  $\mu$ g ml<sup>-1</sup> kanamycin or 20  $\mu$ g ml<sup>-1</sup> tetracycline), in a 250 ml conical flask. The culture was transferred to a 250 ml polypropylene bottle followed by centrifugation in a Sorvall Centrifuge (Thermo Scientific, rotor F14S) for 15 min at 6000g at 4 °C. The plasmid extractions were carried using the Qiagen QIAprep Kit by following the manufacturers' protocol.

### 2.2.4 Isolation of total DNA from plant material

Tobacco leaf or callus tissue (2 g) was ground to a fine powder in a mortar and pestle with liquid nitrogen. The ground, frozen plant tissue was added into 4 ml of extraction buffer (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM EDTA.Na<sub>2</sub> pH 8.0) containing 10 µl of 20 mg ml<sup>-1</sup> proteinase K and 40 µl of 1 M *O*-phenanthroline. The mixture was shaken gently after adding 200 µl of 10 % (w/v) SDS prior to 10 min incubation at 60 °C with occasional mixing. In order to precipitate protein and RNA, 1.35 ml of 5 M Potassium acetate was added and tubes incubated on ice for 30 min. The precipitate was pelleted by centrifugation at 3000g in a Sorvall centrifuge (Thermo Scientific, rotor SA-300) for 15 min. The supernatant was gently extracted twice by addition of equal volume of phenol/chloroform followed by centrifugation at 6000g. The DNA was precipitated by adding 0.54 volumes of isopropanol followed by centrifugation. The DNA pellet was re-suspended in 1.6 ml of 0.5M NaCl. To purify the DNA further, 400 µl of 50 % (w/v) PEG-6000 was added and the DNA left to precipitate on ice for 2 hours. Finally, DNA was pelleted by centrifugation at 6000g and re-suspended in 200-400 µl of TE buffer.

### 2.2.5 Isolation of total DNA from plant material using Qiagen DNeasy Plant Mini Kit

Tobacco leaf or callus tissue (100 mg) was ground to a fine powder in a microfuge tube using a plastic microfuge grinder pestle and liquid nitrogen. The total DNA extractions were carried out using the Qiagen DNeasy Plant Mini Kit by following the manufacturers' protocol.

### **2.2.6 Digestion of plasmid and PCR amplified DNA with restriction endonucleases**

For analytical purposes, digestions were carried out with 0.1 µg of DNA and 1 to 10 units of restriction endonuclease in 20 µl reaction volumes for an appropriate duration (usually 1-2 hours) in a reaction buffer and at a temperature recommended by the manufacturer. For preparative scale, digestions were carried out with 2-20 µg of DNA in larger volumes using greater amounts of restriction endonuclease as necessary but taking care not to exceed a glycerol concentration of 5%.

### **2.2.7 Agarose gel electrophoresis of DNA**

Agarose gel electrophoresis was performed to separate double-stranded DNA fragments using 0.7-2 % (w/v) agarose gels depending on the size of the fragment to be analysed. Agarose gels were prepared in perspex trays from agarose in 1 x TAE buffer containing 0.04 M Tris-acetate and 0.00125 M EDTA with 0.005% SafeView DNA stain and were placed horizontally in SCIE-PLAS gel electrophoresis tanks, containing enough TAE buffer with 0.005 % SafeView DNA stain to submerge the gel. Prior to loading, DNA samples (0.1-10 µg of DNA) were mixed with one tenth volume of agarose loading buffer containing 50 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue in TE buffer. Electrophoresis was carried out under a constant voltage (100 V) for 1-3 hours. Using a suitable standard (Roche DNA molecular weight marker III or IV) fragment sizes were determined after visualising with a UV-transilluminator and taking a digital photograph with a Kodak Gel-Logic Imaging System.

### **2.2.8 Recovery of DNA from agarose gels using Qiagen QIAquick Gel Extraction Kit**

The fragment to be isolated was visualized under 366 nm UV light and excised with a sterile, clean sharp scalpel blade from a 1-2 % Safeview-stained agarose gel. The DNA was purified using Qiagen QIAquick Gel Extraction Kit by following the manufacturers' protocol.

### 2.2.9 Ligation of DNA restriction fragments with vector DNA

Vector DNA (1 to 5 µg) was digested with suitable restriction endonuclease(s) in a total volume of 50 µl to generate a linearised vector which contains compatible ends with the restriction fragments to be ligated. After an appropriate digestion time (2-3 hours), the digestion was incubated at 75 °C for 10 min in order to inactivate restriction enzymes. If the plasmid cleavage was with a single enzyme, this was followed by addition of 20 units of calf intestinal alkaline phosphatase followed by incubation at 37 °C for 1 hour. Cut, dephosphorylated fragments of vector DNA were isolated from agarose gels by the QIAquick gel extraction method (section 2.2.8). DNA fragment inserts and cut, phosphatase-treated vectors (100-200 ng) were mixed 3:1 molar ratio for sticky ends and 7:1 molar ratio for blunt ends followed by addition of 3 µl of 10x Ligase buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 10 mM Dithiothreitol, pH 7.5) and 10-100 units of T4 DNA ligase in 30 µl total reaction volume. The ligation mixture was incubated at 16 °C overnight. Control ligations were also carried out with cut, dephosphorylated vector without the insert DNA. After ligation, 5 µl of ligation mixture was used to transform competent bacterial cells with the ligated product.

### 2.2.10 Transformation of *E. coli* with plasmids

*E. coli* (DH5α) bacteria were transformed with plasmids using a modification of the method of Cohen *et al.*, (1972). LB broth (100 ml) in a 250 ml conical flask was inoculated using 0.5 ml of an *E. coli* overnight culture and incubated at 37 °C for 2-3 hours with continuous shaking at 200 rpm in an orbital shaker to obtain a culture of *E. coli* with an A<sub>600</sub> of approximately 0.5. The cells were then cooled on ice followed by centrifugation for 5 min in a Hitachi Himac-CP at 2000g. The supernatant was discarded and the pellet re-suspended in 10ml of ice-cold 100 mM MgCl<sub>2</sub>. After 15 min on ice the cell suspension was subjected to

centrifugation again and the cells re-suspended in 5 ml of ice-cold 100 mM  $\text{CaCl}_2$ . Cell suspensions were divided into 50  $\mu\text{l}$  aliquots in sterile microfuge tubes and plasmid DNA (10-20 ng) or ligation mixture (5  $\mu\text{l}$ , section 2.2.9) were incorporated and tubes incubated on ice for 30 min. A heat shock was performed by placing the cells/plasmid mixture in a 42 °C water bath for exactly 2 min. The tubes were placed on ice immediately for 2 min and 1 ml of LB broth added followed by incubation at 37 °C for 1 hour to allow expression of the plasmid-encoded antibiotic-resistance gene. The cells were pelleted by centrifugation in a microfuge for 1 min, re-suspended in 100  $\mu\text{l}$  LB broth and spread on an LB plate containing a suitable antibiotic (100  $\mu\text{g ml}^{-1}$  ampicillin or 100  $\mu\text{g ml}^{-1}$  kanamycin), X-gal (100  $\mu\text{g ml}^{-1}$ ) and IPTG (50  $\mu\text{g ml}^{-1}$ ) as appropriate. Plates were incubated overnight at 37 °C.

### **2.2.11 Amplification of DNA**

DNA was amplified by the polymerase chain reaction (PCR) where each 50  $\mu\text{l}$  reaction mix contained 10 pmol forward primers, 10 pmol reverse primers, 10-500 ng of template DNA, PCR grade water and 25  $\mu\text{l}$  of High fidelity PCR master mix (Roche). Solutions added were mixed thoroughly prior to incubation in the PCR thermal cycler (Primus MWG Biotech) at a denaturation temperature of 94 °C for 60 s, annealing temperature of 56 °C for 30 s and extension temperature of 72 °C for 90 s for a total number of 30 cycles with a final extension time of 10 min.

### **2.2.12 DNA hybridization techniques**

#### **2.2.12.1 Preparation of Southern blots**

Total DNA (10  $\mu\text{g}$ ) was digested using appropriate restriction endonucleases and incubated overnight at 37 °C in a volume of 400  $\mu\text{l}$  (section 2.2.6). DNA was precipitated with 2.5



volumes of ice-cold ethanol and 0.4 volumes of 5 M sodium acetate pH 5.5 followed by centrifugation for 10 min at top speed in a microfuge. Precipitated DNA was re-suspended in 20 µl TE buffer and subjected to electrophoresis in a 0.7 % agarose gel containing 0.005 % SafeView DNA stain, for 16 hours at a constant 80 V. After electrophoresis a digital photographic record of the gel was taken on a UV transilluminator. Before DNA transfer the gel was soaked in 500 ml of 0.25 M HCl for 15 min, 500 ml of 1.5 M NaCl, 0.5 M NaOH for 30 min and 500 ml of 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2 for 30 min.

#### **2.2.12.2 Blotting**

Two sheets of 3MM Whatman paper at about 15 mm larger than the gel to be blotted were folded over a rectangular glass plate and the glass plate placed on two dishes containing 20x SSC so that the end of 3MM paper was immersed in the liquid from either side. The agarose gel to be blotted was placed on top of the 3MM paper and a sheet of Saran wrap with a hole cut to the size of the gel placed around the gel to allow the flow of liquid through the gel only. A sheet of Hybond-N+ (Amersham) 1 mm larger than the gel was placed on top of the gel taking care to remove any trapped air bubbles. Three sheets of 3MM paper at slightly larger than the nylon membrane was wetted in 20x SSC and placed on top of the nylon membrane. A stack of paper towels was placed on top of this along with a glass plate and a 1.5 kg weight. The blotting apparatus was left overnight to allow for capillary transfer of DNA fragments to the nylon membrane. After transfer the nylon membrane was removed carefully and soaked in 10x SSC and exposed to 235 nm UV light for 3 min to cross-link DNA fragments to the membrane. The nylon membrane was rinsed in sterile double distilled water and allowed to air dry.

### 2.2.12.3 Random primer labelling of DNA with DIG

A Roche kit was used as described in the manufacturers' protocol. PCR-amplified ADA DNA (1  $\mu$ g) was mixed with 4  $\mu$ l of DIG-High Prime solution (Digoxigenin-11-dUTP, Klenow DNA polymerase enzyme) in a 20  $\mu$ l of reaction mixture prior to overnight incubation at 37 °C. The reaction was stopped by addition of 2  $\mu$ l of 0.2 M EDTA pH 8.0. Determination of the yield of DIG-labelled DNA was carried out with a series of dilutions as shown in table 2.1. Spots of 1  $\mu$ l of dilutions were applied to a nylon membrane followed by exposure to 235 nm UV light for 3 min to cross-link DIG-labelled DNA fragments to the membrane. The membrane was blocked by incubation for 30 min with gentle agitation in 10 ml Blocking Solution (1x Salmon sperm solution, 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5) followed by incubation for 30 min in 10 ml of Antibody Solution containing 1:5000 (150 mU ml<sup>-1</sup>) dilution of Anti-digoxigenin-AP in Blocking Solution. The membrane was washed twice for 15 min in 10 ml of Washing Buffer (0.1 M Maleic acid, 0.15 M NaCl, 0.3 % (v/v) Tween 20; pH 7.5) followed by equilibration for 5 min in 10 ml of Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). Finally, the membrane was incubated in 2 ml of freshly prepared colour substrate solution containing 40  $\mu$ l of 50x NBT/BCIP stock solution in 2 ml of Detection Buffer in the dark for 30 min. The colourimetric reaction was stopped by washing the membrane with TE buffer when the required intensity of the dots was appeared (Approximately 30 min). Then the optimal concentration of the DNA probe to be used was determined by analysing the dot blot.

**Table 2.1: Dilution series of DIG labelled DNA.**

Tube	Dilution	Final concentration (pg $\mu\text{l}^{-1}$ )
1		1000
2	1:100	10
3	1:3.3	3
4	1:10	1
5	1:10	0.3
6	1:10	0.1
7	1:10	0.03
8	1:10	0.01
9	-	0

#### 2.2.12.4 Hybridization of membrane-bound nucleic acid with DNA probes and immunological detection using Roche DIG High Prime Detection Kit

An appropriate volume of *DIG Easy Hyb* pre-hybridization solution (0.1 ml  $\text{cm}^{-2}$  membrane) was pre-heated to 47 °C, added to a nucleic acid blot on nylon membrane and pre-hybridized for 30 min with gentle agitation. A DIG-labelled DNA probe in determined optimal dilution (about 25 ng  $\text{ml}^{-1}$ , section 2.2.12.3) was denatured by boiling for 5 min and rapidly cooled on ice. The denatured DIG-labelled DNA probe was added to pre-heated *DIG Easy Hyb* solution (0.035 ml  $\text{cm}^{-2}$  membrane) and mixed well. The nylon membrane was transferred to the hybridization solution containing probe and incubated at 47 °C overnight with gentle agitation. After hybridization the membrane was washed twice for 5 min with 2x SSC, 0.1 % SDS at room temperature under constant agitation and twice for 15 min with 0.5x SSC, 0.1 % SDS at 65-68 °C under constant agitation. The membrane was rinsed briefly for 1-5 min in Washing Buffer containing 0.1 M Maleic acid, 0.15 M NaCl and 0.3 % (v/v) Tween 20; pH 7.5. The membrane was then blocked by incubation for 30 min with gentle agitation in 100ml Blocking Solution (1x Salmon sperm solution, 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5)

followed by incubation for 30 min in 20 ml of Antibody solution containing 1:5000 (150 mU ml<sup>-1</sup>) dilution of Anti-digoxigenin-AP in Blocking solution. The membrane was washed twice for 15 min in 100 ml of Washing Buffer followed by equilibration for 5 min in 20 ml of Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). Finally, the membrane was incubated in 10ml of freshly prepared colour substrate solution containing 200 µl of 50x NBT/BCIP stock solution in 10ml of Detection Buffer in the dark for 30 min. The colourimetric reaction was stopped by washing the membrane with TE buffer when the required intensity of the bands was achieved. The membrane was placed sandwiched in between two transparency films and photographic record was taken using an Epson image scanner.

## **2.3 RNA methods**

### **2.3.1 Isolation of total RNA using a Sigma Plant total RNA Kit**

Beforehand the glassware was baked overnight at 250 °C and plasticware was thoroughly washed with 0.1 N NaOH/1 mM EDTA then rinsed with diethyl pyrocarbonate (DEPC)-treated water to ensure the elimination of RNases. Prepared solutions were treated with 0.05 % DEPC overnight prior to autoclaving. Plant tissue or calli (2-10 g) were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The ground powder (90-110 mg) was weighed after evaporation of liquid nitrogen in 2 ml microcentrifuge tube pre-chilled in liquid nitrogen. The total RNA extraction was carried out using a Sigma Plant total RNA Kit by following the manufacturers' protocol. Eluted RNA was stored at -20 °C.

### 2.3.2 Gel electrophoresis of RNA

Total RNA was quantified beforehand using a NanoDrop 1000 spectrophotometer, following the manufacture's protocol. RNA loading equivalents were then verified by visualising equal loadings of SafeView-stained ribosomal RNA bands on a separate 1% agarose gel under a UV-transilluminator and taking a photographic record using a Kodak Gel-Logic Imaging System. Equal amounts of total RNA (3000 ng) were denatured by mixing with 15 µl of sample buffer (48 % (v/v) deionised formamide, 40 mM MOPS pH 7.0, 50 mM Na acetate, 5 mM EDTA, 6 % (v/v) formaldehyde, 5 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue). The sample mixture was incubated at 65 °C for 10 min before loading onto a 1.2 % (w/v) agarose gel in 1x running buffer containing 40 mM MOPS pH 7.0, 50 mM Na acetate, 5 mM EDTA, 2% (v/v) formaldehyde and 5 % (v/v) SafeView nucleic acid stain. Electrophoresis, at constant 40 V was carried out for approximately 5 hours in 1 x running buffer.

### 2.3.3 Preparation and hybridization of northern blots

After gel electrophoresis, the gel was equilibrated by incubating twice in 200 ml of 50 mM sodium phosphate buffer pH 6.4 over a period of 40 min. A capillary blot was set up as described in section 2.2.12.2. After blotting, the nylon membrane was removed carefully and soaked in 10x SSC and exposed to 235 nm UV light for 3 min to cross-link the RNA fragments to the membrane. The nylon membrane was rinsed in sterile double distilled water and allowed to air dry (section 2.2.12.2). Hybridization was carried out as described in section 2.2.12.4.

### 2.3.4 Blot analysis

TotalLab Quant software was used to analyse northern blots. The area of ADA mRNA hybridizing bands present on blots were compared to the areas of quantified marker bands in order to calculate approximate relative amounts of ADA mRNA in ng. The intensity of RNA bands on a northern blot was presumed to be directly proportional to the amount of RNA (Wright *et al.*, 1997).

## 2.4 Tobacco plant and tobacco BY-2 cell transformation

DNA constructs were transferred into tobacco BY-2 cell suspension culture and whole plants by *Agrobacterium*-mediated transformation. Binary vector pGreenI0029 constructs were used in transformations, along with the helper plasmid pSoup (Hellens *et al.*, 2000).

### 2.4.1 Transfer of a pGreenI0029 plasmid constructs to *Agrobacterium* by electroporation

*Agrobacterium tumefaciens* AGL1 was grown in 10 ml LB Broth containing 100 µg ml<sup>-1</sup> rifampicin for 24 hours at 28 °C with continuous shaking at 300 rpm in an orbital shaker to obtain a culture of *Agrobacterium* with an A<sub>600</sub> of approximately 1. The cells were then cooled on ice, followed by centrifugation for 5 min in a Hitachi Himac-CP at 2000g. The supernatant was discarded and the pellet was washed in 10 ml, 5 ml, 0.2 ml and 0.2 ml successive volumes of ice-cold 10 % (w/v) glycerol. Finally, the cells were re-suspended in 0.1 ml of ice-cold 10 % (w/v) glycerol, separated into 40 µl aliquots and frozen in liquid nitrogen and stored for several months at -80 °C. Transformation was set up by addition of 10-100 ng of construct in pGreenI0029 vector along with 10-100 ng of pSoup vector in 40 µl of electro-competent *Agrobacterium* cells in a 0.2 cm cuvette. Electroporation was carried out

in a Bio-Rad electroporation apparatus set at 25  $\mu\text{F}$ , 2.5 kV, 400  $\Omega$  followed by dilution of the cells by addition of 950  $\mu\text{l}$  of LB broth prior to incubation for 3 hours at 28 °C with continuous shaking at 300 rpm in an orbital shaker. The cells were pelleted by centrifugation in a microfuge for 1 min, re-suspended in 100  $\mu\text{l}$  of LB broth and spread onto LB plates containing 100  $\mu\text{g ml}^{-1}$  kanamycin / 20  $\mu\text{g ml}^{-1}$  tetracycline followed by incubation for 3 days at 28°C. A transformed *Agrobacterium* colony from the plate was inoculated into 10 ml of LB broth containing 100  $\mu\text{g ml}^{-1}$  kanamycin / 20  $\mu\text{g ml}^{-1}$  tetracycline and the culture incubated for 3 days at 28 °C with vigorous shaking until the absorption  $A_{600}$  had reached 1. The characteristics of the plasmids in the culture were confirmed through plasmid extraction, followed by restriction enzyme digestion and gel electrophoresis (section 2.2.6 and 2.2.7).

#### **2.4.2 Transformation of tobacco BY-2 cell suspensions**

The protocol was developed from the method of Rempel and Nelson, (1995). Using 400  $\mu\text{l}$  of a 3-day-old binary vector transformed *Agrobacterium* culture (section 2.4.1), a new LB broth containing appropriate antibiotics (100  $\mu\text{g ml}^{-1}$  kanamycin / 20  $\mu\text{g ml}^{-1}$  tetracycline) was inoculated and incubated overnight at 28 °C with vigorous shaking at 200 rpm. The absorbance of an overnight-grown culture was measured at  $A_{600}$  and diluted to an  $A_{600}$  of 1 in LB broth. For tobacco BY-2 transformation, 5 ml of 4-day-old propagated BY-2 cell suspension culture containing 5 $\mu\text{l}$  of 20mM acetosyringone was co-cultured with 25  $\mu\text{l}$ , 50  $\mu\text{l}$  and 100  $\mu\text{l}$  of transformed *Agrobacterium* LB broth culture ( $A_{600}=1.0$ ) successively in 100 mm Petri dishes. The dishes were sealed with parafilm and incubated at 25 °C for two days. Co-cultivated tobacco BY-2 cells were transferred from the plate into a 30 ml plastic sterilin tube and kept undisturbed for about 10 min until the cells had settled to the bottom of the tube. The supernatant was discarded and the cells were washed twice with MS media and twice with MS media containing 500  $\mu\text{g ml}^{-1}$  carbenicillin / 500  $\mu\text{g ml}^{-1}$  cefotaxime. Finally, the

cells were re-suspended in 5 ml of MS media containing 500  $\mu\text{g ml}^{-1}$  carbenicillin /500  $\mu\text{g ml}^{-1}$  cefotaxime. Washed cells (2 ml) were spread onto 100 mm Petri dishes containing MS agar, 500  $\mu\text{g ml}^{-1}$  carbenicillin, 500  $\mu\text{g ml}^{-1}$  cefotaxime and 100  $\mu\text{g ml}^{-1}$  kanamycin. Cells were spread by gently rocking and swirling the plates and plates were left uncovered in the laminar flow hood for 5-10 min until the liquid was absorbed. Finally, the plates were sealed with Parafilm and incubated at 25 °C in the dark. Calli were visible after 20-30 days and visible calli were transferred onto new MS agar plates containing carbenicillin/cefotaxime/kanamycin and grown for 7-14 days in the dark at 25 °C prior to expression analysis.

### 2.4.3 Transformation of tobacco and regeneration of whole plants

The following protocol was developed from the method of Horsch *et al.*, (1985). LB broth containing appropriate antibiotics (100  $\mu\text{g ml}^{-1}$  kanamycin / 20  $\mu\text{g ml}^{-1}$  tetracycline) was inoculated with a transformed *Agrobacterium* culture (section 2.4.1) and incubated overnight at 28 °C with vigorous shaking at 200 rpm to obtain a culture of *Agrobacterium* with an  $A_{600}$  of approximately 1. *Agrobacterium* cells were pelleted by centrifugation at 3000 rpm for 15 min and re-suspended in MS medium, pH 5.9 and absorption was measured at  $A_{600}$  and diluted to an  $A_{600}$  of 0.6 by diluting in MS medium. The *Agrobacterium* suspensions (25 ml) were dispensed into 100 mm Petri dishes.

Healthy and fully-extended leaves were dissected from micro-propagated tobacco plant cultures and sterilised by submerging in 30 % sodium hypochlorite solution for 1-2 min. The mid-rib and the outer leaf margin were removed and cut into about 1 cm x 1 cm squares with a sterile scalpel. These were transferred directly to the *Agrobacterium* suspension in a Petri dish abaxial surface lowermost and shaken gently to ensure the full submersion of the cut



edges of the leaves. These explants were maintained in the *Agrobacterium* suspension at room temperature for 20 min, then removed and dabbed on sterile filter paper to remove excess suspension. Explants were transferred to NBM agar, with the abaxial surface of each explant in contact with the medium. Plates were sealed with Parafilm and incubated in a lit ( $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) incubator at 25 °C, 16 hour photoperiod for 3 days. After which the explants were carefully transferred onto NBM agar medium containing  $100 \mu\text{g ml}^{-1}$  kanamycin,  $500 \mu\text{g ml}^{-1}$  cefotaxime and  $500 \mu\text{g ml}^{-1}$  carbenicillin. Shoots started to regenerate from the callus forming on leaf explants after 4-6 weeks. The shoots were cut with flame-sterilised scissors and transferred onto Elongation Medium (MS medium,  $30 \text{ g L}^{-1}$  sucrose,  $8 \text{ g L}^{-1}$  plant agar,  $100 \mu\text{g ml}^{-1}$  kanamycin,  $500 \mu\text{g ml}^{-1}$  cefotaxime,  $500 \mu\text{g ml}^{-1}$  carbenicillin, pH 5.9). Readily rooted stable transgenic plants were formed after 3- 4 weeks, whilst untransformed shoots bleached and died.

## 2.5 Protein methods

### 2.5.1 Isolation of total soluble proteins from plant material

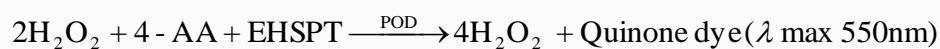
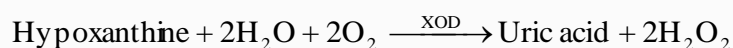
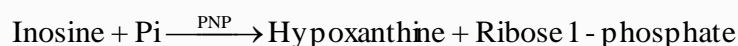
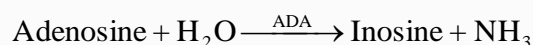
Tobacco leaf or calli tissues (0.1-1 g) were ground to a fine powder in a microfuge tube using a microfuge tube grinder in liquid nitrogen and homogenised in 400  $\mu\text{l}$  of chilled extraction buffer containing 50 mM potassium phosphate pH 7.5, supplemented with  $37.50 \text{ g L}^{-1}$  polyvinylpyrrolidone 40,000MW. The homogenate was then subjected to centrifugation at  $10,000g$  for 10 min at 4 °C to remove plant cell debris.

### 2.5.2 Enzymatic assay for adenosine deaminase

An Adenosine deaminase assay kit (Bioquant, USA) was used to determine levels of ADA in transformed plant and cell extracts (section 2.5.1). The assay is based on the enzymatic conversion of adenosine to inosine by ADA followed by the conversion of inosine to

hypoxanthine by purine nucleoside phosphorylase (PNP). Furthermore, xanthine oxidase (XOD) catalyzes the conversion of hypoxanthine to uric acid and hydrogen peroxide. In the presence of peroxidase (POD), hydrogen peroxide reacts with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) to generate a quinone dye which can be detected in a kinetic manner by measuring the change in absorbance at 550 nm on a Thermo Multiskan plate reader (Thermo, USA).

Principle:



Total soluble proteins (TSP) were isolated from plant tissues or calli using 400 µl of chilled extraction buffer (section 2.5.1). From the soluble protein solution, 5 µl was removed and mixed with 180 µl of R1 solution (50 mM Tris.HCl pH 8.0, 2 mM 4-AA, 0.1 U ml<sup>-1</sup> PNP, 0.2 U ml<sup>-1</sup> XOD, 0.6 U ml<sup>-1</sup> Peroxidase) and incubated for 3 min at 37 °C. R2 solution (90 µl) containing 50 mM Tris.HCl pH 4.0, 10 mM adenosine and 2 mM EHSPT was added and incubated for 5 min at 37 °C. The absorbance was monitored at 550 nm in a Thermo Multiskan plate reader (Thermo, USA) for 3 min with 1 min intervals to obtain absorbance min<sup>-1</sup> values. Assays were performed in triplicate. The average rate of the absorbance change (ΔA min<sup>-1</sup>) was calculated from 3 separate assays per transformants and ADA activity in units per litre protein extract was calculated using the formula below. Similarly, 5 µl of calf

intestinal ADA (Sigma) was analysed as a control. For plants ADA activities from young, medium and old leaf samples were combined and assayed.

$$ADA (\text{Units L}^{-1}) = \frac{\Delta A \text{ min}^{-1} \times T_v}{\epsilon \times S_v \times L} = \Delta A \text{ min}^{-1} \times 1708$$

**T<sub>v</sub>** = Total reaction volume (ml) = 0.275 ml

**S<sub>v</sub>** = Sample volume (ml) = 0.005 ml

**ε** = μmolar extinction coefficient of quinone dye ( $\epsilon = 32.2 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1}$ )

**L** = Cuvette light path length (1.0 cm)

### 2.5.3 Protein Assay

Determination of the amount of total soluble protein from protein extracts (section 2.5.1) were performed using a method based on that of Bradford, (1979). Bovine serum albumin (BSA)  $1 \text{ mg ml}^{-1}$  was prepared in distilled water and diluted to  $0.1 \text{ mg ml}^{-1}$  in protein extraction buffer (section 2.5.1). Diluted BSA ( $100 \mu\text{g ml}^{-1}$ ), distilled water and  $25 \mu\text{l}$  of Bradford reagent (Sigma) was mixed as shown in the table 2.2 in 96 well microtitre plates and the absorbance measured at 590 nm using a Thermo Multiskan plate reader (Thermo, USA). Assays were performed in triplicate and a standard curve was plotted. The protein extract from transformed plants or calli was diluted 10 fold and  $2 \mu\text{l}$  of diluted sample was added to  $98 \mu\text{l}$  of distilled water prior to addition of  $25 \mu\text{l}$  Bradford reagent. Absorbance at 590 nm was measured and the amount of total soluble protein determined from the standard curve.

**Table 2.2: Protein assay.**

BSA 100 $\mu\text{g ml}^{-1}$ ( $\mu\text{l}$ )	H <sub>2</sub> O ( $\mu\text{l}$ )	Bradford ( $\mu\text{l}$ )	total $\mu\text{g}$ BSA
0	100	25	0.0
3	97	25	0.3
6	94	25	0.6
9	91	25	0.9
12	88	25	1.2

#### 2.5.4 SDS-polyacrylamide gel electrophoresis of proteins

The method used was that of Laemmli, (1970) using the Bio-Rad Miniprotein II apparatus. A 12 % (w/v) polyacrylamide gel was prepared in a total volume of 10 ml by adding 20  $\mu\text{l}$  of TEMED and 100  $\mu\text{l}$  of 10 % (w/v) ammonium persulphate to a solution containing 12 % (w/v) acrylamide, 0.4 % (w/v) *NN'*-methylenebisacrylamide, 0.375 M Tris-HCl pH 8.8 and 0.1 % (w/v) SDS. Also a stacking gel was prepared in a total volume of 5 ml by adding 10  $\mu\text{l}$  of TEMED and 50  $\mu\text{l}$  of 10 % (w/v) ammonium persulphate to a solution containing 5 % (w/v) acrylamide, 0.133 % (w/v) *NN'*-methylenebisacrylamide, 0.125 M Tris-HCl pH 6.8 and 0.1 % (w/v) SDS. The gel was prepared in a Bio-Rad gel casting unit using 5 ml of 12 % polyacrylamide gel mixture and 2 ml of 5 % polyacrylamide stacking gel mixture. Sample proteins were diluted at 1:4 ratios with sample buffer containing 0.625 mM HCl, 9.5 % (w/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2- $\beta$ -mercaptoethanol and 0.00125 % (w/v) Bromophenol blue. Proteins containing samples and pre-stained protein standards were separated by electrophoresis through the polyacrylamide gel using a running buffer containing 0.2 M glycine, 0.1 % (w/v) SDS and 0.025 M Tris pH 8.3 at constant voltage (200 V) for one hour.

### 2.5.5 Western blotting

Transfer of proteins from polyacrylamide gels to nitrocellulose was performed by a method based on that of Polvino *et al.*, (1983). The gel was placed on a sheet of Hybond-C (Amersham) nitrocellulose membrane and sandwiched between two sheets of Whatmann 3MM paper soaked in transfer buffer (25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol, 0.1 % (w/v) SDS). The sandwich was then placed on the Mylar mask in a SemiPhore semi-dry transfer unit and two additional pieces of buffer-soaked Whatmann 3MM paper placed on the stack. The transfer of proteins was performed under a constant current of 100 mA for 30 min. After transfer the membrane was immersed in 5% Skimmed milk-TBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) on a plastic tray and incubated overnight at 25 °C, followed by two washes in TBST (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.05 % (v/v) Tween-20). The membrane was incubated for 1 hour with continuous rocking with TBST containing rabbit anti-ADA antibodies at an optimal dilution of 1:3000 (optimal dilution was determined by the dot blot of a series of antibody dilutions). The membrane was then washed twice in TBST and incubated for 1 hour with continuous rocking in TBST containing anti-rabbit IgG conjugated to horseradish peroxidase. Incubation was followed by two washes in TBST prior to incubation for 5 min in 10ml Developing Buffer containing 4 mg ml<sup>-1</sup> luminol in TBS pH 7.5, 0.2 mg ml<sup>-1</sup> 4-iodophenol and 25 µl of 30 % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The membrane was sandwiched between transparency film and hybridizing bands were visualised in Typhoon scanner (Amersham). TotalLab Quant software was used to analyse western blots. The area of hybridizing bands present on blots were compared to the areas of quantified control ADA bands in order to calculate approximate relative amounts of recombinant ADA in micro grams.

### 2.5.6 Thrombin cleavage of ADA-hydroxyproline fusion.

Tobacco BY-2 calli, cell suspension extract or cell suspension media (400 µl) of ADA-hydroxyproline fusion protein was mixed with 20 Units of thrombin prior to incubation at 22 °C for 16 hours. A control ADA lacking hydroxyproline fusion was also incubated.

## 2.6 Statistical analysis

Expression levels of the same transgene in independent transformants do not assume a normal distribution. Therefore, Mann-Whitney  $U$  test, a distribution-free statistical test based on median values was used to evaluate significant difference between any two gene-constructs. Any two gene constructs being compared were combined and ranked by ascending expression levels.  $U$ -values were calculated using the following formulae:

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1 \quad U = n_1 n_2 - \frac{n_2(n_2 + 1)}{2} - R_2$$

Where  $R_1$  is the sum of the ranks assigned to ADA specific-activities from construct-1, whose sample size is  $n_1$  and  $R_2$  is the sum of ranks assigned to ADA specific-activities from construct-2, whose sample size is  $n_2$ . The  $U$  values were compared with a critical region table indicating the level of significance at 5%.

## **Chapter 3**

# **Design and generation of ADA gene expression constructs in pGREEN for tobacco transformation**

### 3.1 Overview

Plants possess an efficient eukaryote protein production mechanism, and with presently accessible gene expression systems, plants can readily produce enormous amounts of recombinant protein (Obembe *et al.*, 2011). Tobacco cells and whole plants are frequently used in foreign gene expression owing to the characteristics of simple, well established transformation and propagation protocols. The recombinant protein gene construct design is an important factor determining the foreign protein yield. For example, choice of promoter affects the yield of recombinant products by influencing the transcription rate. Hence, the choice of promoter should be evaluated on an empirical basis. Since the isolation in the early 1980's by Chua and collaborators at the Rockefeller University, the CaMV 35S promoter is the most extensively used promoter in plant expression systems expressing recombinant proteins at high-levels (Odell *et al.*, 1985; Sanders *et al.*, 1987; Gutierrez-Ortega *et al.*, 2005). The CaMV 35S promoter sequence has been modified by tandem duplication of a 250 bp upstream sequence which in turn enhances the efficiency of expression by approximately 10-fold (Kay *et al.*, 1987). However, subsequently the use of strong promoters has often been associated with posttranscriptional gene silencing and their use is now limited (Que *et al.*, 1997; Schubert *et al.*, 2004). Since first use in the 1980's, the CaMV 35S promoter has been used to produce many proteins of therapeutic and industrial importance, such as Cholera toxin B, SARS virus glycoprotein S, avidin and adiponectin (Jani *et al.*, 2002; Kang *et al.*, 2004c; Li *et al.*, 2006b; Murray *et al.*, 2002). The CaMV 35S promoter has the ability to carry out continuous expression of recombinant proteins and is not greatly influenced by environmental conditions or tissue types, hence it is the promoter of choice in many plant expression systems (Ho *et al.*, 1999; Huang and McDonald, 2009).



Translational regulation is also an important factor in eukaryotic gene expression. Choice of strong translational enhancer/regulation sequences is equally as important as the choice of an efficient promoter when seeking to increase the yield of recombinant products. Most of the regulation in translation occurs at the initiation steps in the 5'-untranslated region (mRNA 5' leader sequence). There are two very important mechanisms by which translational initiation occurs in eukaryotic cells. They are (i) cap-dependent initiation and (ii) internal ribosome entry (Pickering and Willis, 2005). RNA plant viruses, on the other hand, exhibit several different mechanisms by which they regulate gene expression at the translational level. Tobacco mosaic virus (TMV) UTRs represent the most widely used translational regulation sequences for generate high yield constructs in plants (Schmitz *et al.*, 1996).

There are several other plant translational regulator sequences used to make foreign gene constructs, one of which is the RUBISCO small subunit 5' UTR. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) is an enzyme which catalyzes the primary step in carbon fixation in plants (Mazur and Chui, 1985). It is a multimeric enzyme composed of eight large and eight small subunits and is the most abundant protein present in plants. Patel *et al.*, (2004) demonstrated that the untranslated region from C4 *Amaranth* RUBISCO small subunit1 mRNAs confer translational enhancement which leads to increased expression of foreign proteins. Patel *et al.*, (2006) further demonstrated that incorporation of untranslated regions of *Flaveria bidentis* RUBISCO small subunit1 also enhanced translation of associated sequences.

Although, the foreign protein expression in plants can be enhanced by the use of various promoter and UTR sequences, in order to achieve commercial success of plant-based production systems, improvements of foreign protein accumulation is also pivotal (Doran, 2004). Once produced most foreign proteins expressed in heterologous systems undergo

constant degradation due to processes such as proteolysis and irreversible surface adsorption (Doran, 2006). Hence, it is of much importance to take steps in order to reduce foreign protein loss by these processes, in turn leads to accumulation. Conrad and Fielder, (1998) showed that directing produced proteins to the endoplasmic reticulum (ER) effectively reduces the foreign protein degradation and loss. Wirth *et al.*, (2004) showed that recombinant human epidermal growth factor directed to ER increased the yield by a  $10^4$ -fold as compared to cytosolic counterpart. The plant cell ER contains less protease activity and also contains important chaperones (Protein binding motifs BiP) which give a protective environment to foreign proteins (Nuttal *et al.*, 2002). Hence, the use of signal peptides (KDEL or HDEL) in order to retain recombinant proteins in the ER can also be used to reduce protein degradation and direct correct folding of the polypeptide chain.

Certain proteins which contain an *N*-terminal signal peptide sequence undergo secretion into the apoplastic space. Signal sequences such as tobacco pathogenesis related protein (PR1a) when used in conjunction with recombinant proteins can direct foreign proteins into the apoplast (Pen *et al.*, 1993a). Ziegler *et al.*, (2000) effectively produced the catalytic domain of a bacterial thermostable endo-1, 4- $\beta$ -D-glucanase in the apoplast of *Arabidopsis* leaves and BY2 cell suspensions. Incorporation of a PR1a signal peptide at the *N*-terminus increased the accumulation level by up to 26% of TSP (Ziegler *et al.*, 2000). Another apoplast directing signal is found at the *N*-terminus of plant extensins which are well-characterized hydroxyproline-rich glycoproteins forming a major component of the cell wall (Showalter, 1993). Incorporation of an extensin signal peptide sequence at the *N*-terminal of the recombinant protein directs secretion into the apoplast. For example, Francisco *et al.*, (1997) expressed Bryodin 1, a potent ribosome-inactivating protein, as a soluble recombinant protein in BY-2 cell cultures, using the extensin signal peptide sequence to secrete the protein into

the culture medium. Purified Broyodin 1 yield was 30 mg L<sup>-1</sup> of culture and showed identical inhibitory activity to native Bryodin 1 isolated from the roots of *Bryonia dioica*.

Hydroxyproline(Hyp)-rich glycoproteins (HRGPs) are structural glycoproteins distinctively associated with the plant cell wall (Lamport, 1977). HRGP motifs are evolutionary conserved and extremely *O*-glycosylated through the Hydroxyproline (Hyp) residues. Shpak, (2001) reported that two types of glycomodules are resulted by unique post-translational modifications, involving Hyp motifs. One of which is formed by the addition of short unbranched arabinooligosaccharides to a contiguous Hyp residues, whereas the other is formed by the addition of branched arabinogalactan polysaccharides to clustered non-contiguous Hyp residues (Shpak, 2001). This includes repeats of X-Hyp-X-Hyp motifs where X is frequently Ser or Ala and leads to an increase in molecular weight of the protein (Kieliszewski and Lamport, 1994). Chimeric constructs of foreign genes linked to these HRGP motifs at the C-terminal have shown high-level expression of linked therapeutic proteins. For example HRGPs linked to interferon alpha2 (IFN $\alpha$ 2) direct extensive Hyp *O*-glycosylation leading to addition of arabinooligosaccharides resulting in an arabinogalactan protein (AGP) which when, targeted for secretion into the apoplast, increased the yield of secreted recombinant protein by 350–1400-fold as compared to a non-glycosylated IFN $\alpha$ 2 control (Xu, 2008). Targeting recombinant proteins into the apoplast through sub-cellular targeting protects recombinant proteins from proteolytic degradation and accumulation.

The above strategies for stabilizing foreign gene mRNAs and targeting recombinant products have been employed in designing and generating various ADA constructs in order to increase the yield of recombinant human ADA in tobacco whole plants and cell suspensions.

### 3.2 Constructs designed to increase cytosolic levels of human ADA

**Table 3.1: Cytosolic constructs.**

Constructs	Code
Human ADA cDNA	pCDNA_ADA
Minimal 5' UTR	pΔ5'UTR_ADA
TMV Omega 5'UTR	p5'Omega_ADA
TMV Omega 5'_3' UTR	p5'_3' Omega_ADA
RUBISCO SS 5' UTR	pSS 5'UTR_ADA

#### 3.2.1 Human ADA cDNA (pCDNA\_ADA) construct design

In order to generate an ADA cDNA insert with restriction enzyme sites for insertion into the 35S CaMV cassette of pJIT a three step cloning procedure was necessary. Firstly, the ADA cDNA clone (figure 3.1) provided in pCMV6 was isolated from the vector by *NotI* digestion. The *NotI* digested and gel-purified ADA cDNA was inserted into *NotI* digested pUBS and the resulting construct was transformed into *E.coli* and transformants selected. The recombinant plasmid pUBSADA was extracted from one transformant and digested with *XbaI* to produce an insert with *XbaI* ends. The gel- extracted ADA cDNA was ligated into *XbaI* digested pJIT in between the 35S CaMV promoter and terminator cassette. The insertion of ADA cDNA into pJIT was confirmed by restriction enzyme mapping using *XbaI* to release ADA cDNA from pJIT (figure 3.2b). Restriction enzyme digestion of plasmid minipreps of pJITADA digests, (lanes 2 to 9 of figure 3.2b) generated two fragments at 1.4 kbp and 2.9 kbp which confirmed the insertion of ADA (1.4 kbp) into pJIT (2.9 kbp).

Finally, the construct in pJIT 35S cassette was inserted into the pGreenI0029 T-region by digestion of pJITADA and pGreenI0029 with *EcoRV*. *E.coli* DH5 $\alpha$  was transformed with ligated pGreenI0029-35SADA and transformants selected. Putative transformants containing pGreenI0029-35SADA plasmid were isolated and the insertion of 35SADAcDNA into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35SADA cDNA from pGreen (figure 3.3b). Restriction enzyme digestion of isolated pGreenI0029-35SADA with *EcoRV* generated two fragments in all digests (figure 3.3b) at 2.1 kbp and 4.6 kbp. Hence, confirming the insertion of 35SADA (2.1 kbp) into pGreenI0029 (4.6 kbp).

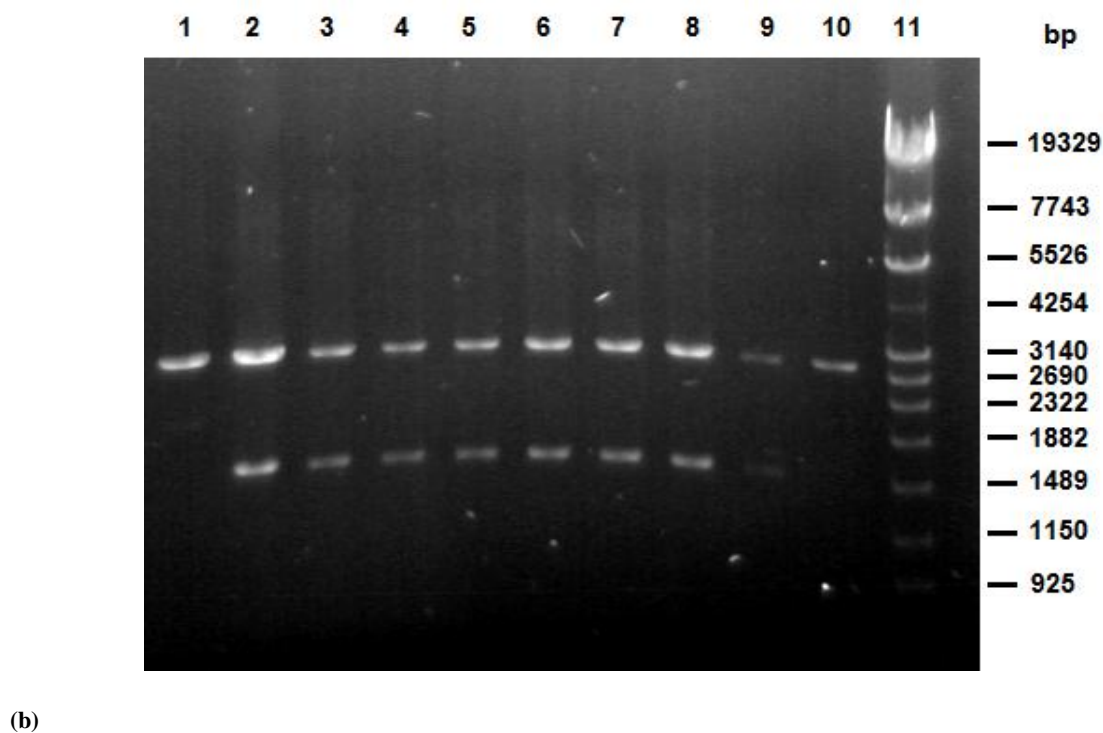
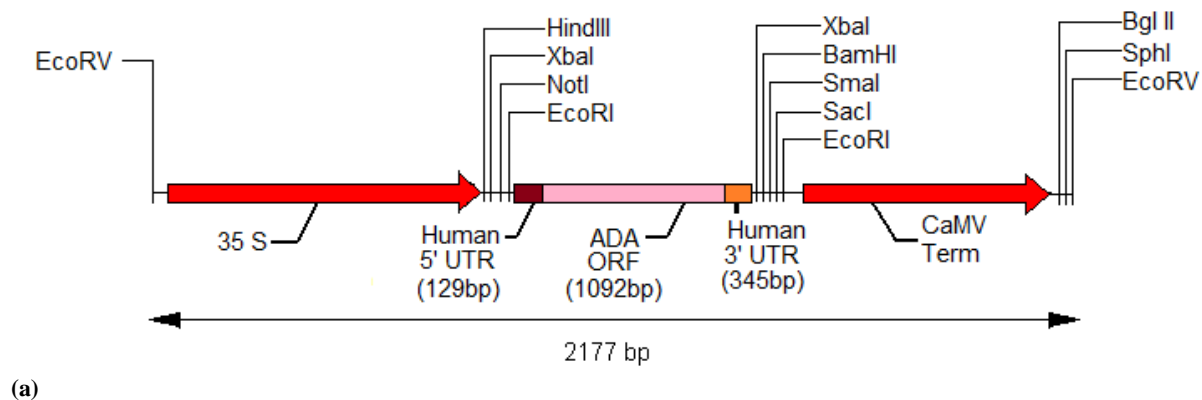
Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029-35SADA along with the helper plasmid pSoup (section 2.4.1). Transformants were selected and transformation was confirmed by remapping of plasmid isolates from *Agrobacterium* transformants using *SalI*. (figure 3.4) *SalI* restriction enzyme digestion of plasmids isolated from transformed *Agrobacterium* clones generated two linearized vector fragments in all digests shown in figure 3.4. DNA fragments running at approximately 6.1 kbp confirmed the presence of pGreenI0029-35SADA plasmid whereas fragments running at approximately 9.7 kbp confirmed the presence of pSoup. Tobacco BY-2 cell suspensions and whole plants were transformed with the pGreenI0029-35SADA construct using *Agrobacterium* mediated transformation (section 2.4.2 and section 2.4.3) and transformants were selected after 5-6 weeks of transformation and ADA activity measured (section 2.5.2).

```

CCCGTTAAGAAAGAGCGTGGCCGGCCGCGGCCACCGCTGGCCCCAGGGAAAGCCGAGCGGC
CACCGAGCCGGGCAGAGACCCACCGAGCGGCGGGCGGAGGGAGCAGCGCCGGGGGCGCACGAG
M A Q T P A F D K P K V E L H V H L
GGCACCATGGCCCCAGACGCCCCGCTTCGACAAGCCCCAAAGTAGAACTGCATGTCCACCTA
D G S I K P E T I L Y Y G R R R G I A L
GACGGATCCATCAAGCCTGAAACCATCTTATACTATGGCAGGAGGAGAGGGATCGCCCTC
P A N T A E G L L N V I G M D K P L T L
CCAGCTAACACAGCAGAGGGGCTGCTGAACGTCATTGGCATGGACAAGCCGCTCACCTT
P D F L A K F D Y Y M P A I A G C R E A
CCAGACTTCCTGGCCAAGTTTGACTACTACATGCCTGCTATCGCGGGCTGCCGGGAGGCT
I K R I A Y E F V E M K A K E G V V Y V
ATCAAAAGGATCGCCTATGAGTTTGTAGAGATGAAGGCCAAAGAGGGCGTGGTGTATGTG
E V R Y S P H L L A N S K V E P I P W N
GAGGTGCGGTACAGTCCGCACCTGCTGGCCAACCTCCAAAGTGGAGCCAATCCCCTGGAAC
Q A E G D L T P D E V V A L V G Q G L Q
CAGGCTGAAGGGGACCTCACCCAGACGAGGTGGTGGCCCTAGTGGGCCAGGGCCTGCAG
E G E R D F G V K A R S I L C C M R H Q
GAGGGGGAGCGAGACTTCGGGGTCAAGGCCCGGTCCATCCTGTGCTGCATGCGCCACCAG
P N W S P K V V E L C K K Y Q Q Q T V V
CCCAACTGGTCCCCCAAGGTGGTGGAGCTGTGTAAGAAGTACCAGCAGCAGACCGTGTA
A I D L A G D E T I P G S S L L P G H V
GCCATTGACCTGGCTGGAGATGAGACCATCCCAGGAAGCAGCCTCTTGCTGCGACATGTC
Q A Y Q E A V K S G I H R T V H A G E V
CAGGCCTACCAGGAGGCTGTGAAGAGCGGCATTACCGTACTGTCCACGCCGGGGAGGTG
G S A E V V K E A V D I L K T E R L G H
GGCTCGGCCGAAGTAGTAAAAGAGGCTGTGGACATACTCAAGACAGAGCGGCTGGGACAC
G Y H T L E D Q A L Y N R L R Q E N M H
GGCTACCACACCCTGGAAGACCAGGCCCTTTATAACAGGCTGCGGCAGGAAAACATGCAC
F E I C P W S S Y L T G A W K P D T E H
TTGAGATCTGCCCCTGGTCCAGCTACCTCACTGGTGCCTGGAAGCCGGACACGGAGCAT
A V I R L K N D Q A N Y S L N T D D P L
GCAGTCATTCCGGCTCAAAAATGACCAGGCTAACTACTCGCTCAACACAGATGACCCGCTC
I F K S T L D T D Y Q M T K R D M G F T
ATCTTCAAGTCCACCCTGGACACTGATTACCAGATGACCAAACGGGACATGGGCTTTACT
E E E F K R L N I N A A K S S F L P E D
GAAGAGGAGTTTAAAAGGCTGAACATCAATGCGGCCAAATCTAGTTTCCTCCAGAAGAT
E K R E L L D L L Y K A Y G M P P S A S
GAAAAGAGGGAGCTTCTCGACCTGCTCTATAAAGCCTATGGGATGCCACCTTCAGCCTCT
A G Q N L -
GCAGGGCAGAACCTCTGAAGACGCGCACTCCTCCAAGCCTTCACCCTGTGGAGTCACCCCA
ACTCTGTGGGGCTGAGCAACATTTTACATTTATTCCTTCCAAGAAGACCATGATCTCAA
TAGTCAGTTACTGATGCTCCTGAACCCTATGTGTCCATTTCTGCACACACGTATACCTCG
GCATGGCCGCGTCACTTCTCTGATTATGTGCCCTGGCCAGGGACCAGCGCCCTTGCACAT
GGGCATGGTTGAATCTGAAACCCTCCTTCTGTGGCAACTTGTAAGTAAATCTGGTGCTC
AATAAAGAAGCCCATGGCTGGTGGCATGCAA

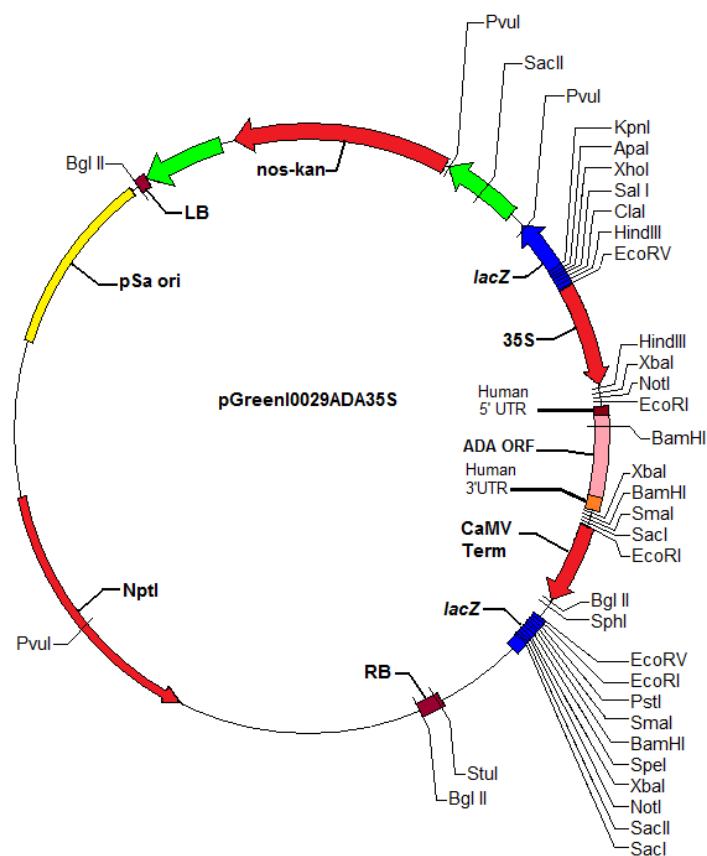
```

Figure 3.1: Human ADA cDNA, DNA sequence in blue, depicts the open reading frame of ADA. Sequence in green before ATG start codon depicts 129 bp 5' UTR region whereas sequence in red depicts 345 bp 3' UTR (Wiginton *et al.*, 1983).

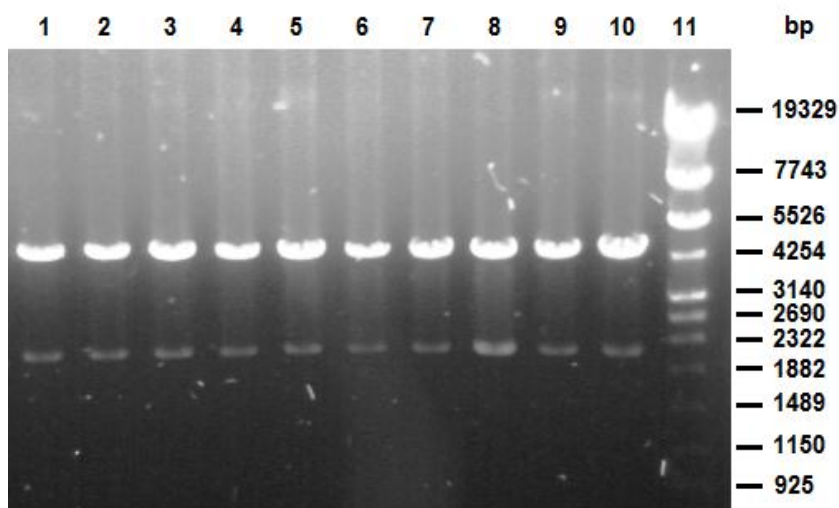


**Figure 3.2:** (a) A human cDNA clone for ADA inserted into pJIT *XbaI* sites. Downstream of the 35S CaMV promoter (b) SafeView<sup>TM</sup> stained 1% agarose gel, lanes 1 to 10 *XbaI* digested pJITADA; lane 11: Roche molecular weight marker IV.





(a)



(b)

Figure 3.3: (a) Human ADA cDNA including 35S CaMV promoter and terminator inserted into the *EcoRV* site of pGreenI0029 (b) SafeView<sup>TM</sup> stained 1% agarose gel, lanes 1 to 10: *EcoRV* digested pGreenI002935SADA; lane 11: Roche DNA molecular weight marker IV.



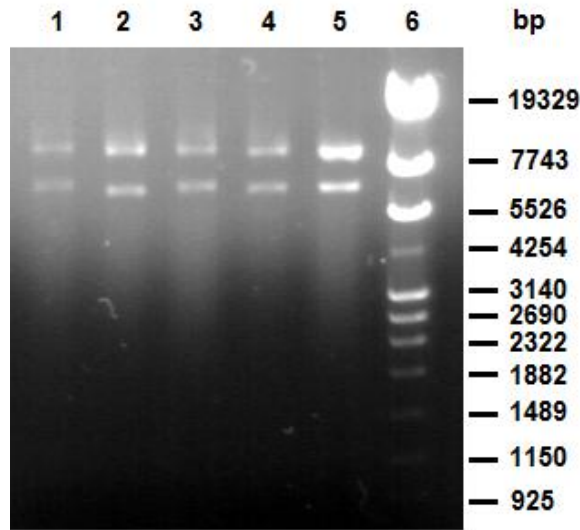


Figure 3.4: SafeView<sup>TM</sup> stained 1% agarose gel, lanes 1 to 5: *SalI* digested pGreenI002935SADA (6193 bp) and pSoup (9274 bp); lane 6: Roche DNA molecular weight marker IV.

### 3.2.2 Minimal 5' UTR (pΔ 5' UTR\_ADA) construct design

The minimal 5' UTR, containing 21 bp was engineered between the 35S CaMV promoter and the ADA ATG initiation codon (figure 3.5). To make this construct, ADA was PCR amplified from pCMV6-ADA cDNA using a forward primer (5'GAAA**TCTAGA**AAA**ATG****GCCCAGACG**3') and reverse primer (5'CTTT**GAATTCTCA****GAGGTTCTGCCCT**3'). Primers were designed to contain an *XbaI* (TCTAGA) restriction site, an ATG initiation codon followed by the next 9 nucleotides of the ADA ORF cDNA sequence in the forward primer. The reverse primer included an *EcoRI* (GAATTC) restriction site, a TCA complimentary stop codon and 13 nucleotides from the 5' end of the complementary ORF of ADA.

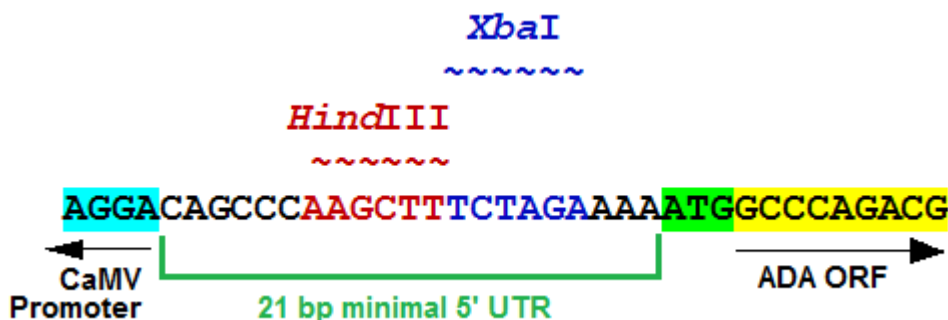


Figure 3.5: Schematic diagram depicting minimal 5' UTR configuration.

PCR-amplified ADA (PCRADA) was digested with *XbaI/EcoRI*, gel-extracted and inserted into *XbaI/EcoRI* digested pJIT in between 35S promoter and terminator cassette (figure 3.6). The resulting construct was transformed into *E.coli* and transformants were selected. Several ampicillin-resistant colonies were chosen and pJIT-Δ 5' UTR-PCRADA was extracted. The insertion of PCR-ADA into pJIT was confirmed by restriction enzyme mapping using *XbaI* and *EcoRI* to release PCR-ADA from pJIT.

Finally, the construct Δ 5' UTR-PCRADA in pJIT, along with the 35S cassette (figure 3.6) was inserted into pGreenI0029 T-region by digestion of pJIT-Δ 5' UTR-PCRADA and pGreenI0029 with *EcoRV*, followed by gel- extraction and ligation of vector and insert DNA. *E.coli* DH5α was transformed with the ligated pGreenI0029-35S-Δ 5' UTR-PCRADA and transformants were selected. The pGreenI0029-35S-Δ 5' UTR-PCRADA plasmid was isolated and the insertion of 35S-Δ 5' UTR-PCRADA into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35S-Δ 5' UTR-PCRADA from pGreen.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029-35S-Δ 5' UTR-PCRADA along with the helper plasmid pSoup as described in section 2.4.1.

Transformants were selected and transformation was confirmed by mapping of plasmid isolates from *Agrobacterium* transformants using *Sall*.

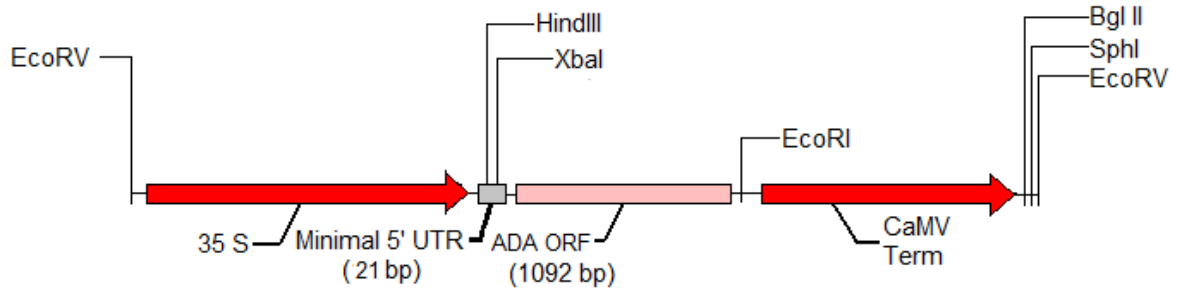


Figure 3.6: Schematic diagram of the gene construct CaMV promoter- pΔ 5' UTR\_ADA.

### 3.2.3 TMV Omega 5' UTR (p5' Omega\_ADA) construct design

A synthetically synthesized 5' TMV Omega ( $\Omega$ ) sequence (Gallie, 2002) (MWG-Biotech AG) (Figure 3.7) containing 80 bp and *HindIII*/*XbaI* restriction sites was inserted upstream (into *HindIII* and *XbaI* sites) of the ADA ORF in pΔ 5' UTR\_ADA construct (figure 3.6). The resulting construct was transformed into *E.coli* and transformants were selected. Several ampicillin-resistant colonies were chosen and putative pJIT-5'  $\Omega$ -PCRADA transformants were extracted for plasmid DNA. The insertion of 5'  $\Omega$  into pΔ 5' UTR\_ADA was confirmed by restriction enzyme mapping using *HindIII* and *EcoRI* to release 5'  $\Omega$ -PCRADA from pJIT, and further confirmed using DNA sequence analysis.

AAGCTTGTATTTTACAACAATTACCAACAACAACAACAACAACATTACAATTACTATTTACAATTACATCTAGA  
*HindIII* *XbaI*

Figure 3.7: Tobacco Mosaic Virus (TMV) 5'Omega UTR DNA sequence with *HindIII* and *XbaI* restriction enzyme sites (Gallie *et al.*, 1987).

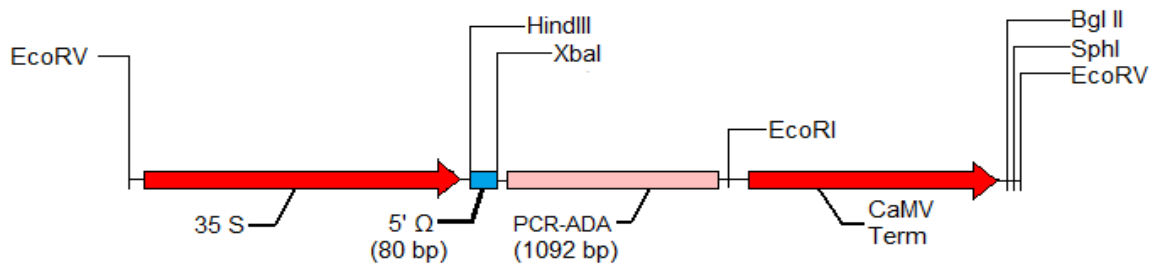


Figure 3.8: A PCR-amplified ADA cDNA and 5'Ω TMV UTR DNA inserted into pJIT, downstream of the 35S CaMV promoter (p5' Omega\_ADA construct).

Finally, the construct p5' Omega\_ADA in pJIT, along with the 35S cassette (figure 3.8) was inserted into pGreenI0029 T-region by digestion of p5' Omega\_ADA and pGreenI0029 with *EcoRV*, followed by gel- extraction and ligation of vector and insert DNA. *E.coli* DH5α was transformed with the ligated pGreenI0029-35S-5'Ω-PCRADA and transformants were selected. The pGreenI0029-35S-5'Ω-PCRADA plasmid was isolated and the insertion of 35S-5'Ω-PCRADA into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35S-5'Ω-PCRADA from pGreen.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029-35S-5'Ω-PCRADA along with the helper plasmid pSoup. Transformants were selected and transformation was confirmed by remapping of plasmid isolates from *Agrobacterium* transformants using *SalI* restriction enzyme.

### 3.2.4 TMV Omega 5' and 3' UTR (p5'\_3' Omega\_ADA) construct design

A synthesized 3' TMV Omega ( $\Omega$ ) sequence (Gallie, 2002) (from MWG-Biotech AG) (figure 3.8) containing 204 bp and *EcoRI* restriction sites was inserted downstream (into *EcoRI* site) of p5'\_Omega\_ADA (figure 3.10). The resulting construct was transformed into *E.coli* and transformants were selected. Several ampicillin-resistant colonies were chosen and putative p5'\_3' Omega\_ADA plasmids were extracted. The insertion of 3' UTR into p 5'\_Omega\_ADA was confirmed by restriction enzyme mapping using *EcoRI* to release 5'  $\Omega$ -PCRADA-3'UTR from pJIT and further confirmed using DNA sequence analysis.

*EcoRI*  
**GAATTC**GGTAGTCAAGATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGC  
 ATAGTGTTTTCCCTCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATA  
 TACATCCGCAGGCACGTAATAAAGCGAGGGGTTCTGAATCCACCCGTTACCCCCGGTAGGGGCC**GAATTC**  
*EcoRI*

Figure 3.9: Tobacco Mosaic Virus (TMV) 3' un-translated region DNA sequence (Gallie and Walbot, 1990).

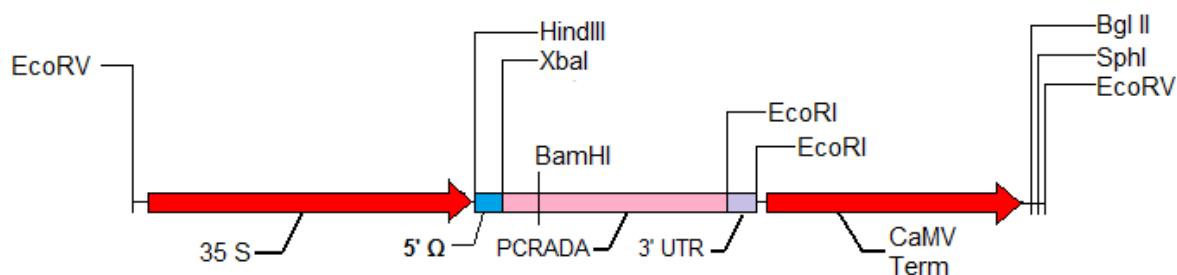


Figure 3.10: TMV  $\Omega$  3'UTR inserted into *EcoRI* site of p5'\_Omega\_ADA construct.

Finally, the construct p5'\_3'Omega\_ADA, along with the 35S cassette was inserted into pGreenI0029 T-region by digestion of p5'\_3'Omega\_ADA construct in pJIT and pGreenI0029 with *EcoRV*, followed by gel-extraction and ligation of vector and insert DNA. *E.coli* DH5 $\alpha$

was transformed with the ligated pGreenI0029-p5' \_3'Omega\_ADA construct and the transformants were selected. The pGreenI0029 plasmid containing p5' \_3'Omega\_ADA construct was isolated and the insertion of p5' \_3'Omega\_ADA construct into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35S-5'Ω-PCRADA from pGreen.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029-35S5' \_3'Ω-PCRADA along with the helper plasmid pSoup. Transformants were selected and transformation was confirmed by remapping of plasmid isolates from *Agrobacterium* transformants using *SalI* restriction enzyme.

### 3.2.5 RUBISCO small subunit 5' UTR (pSS 5'UTR\_ADA) construct design

To engineer pSS 5'UTR\_ADA a synthetically synthesized 5' RUBISCO small subunit DNA sequence (Mazur and Chui, 1985) (figure 3.11) containing 128 bp and *HindIII*/*XbaI* restriction sites was inserted up-stream of the PCR amplified ADA in pΔ5'UTR\_ADA (figure 3.12). The resulting construct was transformed into *E.coli* and transformants were selected. Several ampicillin-resistant colonies were chosen and putative pSS 5'UTR\_ADA transformants were extracted. The insertion of 5' RUBISCO small subunit sequence into pΔ5'UTR\_ADA was confirmed by restriction enzyme mapping using *HindIII* and *EcoRI* to release 5' SS UTR-PCRADA from pJIT and further confirmed using DNA sequence analysis.

***HindIII***

5' **AAGCTT**GAATATGTAAAACCTTATCATTATATATAGAGTGGTGGGCAACTATGCAATGACCATCTTGGA  
AGTTTAAAGGAAAAAAGGAAAGGGAGAAAGAGAAATCTTCTGTCTTAAAGTGTAATTAACA**CTAGA**3'  
***XbaI***

Figure 3.11: 5' Tobacco RUBISCO small subunit 5' UTR DNA sequence (Mazur and Chui, 1985).

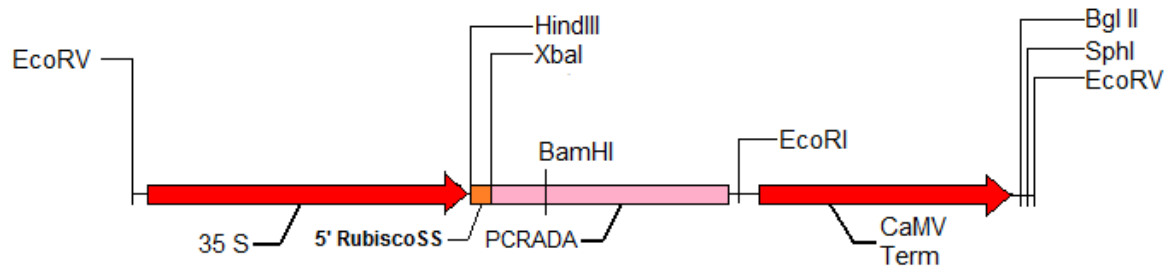


Figure 3.12: A RUBISCO small subunit 5'UTR inserted into p $\Delta$ 5'UTR\_ADA construct (pSS 5'UTR\_ADA construct).

Finally, the construct pSS 5'UTR\_ADA, along with the 35S cassette (figure 3.12) was inserted into pGreenI0029 T-region by digestion of pSS 5'UTR\_ADA construct in pJIT and pGreenI0029 with *EcoRV*, followed by gel-extraction and ligation of vector and insert DNA. *E.coli* DH5 $\alpha$  was transformed with the ligated pGreenI0029- pSS 5'UTR\_ADA construct and the transformants were selected. The pGreenI0029 plasmid containing pSS 5'UTR\_ADA construct was isolated and the insertion of pSS 5'UTR\_ADA construct into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35S-5'SS UTR-PCRADA from pGreen.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029- 35S-5'SS UTR-PCRADA along with the helper plasmid pSoup. Transformants were selected and transformation confirmed by re-mapping of plasmid isolates from *Agrobacterium* transformants using *SalI*.

### 3.3 Constructs designed to increase levels of human ADA in tobacco by directing recombinant protein to the apoplast

**Table 3.2: Subcellular targeting constructs.**

Constructs	Code
TMV Omega 5'UTR PR1a	p5'Omega-PR1a_ADA
TMV Omega 5'UTR Extensin	p5'Omega-Extensin_ADA
3'Hydroxyproline	p5'Omega-Extensin_ADA-3'Hydroxyproline

#### 3.3.1 TMV Omega 5'UTR PR1a and TMV Omega 5'UTR Extensin (p5'Omega-PR1a\_ADA and p5'Omega-Extensin\_ADA) construct design

A PR1a-ADA transcriptional fusion sequence, including 87 bp coding for the *N*-terminal portion of PR1a (Ziegler *et al.*, 2000) and the first 20 5'-terminal codons of the ADA ORF was synthesized (figure 3.13). PR1a-ADA was designed to contain from 5' end an *Xba*I site, an ATG initiation codon, 87 bp of PR1a sequence and the first 60 bp of 5' ADA ORF sequence up to an internal *Bam*HI site in the ADA ORF (figure 3.13). To insert the PR1a sequence 5' to the ADA ORF, the PR1a-ADA fusion sequence was digested with *Xba*I/*Bam*HI, gel-extracted and inserted into *Xba*I/*Bam*HI-digested p5'Omega\_ADA in pJIT (figure 3.15a). *E.coli* DH5 $\alpha$  was transformed with p5'Omega-PR1a\_ADA and transformants were selected. Several ampicillin-resistant colonies were chosen and putative p5'Omega-PR1a\_ADA plasmids were extracted. The insertion of PR1a-ADA fusion into p5'Omega\_ADA was confirmed by DNA sequence analysis and restriction enzyme mapping using *Hind*III and *Eco*RI to release 5' Omega-PR1a-PCRADA from pJIT.

Similarly an extensin-ADA fusion sequence, including a 5' signal sequence from tobacco extensin (Loose *et al.*, 1990) and the 5' end of the ADA ORF sequence up to an internal *Bam*HI site was synthesized (figure 3.14). The extensin-ADA was designed to contain a 5'



*Xba*I site, an ATG initiation codon, 75 bp 5' tobacco extensin sequence and the first 60 bp of 5' ADA ORF sequence up to an internal *Bam*HI site (figure 3.14). To insert the extensin sequence 5' to the ADA ORF, the extensin-ADA fusion sequence was digested with *Xba*I/*Bam*HI, gel-extracted and inserted into *Xba*I/*Bam*HI digested, gel-extracted p5'Omega\_ADA in pJIT (figure 3.15b). *E.coli* DH5 $\alpha$  was transformed with p5'Omega-Extensin\_ADA and transformants were selected. Several ampicillin-resistant colonies were chosen and putative p5'Omega-Extensin\_ADA plasmids were extracted. The insertion of Extensin-ADA fusion into p5'Omega\_ADA was confirmed by DNA sequence analysis and restriction enzyme mapping using *Hind*III and *Eco*RI to release 5' Omega-Extensin-PCRADA from pJIT.

The pJIT constructs, p5'Omega-PR1a\_ADA and p5'Omega-Extensin\_ADA were separately isolated by *Eco*RV digestion of pJIT5'Omega-PR1a\_ADA and pJIT5'Omega-Extensin\_ADA prior to gel-extraction. Isolated 5'Omega-PR1a\_ADA and 5'Omega-Extensin\_ADA fragments were separately inserted into *Eco*RV digested and gel-extracted pGreenI0029 T-region. *E.coli* DH5 $\alpha$  was separately transformed with pGreenI0029-5'Omega-PR1a\_ADA and pGreenI0029-5'Omega-Extensin\_ADA and transformants selected. Transformed colonies were grown separately and putative pGreenI0029-5'Omega-PR1a\_ADA and pGreenI0029-5'Omega-Extensin\_ADA plasmids extracted. Insertion of constructs into plasmids was confirmed by restriction enzyme digestion of both the plasmids with *Eco*RV followed by agarose gel electrophoresis.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed separately with pGreenI0029-5'Omega-PR1a\_ADA and pGreenI0029-5'Omega-Extensin\_ADA along with the helper plasmid pSoup. Transformants were selected and transformation was confirmed by mapping of plasmid isolates from *Agrobacterium* transformants using *Sal*I.



Figure 3.13: Synthesized PR1a-ADA fusion containing 87 bp coding for the N-terminal portion of PR1a and the first 60 bp of 5' ADA ORF sequence up to an internal *Bam*HI site.

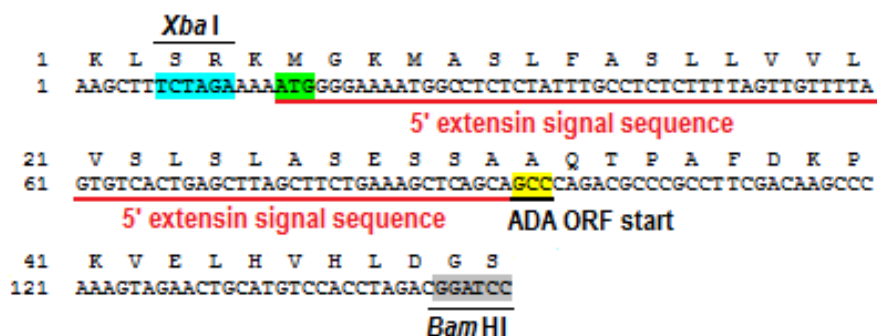
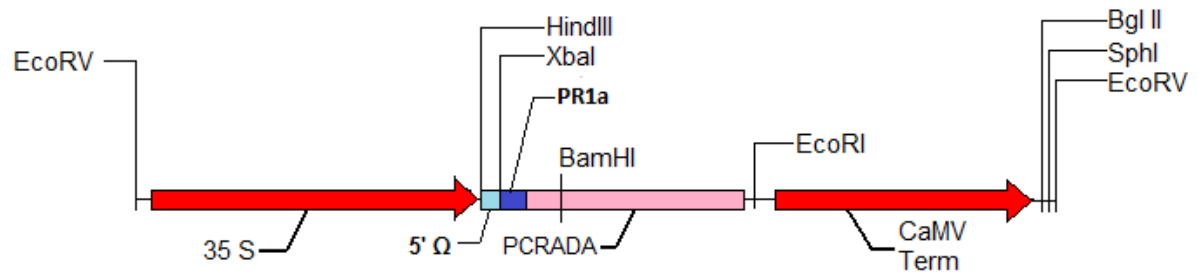
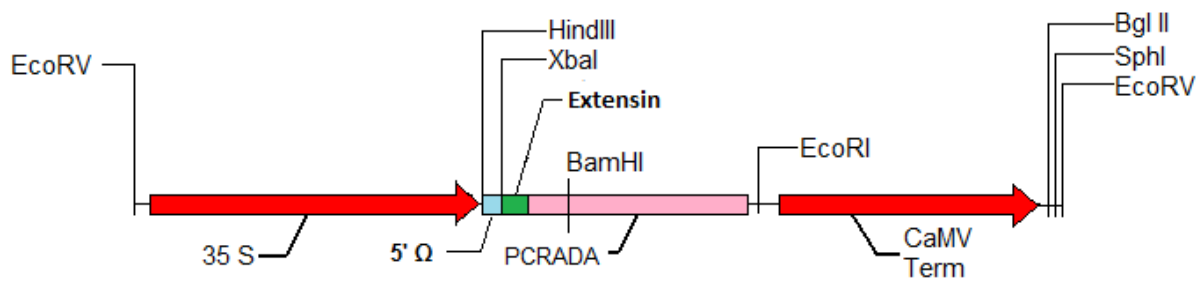


Figure 3.14: Synthesized extensin-ADA fusion containing 75 bp 5' tobacco extensin sequence and the first 60 bp of 5' ADA ORF sequence up to an internal *Bam*HI site.



(a)



(b)

Figure 3.15: (a) Tobacco PR1a signal sequence inserted into p5' Omega\_ADA construct (p5'Omega-PR1a\_ADA construct), (b) Tobacco extensin signal sequence inserted into p5' Omega\_ADA construct (p5'Omega-Extensin\_ADA construct).

### 3.3.2 3’Hydroxyproline (p5’Omega-Extensin\_ADA-3’Hydroxyproline) construct design

A partial ADA ORF- 3’serine-hydroxyproline fusion sequence (Xu *et al.*, 2007) was designed and synthesized (figure 3.16) containing 346 bp from 3’ end of the ADA ORF sequence up to an internal *PsiI* site, a thrombin cleavage sequence, a 63 bp serine-hydroxyproline sequence and a TAG stop codon. A *PsiI* restriction enzyme site (TTATAA) was placed at the 5’ end, and a *SacI* restriction enzyme site (GAGCTC) was placed at the 3’ end. The ADA-3’Hydroxyproline fusion sequence was digested with *PsiI/SacI* and gel-extracted. The ADA-3’Hydroxyproline fragment was inserted into *PsiI/SacI* digested and gel-extracted pCDNA\_ADA in pJIT (figure 3.2a). *E.coli* DH5 $\alpha$  was transformed with pCDNA\_ADA-3’Hydroxyproline and transformants were selected. Several ampicillin-resistant colonies were chosen and the insertion of ADA-3’Hydroxyproline fusion into pCDNA\_ADA was confirmed by DNA sequence analysis and restriction enzyme mapping using *HindIII* and *SacI* to release CDNA\_ADA-3’Hydroxyproline from pJIT.

To engineer an apoplast directing sequence at the 5’ end the construct p5’Omega-Extensin\_ADA (figure 3.15b) was digested with *HindIII/BamHI* and 5’Omega-Extensin-ADA fusion was gel-extracted and inserted into *HindIII/BamHI* digested and gel-extracted pCDNA\_ADA-3’Hydroxyproline. *E.coli* DH5 $\alpha$  was transformed with p5’Omega-Extensin\_ADA-3’Hydroxyproline (figure 3.17) and transformants were selected. Several ampicillin-resistant colonies were chosen and p5’Omega-Extensin\_ADA-3’Hydroxyproline in pJIT was isolated. The insertion of 5’Omega-Extensin-ADA fusion into pCDNA\_ADA-3’Hydroxyproline was confirmed by restriction enzyme mapping using *HindIII* and *SacI* to release p5’Omega-Extensin\_ADA-3’Hydroxyproline from pJIT.

Finally, the construct p5'Omega-Extensin\_ADA-3'Hydroxyproline along with 35S cassette was inserted into pGreenI0029 T-region by digestion of p5'Omega-Extensin\_ADA-3'Hydroxyproline construct in pJIT and pGreenI0029 with *EcoRV*, followed by gel-extraction and ligation of vector and insert DNA. *E.coli* DH5 $\alpha$  was transformed with the ligated pGreenI0029-p5'Omega-Extensin\_ADA-3'Hydroxyproline construct and the transformants were selected. The pGreenI0029 plasmid containing p5'Omega-Extensin\_ADA-3'Hydroxyproline construct was isolated and the insertion of p5'Omega-Extensin\_ADA-3'Hydroxyproline construct into pGreenI0029 was confirmed by restriction enzyme mapping using *EcoRV* to release 35S-5'Omega-Extensin\_ADA-3'Hydroxyproline from pGreen.

Electrocompetent *Agrobacterium tumefaciens* AGL1 were transformed with pGreenI0029-35S-5'omega-Extensin\_ADA-3'Hydroxyproline along with the helper plasmid pSoup. Transformants were selected and transformation was confirmed by mapping of plasmid isolates from *Agrobacterium* transformants using *SalI*.

```

Y N R L R Q E N M H F E I C P W S S Y L
TATAA CAGGCTGCGGCAGGAAAACATGCATTTCGAGATCTGCCCTGGTCCAGCTACCTC
PsiI

T G A W K P D T E H A V I R L K N D Q A
ACTGGTGCCTGGAAGCCGGACACGGAGCATGCAGTCATTTCGGCTCAAAAATGACCAGGCT

N Y S L N T D D P L I F K S T L D T D Y
AACTACTCGCTCAACACAGATGACCCGCTCATCTTCAAGTCCACCCTGGACACTGATTAC

Q M T K R D M G F T E E E F K R L N I N
CAGATGACCAAACGGGACATGGGCTTTACTGAAGAGGAGTTTAAAAGGCTGAACATCAAT

A A K S S F L P E D E K R E L L D L L Y
GCGGCCAAATCTAGTTTCTCCAGAGATGAAAAGAGGGAGCTTCTCGACCTGCTCTAT

K A Y G M P P S A S A G Q N L V P R G S
AAAGCCTATGGGATGCCACCTTCAGCCTCTGCAGGGCAGAACCTCGTCCCAAGGGGGTCA
Thrombin cleavage site
P S P S P S P S P S P S P S P S P S P S P S P -
CCCTCTCCAAGCCCTTCCCATCGCCTAGTCCCTCACCATCCCCTTCTCCAGCCCA TAG
3' serine-hydroxyproline repeats

E L G
GAGCTCGGGG
SacI

```

Figure 3.16: ADA-3' Hydroxyproline fusion containing a *PsiI* restriction enzyme site, 346 bp from 3' end of the ADA ORF sequence, a thrombin cleavage sequence, a 63 bp serine-hydroxyproline sequence, a TAG stop codon and a *SacI* restriction enzyme site.

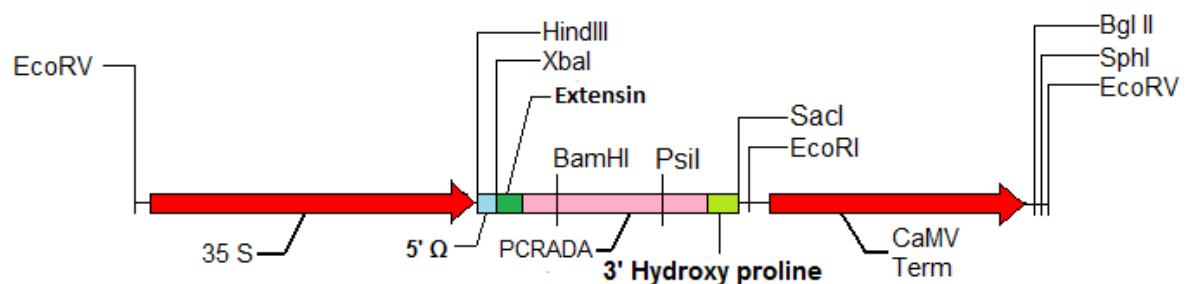


Figure 3.17: p5'Omega-Extensin-ADA-3'Hydroxyproline construct.

Tobacco plants were transformed with the cytosolic constructs and tobacco BY-2 suspensions were transformed with both cytosolic and sub-cellular targeting constructs, using *Agrobacterium* mediated transformation. The transformants were selected after 6-8 weeks of transformation and ADA enzyme-activity of stable transformants were measured (section 2.5.2).

## **Chapter 4**

# **Cytosolic expression of human adenosine deaminase in transgenic tobacco plants and BY-2 calli**



## 4.1 Overview

Tobacco (*Nicotiana tabacum*) is the most widely used organism for biopharmaceutical production in plants owing to well established properties of gene transfer, expression analysis; rapid scale-up potential and high biomass yield (Fischer & Emans, 2000). Stable nuclear transformation is broadly used for production of heterologous proteins and recombinant proteins are generally extracted from leaves. In addition secretory pathway-targeting has been used for excretion of recombinant proteins from roots, leaves and cell suspensions (Komarnytsky *et al.*, 2000). Expression of foreign proteins in plant cell suspensions possess some advantages over whole plants owing to their rapid growth due to short cell doubling times, improved consistency of protein products due to less variation in environmental conditions with controlled bioreactor use and most importantly reduced post-translational silencing effects due to dedifferentiated cells that lack fully functional plasmodesmata (Lee *et al.*, 2000; Su and Lee, 2007).

Adenosine deaminase is found in numerous organisms ranging from bacteria to mammals and catalyses the conversion of cytotoxic adenosine analogues into their non-toxic inosine analogues. However, ADA has been found to be absent in plants (Dancer *et al.*, 1997). In plants, toxic adenosine analogues are converted into non-toxic analogues either by adenosine kinase or by phosphoribosyltransferase (Pelcher, 1995). Absence of ADA in plants provides an excellent platform to produce recombinant ADA in plants.

In this chapter, the expression of recombinant ADA in nuclear transformed plants and calli has been investigated. *Nicotiana tabacum* plants and BY-2 cells were transformed with various ADA constructs designed to express the enzyme in the cytosol. The resulting transgenic plants and calli were assessed for ADA mRNA and enzyme expression levels.

## 4.2 Results

### 4.2.1 Cytosolic expression in transformed plants

*Nicotiana tabacum* plants transformed with ADA cytosolic constructs were assessed for both ADA mRNA and enzyme expression. A non-transformed tobacco plant was also assayed. Young, medium and mature 5-6-week-old leaves of transformed plants were flash frozen in liquid nitrogen, ground to a fine powder and total soluble proteins extracted as described in section 2.5.1. The expression of ADA in plant extracts was tested by adenosine deaminase assay (section 2.5.2) to determine ADA levels and a Bradford assay used to determine total soluble proteins (section 2.5.3). Up to 7 transgenic plants transformed with three cytosolic constructs were analysed for ADA enzyme-activity (figure 4.1 and figure 4.2). ADA specific-activities in Units per milligram ( $\text{U mg}^{-1}$ ) TSP were calculated using ADA-activity and Bradford protein assay data of corresponding transformants. ADA specific-activities of young, medium and mature leaves were measured initially to investigate age-specific activity. An average value was calculated from ADA specific-activities of young, medium and mature leaves, as no consistent correlation of ADA expression was observed with age (figure 4.1). Expression levels of plants transformed with construct p $\Delta$ 5'UTR\_ADA, containing PCR-amplified ADA and a short 21 bp 5' UTR sequence was used as a basal construct to compare the expression levels of two other cytosolic constructs. The mean value for ADA-activity measured in these plants was  $9.1 \times 10^{-6} \text{ U mg}^{-1} \text{ TSP}$ , barely above a background activity of  $1.73 \times 10^{-7} \text{ U mg}^{-1} \text{ TSP}$  measured in non-transformed plants. Expression levels of the same transgene in independent transformants do not assume a normal distribution. Therefore, a distribution-free statistical test, namely the Mann-Whitney  $U$  test, which is based on median values, was used to evaluate difference between two gene-constructs. The  $U$  values were compared with a critical region table indicating the differences at 5 % as shown in table 4.1.

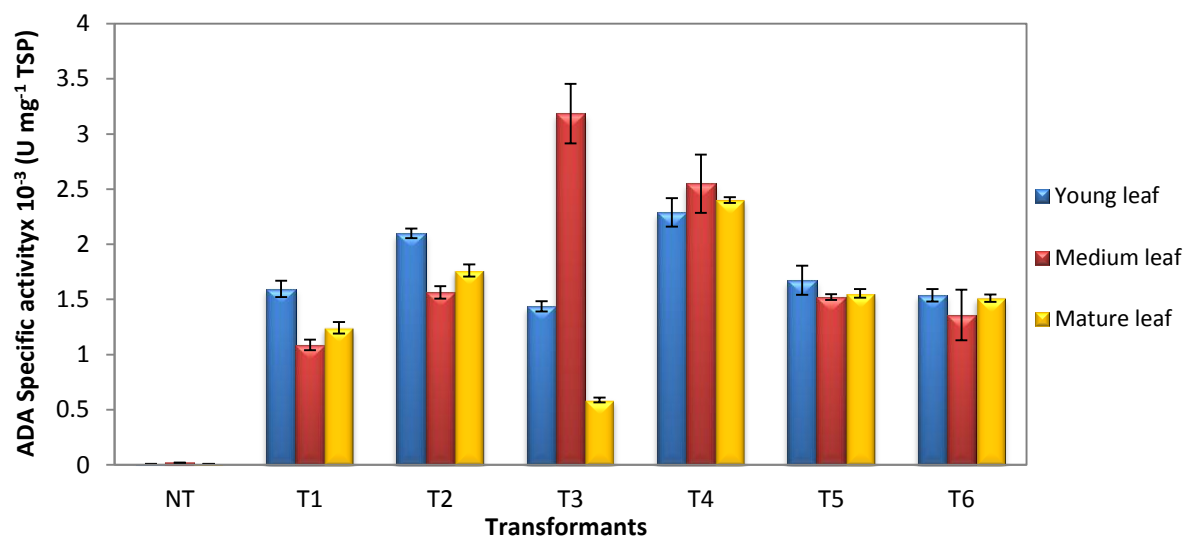


Figure 4.1: ADA specific-activities measured in the leaves of 6 transgenic plant lines transformed with pCDNA\_ADA cytosolic construct.

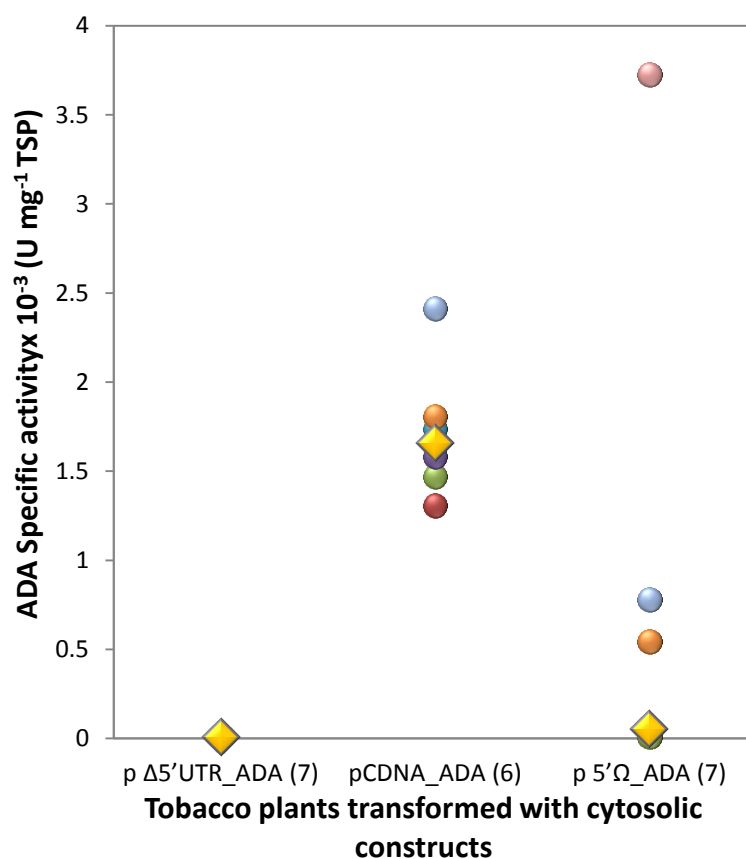


Figure 4.2: ADA specific-activities measured in tobacco plants transformed with various cytosolic constructs. Each circle represents ADA-activity from an independent transformant, and the yellow diamond denotes the median value. The number of plants assayed is shown in brackets after each construct.

**Table 4.1: Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco leaves using Mann-Whitney U test (5 % level).**

Constructs	Population identity	p-Value
pΔ5'UTR_ADA versus pCDNA_ADA	Different	$P < 0.05 = 0.0027$
pΔ5'UTR_ADA versus p5'Ω_ADA	Different	$P < 0.05 = 0.0088$
pCDNA_ADA versus p5'Ω_ADA	Different	$P < 0.05 = 0.0321$

**Table 4.2: Pair-wise comparisons of median ADA specific-activities measured in transgenic tobacco leaves.**

Constructs	Ratio of median ADA specific-activities	Fold increase median expression	Fold increase of highest expressing plants
pΔ5'UTR_ADA versus pCDNA_ADA	$9.13 \times 10^{-6} : 1.66 \times 10^{-3}$	182	273
pΔ5'UTR_ADA versus p5'Ω_ADA	$9.13 \times 10^{-6} : 5.79 \times 10^{-5}$	7	416

A pair-wise comparison of ADA specific-activities measured in transformed tobacco leaves (table 4.1) shows that two of the sample populations are statistically different as compared to plants transformed with construct pΔ5'UTR\_ADA. As shown in figure 4.2 and table 4.1, comparison of the median ADA-activity ratios demonstrates that the presence of the human and TMV omega 5'UTR sequences caused a 7- to 182-fold increase in ADA specific-activity. One outlying transgenic line of p5'Ω\_ADA transformed plants showed the highest ADA specific-activity of  $3.72 \times 10^{-3}$  U mg<sup>-1</sup> TSP (66 ng mg<sup>-1</sup> TSP). The highest ADA specific-activity of a pCDNA\_ADA transformed plant shows  $2.41 \times 10^{-3}$  U mg<sup>-1</sup> TSP (43 ng mg<sup>-1</sup> TSP) (figure 4.2). This is respectively a 416-fold and a 273-fold increase in the ADA specific-activity as compared to the plants transformed with a short 5'UTR construct (table 4.2).

#### 4.2.1.1 Detection of adenosine deaminase protein in transgenic tobacco plants using western blot analysis

Western blot analysis was used to detect adenosine deaminase protein in crude leaf extracts from transgenic tobacco plants. Bovine ADA extracted from calf intestine (100 ng) obtained from Sigma, UK was used as a positive control and 2.1, 2.4 and 2.3 mg of total proteins extracted from respective T1, T2 and T3 transgenic plants, were separated by electrophoresis on a 12 % (w/v) polyacrylamide gel, containing 0.1 % (w/v) SDS as described in section 2.5.4. Gel electrophoresed proteins were transferred to a nitrocellulose sheet (Hybond C) by semi-dry transfer under a constant current of 100 mA for 30 min. The membrane was probed with rabbit anti-ADA antibodies followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The blot (figure 4.3) was developed by Luminol colour substrate solution followed by detection on a Typhoon scanner (Amersham) as described in section 2.5.5. TotalLab Quant software was used to analyse western blots (section 2.5.5). Lanes 3, 4, and 5, of figure 4.3 show that recombinant human ADA has an approximate molecular mass of 42 kDa and bovine ADA in lane 2 of figure 4.3 has a molecular mass of 41 kDa. The reported size of both human and bovine ADA is 41 kDa (Wiginton *et al.*, 1984; Kelly *et al.*, 1996). The apparent 1 kDa size difference of the recombinant ADA could be due to an artefact of the gel system, since large amounts of total proteins are loaded in lane 3-5 compared with lane 2 (figure 4.3). Low molecular weight bands, particularly in lane 5 may be due to protein degradation. High molecular weight bands may be due to some read through of the stop codon to other putative stop codon further downstream (Angenon *et al.*, 1990).

Analysis of the western blot using TotalLab Quant software as described in section 2.5.5, estimated that 1 U of recombinant ADA is equal to approximately 17.7 µg of ADA protein.

This formula was used to calculate approximate estimations of ADA protein amounts in transformed tissues.

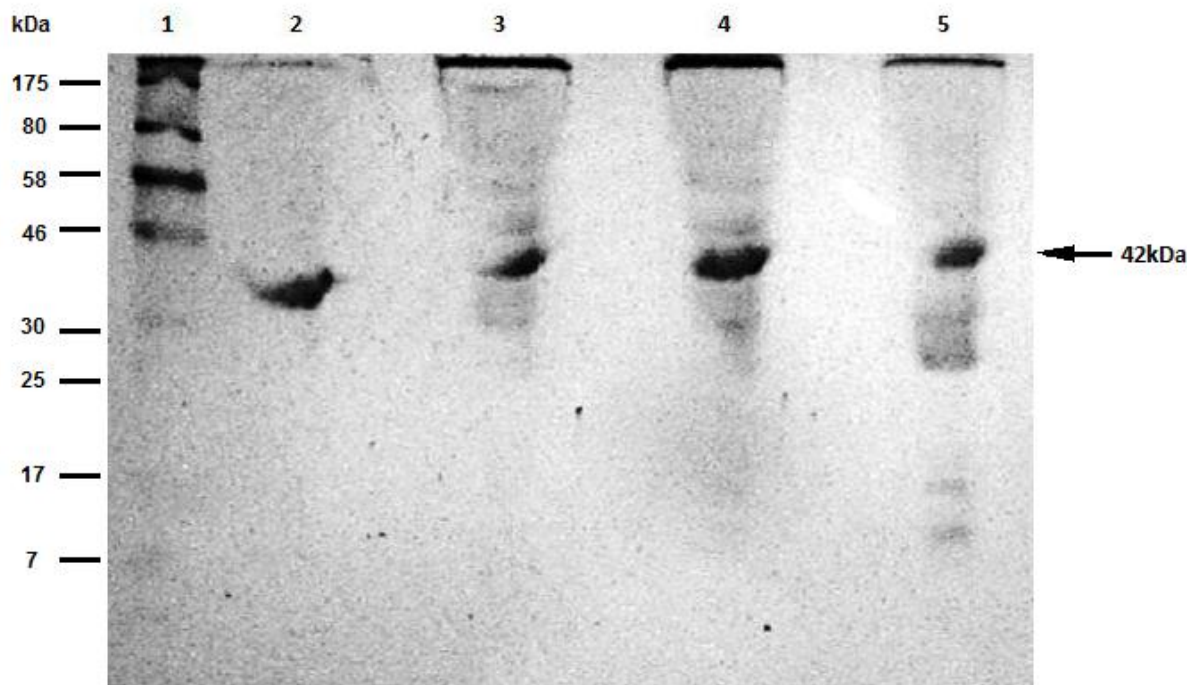


Figure 4.3: Western blot of proteins extracted from the leaves of 3 transgenic tobacco plants transformed with cytosolic construct, pCDNA\_ADA. Lane1: molecular weight marker; lane 2: bovine ADA; lane 3: protein extract from T1 leaves; lane 4: protein extract from T2 leaves and lane 5: protein extract from T3 leaves.

#### 4.2.1.2 Southern blot analysis of genomic DNA from plants transformed with the pCDNA\_ADA construct

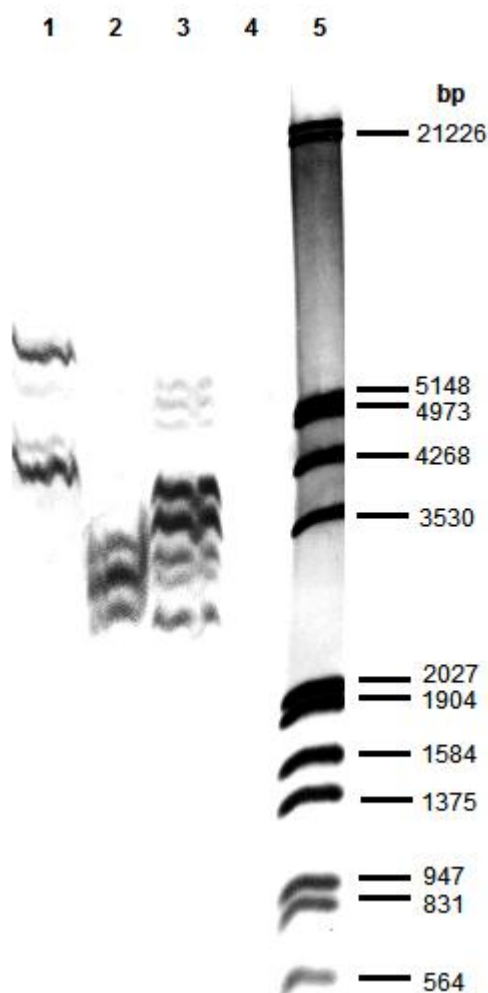
Total DNA was extracted from tobacco leaves of plants transformed with the pCDNA\_ADA construct as described in section 2.2.4. Equal amounts of total DNA (10 µg) from three transformed plants and a non-transformed plant were digested with *KpnI* and *SspI* and fragments separated by electrophoresis on a 0.7 % (w/v) agarose gel. The DNA fragments were transferred to a Hybond-N+ nylon membrane and probed with a DIG-labelled ADA cDNA probe (section 2.2.12.3). High stringency washes (0.1x SSC, 0.1 % (w/v) SDS at 65 °C) were

carried out to remove non-specific nucleic acid binding (section 2.2.12.4). A photographic reproduction of the blot is shown in figure 4.4.

If the inserted construct was a single copy, then the number of expected bands would be one per lane and above 2177 bp in size as there are no internal *KpnI* and *SspI* restriction enzyme sites present on the construct. Hybridization was obtained for 4 bands in lane 1 (fragment sizes: 6.3, 5.6, 4.5, and 4.1 kbp), 3 bands in lane 2 (fragment sizes: 3.2, 2.8 and 2.5 kbp), 8 bands in lane 3 (fragment sizes: 5.7, 5.3, 4.9, 3.9, 3.5, 3.1, 2.9 and 2.5 kbp). There was no specific hybridization in lane 4 (non-transformed plant) as expected and all hybridizing bands were above 2177 bp, the size of the T-DNA region.

Multiple copies of ADA cDNA on the blot can be explained by the random integration of T-DNA into the plant genome by *Agrobacterium*-mediated transformation (DeBeuckeleer *et al.*, 1981). Although the T1 plant genome exhibited the highest copy number (at least 8 copies), it does not correlate with higher ADA expression levels, where the T2 transformant had the highest ADA specific-activity ( $1.81 \times 10^{-3}$  U mg<sup>-1</sup> TSP; table 4.3). The darker bands in figure 4.4 could be due to insertion of tandem arrays of the insert. Hence, gene copy number may be greater than the number of hybridizing bands. The lack of correlation of copy number to ADA enzyme expression levels could be due to integration of the T-DNA inserts into either transcriptionally competent or silent regions of the plant genome (Springer *et al.*, 1995) or alternatively, transgene silencing by DNA methylation (Hepburn *et al.*, 1983). Transgene silencing can also occur post-transcriptionally due to instability of transcribed RNA (Meins F, 2000). Furthermore, *Agrobacterium*-mediated transformation is often associated with transfer of tandem copies of T-DNA at a single locus and it was also found that head to head inverted

T-DNA repeats around the right border are also frequently associated with transgene silencing (Stam *et al.*, 1997).



**Figure 4.4:** Southern blot of genomic DNA (10 µg) of transformed (pCDNA\_ADA) and non-transformed tobacco plant leaves. Lane 1: T3 DNA, Lane 2: T2 DNA, Lane 3: T1 DNA, Lane 4: Non-Transformed plant DNA, and Lane 5: Roche DNA molecular weight marker III.



**Table 4.3: Genome T-DNA copy number and ADA specific-activities of transgenic tobacco plants transformed with the pCDNA\_ADA construct.**

Construct	ADA specific-activity (U mg <sup>-1</sup> TSP)	Minimum number of hybridizing bands
pCDNA_ADA [T1]	1.31 x10 <sup>-3</sup>	8
pCDNA_ADA [T2]	1.81 x10 <sup>-3</sup>	3
pCDNA_ADA [T3]	1.73 x10 <sup>-3</sup>	4

#### 4.2.1.3 Northern blot analysis of ADA mRNA from plants transformed with the pCDNA\_ADA construct

To investigate any possible correlation of ADA mRNA and enzyme expression levels with ADA insert copy number, RNA extracted from leaves of transformed and non-transformed tobacco plants were analysed on northern blots (section 2.3.1). A photographic record of the blot and the stained ribosomal RNA present on an agarose gel is shown in figure 4.5.

Transgenic cell lines were analysed for mRNA amounts using TotalLab Quant software in order to correlate these values with corresponding T-DNA copies and ADA-activities (table 4.4) measured in the same plants (section 2.3.5). The T2 transformant in lane 5 of figure 4.5 showed the highest ADA mRNA levels (27.2 ng; table 4.4) with an estimated 3 copies of the T-DNA insert. Whereas, the T1 transformant in lane 6 showed the lowest ADA mRNA level (7.4 ng) with an estimated 8 copies of the T-DNA insert. The lower ADA expression level of T1 transformants could be due to the presence of silenced copies of ADA T-DNA inserts or posttranscriptional gene silencing (Que *et al.*, 1997; Schubert *et al.*, 2004).

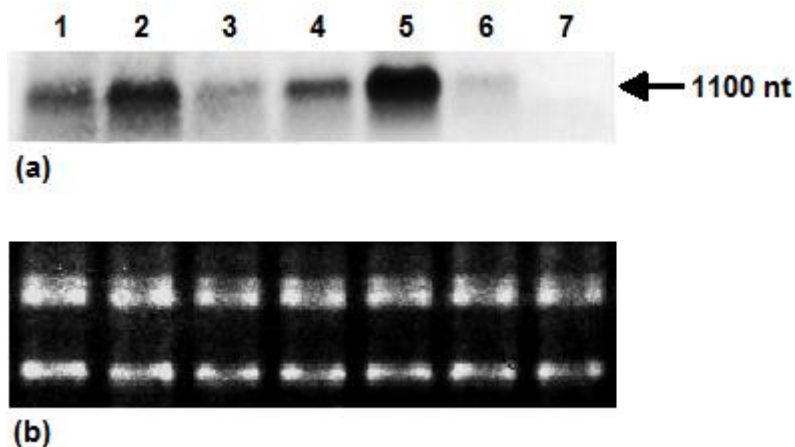


Figure 4.5: (a) Northern blot of total RNA extracted from pCDNA\_ADA transformed and non-transformed tobacco plant leaves. Lane 1: Transformant T6, Lane 2: Transformant T5, Lane 3: Transformant T4, Lane 4: Transformant T3, Lane 5: Transformant T2, Lane 6: Transformant T1 and Lane 7: Non-Transformed plant, (b) SafeView™ stained 2 % agarose gel of rRNA present in lane 1-7.

Table 4.4: ADA specific-activities of tobacco plants transformed with pCDNA\_ADA alongside ADA mRNA amounts measured in northern blots and T-DNA copy number.

Transformant	ADA specific-activity (U mg <sup>-1</sup> TSP)	ADA mRNA (ng)	Estimated genome T-DNA copy numbers
T1	1.31 x10 <sup>-3</sup>	7.4	8
T2	1.81 x10 <sup>-3</sup>	27.2	3
T3	1.73 x10 <sup>-3</sup>	15.7	4
T4	2.41 x10 <sup>-3</sup>	13.1	-
T5	1.58 x10 <sup>-3</sup>	20.1	-
T6	1.47 x10 <sup>-3</sup>	15.5	-

#### 4.2.2 Expression of five ADA cytosolic constructs in tobacco BY-2 calli

Tobacco BY-2 cells are yellow-coloured de-differentiated cells which exhibit rapid growth in culture medium at optimal culture conditions. These cells generally multiply their numbers up to 100-fold within a week and owing to this property and the ability to transfer foreign genes with ease, BY-2 cells are used in many molecular farming experiments. Tobacco BY-2 cells in suspension were transformed with different cytosolic constructs namely: p $\Delta$ 5'UTR\_ADA, pCDNA\_ADA, p5' $\Omega$ \_ADA, p5'\_3'  $\Omega$ \_ADA and pSS 5'UTR\_ADA as described in section 2.4.2. BY-2 calli lines transformed with these constructs were assessed for expression levels of both ADA mRNA and enzyme-activity as described in section 4.2.1. Up to 29 transgenic cell lines of different cytosolic constructs were analysed for ADA enzyme-activity as shown in figure 4.6. A Mann-Whitney statistical test was conducted as described by Pollard (1977). A p $\Delta$ 5'UTR\_ADA construct containing PCR-amplified ADA ORF and a short 21 bp 5' UTR sequence was used as a basal construct to compare other cytosolic constructs. Any two constructs being compared were combined and ranked by ascending expression levels. *U*-values were calculated as describe in section 2.6.

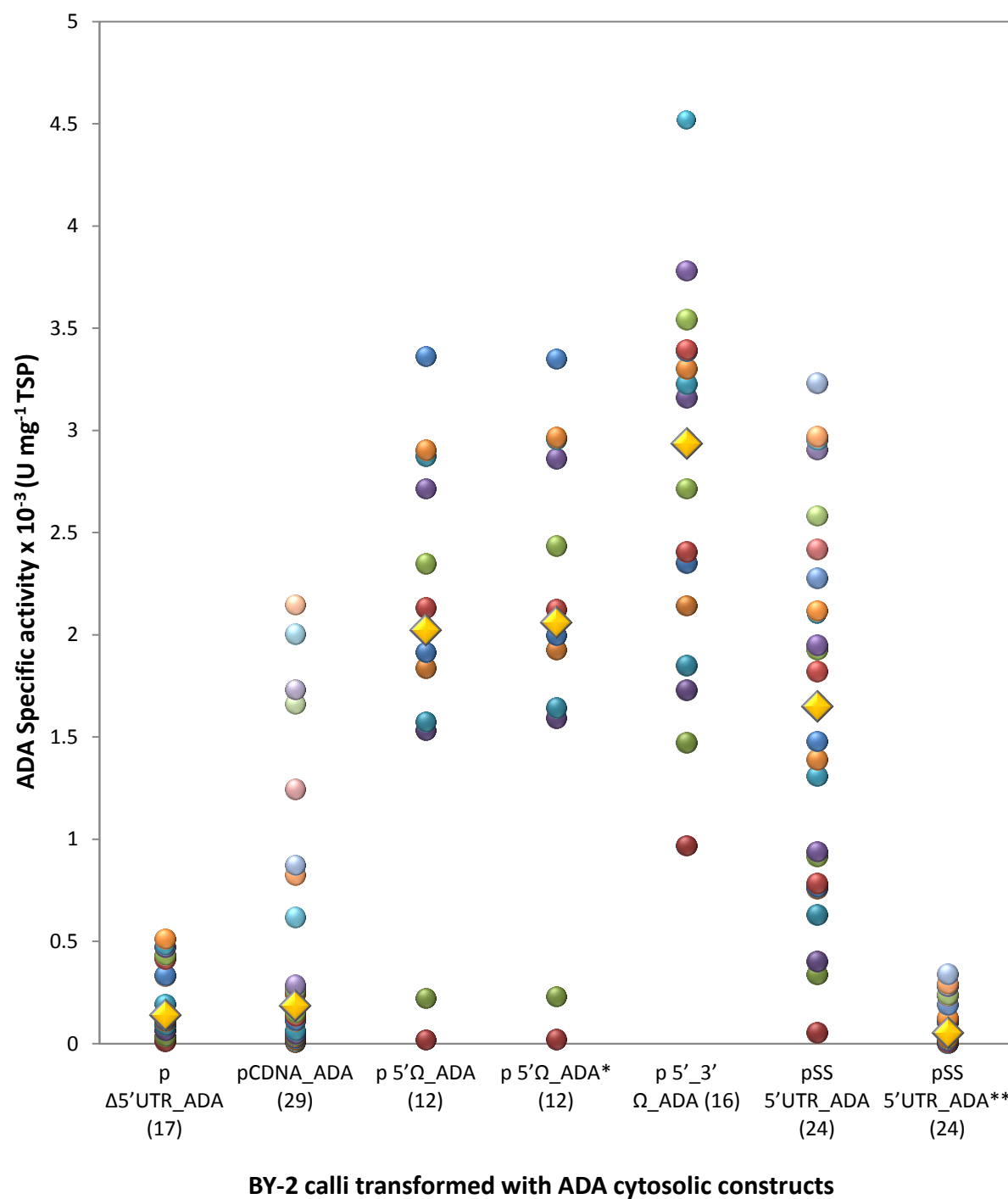


Figure 4.6: ADA specific-activities measured in BY-2 calli transformed with various cytosolic constructs. Each circle represents ADA-activity from an independent transformant. The yellow diamond denotes the median value. The number of calli assayed is shown in brackets after each construct. \* These transformed calli were analysed 9 months after the initial measurements were taken. \*\*These transformed calli were analysed 30 days after the initial measurements were taken.

**Table 4.5: Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco BY-2 calli using the Mann-Whitney U test (5 % level).**

Constructs	Population identity	p-Value
pΔ5'UTR_ADA versus pCDNA_ADA	Same	$p > 0.05 = 0.936515555$
pΔ5'UTR_ADA versus p5'Ω_ADA	Different	$P < 0.05 = 0.000468447$
pΔ5'UTR_ADA versus p5'_3'Ω_ADA	Different	$P < 0.05 = 9.63357 \times 10^{-7}$
pΔ5'UTR_ADA versus pSS 5'UTR_ADA	Different	$P < 0.05 = 2.17074 \times 10^{-6}$
pCDNA_ADA versus p5'Ω_ADA	Different	$P < 0.05 = 0.000424491$
p5'Ω_ADA versus p5'_3'Ω_ADA	Same	$p > 0.05 = 0.051199836$
p5'_3'Ω_ADA versus pSS 5'UTR_ADA	Different	$P < 0.05 = 0.001362336$

**Table 4.6: Pair-wise comparisons of median ADA specific-activities measured in transgenic tobacco BY-2 calli.**

Constructs	Ratio of median ADA specific-activities	Fold increase median expression	Fold increase of highest expressing BY-2 cell line
pΔ5'UTR_ADA versus pCDNA_ADA	$1.43 \times 10^{-4} : 1.87 \times 10^{-4}$	1.3	15
pΔ5'UTR_ADA versus p5'Ω_ADA	$1.43 \times 10^{-4} : 2.02 \times 10^{-3}$	14	25
pΔ5'UTR_ADA versus p5'_3'Ω_ADA	$1.43 \times 10^{-4} : 2.94 \times 10^{-3}$	20.5	32
pΔ5'UTR_ADA versus pSS 5'UTR_ADA	$1.43 \times 10^{-4} : 1.65 \times 10^{-3}$	11.5	22

A pair-wise comparison of ADA specific-activities in transgenic tobacco calli transformed with cytosolic constructs (table 4.5) shows that five out of seven sample populations are statistically non-identical. Comparison of the median ratios of these constructs shows a 1.3 to 20.5-fold increase in ADA specific-activity as compared to calli transformed with pΔ5'UTR\_ADA construct (table 4.6).

Four outlying ADA specific-activities measured in calli transformed with construct p5'\_3'Ω\_ADA showed the highest ADA specific-activities of up to  $4.50 \times 10^{-3}$  U mg<sup>-1</sup> TSP (80 ng mg<sup>-1</sup> TSP). The highest ADA specific-activity of a pCDNA\_ADA, pSS 5'UTR\_ADA

and p5'Ω\_ADA transformed calli showed specific-activities ranging from 2.20 to 3.50 x 10<sup>-3</sup> U mg<sup>-1</sup> TSP (39 to 62 ng mg<sup>-1</sup> TSP) (figure 4.6). This is respectively a 15-fold to 32-fold increase in the ADA specific-activity as compared to the calli transformed with a short 5'UTR construct (table 4.6).

#### **4.2.2.1 Northern blot analysis of transcribed ADA mRNA from tobacco BY-2 calli transformed with five cytosolic constructs**

In order to assess whether ADA mRNA levels correlated with increased levels of ADA enzyme activity, northern blots of transformed tobacco BY-2 calli were performed. Total RNA was extracted as described in section 2.3.1 and equal amounts of RNA (3500 ng) were subjected to electrophoresis under denaturing conditions (section 2.3.2). The RNA was transferred to a Hybond-N+ nylon membrane as described in section 2.3.3 prior to probing with DIG-labelled ADA cDNA (section 2.2.12.3). A photographic reproduction of the blot was taken, as shown in figures 4.7 and 4.8.

As the PCR-amplified ADA ORF is 1092 bp in size, transcribed mRNA which includes 5' and 3' UTR sequences, would be larger than 1092 nucleotides (nt). Observed mRNA sizes were approximately 1100 nt and similar in size in the case of constructs p5'Ω\_ADA and pΔ5'UTR\_ADA (figure 4.7 and 4.8). Transcribed mRNA in construct, pCDNA\_ADA, exhibited a size of 1300 nt. It was assumed that the larger mRNA size in this construct is due to the lengthy 5' and 3' UTR sequences in the human ADA cDNA.

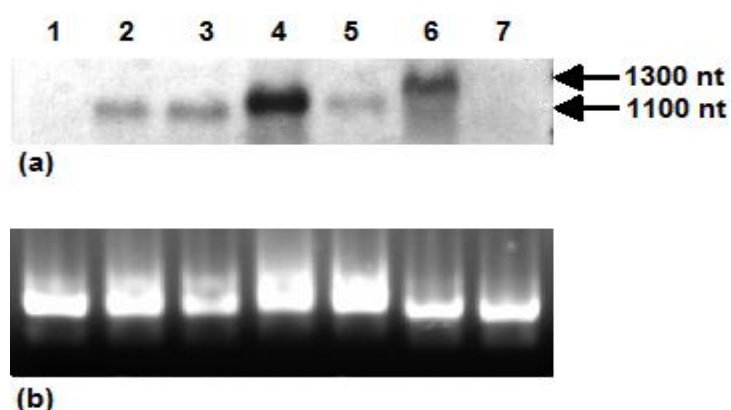
Two transgenic cell lines from each construct were analysed for mRNA levels in order to investigate a possible correlation with their corresponding ADA activities (table 4.7). TotalLab Quant software was used to analyse northern blots (section 2.3.5). Table 4.7 shows ADA

enzyme and mRNA expression levels of constructs used for northern blot analysis. Lane 1 of figure 4.7a, the non-transformed cell line, showed no hybridizing bands. Calli cell lines T3 and T2 transformed with p5'Ω\_ADA in lanes 2 and 3 of figure 4.7a exhibited 2.2 and 3.1 ng of ADA mRNA with respective  $1.84 \times 10^{-3}$  and  $2.91 \times 10^{-3}$  U mg<sup>-1</sup> TSP ADA specific-activities (table 4.7). Whereas one calli cell line transformed with construct, pΔ5'UTR\_ADA [**T15**], showed a significantly higher ADA mRNA level (8.8 ng) but expressed a relatively lower amount of ADA specific-activity ( $4.15 \times 10^{-4}$  U mg<sup>-1</sup> TSP). Lower ADA activities in this construct could be due to the short 5' UTR, which contains only a 21 bp 5' UTR sequence. Pickering and Willis, (2005) demonstrated that most of the regulation in translation occurs at initiation steps in the 5'-untranslated region, which gives rise to the 5' leader sequence in mRNA. Pickering and Willis, (2005) further elaborated the importance of 5' leader sequences in cap-dependent initiation and ribosomal entry sites. Though the mRNA amounts were the highest in the pΔ5'UTR\_ADA [**T15**] construct, a lack of 5'UTR for the generation of an efficient 5' leader sequence appears to have contributed towards the lower amount of expressed ADA enzyme-activity.

Calli cell lines (T2 and T3) transformed with pSS 5'UTR\_ADA, in lanes 2 and 3 of figure 4.8, exhibited respectively 3.9 and 3.3 ng of ADA mRNA and showed relatively high ADA specific-activities ( $1.39$  and  $2.91 \times 10^{-3}$  U mg<sup>-1</sup> TSP respectively) (table 4.7). Although the calli cell lines transformed with pSS 5'UTR\_ADA constructs, initially (5-6 weeks after transformation) showed a high ADA specific-activity, calli tested from subsequent subculturing events showed drastically reduced (approximately 10-fold) ADA expression levels after 30 days as shown in figure 4.6. The decline in ADA enzyme-activity could be explained by degradation of mRNA as shown in figure 4.8. This adverse mRNA degradation in pSS 5'UTR\_ADA construct should be further investigated with new transformed cell lines and may

possibly be due to posttranscriptional gene silencing events. Conversely, calli transformed with construct p5'Ω\_ADA showed no reduction in ADA specific activities after 9 months (figure 4.6), exhibiting stable gene expression over a long period of time.

Calli transformed with construct p5'\_3'Ω\_ADA [T9], also showed a relatively higher ADA specific-activity ( $3.40 \times 10^{-3}$  U mg<sup>-1</sup> TSP) among the transgenic BY-2 cell lines analysed for mRNA levels as shown in table 4.7. However, T9 calli exhibited only a moderate amount of mRNA levels (approx. 4.5 ng) (figure 4.8). As stated previously, the presence of TMV Ω 5' UTR significantly enhances translation of the associated message and further addition of TMV 3' UTR increases the stability of mRNA further and also increases mRNA translation (Gallie and Kado, 1989; Gallie and Walbot, 1990). Hence, enhanced expression of ADA in transgenic BY-2 cell lines of p5'\_3'Ω\_ADA with lower transcribed mRNA levels could be explained by the stabilization and enhancing effects of the incorporated 5' and 3' TMV UTR sequences. However, as shown in table 4.7, comparison of ADA specific-activities with mRNA levels seems to indicate that there is no general correlation between amounts of RNA and ADA enzyme-activities.



**Figure 4.7:** (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli. Lane 1: non-transformed calli, Lane 2: p5'Ω\_ADA [T3], Lane 3: p5'Ω\_ADA [T2], Lane 4: pΔ5'UTR\_ADA [T15], Lane 5: pΔ5'UTR\_ADA [T11], Lane 6: pCDNA\_ADA [T13] and Lane 7: pCDNA\_ADA [T5], (b) SafeView™ stained 2 % agarose gel of rRNA present in lane 1-7.



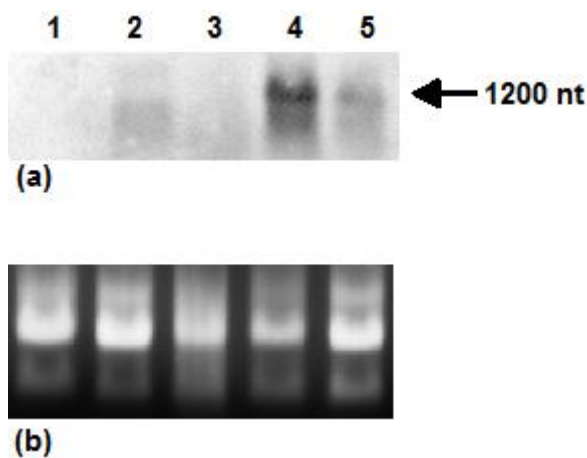


Figure 4.8: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli. Lane 1: non-transformed calli, Lane 2: pSS 5'UTR\_ADA [T3], Lane 3: pSS-5'UTR\_ADA [T2], Lane 4: p5'\_3'Ω\_ADA [T11] and Lane 5: p5'\_3'Ω\_ADA [T9], (b) SafeView™ stained 2 % agarose gel of rRNA present in lane 1-5.

Table 4.7: ADA specific-activities of BY-2 calli transformed with cytosolic constructs alongside ADA mRNA amounts measured in northern blots.

Constructs	ADA specific-activity (U mg <sup>-1</sup> TSP)	ADA mRNA (ng)
p5'Ω_ADA [T3]	1.84 x10 <sup>-3</sup>	2.2
p5'Ω_ADA [T2]	2.91 x10 <sup>-3</sup>	3.1
pΔ5'UTR_ADA [T15]	4.15 x10 <sup>-4</sup>	8.8
pΔ5'UTR_ADA [T11]	4.34 x10 <sup>-4</sup>	2.2
pCDNA_ADA [T5]	1.66 x10 <sup>-3</sup>	7.2
pCDNA_ADA [T13]	2.87 x10 <sup>-4</sup>	1.5
pSS 5'UTR_ADA [T3]	1.39 x10 <sup>-3</sup>	3.9
pSS 5'UTR_ADA [T2]	2.91 x10 <sup>-3</sup>	3.3
p5'_3'Ω_ADA [T11]	2.72 x10 <sup>-3</sup>	10.0
p5'_3'Ω_ADA [T9]	3.40 x10 <sup>-3</sup>	4.5

### 4.3 Discussion

Transgenic tobacco plants and calli were successfully generated following *Agrobacterium*-mediated transformation with different cytosolic constructs of the human adenosine deaminase gene. A number of primary transformants were tested for ADA enzyme-activity and protein using enzymatic assays and western blotting. Southern and northern blots were also performed for comparing insert copy number, levels of mRNA and enzyme activities between transformants.

Initially a western blot was performed with pCDNA\_ADA construct transformed plants. The size of recombinant ADA extracted from transformed tobacco plants was found to be approximately 1 kDa higher than the reported human ADA molecular weight (figure 4.3). Although this may be due to an artefact of the gel system, it is thought that the size of recombinant ADA produced in tobacco could be due to nucleocytoplasmic O-GlcNAcylation. Since, some plant proteins undergo extensive glycosylation after translation including nucleocytoplasmic O-GlcNAcylation (Gomord *et al.*, 2010). This could be investigated further using western blot analysis of transformant extracts treated to remove any associated carbohydrate groups from proteins.

There are many reports that show the expression levels of foreign genes in plants are not directly proportional to T-DNA insert copy number (Vergunst *et al.*, 1998). This is partly due to integration of the insert T-DNA into transcriptionally silent regions of the plant genome (Springer *et al.*, 1995). Transgene silencing by DNA methylation (Hepburn *et al.*, 1983) or by promoter methylation (Meyer, 2000) together with post-transcriptional transgene silencing of transcribed RNA (Meins, 2000; Schubert *et al.*, 2004) could also contribute to lack of correlation of insert copy number to expression levels. Moreover *Agrobacterium*-mediated

transformation is often associated with the transfer of tandem copies of T-DNA at a single locus and head to head inverted T-DNA repeats around the right border are also frequently associated with transgene silencing (Stam *et al.*, 1997). Southern blot analysis confirmed that ADA expression levels in plants transformed with cytosolic constructs are not directly proportional to insert copy number. A transformed plant line with higher insert copy numbers actually showed comparatively lower ADA specific-activities than transformed plant lines with fewer insert copies.

It was postulated that the 5' leader and 3' trailer sequences formed by human 5' UTR and 3'UTR DNA sequences, respectively, would be active to a certain extent in transgenic plants expressing recombinant ADA. And that the equivalent regions from TMV would stimulate gene expression further. To test this hypothesis the median expression levels of ADA in transgenic plants of different constructs were compared. Whole plants transformed with the pCDNA\_ADA construct showed a 182-fold increase in expression levels compared to transgenic plants transformed with the construct containing minimal 5'UTR (p $\Delta$ 5'UTR\_ADA), whereas, plants transformed with the construct containing TMV omega 5'UTR (p5' $\Omega$ \_ADA) showed only a 7-fold increase in ADA-activity. However, one out-lying p5' $\Omega$ \_ADA transformed plant showed the highest ADA specific-activity,  $3.72 \times 10^{-3}$  U mg<sup>-1</sup> TSP (66 ng mg<sup>-1</sup> TSP). Whereas, the highest ADA specific-activity of a pCDNA\_ADA transformed plant was  $2.41 \times 10^{-3}$  U mg<sup>-1</sup> TSP (43 ng mg<sup>-1</sup> TSP) (figure 4.2). This is respectively a 416-fold and a 273-fold increase in the ADA specific-activity compared to plants transformed with the short 5'UTR construct (table 4.2). Because, only small numbers of transgenic plants were assayed, higher ADA-activities of outlying plants do not allow unequivocal conclusions to be drawn. A greater number of transgenic plants should be investigated to clarify this data.

Tobacco BY-2 cells in suspension were also transformed with different cytosolic constructs containing minimal 5'UTR (p $\Delta$ 5'UTR\_ADA), human 5' and 3' UTR (pCDNA\_ADA), TMV omega 5'UTR (p5' $\Omega$ \_ADA), TMV omega 5' and 3' UTR (p5'\_3' $\Omega$ \_ADA) and RUBISCO small sub unit 5' UTR (pSS 5'UTR\_ADA). Stable transformants were tested for ADA-activity using enzymatic assays and northern blots were performed in order to compare the stability of mRNA to ADA enzyme-activities.

Gallie and Kado, (1989) demonstrated that the presence of the TMV 5' untranslated leader sequence ( $\Omega$ ) significantly enhances translation in both eukaryotes and prokaryotes. It was further revealed that the presence of a CAA-rich region within  $\Omega$  is responsible for this translational enhancement (Gallie, 2002). At the other end of TMV RNA genes, the 3' UTR is involved in forming a complex tertiary structure containing a pseudoknot domain and a tRNA-like structure. The addition of 204-bases of this region to foreign mRNAs was shown to increase gene expression by 100-fold as compared to nonadenylated mRNA. The TMV 3'UTR seeming to increase the stability of associated sequences and hence, increasing translation in a similar manner to polyadenylated mRNA (Gallie and Walbot, 1990). Confirming these findings, pair-wise comparisons of ADA specific-activities as shown in table 4.6 revealed that the calli transformed with the pCDNA\_ADA construct, containing human 5' and 3' UTR sequence caused only a 1.3-fold increase in median ADA specific-activity as compared to the calli transformed with construct containing minimal 5'UTR sequence (p $\Delta$ 5'UTR\_ADA). On the other hand, calli transformed with the construct containing TMV omega 5' UTR sequence (p5' $\Omega$ \_ADA) caused a 14-fold increase in median ADA specific-activity and incorporation of both TMV 5' and 3' UTR in the p5'\_3' $\Omega$ \_ADA construct, caused the highest increase in median ADA specific-activity of 20.5-fold. Moreover, one outlying

transgenic line of p5' \_3'Ω\_ADA transformed calli showed the highest ADA specific-activity of  $4.50 \times 10^{-3}$  U mg<sup>-1</sup> TSP (80 ng mg<sup>-1</sup> TSP), a 32-fold increase in ADA specific-activity over short 5'UTR construct (table 4.6).

Patel *et al.*, (2004 and 2006) demonstrated that the 5' UTR from both C4 *Amaranthus* and *Flaveria bidentis* RUBISCO small subunit1 mRNAs confer translational enhancement on associated ORFs which leads to an increase in expression of foreign proteins. Confirming these findings, the calli transformed with construct pSS5'UTR\_ADA, containing RUBISCO small subunit 5' UTR exhibited an 11.5-fold increase of ADA specific-activity as compared to construct pΔ5' UTR\_ADA. One outlying transgenic line of pSS5'UTR\_ADA transformed calli showed the highest ADA specific-activity of  $3.20 \times 10^{-3}$  U mg<sup>-1</sup> TSP (57 ng mg<sup>-1</sup> TSP) (figure 4.6) a 22-fold higher ADA specific-activity as compared to calli transformed with construct pΔ5' UTR\_ADA. Moreover approximately 12 transgenic calli lines of pSS5'UTR\_ADA construct exhibited higher ADA specific-activity as compared to the mean ADA specific-activity of calli lines of pSS5'UTR\_ADA (figure 4.6).

Though the initial ADA-enzyme expression levels were high in calli cell lines transformed with pSS 5'UTR\_ADA construct immediately after selection of transformants, subsequent sub-culturing event showed a much reduced ADA-activity after 30 days of initial measurement. Northern blot analysis revealed that degradation of transcribed mRNA levels in pSS 5'UTR\_ADA construct may have contributed towards the lower ADA expression levels. The reason for mRNA degradation in these calli lines may be due to posttranscriptional gene silencing events, since the nature of mRNA sequence may influence posttranscriptional gene silencing (Schubert *et al.*, 2004). On the other hand the calli transformed with construct

p5'Ω\_ADA showed a stable expression of ADA even after 9 months of sub-culturing events (figure 4.6).

Increased in expression of ADA in different cytosolic constructs containing different 5' UTR sequences and 3' UTR sequences is thought to be due to translational enhancing and mRNA stabilizing properties of these sequences (Gallie and Kado, 1989). The link between untranslated regions and increased expression of ADA was further elaborated by northern blot analysis. Although the mRNA amounts were the highest in the construct containing minimal 5'UTR (pΔ5'UTR\_ADA; table 4.7), a lack of 5'UTR for the generation of 5' leader sequence may have greatly contributed towards the lower amount of expressed ADA enzyme. It was also shown that the p5'\_3'Ω\_ADA, transgenic BY-2 cell lines had the lowest amounts of mRNA but the highest ADA expression, perhaps owing to mRNA stabilizing and translational enhancing effects of incorporated TMV 5' and 3' UTR sequences.

Successful cytosolic expression of a functional human adenosine deaminase in transgenic tobacco plants and calli has been demonstrated in this chapter. Attempts to increase the ADA yield by enhancing expression proved to be successful as it was found that incorporation of translational enhancing sequences in calli, at least, improved ADA expression by a considerable amount. On consideration, only a few transgenic plants were analysed and further generation of plants transformed with cytosolic constructs may reveal results more closely aligned with those found in BY-2 calli. The effect of sub-cellular targeting sequences such as PR1a and extensin in enhancing yield of recombinant ADA further will be discussed in following chapters.

## **Chapter 5**

# **Subcellular directed expression of human adenosine deaminase in transgenic tobacco BY-2 calli**

## 5.1 Overview

Potential use of plant cell cultures as an alternative production system for pharmaceutical proteins is restricted due to low recombinant protein yield and plays a major limitation in commercialising the technique. Hence, wide arrays of strategies have been employed in an attempt to enhance protein yield at the molecular, cell culture and downstream processing steps. Molecular strategies such as enhancing gene transcription, improving translational efficiency, incorporation of novel protein fusion technology and incorporation of secretory pathway targeting technology have been widely used in recent years to enhance the yield of foreign proteins in plant cell cultures (Desai *et al.*, 2010). In this study, the incorporation of novel protein fusion technology and the incorporation of secretory pathway targeting technology have been employed to increase the expression of recombinant ADA in nuclear transformed BY-2 calli. Cells transformed with ADA constructs and the resulting transgenic calli have been assessed for ADA mRNA expression and ADA enzyme activities.



## 5.2 Results

### 5.2.1 Apoplast-directed expression in transformed BY-2 calli

Tobacco BY-2 cells in suspension were transformed with different constructs containing apoplast-directing signals described in chapter 3. These constructs are namely p $\Delta$ 5'UTR-PR1a\_ADA, p $\Delta$ 5'UTR-Ext\_ADA, p5' $\Omega$ -PR1a\_ADA, p5' $\Omega$ -Extensin\_ADA, pSS 5'UTR-PR1a\_ADA, pSS 5'UTR-Extensin\_ADA and p5' $\Omega$ -Extensin\_ADA-3'Hydroxyproline (See section 3 for details). The BY-2 calli lines transformed with ADA apoplast-directed constructs were assessed for expression levels. Five to six-week-old transformed calli were flash-frozen in liquid nitrogen and ground to a fine powder. Total soluble proteins were extracted and the calli extracts were assayed for ADA enzyme-activity to determine ADA levels and a Bradford assay performed to determine total soluble proteins. Up to 30 transgenic cell lines of different apoplast-directed constructs were analysed as shown in figure 5.1. A Mann-Whitney statistical test was conducted as described by Pollard (1977). Calli transformed with various cytosolic constructs including a minimally deleted 5'UTR construct, p $\Delta$ 5'UTR\_ADA and a TMV UTR construct, p5' $\Omega$ \_ADA were used as basal constructs with which to compare ADA levels in the corresponding calli transformed with apoplast-directed constructs. Any two constructs being compared were combined and ranked by ascending expression levels and the *U* values calculated as describe in section 2.6.

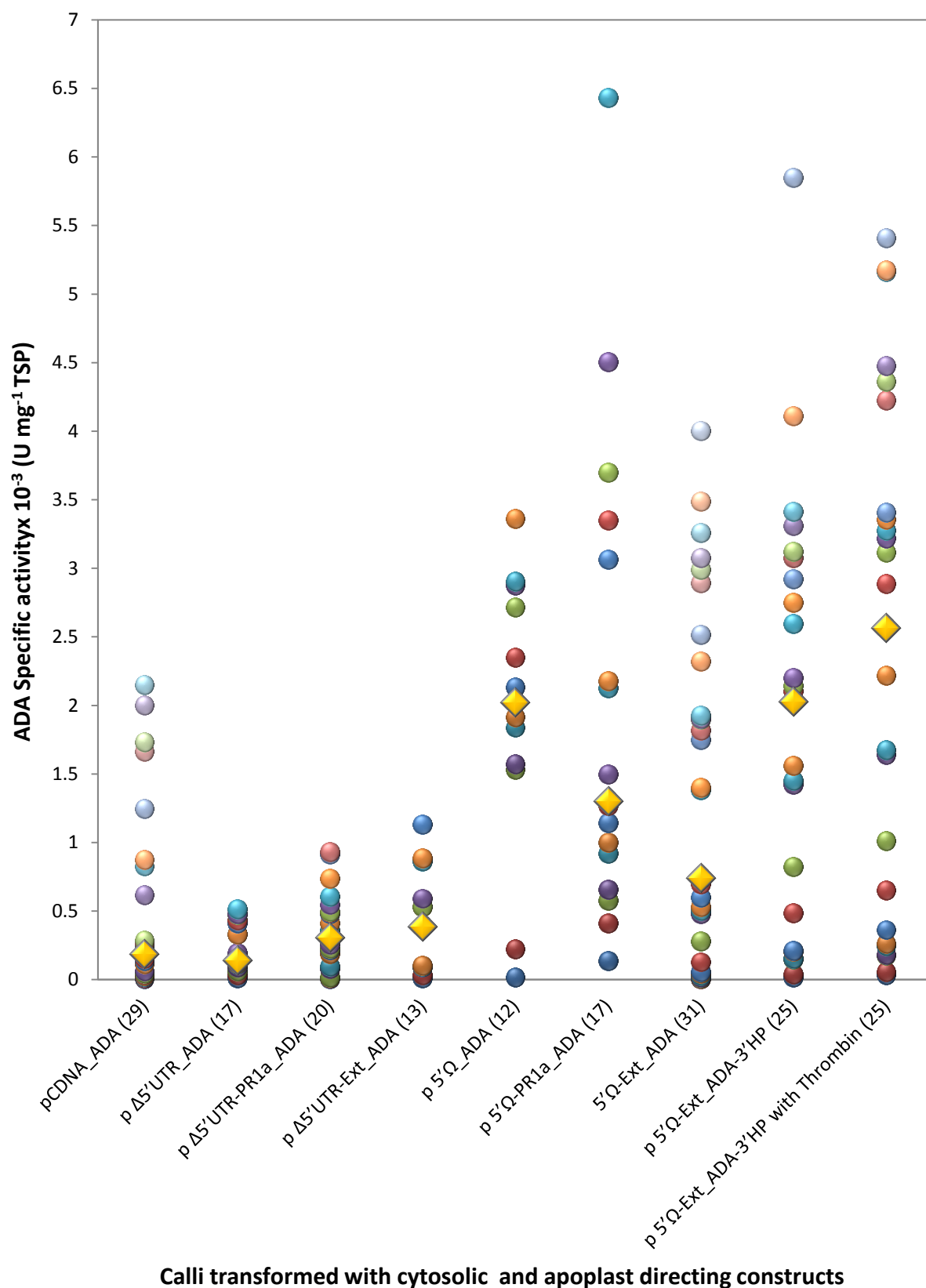


Figure 5.1: ADA specific-activities measured in BY-2 calli transformed with various cytosolic and apoplast directing constructs. Each circle represents ADA-activity from an independent transformant, and the yellow diamond denotes the median value. The number of calli assayed is shown in brackets after each construct.

**Table 5.1a: Pair-wise comparisons of ADA specific-activities measured in transgenic tobacco calli using Mann-Whitney U test (5 % level).**

Constructs	Population identity	p-Value
pΔ5'UTR_ADA versus pΔ5'UTR-PR1a_ADA	Same	$P>0.05=0.17993$
pΔ5'UTR_ADA versus pΔ5'UTR-Ext_ADA	Same	$P>0.05=0.39091$
p5'Ω_ADA versus p5'Ω-PR1a_ADA	Same	$P>0.05=0.59516$
p5'Ω_ADA versus p5'Ω-Ext_ADA	Same	$P>0.05=0.13643$
p5'Ω_ADA versus p5'Ω-Ext_ADA-3'HP	Same	$P>0.05=0.74560$
p5'Ω_ADA versus p5'Ω-Ext_ADA-3'HP Thrombin treated	Same	$P>0.05=0.41730$

**Table 5.1b: Pair-wise comparisons of median ADA specific-activities measured in transgenic tobacco BY-2 calli.**

Constructs	Ratio of median ADA specific-activity	Fold increase median expression	Fold increase of highest expressing BY-2 cell line
pΔ5'UTR_ADA versus pΔ5'UTR-PR1a_ADA	$1.43 \times 10^{-4} : 3.09 \times 10^{-4}$	2.1	1.8
pΔ5'UTR_ADA versus pΔ5'UTR-Ext_ADA	$1.43 \times 10^{-4} : 3.88 \times 10^{-4}$	2.7	2.2
p5'Ω_ADA versus p5'Ω-PR1a_ADA	$2.02 \times 10^{-3} : 1.31 \times 10^{-3}$	0.65	1.9
p5'Ω_ADA versus p5'Ω-Ext_ADA	$2.02 \times 10^{-3} : 7.45 \times 10^{-4}$	0.37	1.2
p5'Ω_ADA versus p5'Ω-Ext_ADA-3'HP	$2.02 \times 10^{-3} : 2.03 \times 10^{-3}$	1.1	1.7
p5'Ω_ADA versus p5'Ω-Ext_ADA-3'HP Thrombin treated	$2.02 \times 10^{-3} : 2.56 \times 10^{-3}$	1.26	1.6

A pair-wise comparison of apoplast-targeted constructs, containing TMV omega 5'UTR with cytosolic omega constructs, shows that ADA levels are not significantly different (table 5.1a). However, when the highest outlying ADA specific-activities of these constructs are compared an increase of ADA specific-activity of between 1.2 to 1.9-fold is observed (table 5.1b). Moreover, 2-5 transgenic lines from each apoplast-targeted construct exhibited a higher ADA specific-activity than the highest expressing p5'Ω\_ADA construct (figure 5.1).

ADA expression data where PR1a and extensin signal sequences are incorporated with the short 21 bp minimally deleted 5'UTR (constructs p $\Delta$ 5'UTR-PR1a\_ADA and p $\Delta$ 5'UTR-Ext\_ADA) also show no significant difference. However again, when the highest expressing lines are compared an increase of ADA specific-activity of between 1.8 to 2.2-fold is observed (table 5.1b). Moreover, 5-6 transgenic lines from each corresponding apoplast-targeted construct exhibited a higher ADA specific-activity than the highest expressing p $\Delta$ 5'UTR\_ADA construct (figure 5.1). The highest outlying ADA specific-activity of p5' $\Omega$ -Ext\_ADA-3'HP transformed calli also showed an increase of 1.7-fold over the highest expressing calli line transformed with p5' $\Omega$ \_ADA.

### **5.2.2 Northern blot analysis of transcribed mRNA from calli transformed with different apoplast-directed constructs**

In order to assess whether mRNA levels correlated with increased levels of ADA enzyme activity, northern blots of transformed and non-transformed tobacco BY-2 calli were performed. Total RNA was extracted as described in section 2.3.1 and equal amount of RNA (3500 ng) were subjected to electrophoresis under denaturing conditions (section 2.3.2). The RNA was transferred to Hybond-N+ nylon membrane as described in section 2.3.3 prior to probing with DIG-labelled ADA cDNA (section 2.2.12.3). A photographic reproduction of the blot was taken, as shown in figures 5.2, 5.3 and 5.4. TotalLab Quant software was used to analyse northern blots (section 2.3.5).

As PCR-amplified ADA is 1092 bp in size, transcribed mRNA would be larger than 1092 nucleotides. Observed mRNA sizes were approximately 1100 nucleotides and similar in size in all constructs apart from construct p5' $\Omega$ -Ext\_ADA-3'HP (figure 5.4, lanes 2 and 3). Transcribed mRNA in construct, p5' $\Omega$ -Ext\_ADA-3'HP, exhibited a size of 1200 nucleotides

and the larger mRNA size in this construct caused by a lengthy DNA sequence (63 bp) coding for serine-hydroxyproline (Ser-Hyp) motifs incorporated at the 3' end of the ADA ORF sequence.

Two transgenic cell lines from each construct were analysed for mRNA levels in order to correlate them with their corresponding ADA activities (table 5.2). No correlation was observed between the levels of ADA mRNA and expressed ADA enzyme-activities, either between transformants from the same constructs or between transformants of different constructs. For example, calli lines transformed with the constructs p $\Delta$ 5'UTR-ADA [T11] and [T15] in figure 5.2, lanes 6 and 7 showed very different mRNA levels of 13.7 and 2.7 ng of ADA mRNA but had very similar ADA specific-activities of about  $4 \times 10^{-4}$  U mg<sup>-1</sup> TSP (table 5.2). Also the faint bands of constructs p $\Delta$ 5'UTR-PR1a-ADA [T3] and [T2] in figure 5.2, lanes 2 and 3 showed approximately 2.0 and 9.2 ng of ADA mRNA with respective  $9.15$  and  $6.05 \times 10^{-4}$  U mg<sup>-1</sup> TSP ADA specific-activities (table 5.2). Comparing this data from different constructs, the cytosolic construct, p $\Delta$ 5'UTR-ADA [T11], showed a 6-fold higher ADA mRNA level but a 2-fold lower ADA specific-activity than the equivalent apoplast construct p $\Delta$ 5'UTR-PR1a-ADA [T3]. Furthermore, the construct p $\Delta$ 5'UTR-Ext-ADA [T11], if compared to the cytosolic construct, p $\Delta$ 5'UTR-ADA [T11], showed very similar amounts of ADA mRNA levels but a 2-fold higher ADA specific-activity. These increased activities of ADA but with lower amounts of mRNA than in corresponding cytosolic constructs may be due to the incorporation of PR1a and extensin signal peptides which direct the ADA enzyme into the apoplastic space which in turn prevents the ADA enzyme from proteolytic degradation in the cytosol. Therefore, higher amounts of ADA mRNA do not necessarily yield higher ADA specific-activities. A similar conclusion can be drawn from PR1a and Hydroxyproline constructs when comparing with equivalent cytosolic constructs (table 5.2).

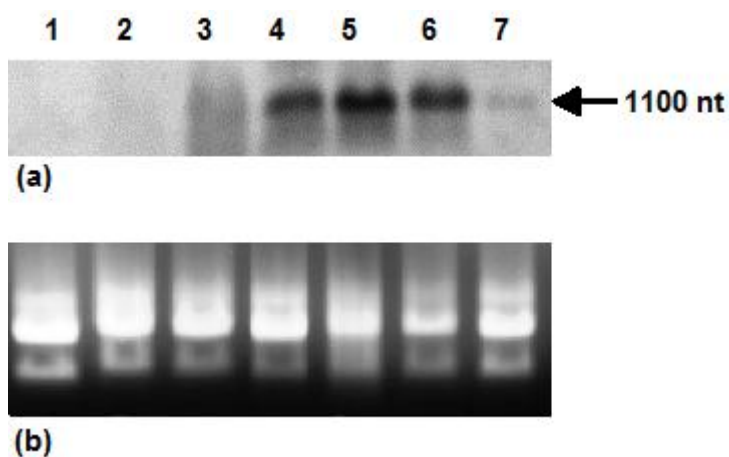


Figure 5.2: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli. Lane 1: non-transformed calli, Lane 2: p $\Delta$ 5'UTR-PR1a\_ADA [T3], Lane 3: p $\Delta$ 5'UTR-PR1a\_ADA [T2], Lane 4: p $\Delta$ 5'UTR-Ext\_ADA [T11], Lane 5: p $\Delta$ 5'UTR-Ext\_ADA [T5], Lane 6: p $\Delta$ 5'UTR\_ADA [T11] and Lane 7: p $\Delta$ 5'UTR\_ADA [T15], (b) SafeView<sup>TM</sup> stained 2 % agarose gel of rRNA present in lane 1-7.

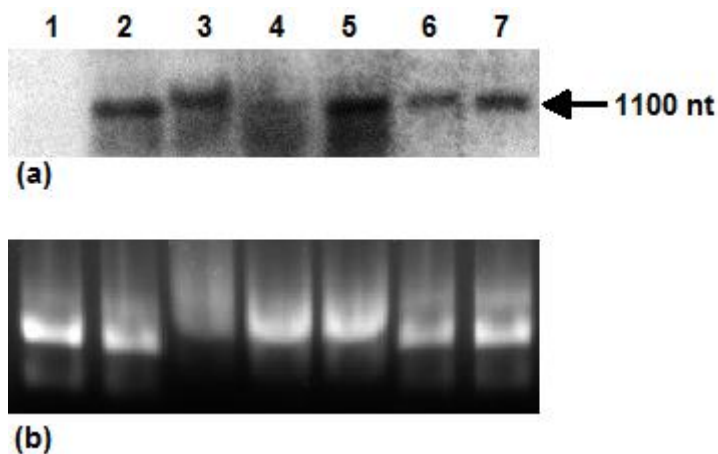
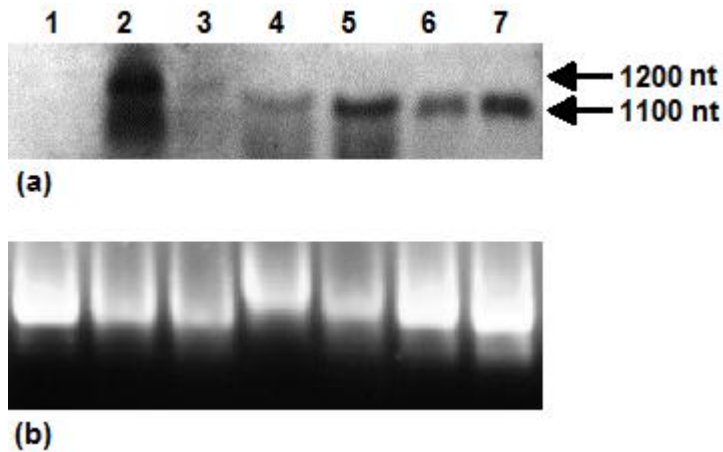


Figure 5.3: (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli. Lane 1: non-transformed calli, Lane 2: p5' $\Omega$ -PR1a\_ADA [T5], Lane 3: p5' $\Omega$ -PR1a\_ADA [T2], Lane 4: p5' $\Omega$ -Ext\_ADA [T14], Lane 5: p5' $\Omega$ -Ext\_ADA [T10], Lane 6: p5' $\Omega$ \_ADA [T3] and Lane 7: p5' $\Omega$ \_ADA [T2], (b) SafeView<sup>TM</sup> stained 2 % agarose gel of rRNA present in lane 1-7.



**Figure 5.4:** (a) Northern blot of total RNA extracted from transformed and non-transformed tobacco BY-2 calli. Lane 1: non-transformed calli, Lane 2: p5'Ω-Ext\_ADA-3'HP [T6], Lane 3: p5'Ω-Ext\_ADA-3'HP [T5], Lane 4: p5'Ω-Ext\_ADA [T14], Lane 5: p5'Ω-Ext\_ADA [T10], Lane 6: p5'Ω\_ADA [T3] and Lane 7: p5'Ω\_ADA [T2], (b) SafeView™ stained 2 % agarose gel of rRNA present in lane 1-7.

**Table 5.2:** ADA specific-activities of tobacco BY-2 calli transformed with cytosolic and apoplast-directing constructs alongside ADA mRNA amounts measured in northern blots.

Constructs	ADA specific-activity (U mg <sup>-1</sup> TSP)	ADA mRNA (ng)
pΔ5'UTR_ADA [T15]	4.34 x10 <sup>-4</sup>	2.7
pΔ5'UTR_ADA [T11]	4.16 x10 <sup>-4</sup>	13.7
pΔ5'UTR-PR1a_ADA [T2]	6.05 x10 <sup>-4</sup>	9.2
pΔ5'UTR-PR1a_ADA [T3]	9.15 x10 <sup>-4</sup>	2.0
pΔ5'UTR-Ext_ADA [T5]	1.13 x10 <sup>-3</sup>	18.8
pΔ5'UTR-Ext_ADA [T11]	8.62 x10 <sup>-4</sup>	13.0
p5'Ω_ADA [T2]	2.91 x10 <sup>-3</sup>	3.3
p5'Ω_ADA [T3]	1.84 x10 <sup>-3</sup>	4.7
p5'Ω-PR1a_ADA [T2]	3.35 x10 <sup>-3</sup>	8.7
p5'Ω-PR1a_ADA [T5]	6.43 x10 <sup>-3</sup>	8.3
p5'Ω-Ext_ADA [T10]	4.00 x10 <sup>-3</sup>	12.1
p5'Ω-Ext_ADA [T14]	2.99 x10 <sup>-3</sup>	10.6
p5'Ω-Ext_ADA-3'HP [T5]	3.36 x10 <sup>-3</sup>	4.7
p5'Ω-Ext_ADA-3'HP [T6]	5.18 x10 <sup>-3</sup>	12.5

### 5.3 Discussion

Transgenic tobacco BY-2 calli were successfully generated following *Agrobacterium*-mediated transformation with different constructs of the human adenosine gene. Stable transformants were assayed for ADA-activity and northern blots were performed to investigate a possible correlation between levels of ADA mRNA and enzyme activities.

Incorporation of TMV 5' omega sequence which gives rise to 5' omega leader in mRNA aids in recruiting translational initiation factors and ribosomes which in turn enhances the translation of omega carrying sequences (Gallie and Walbot, 1992; Schmitz *et al.*, 1996). Another strategy used to increase foreign gene expression is to incorporate signal peptides sequences such as tobacco pathogenesis related protein sequence (PR1a) in conjunction with recombinant ORF to direct foreign proteins into the apoplast (Pen *et al.*, 1993a). Ziegler *et al.*, (2000) successfully expressed the catalytic domain of a thermostable eubacterium endo-1, 4- $\beta$ -D-glucanase in the apoplast of tobacco BY-2 suspension cells and found that incorporation of PR1a signal peptide at the *N*-terminus increased the accumulation endo-1, 4- $\beta$ -D-glucanase proteins. Xu *et al.*, (2002) also demonstrated that the incorporation of PR1a signal peptide enhanced the yield by 2-fold as compared to cells transformed with a corresponding cytosolic construct.

As with PR1a the extensin signal peptide directs the proteins into the cell secretory pathway enhancing the yield presumably by prevention of recombinant protein from proteolytic degradation in the secretory pathway and apoplast or alternatively enabling the protein to fold correctly into a biologically active molecule. Plant extensins are well-characterized hydroxyproline-rich glycoproteins which forms a scaffolding network by covalent extensin-pectin interactions followed by polymerization to provide the required rigidity of the cell wall



(Lamport *et al.*, 2011). Incorporation of the signal peptide sequence of extensin at the *N*-terminus of recombinant proteins directs secretion into the apoplast (Showalter, 1993). Francisco *et al.*, (1997) also demonstrated that incorporation of extensin *N*-terminal signal sequences enhanced the yield of bryodin 1 (ribosome-inactivating protein) in BY-2 cell suspensions, as compared to the corresponding cytosolic construct.

Evolutionary conserved Hydroxyproline(Hyp)-rich glycoproteins (HRGPs) results in two types of glycomodules uniquely involving Hyp contiguity (Lamport, 1977; Shapk, 2001). One of which is formed by the addition of short unbranched arabinooligosaccharides to a contiguous Hyp residues, whereas the other is formed by the addition of branched arabinogalactan polysaccharides to clustered non-contiguous Hyp residues (Shapk, 2001). Defined as the hyperglycosylated arabinogalactan-proteins (AGPs), these often result in an increased molecular mass of the polypeptide (Kieliszewski and Lamport 1994; Kieliszewski and Shapk, 2000). Use of HRGPs for the purpose of enhancing the yield of secreted recombinant protein was demonstrated by Xu *et al.*, (2008). The chimeric expression of HRGPs with recombinant interferon alpha2 (IFN $\alpha$ 2) targeted for secretion using extensin signal peptide has not only increased the yield of recombinant protein by 350- to 1400-fold in the media as compared to a non-glycosylated IFN $\alpha$ 2 control, but also increased the serum half-life and higher receptor binding capacity of IFN $\alpha$ 2 when tested in mice (Xu *et al.*, 2007; 2008)

Confirming these findings the overall spread of data in figure 5.1 seems to suggest that incorporation of apoplast targeting signals gives rise to lines of calli which express ADA at higher levels than calli transformed with a cytosolic counterpart. For example, the range of activities measured in p5' $\Omega$ \_ADA was between 0.02 - 3.4 x 10<sup>-3</sup> U mg<sup>-1</sup> TSP. Whereas, the p5' $\Omega$ -PR1a\_ADA apoplast targeted calli varies from 0.14 - 6.5 x 10<sup>-3</sup> U mg<sup>-1</sup> TSP and for

p5'Ω-Ext\_ADA and p5'Ω-Ext\_ADA-3'Hydroxyproline constructs the range in expression is 0.0061 - 4.0 and 0.034 - 6.0 x 10<sup>-3</sup> U mg<sup>-1</sup> TSP respectively.

Due to either transgene silencing or post-transcriptional gene silencing of inserted constructs, it is possible that the mean and median data points are distorted. It has been shown that the inverted tandem repeats of T-DNA trigger transgene silencing, which results in poorly transcribed genes leading to a poor expression of recombinant proteins (Sijen *et al.*, 2001). It was further found that the transgenes in multiple copies under the control of strong promoters, such as the double CaMV 35S promoter, triggers post-transcriptional gene silencing by surpassing a gene-specific threshold (Vaucheret *et al.*, 2001; Schubert *et al.*, 2004).

The most relevant results to this study were observed in construct p5'Ω-Extensin\_ADA-3'Hydroxyproline, which shows 6 transformed cell lines exhibit a higher ADA-activity than the highest p5'Ω\_ADA cytosolic construct (figure 5.1). Successful expression of a functional human adenosine deaminase in transgenic tobacco BY-2 calli has been demonstrated in this chapter. Attempts to increase the ADA yield by enhancing expression proved to be successful as it was found that incorporation of TMV omega 5' UTR sequences in calli improved ADA yield by a considerable amount and moreover, incorporation of apoplast-directing signals into the same construct produced calli cell lines with almost double the amounts of ADA-activity. Expression of proteins in fusion with AGP glycomodule Ser-Hyp (SO) repeats exhibited the highest mean ADA-activities.

The increased yield of subcellular-directed expression of ADA should be highly pronounced in cell suspensions as subcellular-directed proteins have been found to be secreted into the

culture media (Xu *et al.*, 2010). Hence, the effect of ADA constructs with subcellular-directing sequences such as PR1a, extensin and Ser-Hyp (SO) fusions in transformed BY-2 cell suspensions will be presented in the following chapter.

## **Chapter 6**

# **Expression of human adenosine deaminase in transgenic tobacco BY-2 cell suspension cultures**

## 6.1 Overview

Using plant cell suspension cultures to express foreign proteins encompasses some advantages over whole-plants, microbial and animal cell culture systems (Hellwig *et al.*, 2004). These include a rapid cell doubling time, well established genetic transformation methodologies, low endogenous proteolytic activity, low production of secondary metabolites, capability to carryout post-translational modification and reduced post-translational silencing (Lee *et al.*, 2000; Su and Lee, 2007). Undifferentiated plant calli can be separated and propagated in culture media in a controlled condition to generate cell suspension cultures. Developed in the 1950's, plant cell suspension culture technology has been used for the production of secondary metabolites such as digoxin, paclitaxel, and artemisinin (Georgiev *et al.*, 2009; Huang and McDonald 2009). With increasing demand for therapeutic proteins, as well as modern advances in plant molecular biology, plant cell culture systems have developed as an alternative system for producing therapeutically imperative proteins. The first two therapeutic proteins to be produced in transgenic plant cell suspension cultures were recombinant human serum albumin (Sijmons *et al.*, 1990) and chloramphenicol acetyltransferase (Hogue *et al.*, 1990). A recombinant poultry vaccine produced by Dow AgroSciences was the first plant cell suspension culture-produced therapeutic protein to be approved in February 2006 by the USDA (Kaiser, 2008). Another recombinant protein, human glucocerebrosidase (Taliglucerase alfa) used in ERT to treat Gaucher's disease, has been produced by Protalix Biotherapeutics. This enzyme was the first plant cell-produced human enzyme to be approved by the United States Food and Drug Administration (FDA) in 2012 (Pfizer Press Releases, 2012). Although, many recombinant proteins have been produced in plant cell suspension culture systems, using tomato, rice, soybean and tobacco plants (Huand and McDonald, 2009), tobacco BY-2 cell suspensions are the most commonly used cell system. In this study, BY-2 cell suspension cultures transformed with various ADA

cDNA constructs have been investigated for extra and intra-cellular ADA enzyme-activities over a period of 15 days.

## 6.2 Results

Tobacco BY-2 cells were transformed with cytosolic constructs or different constructs containing apoplast-directing signals. These constructs are namely: pCDNA\_ADA, p5'Ω-PR1a\_ADA, p5'Ω-Extensin\_ADA and p5'Ω-Extensin\_ADA-3'Hydroxyproline (See section 3 for details). The highest expressing transformed BY-2 calli cell lines were selected and suspension cultures were generated in MS media substituted with 0.75 g L<sup>-1</sup> of PVP (M.W 360,000) as described in section 2.1.2. In initial experiments PVP was shown to dramatically improve ADA enzyme-activity when included in the media (figure 6.1). It was subsequently included in all experiments described in this chapter. Aliquots of BY-2 cell suspension cultures were collected on alternate days, starting from day 0 up to day 15. The collected samples were subjected to centrifugation to separate cells from culture media, which was then tested for ADA enzyme-activity using an adenosine deaminase assay to determine the extra-cellular enzyme-activity secreted into the culture medium (section 2.5.2). Intra-cellular ADA-activities were assayed at the same time from pelleted cells, flash frozen in liquid nitrogen as described in section 2.5.1. Eight samples taken over 15 days from BY-2 cell suspensions transformed with various constructs were analysed and ADA-activities are shown in Units L<sup>-1</sup> (figures 6.1 and 6.2). A cytosolic construct, pCDNA\_ADA, containing human 5' and 3' UTR was used as a basal construct with which to compare the corresponding ADA-apoplast-directed constructs.

### 6.2.1 Extra-cellular ADA-activities in BY-2 cell suspensions transformed with ADA expressing constructs

There is between a 31 to 336-fold increase in secreted ADA-activity when apoplast targeting constructs are compared to the cytosolic control on day 7 (figure 6.1 and table 6.1). The highest extra-cellular ADA-activity was measured on the day 7 for p5'Ω-Ext\_ADA-3'HP construct, transformed cell line which measured  $739 \text{ U L}^{-1}$ , a 336-fold increase over the cytosolic construct.

Extra-cellular ADA-activity of construct p5'Ω-Ext\_ADA-3'HP transformed cell line peaked on day 7 followed by a drastic decline in ADA-activity in the next two days and increased ADA-activity thereafter. In a similar experiment investigating the monoclonal antibody levels in *Nicotiana tabacum* cell suspensions, Sharp and Doran, (2001) observed a slight increase in recombinant monoclonal IgG antibody levels in the medium on the day 16 of sampling after a drastic reduction of protein levels in suspension media following peak production on day 7. A similar pattern of ADA-activity was also observed in the construct p5'Ω-PR1a\_ADA transformed cell line. Whereas the ADA-activity of construct p5'Ω-Ext\_ADA transformed cell line showed a constant level of ADA from day 3 to day 11 followed by a steady decrease between days 13-15. The increase in ADA-activity on day 15 is thought to be due to cell death and the stabilizing effect of the PVP on released ADA in the media. As shown in the figure 6.1, the construct p5'Ω-Ext\_ADA-3'HP transformed cell line showed a large sudden increase of ADA levels on day 13 of sampling. This could be due to stabilizing effect by both PVP and also the extensively glycosylated arabinogalactan motifs present on released ADA serving to protect associated ADA from proteolytic degradation. Xu *et al.*, (2007) demonstrated that the incorporation of arabinogalactan motifs increased the resistance of the

recombinant IFN $\alpha$ 2 to chymotryptic digestion by up to 13-fold. This could be further investigated with prolonged incubation times.

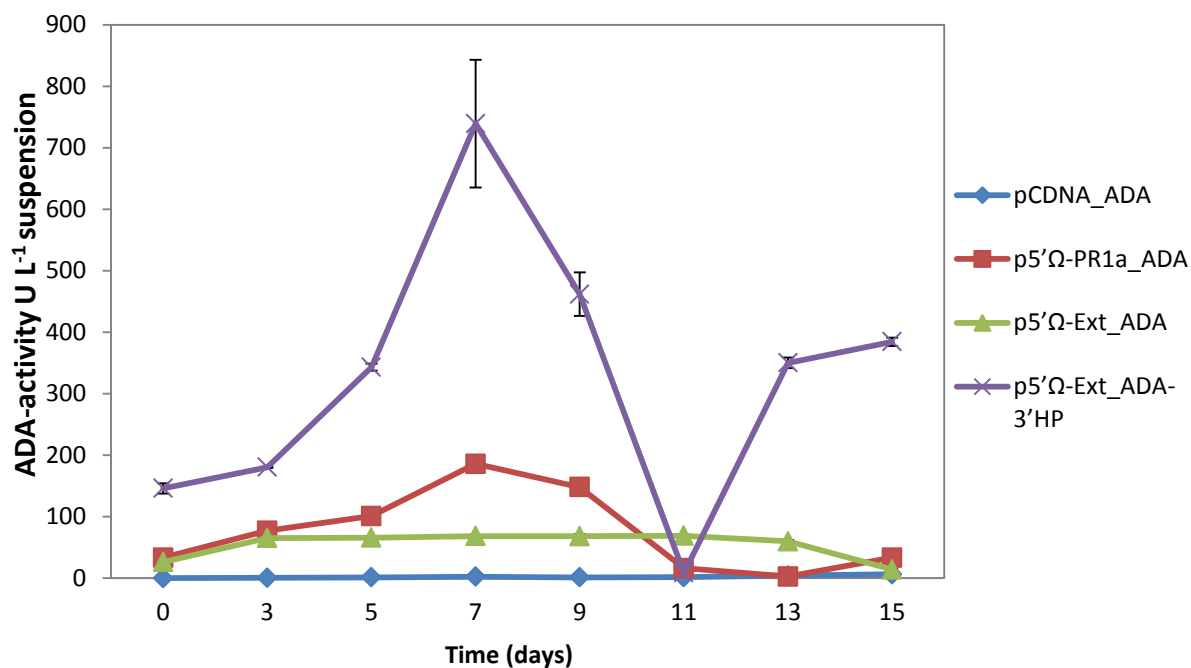


Figure 6.1: Extra-cellular ADA-activities measured in various cytosolic and apoplast-directing constructs in transformed BY-2 cell suspensions.

Table 6.1: Pair-wise comparisons of highest extra-cellular ADA-activities measured in transgenic tobacco BY-2 cell suspensions.

Constructs compared	Ratio of highest ADA-activities (Day 7)	Fold increase of highest ADA-activity on day 7
p cDNA_ADA versus p5'Ω-PR1a_ADA	2.2: 186.0	85
p cDNA_ADA versus p5'Ω-Ext_ADA	2.2: 68.4	31
p cDNA_ADA versus p5'Ω-Ext_ADA-3'HP	2.2: 739.4	336
p5'Ω-Ext_ADA versus p5'Ω-Ext_ADA-3'HP	68.4: 739.4	11



### 6.2.2 Intra-cellular ADA-activities in BY-2 cell suspensions transformed with ADA expressing constructs

There is up to a 1.5-fold increase in the highest recorded intra-cellular ADA-activity when suspensions transformed with apoplast targeting constructs are compared to the cytosolic control construct (figure 6.1 and table 6.1). The highest intra-cellular ADA-activity was measured on day 7 for the p5'Ω-Ext\_ADA-3'HP construct, transformed cell line which measured  $19.1 \text{ U L}^{-1}$ , a 1.5-fold increase over the cytosolic construct.

Unlike extra-cellular ADA-activity, intra-cellular ADA-activity of different constructs peaked on different days, ranging from day 7 to 11. The highest intra-cellular ADA-activity of the cell line containing apoplast targeting construct p5'Ω-PR1a\_ADA, was measured on day 11 of sampling as  $15.2 \text{ U L}^{-1}$ , a 1.2-fold increase over the cytosolic construct (table 6.2). Whereas, the highest intra-cellular ADA-activity of the cell line containing construct p5'Ω-Ext\_ADA, was measured on day 7 of sampling ( $8.4 \text{ U L}^{-1}$ ). Lower intra-cellular ADA-activity in cell suspensions can be explained by the targeting of ADA enzyme by *N*-terminal signal sequences into the secretory pathway, which in turn leads to secretion into the media, leaving little intra-cellular ADA enzyme.

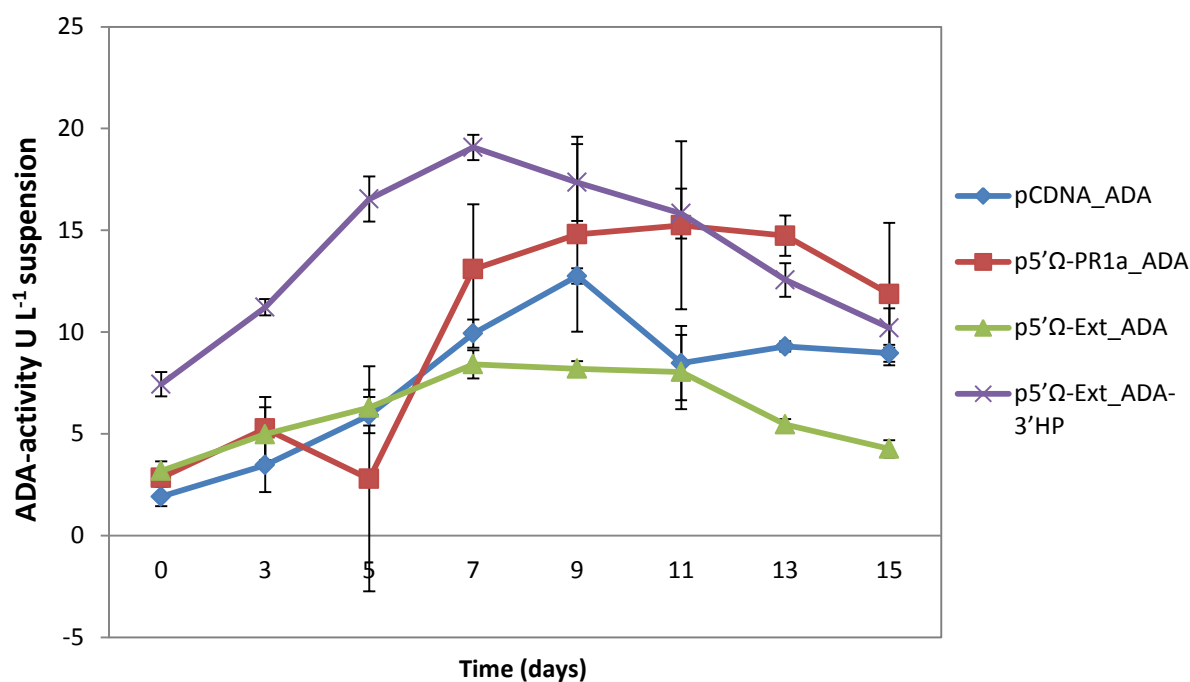


Figure 6.2: Intra-cellular ADA-activities measured in various cytosolic and apoplast-directing constructs in transformed BY-2 cell suspensions.

Table 6.2: Pair-wise comparisons of highest intra-cellular ADA-activities measured in transgenic tobacco BY-2 cell suspensions.

Constructs	Ratio of highest ADA-activities	Fold increase/decrease of highest ADA-activity
p cDNA_ADA versus p5'Ω-PR1a_ADA	12.7: 15.2	1.2
p cDNA_ADA versus p5'Ω-Ext_ADA	12.7: 8.4	0.3
p cDNA_ADA versus p5'Ω-Ext_ADA-3'HP	12.7: 19.1	1.5
p5'Ω-Ext_ADA versus p5'Ω-Ext_ADA-3'HP	8.4: 19.1	2.3

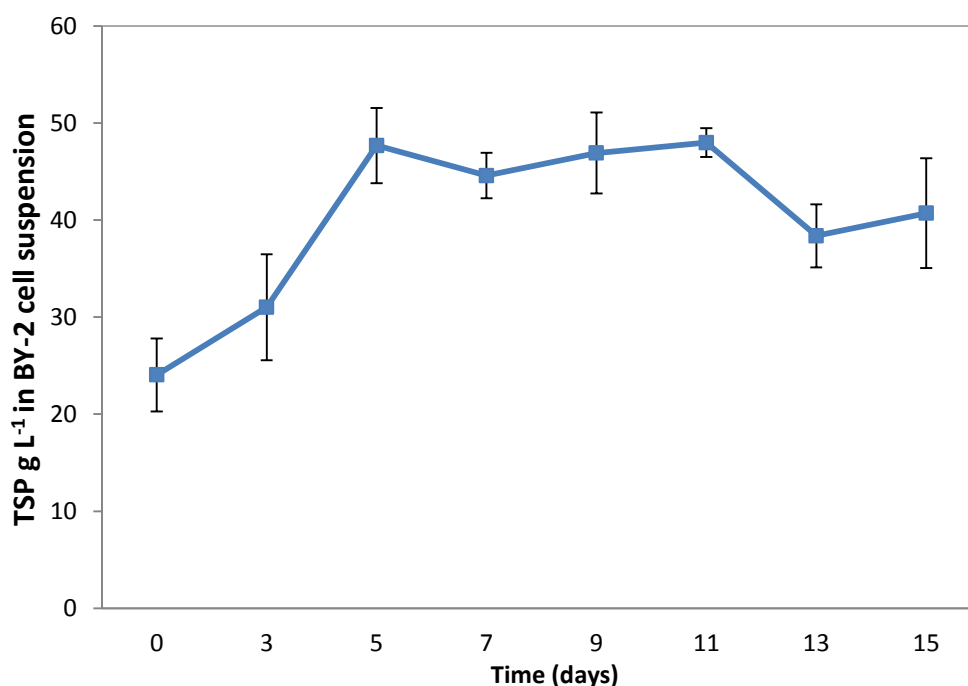


Figure 6.3: Total soluble proteins (g L<sup>-1</sup>) measured in Tobacco BY-2 cell suspension.

Table 6.3: Highest intra and extra-cellular ADA-activities in transgenic tobacco BY-2 suspensions.

Constructs	Intra-cellular (U L <sup>-1</sup> )	Extra-cellular (U L <sup>-1</sup> )	Total ADA in calli and medium (U L <sup>-1</sup> )	Total ADA in calli and medium (mg L <sup>-1</sup> )
p cDNA_ADA	12.7	2.2	14.9	0.3
p5'Ω-PR1a_ADA	15.2	186	201.2	3.6
p5'Ω-Ext_ADA	8.4	68.4	76.8	1.4
p5'Ω-Ext_ADA-3'HP	19.1	739.4	758.4	13.4

As shown in table 6.3, the highest intra-cellular ADA-activity in suspensions was measured in the cell line transformed with construct p5'Ω-Ext\_ADA-3'HP (19.1 U L<sup>-1</sup>/0.3 mg L<sup>-1</sup>). Whereas the highest extra-cellular ADA-activity in the same construct showed a 739.4 U L<sup>-1</sup> (13 mg L<sup>-1</sup>), which is a 39-fold increase in the extra-cellular ADA levels as compared to intra-cellular levels in suspension (table 6.3). The highest intra-cellular ADA-activity of p5'Ω-Ext\_ADA construct measured 8.4 U L<sup>-1</sup> (0.15 mg L<sup>-1</sup>), whereas the extra-cellular ADA-activity of the corresponding constructs measured 68.4 U L<sup>-1</sup> (1.2 mg L<sup>-1</sup>), an 8-fold increase in the extra-cellular ADA levels as compared to intra-cellular levels in suspension. This

suggests that the majority of the produced ADA has been secreted into the medium and also the highly glycosylated ADA containing hydroxyproline motifs seems to be stabilizing the recombinant protein eventually enhancing the yield. Table 6.3 confirms that most of the recombinant ADA targeted to the apoplast by *N*-terminal signal sequences, have been secreted into the medium, leaving little intra-cellular ADA enzyme.

Measurement of TSP in cell suspensions over time is an indicator of cell growth (Lee *et al.*, 2001). As shown in figure 6.3, the amount of total soluble protein measured in tobacco BY-2 cell suspensions shows a rapid increase by up to 2-fold from day 0 to day 5, followed by an almost constant level of TSP until day 11. A reduction in TSP occurred on day 13, followed by a slight increase on day 15. Nagata *et al.*, (1992) and Horemans *et al.*, (2003) also demonstrated that the exponential growth of BY-2 cells in suspension occurs from day 0 to 5, followed by a stationary phase. Sharp and Doran, (2001) reported that the growth of *Nicotiana tabacum* cells in suspension does not peak with foreign protein production, where maximum secreted levels of antibodies were observed on day 7 before maximum biomass was reached on day 14. Hence, higher amounts of biomass (or TSP) do not always peak with higher foreign protein production. Factors such as culture conditions and recombinant construct design determine when peak levels of targeted proteins are secreted into the medium. Confirming this Magnuson *et al.*, (1996) showed that secreted monoclonal antibody heavy chain  $\gamma$  in transgenic *Nicotiana tabacum* cell suspension peaked on day 7 followed by a rapid decrease. Similarly, Lee *et al.*, (2001) showed that murine GM-CSF expressed in transgenic *Nicotiana tabacum* cell suspension reached maximum levels on day 6 followed by a decline. In line with these findings, the highest amounts of secreted ADA were observed on day 7, two days after amounts of TSP had reached maximum levels.

**Table 6.4: High-yield expression of pharmaceutical proteins in plant cell suspension cultures.**

Therapeutic proteins	Host cells	Protein yields and signal used	Promoter	Localization	Reference
Human interferon $\alpha 2b$ (hIFN $\alpha 2$ )	<i>N. tabacum</i> cv BY-2	28 mg L <sup>-1</sup> (Ext)	<i>CaMV35S</i>	Secreted	Xu <i>et al.</i> , (2007)
Human growth hormone (hCH)	<i>N. tabacum</i> cv BY-2	35 mg L <sup>-1</sup> (Ext)	<i>CaMV35S</i>	Secreted	Xu <i>et al.</i> , (2010)
	<i>O. sativa</i> L. cv. Donjin	57 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Kim <i>et al.</i> , (2008a)
Hepatitis B surface antigen (HBsAg)	<i>glycine max</i> cv Williams82 (Soybean)	22 mg L <sup>-1</sup> (ER signal)	(OCS)3mas	Intracellular	Smith <i>et al.</i> , (2002)
mAb against HBsAg	<i>N. tabacum</i> cv BY-2	15 mg L <sup>-1</sup> (Ext)	<i>CaMV35S</i>	~50% secreted	Yano <i>et al.</i> , (2004)
Bryodin 1	<i>N. tabacum</i> cv NT- 1	30 mg L <sup>-1</sup> (Ext)	<i>CaMV35S</i>	Secreted	Francisco <i>et al.</i> , (1997)
Human $\alpha 1$ -antitrypsin (rAAT)	<i>O. sativa</i> (rice)	200 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Huang <i>et al.</i> , (2001)
	<i>O. sativa</i> cv Taipei 309	85 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Terashima <i>et al.</i> , (1999)
	<i>O. sativa</i> (rice)	247 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	McDonald <i>et al.</i> , (2005)
	<i>O. sativa</i> (rice)	110 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Trexler <i>et al.</i> , (2005)
	<i>O. sativa</i> (rice)	50 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Trexler <i>et al.</i> , (2002)
Human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig)	<i>O. sativa</i> (rice)	76.5 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Park <i>et al.</i> , (2010)
Human granulocyte-macrophage colony stimulating factor (hGM-CSF)	<i>O. sativa</i> (rice)	129 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Shin <i>et al.</i> , (2003)
Human lysozyme	<i>O. sativa</i> cv Taipei 309	3%-4% TSP* (RAmy3D)	<i>RAmy3D</i>	Intracellular	Huang <i>et al.</i> , (2002b)
Human serum albumin (HSA)	<i>O. sativa</i> (rice)	77 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Huang <i>et al.</i> , (2005)
Human interleukin-12 (IL-12)	<i>O. sativa</i> (rice)	31 mg L <sup>-1</sup> (RAmy3D)	<i>RAmy3D</i>	Secreted	Shin <i>et al.</i> , (2010b)
Human alkaline phosphatase	<i>N. tabacum</i> cv NT-1	27 mg L <sup>-1</sup>	<i>CaMV35S</i>	Secreted	Becerra-Arteaga <i>et al.</i> , (2006)
Mouse IgG anti <i>Streptococcus</i> surface antigen	<i>N. tabacum</i> cv NT-1	10.8 mg L <sup>-1</sup>	<i>CaMV35S</i>	Secreted	Wongsamuth and Doran (1997)
Human anti-HIV antibody 2G12	<i>N. tabacum</i> cv BY-2	~10 mg L <sup>-1</sup>	<i>CaMV35S</i>	Secreted	Holland <i>et al.</i> , (2010)
Human $\alpha$ -iduronidase	<i>N. tabacum</i> cv BY-2	10 mg L <sup>-1</sup> (proaleurain)	<i>CaMV35S</i>	Secreted	Fu <i>et al.</i> , (2009)
Human lactoferrin	<i>Acanthopanax senticosus</i>	3.6% TSP* (ER signal)	<i>SWPA2</i>	Intracellular	Jo <i>et al.</i> , (2006)
	<i>N. tabacum</i> cv BY-2	4.3% TSP* (ER signal)	<i>SWPA2</i>	Intracellular	Choi <i>et al.</i> , (2003)
<b>Human Adenosine deaminase</b>	<b><i>N. tabacum</i> cv BY-2</b>	<b>13 mg L<sup>-1</sup> (Ext, HP) 3.3 mg L<sup>-1</sup> (PR1a) 1.2 mg L<sup>-1</sup> (Ext)</b>	<b><i>CaMV35S</i></b>	<b>Secreted</b>	<b>Results mentioned in this thesis</b>

\*TSP: total soluble proteins. Modified from Xu *et al.*, (2011)

In line with previous attempts to express biopharmaceuticals in plant cell suspensions using apoplast targeting signals, BY-2 cell suspensions transformed with constructs containing apoplast targeting signals gave considerably higher levels of secreted ADA. Approximate extra-cellular ADA levels of  $13 \text{ mg L}^{-1}$ ,  $3.3 \text{ mg L}^{-1}$  and  $1.2 \text{ mg L}^{-1}$  were observed respectively in BY-2 cell suspensions transformed with p5'Ω-Ext\_ADA-3'HP, p5'Ω-PR1a\_ADA and p5'Ω-Ext\_ADA constructs (table 6.4). These are comparable to the levels of  $30 \text{ mg L}^{-1}$  measured for an extensin-bryodin constructs in tobacco cell suspensions. But lower than for example, hGM-CSF driven by a rice  $\alpha$ -amylase leader measured in rice cell suspension.

### 6.3 Discussion

Tobacco BY-2 cell suspensions were prepared from calli transformed with a cytosolic and three apoplast targeting constructs. The extra- and intra-cellular ADA-activities were measured on alternate days for 15 days in order to investigate the levels of ADA expression in cell suspension over time. The highest ADA enzyme-activities of suspensions transformed with various apoplast targeting constructs were compared to the highest ADA activity of a suspension transformed with a cytosolic construct in order to determine the overall increase in yield.

Incorporation of signal peptide sequences such as tobacco pathogenesis related protein (PR1a) in conjunction with recombinant proteins directs associated proteins into the apoplast leading to higher yield in cell suspensions (Pen *et al.*, 1993a; Ziegler *et al.*, 2000). Confirming these findings a comparison of highest extra-cellular ADA-activity ratios (day 7) shows that the incorporation of a PR1a *N*-terminal signal sequence along with the TMV omega 5'UTR sequence in BY-2 cell suspensions increased the ADA-activity by 85-fold as compared to the pCDNA\_ADA cytosolic construct (table 6.1). Whereas, the intra-cellular ADA levels only increased by 1.2-fold, showing that almost all the ADA enzyme is secreted into the external medium (table 6.2).

As with PR1a, the extensin signal peptide directs linked proteins into the secretory pathway enhancing the yield presumably by directing recombinant proteins away from proteolytic degradation in the cytosol and also in some cases enabling proteins to fold into biologically active structures. Incorporation of plant extensin signal peptide sequence at the *N*-terminus of recombinant proteins has been shown to direct secretion of recombinant proteins into the apoplast, which in turn are secreted into the culture medium of cell suspensions, eventually

enhancing the recombinant protein yield (Showalter, 1993; Francisco *et al.*, 1997). Confirming these findings the comparison of highest extra-cellular ADA activity ratios shows that the incorporation of an extensin *N*-terminal signal sequence along with TMV omega 5'UTR sequence in BY-2 cell suspensions increased the ADA activity by 31-fold as compared to the pCDNA\_ADA cytosolic construct (table 6.1). A decreased ADA activity of 0.3-fold was observed in intra-cellular samples from the same culture (table 6.2).

Another important strategy, by which recombinant proteins can be accumulated at high levels in cell suspension, is to express as a fusion with hydroxyproline (Hyp) residues at the *C*-terminus (Xu *et al.*, 2007). *C*-terminal hydroxyproline (Hyp) residues attract extensive *O*-glycosylation, increasing the molecular size, stability and serum half-life of the associated proteins in a similar way to therapeutic protein PEGylation (Shapk, 2001; Xu *et al.*, 2007). Confirming the findings of Xu *et al.*, (2007; 2008 and 2010), the most relevant results to this study were observed in BY-2 cell suspension containing the construct, p5'Ω-Extensin\_ADA-3'Hydroxyproline, where a comparison of highest extra-cellular ADA-activity (day 7) shows a 336-fold increase in ADA-activity as compared to the cell suspension transformed with the cytosolic construct pCDNA\_ADA (figure 6.1 and table 6.1).

Successful expression of a functional human adenosine deaminase in transgenic tobacco BY-2 cell suspensions has been demonstrated in this chapter. Attempts to increase the ADA yield, by incorporation of apoplast-directing signals in constructs, increased the ADA yield in the culture medium. Expression of proteins in fusion with the AGP glycomodule, Ser-Hyp (SO) repeats, exhibited the highest ADA-activities ( $739.4 \text{ U L}^{-1}/13 \text{ mg L}^{-1}$ ) secreted into culture medium in BY-2 cell suspensions. It's also interesting to note that in cell suspensions of calli transformed with the hydroxyproline construct, ADA levels are more than 10-fold greater



than when the extensin signal is used alone (table 6.3). This magnitude of difference in expression is not the same when ADA amounts are compared between the two corresponding calli (see chapter 5 figure 5.1). Here there is only a 1.6-fold increase in ADA yields between the same two calli lines. Again, this may be due to the stabilizing affect of both the hydroxyproline glycomodule and PVP on secreted ADA, but cell age also seems to be of paramount importance here. Since, cells in suspension are all a few days old, whereas cells in calli range in age from a few days to a few weeks. Hence, in support of the findings of Xu *et al.*, (2007, 2008 and 2010) a possible way forward to enhance the yield of foreign proteins in transgenic plant cell cultures is to express proteins as a fusion with AGP glycomodule Ser-Hyp (SO) repeats targeted to secretion.

# **Chapter 7**

## **Discussion**

Enzyme replacement therapy with polyethylene glycol conjugated bovine ADA (PEG-ADA) has been in use since 1987 for the treatment of ADA deficiency associated severe combined immunodeficiency syndrome (Booth *et al.*, 2007). Although ERT with PEG-ADA is generally well tolerated by patients, some patients develop neutralizing antibodies that hinders catalytic activity and enhances PEG-ADA clearance (Chaffee *et al.*, 1992). Hence, ERT with recombinant human ADA is thought to be an alternative to successfully overcome this problem. Adenosine deaminase catalyses the conversion of cytotoxic adenosine analogues into their non-toxic inosine analogues and is present in numerous organisms ranging from bacteria to mammals with the exception of plants (Dancer *et al.*, 1997). Conversion of toxic adenosine analogues into non-toxic analogues in plants are catalysed either by adenosine kinase or by phosphoribosyltransferase (Pelcher, 1995). Absence of ADA in plants provides an excellent platform to produce recombinant ADA in plants.

Although bacteria are classed as cost-effective and convenient production systems for many small human proteins such as human insulin and growth hormone, inability to carry out post-translational modification and assembly steps which are required for biological activity of most complex proteins, hinders their use for producing such proteins (Sharma and Sharma, 2009). As an alternative production system, plants exhibit an effective and efficient eukaryote protein synthesis mechanism, and with the advent of modern plant biotechnology, plants can be used to produce large quantities of protein with readily established gene expression systems (Shih and Doran, 2009). Plant expression systems are also free of mammalian pathogens and thus offer a prominent advantage over mammalian expression systems. As outlined in chapter 1, a wide array of techniques has been used in recent years to increase the expression levels of foreign genes in plants to ensure commercial feasibility. Moreover, a

readily available range of plant hosts and well established gene expression systems allows a variety of choices depending on the product under production and the site of production.

The recent advances in the use of plant cell cultures producing valuable pharmaceutical proteins highlights their benefits over whole-plants. Cell cultures: exhibit rapid growth due to short cell doubling times; can be used in controlled bioreactors to improve protein consistency; can exert mammalian-like post-translational modifications; and most importantly have reduced post-transcriptional silencing effects due to dedifferentiated cells (Lee *et al.*, 2000; Su and Lee, 2007). Moreover, plant cell suspension cultures can be modified to secrete proteins into media in sterile conditions *in vitro* which in turn reduces the cost of downstream processing. Hence, there is great potential for the production of foreign proteins, cost-effectively in large quantities. Whole plants and other plant cell systems are therefore ideal candidates for the production of human ADA at high-levels.

The work described in this thesis represents a study to assess the possibility of increasing expression levels of a functional human ADA in plants. The highest observed ADA specific-activity of  $3.70 \times 10^{-3} \text{ U mg}^{-1} \text{ TSP}$ , which is  $\sim 65.5 \text{ ng mg}^{-1} \text{ TSP}$  of ADA (0.006 % of TSP) was obtained from plants transformed with a p5'Ω<sub>ADA</sub> cytosolic construct. Stable ADA expression in transgenic plants was achieved without any plant codon optimisation of the gene ORF and only between 6 and 7 transgenic plants were analysed from each construct, therefore there is a potential to further increase expression levels using codon optimisation and analysing greater numbers of transgenic plants.

For a BY-2 cell line transformed with a cytosolic construct p5'\_{3'}Ω<sub>ADA</sub>, the highest observed ADA specific-activity measured was  $4.50 \times 10^{-3} \text{ U mg}^{-1} \text{ TSP}$ , which is  $\sim 80 \text{ ng mg}^{-1}$

TSP of ADA (0.008 % TSP). This consolidates the findings of Schmitz *et al.*, (1996) and Gallie and Walbot, (1990) where incorporation of omega TMV 5' and 3' UTR sequence was shown to increase expression levels of associated sequences. Northern blot analysis of transformed calli confirmed the mRNA stabilizing and translational enhancing effects of incorporated omega TMV 5' and 3' UTR sequences. In addition, southern blot analysis showed that ADA expression levels in plants transformed with cytosolic constructs are not directly proportional to insert copy number, confirming the findings of Jones *et al.*, (1985) amongst many others. Transformed plants with higher insert copy number actually showed lower ADA specific-activities. Western blot analysis of these plants confirmed that the molecular weight of recombinant ADA was very similar to that measured for human ADA.

In an attempt to increase the levels of ADA further, a strategy of targeting expressed proteins into the plant secretory pathway was employed, since cytosolic proteases have been implicated in destruction of over-expressed foreign proteins in the cytosol and some proteins fold up more efficiently into biologically active conformation in the ER (Doran, 2000). For this reason a tobacco pathogenesis related protein (PR1a) signal peptide sequence was placed at the *N*-terminus of the polypeptide sequence to direct associated ADA into the apoplast. This was predicted to increase the protein yield as shown by Pen *et al.*, (1993a) and Ziegler *et al.*, (2000). The plant apoplast is a non-living part of plant tissue, external to the cell membrane and composed of the cell walls, intercellular spaces, and the lumen of dead structures such as xylem cells. Since this region doesn't contain proteases, proteins targeted here are predicted to accumulate. In this study an attempt was made to direct ADA to the apoplast by stable nuclear transformation of BY-2 calli with constructs containing a PR1a *N*-terminal signal sequence. The highest observed ADA specific-activity of  $6.50 \times 10^{-3} \text{ U mg}^{-1} \text{ TSP}$  (~0.012 % of TSP) was obtained from BY-2 calli transformed with a p5'Ω-PR1a\_ADA

subcellular-targeting construct. This is almost 2-fold higher than obtained for the highest performing cytosolic construct. The same construct in cell suspension produced the highest overall ADA-activity of  $201 \text{ U L}^{-1}$  on day 7 of growth (table 7.1 and 7.2). In an attempt to consolidate this data, another apoplast directing signal taken from a tobacco extensin signal sequence was also investigated. Extensin (Ext) *N*-terminal signal sequences have been shown to increase the yield of a ribosome-inactivating protein (bryodin 1) in BY-2 cell suspensions (Francisco *et al.*, 1997). Extensin *N*-terminal signal sequences incorporated into a p5'Ω-Extensin\_ADA subcellular-targeting construct, resulted in ADA specific-activities of up to  $4.30 \times 10^{-3} \text{ U mg}^{-1} \text{ TSP}$ , of ADA ( $\sim 0.0076 \%$  TSP) in one calli cell line. This is only slightly higher than (a 1.2-fold increase) than that found with the highest performing cell line transformed with a cytosolic construct. The highest observed overall ADA-activity of  $76.8 \text{ U L}^{-1}$  was obtained on day 7 for a BY-2 cell suspension transformed with the same construct (table 7.1 and 7.2).

Although ADA levels were almost doubled in transformed BY-2 calli and cell suspensions by use of apoplast targeting signals, higher levels were sought by incorporation of Serine-Hydroxyproline repeats at the *C*-terminus of ADA. Xu *et al.*, (2007); (2008); (2010) demonstrated that the incorporation of the AGP glycomodule, Ser-Hyp (SO) repeats, at the *C*-terminus of recombinant proteins enhances secreted protein yield by between 350- to 1400-fold in BY-2 cell suspension as compared to a non-glycosylated counterpart. Incorporating the AGP glycomodule Ser-Hyp (SO<sub>10</sub>) repeats at the *C*-terminus in the p5'Ω-Ext\_ADA-3'HP subcellular-targeting construct resulted in ADA specific-activities of up to  $6.00 \times 10^{-3} \text{ U mg}^{-1} \text{ TSP}$  in BY-2 calli ( $\sim 0.011 \%$  TSP). This is significantly higher than (a 1.7-fold increase) that measured in the highest performing calli transformed with the cytosolic construct. Cell suspension data revealed that the highest observed combined intra and extra-cellular ADA-

activity of  $758.4 \text{ U L}^{-1}$  was obtained on day 7 in BY-2 cell suspension transformed with p5'Ω-Ext\_ADA-3'HP subcellular-targeting construct (table 7.1 and 7.2). Approximately a 4-fold higher ADA level as compared to a cell suspension transformed with a PR1a construct. The increased yield is thought to be due to the protein stabilizing affect of the hydroxyproline glycomodule. Xu *et al.*, (2007) found that AGP glycomodule Ser-Hyp (SO<sub>10</sub>) repeats not only increased the molecular weight of the protein but also the stability.

Levels of expressed ADA do not always yield significantly more ADA product in calli, when different constructs are compared. However, when ADA production is compared between constructs in transformed cell suspensions much higher secreted levels (up to 7-fold, figure 6.1) are evident. Hence, it is clear that the incorporation of the AGP glycomodule Ser-Hyp (SO<sub>10</sub>) repeats at the C-terminus of ADA ORF along with the protein stabilizing affect of PVP significantly increased the ADA yield in cell suspensions. Another factor taken into consideration here is the cell age difference between cell suspension and calli, where, age of cells in suspension can be measured in days but cells in calli range in age from a few days to a few weeks. Further to these findings, thrombin cleavage of glycosylated motifs didn't increase the ADA-activity considerably. Hence, it would seem that the biological activity of ADA is not significantly hindered by the extensively glycosylated AGP glycomodule. Minute intracellular ADA levels ( $20 \text{ U L}^{-1}$ ) also suggested that more than 95 % of the produced ADA has been secreted into the culture medium.

**Table 7.1: Summary of the highest ADA levels obtained in tobacco plants, BY-2 calli and BY-2 cell suspension transformed with various constructs.**

Construct	ADA U mg <sup>-1</sup> TSP	Estimated ADA ng mg <sup>-1</sup> TSP	ADA as % TSP
Plant cytosolic	3.70 x10 <sup>-3</sup>	65.5	0.006
Calli cytosolic	4.50 x10 <sup>-3</sup>	80	0.008
Calli PR1a	6.50 x10 <sup>-3</sup>	115	0.012
Calli extensin	4.30 x10 <sup>-3</sup>	76	0.0076
Calli AGP	6.00 x10 <sup>-3</sup>	106	0.011
BY-2 suspension cytosolic	1.50 x10 <sup>-3</sup>	26.5	0.002
BY-2 suspension PR1a	1.10 x10 <sup>-2</sup>	195	0.019
BY-2 suspension extensin	8.53 x10 <sup>-3</sup>	151	0.013
BY-2 suspension AGP	4.04 x10 <sup>-2</sup>	715	0.072

**Table 7.2: Summary of the highest combined intra and extra-cellular ADA levels obtained in BY-2 cell suspensions transformed with various constructs.**

Construct	ADA U L <sup>-1</sup>	Estimated ADA mg L <sup>-1</sup>
BY-2 suspension cytosolic	19.2	0.25
BY-2 suspension PR1a	201	3.5
BY-2 suspension Extensin	76.8	1.4
BY-2 suspension AGP	758.4	13.4

Problems regarding expensive and time-consuming downstream processing of plant produced proteins, especially when the host plant contains undesirable toxic compounds, are also addressed in this study where production of proteins in cell suspension provides for a more efficient method of extracting produced protein from the culture medium. Moreover, dedifferentiated BY-2 cells do not produce large amounts of phenolic and other toxic compounds found in whole tobacco plants. Further, the controversy surrounding GM plants grown in non-controlled environments would be irrelevant, as cell suspensions can be entirely contained in a controlled environment and offer a huge potential to produce biopharmaceuticals in a sterile environment (Kaiser, 2008; Shaaltiel *et al.*, 2007). The



productions of the first two USFDA-approved biopharmaceuticals in plant cell culture endorse this approach for the production of future products.

Although some plant glycosylation patterns are highly immunogenic to mammals (Doran, 2000; Leonard *et al.*, 2005), the presence of the AGP glycomodule Ser-Hyp (SO) repeats have been found to be non-immunogenic in mice (Xu *et al.*, 2007; 2010). Further investigations should be conducted in order to categorically confirm this finding, because this novel technique provides a potential plant protocol to produce non-immunogenic proteins including ADA with a greatly enhanced plasma half-life *in vivo* when used therapeutically. It was found that hydroxyproline glycomodule fusion of therapeutic proteins is as effective as PEGylation in enhancing plasma half-life (Yang *et al.*, 2004; Xu *et al.*, 2010)

This study was set up to investigate a range of strategies to increase the production of a functional human adenosine deaminase in plants and plant cell suspensions. Amongst all the strategies employed, incorporation of the AGP glycomodule at the C-terminus of the ADA protein has clearly proven to be the most effective product-yield enhancing strategy. The work described in this thesis leaves scope for future investigation to enhance levels of recombinant human ADA (rhADA) further and to explore protocols for the isolation of a pure product from transgenic plant materials. Purification of ADA is pivotal in order to explore the clinical importance of rhADA. Although the secretion of rhADA into the media of BY-2 cell suspension cultures eliminates most of the difficulties involved in protein purification from plant materials, future research will focus on purification steps necessary of rhADA isolation. Proteins of therapeutic applications require the highest purity (>99 %). Purification of proteins involves a series of enrichment steps including use of column-based methods such as ion exchange chromatography, affinity chromatography, hydrophobic interaction

chromatography and size-exclusion chromatography; also selection-based methods such as microfiltration, ultrafiltration, pertraction and pervaporation. Such steps increase the production costs significantly, where more than 80 % of the total production costs in plant systems accounts for downstream processing (Hellwig *et al.*, 2004). Once purified, investigations can be carried out to further characterise the rhADA using protein sequence analysis and enzyme kinetic studies. Once functionality and characterisation of rhADA are complete, investigation into biological activity of rhADA can be determined using ADA deficient mouse models (Blackburn *et al.*, 1998).

In order to explore the possibility of further increasing the yield of rhADA in plant cell suspensions an ER retention construct containing C-terminal KDEL sequence could be transformed into BY-2 cells and transformants would be analysed for ADA activity. The highest yields of some recombinant proteins were achieved by the retention in ER, rather than secretion into culture media (Doran, 2000). Conrad and Fielder, (1998) demonstrated that the ER retention of single-chain antibody fragments accounted for increased levels of between 10 to 100-fold compared to secreted antibody fragments. Hence, investigation into recombinant ADA retention in the ER could prove to be successful. Glycosylation studies of the ADA produced in different constructs should also be investigated in order to determine the glycosylation patterns generated when ADA is retained in the ER or secreted into the apoplasmic space.

Genomes of different organisms including plants employ codon bias as mechanisms for optimising and regulating protein expression (Gustafsson *et al.*, 2004; Murray *et al.*, 1989). Campbell and Gowri, (1990) reported the use of a preferred 44 codon set in plants, with a preference for codons ending in A or U by dicots such as tobacco. Various codon optimised

foreign genes have shown an enhanced expression in transformed plants including green fluorescent protein (Rouwendal *et al.*, 1997). Hence, it may be possible to enhance ADA expression levels further in tobacco by codon optimisation of the ADA ORF to reflect the codon bias of tobacco.

The chloroplast expression system offers numerous benefits over nuclear expression systems, including high-level expression and stable transformation due to lack of epigenetic interference. Furthermore, because of cytoplasmic localisation, transgenic chloroplasts are not transmitted through pollen grains. Many reports of transgene expression in tobacco chloroplasts show that foreign proteins can be produced in the range of 5-20 % TSP (Maliga, 2004). Hence, expressing ADA in chloroplasts may provide an excellent opportunity to further increase expression levels by very large amounts.

As well as AGP fusions employed in this study, other recently developed protein fusion strategies have been used to increase the accumulation and simplify the purification of recombinant proteins in plants. For example, foreign proteins have been fused to C-terminal elastin-like polypeptides (ELPs), containing pentapeptide repeats of 'VPGXG'. These peptide fusions possess the thermally responsive properties of elastin, which enables easy and inexpensive purification known as inverse transition cycling (Meyer and Chilkoti, 1999). In addition, ELP fusion also enhances recombinant protein accumulation by reducing protein degradation through resisting hydrolysis and proteolytic degradation (Raucher and Chilkoti, 2001; Zhang *et al.*, 1996). ELP fusions have increased the yield of foreign proteins by between 2 to 100-fold (Conley *et al.*, 2011). Interestingly, as with AGP fusions, ELP fusion does not greatly affect the biological activity of associated recombinant proteins (Floss *et al.*, 2009). Similarly, hydrophobin fusions have also been shown to increase associated

recombinant protein yield (Joensuu *et al.*, 2010). Hydrophobins are small surface-active proteins generally found in filamentous fungi (Conley *et al.*, 2011). Due to their surface-active properties, recombinant proteins fused to hydrophobins are easily purified by a surfactant-based aqueous two phase system, ensuring maximum purification (Linder *et al.*, 2004). In addition, hydrophobin fusions enhance the accumulation of associated proteins through protein stabilising effects and have also been found to be inert as fusions do not affect the biological activity of associated proteins. Finally, another strategy used to enhance recombinant protein yield in transgenic plants is to express foreign proteins as a fusion with zeolin. Zeolins are naturally occurring plant seed proteins containing proline-rich motifs (Herman and Larkins, 1999). These proteins have the ability to induce the formation of ER-derived protein bodies in plant tissues which is thought to be the main factor responsible for ER retention (Kogan *et al.*, 2001). Zeolin fusions have been found to increase the yield of foreign proteins by up to 100-fold (Torrent *et al.*, 2009a). Hence, as well as expressing ADA as an AGP fusion protein, other types of fusions could potentially enhance yields further.

# **Chapter 8**

## **References**

- Aerts, J. M. F. G., Yasothan, U. and Kirkpatrick, P. (2010)** 'Velaglucerase alfa', *Nature Reviews Drug Discovery*, 9(11), pp. 837-838.
- Aiuti, A. (2004)** 'Gene therapy for adenosine-deaminase deficient severe combined immunodeficiency', *Clinical Haematology*, 17(3), pp. 505-516.
- Andersson, S., Davis, D., Dahlback, H., Jörnvall, H. and Russell, D. W. (1989)** 'Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme', *The Journal of Biological Chemistry*, 264(14), pp. 8222-8229.
- Anderson, W. F., Blease, R. M. and Culver, K. (1990)** 'The ADA human gene therapy clinical protocol; points to consider response with clinical protocols', *Human Gene Therapy*, 1 (3), pp. 331-362.
- Angenon, G., Van Montagu, M. and Depicker A. (1990)** 'Analysis of the stop codon context in plant nuclear genes', *FEBS Letters*, 271(1-2), pp. 144-146.
- Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrahi, S., Liberman, Y., Freeman, A., Zimran, A. and Galun, E. (2009)** 'A Plant-Derived Recombinant Human Glucocerebrosidase Enzyme-A Preclinical and Phase I Investigation', 4(3), e4792, *PLoS ONE* [Online]. Available at: <http://www.plosone.org/article/> (Accessed: 15 April 2012)
- Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M. A. and Matzke, A. J. M. (1986)** 'The expression of a nopaline synthase-human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue', *Plant Molecular Biology*, 6, pp. 347-357.
- Becerra-Arteaga, A., Mason, H. S. and Shuler, M. L. (2006)** 'Production, secretion, and stability of human secreted alkaline phosphatase in tobacco NT1 cell suspension cultures', *Biotechnology Progress*, 22, pp. 1643-1649.
- Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Herz, S., Fröde, R., Inogés, S., López-Díaz de Cerio, A., Soria, E., Villanueva, H., Vancanneyt, G., McCormick, A., Tusé, D., Lenz, J., Butler-Ransohoff, J.-E., Klimyuk, V. and Gleba Y., (2010)** 'Rapid, high-yield production in plants of individualized idiotypic vaccines for non-Hodgkin's lymphoma', *Annals of Oncology*, 21(12), pp. 2420-2427.

- Benveniste, P.** and Cohen, A. (1995) 'p53 Expression is Required for Thymocyte Apoptosis Induced by Adenosine Deaminase Deficiency', *Proceedings of the National Academy of Sciences*, 92(18), pp. 8373-8377.
- Birnboim, H.C.** and Doly, J. (1979). 'A rapid alkaline extraction procedure for screening recombinant plasmid DNA', *Nucleic Acids Research*, 7(6), pp. 1513–1523.
- Blackburn, M. R., Datta, S. K.** and Kellems, R. E. (1998) 'Adenosine deaminase-deficient mice generated using a two-stage genetic engineering strategy exhibit a combined immunodeficiency', *Journal of Biological Chemistry*, 273, pp.5093–5100.
- Booth, C., Hershfield, M., Notarangelo, L., Buckley, R., Hoenig, M., Mahlaoui, N., Cavazzana-Calvo, M., Aiuti, A.** and Gaspar, H. B. (2007) 'Management options for adenosine deaminase deficiency; proceedings of the EBMT satellite workshop (Hamburg, March 2006)', *Clinical Immunology*, 123(2), pp. 139-147.
- Bordignon, C., Notarangelo, L. D., Nadia, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., Ugazio, A. G.** and Mavilio, F. (1995) 'Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients', *Science*, 270, pp. 475-480.
- Bradford, M.** (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, 72, pp. 248-25.
- Breunig, F.** and Wanner, C. (2003) 'Enzyme replacement therapy for Fabry disease: proving the clinical benefit', *Nephrology Dialysis Transplantation*, 18(1), pp. 7-9.
- Bringloe, D. H., Dyer, T. A.** and Gray, J. C. (1995) 'Developmental, circadian and light regulation of wheat ferredoxin gene expression', *Plant Molecular Biology*, 27, pp. 293-306
- Buckley, R. H., Schiff, S. E., Schiff, R. I., Markert, M. L., Williams, L. W., Roberts, J. L., Myers, L. A.** and Ward, F. E. (1999) 'Hematopoietic Stem-Cell Transplantation for the Treatment of Severe Combined Immunodeficiency', *New England Journal of Medicine*, 340(7), pp. 508-516.

- Chaffee**, S., Mary, A., Stiehm, E. R., Girault, D., Fischer, A. and Hershfield, M. S. (1992) 'IgG antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency', *The Journal of Clinical Investigation*, 89, pp.1643–1651.
- Campbell**, W. H. and Gowri, G. (1990) 'Codon Usage in Higher Plants, Green Algae, and Cyanobacteria', *Plant Physiology*, 92(1), pp. 1–11.
- Chan**, B., Wara, D., Bastian, J., Hershfield, M. S., Bohnsack, J., Azen, C. G., Parkman, R., Weinberg, K. and Kohn, D. B. (2005) 'Long-term efficacy of enzyme replacement therapy for Adenosine deaminase (ADA)-deficient Severe Combined Immunodeficiency (SCID)', *Clinical Immunology*, 117(2), pp. 133-143.
- Choi**, S. M., Lee, O. S., Kwon, S. Y., Kwak, S. S., Yu, D. Y. and Lee, H. S. (2003) 'High expression of a human lactoferrin in transgenic tobacco cell cultures', *Biotechnology Letters*, 25, pp. 213–218.
- Cohen**, S. N., Chang, A. C. Y. and Hsu, L. (1972) 'Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of Escherichia coli by R-Factor DNA', *Proceedings of the National Academy of Sciences*, 69(8), pp. 2110-2114.
- Conley**, A. J., Joensuu, J. J., Richman, A. and Menassa, R. (2011) 'Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants', *Plant Biotechnology Journal*, 9, pp. 419-433.
- Conrad**, U. and Fiedler, U. (1998) 'Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity', *Plant Molecular Biology*, 38, pp. 101-109.
- Cox**, K. M., Sterling, J. D., Regan, J. T., Gasdaska, J. R., Frantz, K. K., Peele, C. G., Black, A., Passmore, D., Moldovan-Loomis, C., Srinivasan, M., Cuisin, S., Cardarelli, P. M. and Dickey, L. F. (2006) 'Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*', *Nature Biotechnology*, 24(12), pp. 1591-1597.
- Dancer**, J.E., Hughes, R.G, and Lindell, S.D. (1997) 'Adenosine-5'-phosphate deaminase: a novel herbicide target', *Plant Physiology*, 114, pp. 119-129



- DeBeuckeleer, M., Lemmers, M., Vos, G., Willmitzer, L., Montagu, M. and Schell, J. (1981)** 'Further insight on the transferred-DNA of octopine crown gall', *Molecular and General Genetics*, 183, pp. 283-288.
- De Marchis, F., Balducci, C., Pompa, A., Riise, S. H. M., Guaragno, M., Pagiotti, R., Menghini, A. R., Persichetti, E., Beccari, T. and Bellucci, M. (2011).** 'Human  $\alpha$ -mannosidase produced in transgenic tobacco plants is processed in human  $\alpha$ -mannosidosis cell lines', *Plant Biotechnology Journal*, 9(9), pp. 1061-1073.
- Desai, P., Shrivastava, N. and Padh, H. (2010)** 'Production of heterologous protein in plants: strategies for optimal expression', *Biotechnology Advances*, 28, pp. 427–435.
- De Zoeten, G. A., Penswick, J. R., Horisberger, M. A., Ahl, P., Schultze, M. and Hohn, T. (1989)** 'The expression, localization, and effect of a human interferon in plants', *Virology*, 172, pp. 213-222.
- Doran, P. M. (2000)** 'Foreign protein production in plant tissue cultures', *Current Opinion in Biotechnology*, 11(2), pp. 199-204.
- Doran, P. M. (2004)** 'Foreign protein expression using plant cell suspension and hairy root cultures', *Molecular Farming*, pp. 13-36.
- Doran, P. M. (2006)** 'Foreign protein degradation and instability in plants and plant tissue cultures', *Trends in Biotechnology*, 24(9), pp. 426-432.
- Doran, P. M. (2006)** 'Loss of secreted antibody from transgenic plant tissue cultures due to surface adsorption', *Journal of Biotechnology*, 122(1), pp. 39-54.
- Faye, L., Boulaflous, A. Benchabane, M., Gomord, V. and Michaud, D. (2005)** 'Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming', *Vaccine*, 23(15), pp. 1770-1778.
- Fedosov, S. N., Laursen, N. B., Nexø, E., Moestrup, S. K., Petersen, T. E., Jensen, E. Ø. and Berglund, L. (2003)** 'Human intrinsic factor expressed in the plant *Arabidopsis thaliana*', *European Journal of Biochemistry*, 270(16), pp. 3362-3367.
- Filpula, D. R. and Sapra, P. (2009)** 'Enzymatic cancer therapy', United States Patent, 20090047270A1.

- Fischer, R., Emans, N., Schuster, F., Hellwig, S. and Drossard, J. (1999a)** 'Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors', *Biotechnology and Applied Biochemistry*, 30, pp. 109–112.
- Fischer, R. and Emans, N. (2000)** 'Molecular farming of pharmaceutical proteins', *Transgenic Reseach*, 9, pp. 279-299.
- Floss, D. M., Sack, M., Arcalis, E., Stadlmann, J., Quendler, H., Rademacher, T., Stoger, E., Scheller, J., Fischer, R. and Conrad, U. (2009)** 'Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus neutralizing antibody', *Plant Biotechnology Journal*, 7, pp. 899–913.
- Fogh, J., Irani, M., Anderson, S. and Weigelt, C. (2003)** 'Production of recombinant human lysosomal alpha-mannosidase', United States Patent, 20030199073A1.
- Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L., Brand, L. A., Fink, C. L., Fry, J. S., Galluppi, G. R., Goldberg, S. B., Hoffmann, N. L. and Woo, S. C. (1983)** 'Expression of bacterial genes in plant cells', *Proceedings of the National Academy of Sciences*, 80, pp. 4803–4807.
- Francisco, J. A., Gawlak, A. L., Miller, M., Bathe, J., Russell, D. and Chace, D. (1997)** 'Expression and characterization of Bryodin 1 and a Bryodin1-based single chain immunotoxin from tobacco cell culture', *Bioconjugate Chemistry*, 8, pp. 708-713.
- Fu, L. H., Miao, Y. S., Lo S, W., Seto, T, C., Sun, S, S, M., Xu, Z, F., Clemens, S., Clarke, L. A., Kermode, A. R. and Jiang, L. (2009)** 'Production and characterization of soluble human lysosomal enzyme alpha-iduronidase with high activity from culture media of transgenic tobacco BY-2 cells', *Plant Science*, 177, pp. 668–675.
- Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. and Wilson, T. M. (1987)** 'The 5'-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*', *Nucleic Acids Research*, 15(8), pp. 3257-3273.
- Gallie, D. R. and Kado, C. I. (1989)** 'A translational enhancer derived from tobacco mosaic virus is functionally equivalent to a Shine-Dalgarno sequence', *Proceedings of the National Academy of Sciences*, 86(1), pp. 129-132.

- Gallie, D. R. and Walbot, V. (1990)** 'RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells', *Genes and Development*, 4, pp. 1149-1157.
- Gallie, D. R. and Walbot, V. (1992)** 'Identification of the motifs within the tobacco mosaic virus 5'-leader responsible for enhancing translation', *Nucleic Acids Research*, 20(17), pp. 4631-4638.
- Gallie, D. R. (2002)** 'The 5'-leader of tobacco mosaic virus promotes translation through enhanced recruitment of eIF4F', *Nucleic Acids Research*, 30(15), pp. 3401-3411.
- Gangi-Peterson, L., Sorscher, D. H., Reynolds, J. W., Kepler, T. B. and Mitchell, B. S. (1999)** 'Nucleotide pool imbalance and adenosine deaminase deficiency induce alterations of N-region insertions during V(D)J recombination', *Journal of Clinical Investigation*, 103(6), pp. 833-841.
- Garger, J. S., Turpen, T. H. and Kumagai M, H. (2005)** 'Production of lysosomal enzymes in plants by transient expression', United States Patent, 6890748.
- Gaspar, H. B., Aiuti, A., Porta, F., Candotti, F., Hershfield, M. S. and Notarangelo, L. D. (2009)** 'How I treat ADA deficiency', *Blood*, 114(17), pp. 3524-3532.
- Georgiev, M. I., Weber, J. and Maciuk, A. (2009)** 'Bioprocessing of plant cell cultures for mass production of targeted compounds', *Applied Microbiology and Biotechnology*, 83, pp. 809–23.
- Gomord, V. and Faye, L. (2004)** 'Post-translational modification of therapeutic proteins in plants', *Current Opinion in Plant Biology*, 7, pp. 171-181.
- Gomord, V., Chamberlain, P., Jefferis, R. and Faye, L. (2005)** 'Biopharmaceutical production in plants: problems, solutions and opportunities', *Trends in Biotechnology*, 23, pp. 559–565.
- Gomord, V., Fitchette, A. C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D. and Faye, L. (2010)** 'Plant-specific glycosylation patterns in the context of therapeutic protein production', *Plant Biotechnology Journal*, 8(5), pp. 564-587.

- Gustafsson, C., Govindarajan, S. and Minshull, J. (2004)** 'Codon bias and heterologous protein expression', *Trends in Biotechnology*, 22(7), pp. 346-353.
- Gutierrez-Ortega, A., Sandoval-Montes, C., de Olivera-Flores, T. J., Santos-Argumedo, L. and Gomez-Lim, M. A. (2005)** 'Expression of functional interleukin-12 from mouse in transgenic tomato plants', *Transgenic Research*, 14, pp. 877–885.
- Haddad, E., Le Deist, F., Aucouturier, P., Cavazzana-Calvo, M., Blanche, S., De Saint Basile, G. and Fischer, A. (1999)** 'Long-Term Chimerism and B-Cell Function After Bone Marrow Transplantation in Patients With Severe Combined Immunodeficiency With B Cells: A Single-Center Study of 22 Patients', *Blood*, 94(8), pp. 2923-2930.
- Hanahan, D. (1985)** 'Techniques for transformation of *E. coli*. In DNA cloning: A Practical Approach', (ed. D.M. Glover), vol. 1 pp. 109-135. IRL Press, Oxford, United Kingdom.
- He, Z., Du, X., Yao, W. and Dai, J. (2008)** 'Pharmaceutical proteins produced in plant bioreactor in recent years', *African Journal of Biotechnology*, 7, pp. 4917–4925.
- Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S. and Mullineaux, P. M. (2000)** 'pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation', *Plant Molecular Biology*, 42(6), pp. 819-832.
- Hellwig, S., Drossard, J., Twyman, R. M. and Fischer, R. (2004)** 'Plant cell cultures for the production of recombinant proteins', *Nature Biotechnology*, 22, pp.1415–22.
- Herman, E. M. and Larkins, B. A. (1999)** 'Protein storage bodies and vacuoles', *Plant Cell*, 11, pp. 601–614.
- Hershfield, M. S. and Mitchell, B. S. (2001)** 'Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency', In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease*, New York: McGraw-Hill, pp. 2585-2625.
- Hershfield, M. S (2011)** 'Adenosine deaminase deficiency', *GeneReviews*, Bookshelf ID: NBK1483 PMID: 20301656. NCBI. [Online]. Available at: <http://www.ncbi.nlm.nih.gov/books/> (Accessed: 20 April 2012).

- Hepburn, A. G., Clarke, L. E., Pearson, L. and White, J. (1983)** 'The role of cytosine methylation in the control of nopaline synthase gene expression in a plant tumor', *Journal of Molecular and Applied Genetics*, 2, pp. 315–329.
- Hiatt, A., Cafferkey, R. and Bowdish, K. (1989)** 'The production of antibodies in transgenic plants', *Nature*, 342, pp.76–78.
- Hirschhorn, R., Tzall, S. and Ellenbogen, A. (1990)** 'Hot Spot Mutations in Adenosine Deaminase Deficiency', *Proceedings of the National Academy of Sciences*, 87(16), pp. 6171–6175.
- Ho, M. W., Ryan, A. and Cummins, J. (1999)** 'Cauliflower Mosaic Viral Promoter - A recipe for Disaster?', *Microbial Ecology in Health and Disease*, 11, pp. 194–197.
- Hogue, R. S., Lee, J. M. and An, G. (1990)** 'Production of a foreign protein product with genetically modified plant cells', *Enzyme and Microbial Technology*, 12(7), pp. 533–538.
- Holland, T., Sack, M., Rademacher, T., Schmale, K., Altmann, F., Stadlmann, J., Fischer, R. and Hellwig, S. (2010)** 'Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture', *Biotechnol and Bioengineering*, 107, pp. 278–289.
- Horemans, N., Potters, G., De Wilde, L. and Caubergs, R. J. (2003)** 'Dehydroascorbate Uptake Activity Correlates with Cell Growth and Cell Division of Tobacco Bright Yellow-2 Cell Cultures', *Plant Physiology*, 133(1), pp. 361–367.
- Horsch, R., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. and Fraley, R. T. (1985)** 'A simple and general method for transferring genes into plants', *Science*, 227, pp. 1229–1231.
- Huang, J., Sutliff, T. D., Wu, L., Nandi, S., Benge, K., Terashima, M., Ralston, A. H., Drohan, W., Huang, N. and Rodriguez, R. L. (2001)** 'Expression and purification of functional human alpha-1-Antitrypsin from cultured plant cells', *Biotechnology Progress*, 17, pp. 126–133.

- Huang, J. M., Wu, L. Y., Yalda, D., Adkins, Y., Kelleher, S. L., Crane, M., Lonnerdal, B., Rodriguez, R. L. and Huang, N. (2002b)** 'Expression of functional recombinant human lysozyme in transgenic rice cell culture', *Transgenic Research*, 11, pp. 229–239.
- Huang, L. F., Liu, Y. K., Lu, C. A., Hsieh, S. L. and Yu, S. M. (2005)** 'Production of human serum albumin by sugar starvation induced promoter and rice cell culture', *Transgenic Research*, 14, pp. 569–581.
- Huang, T. K. and McDonald, K. A. (2009)** 'Bioreactor engineering for recombinant protein production in plant cell suspension cultures', *Biochemical Engineering Journal*, 45(3), pp. 168–184.
- Huang, T. K. and McDonald, K. A. (2012)** 'Bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures', *Biotechnology Advances*, 30(2), pp. 398–409.
- Jani, D., Meena, L. S., Rizwan-ul-Haq, Q. M., Singh, Y., Sharma, A. K. and Tyagi, A. K. (2002)** 'Expression of cholera toxin B subunit in transgenic tomato plants', *Transgenic Research*, 11, pp. 447–454.
- Jo, S. H., Kwon, S. Y., Park, D. S., Yang, K. S., Kim, J. W., Lee, K. T., Kwak, S. S. and Lee, H. S. (2006)** 'High-yield production of functional human lactoferrin in transgenic cell cultures of Siberian ginseng (*Acanthopanax senticosus*)', *Biotechnology and Bioprocess Engineering*, 11, pp. 442–448.
- Joensuu, J. J., Conley, A. J., Lienemann, M., Brandle, J. E., Linder, M. B. and Menassa, R. (2010)** 'Hydrophobin fusions for highlevel transient protein expression and purification in *Nicotiana Benthamiana*', *Plant Physiology*, 152, pp. 622–633.
- Jones, J. D. G., Duismuir, P. and Bedbrook, J. (1985)** 'High level expression of introduced chimeric genes in regenerated transformed plants', *The EMBO Journal*, 4, pp. 2411–2418.
- Kaiser, J. (2008)** 'Is the Drought Over for Pharming?', *Science*, 320, pp. 473–475.
- Kang, T. J., Loc, N., Jang, M. and Yang, M. (2004c)** 'Modification of the cholera toxin B subunit coding sequence to enhance expression in plants', *Molecular Breeding*, 13, pp. 143–15
- Kapusta, J., Modelska, A., Figlerowicz, M., Pniewski, T., Letellier, M., Lisowa, O., Yusibov, V., Koprowski, H., Plucienniczak, A. and Legocki, A. B. (1999)** 'A plant-derived

edible vaccine against hepatitis B virus', *The journal of the Federation of American Societies for Experimental Biology*, 13(13), pp. 1796-1799.

**Kather, H.** (1990) 'Pathways of purine metabolism in human adipocytes. Further evidence against a role of adenosine as an endogenous regulator of human fat cell function', *Journal of Biological Chemistry*, 265(1), pp. 96-102.

**Kay, R., Chan, A., Daly, M. and McPherson, J.** (1987) 'Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes', *Science*, 236(4806), pp. 1299-1302.

**Kelly, M. A., Vestling, M. M., Murphy, C. M., Hua, S., Sumpter, T. and Fenselau, C.** (1996) 'Primary structure of bovine adenosine deaminase', *Journal of Pharmaceutical and Biomedical Analysis*, 14(11), pp. 1513-1519.

**Kieliszewski, M. J. and Lamport, D. T. A.** (1994) 'Extensin: repetitive motifs, functional sites, posttranslational codes and phylogeny', *The Plant Journal*, 5, pp. 157-172.

**Kieliszewski, M. J. and Shpak, E.** (2000) 'Synthetic genes for the elucidation of glycosylation codes for arabinogalactan-proteins and other hydroxyproline-rich glycoproteins', *Cellular and Molecular Life Sciences*, 58, pp. 1386-1398.

**Kim, T. G., Baek, M. Y., Lee, E. K., Kwon, T. H. and Yang, M. S.** (2008) 'Expression of human growth hormone in transgenic rice cell suspension culture', *Plant Cell Reports*, 27(5), pp. 885-891.

**Kogan, M. J., Dalcol, I., Gorostiza, P., Lopez-Iglesias, C., Pons, M., Sanz, F., Ludevid, D. and Giralt, E.** (2001) 'Self-assembly of the amphipathic helix (VHLPPP) 8. A mechanism for zein protein body formation', *Journal of Molecular Biology*, 312, pp. 907-913.

**Komarnytsky, S., Borisjuk, N. V., Borisjuk, L. G., Alam, M. Z. and Raskin, I.** (2000) 'Production of Recombinant Proteins in Tobacco Guttation Fluid', *Plant Physiology*, 124(3), pp. 927-934.

**Kumar, D., Patro, S., Ranjan, R., Sahoo, D. K., Maiti, I. B. and Dey, N.** (2011) 'Development of Useful Recombinant Promoter and Its Expression Analysis in Different Plant Cells Using Confocal Laser Scanning Microscopy', 6(9), e24627, *PLoS ONE* [Online]. Available at: <http://www.plosone.org/article/> (Accessed: 23 March 2012).



- Kumar, V., Abbas, A. K., Fausto, N. and Aster, J. (2009).** Kumar: Robbins and Cotran Pathologic Basis of Disease, Professional Edition, 8th ed. Philadelphia: SAUNDERS.
- LaCount, W., An, G. and Lee, J. M. (1997)** 'The effect of polyvinylpyrrolidone (PVP) on the heavy chain monoclonal antibody production from plant suspension cultures', *Biotechnology Letters*, 19(1), pp. 93-96.
- Laemmli, U. K. (1970)** 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', *Nature*, 227(5259), pp. 680-685.
- Lamport, D. T. A. (1977)** 'Structure, Biosynthesis and Significance of Cell Wall Glycoproteins', *Recent Advances in Phytochemistry*, New York: Plenum, pp.79-115.
- Lamport, D. T. A., Kieliszewski, M. J., Chen, Y. and Cannon, M. C. (2011).** 'Role of the Extensin Superfamily in Primary Cell Wall Architecture', *Plant Physiology*, 156(1), pp. 11-19.
- Lazo, G. R., Stein, P. A. and Ludwig, R. A. (1991)** 'A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*', *Nature Biotechnology*, 9, pp. 963-967.
- Lee, J. H., Kim, N. S., Kwon, T. H., Jang, Y. S. and Yang, M. S. (2002)** 'Increased production of human granulocyte-macrophage colony stimulating factor (hGM-CSF) by the addition of stabilizing polymer in plant suspension cultures', *Journal of Biotechnology*, 96(3), pp. 205-211.
- Lee, J. M., Magnuson, N. S., An, G. and Reeves, R. (2000)** 'Production of secreted foreign polypeptides in plant cell culture', United States Patent, No. 6020169.
- Lee, S. Y., Hur, W., Cho, G. H. and Kim, D. I. (2001)** 'Cultivation of Transgenic *Nicotiana tabacum* Suspension Cells in Bioreactors for the Production of mGM-CSF', *Biotechnology and Bioprocess Engineering*, 6, pp 72-74.
- Leonard, R., Petersen B.O., Himly, M., Kaar, W., Wopfner, N., Kolarich, D., Van Ree, R., Ebner, C., Duus, J. Ø., Ferreira, F. and Altmann, F. (2005)** 'Two novel types of O-glycans on the mugwort pollen allergen Art v 1 and their role in antibody binding', *The Journal of Biological Chemistry*, 280, pp. 7932–7940.



- Li, H. Y., Ramalingam, S. and Chye, M. L. (2006b)** 'Accumulation of recombinant SARS-CoV spike protein in plant cytosol and chloroplasts indicate potential for development of plant-derived oral vaccines', *Experimental Biology and Medicine*, 231, pp. 1346–1352.
- Lim, H., Lee, G., You, Y., Park, E., Song, Y., Yang, D. and Choi, K. (1999)** 'Regerataion and genetic transformation of hot pepper plants', *Acta Horticulturae (International Symposium on Vegetable Quality of Fresh and Fermented Vegetables)*, 483, pp. 387-396.
- Linder, M. B., Qiao, M., Laumen, F., Selber, K., Hyytia, T., Nakari-Setälä, T. and Penttilä, M. E. (2004)** 'Efficient purification of recombinant proteins using hydrophobins as tags in surfactantbased two-phase systems', *Biochemistry*, 43, 11873–11882.
- Lokshin, A., Raskovalova, T. Huang, X., Zacharia, L. C., Jackson, E. K. and Gorelik, E. (2006)** 'Adenosine-Mediated Inhibition of the Cytotoxic Activity and Cytokine Production by Activated Natural Killer Cells', *Cancer Research*, 66(15), pp. 7758-7765.
- Loose, M. D., Gheysen, G., Tiré, C., Gielen, J., Villarroel, R., Genetello, C., Van Montagu, M., Depicker, A. and Inzé, D. (1990)** 'The extensin signal peptide allows secretion of a heterologous protein from protoplasts', *Gene*, 99(1), pp. 95-100.
- Magnuson, N. S., Linzmaier, P. M., Gaob, J.-W., Reeves, R., An, G. and Lee, J. M. (1996)** 'Enhanced Recovery of a Secreted Mammalian Protein from Suspension Culture of Genetically Modified Tobacco Cells', *Protein Expression and Purification*, 7(2), pp. 220-228.
- Maliga, P. (2004)** 'Plastid Transformation in Higher Plants' *Annual Review of Plant Biology*, 55(1), pp. 289-313.
- Markley, N., Nykiforuk, C. Boothe, J. and Moloney, M. (2006)** 'Producing Proteins Using Transgenic Oilbody-Oleosin Technology', *BioPharm International*, 19(6), pp. 34-37.
- Mason, H. S., Lam, D. M. and Arntzen, C. J. (1992)** 'Expression of hepatitis B surface antigen in transgenic plants', *Proceedings of the National Academy of Sciences*, 89(24), pp.11745-11749.
- Mason, H. S., Ball, J. M., Shi, J. J., Jiang, X., Estes, M. K. and Arntzen, C. J. (1996)** 'Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral

immunogenicity in mice', *Proceedings of the National Academy of Sciences*, 93(11), pp. 5335-5340.

**Mason, H. S., Haq, T. A., Clements, J. D. and Arntzen, C. J. (1998)** 'Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene', *Vaccine*, 16(13), pp. 1336-1343.

**Mazur, B. J. and Chui, C. F. (1985)** 'Sequence of a genomic DNA clone for the small subunit of ribulose bis-phosphate carboxylase-oxygenase from tobacco', *Proceedings of the National Academy of Sciences*, 13, pp. 2373-2386.

**McDonald, K. A., Hong, L. M., Trombly, D. M., Xie, Q. and Jackman, A. P. (2005)** 'Production of human alpha-1-antitrypsin from transgenic rice cell culture in a membrane bioreactor', *Biotechnology Progress*, 21, pp. 728-734.

**Medin, J. A., Hunt, L., Gathy, K., Evans, R. K. and Coleman, M. S. (1990)** 'Efficient, low-cost protein factories: Expression of human adenosine deaminase in baculovirus-infected insect larvae', *Proceedings of the National Academy of Sciences*, 87, pp. 2760-2764.

**Meins, F. (2000)** 'RNA degradation and models for post-transcriptional gene silencing', *Plant Molecular Biology*, 43, pp. 261-273.

**Meyer, D. E. and Chilkoti, A. (1999)** 'Purification of recombinant proteins by fusion with thermally-responsive polypeptides', *Nature Biotechnology*, 17, pp. 1112-1115.

**Meyer, P. (2000)** 'Transcriptional transgene silencing and chromatin components', *Plant Molecular Biology*, 43, pp. 221-234.

**Modelska, A., Dietzschold, B., Sleysh, N., Fu, Z. F., Stepkowski, K., Hooper, D. C., Hilary, K. and Yusibov, V. (1998)** 'Immunization against rabies with plant-derived antigen', *Proceedings of the National Academy of Sciences*, 95(5), pp. 2481-2485.

**Murray, C., Sutherland, P. W., Phung, M. M., Lester, M. T., Marshall, R. K. and Christeller, J. T. (2002)** 'Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences', *Transgenic Research*, 11, pp. 199-214.

**Murray, E. E., Lotzer, J. and Eberle, M. (1989)** 'Codon usage in plant genes', *Nucleic Acids Research*, 17(2), pp. 477-498.

- Nagata, T.**, Nemoto, Y. and Hasezawa, S. (1992) 'Tobacco BY-2 Cell Line as the “HeLa” Cell in the Cell Biology of Higher Plants. International Review of Cytology', *International Review of Cytology*, 132, pp. 1-30.
- Nuttall, J.**, Vine, N., Hadlington, J. L., Drake, P., Frigerio, L. and Ma, J. K. (2002) 'ER-resident chaperone interactions with recombinant antibodies in transgenic plants', *European Journal of Biochemistry*, 269, pp. 6042-6051.
- Nykiforuk, C. L.**, Boothe, J. G., Murray, E. W., Keon, R. G., Goren, H. J., Markley, N. A. and Moloney, M. M. (2006) 'Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds', *Plant Biotechnology Journal*, 4(1), pp. 77-85.
- Obembe, O. O.**, Popoola, J. O., Leelavathi, S. and Reddy, S. V. (2011) 'Advances in plant molecular farming', *Biotechnology Advances*, 29(2), pp. 210-222.
- Odell, J. T.**, Nagy, F. and Chua, N. H. (1985) 'Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter', *Nature*. 313, pp. 810–812.
- Pacheco, R.**, Martinez-Navio, J. M., Lejeune, M., Climent, N., Oliva, H., Gatell, J. M., Gallart, T., Mallol, J., Lluís, C. and Franco, R. (2005) 'CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse', *Proceedings of the National Academy of Sciences*, 102(27), pp. 9583-9588.
- Panahi, M.**, Alli, Z., Cheng, X., Belbaraka, L., Belgoudi, J., Sardana, R., Phipps, J. and Altosaar, I. (2004) 'Recombinant Protein Expression Plasmids Optimized for Industrial *E. coli* Fermentation and Plant Systems Produce Biologically Active Human Insulin-like Growth Factor-1 in Transgenic Rice and Tobacco Plants', *Transgenic Research*, 13(3), pp. 245-259.
- Park, C. I.**, Lee, S. J., Kang, S. H., Jung, H. S. and Kim, D. I. (2010) 'Fed-batch cultivation of transgenic rice cells for the production of hCTLA4Ig using concentrated amino acids', *Process Biochemistry*, 45, pp. 67–74.
- Pastores, G.**, and Hughes, D. (2011) 'Gaucher Disease', *GeneReviews*, Bookshelf ID: NBK1269 PMID: 20301446. NCBI [Online]. Available at: <http://www.ncbi.nlm.nih.gov/books/> (Accessed: 20 April 2012).

- Patel, M., Corey, A.C., Yin, L. P., Ali, S., Taylor, W. C. and Berry, J. O. (2004)** 'Untranslated regions from C4 *Amaranth* AhRbcS1 mRNAs confer translational enhancement and preferential bundle sheath cell expression in transgenic c4 *Flaveria bidentis*', *Plant physiology*, 136, pp. 3550-3561.
- Patel, M., Siegel, A. J. and Berry, J. O. (2006)** 'Untranslated regions of FbRbcS1 mRNA mediate bundle sheath cell-specific gene expression in leaves of a C4 plant', *The Journal of Biological Chemistry*, 281, pp. 25485–25491.
- Paul, M. and Ma, J. K. C. (2011)** 'Plant-made pharmaceuticals: Leading products and production platforms', *Biotechnology and Applied Biochemistry*, 58(1), pp. 58-67.
- Pelcher, L. (1995)** 'Selectable/reporter gene for use during genetic engineering of plants and plant cells', United States Patent, 5474929.
- Pen, J., van Ooyen, A. J. J., van den Elzena, P. J. M., Quaxb, W. J. and Hoekema, A. (1993a)** 'Efficient production of active industrial enzymes in plants', *Industrial Crops and Prod. Science*, 1, pp. 241-250.
- Petolino, J. F., Young, S., Hopkins, N., Sukhapinda, K., Woosley, A., Hayes, C. and Pelcher, L. (1999)** 'Expression of murine adenosine deaminase (ADA) in transgenic maize', *Transgenic research*, 9, pp. 1-9.
- Petrucelli, S., Otegui, M. S., Lareu, F., Tran Dinh, O., Fitchette, A. C., Circosta, A., Rumbo, M., Bardor, M., Carcamo, R., Gomord, V. and Beachy, R. N. (2006)** 'A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds', *Plant Biotechnology Journal*. 4(5), pp. 511-527.
- Pfizer Press Releases, (2012.)** 'Pfizer And Protalix BioTherapeutics Announce FDA Approval Of Elelyso™ (taliglucerase alfa) For The Treatment Of Gaucher Disease', [Online]. Available at: [http://www.pfizer.com/news/press\\_releases/](http://www.pfizer.com/news/press_releases/) (Accessed: 02 May 2012).
- Pickering, B. M. and Willis, A. E. (2005)** 'The implications of structured 5' untranslated regions on translation and disease', *Seminars in Cell & Developmental Biology*, 16(1), pp. 39-47.

- Pogue, G. P.,** Vojdani, F., Palmer, K. E., Hiatt, E., Hume, S., Phelps, J., Long, L., Bohorova, N., Kim, D., Pauly, M., Velasco, J., Whaley, K., Zeitlin, L., Garger, S. J., White, E., Bai, Y., Haydon, H. and Bratcher, B. (2010) 'Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems', *Plant Biotechnology Journal*, 8(5), pp. 638-654.
- Pollard, J. H.** (1977) *A Handbook of Numerical and Statistical Techniques with Examples Mainly from the Life Sciences*, pp. 116-127, Cambridge UK: Cambridge University Press.
- Polmar, S. H.,** Stern, R. C., Schwartz, A. L., Wetzler, E. M., Chase, P. A. and Hirschhorn, R. (1976) 'Enzyme Replacement Therapy for Adenosine Deaminase Deficiency and Severe Combined Immunodeficiency', *New England Journal of Medicine*, 295(24), pp. 1337-1343.
- Polvino, W. J.,** Saravis, C. A., Sampson, C. E. and Cook, R. B. (1983) 'Improved protein analysis on nitrocellulose membranes', *Electrophoresis*, 4(5), pp. 368-369.
- Qi, W.,** Fong, C. and Lamport, D. T. A. (1991) 'Gum Arabic glycoprotein is a twisted hairy rope: a new model based on O-galactosylhydroxyproline as the polysaccharide attachment site', *Plant Physiology*, 96, pp. 848-855.
- Que, Q.,** Wang, H. Y., English, J. J. and Jorgensen, R. A. (1997) 'The Frequency and Degree of Cosuppression by Sense Chalcone Synthase Transgenes Are Dependent on Transgene Promoter Strength and Are Reduced by Premature Nonsense Codons in the Transgene Coding Sequence', *The Plant cell*, 9(8), pp. 1357-1368.
- Raucher, D.** and Chilkoti, A. (2001) 'Enhanced uptake of a thermally responsive polypeptide by tumor cells in response to its hyperthermia-mediated phase transition', *Cancer Research*, 61, pp. 7163-7170.
- Rempel, H. C.** and Nelson, L. M. (1995) 'Analysis of conditions for *Agrobacterium*-mediated transformation of tobacco cells in suspension', *Transgenic Research*, 4, pp. 199-207.
- Rouwendal, G. J. A.,** Mendes, O., Wolbert, E. J. and Douwe de Boer, A. (1997) 'Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage', *Plant Molecular Biology*, 33(6), pp. 989-999.

- Rubocki**, R. J., Parsa, J. R., Hershfield, M. S., Sanger, W. G., Pirruccello, S. J., Santisteban, I., Gordon, B. G., Strandjord, S. E., Warkentin, P. I. and Coccia, P. F. (2001) 'Full hematopoietic engraftment after allogeneic bone marrow transplantation without cytoreduction in a child with severe combined immunodeficiency', *Blood*, 97(3), pp. 809-811.
- Rybicki**, E. P. (2010) 'Plant-made vaccines for humans and animals', *Plant Biotechnology Journal*, 8(5), pp. 620-637.
- Saint-Jore-Dupas**, C., Faye, L. and Gomord, V. (2007) 'From planta to pharma with glycosylation in the toolbox', *Trends in Biotechnology*, 25, pp. 317-323.
- Samyn-Petit**, B., Gruber, V., Flahaut, C., Wajda-Dubos, J. P., Farrer, S., Pons, A., Desmaizieres, G., Slomianny, M. C., Theisen, M. and Delannoy, P. (2001) 'N-glycosylation potential of maize: The human lactoferrin used as a model', *Glycoconjugate Journal*, 18(7), pp. 519-527.
- Sanders**, P. R., Winter, J. A., Barnason A. R., Rogers, S. G. and Fraley R. T. (1987) 'Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants' *Nucleic Acid Research*, 15(4), pp. 1543-1558.
- Santisteban**, I., Arredondo-Vega, F. X., Daniels, S. and Hershfield, M. S. (2003) '*E. coli* Expression System for Identifying Folding Mutations of Human Adenosine Deaminase', *Protein Misfolding and Disease Principles and Protocols*. P. Bross and N. Gregersen, New Jersey: Humana Press, 232, pp. 175-182.
- Schmitz**, J., Prüfer, D., Rohde, W. and Tacke, E. (1996) 'Non-canonical translation mechanisms in plants: efficient *in vitro* and in planta initiation at AUU codons of the tobacco mosaic virus enhancer sequence', *Nucleic Acids Research*, 24(2), pp. 257-263.
- Schubert**, D., Lechtenberg, B., Forsbach, A., Gils, M., Bahadur, S. and Schmidt, R. (2004) 'Silencing in Arabidopsis T-DNA Transformants: The Predominant Role of a Gene-Specific RNA Sensing Mechanism versus Position Effects', *The Plant Cell*, 16(10), pp. 2561-2572.
- Shaaltiel**, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., Dym, O., Boldin-Adamsky, S. A., Silman, I., Sussman, J. L., Futerman, A. H. and Aviezer, D. (2007) 'Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement

therapy of Gaucher's disease using a plant cell system', *Plant Biotechnology Journal*, 5(5), pp. 579-590.

**Sharma, A. K. and Sharma, M. K.** (2009) 'Plants as bioreactors: recent developments and emerging opportunities', *Biotechnology Advances*, 2, pp. 811-832.

**Sharp, J. M. and Doran, P. M.** (2001) 'Strategies for Enhancing Monoclonal Antibody Accumulation in Plant Cell and Organ Cultures', *Biotechnology Progress*, 17(6), pp. 979-992.

**Shih, S. M. H. and Doran, P. M.** (2009) 'Foreign protein production using plant cell and organ cultures: Advantages and limitations', *Biotechnology Advances*, 27(6), pp. 1036-1042.

**Shin, Y. J., Hong, S. Y., Kwon, T. H., Jang, Y. S. and Yang, M. S.** (2003) 'High level of expression of recombinant human granulocyte-macrophage colony stimulating factor in transgenic rice cell suspension culture', *Biotechnology and Bioengineering*, 82(7), pp. 778-783.

**Shin, Y. J., Lee, N. J., Kima, J., An, X. H., Yang, M. S. and Kwon, T. H.** (2010b) 'High-level production of bioactive heterodimeric protein human interleukin-12 in rice', *Enzyme and Microbial Technology*, 46, pp. 347–351.

**Shoji, Y., Farrance, C. E., Bautista, J., Bi, H., Musiyчук, K., Horsey, A., Park, H., Jaje, J., Green, B. J., Shamloul, M., Sharma, S., Chichester, J. A., Mett, V. and Yusibov, V.** (2012) 'A plant-based system for rapid production of influenza vaccine antigens', *Influenza and Other Respiratory Viruses*, 6(3), pp. 204-210.

**Showalter, A. M.** (1993) 'Structure and function of plant cell wall proteins', *Plant Cell*, 5, pp. 9-23.

**Showalter, A. M.** (2001) 'Arabinogalactan-proteins: structure, expression and function', *Cellular and Molecular Life Sciences*, 58, pp. 1399–1417.

**Shpak, E., Barbar, E., Leykam, J. F. and Kieliszewski, M. J.** (2001) 'Contiguous hydroxyproline residues direct hydroxyproline arabinosylation in *Nicotiana tabacum*', *The journal of biochemistry*, 276(14), pp. 11272-11278.



- Sijen, T.**, Vijn, I. Rebocho, A., van Blokland, R., Roelofs, D., Mol, J. N. M. and Koote, J. M. (2001) 'Transcriptional and posttranscriptional gene silencing are mechanistically related', *Current biology*, 11(6), pp. 436-440.
- Sijmons, P. C.**, Dekker, B. M. M., Schrammeijer, B., Verwoerd, T. C., van den Elzen, P. J. M. and Hoekema, A. (1990) 'Production of Correctly Processed Human Serum Albumin in Transgenic Plants', *Nature Biotechnology*, 8(3), pp. 217-221.
- Sitkovsky, M.** and Lukashev, D. (2005) 'Regulation of immune cells by local-tissue oxygen tension: HIF1[alpha] and adenosine receptors', *Nature Reviews Immunology*, 5(9): 712-721.
- Smith, M. L.**, Mason, H. S. and Shuler, M. L. (2002) 'Hepatitis B surface antigen (HBsAg) expression in plant cell culture: kinetics of antigen accumulation in batch culture and its intracellular form', *Biotechnology and Bioengineering*, 80, pp. 812-822.
- Springer, P. S.**, McCombie, W. R., Sundaresan, V. and Martienssen, R. A. (1995) 'Gene trap tagging of PROLIFERA, an essential MCM2-3-5-like gene in *Arabidopsis*', *Science*, 268, pp. 877-880.
- Stam, M.**, De Bruijn, R., Kenter, S., Van Der Hoorn, R. A. L., Van Blokland, R., Mol, J. N. M. and Kooter, J. M. (1997) 'Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats', *The Plant Journal*, 12, pp. 63-82.
- Streatfield, S. J.** (2006) 'Mucosal immunization using recombinant plant-based oral vaccines', *Methods*, 38(2), pp. 150-157.
- Su, W. W.** and Lee, K. T. (2007). 'Chapter 10 - Plant Cell and Hairy Root Cultures-Process Characteristics, Products, and Applications', *Bioprocessing for Value-Added Products from Renewable Resources*, Y. Shang-Tian. Amsterdam: Elsevier: pp. 263-292.
- Tacket, C. O.**, Pasetti, M. F., Edelman, R., Howard, J. A. and Streatfield, S. (2004) 'Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn', *Vaccine*. 22(31-32), pp. 4385-4389.
- Terashima, M.**, Murai, Y., Kawamura, M., Nakanishi, S., Stoltz, T., Chen, L., Drohan, W., Rodriguez, R. L. and Katoh, S. (1999) 'Production of functional human alpha(1)-antitrypsin by plant cell culture', *Applied Microbiology and Biotechnology*, 52(4), pp. 516-523.



- Torrent, M.**, Llompart, B., Lasserre-Ramassamy, S., Llop-Tous, I., Bastida, M., Marzabal, P., Westerholm-Parvinen, A., Saloheimo, M., Heifetz, P. B. and Ludevid, M. D. (2009a) 'Eukaryotic protein production in designed storage organelles', *BMC Biology*, 7, 5.
- Torres, E.**, Vaquero, C., Nicholson, L., Sack, M., Stöger, E., Drossard, J., Christou, P., Fischer, R. and Perrin, Y. (1999) 'Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies', *Transgenic Research*, 8(6), pp. 441-449.
- Trexler, M. M.**, McDonald, K. A. and Jackman A. P. (2002) 'Bioreactor production of human alpha(1)-antitrypsin using metabolically regulated plant cell cultures', *Biotechnology Progress*, 18, pp. 501-508.
- Trexler, M. M.**, McDonald, K. A. and Jackman A. P. (2005) 'A cyclical semicontinuous process for production of human alpha(1)-antitrypsin using metabolically induced plant cell suspension cultures', *Biotechnology Progress*, 21, pp. 321-328.
- Tsuboi, I.**, Sagawa, K., Shichijo, S., Yokoyama, M. M., Ou, D. W. and Wiederhold, M. D. (1995) 'Adenosine deaminase isoenzyme levels in patients with human T-cell lymphotropic virus type 1 and human immunodeficiency virus type 1 infections', *Clinical and Diagnostic Laboratory Immunology*, 2(5), pp. 626-630.
- Van de Wiele, C. J.**, Vaughn, J. G., Blackburn, M. R, Ledent, C. A, Jacobson, M., Jiang, H. and Thompson, L. F. (2002) 'Adenosine kinase inhibition promotes survival of fetal adenosine deaminase-deficient thymocytes by blocking dATP accumulation', *The Journal of Clinical Investigation*, 110, pp. 395-402.
- Vaucheret, H.**, Béclin, C. and Fagard, M. (2001) 'Post-transcriptional gene silencing in plants', *Journal of Cell Science*, 114(17), pp. 3083-3091.
- Vergunst, A. C.**, Jansen, L. E. T. and Hooykaas, P. J. J. (1998) 'Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase', *Nucleic Acids Research*, 26, pp. 2729-2734.
- Wiginton, D. A.**, Adrian, G. S., Friedman, R. L., Suttle, D. P. and Hutton, J. J. (1983) 'Cloning of cDNA sequences of human adenosine deaminase', *Proceedings of the National Academy of Sciences*, 80(24), pp. 7481-7485

- Wiginton, D. A., Adrian, G. S. and Hutton, J. J. (1984)** 'Sequence of human adenosine deaminase cDNA including the coding region and a small intron', *Nucleic Acids Research*, 12(5), pp. 2439-2446.
- Winslow, L. C. and Kroll, D. J. (1998)** 'Herbs as medicines', *Archives of Internal Medicine*, 158, pp. 2192–2199.
- Wirth, S., Calamante, G., Mentaberry, A., Bussmann, L., Lattanzi, M., Barañao, L. and Bravo-Almonacid, F. (2004)** 'Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems', *Molecular Breeding*, 13, pp. 23-35.
- Wongsamuth, R. and Doran, P. M. (1997)** 'Production of monoclonal antibodies by tobacco hairy roots', *Biotechnology and Bioengineering*, 54, pp. 401-415.
- Woods, R., Geyer, B. C. and Mor, T. S. (2008)** 'Hairy-root organ cultures for the production of human acetylcholinesterase', *BMC Biotechnology*, 8(1), 95.
- Wright, P. S., Loudy, D. E., Cross-Doersen, D. E., Montgomery, L. R., Sprinkle-Cavallo, J., Miller, J. A., Distler, C. M., Lower, E. E. and Woessner, R. D. (1997)** 'Quantitation of vascular endothelial growth factor mRNA levels in human breast tumors and metastatic lymph nodes', *Experimental and Molecular Pathology*, 64, pp. 41–51.
- Xu, H., Montoya, F. U., Wang, Z., Lee, J. M., Reeves, R., Linthicum, D. S. and Magnuson, N. S. (2002)** 'Combined Use of Regulatory Elements within the cDNA to Increase the Production of a Soluble Mouse Single-Chain Antibody, scFv, from Tobacco Cell Suspension Cultures', *Protein Expression and Purification*, 24(3), pp. 384-394.
- Xu, J., Tan, L., Goodrum, K. J. and Kieliszewski, M. J. (2007)** 'High-yields and extended serum half-life of human interferon  $\alpha 2b$  expressed in tobacco cells as arabinogalactan-protein fusions', *Biotechnology and Bioengineering*, 97(5), pp. 997-1008.
- Xu, J., Tan, L., Lamport, D. T., Showalter, A. M. and Kieliszewski, M. J. (2008)** 'The *O*-Hyp glycosylation code in tobacco and Arabidopsis and a proposed role of Hyp-glycans in secretion', *Phytochemistry*, 69(8), pp. 1631-1640.

- Xu, J.**, Okada, S., Tan, L., Goodrum, K. J., Kopchick, J. J. and Kieliszewski, M. J. (2010) 'Human growth hormone expressed in tobacco cells as an arabinogalactan-protein fusion glycoprotein has a prolonged serum life', *Transgenic Research*, 19(5), pp. 849–867.
- Xu, J.**, Ge, X. and Dolan, M. C. (2011) 'Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures', *Biotechnology Advances*, 29(3), pp. 278-299.
- Yang, Z.**, Wang, J., Lu, Q., Xu, J., Kobayashi, Y., Takakura, T., Takimoto, A., Yoshioka, T., Lian, C., Chen, C., Zhang, D., Zhang, Y., Li, S., Sun, X., Tan, Y., Yagi, S., Frenkel, E. P. and Hoffman, R. M. (2004) 'PEGylation Confers Greatly Extended Half-Life and Attenuated Immunogenicity to Recombinant Methioninase in Primates', *Cancer Research*, 64(18), pp. 6673-6678.
- Yano, A.**, Maeda, F. and Takekoshi, M. (2004) 'Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen', *Journal of Medical Virology*, 73, pp. 208–215.
- Yusibov, V.**, Modelska, A., Steplewski, K., Agadjanyan, M., Weiner, D., Hooper, D. C. and Koprowski, H. (1997) 'Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1', *Proceedings of the National Academy of Sciences*, 94(11), pp. 5784-5788.
- Yusibov, V.**, Streatfield, S. J. and Kushnir, N. (2011) 'Clinical development of plant-produced recombinant pharmaceuticals: Vaccines, antibodies and beyond', *Human Vaccines*, 7(3), pp. 313-321.
- Yusibov, Y.**, Mett, V. and Musiychuk, K. (2012) 'Influenza vaccines, antigens, composition and methods', United States Patent, 20120034253A1.
- Zhang, X.**, Urry, D. W. and Daniell, H. (1996) 'Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plants', *Plant Cell Reports*, 16, 174–179.
- Ziegler, T. M.**, Thomas, S. R. and Danna, K. J. (2000) 'Accumulation of a thermostable endo-1,4- $\beta$ -D-glucanase in the apoplast of *Arabidopsis thaliana* leaves', *Molecular Breeding*, 6, pp. 37-46.

**Zhong, Q.**, Gu, Z. and Glatz, C, E. (2006) 'Extraction of Recombinant Dog Gastric Lipase from Transgenic Corn Seed', *Journal of Agricultural and Food Chemistry*, 54(21), pp. 8086-8092.

# **Chapter 9**

## **Appendices**

## **Appendix A- Presentations, posters and abstract publications**

### **Oral presentations**

Annual PGR conference at School of Health and Biosciences, University of East London,  
June 2010

*Oral presentation on “Expression of ADA in plant cell cultures”*

Annual PGR conference at School of Health and Biosciences, University of East London,  
June 2009

*Oral presentation on “Expression of ADA in plant cell culture”*

Annual PGR conference at School of Health and Biosciences, University of East London,  
July 2008

*Oral presentation on “Expression of ADA in plant cell culture”*

## Poster presentations

Society of Experimental Biology annual conference, Glasgow (28<sup>th</sup> June- 1<sup>st</sup> July 2009)

Poster presentation: "Expression of functional human adenosine deaminase in tobacco plant cell suspensions and whole plants"

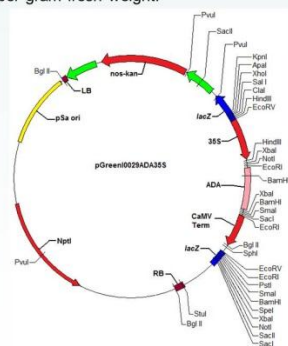
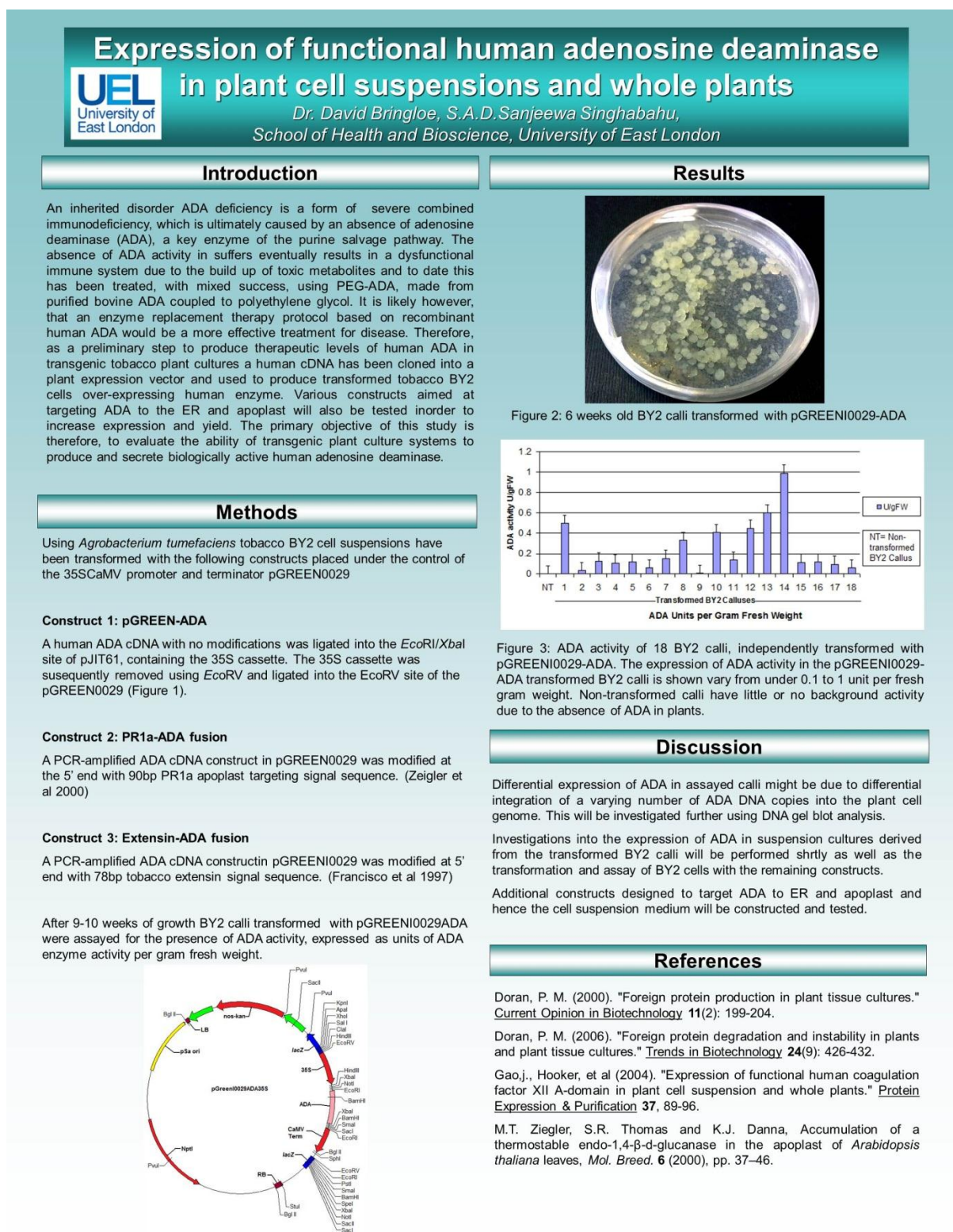


Figure 1: pGREENI0029-ADA

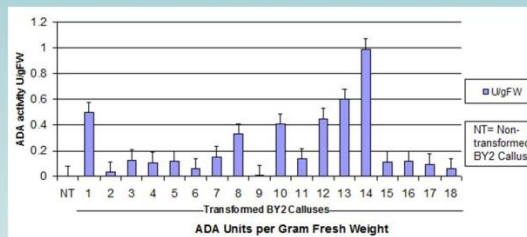


Figure 3: ADA activity of 18 BY2 calli, independently transformed with pGREENI0029-ADA. The expression of ADA activity in the pGREENI0029-ADA transformed BY2 calli is shown vary from under 0.1 to 1 unit per fresh gram weight. Non-transformed calli have little or no background activity due to the absence of ADA in plants.



Recombinant pharmaceutical manufacturing from plants - the future of molecular farming held at Euroscicon, Hertfordshire in 15<sup>th</sup> October 2010

Poster presentation: "Expression of a functional human adenosine deaminase in tobacco plant cell suspensions and whole plants"

Winner of the *best poster price*

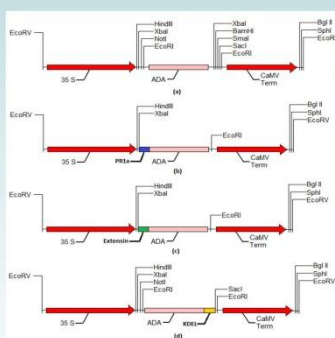
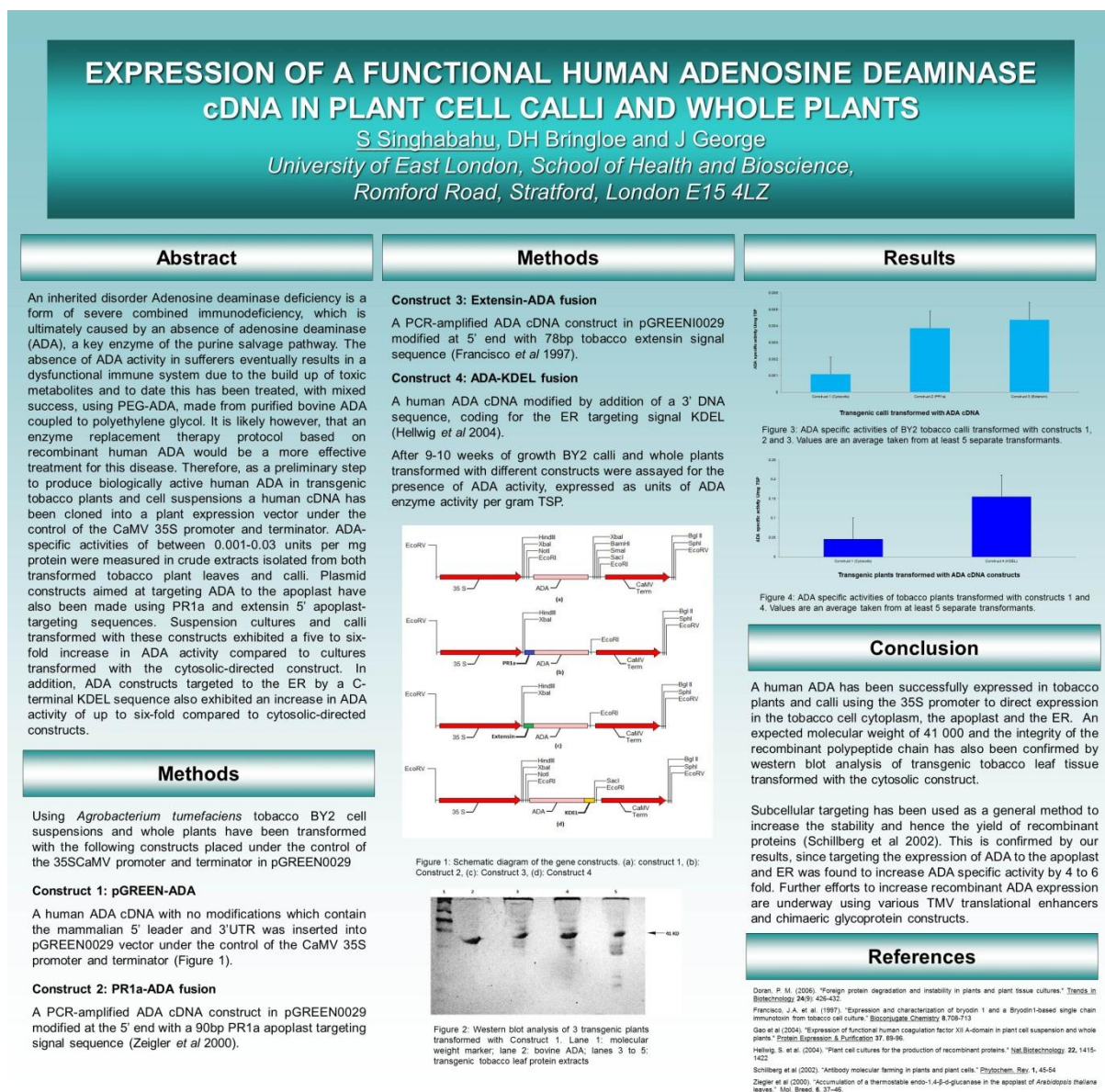


Figure 1: Schematic diagram of the gene constructs. (a): construct 1, (b): Construct 2, (c): Construct 3, (d): Construct 4

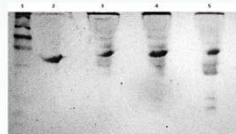


Figure 2: Western blot analysis of 3 transgenic plants transformed with Construct 1: Lane 1: molecular weight marker; lane 2: bovine ADA; lanes 3 to 5: transgenic tobacco leaf protein extracts