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

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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; Culianez-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.

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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
bla	gene encoding β-lactamase which confers resistance to ampicillin and carbenicillin
bp	base-pair
BSA	bovine serum albumin
°C	degree Celcius
Cb	carbenicillin
Ci	curie (3.7×10^{10} nuclear disintegrations 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(Na_2)	ethylenediaminetetraacetic acid, disodium salt
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
g	gram
g	acceleration due to gravity (9.81 m s^{-2})
GUS	β-glucuronidase
gusA	gene encoding β-glucuronidase (syn. *uidA*)
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
Hoechst 33258	2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bis-1H-benzimidazole; bisbenzimide
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2ip	6-(γ-γ-dimethylallylamino)purine
IPTG	isopropyl-β-D-thiogalactopyranoside

kΩ	kiloohm
kb	kilobase-pairs
Kinetin	6-fufurylaminopurine
Km	kanamycin
kV	kilovolt
LB	left border from T-DNA of *Agrobacterium tumefaciens*
l	litre
M	Molar, moles per litre
mcs	multiple cloning site
mcs-P_{35S}-*nptII*	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
μF	microFarad (capacitance) (A s V^{-1})
μg	microgram
μm	micrometre
mg	milligram
min	minute
MilliQ water	water that has been purifed 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
nptII	gene from Tn5 coding for neomycin phosphotransferase
NPTII	neomycin phosphotransferase which confers resistance to kanamycin
OD	*overdrive*
OD_{600}	optical density at 600 nm in a 1 cm light path
Ω	ohm (electrical resistance) (V A^{-1})
*ocs*3′	transcription-termination sequence of the octopine synthase gene
oriV	origin of replication
P_{35S}	the promoter of the Cauliflower Mosaic Virus 35S RNA subunit
P_{35S}-*nptII*	an NPTII coding gene under the control of the P_{35S} promoter
P_{nos}	the promoter of the plant-expressed nopaline synthase gene from *Agrobacterium tumefaciens*
P_{nos}-*nptII*	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 *Agrobacterium tumefaciens*
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	*N,N,N′,N′*-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 (m^2 kg s^{-3} A^{-1})
v/v	volume per volume
vol	volume
W	watt (m^2 kg s^{-3}) or (V A)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
Zeatin	6-(4-hydroxy-3-methyl-but-2-enylamino)purine

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