

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**A NOPALINE-TYPE *OVERDRIVE* ELEMENT,  
AND ITS INFLUENCE UPON *AGROBACTERIUM*-MEDIATED  
TRANSFORMATION FREQUENCY AND T-DNA COPY NUMBER IN  
*NICOTIANA TABACUM***

A Thesis presented in partial fulfilment of  
the requirements for the degree of  
Doctor of Philosophy in Molecular Genetics  
at Massey University, Palmerston North,  
New Zealand/Aotearoa

**ANDREW GILBERT GRIFFITHS**

**1996**

## **Dedication**

This Thesis is  
dedicated to my parents

Juliet, Merv and Pauline

and to Tina

## ABSTRACT

*Overdrive* is an enhancer element located outside and adjacent to the right border of the T-DNA in *Agrobacterium tumefaciens* octopine-type tumour-inducing (Ti) plasmids. This element is necessary for maximal enhancement of T-strand production and subsequent *A. tumefaciens*-mediated plant transformation frequency, and only the octopine-type *overdrive* had been characterised in any detail. A putative *overdrive* has been identified in the nopaline-type Ti-plasmid pTiT37 on the basis of its homology with known octopine-type *overdrive* sequences, particularly the eight base-pair so-called *overdrive* consensus core. The putative nopaline-type *overdrive* core, however, is only 75% homologous to that of all known *overdrive* core regions. Furthermore, as there are other sequences throughout the nopaline-type T-region that share 75% homology with the *overdrive* consensus core, the precise location of the nopaline-type *overdrive* is undetermined, although all nopaline-type T-region fragments exhibiting *overdrive*-like activity contained the putative *overdrive* core adjacent to the right border. The role of this particular putative core in T-DNA transfer has never been established.

Deletions were made in the putative nopaline-type *overdrive* consensus core adjacent to the right border of a binary plant transformation vector derived from pTiT37. This was to establish whether this putative *overdrive* core does have a role as a transmission enhancer as proposed (Peralta *et al.*, 1986; Van Haaren *et al.*, 1988; Cullianez-Macia and Hepburn, 1988). Two deletions were selected for the full study. The first encompassed the putative nopaline-type *overdrive* core flanked by 3 bp (5') upstream, and 4 bp (3') downstream, and was located in pANDY9. The other, located in pANDY10, encompassed the putative consensus core plus the entire region sharing homology with the octopine-type *overdrive*. This second deletion was to determine whether the core alone could account for *overdrive*-like activity, or whether further sequences are necessary to produce the effect. The vector with no deletions in the putative nopaline-type *overdrive* region was pANDY8.

As determined by quantitative *Nicotiana tabacum* transformation assays, both deletions of the putative nopaline-type *overdrive* core (pANDY9, pANDY10) equally decreased the rate at which calli appeared, and equally decreased transformation frequency by 47% compared with that of pANDY8. That deletion of the putative core influenced plant transformation frequency provided strong evidence that it was indeed an *overdrive*-like core. Furthermore, in a *virC2* mutant environment, the plant transformation frequency was reduced markedly for all three plasmids (approximately

90% reduction compared to when in the wild-type *vir* environment). However, there was no difference in the plant transformation frequencies of the pANDY8-10 series in a *virC2* environment. This indicated that the mechanism by which the deletions influenced plant transformation frequency did not act independently of the *virC* operon, which is further evidence of *overdrive*-like activity.

The type of *vir* regulon influenced the effect of the deletions in the putative *overdrive*. The transformation frequency of the plasmid with the intact putative *overdrive* region (pANDY8) was very similar in both an octopine-type *vir* environment (21.7 organogenic calli per 10 leaf discs in LBA4404) and a nopaline-type *vir* environment (18.7 organogenic calli per 10 leaf discs). However, in an octopine-type *vir* environment, deletions in the putative core resulted in a 47% decrease in transformation frequency, whereas in a nopaline-type *vir* environment the deletions had no effect upon transformation frequency. This may be due to a higher level of *vir* gene products (a feature associated with nopaline-type *vir* regulons), particularly VirD1 and VirD2 compensating for the lack of a fully active putative *overdrive*.

Southern analysis of plants arising from the transformation experiments (in an octopine-type *vir* environment) revealed that removal of the putative nopaline-type *overdrive* core halved the incidence of multiple T-DNA insertion events from 34.7% (pANDY8, intact nopaline-type *overdrive*) to 12.2% (pANDY9) and 14.3% (pANDY10). Deletion of the nopaline-type *overdrive* core also restricted the insert number to a maximum of two, rather than four or more. This is the first time that deletions in the regions outside the T-DNA have been shown to influence T-DNA copy number.

## ACKNOWLEDGEMENTS

I wish to thank my supervisors, Dr Derek White and Professor Barry Scott for encouragement and guidance through the course of this project. I would particularly like to thank Dr Derek White for the use of facilities and resources in the Plant Molecular Genetics Laboratory at AgResearch Grasslands, as well as financial assistance for the preparation of this thesis. I am also grateful for the financial assistance provided by a University Grants Committee post-graduate scholarship, and a William Georghetti scholarship, during the course of this study.

I would like to thank Professor Paul Hooykaas for the gift of *Agrobacterium tumefaciens* strains, MOG1010 and MOG1010-C, and Paul Sanders for the gift of *Arabidopsis thaliana* ecotype, No-0, and particularly Donald Kerr for the generous gift of his promoter-tagging vector, pBIN19/GTG.

Thanks to Nick Ellison for brilliant Mac support, and Helen Dick for the generous loan of a computer, and both Helen and Bob Fletcher for statistical guidance. And to Steven, Ann, Sarah and Barbara of the CRI Campus Library-thanks for your efforts and humour-they don't pay you enough!!

A special thanks to Peter Spring, as well as Derek Charlton for photographic work.

And to all those past and present comprising the Plant Molecular Genetics Laboratory: Derek, Jacqui, Donald, Roy, Thomas, Marg (Xena), Feri, Brigi, Shashi, Erika, Nick, Dale, Alicia, Kez, Dorothy, Don and Trish, and especially Fran, Anya and Blackie, Lorelle, Bron and Andrew, Wendy and Sarah (honourary members). I wish to thank you all for your knowledge, friendship and encouragement, and for helping make science a fun place to be.

A special thank you to Mum, Dad, Pauline, Gran, Dean, Nils, John and Kaisu, just for being there.

Thanks too to the Voisey clan: Hilly, Peter, Jane, Pole, Lindy, Susie and Graham, (and Oscar!!) for the generous welcome, and for bravely leaving a daughter/sister/aunt on this side of the planet.

And I am especially grateful to my partner in crime and in life, Dr Christine Voisey, not only for her excellent proofing skills, but for her support, encouragement and love.

## ABBREVIATIONS

A	ampere
$A_{260}$	absorbance [ $\log(I_0/I)$ ] in a 1 cm light path at 260 nm
Ap	ampicillin
ATP	adenosine 5'-triphosphate
BAP	6-benzylaminopurine
<i>bla</i>	gene encoding $\beta$ -lactamase which confers resistance to ampicillin and carbenicillin
bp	base-pair
BSA	bovine serum albumin
$^{\circ}\text{C}$	degree Celcius
Cb	carbenicillin
Ci	curie ( $3.7 \times 10^{10}$ nuclear disintegrations $\text{s}^{-1}$ ; 37 GBq)
cpm	counts per minute
CTAB	hexadecyltrimethylammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEAE	diethylaminoethyl
DMF	dimethyl formamide
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EDTA(Fe)	ethylenediaminetetraacetic acid, ferric-sodium salt
EDTA( $\text{Na}_2$ )	ethylenediaminetetraacetic acid, disodium salt
EGTA	ethylenebis(oxyethylenitrilo)tetraacetic acid
g	gram
<i>g</i>	acceleration due to gravity ( $9.81 \text{ m s}^{-2}$ )
GUS	$\beta$ -glucuronidase
<i>gusA</i>	gene encoding $\beta$ -glucuronidase (syn. <i>uidA</i> )
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
Hoechst 33258	2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bis-1H-benzimidazole; bisbenzimidazole
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2ip	6-( $\gamma$ - $\gamma$ -dimethylallylamino)purine
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside

k $\Omega$	kiloohm
kb	kilobase-pairs
Kinetin	6-furfurylaminopurine
Km	kanamycin
kV	kilovolt
LB	left border from T-DNA of <i>Agrobacterium tumefaciens</i>
l	litre
M	Molar, moles per litre
mcs	multiple cloning site
mcs- $P_{35S}$ - <i>nptII</i>	an NPTII-encoding gene under the control of a $P_{35S}$ promoter with a pUC18 mcs located 5' of the $P_{35S}$
MES	2-[N-morpholino]ethanesulphonic acid
$\mu$ F	microFarad (capacitance) ( $A\ s\ V^{-1}$ )
$\mu$ g	microgram
$\mu$ m	micrometre
mg	milligram
min	minute
MilliQ water	water that has been purified by passing through a MilliQ ion exchange column
mM	millimolar
mm	millimetre
mol	mole
$M_r$	relative molecular mass ( $g\ mol^{-1}$ )
ms	millisecond
ng	nanogram
<i>nptII</i>	gene from Tn5 coding for neomycin phosphotransferase
NPTII	neomycin phosphotransferase which confers resistance to kanamycin
OD	<i>overdrive</i>
OD <sub>600</sub>	optical density at 600 nm in a 1 cm light path
$\Omega$	ohm (electrical resistance) ( $V\ A^{-1}$ )
<i>ocs3'</i>	transcription-termination sequence of the octopine synthase gene
<i>oriV</i>	origin of replication
$P_{35S}$	the promoter of the Cauliflower Mosaic Virus 35S RNA subunit
$P_{35S}$ - <i>nptII</i>	an NPTII coding gene under the control of the $P_{35S}$ promoter
$P_{nos}$	the promoter of the plant-expressed nopaline synthase gene from <i>Agrobacterium tumefaciens</i>
$P_{nos}$ - <i>nptII</i>	an NPTII coding gene under the control of the $P_{nos}$ promoter
PEG	poly(ethylene glycol)
PVP	polyvinylpyrrolidone
RB	right border from T-DNA of <i>Agrobacterium tumefaciens</i>
Rf	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute



---

s	second (time)
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
SSPE	saline, sodium phosphate, and EDTA buffer
Tc	tetracycline
TE	Tris (10.0 mM), EDTA (1.0 mM) pH 8.0
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
Tm	timentin
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	octylphenoxy polyethoxyethanol
U	units
UV	ultraviolet light
UV-A	near UV (315-400 nm)
V	volt ( $\text{m}^2 \text{kg s}^{-3} \text{A}^{-1}$ )
v/v	volume per volume
vol	volume
W	watt ( $\text{m}^2 \text{kg s}^{-3}$ ) or (V A)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide
Zeatin	6-(4-hydroxy-3-methyl-but-2-enylamino)purine

## TABLE OF CONTENTS

	Page
ABSTRACT .....	iii
ACKNOWLEDGEMENTS .....	v
ABBREVIATIONS .....	vi
TABLE OF CONTENTS .....	ix
LIST OF TABLES .....	xvi
LIST OF FIGURES .....	xvii
CHAPTER 1 INTRODUCTION .....	1
OVERVIEW .....	1
1.1 <i>AGROBACTERIUM TUMEFACIENS</i> : GENETIC COLONISER ...	4
1.2 <i>A. TUMEFACIENS</i> : PLANT GENETIC ENGINEER .....	7
1.3 <i>A. TUMEFACIENS</i> -MEDIATED PLANT TRANSFORMATION ...	8
1.3.1 Ti-plasmid Structure .....	8
1.3.2 Induction of <i>vir</i> Genes .....	11
1.3.3 T-DNA Generation .....	17
1.3.4 T-complex Transfer .....	19
1.3.5 Nuclear Localisation .....	20
1.3.6 Integration into Plant Nuclear DNA .....	22
1.4 THE <i>OVERDRIVE</i> T-DNA TRANSMISSION ENHANCER .....	23
1.4.1 Discovery of <i>Overdrive</i> .....	23
1.4.3 Characterising <i>Overdrive</i> .....	26
1.4.4 Role of <i>Overdrive</i> .....	28
1.4.5 <i>Overdrive</i> and the <i>virC</i> Operon .....	29
1.4.6 Is There a Nopaline-type <i>Overdrive</i> ? .....	31
1.5 <i>OVERDRIVE</i> AND MULTIPLE T-DNA COPIES IN TRANSGENIC PLANTS .....	35
1.6 AIMS .....	36

---

CHAPTER 2	MATERIALS AND METHODS	37
<b>2.1</b>	<b>BACTERIAL PROCEDURES</b>	37
2.1.1	Growth of Bacteria	37
2.1.2	Bacterial Strains and Plasmids	37
2.1.3	Transformation of <i>A. tumefaciens</i> : Tri-parental Mating	37
2.1.4	Transformation of <i>A. tumefaciens</i> : Electroporation	40
2.1.5	Preparation of Electrocompetent <i>A. tumefaciens</i> Cells	41
2.1.6	Transformation of <i>E. coli</i> : CaCl <sub>2</sub> Method	41
2.1.7	Preparation of Competent <i>E. coli</i> Cells with CaCl <sub>2</sub>	41
2.1.8	Transformation of <i>E. coli</i> : Electroporation	42
2.1.9	Preparation of Electrocompetent <i>E. coli</i> Cells	43
<b>2.2</b>	<b>DNA PROCEDURES</b>	43
2.2.1	Plasmid Isolation from <i>A. tumefaciens</i> : Alkaline Lysis	43
2.2.2	Plasmid Isolation from <i>E. coli</i> : STET Method	44
2.2.3	Plasmid Isolation from <i>E. coli</i> : Large Scale Alkaline Lysis	44
2.2.4	Precipitation of DNA	45
2.2.5	DNA Quantification by Spectrophotometry	45
2.2.6	DNA Quantification by Serial Dilution on Agarose Gels	45
2.2.7	DNA Quantification by Fluorometry	46
2.2.8	Determining DNA Fragment Size	46
2.2.9	Agarose Gel Electrophoresis of DNA	46
2.2.10	DNA Recovery from Agarose Gels: DEAE-Cellulose Method	47
2.2.11	DNA Recovery from Agarose: Silica Powder Method	48
2.2.12	DNA Recovery from Agarose: Spin-Column Method	48
2.2.13	Restriction Digestion of Plasmid DNA	49
2.2.14	Partial <i>Eco</i> RI Digestion of <i>Hind</i> III-linearised Plasmid DNA	49
2.2.15	Exonuclease BAL 31 Deletions in pANDY6	49
2.2.16	End-filling 5' Overhangs	50
2.2.17	Recessing 3' Overhangs	51
2.2.18	CAP-Treatment of Vector DNA	51
2.2.19	DNA Ligation	51
2.2.20	Screening Recombinant Plasmids: $\beta$ -Galactosidase Method	52
2.2.21	Screening Recombinant Plasmids: Rapid Colony Lysis	52
2.2.22	DNA Sequence Analysis: Sequencing Reactions	52
2.2.23	DNA Sequence Analysis: Sequencing Gels	53

<b>2.3</b>	<b>PLANT PROCEDURES</b> .....	54
2.3.1	Growth of <i>Arabidopsis thaliana</i> .....	54
2.3.2	<i>A. thaliana</i> : Bulk Seed Production .....	54
2.3.3	<i>A. thaliana</i> : Seed Sterilisation and Germination .....	54
2.3.4	<i>A. thaliana</i> : Producing Root Explants .....	55
2.3.5	Growth of <i>Nicotiana tabacum</i> .....	55
2.3.6	<i>N. tabacum</i> : Producing Leaf Disc Explants .....	55
2.3.7	Plant Transformation .....	55
2.3.8	Plant Transformation: <i>Arabidopsis thaliana</i> .....	56
2.3.9	Plant Transformation: <i>Nicotiana tabacum</i> .....	57
<b>2.4</b>	<b>SOUTHERN ANALYSIS</b> .....	58
2.4.1	Isolation of <i>N. tabacum</i> Genomic DNA .....	58
2.4.2	Restriction Digestion and Electrophoresis of Genomic DNA ..	58
2.4.3	Southern Blotting: Conventional Alkaline Transfer .....	59
2.4.4	Southern Blotting: Downward Alkaline Transfer .....	59
2.4.5	Preparing [ <sup>32</sup> P]-Labelled Probe DNA .....	60
2.4.6	Purifying [ <sup>32</sup> P]-Labelled Probe DNA .....	61
2.4.7	Hybridisation: SSPE Method .....	61
2.4.8	Hybridisation: SDS Method .....	62
2.4.9	Stripping Blots .....	62
CHAPTER 3	RESULTS .....	63
PART I	VECTOR CONSTRUCTION .....	63
<b>3.1</b>	<b>DELETING THE PUTATIVE <i>OVERDRIVE</i> CORE</b> .....	63
3.1.1	Overview .....	63
3.1.2	Optimising Partial <i>EcoRI</i> Digestion of pBIN19/GTG .....	63
3.1.3	Removal of <i>P<sub>nos</sub>-nptII</i> fusion to create pANDY6 .....	68
3.1.4	Optimising BAL 31 Reaction Conditions .....	68
3.1.5	Identifying Clones with Putative Deletions in <i>Overdrive</i> .....	73
3.1.6	Sequencing Deletions in the Putative <i>overdrive</i> Region .....	73
<b>3.2</b>	<b>MODIFYING <i>P<sub>35S</sub>-nptII</i> PLANT SELECTABLE MARKER</b> .....	78
3.2.1	Overview .....	78
3.2.2	Preparing <i>P<sub>35S</sub>-nptII</i> Fusion for Attachment of an mcs .....	78
3.2.3	Attaching pUC18 mcs to <i>P<sub>35S</sub>-nptII</i> Fusion .....	82

3.2.4	Preparing <i>mcs-P<sub>35S</sub>-nptII</i> for Insertion into pANDY6, pANDYOD Vector Series . . . . .	85
3.2.5	Sequencing Junctions of <i>mcs-P<sub>35S</sub>-nptII</i> in pANDY4 . . . . .	87
<b>3.3</b>	<b>COMPLETION OF pANDY VECTOR SERIES . . . . .</b>	<b>92</b>
3.3.1	Overview . . . . .	92
3.3.2	Construction of pANDY8, pANDY9, and pANDY10 . . . . .	92
3.3.3	Sequencing Junctions of <i>mcs-P<sub>35S</sub>-nptII</i> in pANDY8 to pANDY10 . . . . .	92
3.3.4	Transferring pANDY8-10 Series to <i>A. tumefaciens</i> . . . . .	97
<b>3.6</b>	<b>SUMMARY OF VECTOR MODIFICATIONS . . . . .</b>	<b>100</b>
PART II	PLANT TRANSFORMATION EXPERIMENTS . . . . .	101
<b>3.7</b>	<b>OVERDRIVE AND PLANT TRANSFORMATION . . . . .</b>	<b>101</b>
3.7.1	<i>N. tabacum</i> Transformation Experimental Design . . . . .	101
3.7.2	<i>Overdrive</i> and Callus Production in <i>N. tabacum</i> : a Time-Course . . . . .	101
3.7.3	The <i>overdrive</i> and Production of Callus, Green Callus, and Organogenic Callus in <i>N. tabacum</i> . . . . .	103
3.7.4	<i>Overdrive</i> and Other Strains of <i>A. tumefaciens</i> . . . . .	106
3.7.5	<i>Overdrive</i> and <i>vir</i> Environment . . . . .	106
3.7.6	<i>Overdrive</i> and the <i>virC</i> Operon . . . . .	111
3.7.7	<i>Overdrive</i> and <i>Arabidopsis thaliana</i> . . . . .	111
<b>3.8</b>	<b>DETERMINING T-DNA COPY NUMBER IN <i>N. TABACUM</i> . . . . .</b>	<b>114</b>
3.8.1	Determining T-DNA Copy Number . . . . .	114
3.8.2	T-DNA Copy Number . . . . .	123
3.8.3	Spread of T-DNA Copy Number . . . . .	123
CHAPTER 4	DISCUSSION . . . . .	126
<b>4.1</b>	<b>ANALYSING THE PUTATIVE NOPALINE-TYPE OVERDRIVE . . . . .</b>	<b>126</b>
4.1.1	Identification of Regions Necessary for Function of the Putative Nopaline-type <i>Overdrive</i> . . . . .	127
4.1.2	The Putative Nopaline-type <i>Overdrive</i> does not Influence Processes after T-DNA Transfer . . . . .	128
4.1.3	Nopaline vs Octopine-type <i>vir</i> Environment . . . . .	129

---

4.1.4	Was Putative Nopaline-type <i>Overdrive</i> Activity Abolished? .	130
4.1.5	Importance of Sequences between the Right Border and the <i>Overdrive</i> Region . . . . .	133
4.1.6	Importance of the <i>Overdrive</i> Consensus Core . . . . .	136
4.1.7	Is the Putative Nopaline-Type <i>Overdrive</i> Core an <i>Overdrive</i> Core? . . . . .	136
4.1.8	Biological Significance of <i>Overdrive</i> . . . . .	138a
4.1.9	<i>Overdrive</i> in other DNA Transfer Systems . . . . .	138a
<b>4.2</b>	<b>INFLUENCE OF <i>OVERDRIVE</i> ON T-DNA COPY NUMBER . .</b>	<b>139</b>
<b>4.3</b>	<b>IMPLICATIONS OF T-DNA COPY NUMBER . . . . .</b>	<b>142</b>
4.3.1	T-DNA Copy Number and Transformation Frequency in Plant Transformation Systems . . . . .	142
4.3.2	T-DNA Copy Number and Transformation Frequency in Gene-Tagging . . . . .	143
<b>4.4</b>	<b>FUTURE DIRECTIONS . . . . .</b>	<b>145</b>
<b>4.5</b>	<b>SUMMARY . . . . .</b>	<b>145</b>
	REFERENCES . . . . .	146
	APPENDICES . . . . .	163
	<b>APPENDIX 1: BACTERIAL MEDIA . . . . .</b>	<b>163</b>
A1.1	AB Minimal Medium . . . . .	163
A1.2	LB (Luria-Bertani) Medium . . . . .	163
A1.3	SOB Medium . . . . .	163
A1.4	SOC Medium . . . . .	163
A1.5	TY Medium . . . . .	164
A1.6	YEB Medium . . . . .	164
A1.7	YM Medium . . . . .	164
	<b>APPENDIX 2: PLANT MEDIA . . . . .</b>	<b>165</b>
A2.1	GM Medium . . . . .	165
A2.2	ARM Media Base . . . . .	165
A2.3	ARM I Medium . . . . .	165
A2.4	ARM II Medium . . . . .	165

---

A2.5	ARM III Medium	166
A2.6	½MS Medium	166
A2.7	Nic I Medium	166
A2.8	Nic II Medium	166
A2.9	Nic III Medium	166
<b>APPENDIX 3:</b>	<b>MEDIA STOCK SOLUTIONS</b>	<b>167</b>
A3.1	Antibiotics	167
A3.2	Phytohormones	167
A3.3	MS Stock Solutions	167
A3.4	Acetosyringone	168
<b>APPENDIX 4:</b>	<b>SOLUTIONS AND BUFFERS FOR DNA</b>	<b>169</b>
A4.1	1 M Tris-HCl, pH 8.0	169
A4.2	0.5 M EDTA(Na <sub>2</sub> ), pH 8.0	169
A4.3	TE Buffer	169
A4.4	DNase-Free RNase-A	169
A4.5	Cell Resuspension Buffer	169
A4.6	Cell Lysis Solution	169
A4.7	STET Lysis Buffer	169
A4.8	Equilibrated Phenol	170
A4.9	TNE Fluorometry Buffer	170
A4.10	DNA Fragment Size Standards	170
A4.11	TAE Buffer	170
A4.12	SUDS	170
A4.13	GLB	171
A4.14	DEAE Elution Buffer	171
A4.15	Restriction Endonuclease Dilution Buffer	171
A4.16	5× Blunt End Ligation Buffer	171
A4.17	2× Cracking Buffer	171
A4.18	TBE Buffer for Sequencing Gels	171
A4.19	40% Acrylamide Solution	171
A4.20	Nucleotide TLC Phosphate Buffer	172
A4.21	TE-Equilibrated Sepharose CL-6B Resin	172
A4.22	20× SSPE	172
A4.23	100× Denhardt's Solution	172
A4.24	SSPE Hybridisation Solution	172
A4.25	Fragmented Herring Testes DNA	172

---

A4.26	2× SSPE Wash	173
A4.27	1× SSPE Wash	173
A4.28	0.1× SSPE Wash	173
A4.29	1 M Disodium Phosphate Buffer, pH 7.2	173
A4.30	SDS Hybridisation Solution	173
A4.31	SDS Wash Solution	173
A4.32	Genomic Extraction Buffer	173
<b>APPENDIX 5: SEQUENCE DATA FROM pANDY4</b>		<b>174</b>
A5.1	Sequence data from the junction of pMTL25P through the pUC18 mcs to the $P_{mas2}$ promoter of the mcs- $P_{35S}$ - <i>nptII</i> fusion	174
A5.2	Sequence data from the junction of pMTL25P through to the <i>ocs3'</i> terminator of the mcs- $P_{35S}$ - <i>nptII</i> fusion	175
<b>APPENDIX 6: PLASMID MAPS</b>		<b>176</b>
A6.1	pBIN19/GTG	176
A6.2	pANDY6	177
A6.3	pANDYOD-2 and pANDYOD-3	178
A6.4	pSLJ491	179
A6.5	pMTL22P	180
A6.6	pMTL25P	181
A6.7	pUC18	182
A6.8	pANDY1	183
A6.9	pANDY2	184
A6.10	pANDY3	185
A6.11	pANDY4	186
A6.12	pANDY8, pANDY9, and pANDY10	187
A6.13	pJIT166	188
<b>APPENDIX 7: SOUTHERN ANALYSIS DATA</b>		<b>189</b>



## LIST OF TABLES

	Page
Table 1.	Bacterial strains and plasmids used in this study. . . . . 38
Table 2.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>nptII</i> left border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY8. . . . . 189
Table 3.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>nptII</i> left border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY9. . . . . 192
Table 4.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>nptII</i> left border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY10. . . . . 194
Table 5.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>gusA</i> right border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY8. . . . . 195
Table 6.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>gusA</i> right border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY9. . . . . 196
Table 7.	Southern analysis using a [ <sup>32</sup> P]-labelled <i>gusA</i> right border probe with <i>HindIII</i> and <i>EcoRI</i> -digested genomic DNA from plants transformed with pANDY10. . . . . 197

## LIST OF FIGURES

	Page
Figure 1A-B. Comparison of the octopine and nopaline-type Ti-plasmids. . . .	9
Figure 2. Overview of the molecular processes involved in <i>Agrobacterium tumefaciens</i> -mediated plant transformation. . .	13
Figure 3. Comparison of <i>overdrive</i> -like sequences in right border regions of the octopine-type pTiA6NC T <sub>L</sub> - and T <sub>R</sub> -DNA, the nopaline-type pTiT37, and the <i>Agrobacterium rhizogenes</i> pRiA6 T <sub>L</sub> -DNA. . . . .	24
Figure 4. Characterising the octopine-type <i>overdrive</i> . . . . .	27
Figure 5. Comparison of <i>overdrive</i> -like sequences in right border regions of a range of Ti- and Ri-plasmids. . . . .	32
Figure 6. Summary of construction of pANDY6, pANDYOD <sup>-</sup> 2, and pANDYOD <sup>-</sup> 3. . . . .	64
Figure 7. Fragments generated by a partial <i>Eco</i> RI digestion of <i>Hind</i> III-linearised pBIN19/GTG. . . . .	65
Figure 8. Establishing the optimal <i>Eco</i> RI concentration for production of partially <i>Eco</i> RI-digested fragments of <i>Hind</i> III-linearised pBIN19/GTG. . . . .	67
Figure 9A-B. Identification of pANDY6. . . . .	69
Figure 10A-C. Optimising BAL 31 reaction conditions. . . . .	71
Figure 11. Identification of clones with deletions in the putative nopaline-type <i>overdrive</i> core. . . . .	74
Figure 12A-B. Sequence data detailing various deletions centred upon the putative nopaline-type <i>overdrive</i> core of the pANDYOD <sup>-</sup> series. . . . .	76

---

Figure 13.	Summary of modification of the $P_{35S}$ - <i>nptII</i> fusion prior to its insertion . . . . .	79
Figure 14A-C.	Determining orientation of the $P_{35S}$ - <i>nptII</i> insert with regard to the pUC18 mcs during construction of pANDY2. . . . .	83
Figure 15.	Restoration of the <i>EcoRI</i> site during the construction of pANDY4. . . . .	88
Figure 16A-C.	Nucleotide sequence of the junctions of the mcs- $P_{35S}$ - <i>nptII</i> fusion with pMTL25P in pANDY4. . . . .	90
Figure 17.	Summary of construction of pANDY8, pANDY9, and pANDY10 . . . . .	93
Figure 18A-D.	Identification of plasmids pANDY8, pANDY9, and pANDY10. . . . .	95
Figure 19A-D.	Identification of <i>A. tumefaciens</i> strain LBA4404 colonies harbouring either pANDY8, pANDY9, or pANDY10. . . . .	98
Figure 20.	Deletions in the putative nopaline-type <i>overdrive</i> region of plant transformation vectors, and their influence upon mean cumulative callus production (calli per 10 leaf discs) plotted against time (days after cocultivation). . . . .	102
Figure 21.	Deletions in the putative nopaline-type <i>overdrive</i> , and their influence upon the production of callus, green callus, and organogenic callus (transformation frequency), after a 60 day cultivation period on a selective medium. . . . .	104
Figure 22.	Deletions in the putative nopaline-type <i>overdrive</i> , and their influence upon the proportion (%) of <i>N. tabacum</i> calli that became green, and the proportion (%) of green calli that became organogenic, after a 60 day cultivation period on a selective medium. . . . .	104

---

Figure 23A-C. Deletions in the putative nopaline-type <i>overdrive</i> region of the plant transformation vectors (pANDY8-10), and their influence upon callus production in <i>N. tabacum</i> , when harboured by LBA4404. . . . .	107
Figure 24A-C. Deletions in the putative nopaline-type <i>overdrive</i> region of the plant transformation vectors (pANDY8-10), and their influence upon callus production in <i>N. tabacum</i> , when harboured by MOG1010. . . . .	109
Figure 24D-F. Deletions in the putative nopaline-type <i>overdrive</i> region of the plant transformation vectors (pANDY8-10), and their influence upon callus production in <i>N. tabacum</i> , when harboured by LBA4404, MOG1010, GV3101, and MOG1010-C. . . . .	110a
Figure 25A-C. Deletions in the putative nopaline-type <i>overdrive</i> region of the plant transformation vectors (pANDY8-10), and their influence upon callus production in <i>Arabidopsis thaliana</i> , when harboured by LBA4404. . . . .	113
Figure 26A-C. Determining T-DNA copy number with <i>Hind</i> III digested plant genomic DNA. . . . .	115
Figure 27. Representative <i>Hind</i> III digest of <i>N. tabacum</i> genomic DNA. . . . .	117
Figure 28. Representative autoradiograph of <i>Hind</i> III-digested <i>N. tabacum</i> genomic DNA hybridised with [ <sup>32</sup> P]-labelled <i>nptII</i> probe. . . . .	118
Figure 29A-C. Determining completeness of genomic digestion using <i>Eco</i> RI. . . . .	120
Figure 30. Representative autoradiograph of <i>Eco</i> RI-digested <i>N. tabacum</i> genomic DNA hybridised with [ <sup>32</sup> P]-labelled <i>nptII</i> probe. . .	122

---

Figure 31.	The effect of deletions in the <i>overdrive</i> region upon the incidence of multiple T-DNA insertion events in transgenic <i>N. tabacum</i> . . . . .	124
Figure 32.	The effect of deletions in the <i>overdrive</i> region upon the range of T-DNA copy number in transgenic <i>N. tabacum</i> . . . . .	125
Figure 33.	The top and bottom strand sequence of the T <sub>L</sub> -DNA right border and <i>overdrive</i> region of the octopine-type Ti-plasmid pTiA6 that is protected by VirC1 in DNase I footprint analysis. . . . .	135