Plant transformation using an agrobacterium. Tumefadens ti-plasmid vector system

Edwards, Glyn Alyn

PLANT TRANSFORMATION USING AN *Agrobacterium tumefaciens* Ti-PLASMID VECTOR SYSTEM

A Thesis Submitted by:

GLYN ALYN EDWARDS B.Sc. A.R.C.S.

In accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham

Department of Botany. September 1988.

DECLARATION

I declare that no part of this thesis has previously been submitted for a degree at this or any other university. Also that, unless otherwise indicated, the work presented in this thesis is entirely my own.

[†] This thesis contains information of potential commercial value. The circulation of this thesis has been restricted and, until further notice, should not be distributed to any persons without written permission from the author.

ABSTRACT

A plasmid, pDUB116 was constructed, containing the Nos-NPT dominant selectable marker for expression in plants which was capable of being mobilised to *Agrobacterium tumefaciens* and forming cointegrates with pGV3850 and pGV3851. The frequencies of cointegrate formation of these plasmids were determined and the cointegrate structure established by Southern blotting. Inoculation of *Kalanchoe diaigremontiana* leaves and *Nicotiana tabacum* stems *in vivo* showed that pGV3851 is only weakly oncogenic. A fully oncogenic Ti plasmid (pTiGE1) was constructed which was capable of forming cointegrates with pDUB116 and suitable for use in *in vivo* plant transformation systems.

A. tumefaciens GV3101 [pGV3850] was found to be highly resistant to cefotaxime and carbenicillin in plant tissue culture media, preventing its use in *in vitro* plant transformation proceedures. *A. tumefaciens* strains were therefore screened for sensitivity to a number of antibiotics, two of which, augmentin and timentin, were found to be inhibitory to the growth of *A. tumefaciens* but non-inhibitory to callusing, shooting or rooting of *N. tabacum* in tissue culture.

The effect of the SV40 enhancer on the expression of the Nos promoter was investigated by constructing integrating plasmids (pDUB116 derivatives) with the SV40 enhancer 5′ and 3′, and in both orientations with respect to the Nos-NPT gene. These plasmids were mobilised to *A. tumefaciens* and cointegrates with pTiGE1 selected, characterised by Southern blotting, and inoculated *in vivo* onto leaves of *K. diaigromentiana*. Extracts from the resultant callus tissues were found to contain no detectable NPT activity in all cases. The same constructs were used to transform *N. tabacum in vitro* by a leaf disc transformation method and callus was selected on hormone free media and kanamycin. The callus induced by the constructs containing the SV40 enhancer showed no significant increase over the control construct, indicating that the SV40 enhancer does not function in these plants. Further improvements to the pBR322-homology mediated Ti vector system were made by constructing a new oncogenic Ti vector, pTiGE2, which has a smaller T-DNA containing a single copy of pBR322, giving a more defined T-DNA which is easier to analyse after cointegrate formation. New T-DNA integrating vectors containing the LacZ insertional inactivation region from pUC18 were constructed giving more unique restriction enzyme sites and making the selection of recombinants easier.

A chimaeric CAMV-pea lectin gene was constructed and subcloned into a T-DNA integrating vector. Resultant cointegrates with pGV3850 were characterised and transgenic *N. tabacum* plants regenerated. The T-DNA structure and copy number in the transgenic plants were investigated. Expression of the CAMV-pea lectin gene was characterised by northern blotting, western blotting, haemagglutination and ELISA and showed the gene to be expressed constitutively at high levels and the protein to be processed correctly. The subcellular site of lectin deposition in transgenic tabacco root was found to be the vacuole. Plants expressing lectin at high level were screened for resistance to a root-knot nematode and found not to be resistant to infection.

ABBREVIATIONS

Abbreviations are used as recommended in the 'Biochemical Journal instructions to authors'(Biochemical Society , 1975) with the additions listed below.

bp	:	base pairs
kb	:	Kilobase pairs
BAP	:	6-benzylaminopurine
BSA	:	bovine serum albumin (Pentax fraction 5)
DAB	:	3,3 diaminobenzidine
DABA	:	3,5-diaminobenzoic acid
DEAE	:	diethylaminoethane
DMSO	:	dimethylsulphoxide
ss-DNA	:	single stranded DNA
ds-DNA	:	double stranded DNA
dNTP	:	deoxynucleotide triphosphate
DTT	:	dithiothreitol
EDTA	:	ethylenediaminetetra-acetic acid
EGTA	:	ethylenebis(oxyethylene nitrilo)tetracetic acid
ELISA	:	enzyme-linked immunosorbant assay
EtBr	:	ethidium bromide
FITC	:	fluorosceine isothiocyanate (isomer 1)
IPTG	:	isopropyl-β-D-thiogalactopyranoside
NAA	:	1-napthylacetic acid
Nos	:	nopaline synthase
NPT	:	neomycin phosphotransferase
2-ME	:	2-mercaptoethanol
OD	:	optical density
ONPG	:	ortho-nitrophyl-β-D-galactopyranoside
PEG	:	polyethylene-glycol
PPO	:	2,5-diphenyloxazole
POPOP	:	1,4-di[2-(5-phenyloxazole)]-benzene
PVP	:	polyvinylpyrrolidone
SDS	:	sodium dodecyl sulphate
TEMED	:	NNN'N'-tetramethylethylene-diamine
Tris	:	(hydroxymethyl)-aminomethane
UV	:	ultra-violet
5′	:	5′ terminal phosphate of a DNA or RNA molecule
3′	:	3′ terminal hydroxyl of a DNA or RNA molecule
X-gal	:	5-bromo-4-chloro-3-indolyl-β-D galactopyranoside

* **Note on nomenclature. For reproduction purposes, nomenclature for some enzymes and for all restriction enzymes in this text uses Arabic instead of Roman numerals.**

ACKNOWLEDGMENTS

I would like to thank Dr. John Gatehouse for his excellent supervision throughout the course of this work.

Thanks are also due to Professor Donald Boulter, Dr. Ron Croy, Dr. Marta Evans, Dr. Charlie Shaw, Dr. Vaughan Hilder Dr. Anharad Gatehouse and Dr. Nick Harris for their interest, constructive advice, encouragment and sufferance during the past three years.

I am gratefull to the following people for giving advice and practical help with various laboratory techniques; Dave Jobes and Paul Simpson (plasmid preparations), Dr. Anil Shirsat (Southern blotting), Dr. Marta Evans and Andy Thompson (RNA work), Drs. Andy Hepher and Ray Ellis (see page 27 for current addresses) (plant tissue culture and transformation), Dr. Charlie Shaw (bacteriological techniques) and, lastly, Paul 'Doc'Preston for supplying glassware, solutions, sterile equipment and a ready supply of unrepeatable jokes (the one about the gorrilla being particularly memorable).

I am deeply indebted to Alan Hazell (of Sensititre Ltd, East Sussex UK.) for conducting the *A. tumefaciens* antibiotic resistance studies, to Laurence Smith (of Newcastle University) for carrying out the nematode resistance work, and to Hellen Grindley for conducting the *in situ* cytological detection experiments.

Thanks are also due to those who helped with the production of this manuscript: Drs. John Gatehouse, Marta Evans, Ron Croy Vaughan Hilder and Prof. Boulter, Ron Croy for donating the computer programme and help in producing the diagrams, Paul Sidney for doing the photographic work and the staff of Durham University computer centre (especially Mike Cawley) for help with word-proccessing and reproduction of the text.

(Unless otherwise specified, all the aforementioned are at Durham University).

This thesis would not be complete without acknowledging the contribution of Miss Geraldine Dowling SRN, who over the past year has done my washing, ironing, shopping, cooking and given great moral and financial support.

CONTENTS

TABLES

FIGURES

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

In plants the level of molecular, biochemical and genetic understanding is generally less than that available for microbial and animal systems. However, the recent availability of techniques to produce transgenic plants has enabled novel research directed at understanding the regulation and expression of plant genes and their products to be conducted and this has resulted in a surge of interest in research in these areas. The ability to produce large numbers of transgenic plants with relative ease, the availability of methods to regenerate and clone plants *in vitro* and the lack of controversy and legislation compared to that associated with e.g. mammalian research, have all contributed to this increase in the use of plant systems as research tools in molecular biology. However, the major impetus for the increased research effort in the molecular biology and physiology of plants is due to the acknowledgment that the methods for producing transgenic plants could be used to improve the agronomic traits of the major crop plants on which we depend for our food *etc.* The different objectives, requirements and problems involved in these two applications of transgenic plants are reflected in the strategies and techniques currently being employed and developed in plant genetic engineering.

The requirements of plant transformation systems for use in the study of plant gene expression are that the systems employed rapidly yield reproducible results using easily handled (i.e. non-labour intensive and simple) methods. In practical terms the ideal plant transformation system should have the attributes of a high frequency of transformation, ease of selection or identification of transformants, good tissue culture characteristics of the plant material used so as to enable recovery of the desired tissues or plants, and the ability to rapidly assay the desired gene product or phenotype. A wide variety of transformation systems are available to chose from in order to attempt to satisfy these criteria, but in practice those employed most frequently, particularly when callus or regenerated plants are required, use *Agrobacterium tumefaciens* Ti-plasmid vectors for the delivery of DNA into a limited number of species of the *Solanaceae*. Plant transformation systems using these methods are now in widespread use in a large number of laboratories and can now be viewed as routine research tools in plant molecular biology.

Applied research directed towards producing transgenic crop plants with improved agronomic traits falls into two main areas; firstly identifying and isolating genes which may confer these traits and secondly developing methods which have the potential of delivering these genes with subsequent regeneration, concentrating on the major crop plants of the developed world, particularly members of the *Gramineae*. It is the regeneration of transformed tissues into whole, viable plants which has been the major stumbling block in attempts to produce transgenic crop plants. Whereas some members of the *Solanaceae*, such as those used in the 'routine' assay systems, are very amenable to regenerating viable plants from the cells of many tissues, the major crop plants have proved to be much less tractable to conventional *in vitro* tissue culture techniques. To circumvent these problems, many transformation techniques have been employed in an attempt to deliver DNA into the cells

of tissues which are amenable to regeneration either *in vitro* or *in vivo*. Amongst the panopoly of techniques and strategies which have been tried, *A. tumefaciens* Ti-plasmid vectors have featured strongly. Also, because of the difficulty in regenerating the major crop plants the genes which have the potential to give desirable agronomic properties have, to date, been assayed for expression and biological activity not in the major crop plants themselves but in the members of the *Solanaceae* which are easily regenerated, using Ti-plasmid vectors as the method of transformation.

Ti-plasmid vectors can be seen to play a key role in both pure and applied research in plant genetic engineering. This role has been made possible by the availability of Ti-plasmid based cloning vectors which are easily manipulated in the laboratory and which can satisfy the requirements of plant transformation in a wide variety of situations.

1.2 *Agrobacterium* AND THE Ti PLASMID

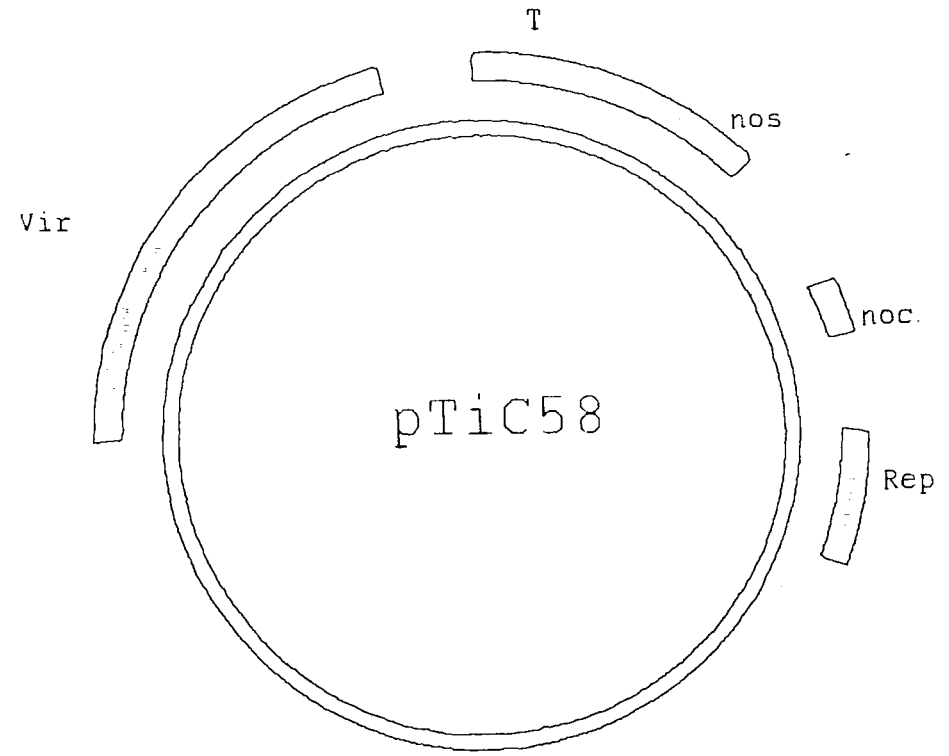
The gram negative soil organism *Agrobacterium* is a member of the family Rhizobiaceae and contains species which are peritrichously flagellated (motile) rods. *A. tumefaciens* is the causative agent of the plant disease crown gall (Smith and Townsend, 1907), while the related *A. rhizogenes* provokes hairy roots in plants (Riker, 1930). The crown gall disease is characterised by the formation of tumours called crown galls and hairy root by the production of abundant root proliferations, both occuring at infected wound sites. These diseases are neoplasic with crown gall and hairy root cells both being characterised by an ability to proliferate unlimitedly and autonomously in the absence of added phytohormones which are needed for the growth of normal plant cells. In the absence of phytohormones (in *in vitro* culture) crown gall cells form amorphous calli, whereas hairy root cells differentiate into roots (Tepfer, 1984). A common feature of both crown gall and hairy root cells is the production and excretion of compounds (collectively termed opines) which are not found in normal plant cells (reviewed by Tempe and Goldmann, 1982). During crown gall and hairy root induction, part of a large plasmid which is present in virulent strains (the tumour inducing (Ti) plasmid of *A. tumefaciens* and the root inducing (Ri) plasmid of *A. rhizogenes*) is integrated and expressed in the nuclear genome of plant cells at the infection sites. Expression of this transfered DNA (T-DNA) in plant cells results in their transformation into opine producing tumour cells. The opines that are produced can be catabolised by the virulent *Agrobacterium* which induced tumour formation, but not by most other soil organisms and thus the inciting organism creates a favourable niche for itself. This process, which has been termed 'genetic colonisation'(Schell *et el.* 1979) is unique and lends itself to be manipulated for use in plant genetic engineering. A vast amount is known and has been written about *Agrobacterium* and the Ti and Ri plasmids (for reviews see Bevan and Chilton (1982a), Schell and Van Montagu (1983), Mattysse (1986), Fraley *et al.* (1986), Klee *et al.*, (1987) and Melchers and HooyKaas (1987)), but in this work, emphasis is placed upon those aspects of the wild-type *Agrobacterium* Ti-plasmid system important in its use as a DNA delivery system in plant genetic engineering.

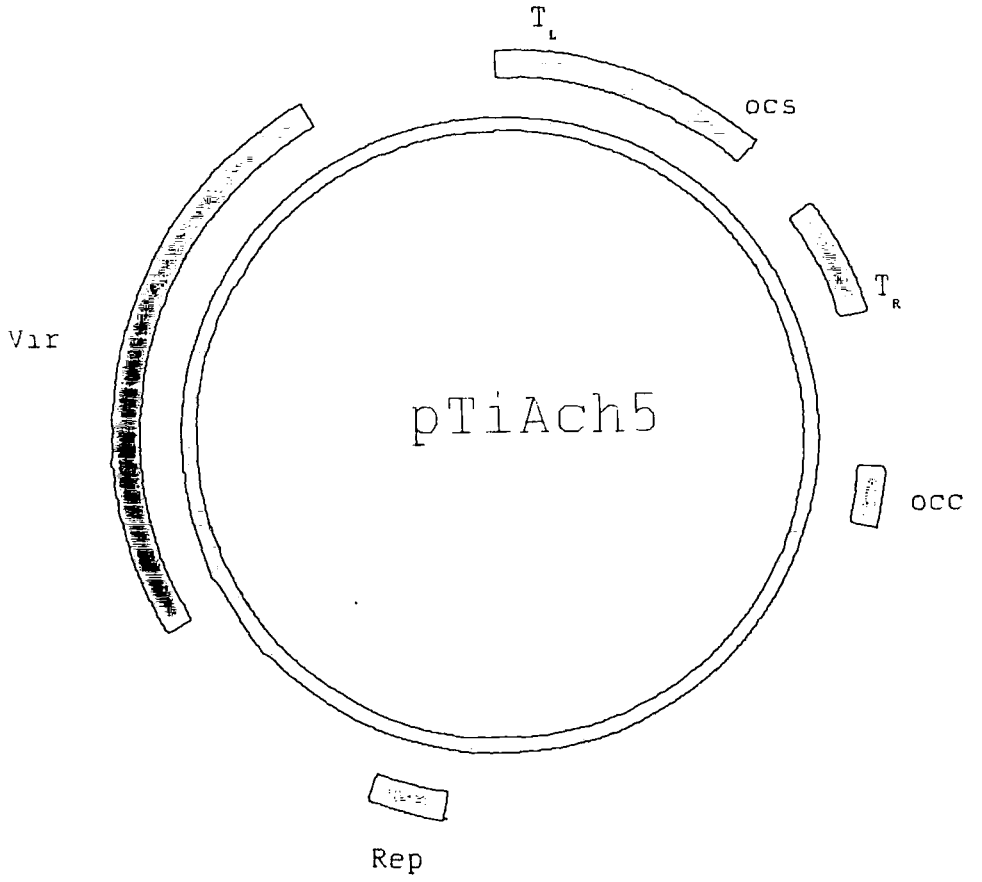
The majority of Ti plasmids isolated to date are between 190 and 240kb in size and have

traditionally been classified on the basis of the type of opine produced in the induced callus. Of the four types of Ti plasmid the octopine and nopaline types have been the most extensively studied. The octopine class of Ti plasmids such as pTiAch5, pTiB6 and pTiA6 form a relatively homogenous group as determined by restriction mapping and homology, whereas the nopaline class of Ti plasmids are more heterogenous (Melchers and Hooykaas, 1987), although the nopaline plasmids pTiC58 and pTiT37 are closely related (Depicker *et al.*. 1980). The Ti plasmids confer a number of properties on *A. tumefaciens.* Besides possesing an origin of replication and accompanying incompatibility functions, the plasmids have genes for opine transport and catabolism and sometimes for amino acid metabolism (Dessaux *et al.*, 1986). The plasmids are also capable of conjugative transfer via inducible *tra*-genes (Ellis *et al.*, 1982) and sometimes contain functions which inhibit phage reproduction (Van Larebeke *et al.*, 1977). Some Ti plasmids also contain genes located outside the T-DNA which are involved in the production of phytohormones in the bacterium (Beaty *et al.*, 1986). However the two regions of the Ti plasmid which are essential for tumorigenesis are the T-DNA and the virulence (Vir) region.

The organisation of the T-DNA in octopine and nopaline type plasmids is different. Octopine plasmids such as pTiAch5 have two T-regions called T_L and T_R. Nopaline plasmids such as pTiC58 have only one T-DNA (see figure 1.1). All of these T-DNA's are flanked by two 25bp (imperfect) direct repeat sequences. Sequence analysis of the border regions integrated into the plant genome and comparison with the plasmid sequences has established the ends of the T-DNA. In the majority of cases the integrated T-DNA ends within the 25bp border sequences (Holsters *et al.*, 1983; Yadav *et al.*, 1982). Deletion analysis has shown that the right border is essential for tumour formation in most plant species (Shaw *et al.*. 1984a), but that the deletion of the left border has little effect (Joos *et al.*, 1983). The right border repeat is strongly dependent on its correct orientation for efficient T-DNA transfer (Van Haaren *et al.*. 1986). Enhancer like sequences have been found in the octopine Ti plasmids which lie close to the right borders and increase the efficiency of T-DNA transfer (Van Haaren *et al.*, 1987). A similar sequence is also present at the right border of the nopaline plasmid pTiC58 (C.H Shaw, personal communication). These border sequences form the signals which are recognised by proteins of the transfer system of *Agrobacterium.* Genes products of the Vir-region (see later) introduce nicks in the bottom strand of the of the 25bp border repeats (Yanofski *et al.*. 1986. Wang *et al.*. 1987) and a single stranded linear T-DNA molecule is formed (Stachel *et al.*. 1986), possibly by excission in a process similar to that occuring during DNA repair or by displacement by new strand synthesis from the right border nick. Although the mechanism of transfer the T-DNA to the plant genome is not known, it is thought that these single stranded T-DNA's may be important intermediates (Stachel *et al.*. 1987).

During tumour induction the T-DNA becomes integrated into the plant nuclear DNA, although one report sugests that it can also enter the chloroplast but is unstable (De Block *et al.*. 1985). The T-DNA structure in different tumour lines varies considerably. In most tumour lines only one or two T-DNA's are present but others may contain multiple copies, which can be complete





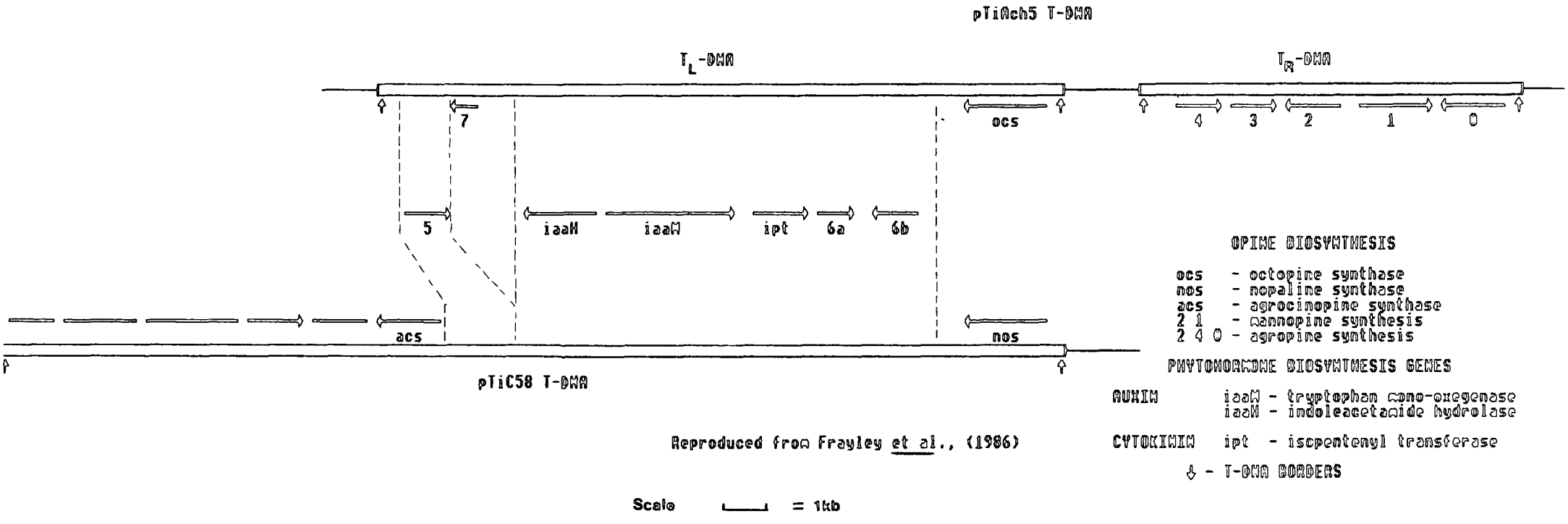
igure 1.1 Genetic Organisation of pTiC58 and pTiAch5
ey: ocs - octopine synthase nos - nopaline synthase
occ - octopine catabolism noc - nopaline catabolism
Rep - origin of replication See text for further details.

or truncated, at one or a number of loci. If multiple copies are present at one locus then these can be arranged as tandem or inverted repeats (Melchers and Hooykas, 1987; and references therein). The T-DNA may integrate at different sites in the plant genome and these have been found both in repetitive and single copy DNA (Zambryski *et al.*, 1982) and when multiple copies are present, the T-DNA can be on a number of different chromosomes (Chyi *et al.*, 1986; Wallroth *et al.*, 1986).

When integrated into the plant genome, the expression of the T-DNA genes determine some of the properties of crown gall cells, including opine synthesis and excretion and phytohormone independent growth. Thirteen genes have been identified in the 23kb T-DNA of the nopaline plasmid pTiC58 and in the octopine plasmid pTiAch5, (eight on the 13.2kb T_L-DNA and five on the 7.9kb T*R*-DNA (Frayley *et al.*, 1986)). A variety of techniques have been used to identify the functions of these genes, including transposon insertion (Holsters *et al.*, 1980; Garfinkel *et al.*, 1981)., deletion analysis (Joos *et al.*, 1983), transcript mapping (Bevan and Chilton, 1982b; Willmitzer *et al.*, 1983) and expression of genes in *Escherichia coli* (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchman *et al.*, 1985; Thomashow *et al.*, 1986). The *cyt* or *tmr* (tumour morphology root) locus is expressed as one poly-adenylated RNA of 1200 bases and codes for the enzyme isopentenyl transferase (*ipt*) which catalyses the reaction between isopentenyl-pyrophosphate and AMP to yield isopentenyl-AMP, which exhibits cytokinin activity. The *aux* or *tms* (tumour morphology shoot) locus gives two polyadenylated RNA's of 2700 and 1600 bases which code for the enzymes tryptophan mono-oxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*) respectively. These enzymes are involved in the catalysis of tryptophan to indole acetic acid via the intermediate, indoleacetamide. The production of these phytohormones explains why the cells containing T-DNA grow as tumour cells. Deletion of all other genes within the T-DNA does not inhibit tumour formation and hence are not essential for tumourigenesis. Two transcripts (5 and 6b) located in the nopaline T-DNA and in the T_L-DNA of octopine plasmids can slightly modify tumour formation but this effect is only detected in the presence of mutations in the *aux* or *cyt* loci (Joos *et al.*, 1983). Gene 6a is involved in opine secretion (Messens *et al.*, 1985). The remaining genes are either involved in opine synthesis or their functions are unknown. Figure 1.2 shows the organisation of these genes in both a nopaline and an octopine T-DNA and the maps are aligned to indicate the region of homology (the common region). The 5'and 3'regulatory sequences of some of these genes have been defined and they depend on typical eukaryotic expression signals (reviewed by Heidecker and Messing, 1986). None of the genes of the T-DNA are involved in the transfer process but only in maintaining the properties of the crown gall cells and the only sequences of the T-region which are essential for the transfer of the T-DNA to the plant cell are the border sequences (Leemans *et al.*, 1982; Zambryski *et al.*, 1983).

The *vir*-genes of the Vir-region are essential for the transfer of the T-DNA to plant cells. The gene products of this region act in *trans* and direct the events involved in the transfer process (Hoekema *et al.*, 1983; De Framond *et al.*, 1983). The 40kb Vir-region is located adjacent to the left T-DNA border (see figure 1.1). Seven transcription units have been identified in the octopine plasmid pTiAch5 which are named *virA-G* (Stachel and Nester, 1986). The nopaline plasmid pTiC58 has

FIGURE 1.2 GENETIC ORGANISATION OF T-DNA OF pTiAch5 AND pTiC58



Reproduced from Frayley et al., (1986)

corresponding loci except for *virF* (Rogowsky *et al.*, 1987). Mutation analysis of these loci has shown that four of these loci (*virA*, *B*, *D* and *G*) are essential for T-DNA transfer whereas mutations in the remaining loci result in a reduction in the host range for tumour induction (Melchers and Hooykas, 1987). Of the essential loci *virA* and *virG* code for one protein each. *virB* and *virD* are polycistronic and contain genes which code for 11 and 4 proteins respectively (Melchers and Hooykas, 1987). In the 'uninduced'state (i.e. when grown on routine bacterial media) *virA* and *virG* are the only *vir*-genes expressed at a significant level and these two gene products are involved in the regulation of transcripion of the remaining *vir* operons (Stachel and Nester, 1986) The *virA* protein is a membrane bound protein situated in the inner membrane and probably functions as the environmental sensor of plant derived inducer molecules (Leroux *et al.*, 1987). The *virG* gene product has homology to a number of known positive regulators of bacterial transcription and is thought that this protein is activated (by an unknown mechanism) by the *virA* gene product after induction (Winans *et al.*, 1986). When *Agrobacterium* is exposed to plant exudates the other *vir*-genes then become expressed at high levels, this increase starting 1h after induction, reaching a maximum after 6-12h and the expression returning to the basal (pre-induced) level 48h after induction (Winans *et al.*, 1986). Plant exudates have been fractionated to identify the inducer compounds and acetosyringone and a group of related phenolic compounds were identified (Stachel *et al.*, 1985). Thus the current model for the induction of expression of the *vir*-genes is that the *virA* gene product detects the presence of these plant phenolics and activates *virG* either directly or via some intermediary mechanism. The activated *virG* protein then acts upon the remaining *vir*-promoters and positively regulates transcription. The *virA/G* regulatory system has also been demonstrated to be involved in the chemotactic response of *Agrobacterium* to acetosyringone but that the system responds to much lower concentrations of the inducer than required for activation of *vir*-gene expression (Shaw *et al.*, 1988). Of the remaining *vir*-genes, the functions of only two proteins *virD1* and *virD2* are known. These gene products are involved in nicking at the border sequences of the T-DNA and the formation of the single stranded T-DNA (Stachel *et al.*, 1987).

Functions involved in the process of tumourigenisis are also encoded by chromosomal genes. Some of these genes are involved in attachment of *Agrobacterium* to cells at the wound sites, either via the expression of specific proteins (Mathysse, 1987) or via the synthesis of cellulose fibrils (Deasey and Mathysse, 1984). Other chromosomal loci have been identified which, when deleted, have pleotrophic effects and such mutants lack flagella, are defective in plant cell attatchment and fail to produce extracellular polysaccharides. Loss of these functions may not always prevent tumourigenisis but the virulence and host range may be affected (Melchers and Hooykaas, 1987).

1.3 *A. tumefaciens* Ti VECTORS

The essential elements of any Ti plasmid-based plant transformation vector are a virulent *A. tumefaciens* strain which is compatible with the plant species to be transformed, a functional Vir-region compatible both with the bacterial strain and the plant species, the T-DNA border(s),methods

for cloning the desired genes between these borders and the presence of genes within the T-DNA which allow the transformed plant material to be recovered or identified either by direct selection or by screening.

Selectable and screenable markers are required in a plant transformation system to enable transgenic plant material to be identified and recovered from amongst the background of untransformed material. The first selectable markers used in Ti plasmid vectors were the phytohormone genes of the T-DNA, with the transformed material being identified by the resultant phytohormone independent growth (Hernalsteens *et al.*, 1980. Garfinkel *et al.*, 1981). Ti plasmid vectors based on this selection method are called 'oncogenic'vectors. The presence of the Ti phytohormone genes prevents the regeneration of whole plants from transformed tissue and only allows undifferentiated callus tissue to be propagated (Frayley *et al.*, 1986). A number of dominant selectable marker genes have been constructed for use in Ti plasmid vectors where the phytohormone genes have been deleted, these being termed 'disarmed'vectors. These chimaeric genes enable transformed tissue to be selected for *in vitro* and the absence of the phytohormone genes enables the regeneration of whole viable plants. Genes used as selectable markers must have the property of conferring a marked increase in the level of resistance against the selective agent to the transformed plant and the agent itself must not affect the transformed plant material through secondary effects such as inducing untransformed plant cells to produce toxic compounds such as phenolics.

One gene which has been successful as a dominant selectable marker in the transformation of a large number of plant species is the neomycin phosphotransferase 11 (NPT 2) gene from the prokaryotic transposon TN5 (Beck *et al.*, 1982) which detoxifies the aminoglycoside antibiotics, such as kanamycin, neomycin and G418 by phosphorylation. For expression in plant cells the coding region has been fused to a variety of 5′ and 3′ sequences (reviewed by Fraley *et al.*, 1986) from T-DNA and viral genes to give the constitutive expression needed for selectable markers. Similarly, a variety of other dominant selectivable chimaeric genes have been constructed including genes which give resistance to such agents as chloramphenicol, methotrexate, aminoethyl cysteine, hygromycin B, (reviewed by Fraley *et al.*, 1986) as well as bleomycin (Hille *et al.*, 1986) and a number of herbicides such as phospinothricin and bialaphos (De Block *et al.*, 1987), glyphosate (Shah *et al.*, 1986), and the sulphonylurea herbicides (Haughn *et al.*, 1988). Screenable markers can in theory be used to identify transformed plant material and several groups are currently investigating the possibility of using screenable markers as the primary method of identifying plant material transformed with Ti plasmid vectors (various personal communications), but routine transformation systems are usually only used to confirm the presence of the T-DNA after initial selection. Various screenable markers are available for use in Ti plasmid vector systems including nopaline synthase, octopine synthase as well as chimaeric constructs using a β-galactosidase gene (Helmer *et al.*, 1984), a luciferase gene (Ow *et al.*, 1986) and a β- glucuronidase gene (Jefferson *et al.*, 1987).

The aim of Ti plasmid vector systems is to place the gene(s) to be transferred into the

T-DNA so as reside alongside a suitable dominant selectable marker and, if required a screenable marker. Subsequent transfer of the T-DNA to the plant cells and selection for the marker identifies or recovers only transformed tissues and the linked nature of the selectable marker to the other genes within the T-DNA ensure a high frequency of co-transformation. However, the large size of the Ti plasmid makes it unfeasible to clone genes directly between the borders of an intact Ti plasmid. Two strategies have been employed to overcome this problem which are in common use today. Both use small plasmids which can replicate in *E. coli* and are amenable to *in vitro* genetic manipulation techniques (Maniatis *et al.*, 1982). The 'binary'vector systems take advantage of the fact that the *vir*-gene products can act in *trans*. By using a broad host range origin of plasmid replication on the small (cloning) plasmid, genes can be introduced directly into the plasmid in between two T-DNA borders and alongside a suitable marker and cloned in *E. coli*. This plasmid can then be transferred to *Agrobacterium*, usually by conjugation, which contains a Ti plasmid (usually with the T-region deleted) possessing a suitable Vir region.The two plasmids are then maintained as separate replicons and the T-DNA is available for transfer to the plant by the action of the *vir*-genes on the T-DNA borders. A number of binary vectors have been constructed to date including Bin 19 (Bevan, 1984), the pAGS vectors (Van den Elzen *et al.*, 1985) and pGA471 (An *et al.*, 1985).

Cis or 'monomeric'Ti plasmid cloning systems use a small cloning plasmid which can replicate only in *E. coli*. Genes for delivery to the plant are cloned into this plasmid. When this plasmid (the 'integrating'plasmid) is introduced into *A. tumefaciens* containing a suitable Ti plasmid it is only recovered if it integrates into the T-DNA of the Ti plasmid by the process of homologous recombination. In this work, a monomeric (*cis*) vector system is used which is based upon the pGV3850/pGV3851 vector system described by Zambryski *et al.*, (1983, 1984). To function in this system the integrating plasmid (small cloning plasmid) has to have the following properties; pBR322 homology: an origin of replication which functions in *E. coli* but not in *A. tumefaciens* (i.e pBR322); a *bom* (basis of mobilisation) sequence; an antibiotic resistance marker (other than ampicillin, neomycin and tetracycline) expressed in *A. tumefaciens*; and a dominant selective marker expressed in plant cells. After cloning the desired genes into the integrating vector, it is then introduced into the *E. coli* strain GJ23 for subsequent delivery to *A. tumefaciens*. This strain contains two plasmids, R64*drd11* and pGJ28, which can replicate in *E. coli* but not in *A. tumefaciens*. R64*drd11* is a fully autotransferable plasmid which encodes all the functions required for its transfer to *A. tumefaciens* by conjugation and also posesses a tetracycline resistance gene for its selection in *E. coli*. GJ23 is not autotransferable but is capable of being mobilised to *A. tumefaciens* in the presence of an autotransferable plasmid such as R64*drd11*. pGJ28 can do this because it contains both a *mob* gene and a compatible *bom* sequence and the products of the *mob* gene act in *trans* and recognise the *bom* site, initiating transfer of the plasmid to the recipient strain (Van Haute *et al.*, 1983). pGJ28 also contains an antibiotic resistance gene (kanamycin/neomycin) for selection in *E. coli*. pBR322 (and hence the integrating plasmid) contains a *bom* site which is compatible with the *mob* gene of pGJ28 and during conjugation is also transferred to *A. tumefaciens*. After transfer to *A. tumefaciens*, the integrating plasmid is 'rescued'by pBR322 mediated homologous recombina-

tion between the integrating plasmid and the Ti (acceptor) plasmid. The pBR322 homology in the Ti plasmid is supplied by the Ti plasmids pGV3850 and pGV3851 which were constructed using double homologous recombination events to effectively replace part of the T-DNA with pBR322. pGV3850 is a fully disarmed Ti plasmid derived from pTiC58 and the T-DNA region comprises *Hind 3* fragment 23 (using the numbering system of Depicker *et al.*, 1980) which contains the right T-DNA border sequence and nopaline synthase gene, a full length copy of pBR322 inserted via the *Hind 3* site and *Hind 3* fragment 10 which contains the left T-DNA border sequence. pGV3851 is essentially the same but has less of the T-DNA deleted and still contains the T-DNA *Hind 3* fragments 32, 22, 41 and a small part of fragment 19 (see figure 1.3). The *iaaH* is deleted entirely but the *cyt* locus (*ipt* gene) is still present, a genotype which induces attenuated tumour formation with tumour having a shooty phenotype (Joos *et al.*, 1983; Zambryski *et al.*, 1984). Cointegrate formation between these Ti plasmids and the integrating plasmid can be detected by selection for the antibiotic resistance marker carried on the integrating vector and continued selection during the growth of the cointegrate-containing strain prevents any recombination between the resultant direct repeats of pBR322 which would lead to the loss of integrating plasmid. Figure 1.4 gives a diagramatic representation of the single cross-over event between the integrating vector and pGV3850 and shows the structure of the resultant cointegrate.

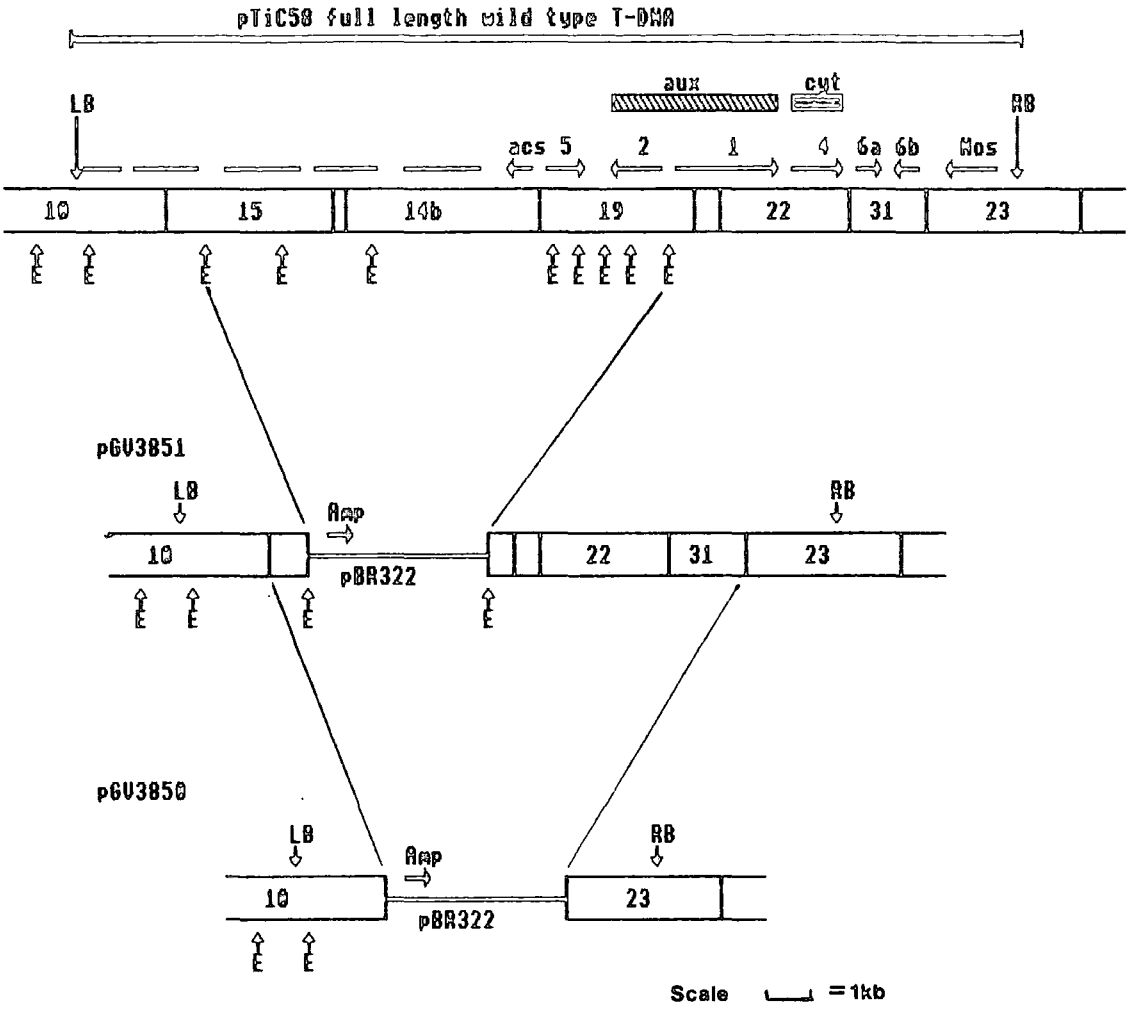
This type of Ti plasmid vector system has the advantage that any one integrating vector construct can be used either in a disarmed vector system or in an oncogenic vector system depending on the nature of the Ti acceptor plasmid and this allows a greater flexibility in the choice of tissue culture strategies which can be employed in plant transformation.

1.4. PLANT TRANSFORMATION TECHNIQUES USING Ti VECTORS.

Although a large number of individual protocols are have been developed for the transformation of plants, those used most frequently in the routine transformation systems of plant genetic engineering fall into three major categories.

Whole plant infection involves the inoculation of Agrobacterium containing oncogenic Ti plasmid vectors onto wound sites of plants *in vivo*. One plant which has frequently been used is *Kalanchoe diaigremontana* which has thick leaves suitable for wounding and supporting abundant callus growth (Garfinkel and Nester, 1980; Klee *et al.*, 1982; Shaw *at al.*, 1984b), although inoculation of tobacco and sunflower stems has also been used (Klee *et al.*, 1982; Murai *et al.*, 1984). Some allied *in vitro* techniques, using surface sterilised explants such as carrot root slices and tobacco stem sections (Barton *et al.*, 1983) have also been used with oncogenic Ti plasmid vector systems. However, these simple *in vitro* techniques do not use complex plant growth media and phytohormones and do not require the addition of antibiotics to inhibit the growth of the tumour inciting *Agrobacterium* strain as they rely on spacial separation of the growth media and the inoculation site. These methods have the disadvantage that they employ oncogenic Ti vector systems and only undifferentiated callus can be recovered but have the advantage that transformed tissue can be re-

FIGURE 1.3 T-DNA STRUCTURE OF pGV3850 AND pGV3851



KEY

LB = left 25bp border sequence
RB = right 25bp border sequence
E = Eco R1
ags = agrocinopine synthase
Nos = nopaline synthase
T-DNA transcripts are numbered by the numbering system of Willmitzer et al., (1983)

Figures in boxes represent Hind 3 restriction fragments using the numbering system of Depicker et al., (1980)

aux and cyt loci are indicated in the wild-type T-DNA

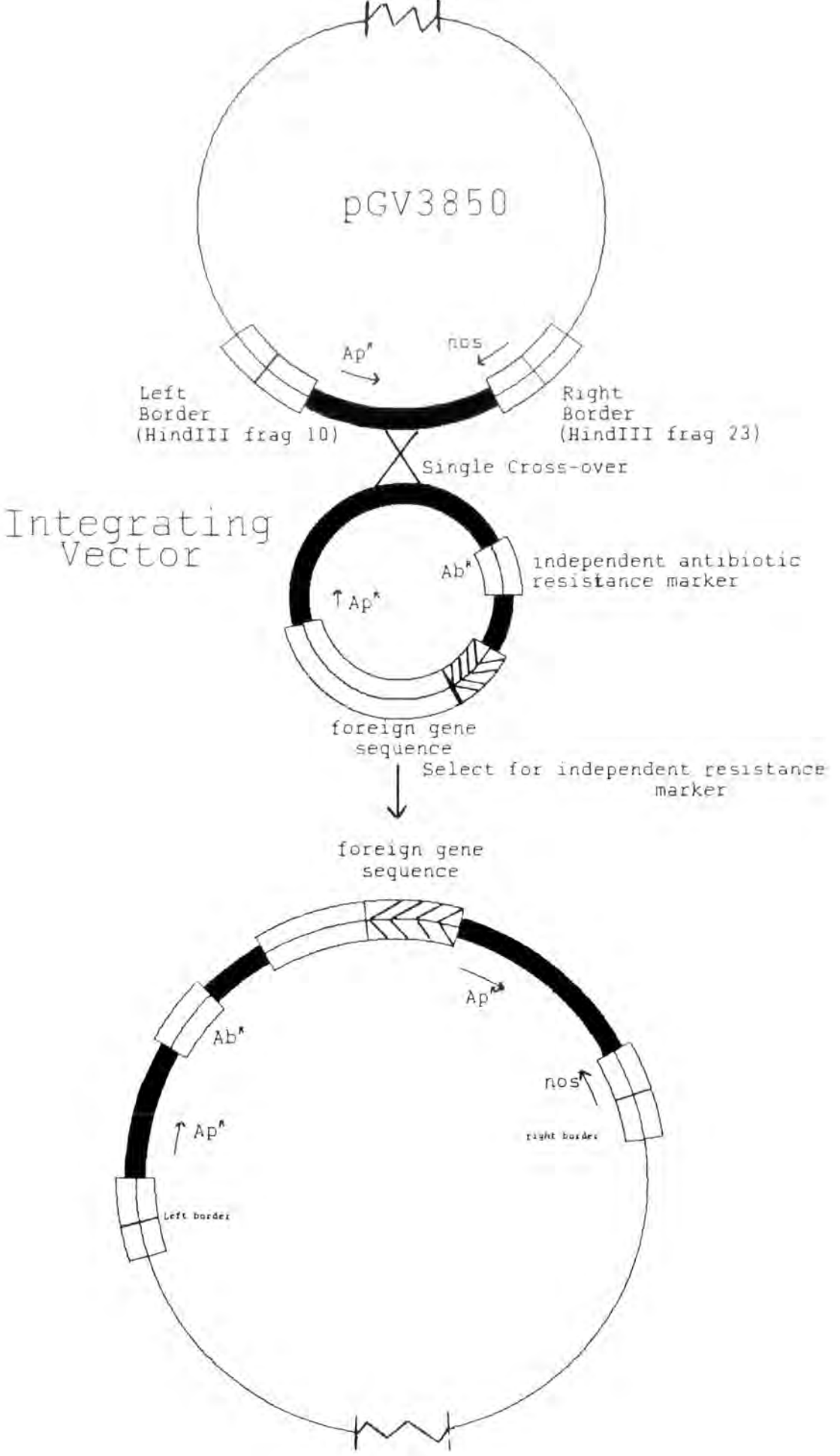


Figure 1.4: Co-integrate Formation between T-DNA Integrating Vector and pGV3850

covered rapidly without the need for any complex tissue culture techniques which can be extremely labour intensive to both develop and conduct.

Protoplast and leaf-disc (type) transformation techniques are all compatible with disarmed Ti plasmid vectors and enable selection for dominant selective markers present in transformed plant cells to be conducted *in vitro*. These techniques all neccessitate the use of surface sterilised or axenic starting material and use complex plant tissue culture media for the propagation of the transformants. Using differing plant growth media and phytohormone supplements, either undifferentiated callus or regenerated plants (some species) can be obtained from cells transformed with disarmed Ti vectors and the methods can also be used with oncogenic Ti vectors, with transformed plant cells being selected on phytohormone free plant growth media. The methods involve cocultivating the Ti vector-containing *Agrobacterium* strain and the plant tissues or cells to allow delivery of the T-DNA to the plant cells. After this step, antibiotic treatments are used to prevent the *Agrobacterium* strain overgrowing, and hence killing, the transformed plant cells (see later).

Cocultivation of *Agrobacterium* with cell-wall regenerating protoplast derived plant cells has been used extensively to generate transformed plant material (Marton *et al.*, 1979; Herrera-Estrella *et al.*, 1983; Frayley *et al.*, 1983). The procedure involves incubating a suspension of 2-3 day old protoplast derived cells at a titre of approximately $10^4 - 10^5$/ml with the bacteria for 36-48h followed by washing and antibiotic treatment to remove the bacteria. The cells are then grown into microcolonies and placed on a selective medium where the transformed microcolonies continue to grow. After a sufficient period, the resultant callus can be transferred to a medium which promotes shoot formation, or alternatively the transformed tissue can be continued to be propogated as callus. This method is suitable for the generation of a large number of independant transformants, and transformation frequencies as high as 50% have been reported (An, 1985), but suffers from the disadvantage that for plant regeneration the incubation periods required to obtain enough callus to support shoot formation are relatively long and can cause somaclonal variation in transformants (Shepard *et al.*, 1980; Peerbolte *et al.*, 1987). Fusion of *A. tumefaciens* sphaeroplasts with plant cell protoplasts has also been reported (Hain *et al.*, 1984) but this process has not been extensively used and is more comparable to liposome fusion than to cocultivation.

Leaf-disc (type) transformation methods exclude the need for protocols to prepare and culture protoplasts. The methods combine the ability of explants (of some species) to produce callus or adventitious shoots from micro-calli at wound sites with the ability of *A. tumefaciens* to infect those cells at the wound site. The method involves infecting a small explant with a suitable *Agrobacterium* cell suspension after which the explant is placed onto a solid plant growth medium for 1-2 days to allow T-DNA delivery. After washing and treatment to remove the bacteria the explants are placed directly onto solid media containing the selective agent, antibiotics for the prevention of growth of *Agrobacterium* and supplemented with phytohormones for the propagation of either callus or shoots. In the latter case shoots of some Solanaceous species such as tobacco, petunia and tomato

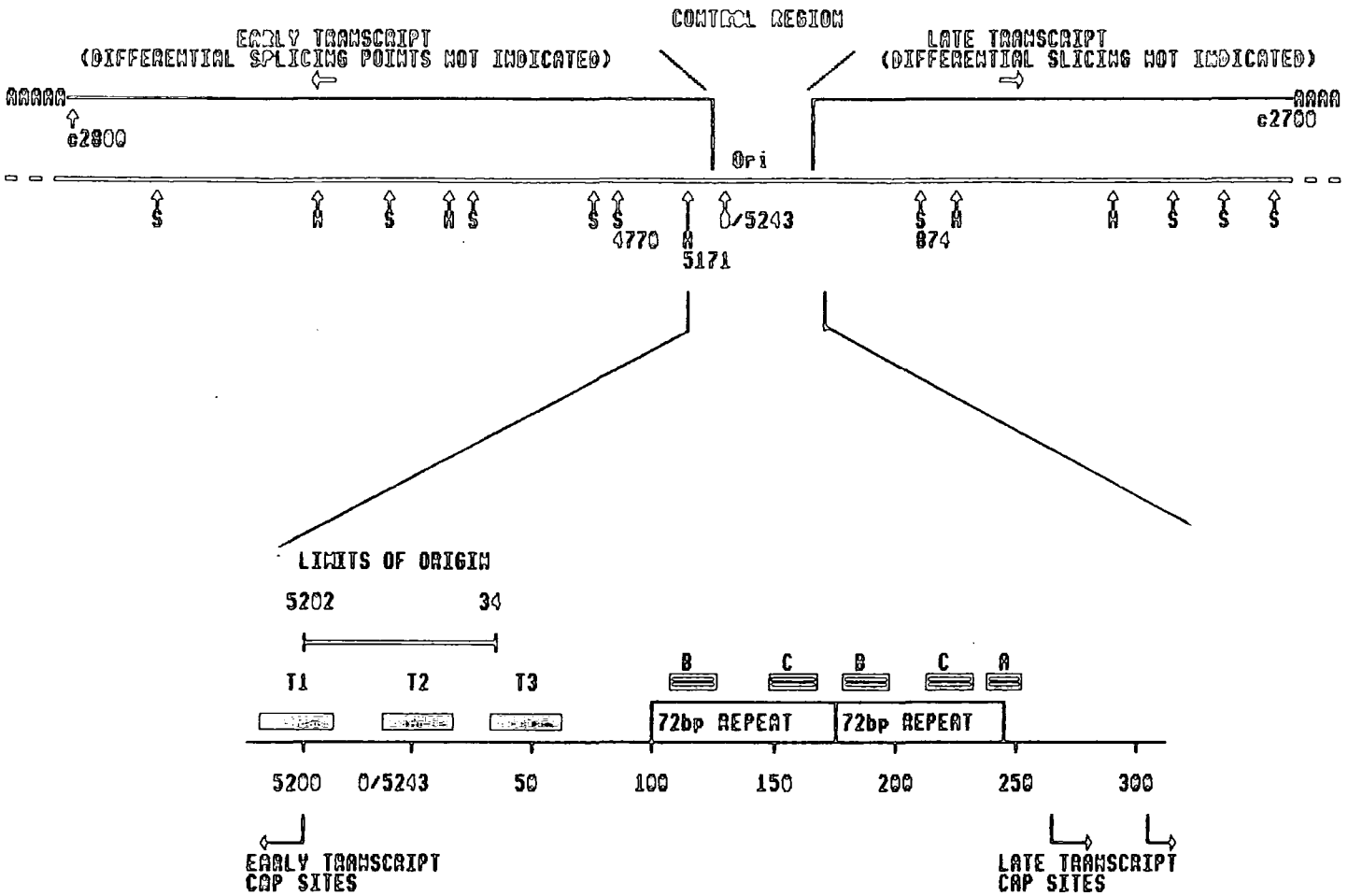
can be excised for rooting within 2-4 weeks after infection. This type of transformation technique takes its name from the prototype method of Horsch *et al.*, (1985), where leaf discs were used, and because in the *Solanaceae* leaf discs are still the most commonly used explant. However, this approach is being increasingly used with other tissue explants in a variety of species, such as *Brassica napus* stem section (Fry *et al.*, 1987), and a more fitting description for these techniques would be *in vitro* explant transformation.

1.5 THE SV40 ENHANCER

Enhancers are *cis*-acting DNA elements essential for the efficient transcription of many viral and cellular genes and differ from other *cis*-acting elements in that they can function in any orientation and in a position independent manner. The first enhancer to be discovered was that of the Simian virus 40 (SV40). This virus, a member of the papovaviruses, has a circular dsDNA genome of 5243bp and contains two transcription units (see figure 1.5). The early transcription unit is expressed during the initial phase of viral infection and codes for the small *t* and the large T antigens, both being translated from separate RNAs formed by differential splicing of the early transcript. The late transcript encodes for the viral coat proteins (VP_1-VP_3) which are translated from two RNAs, again created by differential splicing of the transcipt, with VP_2 and VP_3 being translated from the same message but using different reading frames (for reveiws, see Tooze, 1981; and Shaffner, 1985). Between the two start sites of transcription lies the regulatory region controlling the expression of the transcripts and the origin of replication. Sequences required for the efficient expression of both the early and late transcripts show considerable overlap. For instance, the T-antigen binding sites (T1, T2 and T3) which are required for high level expression of the late transcript are located between the early transcript start sites and the 72bp direct repeats, while sequences required for the efficient expression of the early transcript are located between the 72bp direct repeats and the late transcript start sites (Benoist and Chambon, 1981., Rio and Tjian, 1984). When a 360bp DNA fragment containing this regulatory region was placed adjacent to the rabbit β globin gene, an increase in transcription of more than two orders of magnitude was observed in HeLa cells (Banerji *et al.*, 1981). The enhancer effect of this DNA sequence has also been demonstrated on a variety of other genes (for reveiw see Shaffner, 1985) and the position/orientation independent action demonstrated. The enhancer effect has also been shown to occur over distances exceeding 3kb away from the reporter gene transcription start site (Fromm and Berg, 1983). Deletion and reconstruction analysis of this regulatory region has shown that the major contributors of the enhancer effect are the 72bp direct repeats with some sequences upstream of the repeat (with respect to the early transcript) also being involved (Herr *et al.*, 1985; Herr and Gluzman, 1985; Ceregini *et al.*, 1985).

Since the discovery of the enhancer effect, many other enhancers from both viral and cellular genes have been isolated. Enhancers have been found 5′ of the protein coding sequence, as with the SV40 enhancer; 3′, as in the bovine papilloma virus early transcription unit (Lusky *et al.*, 1983); within an intron, as in the immunoglobin heavy chain genes (Banerji *et al.*, 1983) and also within the

FIGURE 1.5. GENETIC ORGANISATION OF THE SV40 VIRUS GENOME



T1, T2 and T3; T-ANTIGEN BINDING SITES

A, B AND C; ENHANCER ELEMENTS WITHIN 72bp REPEAT (SEE TEXT)

H = Hind 3

S = Sau 3a

coding region, as in the the Adenovirus E1A genes (Osborne *et al.*, 1984). Characterisation of these enhancers has shown that individual enhancers show promoter specificity and differentially increase the level of transcription in different genes. For instance, the SV40 enhancer has no effect on the level of transcription from the Herpes Simplex virus thymidine kinase gene but does dramatically increase the level of transcription of the SV40 early gene in the same host cells (Robbins and Botcham, 1985). In addition to promoter specificity, enhancers also show varying degrees of cell type specificity with the majority of known enhancers being functional in only one or a small range of host cell lines. In contrast, the SV40 enhancer is functional in a broad range of mammalian cell lines and has also been shown to be functional in amphibian cells (Shaffner., 1985) and in algal cells (Neuhaus *et al.*, 1984). One possible reson for this is the recent discovery that the SV40 enhancer is composed of three elements, A B and C, of 21, 22 and 15 bp respectively with each element containing an 8bp core concensus sequence which is present in other enhancers. The respective positions of these elements with respect to the 72bp direct repeats are shown in figure 1.5. Oligonucleotide synthesis of the elements and subsequent reconstitution experiments have shown that the elements are not functional as single copies, that each element can compensate for the loss of one of the others and that elements B and C give different enhancement of transcription in different cell lines (Ondek *et al.*, 1987). These results indicate that the SV40 enhancer is composed of three separate enhancer elements which confer different cell type specificities and may explain the broad host cell range of the wild type enhancer region in comparison to other (single element) enhancers.

Although the mechanism of action of enhancers is not known, the increase in transcription rates in the presence of enhancers is thought to be mediated via the binding of specific *trans* acting proteins. No SV40 enhancer-binding proteins have been isolated to date but the function of the SV40 enhancer in other species which are highly evolutionary diverged implies that these proteins are present and has important implications in understanding the mechanisms of control of expression of eukaryotic genes. On a practical level enhancers can also be used as tools in genetic engineering to increase the level of gene expression in transgenic organisms.

1.6 PEA LECTIN

Lectins, proteins capable of binding and cross-linking monosaccharides and oligosaccharides are widely distributed in nature but are found most abundantly in the seeds of legumes. The lectin from *Pisum sativum* belongs to a group of structurally related lectins which include lentil lectin (from *Lens culinaris*), favin (from *Vicia faba*), and concanavalin A (from *Canavalia ensiformis*). This group of lectins are also related in their sugar binding specificities and bind *D*-glucose and *D*-mannose, and have all been shown to be mitogenic (Lis and Sharon, 1986).

Comparison of the cDNA sequence of the pea lectin gene (Higgins *et al.*, 1983a) to the sequence of a genomic clone (Gatehouse *et al.*, 1987) has shown that, in common to the related lectins, the gene has no introns. The gene copy number was also determined in the latter work and was found to be a single copy gene (1 gene/haploid genome), this finding being significant because it

indicates that the clones do represent active genes and that all forms of the gene product ('isolectins') are derived from a single identical precursor in homozygous plants. Quantitative studies on pea lectin levels in developing cotyledons suggest that its expression is typical of the major storage proteins such as legumin and vicillin, with a maximum lectin content of 0.7% of dry seed weight (approx. 2% of total seed protein) being present at seed maturation (Gatehouse *et al.*, 1986). The structure of lectin from pea cotyledons has been determined (Trowbridge, 1974) and found to be a non-covalently bound tetramer of M_r47000* composed of two large (β subunits) and two small (α subunits) of M_r17000 and 6000 respectively, containing less than 0.5% carbohydrate, and possessing two sugar binding sites. Pulse-chase experiments (Higgins *et al.*, 1983a and 1983b) have shown that the lectin is initially synthesised in the cotyledon as a M_r28000 preprolectin of the form NH_2-leader-β chain-α chain-COOH, which is cotranslationally modified in the ER to give a M_r25000 prolectin which is transported, via the golgi apparatus to the protein bodies where it is slowly processed by proteolytic cleavage to give rise to α and β chains of 58 and 187 amino acids respectively. These chains are then further processed at the C-termini to give rise to the most abundant lectin molecules found within the cotyledons (see figure 1.6). The β subunit is processed to give a 179 amino acid chain which is present in all the isolectins (Trowbridge, 1974; Higgins *al al.*, 1983b). The α subunit is C-teminally processed to give subunits of both 54 and 52 amino acids (Rini *et al.*, 1987) and it is the mass/charge heterogeneity of the α subunits which give rise to the different isolectins in the pea cotyledon. The smaller of the two subunits (α_A) is more acidic than the 54 amino acid subunits(α_B) due to the loss of a lysine residue during the C-terminal processing. This results in three isolectin forms found in the cotyledon, isolectin 1 with a subunit structure $(\alpha_B)_2\beta_2$, isolectin 2 with a subunit structure $(\alpha_A)_2\beta_2$ and a third (unnamed) isolectin with the structure $\alpha_A\alpha_B\beta_2$. Estimates of the pI values of these isolectins vary, with isolectin 1 and 2 having quoted values of 7.0 and 6.0 respectively (Gatehouse and Boulter. 1980). and 7.6 and 6.8 respectively (Rini *et al.*, 1987). However. both of these studies agree that the differences in pI between these isolectin forms is in the region of one pI unit and that the isolectin with the subunit composition $\alpha_A\alpha_B\beta_2$ (hereafter to be referred to as isolectin 3) has an intermediate pI value.

Pea lectin has also been detected in young pea roots (Gatehouse and Boulter, 1980; Diaz *et al.*, 1984), being present at a 5-10 fold lower concentration than in the seed, with only isolectin 2 having been detected. The lectin present has been detected in both the root slime and bound to the surface of the root hairs. *In situ* localisation of the cell wall lectin suggests that the lectin is situated only on the surface of elongating root hairs and on epidermal cells located just below the young hairs which are on the (proto)xylemic poles of the roots (Diaz *et al.*. 1986).

With one exception (Gatehouse and Boulter. 1980), root lectin (isolectin 2) from pea has been shown to have similar sugar-binding specificities and dissociation constants to (predominantly isolectin 1) seed lectin (Kijne *et al.*. 1983; Hosselet *et al.*, 1983; Diaz *et al.*, 1984; Stubbs *et al.*, 1986). The prolectin isolated from pea seeds (Higgins *et al.*, 1983a) and a chimaeric preprolectin/bacterial protein expressed in *E. coli* (Stubbs *et al.*, 1986) were also found to be unchanged with respect

to sugar-binding specificities and dissociation constants indicating that correct posttranslational processing is not a pre-requisite for the activity of this protein.

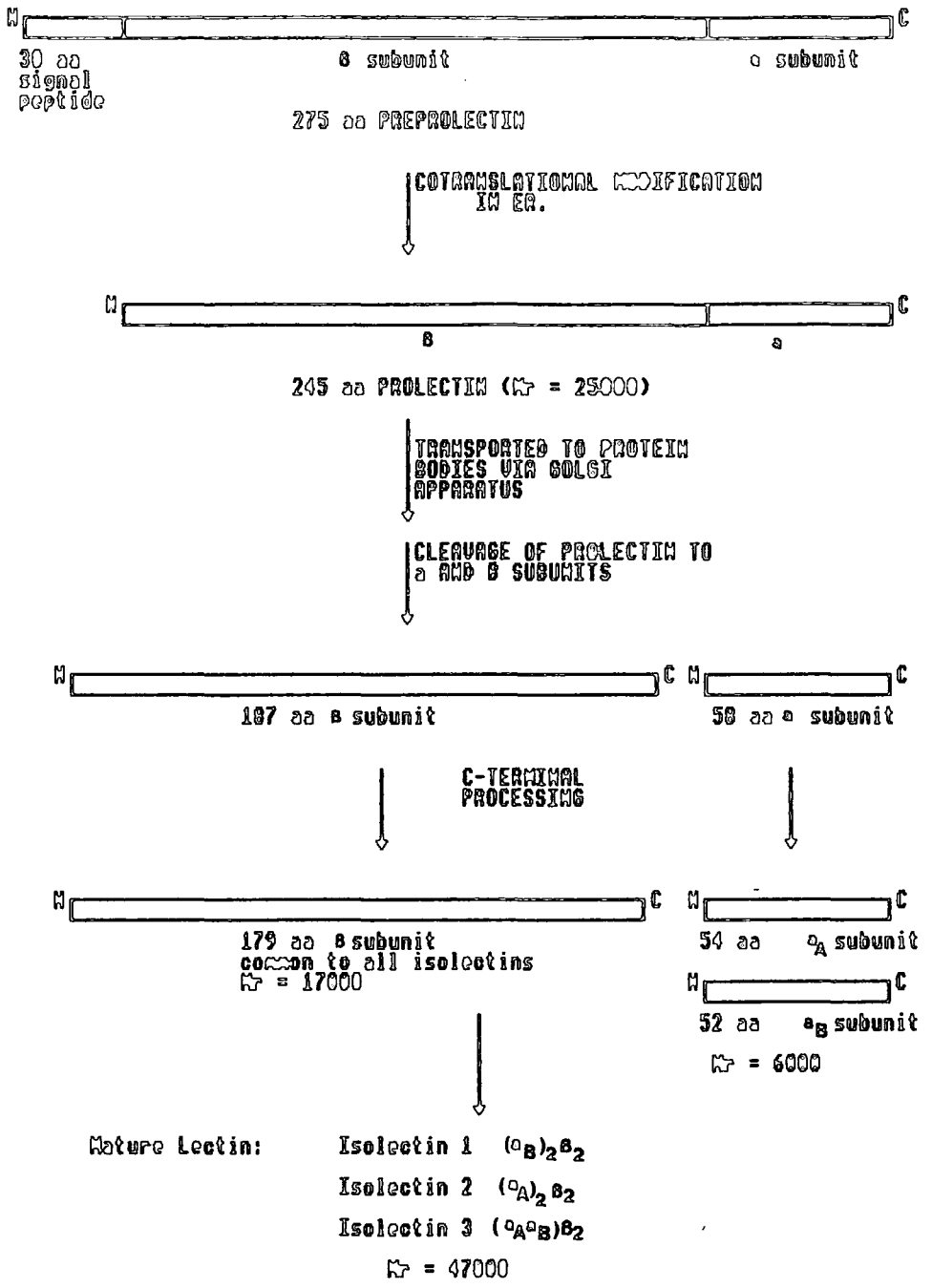
1.6.1 THE BIOLOGICAL ROLE OF PEA LECTIN

No direct evidence for the biological role of pea lectin exists, but circumstantial evidence indicates that pea lectin does have a number of possible functions. The root lectins of legumes have been implicated in *Rhizobium* binding. The root lectins of clover (Truchet *et al.*, 1986), soya bean (Sin-Cheong *et al.*, 1986), *Lotononis bainesii*, (Law and Strijdom, 1984) as well as pea (Chahal and Vikhu, 1985. Diaz *et al.*, 1986) have been shown to bind specifically to those strains of *Rhizobium* capable of inciting nodule formation and also to be present at the site of infection, the root hairs. A correlation between the ratio of pea lectin in the cell walls to the root slime content with nitrate concentration in the growth medium has been established in 7 day old pea plants. The overall lectin content of the roots remains constant with increasing nitrate concentration but the ratio of root slime lectin to cell wall lectin increases dramatically when the nitrate concentration exeeds 20mM, a concentration known to inhibit infection by *R. leguminoasarum* (Diaz *et al.*, 1984).

It has also been suggested that lectins are protective agents against phytopathogens. This hypothesis was based on the observations that wheat germ agglutinin inhibits the growth of *Fusarium* and *Trichoderma* species in vitro (Mirelmar *et al.*, 1978) and that this lectin also inhibits the germination of *Penicillium* and *Aspergillus* spores (Barkai-Golan *et al.*, 1978), although no evidence exists that pea lectin also has anti-fungal properties. Another possible function of plant lectins is as protective agents against nematodes. Two lectins closely related to pea lectin, concanavalin A and lentil lectin, have been shown to be inhibitory to the chemotactic response of the bacteriophagous nematodes. *Caenorhabditus elegans* and *Panagrellus redivivus* (Jeyaprakash *et al.*, 1984). Application of concanavalin A by watering with dilute solutions has shown that this lectin can give significant control of galling in tomatoes by the root-knot nematode *Meloidogyne incognita* with as little as 3μg/250ml soil being effective (Marban-Mendoza *et al.*, 1987).

Plant lectins have also been shown to have insecticidal properties. The lectin from *Phaseolus vulgaris* lectin has been shown to be toxic against the bruchid beetle *Callosobruchus maculatus* with an LD_{50} value of 0.6% of dietary protein. The mechanism of action was also investigated and it was found that the lectin bound to the mid-gut epithelium of the insect, where it is thought to induce changes in the membranes causing mixing of body fluids with gut contents (Gatehouse *et al.*, 1984). The toxicity of pea lectin to *C. maculatus* has also been determined, and has an LD_{50} value of 1.5% of dietary protein (Boulter, 1986). Whilst this value is higher than that for *P. vulgaris* lectin, pea lectin has the advantage that it has low toxicity to humans (Begbie and King, 1985) and the gene is therefore a candidate for use in transgenic crop plants to confer agronomically useful traits.

FIGURE 1.6 PROCESSING OF LECTIN IN PEA COTYLEDONS.



o Molecular weights given are those estimated by Trowbridge (1974).

1.7 AIMS OF THE PROJECT

This project was conceived initially during the phase of Ti plasmid vector development in plant molecular biology, to enable a *cis* (monomeric) Ti plasmid cloning system to be available for use in this laboratory. The pGV3850/3851 Ti plasmid vector system was chosen as this enables both *in vivo* and *in vitro* plant transformation techniques to be employed using similar integrating vectors and methods for delivery into either the disarmed (pGV3850) or the oncogenic (pGV3851) acceptor plasmids. The aims of this project, therefore, were to construct an integrating plasmid suitable for use in the pGV3850/3851 Ti vector system and to gain experience and develop methods for the selection and characterisation of the resultant cointegrates. The partially oncogenic Ti vector pGV3851 was to be investigated to determine its suitability as an oncogenic vector for use in *in vivo* transformation systems and, if found necessary, a fully oncogenic Ti acceptor plasmid would be constructed as a replacement. *In vitro* transformation and tissue culture methods were to be investigated for compatibility with the disarmed (pGV3850) Ti acceptor plasmid and new methods developed where required.

Once these methods had been developed, the aim was to investigate the effect of the SV40 enhancer sequence on the expression of the Nos promoter in plants using the oncogenic Ti plasmid vector in an *in vivo* transformation system. To do this, the SV40 enhancer sequence would be cloned in both orientations 5′ and 3′ with respect to a chimaeric Nos-NPT gene construct and the level of expression of this gene determined using an assay for the NPT enzyme.

This project also aims to use the disarmed Ti plasmid pGV3850 to deliver a chimaeric gene for the expression of pea lectin in tobacco, to regenerate tobacco plants containing this gene, to investigate the structure of the T-DNA in the resultant plants and characterise the expression of the chimaeric gene.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. GLASSWARE AND PLASTICWARE

All glassware and plasticware used for handling nucleic acid samples was autoclaved and siliconised before use.

2.1.2. CHEMICALS AND BIOLOGICAL REAGENTS

Reagents, unless otherwise indicated, were obtained from BDH Chemicals Ltd., Poole, Dorset, UK. and were of analytical grade or the best available. The following materials were purchased from the designated sources.

Acrylamide, bis-acrylamide, BSA, Coomassie blue, DTT, EtBr, ATP, dNTPs, RNase A, DAB, pronase, protease K, lysozyme, ampicillin, chloramphenicol, carbenicillin, gentamycin, kanamycin, neomycin, tetracyclin and FITC conjugated goat anti-rabbit IgG antibodies were from Sigma Chemical Co., Poole, Dorset, UK.

Augmentin and timentin were from Beechams Pharmaceuticals, Brentford, Middlesex, UK. All other antibiotics were obtained from Sensititre Ltd., East Grinstead, Sussex, UK.

3MM, DEAE cellulose (DE81), and P81 paper were from Whatman Ltd., Maidstone, Kent, UK. Nitocellulose filters were from Schliecher and Schuell GmbH., Dassel, W.Germany.

MS medium and 7X detergent were from Flow Laboratories, Rickmansworth, Herts, UK. *Hae 11* linkers, Sephadex G-50 and Ficoll-400 were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Triton X-100, PPO and POPOP were from Koch-Light Ltd., Colnbrook, Berks, UK. Agarose and high gelling temp. agarose were from BRL Ltd., Uxbridge, Middlesex, UK.

Yeast extract was from Biolife S.r.l., Milan, Italy. Bacto-agar was from Difco, Detroit, Michigan, USA. Bacto-tryptone and beef extract were from Oxoid Ltd, Basingstoke, Hants, UK.

Radiolabelled chemicals, nick translation kit, ONPG, and β- galactosidase conjugated donkey anti-rabbit IgG antibodies were from Amersham International Plc, Amersham, Oxon, UK.

Restriction endonucleases and DNA modifying enzymes were from Boehringer Manheim GmbH, Manheim, W.Germany., New England Biolabs, Beverly, MA., USA. and Northumberland Biologicals Ltd, Cramlington, UK. CsCl, X-gal and IPTG were also from Boeringer Manheim.

DAB was from Aldrich Chemical Co. Gillingham, Dorset UK. Perlite was from Silvaperl

Products Ltd. Harrogate, Yorks, UK.

Horse raddish peroxidase conjugated goat anti-rabbit IgG antibodies were from Bio-Rad, Richmond, Calif., USA. Gold labelled donkey anti-rabbit IgG antibodies were from Bioclin services, Cardiff, U.K. LR white resin was from Agar Aids Ltd., Stanstead, U.K.

Lectin from *Pisum sativum* L. cv. Feltham First seeds, purified by the method of Trowbridge (1974) and rabbit anti-Lectin antibodies, purified by the method of Livingstone (1974), were obtained from J.Gatehouse and R.Croy†. Total RNA from developing pea cotyledons was from M.Evans†.

2.1.3. PLANT MATERIAL

Nicotiana tabacum cv. Petit Havana Str-r_1, a streptomycin resistant tetraploid formed by spontaneous mutation and polyploidy of a diploid cell culture line, Maliga *et al.* (1973), and *N.tabacum* cv. Wisconsin callus transformed with the NOS:NPT gene were obtained from A.Hepher*. Untransformed callus was obtained from J.R. Ellis#. *Kalanchoe diaigremontiana* plants were obtained from C.Shaw†.

2.1.4. BACTERIAL STRAINS AND PLASMIDS

All bacterial strains and plasmids used are described in Table 2.1.

Table 2.1 *Escherichia coli* **and** *Agrobacterium tumefaciens* **strains and plasmids**

Bacterial strains	**Genotypes/characteristics**	**Ref/source**
E. coli		
DH1	F-,*recA1, endA1,supE44*, thi,hsdR17, λ-	Yanish-Perron *et al.* (1985)
JM109	*recA1,endA1,gyrA96,thi, hsdR17,supE44*, λ-,Δ *(lac-proAB)* [F',*traD36,proAB,lacI*^{q}Δ M15]	Yanish-Perron *et al.* (1985).
JC2926	F-,*recA1,supE44,thi,leu, lacY,gal,ara,pro,arg,his*	Warren *et al.* (1978)
GJ23	JC2926 [R64*drd11*,pGJ28]	Van Haute *et al.* (1978)
A. tumefaciens		
GV3101	(C58C^1 Rif^r), Ti-cured Rif^r C58 derivative.	Holsters *et al.* (1980)
LBA4404	ACH5 [pAL4404]	De Framond *et al.*

		(1983)
Plasmids		
pBR322	Ap^r,Tc^r ColE1 replicon *bom*$^+$	Bolivar *et al.* (1977)
pGJ28	Kn/Nm^r,ColD replicon, ColE1 *bom*$^+$,*mob*$^+$	Van Haute *et al.* (1983)
R64*drd11*	Tc^r,*Iα* type autotransferable plasmid	Warren *et al.* (1978)
pDUB1114	pBR322 containing Km /Gm^r gene from Mini Sa	C.Shaw† Leemans *et al.* (1982)
pUC 18/19	Ap^r, lacZ cloning region from M13 mp18/19	Yanish-Perron *et al.* (1985)
pNOS-NPT	Nos promoter/NPT 2 gene fusion in pUC 9	Bevan (1984)
Bin 19	NOS-NPT 2 gene on broad host range cloning vector	Bevan (1984)
pAL4404	Str^r pTiACH5 with T-DNA deleted	De Framond *et al.* (1983)
pGV3100	pTiC58 derepressed for autotransfer	Holsters *et al.* (1980)
pGV3850	pGV3100,pBR322 replacing T-DNA between *Hind 3* fragments 23-10	Zambryski *et al.* (1983)
pGV3851	pGV3100. pBR322 replacing T-DNA *EcoR 1* fragment 14	Zambryski *et al.* (1984)
pGV0342	pBR322 containing pTiC58 T-DNA *Hind 3* fragments 10-14b	Depicker *et al.* (1980)
pGV0319	pBR322 containing pTiC58 T-DNA *Hind 3* fragments 31-14b	Depicker *et al.* (1980)
pDUB1105	pBR322 containing TN5 NPT 2 gene	C.Shaw†
pDUB80	pUC18 containing pea LecA Gene (pDJ20)	R.Croy†
pDB7.01A	pUC18 containing SV40 enhancer sequence	D.Bown†
pCaMV	800bp 35S promoter from ROK2 (a derivative of ROK1) in pUC 18	M.Bevan and T. Kavenagh‡ Baulcombe *et al.* (1986)

Key: Amp^r, ampicillin resistance. Gm^r, gentamycin resistance. Km^r, kanamycin resistance. Nm^r, neomycin resistance. Rif^r, rifampicin resistance. Str^r, streptomycin resistance. Tc^r,

tetracyclin resistance. *RecA*, recombination deficient. [F], strain containing plasmid F. F', episomal F plasmid. Δ, deletion. ΔM15, deletion of first 15 codons at 5' of *lacZ*. *traD*, transfer deficient plasmid. *hsdR* endonuclease R host restriction activity. *supE* , supressor of amber mutations. *endA1*, DNA specific endonuclease 1 deficient. *lacY*, lactose permease deficient. bom^+, containing basis of mobilisation site. mob^+, containing mobilising protein gene. *leu, pro, arg, his, thi*, auxotrophic for leucine, proline, arginine, histidine and thiamine respectively. *gal, ara*, deficient for galactose and arabinose catabolism respectively.

† Present address: Dept. Botany, University of Durham, UK. ‡ Present address: Plant Breeding Institute, Cambridge, UK. * Present address: Shell Research station, Sittingbourne, Kent. # Present address: ICI, Jealbts Hill research station, Bracknell, Berks.

2.1.5. BACTERIAL CULTURE MEDIA AND ANTIBIOTICS

The following media were used for the growth of bacterial cultures: 2xYT medium: 6g/l bacto-tryptone,10g/l yeast extract, 5g/l NaCl. (Miller, 1972)

L-broth :10g/l bacto-tryptone,5g/l yeast extract,0.5g/l NaCl, pH to 7.0 with NaOH,10ml 20% sterile glucose solution added after autoclaving.(Miller, 1972)

YEB-broth: 5g/l bacto-tryptone, 1g/l yeast extract, 5g/l beef extract, $MgSO_4$ to 2mM, pH to 7.2 with NaOH, 10ml 50% sterile sucrose solution added after autoclaving. (Vervliet *et al.*, 1975)

For solid media. 15g/l agar was added before autoclaving. After autoclaving, (121°C,15psi.), and the media cooled to 55°C before antibiotics were added (see Table 2.2). For the detection of functional β-galactosidase in *E.coli*, 2xYT agar was supplemented with 40μg/ml X-gal and 0.1mM IPTG.

Table 2.2 Antibiotic concentrations used in bacterial culture media

Antibotic	abbv.	*A. tumefaciens* μg/ml	*E. coli* μg/ml
ampicillin	Ap	-	50
carbenicillin	Cb	100	-
gentamycin	Gm	10	2.5
kanamycin	Km	-	20
neomycin	Nm	-	25
rifampicin	Rif	100	-
tetracyclin	Tc	-	10

2.2 METHODS

2.2.1. BIOCHEMICAL TECHNIQUES

2.2.1.1. STORAGE OF BACTERIA

Bacterial cultures were stored at 4°C for up to 6 weeks on inverted agar plates sealed with Nescofilm. For long term storage, bacterial lawns grown from single colonies on selective agar plates were transferred to sterile 2ml aliquots of a solution containing 60% L broth and 40% glycerol, mixed thoroughly by vortexing, and stored at −80°C.

2.2.1.2. ALCOHOL PRECIPITATION OF DNA

0.1 volumes of 3M sodium acetate pH 5.2 and 2.0 volumes of ethanol were added to the DNA solution and kept at −70°C for 30min, or at −20°C overnight. The precipitated DNA was pelleted by centrifugation at 12000g for 10min (MSE Micro Centaur microcentrifuge) for small samples, or at 25000g for 30min (Sorvall RC-5B centrifuge) for larger samples. The pellet was washed twice in 70% (v/v) ethanol, dried briefly under vacuum and redissolved in a small volume of water or TE (10mM Tris-HCl pH7.4, 1mM EDTA). To minimize the volume of solution to be centrifuged, isopropanol was sometimes used instead of ethanol. In these cases, 0.6 -1.0 volume of isopropanol was added to the DNA solution and the mixture was kept at −20°C for 30min.

2.2.1.3. DEPROTEINISATION OF DNA SAMPLES USING PHENOL

Solutions of DNA were deproteinised by two successive extractions with phenol-chloroform-isoamyl alcohol (25:24:1 v/v)-henceforth refered to as "phenol". 1.5 volumes of phenol were added to the DNA sample and mixed by vortexing. The aqueous phase and phenolic phase were separated by a brief centrifugation (approx 30s) in a microcentrifuge. The upper phase was transfered to a fresh tube and the phenol extraction was repeated. When extracting small amounts of valuable DNA, the phenol phase was back extracted with an equal volume of TE buffer, and the resultant aqueous phase was combined with the original aqueous phase. Phenol extractions were followed by two extractions with an equal volume of chloroform-isoamyl alcohol (24:1, v/v) to remove the remaining traces of phenol. In some cases, when the DNA sample was very viscous, the remaining traces of phenol were removed by three or four extractions of an equal volume of diethyl ether. Traces of diethyl ether were then removed by aspirating the sample with nitrogen for 5min. After deproteinisation, DNA was recovered by alcohol precipitation.

2.2.1.4 DIALYSIS OF DNA SOLUTIONS

Suitable lengths of dialysis tubing were boiled for 20min in 10mM EDTA, then thoroughly rinsed in distilled water. The sealed dialysis tube containing the DNA solution was then placed in

5l TE buffer (10mM Tris-HCl pH 7.4, 1mM EDTA) and stirred at 4°C. The TE buffer was changed 2-3 times over a period of 24h.

2.2.1.5. SPECTROPHOTOMETRIC ANALYSIS OF NUCLEIC ACID SOLUTIONS

The optical densities (OD) of nucleic acid solutions in 1cm quartz cells were recorded from 320 to 230 nm in a Pye Unicam SP8-150 UV/vis spectrophotometer operated in the scanning mode. An OD_{260} of 0.02 corresponds to a DNA concentration of 1μg/ml. An OD_{260} of 0.024 corresponds to an RNA concentation of 1μg/ml.

The purity of the nucleic acid was determined by the $OD_{260/280}$ ratio (1.8 for pure DNA, 2.0 for pure RNA) and by comparison the $OD_{260/235}$ ratio should be higher than the $OD_{260/280}$ ratio.

2.2.1.6. DETERMINATION OF DNA CONCENTRATION BY FLUORIMETRIC ANALYSIS

The method was that of Thomas and Farquar (1978) and was used to determine low concentrations of DNA fragments used as gene copy reconstructions in Southern blots.

1-10μl samples of DNA solutions of unknown concentrations were placed in Eppendorf tubes, centrifuged briefly, and dried down under vacuum. 20μl DABA solution (400mg/ml, purified and recrystallised as described in the reference) was added to each tube, centrifuged briefly, incubated at 60°C for 30min and 1.6ml 1M HCl added and mixed thoroughly. The relative fluorescence was read on a Baird-Atomic *Fluoripiont* fluorimeter at 405nm excitation and 505nm emission. A standard curve was constructed using pure herring sperm DNA ($OD_{260/280}$ =1.8) with standards containing 20ng-1μg DNA.

2.2.2. NUCLEIC ACID ISOLATION

2.2.2.1. RAPID MINI-PREPARATION OF PLASMID DNA

The method used was that of Birnboim and Doly (1979) with minor modifications as described below. The plasmid- bearing strain of *E.coli* was grown to saturation at 37°C in 10ml of L-broth containing appropriate antibiotics. The cells were pelleted by centrifugation at 6000g for 5min in an MSE bench centrifuge (using the culture bottles as centrifuge tubes), and resuspended by vortexing in 200μl of freshly prepared 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 4mg/ml lysozyme. The suspension was transfered to a 1.5 ml Eppendorf tube and placed on ice for 30min. 400μl of freshly prepared 0.2M NaOH, 1% SDS was added, mixed gently by inversion, and kept on ice for 5min. 300μl of 3M sodium acetate pH 4.8 was added and thoroughly mixed. The mixture was placed on ice and agitated every 5min for a total period of 30min. The sample was centrifuged for 15min at 12000g. The supernatant was phenol extracted once, followed by two

chloroform extractions. The DNA was precipitated using isopropanol, redissolved in 300μl of water, precipitated with ethanol and the pellet washed twice in 70% ethanol followed by a brief drying under vacuum. The pellet was dissolved in 200μl TE buffer and stored at −80°C.

2.2.2.2. LARGE SCALE PREPARATION OF PLASMID DNA FROM *E. coli*

The method used was essentially that of Godson and Vapnek (1973) and involved lysis of bacterial sphaeroplasts with SDS. The method was modified by using potassium acetate instead of NaCl in the initial precipitation of cell debris (as described in the BRL NACS Application guide, 1981).

The plasmid-containing strain of *E. coli* was grown in 1l L-broth, containing appropriate antibiotics, at 37°C with vigorous shaking to an OD_{650} of 0.9-1.0. Chloramphenicol was added to a final concentration of 170μg/ml (N.B. all *E. coli* plasmids used were pBR322 or pUC based and therefore amplifiable) and incubation continued for 16-20h. The cells were harvested by centrifugation at 6000g for 10min and the pellet washed in a small volume of 25mM Tris-HCl pH8.0, 10mM EDTA, and the washings discarded. The pellet was resuspended in lysozyme buffer (15% sucrose, 50mM Tris-HCl pH8.0, 50mM EDTA) and placed on ice. 4ml of lysozyme buffer containing 10mg/ml lysozyme was added to the cell suspension, mixed gently and placed on ice for 15min with occasional gentle shaking. 6ml of 10% SDS solution was added, mixed, and the suspension placed on ice for 10min. 12ml of ice cold K-acetate was added to the lysate, mixed thoroughly, and placed on ice for 30min, followed by centrifugation at 27000g for 30min at 4°C. The supernatant was recovered, the volume estimated, and solid NaCl and 50% (w/v) PEG solution added to give final concentrations of 3.6g/l and 10% (w/v), respectively. The mixture was placed on ice for 2h, followed by centrifugation at 6000g for 10min at 4°C. The precipitate was dissolved in 10ml TE (10mM Tris-HCl pH7.4, 1mM EDTA) and 0.3ml 10mg/ml EtBr was added, followed by the addition of CsCl to a final concentration of 48.4% (w/v). The solution was stored on ice for 30min followed by centrifugation at 27000g for 30min. The red pellicle on the surface was removed and the solution centrifuged at 44000g for 18-24h at 15°C in a Beckman VTi50 vertical rotor. The lower plasmid band, as seen under 300nm UV light, was removed using a syringe and purified on a second CsCl gradient, as previously described. After recovery of the plasmid, the EtBr was removed by extraction with CsCl saturated isopropanol, and the CsCl removed by dialysis against TE. The DNA was precipited with ethanol, dissolved in a suitable volume of TE and the DNA concentration determined by spectrophotometric analysis. The DNA was stored at -80°C.

2.2.2.3. *A. tumefaciens* TOTAL DNA PREPARATION

The method used was essentially that of Dhaese *et al.* (1979). *A.tumefaciens* strains were grown in 5ml YEB-broth containing the appropriate antibiotics for 24-48h at 27°C with vigorous shaking. 1.5ml of culture was centrifuged for 30s at 12000g in an Eppendorf tube and the cells resuspended in 380μl pronase buffer (50mM Tris-HCl pH8.0, 20mM EDTA, 0.8% Na-lauroyl sarcosi-

nate). 20μl of a 20mg/ml solution of pronase, previously incubated at 42°C for 2h, was added to the resuspended cells, mixed thoroughly and incubated at 37°C for 1h. The resulting lysate was sheared by passing it through a Pasteur pipette 4-5 times. The sheared lysate was deproteinised twice by extraction with phenol followed by four extractions with diethyl ether. The DNA was precipitated with alcohol, redissolved in 200μl TE, reprecipitated, and washed twice in 70% ethanol. The DNA pellet was dissolved in 100μl TE and stored at −70°C.

2.2.2.4. EXTRACTION OF HIGH MOLECULAR WEIGHT DNA FROM *Nicotiana tabacum* LEAVES

The method employed was that of Graham (1978) with additional CsCl centrifugation steps included to further purify the DNA.

Healthy leaves were excised from plants, the midribs removed and discarded, and the remaining leaf material wrapped in foil and placed in liquid nitrogen. This material was stored at -80°C or extracted immediately. 5g of frozen leaf was ground to a fine powder in a pre-cooled (-20°C) mortar and warmed to -5°C in a ice/saltwater bath. 5ml of homogenising buffer (0.1M NaCl, 25mM EDTA, 2% (w/v) SDS) were added and mixed with the powder, rapidly followed by 1.25ml 5M Na-perchlorate, 5ml phenol and 5ml 1% (v/v) octanol in chloroform. The resulting suspension was shaken on a rotary shaker for 1.5h at 4°C followed by centrifugation at 5000g for 10 min at 4°C. The clear aqueous phase was recovered and extracted with an equal volume of 1% (v/v) chloroform. The DNA was precipitated by the addition of 2.5 volumes of -20°C ethanol and the DNA spooled on a spatular. The spool was drained of excess fluid and dissolved in 2ml 50mM Tris-HCl pH8.0, 10mM EDTA at 4°C for 12-16h. 50μl of pronase solution (20mg/ml, pre-incubated for 2h at 37°C) were added and the solution incubated for 2h at 37°C.

The DNA preparation was further purified by two CsCl centrifugation steps , dialysed, recovered by ethanol precipitation, re-dissolved and the concentration determined as described in section 2.2.1.5. Yields of 10 − 40μg DNA/g plant leaf tissue were obtained using this method.

2.2.2.5. TOTAL RNA EXTRACTION FROM *Nicotiana tabacum* LEAVES

The method used was a modification of that of Hall *et al.* (1978).

Leaf tissue was harvested, frozen and ground as described in section 2.2.2.4. 2g of leaf material being used for each extraction. The mortar was warmed to -5°C in an ice/salt water bath and 10ml of hot (100°C) extraction buffer (0.2M Na-borate, 1%(w/v)SDS, 30mM EGTA, pH9.0) added and thoroughly mixed. A few drops of isoamyl alcohol were added to prevent foaming. The homogenate was cooled to 37°, 100μl of proteinase K solution (20mg/ml) added, mixed, and incubated at 37°C for 1h. 0.8ml 2M KCl was added and the homogenate placed on ice for 10min followed by centrifugation at 5000g for 10min at 4°C (all subsequent centrifugation steps were

carried out under these conditions and are refered to simply as 'centrifugation'). The supernatant was recovered, the volume estimated, solid LiCl added to a final concentration of 2M and the solution incubated for 12-16h at 4°C. The pellet was recovered by centrifugation and washed twice in ice cold 2M LiCl, the pellet being recovered after each wash by centrifugation. The pellet was dissolved in 0.2M K-acetate pH5.5, centrifuged, the supernatant recovered and the RNA precipitated by the addition of 2.5 volumes of ethanol and storage at -20°C for a minimum of 2h. The RNA was recovered by centrifugation, redissolved in K-acetate as previously described, extracted twice with phenol/chloroform/isoamyl-alcohol (25:25:1), recovered by ethanol precipitation and dissolved in a suitable volume of H_2O. The RNA concentration was determined by spectrophotometric analysis and the RNA stored under liquid nitrogen. Yields of approximately 500μg RNA/g of leaf tissue were obtained using this method.

2.2.3. ENZYMIC REACTIONS USED ROUTINELY IN DNA MANIPULATIONS

2.2.3.1. RESTRICTION WITH ENDONUCLEOLYTIC ENZYMES

DNA molecules were digested with type 2 restriction endonucleases in one of the four buffers recommended by Maniatis *et al.* (1982). The buffers, modified to include spermidine, are shown in Table 2.3.

Table 2.3 Endonuclease Digestion Buffers

Buffer	Components (mM)					
	Tris-HCl pH7.5	$MgCl_2$	DTT	Spermidine	NaCl	KCl
Low Salt	10	10	1.0	2.0	-	-
Medium Salt	10	10	1.0	2.0	50	-
High Salt	50	10	1.0	2.0	100	-
SmaI	10(pH8.0)	10	1.0	2.0	-	20

Generally, the enzymes were used at a concentration of 2-5U/μg DNA and incubated at the temperature recommended by the manufacturers for 1-3h. Many of the enzymes have been shown to work adequately at different NaCl concentrations (New England Biolabs 1985/86 catalogue) and hence multiple digestions could usually be performed simultaneously in the same buffer. For digestion of mini-prep plasmid DNA to be analysed on agarose gels, 100μg/ml pancreatic RNase (RNase A), previously boiled for 30min to inactivate contaminating DNases, was included in the reaction mixture.

2.2.3.2. 5'-DEPHOSPHORYLATION OF DNA USING ALKALINE PHOSPHATASE

The 5' phosphate groups of DNA molecules were removed by treatment with calf intestine alkaline phosphatase in 50mM Tris- HCl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine (Maniatis *et al.*, 1982). For fragments with protruding 5' ends, the reaction mixture was incubated for

1h at 37°C with 0.2U enzyme/μg of DNA. To dephosphorylate blunt ended molecules, the reaction was incubated for 15min periods first at 37°C, then at 56°C. A second aliquot of phosphatase was then added and the incubations at both temperatures repeated.

2.2.3.3. CONVERSION OF 3' RECESSED ENDS OF dsDNA TO BLUNT ENDS

The method used was that of Wartell and Reznikoff (1980) and a typical reaction mixture contained the following constituents: 1μg of DNA in TE buffer in a volume of up to 20μl, 2.5μl of 10x nick translation buffer (0.5M Tris-HCl pH7.2, 0.1M $MgSO_4$, 1mM DTT , 500μg/ml BSA), 1μl of a 2mM solution each of dATP, dCTP, dGTP and dTTP, H_2O to a final volume of 25μl and 1 unit of the large fragment (Klenow fragment) of *E.coli* DNA polymerase I. The reaction mixture was incubated at 22°C for 30min and the reaction terminated by the addition of 1μl of 0.5M EDTA. Alternatively the reaction buffer used was the commercially available Amersham 5x nick translation buffer supplemented with dCTP to a final concentration of 80μM.

2.2.3.4. DNA LIGATION

ds-DNA molecules with compatible, protruding ends or blunt ends were covalently joined by treatment with T4-DNA ligase in a minimal volume of ligase buffer (20 mM Tris HCl pH7.6, 10mM $MgCl_2$, 10mM DTT, 0.6mM ATP). Cohesive termini were ligated at 4°C for 12-16h using 1U enzyme/μg DNA. Blunt ended molecules were ligated at 4° or room temperature for 12-16h using 2U enzyme/μg DNA. A further aliquot of ligase and ATP were added and incubation continued for a 12-16h at room temperature. The efficiency of ligation was determined on agarose minigels.

2.2.4. INTRODUCTION OF DNA INTO BACTERIA

2.2.4.1. TRANSFORMATION OF *E. coli* CELLS WITH PLASMID DNA

E.coli cells were rendered competent for DNA transformation by the procedure of Dagert and Ehrlich (1979). 50ml of *E.coli* culture were grown at 37°C in L-broth to an OD_{650} of 0.2. The culture was chilled on ice for 10min and the cells were pelleted by centrifugation at 6000g at 4°C. The pellet was resuspended in 20ml ice cold 0.1M $CaCl_2$ and placed on ice for 30min. The cells were reharvested by centrifugation, resuspended in 2ml 0.1M $CaCl_2$, and stored on ice until required. The maximum transformation efficiency was obtained after 24h on ice. For transformation, the DNA, dissolved in 5 – 10μl water or TE buffer, was added to 100μl of the competent cell suspension in a 1.5ml Eppendorf tube and placed on ice for 30min. The suspension was then heated to 42°C for 2min and replaced on ice for a further 10min. 0.5ml of L-broth (or 2xYT broth in the case of JM109) was added and the suspension incubated at 37°C on a rotary shaker for 30min, after which 20 – 200μl of the suspension was spread on appropriate selective agar plates.

2.2.4.2. BACTERIAL CONJUGATIONS

The method used was essentially that of Van Haute *et al* (1983) with minor modifications. Cultures of donor and acceptor bacteria were grown overnight to saturation in liquid medium (*E. coli* in L-broth and *A. tumefaciens* in YEB-broth, supplemented with the appropriate antibiotics). The cells were harvested by centrifugation and washed twice in phage buffer (10mM Tris-HCl pH 7.5, 10mM $MgSO_4$) and resuspeded to the original cell density. 0.5ml of donor and acceptor strains were mixed and concentrated on a 2.5cm, 0.45μm pore size nitrocellulose filter and the filter placed on an L-agar plate. *E. coli/E. coli* crosses were incubated at 37°C for 6h and *E. coli/A. tumefaciens* crosses at 28°C overnight. After incubation, cells were resuspended in phage buffer, diluted, and plated on appropriate selective agar plates to select for transconjugants and to determine donor and acceptor cell concentrations. The efficiency of transfer was determined by the exconjugant over acceptor ratio.

2.2.5. GEL ELECTROPHORESIS

2.2.5.1. AGAROSE GEL ELECTROPHORESIS OF DNA

The methods used for the preparation of gels and subsequent electrophoresis of DNA samples were as described by Maniatis *et al.* (1982). Briefly, the correct amount of agarose (0.5-1.5% (w/v) depending on the size of DNA to be resolved) was added to Alex's gel buffer (40mM Tris-acetate pH7.7, 2mM EDTA) and boiled until the agarose dissolved. The solution was cooled to 50−60°C and EtBr added to a final concentration of 0.5μg/ml. The solution was allowed to set in a Perspex mould (190x150x6mm) adhered to a glass plate using silicone grease, and containing a suitable well forming comb. The formed gel was transferred to an electrophoresis tank and Alex's gel buffer containing 0.5μg/ml EtBr added to a level 1-2mm above the surface of the gel. The DNA samples, containing 20% loading buffer (0.25%(w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol, 10mM EDTA) were loaded into the wells and electrophoresis performed, usually at 50mA (30V) overnight. The DNA was visualised under 300nm UV light and photographed with a Polaroid MP-4 Land camera through a Kodak 23A Wrattan filter, using Polaroid type 667 film with an exposure of 10s at f5.6.

Minigels were used to estimate DNA concentrations and to monitor the progress of reactions as small amounts of DNA can be detected (less than 10ng) and separated rapidly. These were essentially the same as the gels already described except 100x80x5mm gel moulds and Tris-borate electrophoresis buffer (0.089M Tris-borate, 0.089M boric acid, 2mM EDTA) were used. The gels were run for 1-3h at 50mA (15V).

2.2.5.2. AGAROSE GEL ELECTROPHORESIS OF RNA

The method used was that of McMasters and Carmichael (1977).

RNA samples and DNA size markers were glyoxalated by mixing the following solutions (in order): 20μl DMSO, 2μl 0.2M Na-phosphate buffer pH7.0, 5.7μl 30% (w/v) glyoxal (previously deionised with Amberlite resin), 12.3μl RNA or DNA in H_2O, and incubating at 50°C for 1h. Glyoxalated samples, containing 20% (v/v) loading buffer (30%(v/v) glycerol, 50mM Na-phosphate pH7.0, 25μg/ml bromophenol blue), were loaded onto a 1.5% agarose gel (high gelling temp. agarose) containing 10mM Na-phosphate buffer pH7.0, cast on a piece of Gelbond. Gels were electrophoresed in 10mM Na-phosphate buffer pH7.0 at 100v for 4h, with the buffer being re-circulated and mixed. Gels were bisected and the section containing the size markers stained in 30mg/l acridine orange in gel running buffer for 10min, destained in the same buffer, and photographed as described in section 2.2.5.1. The remainder of the gel was used for northern transfer of RNA to nitrocellulose filters (see section 2.2.10.).

2.2.5.3. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF PROTEINS

The method employed was essentially that of Laemmli (1970). Recipes for the preparation of the resolving and stacking gels are given in Table 2.4. Gels, 150 x 180 x 15mm were constructed and run in a Studier-type gel apparatus as described by Hames (1981). The reservoir buffer comprised 192mM glycine, 25mM Tris base. Three drops of tracking dye (1%(w/v) bromophenol blue in ethanol) were added to the buffer in the upper reservoir prior to the start of electrophoresis. Unless specified otherwise, proteins were extracted from plant tissue by freezing the tissue in liquid nitrogen, grinding to a fine powder and lyophilising for 12-16h. Extraction buffer (see results section) was added and the mixture shaken at 4°C for 1h. The homogenate was sonicated for 1min (Kerry Ultrasonics KS101 sonicator) and shaking continued for 1-2h. The homogenate was centrifuged (5000g, 4°C) for 5min and the protein content of the supernatant determined as described in section 2.2.19. Extracts were mixed with loading buffer to give a final concentration of 10% sucrose in the case of non-denaturing PAGE, or 10% sucrose, 2% SDS for use in SDS-PAGE. SDS-PAGE samples were boiled for 5min prior to loading. Gels were electrophoresed at 8mA overnight or at 30mA for approximately 4h at 4°C. On completion of electrophoresis, the gels were either processed for use in the NPT assay (see section 2.2.21), western blotting (see section 2.2.23.) or the proteins visualised by staining the gel for 2-3h in 200ml kenacid blue stain (0.05%(w/v) kenacid blue R in 50% (v/v) methanol, 7% (v/v) acetic acid). Excess stain was removed by soaking the gel in 2-3 changes of destain solution (50% (v/v) methanol, 7%(v/v) acetic acid) for 8-12h.

Table 2.4 Recipes for the preparation of polyacrylamide gels using the discontinuous buffer system

	Final acrylamide concentration (%)		
	3[a]	10[b]	17[b]
Components*	Volume(ml)		
30% acrylamide stock[c]	2.0	20	34
1.0M Tris-HCl pH8.8	-	22.5	22.5
1.0M Tris-HCl pH6.8	2.5	-	-
H_2O to final volume	20	60	60
	Mix and deaerate under vacuum		
1.5% Ammonium persulphate	0.5	1.5	1.5
TEMED	0.02	0.02	0.02
	Mix and pour immediately		

a. Recipe for stacking gel

b. Recipe for resolving gel

c. 30% acrylamide stock : 30%(w/v) acrylamide, 1%(w/v) bisacrylamide

* 0.1% (w/v) SDS was included in the stacking gel, resolving gel and running buffer for SDS-PAGE.

2.2.6. RECOVERY OF DNA FROM AGAROSE GELS

The method of Dretzen *et al.* (1981) was used with minor modifications. Strips of DEAE-cellulose paper (Whatman DE81) were processed by soaking for several hours in 2.5M NaCl, washed thoroughly with water, and stored dry between sheets of 3MM paper at room temperature. After gel electrophoresis, strips of the DEAE-cellulose paper were inserted into slits cut immediately in front and behind the desired DNA fragment. Electrophoresis was resumed until the fragment had completely entered the paper. The strip of paper inserted behind the band served to prevent contamination by larger bands and was subseqently discarded. The DEAE-cellulose paper containing the desired fragment was blotted dry on 3MM paper. Immobilised DNA was located on the paper by UV-fluorescence and the excess paper trimmed off. A 0.6ml polypropylene microcentrifuge tube was punctured through the base with a fine needle, plugged with siliconised glass-wool and the lid was removed. The DEAE-cellulose paper was placed in the tube, and the whole assembly was placed in a 1.5ml Eppendorf tube with the lid removed. The paper was washed twice in 100μl water, the water being removed from the paper by a 10sec centrifugation at low speed in a microfuge, the washings being collected in the lower tube and discarded. 150μl of elution buffer (1.5M NaCl, 20mM Tris-HCl pH 7.5, 1mM EDTA) was added to the paper and incubated at 37°C for 1h. The elution buffer was recovered in the 1.5ml Eppendorf by centrifugation as previously described. This

step was repeated twice and the total eluate was combined, centrifuged at 12000g for 3min, and the supernatant transferred to a fresh tube. It was then extracted with two volumes of isoamyl alcohol saturated elution buffer and the DNA recovered by ethanol precipitation. DNA recovered by this procedure required no further purification before subsequent enzymic reactions. Recovery was estimated to be 70-90% for linear molecules of 0.1-6.0 kb.

2.2.7. ANNEALING OF *Hha 1* LINKERS TO *Hae 2* RESTRICTION FRAGMENTS

Hha 1 linkers in H_2O were heated to 80°C, cooled rapidly to 65°C and mixed with the *Hae 2* DNA fragment. The mixture was cooled slowly to 0°C over a period of 1h. A 3ml G-50 Sephadex column was poured and equilibrated with TE at 4°C. The void volume and the internal volume were determined by running blue-dextran and a small amount of ^{32}P-dCTP on the column. The anealled linkers-*Hae 2* fragment were loaded on the column and 50μl aliquots recovered. The aliquots were analysed for the presence of the fragment on a minigel and suitable aliquots were used directly in a ligation mix containing the blunt ended vector. Ligation was conducted at 4°C.

2.2.8. PROCESSING OF BACTERIA FOR *in situ* COLONY HYBRIDISATION OF *E. coli*

The procedure used was based on that of Grunstein and Hogness (1975) with modifications as described by Maniatis *et al.* (1982).

Bacterial colonies were replica-plated in a grid pattern onto two L-agar plates containing appropriate antibiotics, one of which was overlaid with a 0.45μm pore size nitrocellulose filter, and grown at 37°C overnight. The "master" plate was sealed and stored at 4°C. The filter, with resident colonies, was transfered to a fresh L-agar plate containing 170μg/ml chloramphenicol and incubated overnight to amplify the plasmid. The filters were then processed by placing them, with colonies uppermost and for 7min in each case, on 3 layers of 3MM paper soaked in the following solutions i) 10% SDS. ii) 0.5M NaOH, 1.5M NaCl. iii) 1M Tris-HCl pH7.5. iv) 3xSSC (0.45M NaCl, 0.045M Na-Citrate pH7.0). The filters were air dried, followed by baking at 80°C under vacuum. Filters were stored under vacuum prior to hybridisation. Positive clones identified after hybridisation and subsequent autoradiography were obtained by subculturing the corresponding colony from the stored master plate.

2.2.9. SOUTHERN TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE FILTERS

The method was modified from that of Southern (1975). After photography, the DNA was depurinated by agitating the gel for 10min in 1% HCl. The gel was transferred to denaturation solution (1.5M NaCl, 0.5M NaOH, 1mMEDTA) and agitated for 30min with one change of solution. The gel was then agitated for 45min in neutralisation buffer (3.0M NaCl, 0.5M Tris-HCl pH 7.0,

1mM EDTA) with one change of buffer and rinsed in 20x SSC (3.0M NaCl, 0.3M Na-citrate pH 7.0). The gel was transferred to a capillary blotting apparatus consisting of a tray containing sponges overlaid with 3 layers of 3MM paper and filled to a level a few cm below the top of the sponges with 20x SSC. Clingfilm was arranged around the gel to prevent bypass of buffer and nitrocellulose paper (0.45μm pore size, pre-soaked in 20x SSC and cut to the dimensions of the gel) placed over the gel. The nitrocellulose filter was overlaid with 3 layers of 3MM paper followed by 3 layers of disposable nappies and a 1Kg weight was placed on top of the apparatus. Transfer of DNA was continued for 6-20h at 4°C. After transfer, the positions of the gel loading wells were marked on the filter with a ball point pen and the filter rinsed in 3x SSC, air dried and baked at 80°C under vacuum for 1h. Filters were stored under vacuum prior to hybridisation with ^{32}P labelled probes (see section 2.2.2.11.).

2.2.10. TRANSFER OF RNA TO NITROCELLULOSE FILTERS BY NORTHERN BLOTTING

The method used was that of Thomas (1980). Glyoxalated RNA, separated on 1.5% agarose gels as described in section 2.2.5.2, was transferred directly to nitrocellulose filters on a blotting apparatus and using the same buffer as described for Southern transfer of DNA. After transfer the filters were baked at 80°C under vacuum for 1h and stored under vacuum prior to hybridisation.

2.2.11. ^{32}P-LABELLING OF DNA BY NICK-TRANSLATION

In vitro labelling of DNA was based on the method of Rigby *et al.* (1977) and was performed using the Amersham nick translation kit. The method used was as described in the instructions. A typical reaction for labelling DNA to a specific activity of 10^7dpm/μg contained 0.5μg DNA, 10μl nucleotide/buffer solution (100μM each of dATP, dTTP and dGTP containing Tris-HCl pH7.8, $MgCl_2$ and β-mercaptoethanol in unspecified concentrations),5μl (50μCi;125pmole) of [α^{32}P]-dCTP, water to 45μl, and 5μl of enzyme solution (2.5 units DNA polymerase I and 50pg DNaseI in a buffer containing Tris-HCl, $MgCl_2$,glycerol and BSA in unspecified concentrations). The mixture was incubated at 14°C for 2h and the reaction terminated and unincorporated label removed by chromatography on a Sephadex G50 (superfine grade) column equilibriated and eluted with 50mM Tris-HCl pH7.5, 0.1% SDS, 150mM NaOH, 10mM EDTA. 1μl of the pooled labelled DNA (0.5ml) was dispersed in 5ml scintillation fluid (3.37g PPO, 667ml toluene, 333ml Triton-X/1) and the radioactivity determined on a Packard Tri-carb Prias scintillation counter.

2.2.12. HYBRIDISATION OF ^{32}P-LABELLED PROBES TO FILTER BOUND DNA

This technique was used to detect homologous DNA sequences transferred to nitrocellulose filters by Southern transfer and in situ colony transfer (see sections 2.2.9. and 2.2.10. respectively)

Filters were equilibriated in solution containing 3x SSC (0.45M NaCl, 0.045M Na-citrate pH

7.0), 5x Denhardt's solution (0.1% (w/v) each of Ficoll 400, BSA, and PVP), 0.1% (w/v) SDS, and 100μg/ml sheared and denatured herring sperm DNA, at 65°C with shaking for 1-3h in a heat-sealed plastic bag. 0.5-1.0ml of this solution was used/cm^2 of filter. After equilibration, ^{32}P-labelled DNA (see section 2.2.11.), previously denatured by boiling for 5min, was added and incubation continued, as previously described, for 8-20h. After incubation, the hybridisation solution was removed and the filter washed twice for 10min at 65°C in solutions containing 0.1% SDS and varying concentrations of SSC, depending on the stringency required. In most cases, where probe was fully homologous to sequences to be detected, two 10min washes in each of 3x SSC, 1x SSC and 0.1x SSC, were performed. After washing, the filters were air dried and the presence and position of annealed radioactive probe detected by autoradiogrphy.

2.2.13. HYBRIDISATION OF ^{32}P LABELLED PROBES TO FILTER- BOUND RNA

The method used was that of Thomas (1980). Nitrocellulose filters containing bound RNA were boiled for 5min in 20mM Tris-HCl pH8.0. The filters were prehybridised in a solution (0.5-1.0ml/cm^2 filter) containing 50% de-ionised formamide, 5x Denhardt's solution (0.1/5x SSC (0.75M NaCl, 0.075M Na-citrate pH7.0) and 100μg/ml sheared and denatured herring sperm DNA, for 4h at 42°C with shaking. Hybridisation was carried out under similar conditions, except 2x Denhardt's solution and 200μg/ml herring sperm DNA was used. ^{32}P-labelled DNA probe was boiled for 5min before addition. Hybridisation was carried out at 42°C for 48h. The filters were washed with two washes of 10min each in 3x SSC, 1xSSC and 0.1xSSC, containing 0.1%SDS. The filters were air dried and autoradiographed.

2.2.14. AUTORADIOGRAPHY

Autoradiography for ^{32}P was carried out using a preflashed film (Fuji-RX, Fujimex, Swindon, Wilts, UK.) and an intensifying screen (Dupont, Wilmington, Delaware, USA.), exposing the autoradiograph at −80°C for appropriate times.

2.2.15. DETERMINATION OF THE RESISTANCE OF *A. tumefaciens* STRAINS TO ANTIBIOTICS

Approximately 10^5 bacterial cells in 0.1ml 0.5x concentration YEB were added to the wells of a microtitre plate containing lyophilised serial dilutions of a range of antibiotics. The plates were incubated at 27° for 72h and the presence or absence of growth (determined by the OD_{650} reading in a plate reader) and the minimum inhibitory concentrations of the antibiotics were determined.

2.2.16. SURFACE STERILIZATION OF PLANT MATERIAL

The method employed was essentially that of George and Sherrington (1984). Leaves were cut from healthy plants and the cut ends of the petioles sealed with warm, molten sealing wax.

The leaves were then immersed in 70% ethanol and gently agitated for 30s and rinsed in sterile distilled water. The leaves were transferred to 5% (w/v) $Ca(OCl)_2$ containing 0.1% 7X detergent for 15min with gentle agitation given every 5 min, followed by 3 washings in sterile distilled water. The disinfected leaves were transferred to a sterile petri dish and 5-10mm square explants cut from undamaged areas using a flamed and cooled scalpel.

N. tabacum seeds were surface sterilized by a similar method except the seeds were immersed in the $Ca(OCl)_2$ solution for 1h prior to rinsing.

2.2.17. PRODUCTION OF TRANSGENIC PLANT MATERIAL BY COCULTIVATION WITH *A. tumefaciens*

The method employed was based on the leaf disk cocultivation method of Horsch *et al.* (1985) as modified by J.R. Ellis (personal communication). The relevant strain of *A. tumefaciens* was grown overnight at 27°C in 50ml YEB-broth containing appropriate antibiotics with vigorous shaking. The cells were harvested by centrifugation and washed three times in 2mM $MgSO_4$, the cells being resuspended, reharvested and the supernatant discarded in each case. After the final wash, the cells were resuspended in 50ml co-cultivation media (details of constituents of all the media used in the various experiments are given in Table 2.5) to give a cell density of approximately 10^{10} cells/ml. Disinfected leaf explants (see section 2.2.16.) were floated on the surface of the culture for 10min and then incubated on co-cultivation agar for 48h at 25°C in a plant growth cabinet with a 16h (9000 lux) photoperiod. The explants were then washed overnight in explant washing medium with gentle shaking, 8 explants being washed in 30ml of media. The explants were briefly washed again in fresh explant washing media, blotted on sterile filter paper and placed on selective agar plates. The plates were sealed and incubated under the conditions previously described.

Transformed callus obtained was subcultured on Selective Agar plates 4-6 weeks after initiation and subcultured every 4 weeks thereafter. Shoots obtained in the relevent experiments were excised afer 4-6 weeks at the second or third internode. The base of the stems was cut at an angle to maximise the surface area available for rooting and placed, with the stem a few mm in the agar, on rooting Agar (25ml) in 60ml poly-carbonate containers. When an extensive root system had developed, the plants were potted out in a 1:1 mixture of Perlite/Levington compost, and covered with a plastic bag. The bag was slit after 3 days and completely removed after 1 week. All plants were grown at 25°C with a 18h photoperiod (6000 lux).

Table 2.5 Plant tissue culture media constituents

Plant species	N.tabacum cv. Petit Havana Str-r1	
Transformed plant material required	regenerated plants	callus tissue
A.tumefaciens plasmid system used	disarmed (pGV3850 based)	oncogenic (pTiGE1 based)
Selection of transformed tissue	NPT II conferred kanamycin resistance	hormone independent callusing, NPT II kanamycin resistance
cocultivation medium	1xMS medium (4.71g/l) Murashige and Skooge (1962) 2%(w/v) sucrose 1.0mg/l BAP,0.1mg/l NAA pH to 5.8 with HCl hereafter called MS2SBN	1xMS medium 3% sucrose pH to 5.8 with HCl hereafter called MS3S
cocultivation agar	as MS2SBN with 0.8% agar	as MS3S with 0.8% agar
washing medium	as MS2SBN with added anti-*A.tumefaciens* antibiotics. (see section 3.1.6.1)	as MS3S with added anti-*A.tumefaciens* antibiotics
selection medium	as MS2SBN. 0.8% agar. anti-*A. tumefaciens* antibiotics, 100μg/ml kanamycin sulphate	as MS3S, 0.8% agar, anti-*A.tumefaciens* antibiotics, 100μg/ml kanamycin sulphate
rooting medium	0.5xMS medium, 100μg/ml kanamycin sulphate, 0.8% agar, anti-*A.tumefaciens* antibiotics	
seed germination media	0.5x MS medium 0.8% agar	

All media were autoclaved for 15min at 15p.s.i. (121°C) before use, and cooled to 50°C before antibiotics were added.

2.2.18. INOCULATION OF PLANTS WITH ONCOGENIC *A. tumefaciens* STRAINS

A.tumefaciens strains were grown overnight in 5ml YEB-broth, containing appropriate antibiotics, at 27°C with vigorous shaking. The cells were harvested by centrifugation (6000g for 5min), washed twice and resuspended in 5ml 2mM $MgSO_4$. Healthy, expanding leaves of *K. diaigremontiana* were wounded by scraping the underside with a needle and inoculated by applying the bacterial culture (30μl) with a micro- pipette. Stems of *N. tabacum* were similarly inoculated.

2.2.19. DETERMINATION OF PROTEIN CONTENT OF PLANT EXTRACTS

The method used was a scaled down version of the Coomassie brilliant blue binding assay of Bradford (1976). The method was used directly on extracts (or diluted extracts) prepared for use in ELISA, the NPT assay, nopaline assay, haemagglutination, and in western blots (see relevant method sections). Protein assay reagent was prepared by dissolving 20mg Coomassie brilliant blue G250 in 10ml 95% (v/v) ethanol. 20ml 85% (w/v) phosphoric acid was then added to this solution and the resultant solution diluted with water to a final volume of 200ml.20μl of plant extract was mixed with 1ml of the reagent and the absorbance at 595nm measured after 2min in a 1cm cuvette against a blank containing 20μl of the appropriate plant tissue extraction buffer and 1ml of reagent. Protein concentrations were determined by extrapolation from a standard curve constructed using BSA standards in the concentration range of 0 – 500μg/ml dissolved in the appropriate buffer.

2.2.20. DETECTION OF NOPALINE IN TRANSFORMED PLANT TISSUE BY PAPER ELECTROPHORESIS

The method employed was based on that of Leon and Schilperoort (1978), with the electrophoresis conditions modified to give better separation from interfering plant metabolites (Ellis *et al.*, 1988).

Tissue was frozen in liquid nitrogen, ground to a fine powder, freeze dried, resuspended in water and extracted for 2-3h at 4°C on a rotary shaker. The homogenate was centrifuged at 12000g for 5min and the protein content of the supernatant determined (see section 2.2.19.). Extract containing a known amount of protein was freeze dried and redissolved in 5μl H_2O. The samples were spotted onto the centre of a Whatman 3mm chromatography paper (20 x 35cm) in 1μl aliquots, the spots being dried under a hot airstream between each application. Nopaline standard (1μl of a 0.5mg/ml solution) and marker dye (1μl of a 10mg/ml bromophenol blue, 10mg/ml xylene orange FF, 20mg/ml fast orange solution) were also applied to the paper. Electrophoresis was conducted at 400v for 3h in 0.4% (v/v) acetic acid, 0.96%(v/v) pyridine buffer. The electrophorogram was dried under a hot airstream for 1h and sprayed with phenanthraquinone reagent (freshly prepared by mixing a solution of 0.02%(w/v) phenanthraquinone in ethanol with an equal volume of 10%(w/v) NaOH in 60% ethanol). The electrophorogram was dried under a cold airstream and viewed and photographed under UV (350nm) illumination.

2.2.21. NEOMYCIN PHOSPHOTRANSFERASE 2 ASSAY

The method was adapted from Reiss *et al.* (1982). 100mg of plant tissue was placed in an Eppendorf tube and frozen in liquid nitrogen. 20μl of extraction buffer (10% glycerol, 0.062M Tris-HCl pH6.8, 5% 2-ME) and a small amount of acid washed sand were added and crushed using a 1ml pipette with a melted end and sonicated for 1min in a Kerry Ultrasonics KS101 sonicator. The extract was centrifuged at 12000g for 5min in a microfuge and protein content of the supernatant determined. The extract was loaded on a 10% non-denaturing polyacrylamide gel (see section 2.2.5.2) along with a bacterial NPT 2 control. This was produced by spinning down 100μl of an overnight culture of *E.coli* DH1 [pDUB 1105], grown in L-broth suplemented with 25μg/ml kanamycin sulphate, resuspending in 30μl extraction buffer, sonicating for 1min and centrifuging at 12000g for 5min. 20μl of the supernatant was loaded on the gel. The gel was run at 30mA at 4°C until the bromophenol blue was a few cm from the bottom of the gel. The gel was removed from the glass plates and washed twice and equilibrated for 15min in NPT reaction buffer (67mM Tris-malate pH7.1, 42mM $MgCl_2$, 400mM NH_4Cl), containing 50μg/ml kanamycin sulphate. The gel was transfered onto a glass plate and a mould constructed around it using 4mm thick strips of plastic held in place with silicone grease. A 1% agarose solution in NPT buffer was prepared (30ml) and cooled to 42°C. 125μCi [$\gamma-^{32}$P]-ATP (5000-7000 Ci/mmol) and kanamycin sulphate to 50μg/ml were added and the solution poured over the polyacrylamide gel to form a 2mm thick overlay. The gels were incubated at 37°C for 1h, after which the mould was removed and the gel overlaid with Whatman P81 chromatography paper cut to the size of the gel. Three layers of 3MM paper and a layer of disposable nappies were placed over the P81 paper and a 1Kg weight placed on the apparatus. Transfer of labelled kanamycin was allowed to continue for 4h, after which the P81 paper was removed and washed 5x in water at 80°C for 5min per wash. The paper was air-dried and labelled kanamycin detected by autoradiography. Radioactive bands were cut from the paper and placed in scintillation fluid (3.0g/l PPO, 300mg/l POPOP in toluene) and counted on a liquid scintillation counter (see section 2.2.11.).

2.2.22. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) OF PEA LECTIN IN TRANSFORMED PLANTS

ELISA methods have been reviewed by Engvall (1980). Leaf material was frozen in liquid nitrogen, finely ground, and lyophilised for 12-16h. The material was resuspended in extraction buffer (50mM Tris-HCl pH9.5), shaken at 4°C for 1h, sonicated, and shaking continued for 1-2h. The homogenate was centrifuged at 5000g for 5min and the protein content of the supernatant determined (see section 2.2.19.). The extract was diluted in extraction buffer, and 200μl of suitable dilutions transferred to the wells of a polystyrene microtitre plate (Nunc Immuno-F1) along with suitable control dilutions of purified lectin dissolved in extraction buffer. The plates were incubated overnight at 4°C, emptied, and the wells washed 3x with 300μl phosphate buffered saline (PBS),(8g NaCl, 0.20g KCl, 1.15g Na_2HPO_4, 0.20g KH_2PO_4 /l), containing 0.1% Tween 20, this step referred to as 'washing'hereafter. 250μl of PBS containing 1% BSA was added to the wells and the plates

incubated for 1h, removed and the wells washed. 200μl primary antibody (rabbit anti-pea lectin) dissolved in PBS containing 1% BSA, 0.1% Tween 20,10mM $MgCl_2$, 1mM 2-ME, was added to the wells and incubated for 1h, removed and the wells washed. 150μl of β-galactosidase-conjugated donkey anti-rabbit IgG anti-body, dissolved in the same solution as primary antibody, was added to the wells, incubated for 1h, removed, and the wells washed. 200μl of substrate solution (3mM ONPG, in PBS containing 10mM $MgCl_2$,0.1M 2-ME) was added to the wells and the plates incubated at 37°C for up to 3h. The $O.D._{405}$ of the wells was read on a Titertek Multiscan MCC (Flow Laboratories Ltd, UK.) plate reader and the concentration of pea lectin in the transformed tissue estimated by comparison to the controls.

2.2.23. WESTERN BLOTTING: ELETROPHORETIC TRANSFER OF PROTEINS FROM POLYACRYLAMIDE GELS TO NITROCELLULOSE FILTERS

The method used was an adaptation of that of Towbin *et al.* (1979), using the semi-dry electroblotting method of Kyse-Andersen (1974).

Transfer was conducted on a Sartorius SM electroblotter according to the instructions supplied. Briefly, the following components were stacked on the graphite anodic plate in the order described. Two sheets of Whatman 3MM paper soaked in anode buffer 1 (0.3M Tris, 20%(v/v) methanol, pH 10.4); one sheet of 3MM paper soaked in anode buffer 2 (25mM Tris, 20% methanol, pH 10.4); a sheet of nitrocellulose paper soaked in H_2O; the polyacrylamide gel; one sheet of 3MM paper soaked in cathode buffer (25mM Tris, 40mM 6-amino hexanoic acid, 20% methanol, pH 9.4); a sheet of dialysis membrane soaked in H_2O and finally, two sheets of 3MM soaked in cathode buffer. The cathodic graphite plate was placed on top of the stack and the apparatus run at 0.8mA/cm^2 of gel for 1.5h. After transfer the filters were processed for the immunological detection of pea lectin as described in section 2.2.24.

2.2.24. IMMUNOLOGICAL DETECTION OF LECTIN IMMOBILISED ON NITROCELLULOSE FILTERS

The method used is modified from that of Towbin *et al.* (1979). BLOTTO (bovine lacto transfer technique optimiser) was used as the blocking agent as described by Johnson *et al.* (1984).

All incubations described were at 40°C with shaking and all washing steps conducted in the same conditions for 10min each. Filters were incubated and washed once in BLOTTO (5%(w/v) non-fat dried milk powder in 20mM Tris-HCl pH7.2, 0.9%(w/v) NaCl). Filters were then incubated with rabbit anti-pea lectin antibodies (50μl/ 50ml BLOTTO) for 2h, washed three times in BLOTTO, and incubated with horse-raddish peroxidase linked goat anti-rabbit IgG antibodies (20μl/ 50ml BLOTTO). Filters were washed three times in BLOTTO and once with 20mM Tris-HCl pH7.2, 0.9% NaCl. 50mg DAB was dissolved in 100ml 20mM Tris-HCl pH7.2, 0.9% NaCl, and 100μl of 30% (v/v) hydrogen peroxide and 3ml 1% CoCl added. The reagent was filtered onto the nitro-

cellulose and the reaction terminated by rinsing with distilled water when the bands were sufficiently developed.

2.2.25. HAEMAGGLUTINATION ACTIVITY OF PLANT EXTRACTS

The method used was that of Sharon and Lis (1972).

Blood from New Zealand White rabbits was mixed with an equal volume of Alsever's solution (2% (w/v) glucose, 0.8% (w/v) Na-citrate, 0.42% NaCl, pH to 6.1 with citric acid) in a heparinised tube (Sarstedt Ltd. Leicester, UK.) and stored at 4°C. A working strength red blood suspension was prepared by washing the cells in PBS three times and resuspending at 2% of the original cell density in PBS.

Plant tissue extracts were prepared by freezing tissue in liquid nitrogen, grinding to a fine powder and freeze drying. A suitable volume of PBS was added and the homogenate shaken on a rotary shaker for 1h, sonicated, and shaking continued for 1-2h. The homogenate was centrifuged at 12000g for 5min and the protein content of the supernatant determined (see section 2.2.19.)

Two-fold serial dilutions of the plant extact were prepared in PBS and 100μl of each dilution placed (in duplicate) in the wells of a mico-titre plate with U-shaped wells (Sarstedt Ltd.). Suitable dilutions of purified pea lectin in PBS were also included in the assay. 100μl of the red blood cell preparation was added to the wells and mixed and the plates incubated at room temperature for 2-3h. The end point of the haemagglutination assay was defined as the lowest dilution of sample where the agglutinated blood covered the whole of the bottom of the well.

2.2.26. BINDING OF RABBIT RED BLOOD CELLS TO ROOTS OF LECTIN EXPRESSING TRANSGENIC PLANTS

The method used was essentially that of Hamblin and Kent (1973) except that rabbit red blood cells were used instead of those of sheep.

Rabbit red blood cell suspensions were prepared as described in section 2.2.25. Transgenic tobacco roots (the final 1.5cm, containing the region of elongating and elongated root hairs) were incubated with the red blood cell suspension for 30min at room temperature with sufficient shaking to keep the cells suspended. The roots were washed 3 times in PBS (5min/wash with gentle shaking) and the roots examined under the microscope.

2.2.27. DETECTION OF LECTIN ON THE SURFACE OF TRANSGENIC TOBACCO ROOTS BY FLUORESCENCE MICROSCOPY

The method used was essentially that of Diaz *et al.* (1986).

Root tips, consisting of 1.5cm of root containing regions of elongating and elongated root hairs from both control plants and lectin expressing transgenic tobacco plants, were incubated with rabbit anti-lectin antibodies (1/10 dilution in PBS) or rabbit pre-immune antibodies for 1h at room temperature with gentle shaking. The roots were then washed 3 times in PBS containing 0.1% (v/v) Tween 20 (5 min/wash) followed by incubation with FITC conjugated goat anti-rabbit IgG antibodies (1/10 or 1/100 dilution in PBS) for 1h at room temperature with gentle shaking. The roots were washed 3 times as previously described and the roots examined by flourescence microscopy using 450-490nm incident light.

2.2.28. SUBCELLULAR LOCALISATION OF LECTIN IN THE ROOTS OF TRANSGENIC TOBACCO PLANTS BY IMMUNO-GOLD LABELLING

The method used was that of Vanden-Bosch (1986). 1mm sections of transgenic tobacco root from the region of root hair elongation were fixed in 3% paraformaldehyde and 1.25% glutaraldehyde in 0.05M phosphate buffer pH7.0 for 16h. The tissue was washed 3x in 0.05M phosphate buffer pH7.0 and once in distilled water before dehydration in the following ethanol solutions (v/v): 12.5%, 25%, 50%, 75% and with two 30min immersions at each concentration and a final dehydration step of 4x 30min immersions in 100% ethanol. The tissue was removed and placed in a 1:1 mix of ethanol and LR-white resin for 16h followed by 3x 12-16h treatments in 100% LR-white. The tissue was embeded in the resin by placing the tissue in LR-white in an air tight capsule and polymerising in a 70° oven overnight. 100nm sections were prepared using an LBK 4801A microtome and the sections picked up on formvar coated 200 mesh copper grids. The sections were blocked for 10min with 1% BSA, washed 3x in distilled water and incubated with rabbit anti-lectin antibodies (1/100 dilution in PBS, 0.1% Tween 20) for 1h. Controls were incubated with pre-immune serum at the same concentration. The sections were washed 10x in PBS containing 0.1% Tween 20 before incubation with gold labelled (20nm) goat anti-rabbit IgG antibodies (1/20 dilution as before). The sections were washed 10x as described, washed in water, and stained in saturated aqueous uranyl acetate for 15min and finally washed in water. Sections were examined by electron microscopy (Phillips EM400T/ST).

2.2.29. SCREENING OF TRANSGENIC TOBACCO PLANTS FOR RESISTANCE TO THE ROOT-KNOT NEMATODE *Meloidogyne incognita*

Transgenic tobacco plants were transplanted from rooting medium into compost (see section 2.2.17.) and grown for two weeks as previously described. The plants were removed from the compost and the excess compost surrounding the root balls shaken free. The plants were repotted, with a small amount of compost containing eggs of *Meloidogyne incognita* placed adjacent to the root

ball. The plants were then grown for a further four weeks prior to inspection of the plants for any differences in growth rates or morphology. The plants were then removed from the compost, the roots washed, and the extent of infection determined by visual inspection.

3. RESULTS

3.1 INITIAL CHARACTERISATION AND DEVELOPMENT OF *A. tumefaciens* pGV3850 and pGV3851 CLONING SYSTEM

3.1.1. CONSTRUCTION OF pDUB116: A T-DNA INTEGRATING VECTOR

A plasmid, pDUB116, was constructed which contained all the sequences necessary for mobilisation to *A. tumefaciens* (bom site), selection of cointegrates with pGV3850 and pGV3851 (Km/Gm gene from Mini-Sa) and selection of transgenic plant tissue (Nos-NPT gene). Its construction involved the ligation of a 1.5kb *Hind111/EcoR1* fragment containing the chimaeric Nos-NPT gene from pNos-NPT into the plasmid pDUB114, a pBR322 derivative containing a Km/Gm resistance marker from Mini-Sa. A schematic diagram of the steps involved is shown in figure 3.1.

E.coli DH1 was transformed with the ligation mix and ampicillin resistant colonies were selected on L-agar containing 50μg/ml ampicillin. A control ligation mix containing phosphorylated vector alone gave no resistant colonies following transformation. Mini-preped plasmid DNAs from four randomly chosen ampicillin resistant colonies were digested with *Eco R1/Hind 3* and analysed by agarose gel electrophoresis. All four plasmid DNAs contained the 1.5kb *Eco R1/Hind 3* fragment. One plasmid isolate was digested with *Bam H1, Eco R1, Hind 3, Kpn 1, Pst 1, Sst 2* and *Kpn 1/ Bam H1* and further analysed by agarose gel electrophoresis (results not shown). The banding pattern obtained agreed with the predicted banding pattern determined from the published restriction maps (see section 2.1.4 for references) except for an anomalous *Bam H1* site. This site was mapped to the Nos-NPT gene and was previously supposed to have been deleted (Bevan, 1984), but digestion of pNos-NPT showed the site was present in the supplied construct (results not shown).

pDUB116 is a 7.5kb plasmid, containing two unique restriction enzyme sites, *Eco R1* and *Hind 3*, suitable for cloning DNA for delivery to plants.

3.1.2. TRANSFER OF pDUB116 TO *A. tumefaciens* AND SELECTION OF pGV3850:: pDUB116 AND pGV3851::pDUB116 COINTEGRATES

E.coli GJ23 was conjugated with *E.coli* DH1 [pDUB116] and the conjugation mix diluted to 10^{-8}. Suitable dilutions were plated (in duplicate) on L-agar plates containing the following antibiotics and the cell titres determined:

a) 50μg/ml ampicillin (for selection of exconjugants plus acceptors).

b) 25μg/ml neomycin, 10μg/ml tetracycline and 50μg/ml ampicillin (for selection of exconjugants alone).

The transmission frequency of R64*drd11* and pGJ28 to *E.coli* DH1 [pDUB116] (exconjugants over acceptor ratio) was 55%. Control platings of donor and acceptor strains alone on exconjugant

selection media gave no resistant colonies.

An ampicillin, neomycin and tetracycline resistant colony was chosen at random and conjugated with *A. tumefaciens* GV3101 [pGV3850]. The conjugation mix was diluted to 10^{-4} and plated out on YEB-agar containing 100μg/ml rifampicin, 100μg/ml carbenicillin and 25μg/ml kanamycin. The conjugation mix gave two distinct types of colonies; many small white colonies and a few large mucoid pink colonies. *E.coli* DH1 [R64*drd11*, pGJ28, pDUB116] plated alone on this medium also gave small white colonies at a frequency of between 1 x 10^{-5} and 1 x 10^{-7} of the original cell titre. *A. tumefaciens* GV3101 [pGV3850] plated on this medium gave no resistant colonies. The results indicate that the small white colonies obtained on this medium are rifampicin resistant mutants of the *E.coli* strain and that the large pink mucoid colonies are GV3101 [pGV3850::pDUB116] exconjugants/recombinants.

One colony of the putative exconjugant/recombinant and one colony of the rifampicin resistant *E.coli* strain were selected and streaked onto YEB-agar plates containing 100μg/ml rifampicin, 100μg/ml carbenicillin and gentamycin in the concentration range $5-30\mu$g/ml. Rifampicin resistant *E.coli* [R64*drd11*, pGJ28, pDUB116] failed to grow on any of the plates. *A. tumefaciens* GV3101 [pGV3850::pDUB116] grew on plates containing all levels of gentamycin. The results show that the level of gentamycin resistance derived from the Km/Gm resistance gene is much lower in the *E.coli* strain than in the *A. tumefaciens* strain and that this fact can be used to screen out any rifampicin resistant *E.coli* mutants present in the conjugation mix.

E.coli DH1 [R64*drd11*, pGJ28, pDUB116] was conjugated with *A.tumefaciens* GV3101 [pGV3850] and GV3101 [pGV3851], the conjugation mixes diluted to 10^{-8} and plated out on YEB-agar plates containing :

a) 100μg/ml rifampicin and 100μg/ml carbenicillin (for selection of acceptors plus exconjugants)

b) as a) but also containing 10μg/ml gentamycin (for selection of exconjugants).

The frequencies of transmission/recombination of gentamycin resistance to *A. tumefaciens* GV3101 [pGV3850] and [pGV3851] were 8.1 x 10^{-7} and 6.3 x 10^{-7} respectively.

3.1.3. ANALYSIS OF T-DNA OF pGV3850::pDUB116 and pGV3851::pDUB116 CO-INTEGRATES

Five isolates each of *A. tumefaciens* GV3101 [pGV3850::pDUB116] and [pGV3851::pDUB116], isolated as described in the previous section, were chosen at random and total DNA prepared as descibed in section 2.2.2.3. 10μl (out of 100μl total volume) aliquots of the DNA preparations and control DNAs (GV3101 [pGV3850] and GV3101 [pGV3851] were digested with *Eco R1* and *Hind 3*

FIGURE 3.1 CONSTRUCTION OF pDUB116

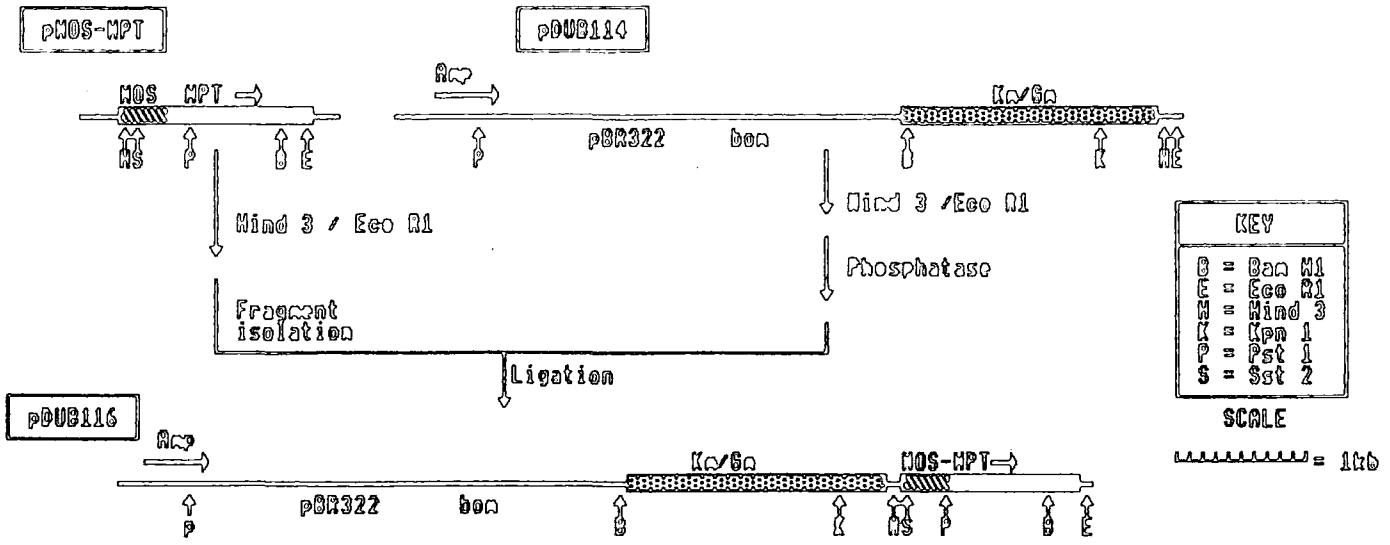
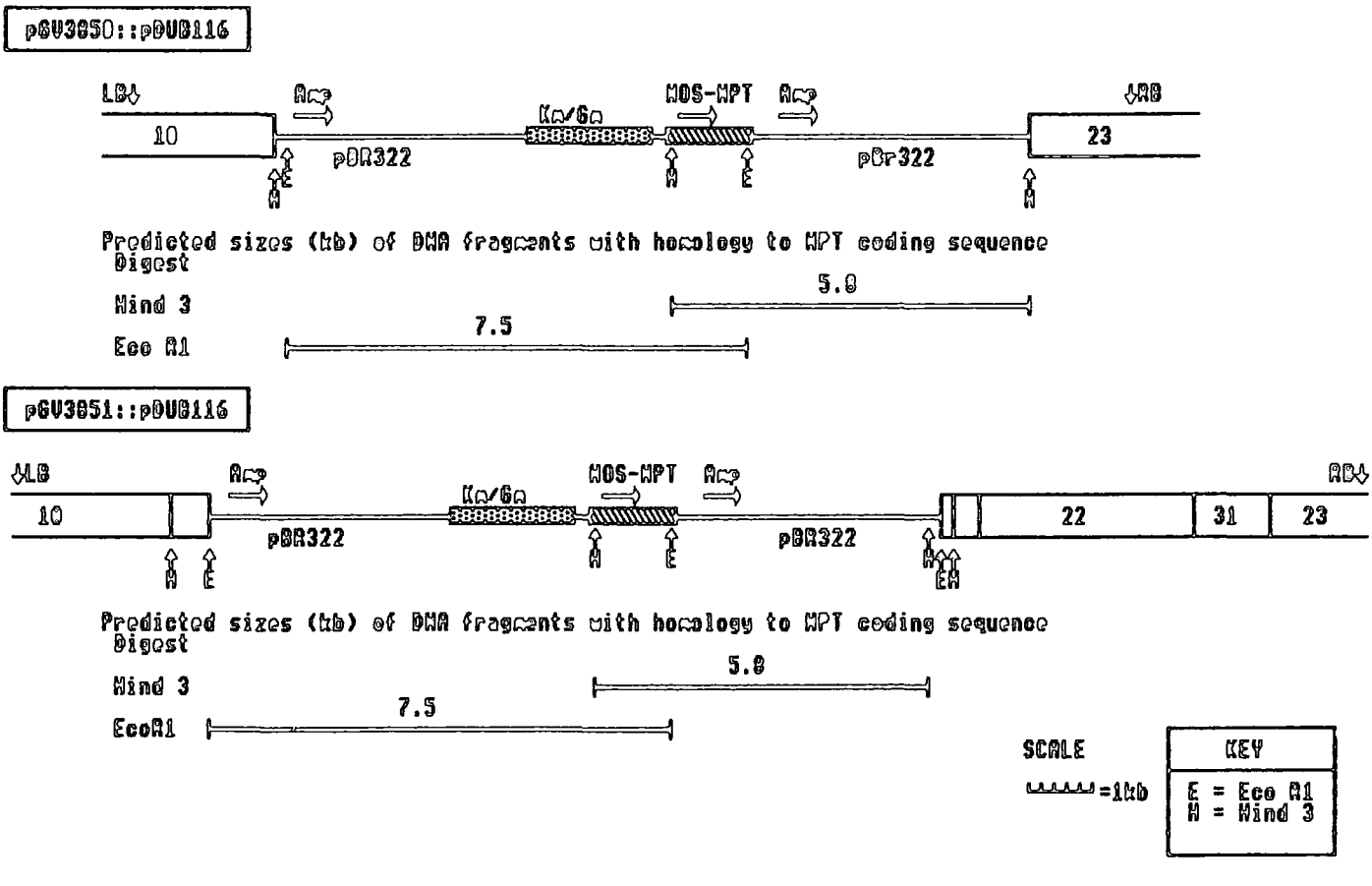


FIGURE 3.2 T-DNA STRUCTURE OF pGV3850::pDUB116 AND pGV3851::pDUB116



Figures in boxes represent Hind 3 restriction fragments of native (pTiC58) Ti-plasmid using the numbering system of Depicker et al (1980).

and separated by agarose gel electrophoresis (0.7% gel) h and the DNA transferred to nitrocellullose filters (see section 2.2.9). The DNA was hybridised with a ^{32}P labelled 1.1kb *Pst 1/Hind 3* fragment encoding the coding region of the Nos-NPT gene. The filters were washed to 0.1xSSC, 65°C stringency and autoradiographed (results not shown). DNA from all isolates gave single bands of hybridisation of 7.4kb (*Eco R1* digested) and 5.7kb (*Hind 3* digested). No bands of hybridisation were detected in control DNA. Figure 3.2 shows the predicted structures of the cointegrates along with the predicted sizes of DNA fragments with homology to the probe. The sizes of bands of homology obtained experimentally are close to the predicted sizes and the results show that the integrating vector integrates into the desired region of T-DNA at a sufficiently high frequency.

3.1.4. INOCULATION OF PLANTS WITH ONCOGENIC *A. tumefaciens* STRAINS

Leaves of three *K. diaigremontiana* plants and stems of three *N. tabacum* cv. Petit Havana Str-r_1 plants were inoculated with *A. tumefaciens* strain GV3101 containing the following plasmids: pGV3100 (wild type T-DNA), pGV3851 and pGV3851::pDUB116. One month after inoculation only the wild type plasmid had produced detectable callus. Six weeks after inoculation one small (less than 1mm diameter) callus was present at a GV3101 [pGV3851] wound site on *N. tabacum* (of 6 sites inoculated). No other callus could be detected at other inoculation sites with GV3101 [pGV3851] or its derivative. Because of this low level of oncogenicity, an oncogenic vector containing a full complement of phytohormone genes and containing pBR322 homology was constructed.

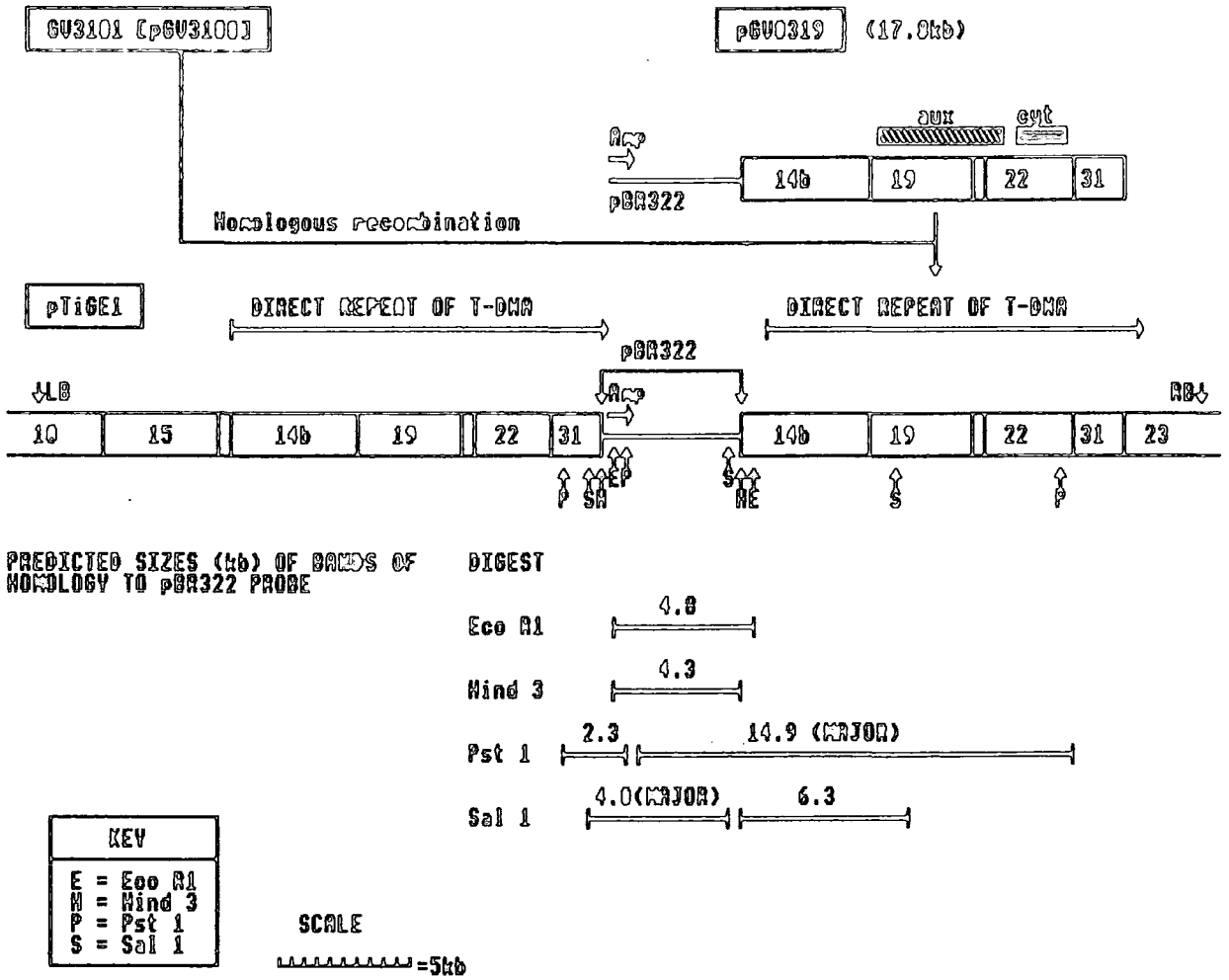
3.1.5. CONSTRUCTION OF pTiGE1: A TRULY ONCOGENIC pGV3851 REPLACEMENT

The strategy employed to construct an oncogenic Ti-plasmid containing pBR322 homology was to mobilise a plasmid with T-DNA homology on a pBR322 replicon into *A. tumefaciens* GV3101 containing a fully oncogenic Ti-plasmid. Recombination via the T-DNA homology results in a direct repeat of T-DNA flanking the pBR322 moeity. A schematic diagram of the construction of pTiGE1 is shown in figure 3.3.

pGV0319, a plasmid containing *Hind 3* fragments 14b, 19, 41, 22 and 31 of wild type pTiC58 T-DNA on a pBR322 replicon was introduced into *E.coli* DH1 by transformation. This strain was conjugated with *E.coli* GJ23 and exconjugants (DH1 [R64 *drd11*, pGJ28, pGV0319]) selected as previously described. This strain was conjugated with *A. tumefaciens* GV3101 [pGV3100] and exconjugants/recombinants selected on YEB-agar containing 100μg/ml rifampicin and 100μg/ml carbenicillin. The plated conjugation mixture contained large pink mucoid colonies (exconjugants) amongst rifamipicin resistant *E.coli* mutants. The transmission/recombination frequency of pGV0319 to GV3101 [pGV3100] was approximately 2×10^{-6}.

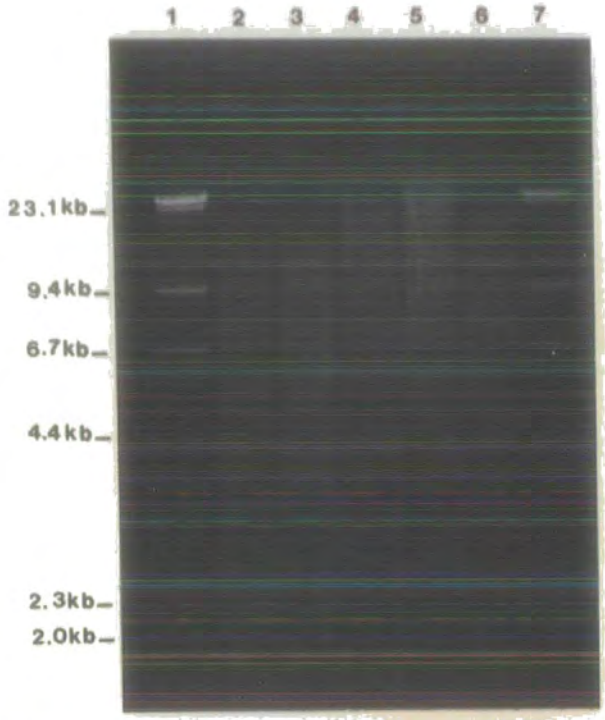
Five putative exconjugants were chosen at random and the presence of pBR322 determined by the ability of the isolates to undergo recombination with pDUB116 after conjugation with the

FIGURE 3.3. CONSTRUCTION OF pTiGE1 SHOWING STRATEGY USED IN SOUTHERN BLOT ANALYSIS



Figures in boxes represent Hind 3 restriction fragments of native (pTiC58) Ti-plasmid using the numbering system of Depicker et al. (1980).

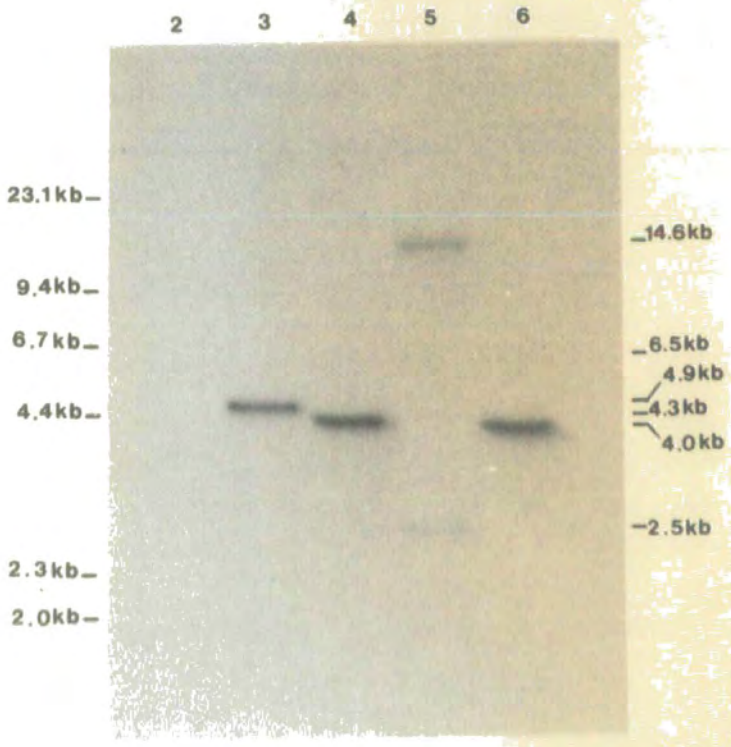
FIG 3.4



Analysis of pTiGE1. Lanes 1 and 7; λ *Hind 3* size marker. Lane 2; *Eco R1* digested total DNA from *A. tumefaciens* GV3101 [pGV3100]. Lanes 3-6; Total DNA from *A. tumefaciens* GV3101 [pTiGE1] digested with *Eco R1, Hind 3, Pst1* and *Sal 1* respectively.

FIG 3.5

Autoradiograph of the gel shown in figure 3.4 after Southern transfer and hybridisation with ^{32}P-labelled pBR322. Details of lanes are as described for figure 3.4.



mobilising strain and hence express gentamycin resistance. Four of the isolates proved capable of recombining with pDUB116. Total DNA was prepared from one of these isolates (renamed pTiGE1) and 10μl aliquots (of 100μl total) were digested with *Eco R1, Hind 3, Pst 1* and *Sal 1* and separated, along with control DNA (*Eco R1* digested GV3101 [pGV3100]), by agarose gel electrophoresis (0.7% gel). The gel obtained is shown in figure 3.4. The DNA was transferred to a nitro-cellulose filter and hybridised with 0.5μg ^{32}P labelled pBR322 DNA (8x 10^6 c.p.m./μg specific activity). The filter was washed to 0.1 x SSC, 65°C stringency and autoradiographed. Figure 3.5. shows the resultant autoradiograph. Table 3.1 gives a summary of the sizes of bands of hybridisation obtained.

Table 3.1 Sizes of bands of hybridisation in Southern analysis of pTiGE1.

Digest	Sizes (kb) of bands of hybridisation	
	Major	Minor
Eco R1	4.9	-
Hind 3	4.3	-
Pst 1	14.6	2.5
Sal 1	4.0	6.5

The results of the Southern analysis compare favourably with the predicted results (see figure 3.3), indicating that the desired construct was obtained.

The oncogenicity of pTiGE1 was compared with pGV3100 by inoculation of *K. diaigremontiana* and *N. tabacum* as previously described. No differences in the amount of callus or the callus morphology were seen.

3.1.6. REGENERATION OF *N. tabacum* AFTER INFECTION WITH *A. tumefaciens* GV3101 [pGV3850::pDUB116]

3.1.6.1. INHIBITION OF GROWTH OF *A.tumefaciens* IN TISSUE CULTURE

After cocultivation of *A. tumefaciens* with explants, the growth of the infecting strain must be inhibited using antibiotics to enable transgenic plant material to be cultured *in vitro* without bacterial overgrowth. The antibiotics used must not affect development of callus and subsequent shoots at concentrations inhibitory to the bacterial growth. Cefotaxime (a cephalosporin) has been reported to be inhibitory to growth of *A. tumefaciens* GV3101 containing a pGV3850 derivative at 0.5mg/ml enabling transgenic plants to be regenerated (Zambryski *et al.*, 1984). Carbenicillin has been used to inhibit the growth of LBA4404 containing a binary vector system at 0.5mg/ml and enabled transgenic tobacco plants to be regenerated (Bevan. 1984).

In an attempt to repeat these results, *N. tabacum* cv. Petit Havana Str-r_1 explants were

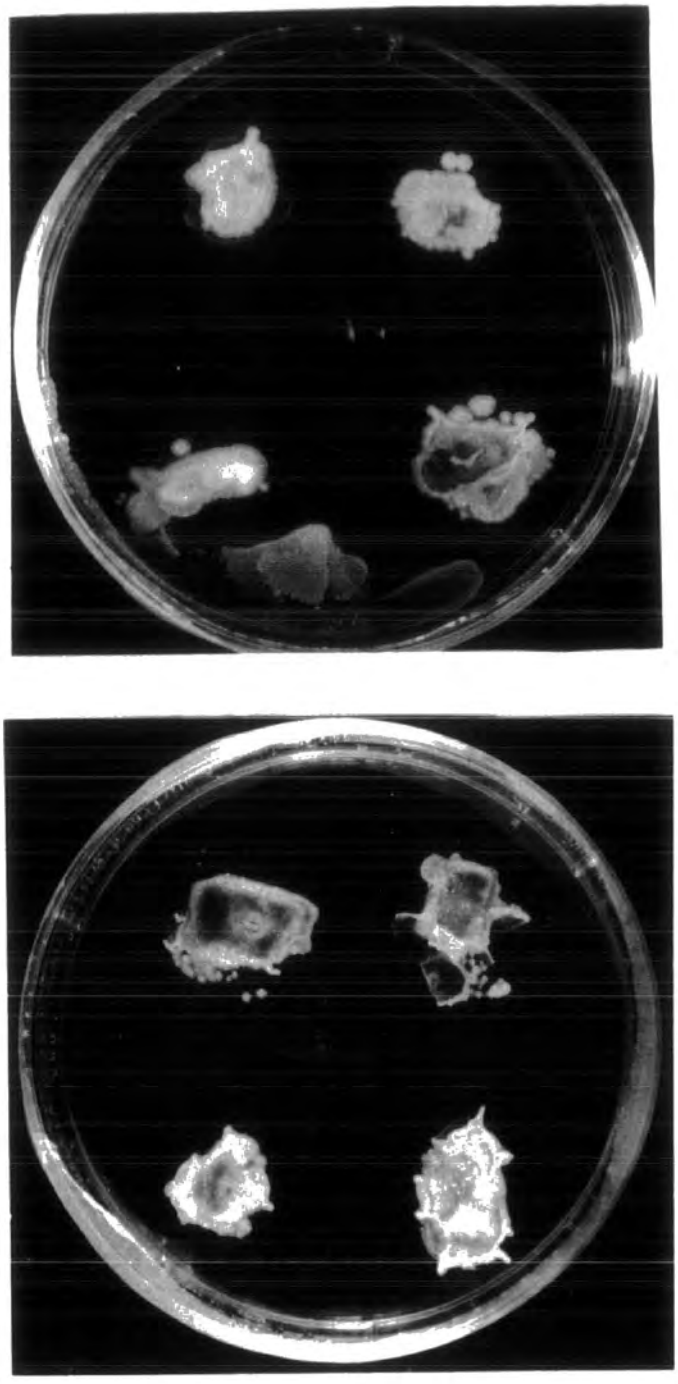
infected with GV3101 [pGV3850::pDUB116] by the leaf disc co-cultivation method described in section 2.2.17. After 48h incubation on co-cultivation medium, the explants were washed in explant washing medium containing 0.5mg/ml cefotaxime and plated onto selection medium containing 0.5mg/ml cefotaxime. Within four days of plating the explants were overgrown with bacterial contamination. When streaked on YEB-agar containing the appropriate selective antibiotics, the bacteria grew normally and showed the typical morphology of *A. tumefaciens* GV3101, indicating that the contamination was due to uncontrolled growth of inoculum. The procedure was repeated using 1mg/ml cefotaxime and in both washing and selection media. Washing of explants was also made more stringent by changing the media 3 times over a 24h period. Similar treatments were conducted using 1mg/ml carbenicillin and 0.5mg/ml of both antibiotics. In all cases signs of bacterial growth could be seen after 6 days and explants were overgrown after 12 days, necrosis occuring shortly afterwards. Bacterial growth was inhibited using 1.5mg/ml of cefotaxime and 0.75mg/ml of both cefotaxime and carbenicillin, but explants produced no callus and subsequent shoots. 1.5mg/ml carbenicillin was not inhibitory to bacterial growth. Figure 3.6 shows explants overgrown with inoculum on plates containing 1mg/ml of cefotaxime and carbenicillin after 12 days of incubation on selection medium.

The results show that the work of Zambryski *et al.* (1983) cannot be repeated in this laboratory and that cefotaxime and carbenicillin are not effective at inhibiting the growth of *A. tumefaciens* GV3101 in concentrations low enough to be of use in tissue culture.

3.1.6.2. SCREENING OF ANTIBIOTICS FOR ACTIVITY AGAINST *A. tumefaciens* STRAINS

GV3101 [pGV3850::pDUB116] was screened for sensitivity to a wide range of antibiotics inhibiting cell wall synthesis by the method described in section 2.2.15. LBA4404 [pAL4404, BIN19] was also screened for comparison. The minimum inhibitory concentrations of antibiotic (μg/ml) after 72h incubation are shown in Table 3.2.

The results show that GV3101 [pGV3850::pBUB116] is more resistant to a wide range of penicillins and cephalosporins than LBA4404 [pAl4404, BIN19]. The results show that both strains are relatively sensitive to one penicillin (imipenin), two cocktail antibiotics containing penicillin and the β-lactamase inhibitor clavulanic acid (augmentin and timentin) and the research drug Schering 34343. These antibiotics were selected for further investigation to determine their suitability for use in plant tissue culture.



Over-growth of *N. tabacum* leaf explants by *A. tumefaciens* GV3101 [pGV3850::pDUB116] on shoot selection media containing 1mg/ml carbenicillin (top) and 1mg/ml cefotaxime (bottom).

Table 3.2 Minimum inhibitory concentrations (μg/ml) of antibiotics of growth on *A. tumefaciens* strains

Antibiotic	Type	72h MIC strain1	72h MIC strain2	Antibiotic	Type	72h MIC strain1	72h M IC strain2
amoxacillin	P	>32	8	ampicillin	P	>64	8
apalcillin	P	>256	16	augmentin	P	1	2
azlocillin	P	>256	16	azthreonam	R	>32	8
carbenicillin	P	>256	4	cefamandiole	C	>128	8
cefadroxyl	C	>64	1	cefazolin	C	>32	8
cefoperazone	C	>128	1	cefonical	C	>32	2
cefotaxime	C	>32	0.5	cefotoxitin	C	32	2
cefsulfodin	C	>64	>64	ceftazidine	C	>64	>64
ceftizoxime	C	>32	16	cefpiramide	C	>64	32
ceforamide	C	>64	0.5	cefuroxime	C	>32	0.5
ceftriaxone	C	>32	0.25	ceftrioxime	C	64	16
cefotefan	C	>128	1	cephalothin	C	>64	32
cephaloridine	C	64	32	cephalchlor	C	>16	2
cephradine	C	32	2	cephaloridine	C	64	4
cephapirin	C	>256	8	cephalexin	C	>128	32
cloxacillin	P	>32	16	flucloxacillin	P	>128	32
imipenin	P	0.125	0.25	moxalactam	P	>32	16
nafcillin	P	>32	>32	oxacillin	P	>16	>16
penicillin G	P	>16	4	piperacillin	P	>256	2
Schering 34343	R	<0.5	<0.5	ticarcillin	P	>128	1
timentin	P	0.5	0.5	vancomycin		32	8

Key MIC = minimum inhibitory concentration (μg/ml). C = cephalosporin and cephamycin group of antibiotics. P = penicillin group of antibiotics. R = research drug of unknown structure. Strain 1 = GV3101 [pGV3850::pDUB116]. Strain 2 = LBA4404 [pAL4404, BIN19].

3.1.6.3. EFFECT OF NOVEL ANTIBIOTICS ON THE SHOOTING RESPONSE OF *N. tabacum* LEAF EXPLANTS

The effect of augmentin, timentin, imipenin and Schering 34343 on the shooting response of *N. tabacum* was examined by placing surface sterilised leaf explants on MS2SBN-agar containing 50, 100, 250 and 500μg/ml of each of the antibiotics. A control of explants on MS2SBN-agar alone was also prepared.

Explants on media containing Schering 34343 gave no preliminary callusing and subsequent shooting. The plates became heavily discoloured and the explants on 500μg/ml became necrotic after approximately 14 days of incubation. Explants on 50μg/ml imipenin gave some initial callusing and

a limited amount of shooting (average of 2 shoots/explant) after 4 weeks of incubation.

Explants on media containing augmentin and timentin gave a shooting response comparable to that of the control. Figure 3.7 shows explants on media containing 500μg/ml of timentin and augmentin along with the control after three weeks of incubation. The results show that augmentin and timentin have low toxicity to plant tissues and do not inhibit shooting of explants *in vivo*. These antibiotics were tested further to determine whether they could be used to inhibit the growth of bacterial inoculum at these non-physiologically active concentrations.

3.1.6.4. REGENERATION OF TRANSGENIC *N.tabacum* USING AUGMENTIN AND TIMENTIN AS BACTERIAL GROWTH INHIBITORS

N. tabacum leaf explants were infected and co-cultivated with GV3101 [pGV3850::pDUB116] as described previously. Explants were washed over-night in MS2SBN containing a 2x final concentration of each antibiotic and plated onto MS2SBN-agar, 100μg/ml kanamycin supplemented with 50, 100, 250 or 500μg/ml augmentin or timentin. A selection control of uninfected explants on MS2SBN, 100μg/ml kanamycin was also prepared.

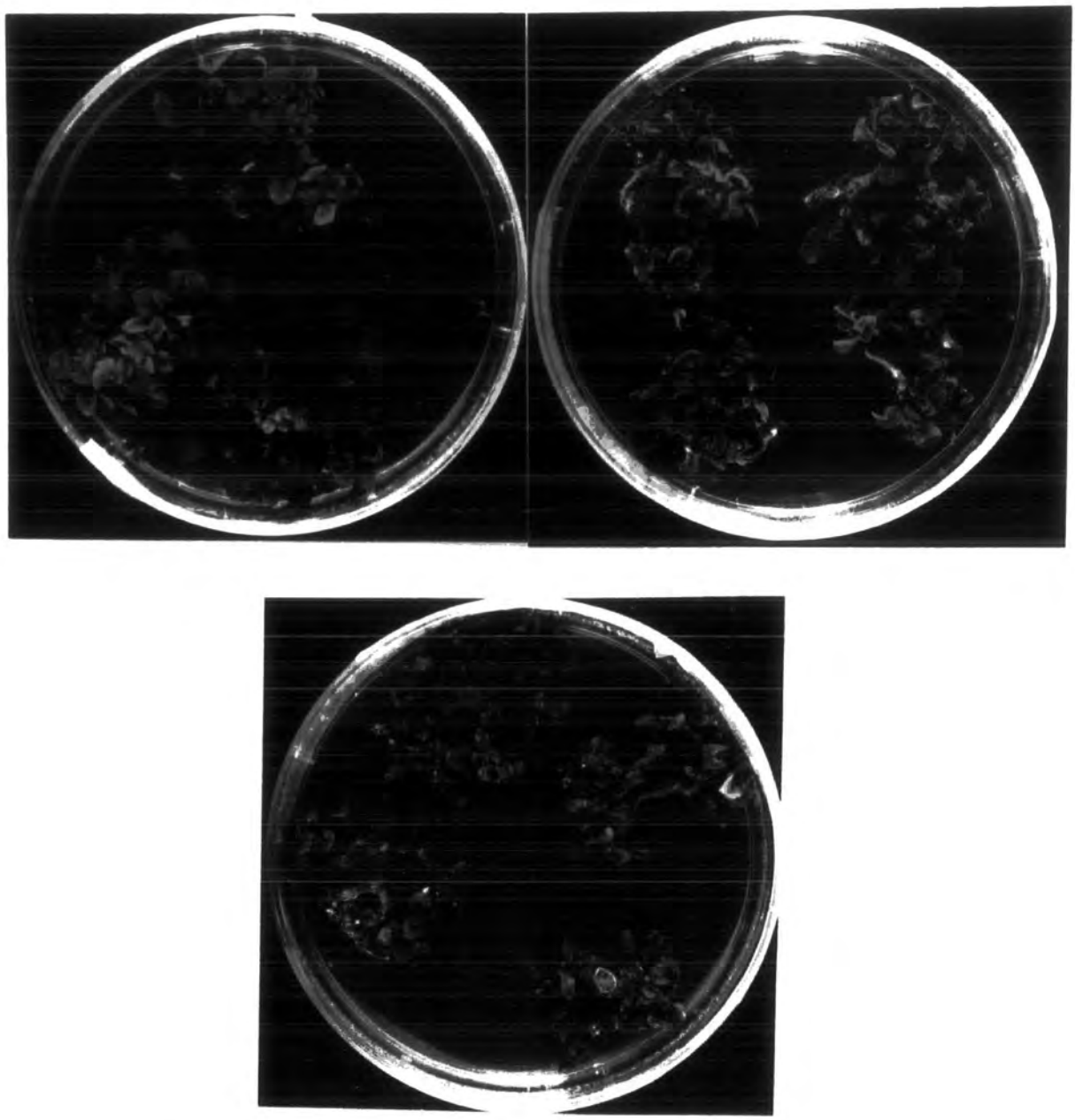
After 1 month incubation, no bacterial contamination could be seen on any of the explants. Callus had formed at discrete points (approx. 6/explant) around the periphery of the explants and shoots had developed from this callus. No callus had grown on the selection control. Figure 3.8 shows developing shoots on media containing 100μg/ml augmentin and timentin.

The results show that these antibiotics are superior to cefotaxime and carbenicillin and enable the growth of *A. tumefaciens* GV3101 to be controlled in plant tissue culture.

3.1.6.5. THE EFFECT OF AUGMENTIN AND TIMENTIN ON THE ROOTING OF TRANSGENIC *N. tabacum* SHOOTLETS.

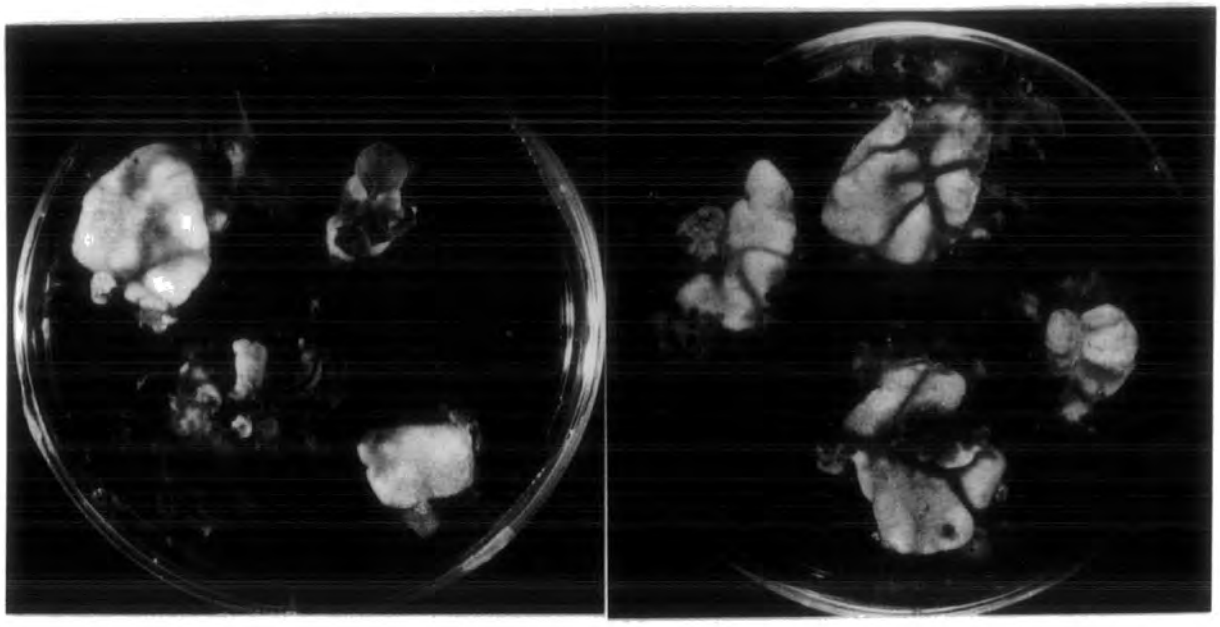
Transformed (kanamycin resistant) shootlets produced on media containing 100μg/ml augmentin as described in the previous section were excised from callus tissue and placed on rooting media (0.5xMS, 100μg/ml kanamycin) supplemented with 0, 50 or 100μg/ml augmentin. Similarly, shootlets produced on 100μg/ml timentin were excised and placed on rooting media containing 0, 50 or 100μg/ml timentin. Of the 10 shootlets regenerated on augmentin and transferred to media containing no augmentin, 6 developed serious bacterial contamination within 1 week of transfer. In the analogous experiment with shootlets regenerated on timentin, 9 out of 10 shootlets developed serious contamination. These results indicate that the antibiotics are having a bacteriostatic effect, and do not eradicate the bacteria at this concentration. No bacterial growth occured on shoots transferred to media containing augmentin or timentin. Table 3.3 shows the numbers of shootlets forming roots at various times after transfer to rooting medium. The results show that a high proportion of shootlets can be rooted on 50 and 100μg/ml augmentin. Timentin appears to delay rooting by about

FIG 3.7.



Shooting of *N. tabacum* leaf explants on media containing 500μg/ml augmentin (top left), 500μg/ml timentin (top right) and no added antibiotics (centre).

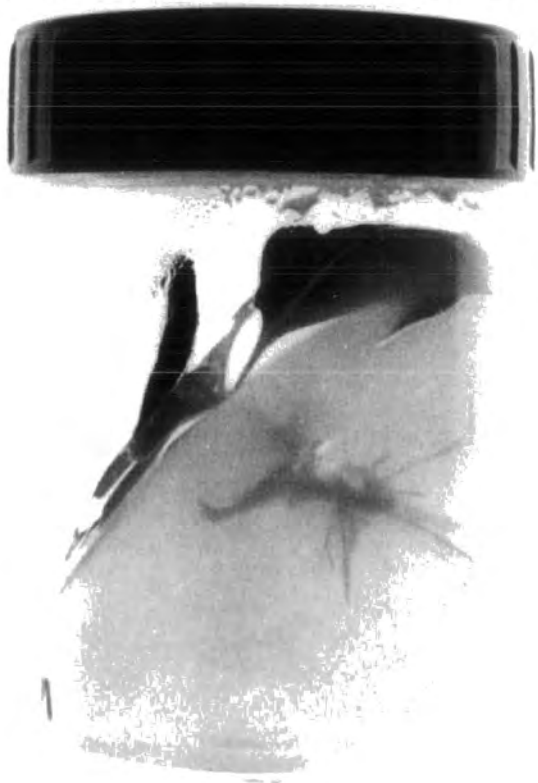
FIG 3.8



Shooting of transformed *N. tabacum* explants on shoot selection agar containing $100\mu g/ml$ augmentin (left) and $100\mu g/ml$ timentin (right).

FIG 3.9

Rooting of a transformed *N. tabacum* shoot on rooting media containing $50\mu g/ml$ augmentin.



a week at both 50 and 100μg/ml, with a significantly lower proportion of shootlets rooting after 28 days at 100μg/ml. Augmentin therefor seems to be the antibiotic of choice in rooting media using this cultivar. Figure 3.9. shows a kanamycin resistant shoot rooting on media containing 50μg/ml augmentin.

The results indicate that pGV3850::pDUB116 can transform *N. tabacum* to kanamycin resistance and that the new antibiotic regimes using augmentin and timentin enable transgenic plants to be recovered using this system.

Table 3.3. Times of root formation after transfer of shoots to rooting media containing augmentin and timentin.

Days after transfer		7	11	18	26
		No. of shoots rooting out of (total=10)			
Suppllementation of rooting media (μg/ml)					
Augmentin	50	2	5	8	9
	100	0	4	7	8
Timentin	50	0	1	4	7
	100	0	0	3	5

3.2. USE OF AN *IN VIVO* ONCOGENIC CLONING SYSTEM TO INVESTIGATE THE FUNCTIONALITY OF THE SV40 ENHANCER SEQUENCE IN PLANTS

3.2.1. CONSTRUCTION OF pDUB116 DERIVATIVES CONTAINING THE SV40 ENHANCER SEQUENCE

The plasmid pDB7.01A was previously constructed by cloning a 1347bp DNA fragment from SV40 (containing sequences from 4770 to 847bp using the nucleotide numbering system of Tooze, (1981) into pUC8. This resulted in one *Bam H1* site being regenerated in the final construct as shown in figure 3.10. (D. Bown, personal communication). pDB7.01A was digested with *Hind 3*, yielding a 0.97kb fragment consisting of SV40 sequence from nucleotides 5171 to 874 and an assymetric *Bam H1* site adjacent to the *Hind 3* site originating from pUC8. This DNA fragment contains the SV40 origin of replication, the 72bp direct repeat enhancer sequence and the start sites of both early and late transcripts (see figure 1.5).

The 0.97kb *Hind 3* fragment was cloned 5′ with repect to the Nos-NPT gene by ligation to *Hind 3* digested, phosphatased pDUB116. *E.coli* DH1 was transformed to ampicillin resistance with the ligation mix and plasmid DNA from 6 randomly chosen isolates was prepared. Isolated plasmid DNA was analysed by digestion with *Eco R1, Hind 3* and *Bam H1* and agarose gel electrophoresis (results not shown). Two isolates giving the correct size DNA after *Eco R1* digestion (8.5kb) but

with different *Bam H1* banding patterns indicating opposite orientations of insert were selected and named pDUB118a and pDUB118b.

pDUB116 derivatives containing the SV40 sequence 3′ in relation to the Nos-NPT gene were constructed by 'blunt ending 'the 0.97kb SV40 DNA fragment by filling in the 5′ overhanging termini with the Klenow fragment of *E.coli* DNA polymerase 1, followed by ligation to *Eco R1* digested, blunt ended pDUB116. *E. coli* DH1 was transformed with the ligation mix and 1000 ampicillin resistant colonies isolated, replica plated, transferred to nitro-cellulose and processed for colony hybridisation using the methods previously descibed (see methods section). The filters were hybridised with 0.5μg ^{32}P-labelled 0.97kb *Hind 3* SV40 fragment (9 x 10^6 c.p.m./μg DNA) and washed to 0.1xSSC, 65°C stringency and autoradiographed. Of the 110 colonies giving spots of hybridisation, 6 were chosen at random, and plasmid DNA prepared. The DNA was digested with *Hind 3* and *Bam H1* and analysed by agarose gel electrophoresis (results not shown). Two isolates giving a single *Hind 3* band of the expected size (8.5kb) but with a *Bam H1* banding pattern indicating opposite orientations of insert were chosen and named pDUB119a and pDUB119b. A third isolate, giving a *Hind 3* band of 9.4kb and a *Bam H1* banding pattern indicating two fragments had ligated into the vector as a direct repeat was named pDUB119c.

Figure 3.10 shows the steps used in the construction of these plasmids, and indicates the orientation of the SV40 insert with respect to the early RNA promotor of SV40.

3.2.2. CHARACTERISATION OF COINTEGRATES BETWEEN pTiGE1 AND SV40 ENHANCER-CONTAINING PLASMIDS

E.coli DH1 [pDUB116] and [pDUB118a]-[pDUB119c] were conjugated with GJ23 and exconjugants selected as previously described. The plasmids were mobilised to *A. tumefaciens* GV3101 [pTiGE1] and exconjugants/recombinants selected on YEB-agar containing 100μg/ml of both carbenicillin and rifampicin and 10μg/ml gentamycin. Transmission/integration frequencies were in the range 5 x 10^-7 to 1 x 10^-6. One isolate of each putative co-integrate was selected at random and the DNA prepared. 10μl aliquots of the DNA preparations (of 100μl total prepared) were digested with *Bam H1* and *Eco R1*, the DNA separated by agarose gel electrophoresis (see figure 3.11) and transferred to a nitro-cellulose filter. The bound DNA was hybridised with 0.5μg ^{32}P-labelled 0.97kb *Hind 3* SV40 DNA fragment from pDB7.01A (8 x 10^6c.p.m./μg DNA) and washed to a stringency of 0.1xSSC, 65°C. Figure 3.12 shows the autoradiograph obtained.

pTiGE1::pDUB119a and pTiGE1::pDUB119c both gave bands of homology after *Eco R1* digestion which were larger than predicted (see later). The DNA was further analysed by Southern blotting as described above after digestion with *Sma 1* and *Bam H1*, and the autoradiograph obtained is shown in figure 3.13. Table 3.4 summarises the results obtained from both analyses and also gives the predicted sizes of bands of homology to the probe as shown diagramatically in figure 3.14.

The results show that bands of homology to the probe obtained after digestion with *Bam H1* are in agreement with those predicted, indicating that the correct constructs are in the co-integrates. Anomalous bands which occured in pTiGE1::pDUB118b, pDUB119a and pDUB119c after digestion with *Sma 1* can be explained as direct repeats of integrating vector being present i.e more than one integrating vector inserted into the region of pBR322 homology. This was seen because *Sma 1* does not cut within pBR322 or the integrating vector but cuts in the flanking DNA and hence the region of direct repeats was excised as a single band. The anomalous bands of hybridisation in pTiGE1 co-integrates with pDUB119a and 119c were also seen after digestion with *Eco R1* because the *Eco R1* site was destroyed in the construction of pDUB119a-119c, and hence *Eco R1* cuts only at sites outside the direct repeats. pTiGE1::pDUB118a contains a direct repeat of pDUB118a, as seen after *Sma 1* digestion. This was not detected after digestion with *Eco R1* however, because this enzyme cuts within the integrating vector and the direct repeats were excised as intact monomers, hence giving a single band of hybridisation. The results indicate that pTiGE1::pDUB118b, pDUB119a and pDUB119c contain dimers of the respective integrating vectors as the size of the *Sma 1* bands with homology to the probe larger than expected by approximately the size of one integrating vector.

The presence of two bands of hybridisation in pTiGE1::pDUB119c indicates that the problem of multiple integration has been compounded. After construction of pTiGE1, the method of analysis used would not have detected multiple integration events of pGV0319. The presence of two bands of hybridisation in this construct indicate that two or more regions of pBR322 homology are present in pTiGE1 and that one copy of pDUB119c has integrated at one region and two copies at another. Although these results indicate that the structure of the T-DNA is relatively undefined, the *Bam H1* digestion results indicate that the SV40 sequence is still linked to the Nos-NPT gene. Any pronounced effect of increased gene expression due to the presence of this sequence should be detectable and it was decided to proceed with the experiment using the isolates described.

Table 3.4 Summary of results of Southern analysis of pTIGEI::[pDUB118a]-[pDUB119c]

	sizes (kb) of bands of hybridisation					
	obtained			predicted		
digest	B	E	S	B	E	S
construct						
pTiGE1::pDUB118a	3.0	8.7	17.5	3.0	8.5	17.7
pTiGE1::pDUB118b	2.1	8.7	c25	2.1	8.5	17.7
pTiGE1::pDUB119a	1.2	c25	c25	1.3	13.4	17.7
pTiGE1::pDUB119b	4.8	13.5	17.5	5.0	13.4	17.7
pTiGE1::pDUB119c	1.0	14.3	18.9	1.0	14.1	18.7
	4.9	22.5	c27	5.0	-	-

Key: B = *Bam H1*, E = *Eco R1*, S = *Sma 1*, c = *circa*.

FIGURE 3.10. CONSTRUCTION OF pDUB116 DERIVATIVES CONTAINING THE SV40 ENHANCER SEQUENCE

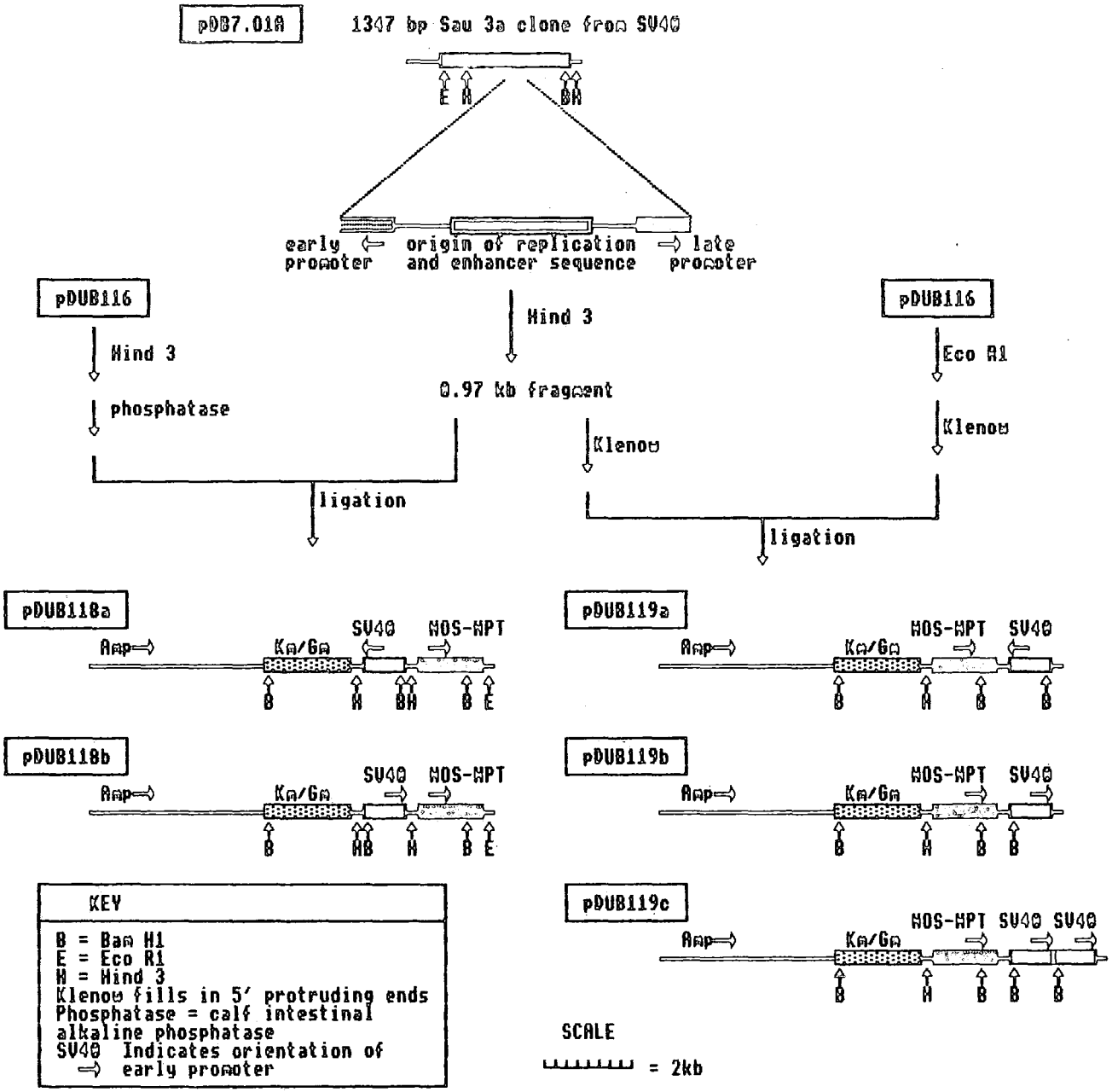
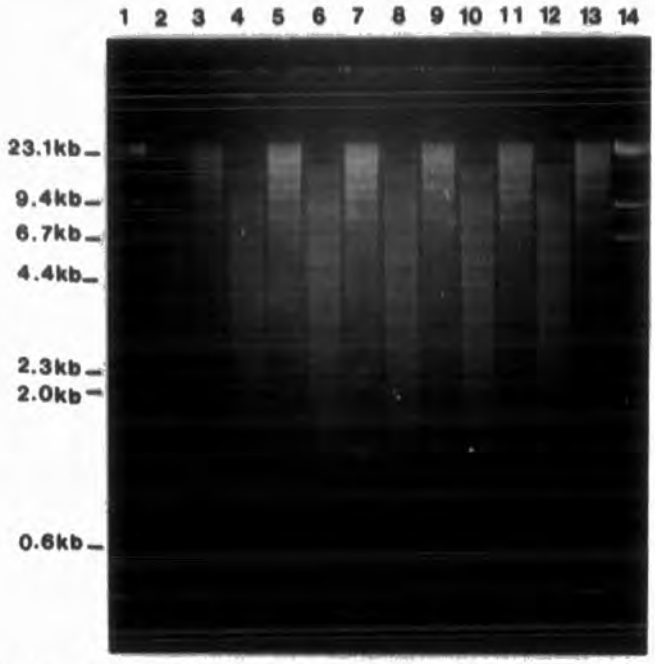


FIG 3.11



Analysis of cointegrates of pTiGE1 and SV40 enhancer-containing plasmids. Lanes 1 and 14; 1μg λ *Hind 3* size marker. Lanes 2-13 inclusive; total DNA from *A. tumefaciens* GV3101 containing the following Ti plasmids. Lanes 2 and 3; pTiGE1. Lanes 4 and 5; pTiGE1::pDUB118a. Lanes 6 and 7; pTiGE1::pDUB118b. Lanes 8 and 9; pTiGE1::pDUB119a. Lanes 10 and 11; pTiGE1::pDUB119b. Lanes 12 and 13; pTiGE1::pDUB119c. All DNA's digested with *Eco R1* (even numbered lanes) and *Bam H1* (odd numbered lanes).

FIG 3.12

Autoradiograph of the gel shown in figure 3.11 after Southern transfer and hybridisation with ^{32}P-labelled 0.97kb *Hind 3* DNA fragment from pDB7.01 containing the SV40 enhancer. Details of lanes are as described for figure 3.11.

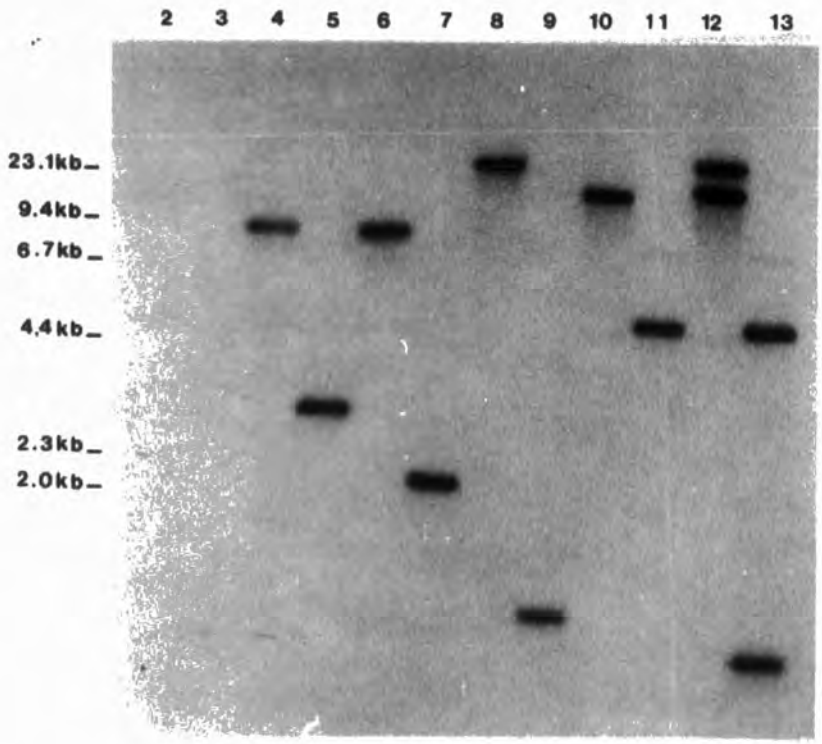
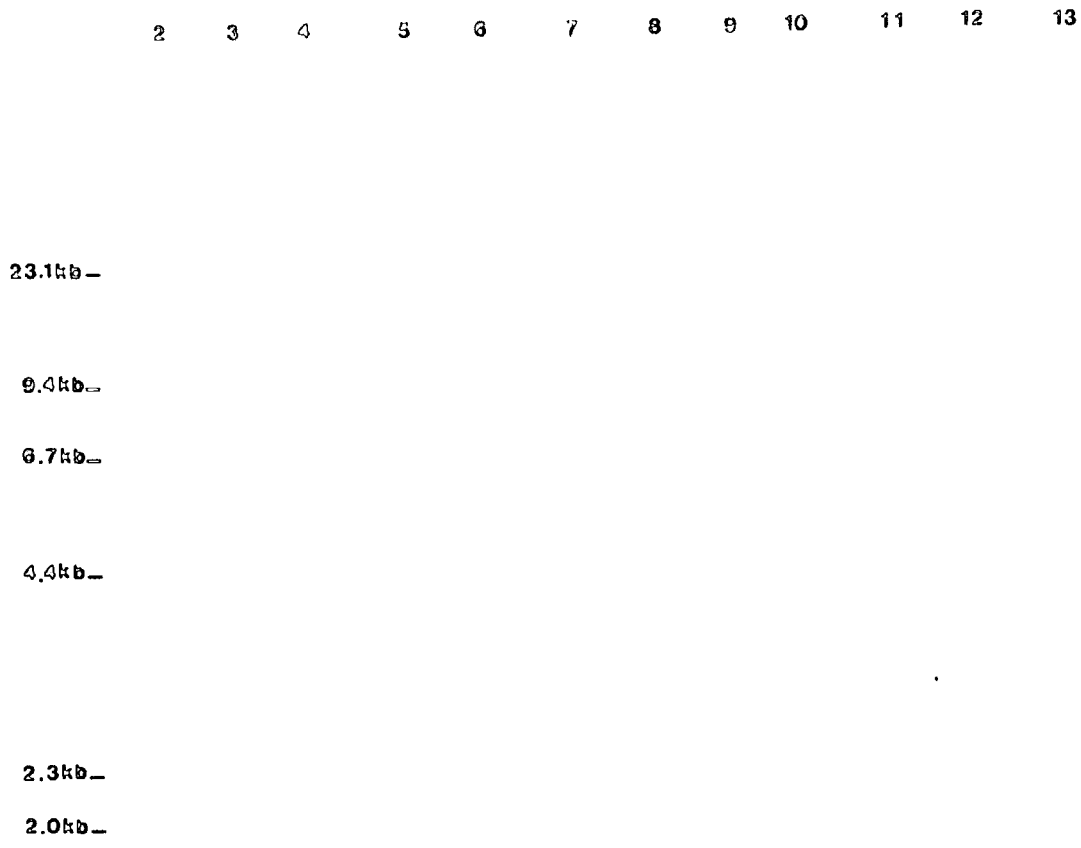
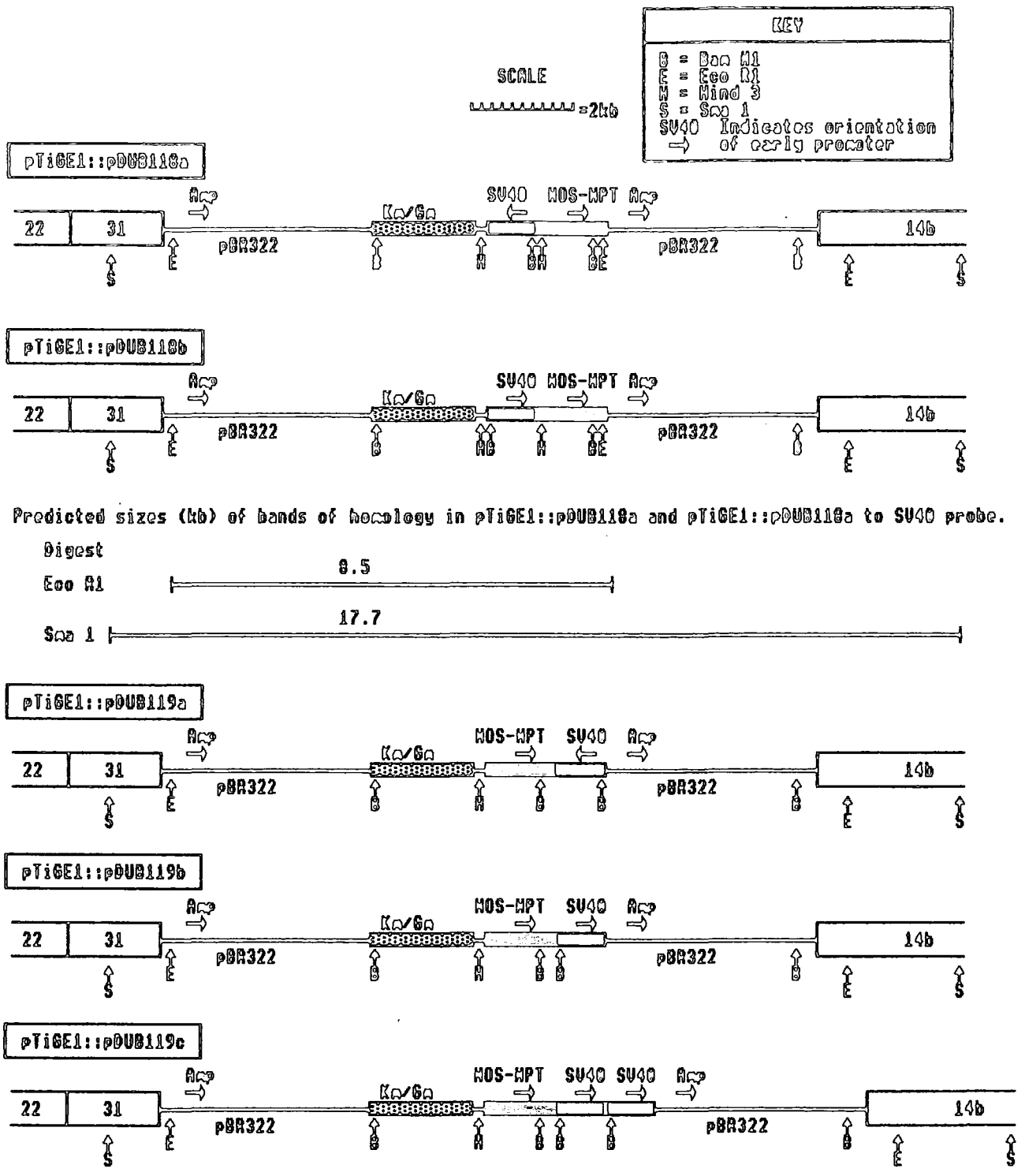


FIG 3.13



Analysis of cointegrates of pTiGE1 and SV40 enhancer-containing plasmids continued. Autoradiograph obtained after Southerntransfer and hybridisation with ^{32}P-labelled 0.97kb *Hind 3* DNA fragment from pDB7.01 containing the SV40 enhancer. The gel (not shown) was similar to that shown in figure 3.11 and the constructs were loaded in the same order. The DNA's were digested with *Bam H1* (even numbered lanes) and *Sma 1* (odd numbered lanes).

Figure 3.14. STRUCTURE OF T-DNA IN pTiGE1 COINTEGRATES CONTAINING THE SV40 ENHANCER



Predicted sizes (kb) of bands of homology in pTiGE1::pDUB118a and pTiGE1::pDUB118a to SV40 probe.

Digest	
Eco R1	8.5
Sma 1	17.7

Predicted sizes (kb) of bands of homology in pTiGE1::pDUB119a and pTiGE1::pDUB119b to SV40 probe.

Digest	
Eco R1	13.4
Sma 1	17.7

N.B. Sizes of these bands are 1.0kb larger in pTiGE1::pDUB119c due to the SV40 sequence direct repeat.

3.2.3. INITIAL CHARACTERISATION OF THE NPT ASSAY

3.2.3.1. LINEARITY OF DOSE RESPONSE

An NPT extract of *E.coli* DH1 [pDUB1105] was prepared as described in section 2.2.21. 5, 10, 20, 30, 40, and 60μl of the extract were loaded onto a non-denaturing polyacrylamide gel in separate wells, separated by electrophoresis and a NPT assay conducted on the separated proteins. The resultant autoradiograph is shown in figure 3.15. Radioactive bands containing bound ^{32}P-phosphorylated kanamycin and background controls were cut from the P-81 paper and counted on a scintillation counter. The results obtained are shown in graphical form in figure 3.16.

The results show that assay gives good dose linearity over the range of protein loaded, to a maximum of 2.6 x 10^4 c.p.m./band of NPT.

3.2.3.2. LOSS OF NPT ACTIVITY DUE TO INACTIVATION IN PLANT CELL EXTRACTS

A bacterial NPT extract was prepared from *E. coli* DH1 [pDUB1105] and stored on ice. 20μl of this extract was added to 50μl of protein extract (as prepared for the NPT assay) from callus of untransformed *N. tabacum* cv. Wisconsin. Similarly 20μl of bacterial extract was added to 50μl of protein extract from *K. diaigremontiana* callus induced by infection with *A. tumefaciens* GV3101 [pGV3100]. These mixtures were incubated at 25°C for 30min, along with 10μl of the bacterial extract alone. After incubation the extracts were separated by non-denaturing PAGE and a NPT assay conducted on the gel. Following autoradiography (autoradiograph not shown) the radioactive bands were cut from the P-81 paper and counted on a scintillation counter. The results obtained are shown in table 3.5.

The results indicate that inactivation of NPT in the plant extracts tested is not a major problem. Under the usual extraction conditions, where extracts are stored on ice and loaded onto the gel soon after extraction, the amount of inactivation of NPT should be considerably less than under the harsh conditions used in this experiment. Extra precautions to limit degradation, such as the addition of protease inhibitors, were therefore not required.

Table 3.5 Inactivation of NPT in plant extracts

extract	c.p.m. ^{32}P bound†	% reduction in activity
bacterial extract (kept on ice)	16490	-
incubated bacterial extract	16120	-
'spiked '*N. tabacum* extract	13850	14
'spiked '*K. diaigremontiana* extract	15480	4

† c.p.m. corrected for background (average = 230 c.p.m. ±40, 4 pieces of paper counted).

3.2.3.3. REPRODUCIBILITY OF NPT ASSAY

Reproducibility of response within any one NPT assay was investigated by conducting a NPT assay on one line of clonal callus tissue. This callus tissue was produced by direct gene transfer of pNos-NPT to *N. tabacum* cv Wisconsin protoplasts and subcultured (under identical conditions) from a single micro-callus. Southern analysis of this callus (line 2) showed the callus tissue to be derived from a single transformed protoplast (A. Hepher, unpublished results).

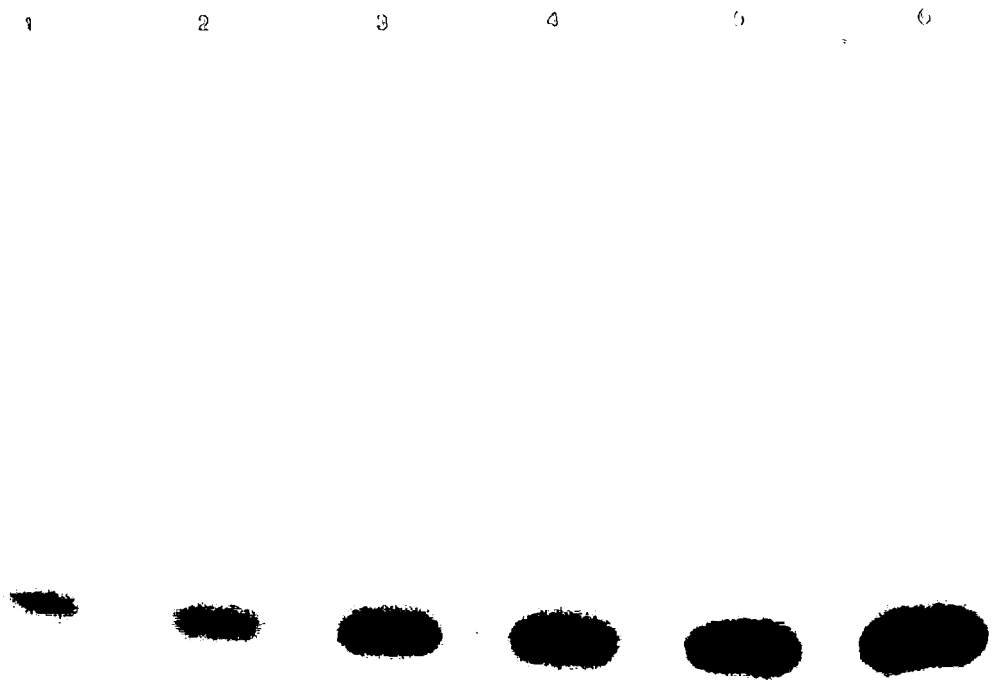
NPT extracts from 5 x 100mg samples of callus were prepared as previously described. An NPT assay was conducted using 20μl of each extract. The resultant autoradiograph is shown in figure 3.17. Table 3.6. shows the level of bound ^{32}P in each NPT band and the NPT activity for each extract corrected for the amount of protein loaded.

The ratio of the highest activity to the lowest activity (lane 9/lane 11) is 1.6.

Table 3.6 Summary of results of reproducibility of NPT assay expt.

lane	c.p.m. ^{32}P bound†	μg protein loaded (to nearest 0.5μg)	NPT activity (c.p.m. bound/mg protein)
3	728	7.5	9.7×10^4
5	815	9	9.1×10^4
7	1123	9.5	1.3×10^5
9	1345	9.5	1.4×10^5
11	670	7.5	8.9×10^5

† c.p.m. corrected for background (average = 730 c.p.m. ±60, 4 filter pieces counted).



Autoradiograph of NPT assay showing linearity of dose response. Lanes 1-6; 5, 10, 20, 30, 40 and 60μl of *E. coli* DH1 [pDUB1105] extract respectively.

FIGURE 3.16. LINEARITY OF DOSE RESPONSE OF NPT ASSAY

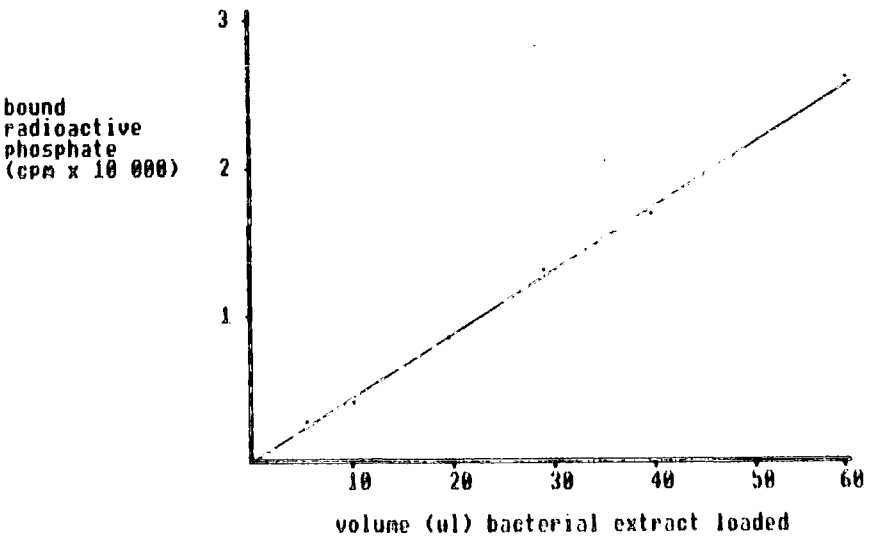
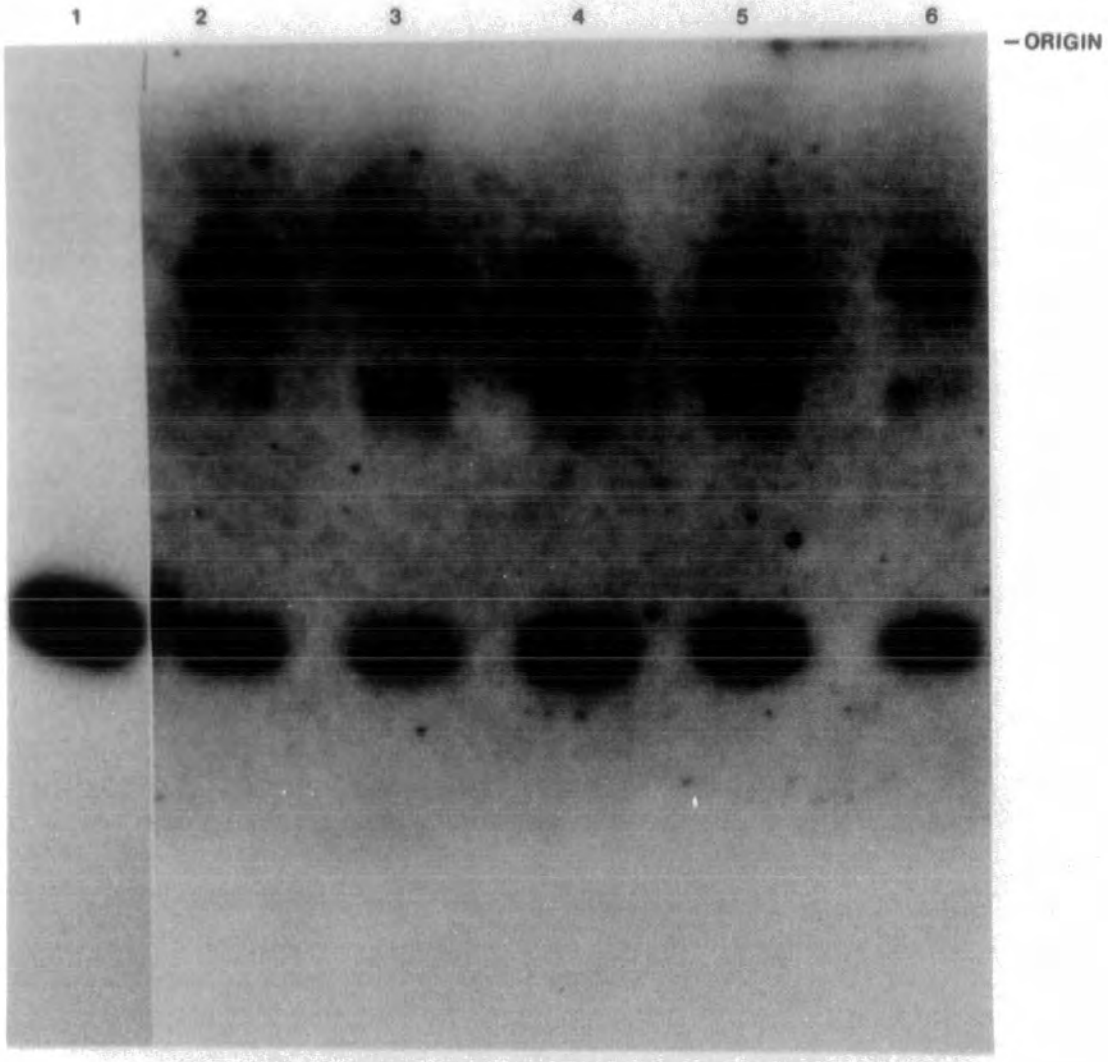


FIG 3.17



Autoradiograph showing reproducability of the NPT assay. Lane 1; 20μl of extract from *E.coli* DH1 [pDUB1105] (short exposure). Lanes 2-6; 20μl of separate extracts from a clonal line of kanamycin resistant *N. tabacum* cv. Wisconsin callus.

3.2.4. VARIATION OF NPT ACTIVITY IN POOLED CALLUS SAMPLES INDUCED BY A SINGLE CONSTRUCT

In order to determine the variation of NPT expression from the Nos-NPT gene in pooled samples of of *K. diaigremontiana* callus, an NPT assay was conducted on protein extracts of callus induced by inoculation with a single construct. The extent of variation, due to the so called positional effects (see discussion) must be quantified so that any significant variations in gene expression due to transformation with different gene constructs can be detected amongst this background variation.

Three leaves each of 5 plants were inoculated with GV3101 [pTiGE1::pDUB116], each leaf being inoculated on both sides of the midrib at a wound running parallel to the midrib and running the entire length of the leaf. 50mg of callus tissue was harvested from each wound site one month after inoculation and the havested callus from one wound site from each plant pooled to give six callus samples each of 250mg. This laborious sampling stratagy was used to cancel any variations between plants and leaves whilst pooling large amounts of callus to minimise the detected variable expression due to the positional effects by an averaging process.

NPT extracts were prepared from these pooled samples and 20μl of each extract (corresponding to between 5.5 and 7.5μg protein) were loaded on the gel and an NPT assay conducted. After five days exposure of the P-81 paper to autoradiographic film no radioactive bands co-responding to NPT activity could be seen. Background radioactivity levels prevented longer exposure. Fresh extracts were prepared as described and the NPT assay repeated on 60μl of extract (15-21μg of protein). Again, no NPT activity could be detected (results not shown).

3.2.5. DETERMINATION OF NPT ACTIVITY IN CALLUS INDUCED BY CONSTRUCTS CONTAINING THE SV40 ENHANCER SEQUENCE

The effect of the SV40 enhancer on the expression of NPT in *K. diaigremontiana* was determined by inoculating 3 leaves each of 5 plants with GV3101 containing co-integrates of pTiGE1 and pDUB116 and pDUB118a-pDUB119c. The six constructs were inoculated as described in the previous section in a pattern so that each construct was inoculated on one leaf of each plant, and each construct was inoculated next to a different construct on each of the 5 leaves. After one month of growth, no qualitative or quantitative differences in callus derived from different constructs could be seen. Callus was harvested as described in the previous section and callus derived from similar constructs pooled. 60μl of extract of each pool was used for an NPT assay (containing 17-22.5μg protein). After exposure of the P-81 paper to autoradiographic film for six days, no NPT activity was detected (results not shown). The results indicate that the presence of the SV40 enhancer does not increase the expression of the Nos-NPT gene to detectable levels.

3.2.6. NOPALINE ASSAY OF EXTRACTS OF CALLUS INDUCED BY CONSTRUCTS CONTAINING THE SV40 ENHANCER SEQUENCE

Callus extracts containing 20μg of protein prepared for the NPT assay in the previous section and control extracts prepared from untransformed *K. diaigremontiana* leaves were freeze dried, redissolved in 5μl H_2O and a nopaline assay conducted. The resultant electrophorogram is shown in figure 3.18.

Although the results are non-quantitative, they do not indicate any dramatic increase in the level of nopaline present in callus derived from constructs containing the SV40 enhancer compared to the control construct.

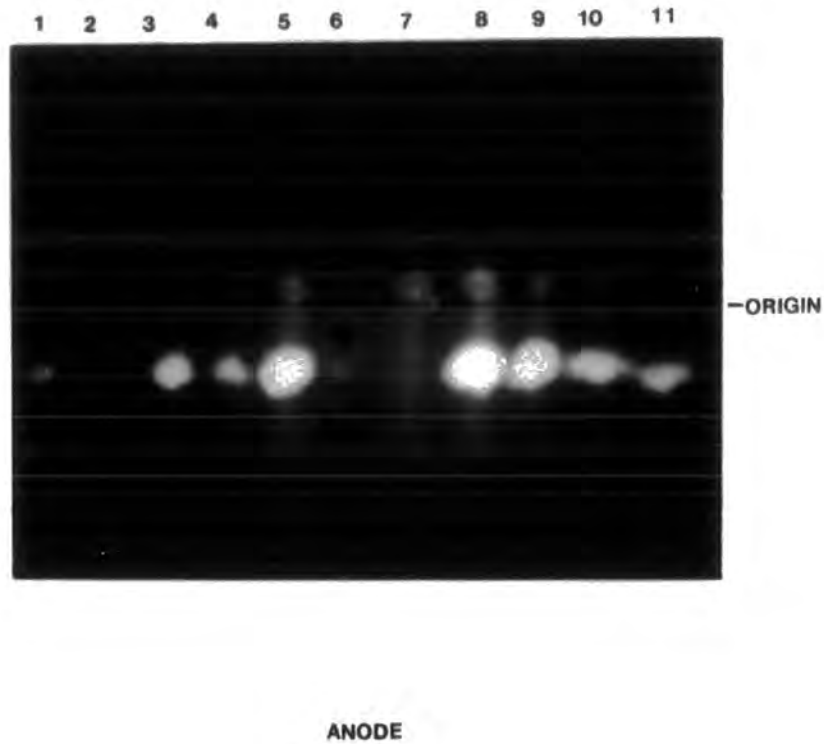
3.3. INVESTIGATION OF THE FUNCTIONALITY OF THE SV40 ENHANCER SEQUENCE IN *N. tabacum* CALLUS PRODUCED *in vitro*

3.3.1. VARIATION OF NPT ACTIVITY IN POOLED KANAMYCIN RESISTANT CALLUS SAMPLES INDUCED BY A SINGLE CONSTRUCT

N. tabacum cv. Petit Havana Str_1 leaf explants were co-cultivated with GV3101 [pTiGE1::pDUB116], washed in media containing 100μg/ml augmentin and plated on MS3S-agar containing 100μg/ml of both kanamycin and augmentin. Each explant gave an average of 7 regions of callusing at discrete positions around the explant periphery. After 5 weeks of growth the friable callus derived from five explants was harvested, broken up, and mixed thoroughly. Extracts for the NPT assay were prepared from 6 of these pools using 250mg of the callus tissue in each case. An NPT assay was conducted on 40μl of each extract (autoradiograph not shown). A summary of the results is shown in table 3.7.

The results show that even though callus from many different (approx. 35) transformation events was pooled, a 16 fold difference between the highest and lowest NPT activities was obtained (pool 6/pool 1). This high level of variability in pooled callus suggests a far greater variability of NPT activity in individual (clonal) callus derived from particular transformation events. The 16 fold difference in NPT activity obtained may be an under-estimate as the amount of ^{32}P bound to the P-81 paper is higher than the maximum bound during the expt. to determine the linearity of response (see section 3.2.3.1) and the values obtained may be on a non-linear part of the curve.

FIG 3.18



Electrophorogram of nopaline in callus. Lanes 1 and 11; nopaline standards. Lanes 2 and 7; extract containing 20μg of protein from leaves of untransformed *K. diaigremontiana*. Extract containing 20μg protein from *K. diaigremontiana* callus induced by: Lane 3; pTiGE1::pDUB116. Lane 4; pTiGE1::pDUB118a. Lane 5; pTiGE1::pDUB118b. Lane 8; pTiGE1::pDUB119a. Lane 9; pTiGE1::pDUB119b. Lane 10; pTiGE1::pDUB119c.

Table 3.7 Variation of NPT activity in pooled kanamycin resistant callus induced by a single construct

pool no.	c.p.m. ^{32}P bound†	μg protein loaded (to nearest 0.5μg)	NPT activity (c.p.m. bound/mg protein)
1	708	22	3.21×10^4
2	1619	17	9.52×10^4
3	2657	23	1.15×10^5
4	6972	15.5	4.50×10^5
5	4006	24	1.67×10^5
6	11230	22	5.10×10^5

† c.p.m. corrected for background (average = 814 c.p.m. ±60, 4 filter pieces counted).

3.3.2. NPT ACTIVITY IN EXTRACTS OF KANAMYCIN RESISTANT CALLUS INDUCED BY CONSTRUCTS CONTAINING THE SV40 ENHANCER SEQUENCE

N. tabacum leaf explants were co-cultivated with GV3101 containing pTiGE1::pDUB116 and pTiGE1::pDUB118a-119c and kanamycin resistant callus selected and cultured as described in section 3.3.1. The callus obtained from each construct showed no qualitative or quantitative differences, an average of 7 discrete areas of callus growth being present on each explant. Callus from 5 explants derived from each construct was pooled and NPT extracts prepared as described in section 3.3.1. An NPT assay was conducted on 40μl of extract of each pool (assay 1). A second assay was similarly conducted using extracts prepared from different callus pools (assay 2). The autoradiographs obtained are shown in figure 3.19. The NPT activities obtained are shown in table 3.8.

When placed in rank of decreasing activity, the control construct without the SV40 enhancer present is fifth highest in assay 1, and fourth highest in assay 2. The ratio of the highest activity obtained in pooled callus induced by a construct containing the SV40 enhancer to the activity in callus induced by the control construct in assay 1 (pTiGE1::pDUB119b/ pTiGE1::pDUB116) is 4.1. The ratio in assay 2 is 3.6, with pTiGE1::pDUB119b also giving the highest activity.

The fact that some callus induced by constructs containing the SV40 enhancer sequence gave lower activities than the control construct and that the variation of activities obtained between callus induced by SV40 enhancer containing constructs and the control is within the limits of variation encountered within callus samples derived from a single construct (see section 3.3.1.) indicate that the SV40 enhancer sequence does not exert a detectable effect on plant gene expression in this system.

Table 3.8 NPT activities in pooled kanamycin resistant callus induced by constructs containing the SV40 enhancer sequence

construct	c.p.m. ^{32}P bound†	μg protein loaded (to nearest 0.5μg)	NPT activity (c.p.m. bound/mg protein)
assay 1			
pTiGE1::pDUB116	694	19	3.65×10^4
pTiGE1::pDUB118a	3516	28.5	1.23×10^5
pTiGE1::pDUB118b	1536	18	8.53×10^4
pTiGE1::pDUB119a	2345	22	1.07×10^5
pTiGE1::pDUB119b	4383	29	1.51×10^5
pTiGE1::pDUB119c	674	21	3.21×10^4
assay 2			
pTiGE1::pDUB116	2579	28	9.21×10^4
pTiGE1::pDUB118a	317	18	1.76×10^4
pTiGE1::pDUB118b	2884	28	1.03×10^5
pTiGE1::pDUB119a	3802	22	1.72×10^5
pTiGE1::pDUB119b	9660	29	3.33×10^5
pTiGE1::pDUB119c	1420	19	7.47×10^4

† c.p.m. corrected for background (average for assay 1= 62 c.p.m. ±8, average for assay 2= 74 c.p.m. ±12, 4 filter pieces counted).

3.4. FURTHER IMPROVEMENTS TO THE HOMOLOGY MEDIATED Ti-PLASMID CLONING SYSTEM

3.4.1. CONSTRUCTION OF pTiGE2: AN ONCOGENIC Ti-PLASMID WITH A SINGLE COPY OF pBR322 HOMOGOLOY

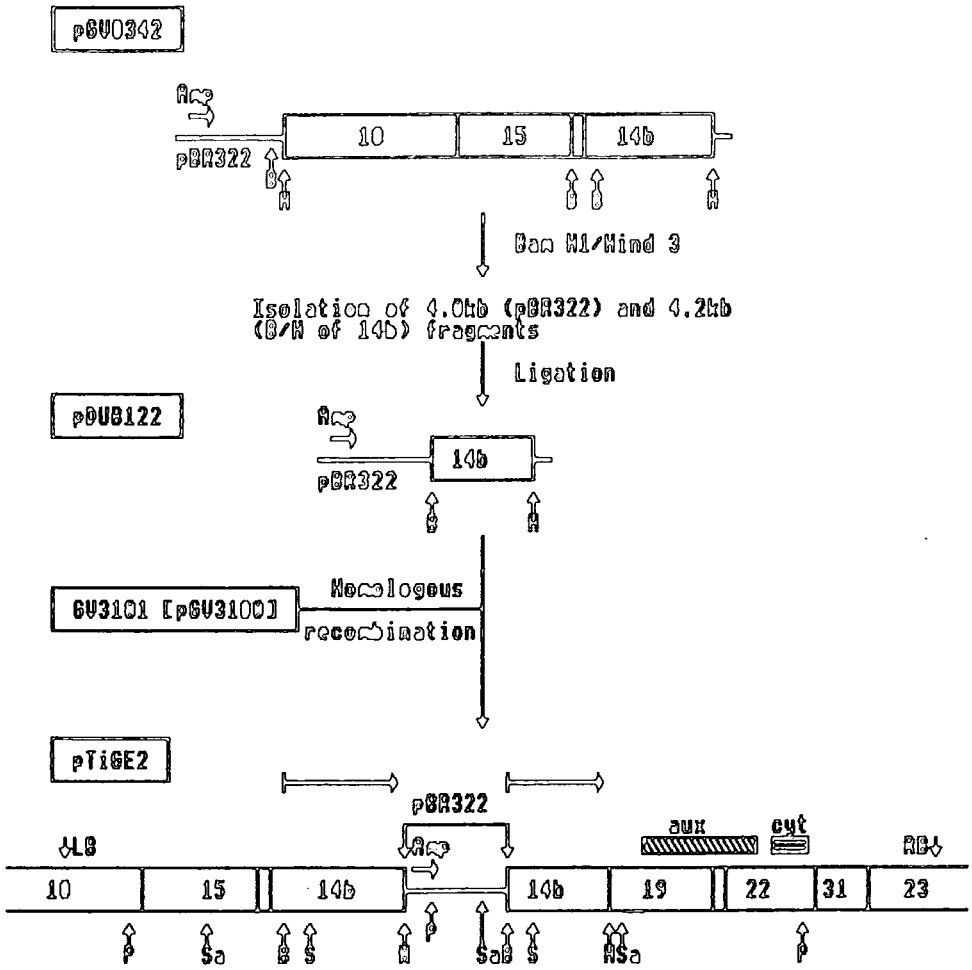
A new oncogenic vector capable of undergoing pBR322 mediated co-integrate formation but having a single region of pBR322 homoloy and a smaller region of T-DNA direct repeat was constructed using the strategy shown in figure 3.20.

A 4.2kb *Bam H1/Hind 3* DNA fragment originating from the *Hind 3* fragment 14b of pTiC58 was cloned into the *Bam H1/Hind 3* sites of pBR322 by isolation and ligation of the relevant fragments from pGV0342. *E.coli* DH1 was transformed to ampicillin resistance with the ligation mix and plasmid DNAs prepared. One isolate giving the correct banding pattern on restriction analysis was named pDUB122. This plasmid was transferred to *A. tumefaciens* GV3101 [pGV3100] and exconjugants/recombinants selected as described in section 3.1.5. Five carbenicillin resistant colonies were chosen at random and the DNA prepared. The DNA was digested with *Pst 1* and analysed by Southern blot analysis using 0.5μg ^{32}P-labelled pBR322 (9.5 x 10^6 c.p.m./μg DNA) and washing to a stringency of 0.1 x SSC. 65°C.

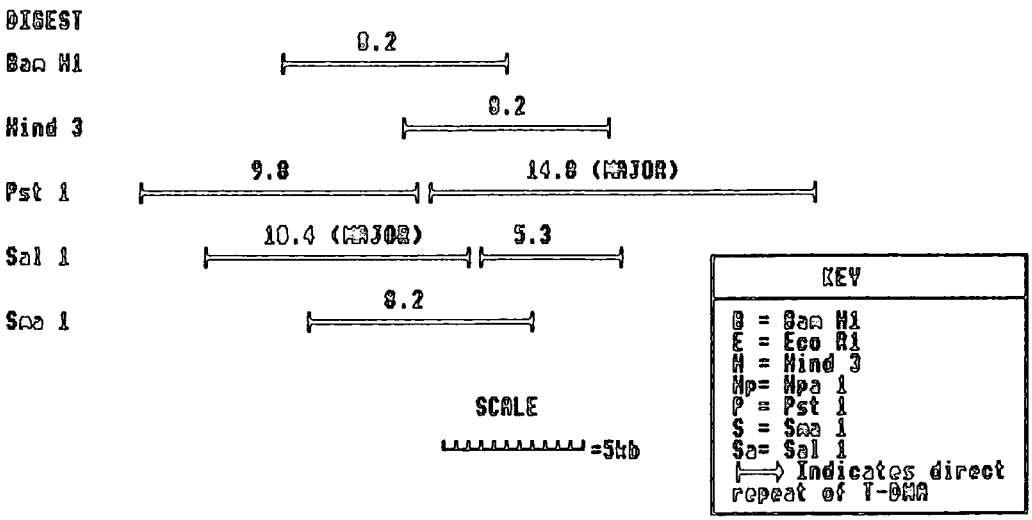
Of the five isolates, two lacked an 8.2kb *Pst 1* band of hybridisation which is diagnostic for multiple copies of pDUB122 being present in the co-integrate (results not shown). DNA from one of these two isolates was further analysed by Southern blotting (using the same conditions and probe as detailed above) after digestion with *Bam H1, Hind 3, Pst 1. Sal 1* and *Sma 1.* The agarose gel and autoradiograph are shown in figures 3.21. and 3.22. respectively. The banding pattern obtained agrees with the predicted results (see figure 3.20.) and indicates that the desired construct was obtained. This plasmid was named pTiGE2.

Inoculation of *K. diaigremontiana* with GV3101 [pTiGE2] showed the construct to be fully oncogenic and no qualitative or quantitative differences were seen in comparison with callus induced by GV3101 [pGV3100]. Conjugation of GV3101 [pTiGE1] with the *E. coli* mobilising strain containing pDUB116 gave a transmission/recombination frequency of 5 x 10^{-6} indicating that pTiGE2 is capable of undergoing recombination with the integrating plasmid with a frequency comparable to pGV3850 and pGV3851.

FIGURE 3.20. CONSTRUCTION OF pTiGE2 SHOWING STRATEGY USED IN SOUTHERN BLOT ANALYSIS



PREDICTED SIZES (kb) OF BANDS OF HYBRIDISATION USING pBR322 PROBE



Figures in boxes represent Hind 3 restriction fragments of native (pTiC58) Ti-plasmid using the numbering system of Depicker et al (1980).

3.4.2. CONSTRUCTION OF pDUB116 DERIVATIVES CONTAINING THE pUC18 INSERTIONAL INACTIVATION CLONING REGION

Plasmid pDUB116 is limited in its use as a cloning vector as it has only two unique restriction enzyme sites suitable for cloning DNA for subsequent delivery to plants. Detection of these recombinant plasmids is difficult and may require time consuming procedures such as colony hybridisation (as had to be used in section 3.2.1.). In order to increase the number of restiction enzyme sites available for cloning and to make the subsequent detection of recombinant clones easier, the *lacZα* gene containing a multiple cloning site polylinker was cloned from pUC18 into pDUB116 derivatives as shown diagramaticaly in figure 3.23.

The two *Bam H1* sites in pDUB116 were destroyed by digestion with *Bam H1* and treatment of the digestion mix with the Klenow fragment of *E. coli* DNA polymerase 1. After religation of the two blunt ended molecules, the DNA was again treated with *Bam H1* to select against recovery of plasmids where the *Bam H1* sites had not been destroyed by filling in. *E. coli* DH1 was transformed with the treated DNA and plated ón L-agar containing 20μg/ml kanamycin. The two *Bam H1* sites in pDUB116 flank the Km/Gm resistance gene and by selecting on kanamycin, only plasmids containing the two blunt ended fragments were recovered. Restriction analysis was conducted on plasmid DNA from six isolates (results not shown) and an isolate containing the Km/Gm gene and 5′ region of the Nos-NPT gene in the correct orientation (the same orientation as in pDUB116) as determined by digestion with *Kpn 1/Eco R1*, and with the *Bam H1* sites destroyed was chosen and named pDUB123.

The *Eco R1* site of pDUB123 was destroyed by filling in to produce pDUB124, and similarly the *Hind 3* site was destroyed to produce pDUB125.

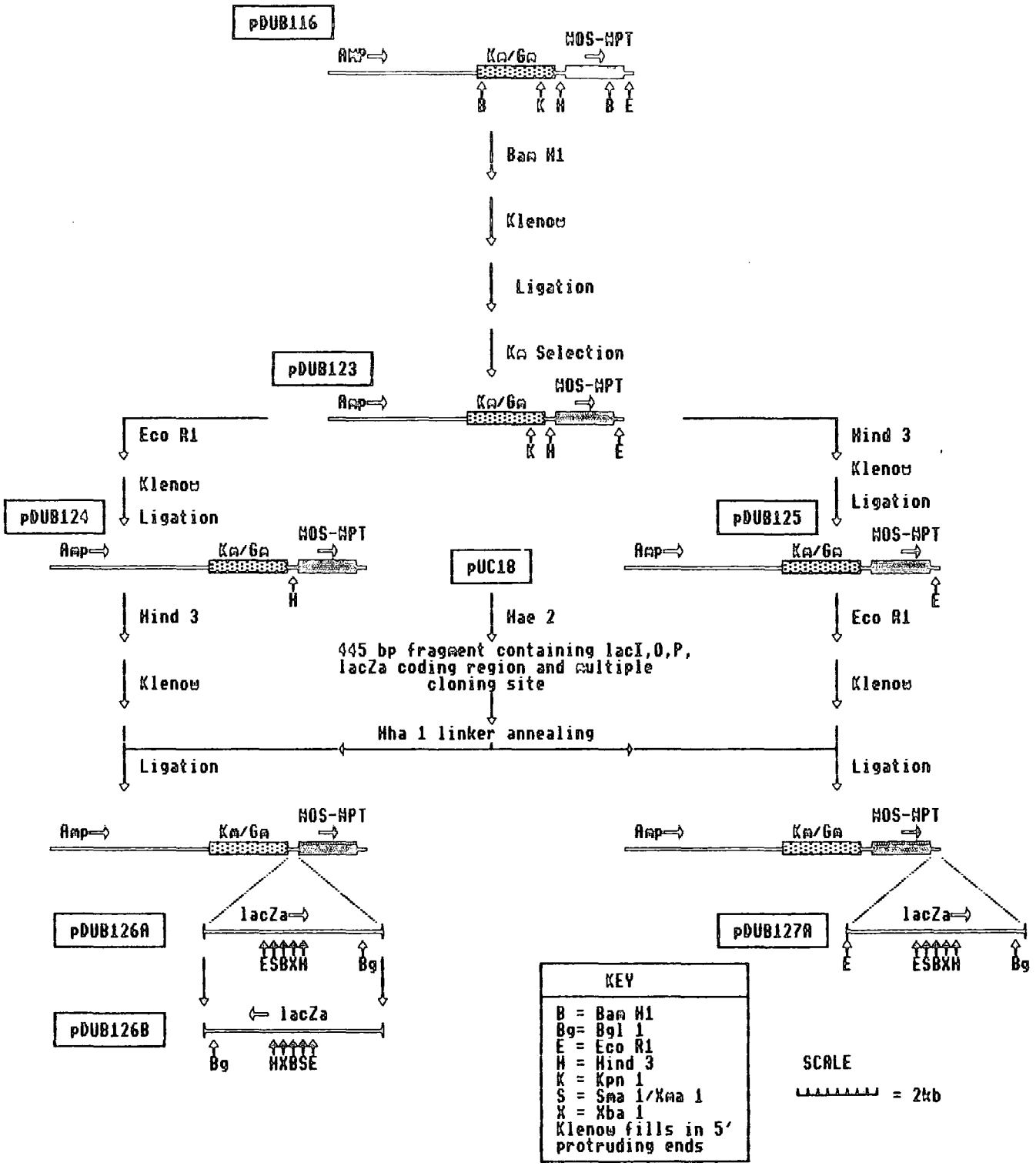
The 440bp *Hae 2* fragment of pUC18 was prepared and hybridised to denatured *Hha 1* linkers (see section 2.2.7.) and ligated into both *Hind 3* cut filled in pDUB124 and *Eco R1* cut filled in pDUB125. Blue, ampicillin resistant colonies were selected on 2xYT-agar supplemented with X-gal and IPTG (see section 2.1.5). Plasmid DNA was analysed by restiction enzyme analysis (results not shown). Two constructs with the 440bp *Hae 2* fragment 5′ to the Nos-NPT gene but with the fragment in opposite orientations (as determined by the *Bgl 1* banding pattern) were selected and named pDUB126a and pDUB126b. The orientations of the fragment in these constructs is indicated by the direction of transcription of the *lacZα* gene, and the order of the restriction enzyme sites in the poly-linker. These vectors have unique restriction enzyme sites for *Hind 3*, *Sma 1/Xma 1*, *Bam H1*, *Xba 1* and *Eco R1* within the polylinker and recombinant plasmids can be detected by the insertional inactivation of the *lac Z* gene when plated on a medium containing a chromogenic substrate such as X-gal.

Repeated attempts to clone the 440bp fragment into the filled in *Eco R1* site of pDUB125 resulted in only 3 ampicillin resistant blue colonies being obtained. On restriction analysis, one of

these isolates contained the two copies of the 440bp fragment in opposite orientations. The two remaining isolates, with the insert in the same orientation, had two *Eco R1* sites, indicating that one of the sites previously filled in had been regenerated during the cloning step. One of these isolates was named pDUB127a and has unique restriction enzyme sites in the polylinker for *Hind 3, Sma 1/Xma 1, Bam H1* and *Xba 1*

These plasmids proved capable of transferring Gm resistance to *A. tumefaciens* GV3101 [pGV3850], and of giving 99%+ blue colonies after digestion with *Bam H1*, and *Sma 1* and subsequent religation, transformation and selection, using the bacterial strain and media previously described.

FIGURE 3.23. CONSTRUCTION OF pDUB116 DERIVATIVES CONTAINING THE pUC18 LacZ INSERTIONAL INACTIVATION CLONING REGION



3.5. REGENERATION OF TRANSGENIC *N. tabacum* PLANTS WITH IMPROVED AGRONOMIC POTENTIAL

3.5.1. CONSTRUCTION OF CaMV-PEA LECTIN CHIMAERIC GENE

The steps involved in the construction of the cauliflower mosaic virus (CaMV) 35S promoter-pea lectin chimaeric construct are shown diagramatically in figure 3.24.

A 1.2kb *Sph 1/Eco R1* DNA fragment consisting of the coding region, 403 bp of 3′ sequence (including a polyadenylation site) and 20bp of 5′ untranslated sequene, was cloned from the pea *LecA* gene in pDUB80 into the *Sph 1/Eco R1* sites of pUC18 to give the plasmid pDUB128. This cloning step removes the native *LecA* gene transcriptional start (cap site) and 5′ controlling sequences and places the ATG translational start close to the *Hind 3* site present in the pUC18 polylinker. pDUB128 was digested with *Hind 3*, the 5′ protruding termini filled in with the Klenov fragment of *E. coli* DNA polymerase 1 and the DNA digested with *Eco R1*. The resultant 1.2kb blunt-ended/*Eco R1* fragment was then cloned into the *Sma 1/Eco R1* sites of pUC18 to give the plasmid pDUB129. The resultant chimaeric gene construct consists of an 800bp fragment containing the CaMV 35S promotor, including the CAMV transcriptional start (cap site). The 5′ untranslated leader sequence between this cap site and the in-frame ATG codon of the *LecA* coding sequence contains 10bp derived from the CaMV gene, 12bp derived from the pUC18 cloning region and filling-in step and 20bp derived from the 5′ of the *LecA* gene. This 20bp sequence contains an extra ATG codon (out of frame to the *LecA* coding sequence, see diagram 3.24) followed by codons for 2 amino acids and finally a translational stop. It was not thought that the presence of this extra ATG codon would affect translation of the RNA as the gene is expressed *in vivo* in pea and also the sequence surrounding the codon does not have good homology to the consensus translational start found in plant genes (Heidecker and Messing, 1986).

The construct was cloned from pDUB129 into the *Hind 3/Eco R1* sites of pDUB126a to give the T-DNA integration vector pDUB130.

3.5.2. SELECTION AND CHARACTERISATION OF pGV3850::pDUB130 COINTEGRATES

pDUB130 was mobilised to *A. tumefaciens* GV3101 [pGV3850] as previously described (see section 3.1.2). pDUB126a was also mobilised to this strain for use as a negative control (see later). Co-integrates were selected on YEB-agar containing 100μg/ml of both rifampicin and carbenicillin and 5μg/ml gentamycin. The concentration of gentamycin was reduced from the level previously used (see section 3.1.2) in an attempt to prevent selection of co-integrates with only multiple copies of integrating vector because of gene dosage affects.

Total DNA was prepared from 5 isolates from each mating. DNA from isolates containing

pGV3850::pDUB130 and pGV3850::pDUB126a was digested with *Kpn 1* and *Sma 1* respectively, and Southern analysis conducted using ^{32}P-labelled pBR322 probe (gel and autoradiograph not shown). These enzymes cut the integrating vector once and hence a band of hybridisation the size of the integrating vector is diagnostic for multiple copies being present. The enzymes also cut the Ti-plasmid DNA outside the T-DNA and hence the analysis also ensures that the correct Ti-plasmid is present in the co-integrate.

Four of the five pGV3850::pDUB126a co-integrates gave a 7.9kb band of hybridsation diagnostic for multiple copies of integrating vector being present. The isolate containing a single copy was subsequently used to regenerate plants (see section 3.5.3). Four of the five pGV3850::pDUB130 co-integrates gave a 10kb bands of hybridisation of intensities indicating that two or more copies of the integrating vector were present. The remaining isolate gave a 10kb band of hybridisation of low intensity indicating that multiple copies of integrating vector were present in only a subset of the population of cells used for the DNA prep. DNA from this isolate was further analysed by Southern analysis after digestion with *Kpn 1, Bam H1, Eco R1, Hind 3* and *Eco R1/Hind 3*. Figure 3.25 shows the resultant agarose gel. After transfer of the DNA to a nitrocellulose filter, the filter was bisected and one half hybridised with 0.5μg of a ^{32}P-labelled 800bp *Eco R1/Hind 3* fragment from pCaMV containing the 35S promoter (1.1 x 10^7 c.p.m./μg DNA). The other half of the filter was hybridised with 0.5μg of a ^{32}P-labelled 1.2kb *Sph 1/Eco R1* fragment from pDUB80 containing the *LecA* coding region and 3′ sequence (1.4 x 10^7 c.p.m./μg DNA). The filters were washed to a stringency of 0.1 x SSC, 65°C and the resultant autoradiographs are shown in figure 3.26.

Table 3.9 gives a summary of the results of the Southern analysis of pGV3850::pDUB130 and shows the sizes of the major bands of hybridisation obtained and also the predicted sizes from the predicted T-DNA structure (see figure 3.24). The sizes of the major bands of hybridisation indicate that the desired construct has been obtained. The weak bands of hybridisation obtained (these bands are not recorded in table 3.9) could possibly be due to the integrating vector increasing in copy number during the growth of the culture used for the DNA prep. This would explain why the band of hybridisation is weak as only some of the cells in the culture contain the multiple copies. The presence of gentamycin in the liquid culture media may enrich the culture for cells containing Ti-plasmids with a higher gentamycin resistance gene copy number due to increased resistance and therefore faster growth rates than cells containing Ti-plasmids with a single gene. The stock culture of this isolate may not contain significant numbers of cells with multiple copies of the integration vector (see later), and hence this isolate was used for plant regeneration.

Table 3.9 Results of Southern analysis of pGV3850::pDUB130

	Sizes (kb) of bands of hybridisation	
digest and probe	obtained	predicted
pBR322		
Kpn 1	12.2, 6.2	12.4, 6.1
CaMV 35S promoter		
Bam H1	6.7	6.5
Eco R1	16.5	16.3
Hind 3	8.6	8.4
Kpn 1	12.6	12.4
Eco R1/Hind 3	2.1	2.1
LecA coding region		
Bam H1	0.5, 0.7	0.5, 0.7
Eco R1	16.5	16.3
Hind 3	8.6	8.4
Kpn 1	12.6	12.4
Eco R1/Hind 3	2.1	2.1

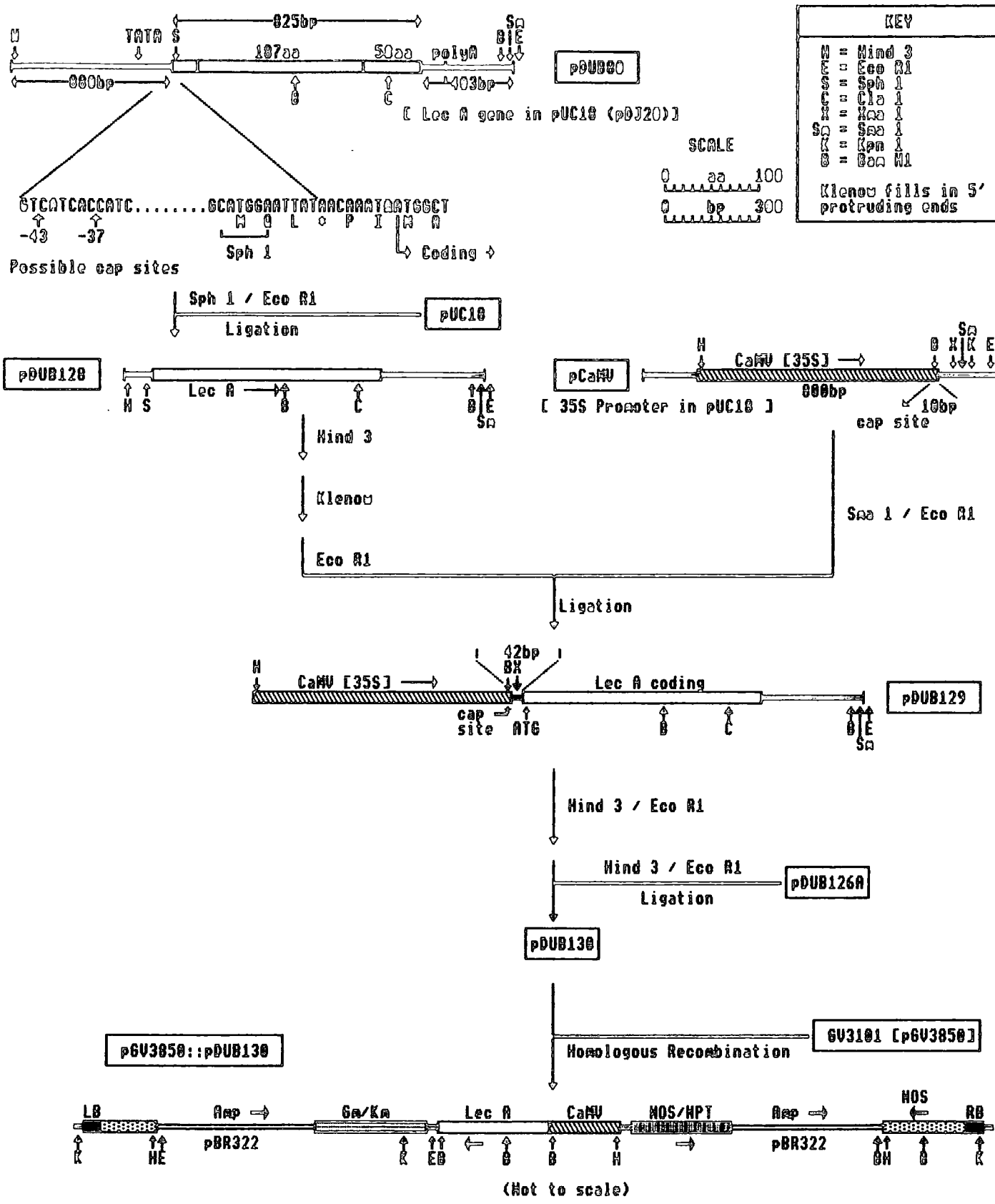
3.5.3. REGENERATION OF TRANSGENIC *N. tabacum* PLANTS CONTAINING THE CaMV-*LECA* CHIMAERIC GENE

N. tabacum cv. Petit Havana Str-r_1 explants were co-cultivated with GV3101 [pGV3850::pDUB130] and GV3101 [pGV3850::pDUB126a] and transgenic plants regenerated using the methods described, 100μg/ml and 50μg/ml augmentin being used as bacterial growth inhibitors in the shoot selection and rooting media respectively.

Twelve kanamycin resistant shootlets transformed by GV3101 [pGV3850::pDUB130] (L1-L12) and 4 transformed with the control strain (C1-C4) were potted out and grown under the conditions described (see section 2.2.17.). All plants grew normally with no differences being apparent between the putative lectin expressing plants and the controls. Figure 3.27. shows the plants 8 weeks after being potted out.

All the plants were self-fertile and selfed seeds from all plants had a germination rate of over 85% (20 seeds from each plant tested). The resultant seedlings grew normally and had similar morphology to untransformed seedlings.

FIGURE 3.24. CONSTRUCTION OF CaMV-PEA LECTIN CHIMERIC GENE AND STRUCTURE OF pGV3850 COINTEGRATE



3.5.4. DETECTION OF PEA LECTIN IN TRANSGENIC PLANTS

3.5.4.1. PROTEIN BINDING CAPACITY OF ELISA MICROTITRE PLATES

A competition assay was conducted to determine the protein binding capacity of the microtitre plate wells. Dilutions of an extract of untransformed *N. tabacum* cv. Petit Havana Str-r_1 leaf containing between 0.1-20μg/ml protein were prepared (see section 2.2.22), and each dilution supplemented ('spiked ') with purified pea lectin to a final concentration of 50ng/ml. 2 x 200μl aliquots of each spiked dilution and 2 x 200μl aliquots of unspiked extract containing 20μg/ml protein (background control) were placed in the wells of a micro-titre plate and an ELISA assay conducted. Figure 3.28 shows the standard curve obtained. Background β-galactosidase activity corresponding to 9μg/ml lectin was detected in the unspiked plant extract and the apparent concentration of lectin in the spiked dilutions (corrected for background) plotted against total protein concentration are shown in figure 3.29. These results show an increasing response to the added lectin with decreasing protein concentration, indicating that the plant proteins are competing with the added lectin for the available binding sites on the polystyrene. The response reaches a peak at a total protein concentration of approximately 1.5μg/ml indicating that this is the maximum amount of protein capable of being bound to the wells. Below this concentration the apparent lectin concentration decreases as the β-galactosidase activity of the plant extract is diluted out (this effect is seen as the results have been corrected for the undiluted background level).

The maximum detected concentration of lectin is approx. 30μg/ml, a loss of 40% of the added concentration, this possibly being due to degradation of the protein or the masking of antigenic sites on the lectin by specific or non-specific binding of constituents of the plant extract.

3.5.4.2. DETECTION AND ESTIMATION OF LECTIN IN TRANGENIC TOBACCO PLANTS BY ELISA

The stratagy used to screen the transgenic plants was to add an excess of protein to the micro-titre plate wells as this assures maximum protein binding and therefore maximum response in the assay.

Leaf extracts from plants L1-L12, C1-C4 (see section 3.5.3) and a further 8 plants (U1-U8) grown from seed and at a similar stage of development (4 weeks after being potted out in the case of the transgenic plants) were prepared and diluted to contain a final concentration of 3μg/ml protein. 200μl aliquots of each diluted extract were transferred (in duplicate) to the wells of a micro-titre plate and an ELISA assay conducted, the assay also being conducted in duplicate.

The apparent lectin concentration, determined from comparison to the standard curves (not shown) in the control and putative lectin expressing plants are shown in table 3.10. (concentrations shown are the averages of the 4 values obtained). The results are also shown graphically in figure 3.30.

The results show that the putative lectin expressing plants do show an increase in the response of the assay. The average increase in apparent lectin content in plants L1-L12 compared to the control plants is approximately 6ng/ml, which, using the maximum protein binding capacity determined in the previous section, gives an average level of expression of approximately 0.4% of total soluble leaf protein (this figure assumes that all the plants are expressing the gene and is an under-estimate, see later).

These results suggest that the introduced CaMV-*LecA* gene is being expressed in the transgenic plants but because of the high backgrounds of β-galactosidase activity in the extracts and the resultant low signal/noise ratio of the assay it is not possible to assign a definite phenotype with respect to lectin expression to all of the plants, as the response to the assay of the two populations has considerable overlap.

Table 3.10. Apparent lectin content of transgenic and control plants

Plant	Apparent lectin content (ng/ml)	Plant	Apparent lectin content (ng/ml)
L1	9	C1	3.5
L2	13	C2	7
L3	15.5	C3	10
L4	14.5	C4	9.0
L5	9.5	U1	4.5
L6	6.5	U2	8.5
L7	11.5	U3	5
L8	12.5	U4	6.5
L9	16	U5	4
L10	15.5	U6	5.5
L11	12.5	U7	7.5
L12	10	U8	6

3.5.5. SCREENING OF TRANSGENIC PLANTS FOR LECTIN EXPRESSION USING WESTERN BLOTTING

Leaf extracts from plants L1-L12 and C1 were prepared as for the ELISA assay and extracts containing 200μg protein were separated on an 11% non-denaturing polyacrylamide gel. 200ng purified pea lectin was also run as a positive control. The proteins were transferred to a nitrocellulose filter and detected immunologically (see sections 2.2.23 and 2.2.24 respectively), and figure 3.31. shows the lectin banding pattern obtained.

The results show that all the plants transformed with the CaMV-*LecA* containing construct

express the gene product with the exception of plant L11. The banding pattern shows that the lectin expressed in the tobacco plant leaves has electrophoretic mobilities corresponding to the two faster migrating isolectins from pea cotyledon. Although the results obtained do not allow the level of expression to be determined, the relative intensities of the bands indicates that plants L3, L4, L7, L8, L9 and L10 express the gene product at a higher level than plants L1, L2, L5, L6 and L12.

3.5.6. SCREENING OF TRANSGENIC PLANTS FOR NOPALINE

Nopaline assays were conducted on leaf extracts of plants L1-L12 and the control plants C1 and C2. Noplaline was detected in all plants except plants L6 and L11 (results not shown).

FIGURE 3.28. ELISA STANDARD CURVE.

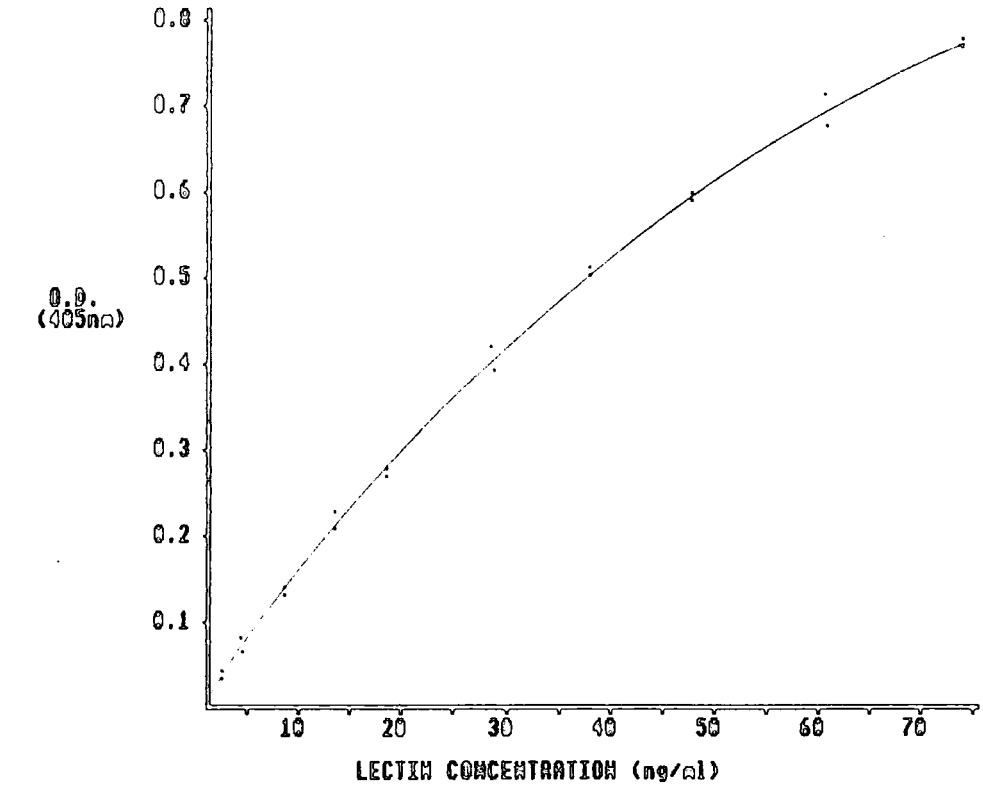


FIGURE 3.29. RESULTS OF COMPETITION ASSAY

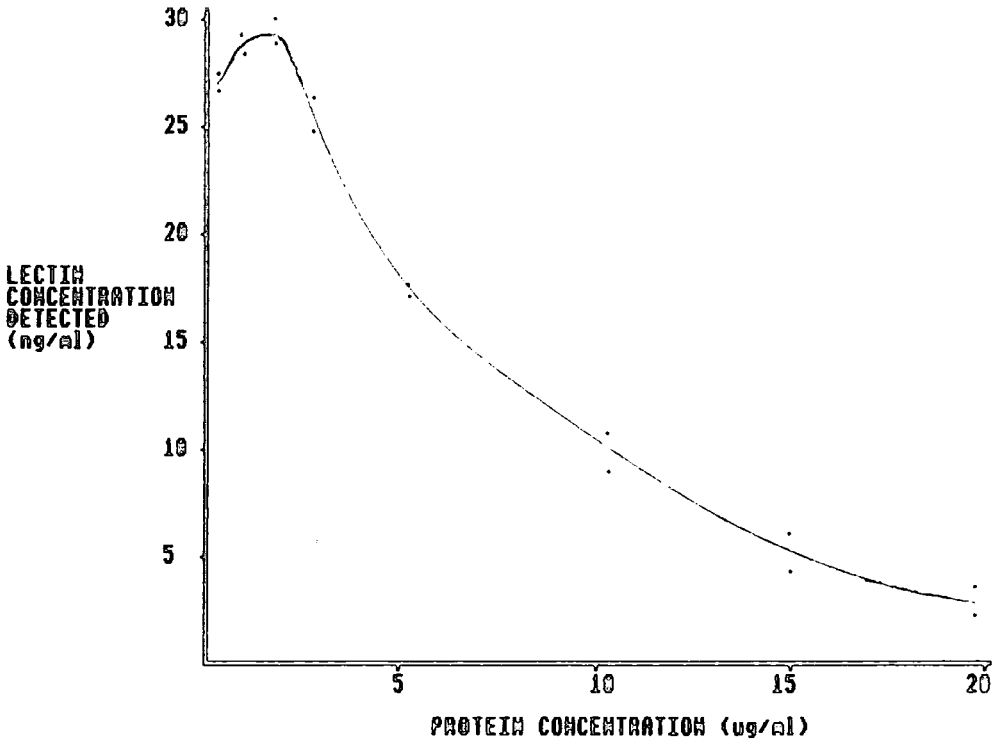


FIGURE 3.30. RESULTS OF SCREENING TRANSGENIC PLANTS FOR LECTIN CONTENT BY ELISA.

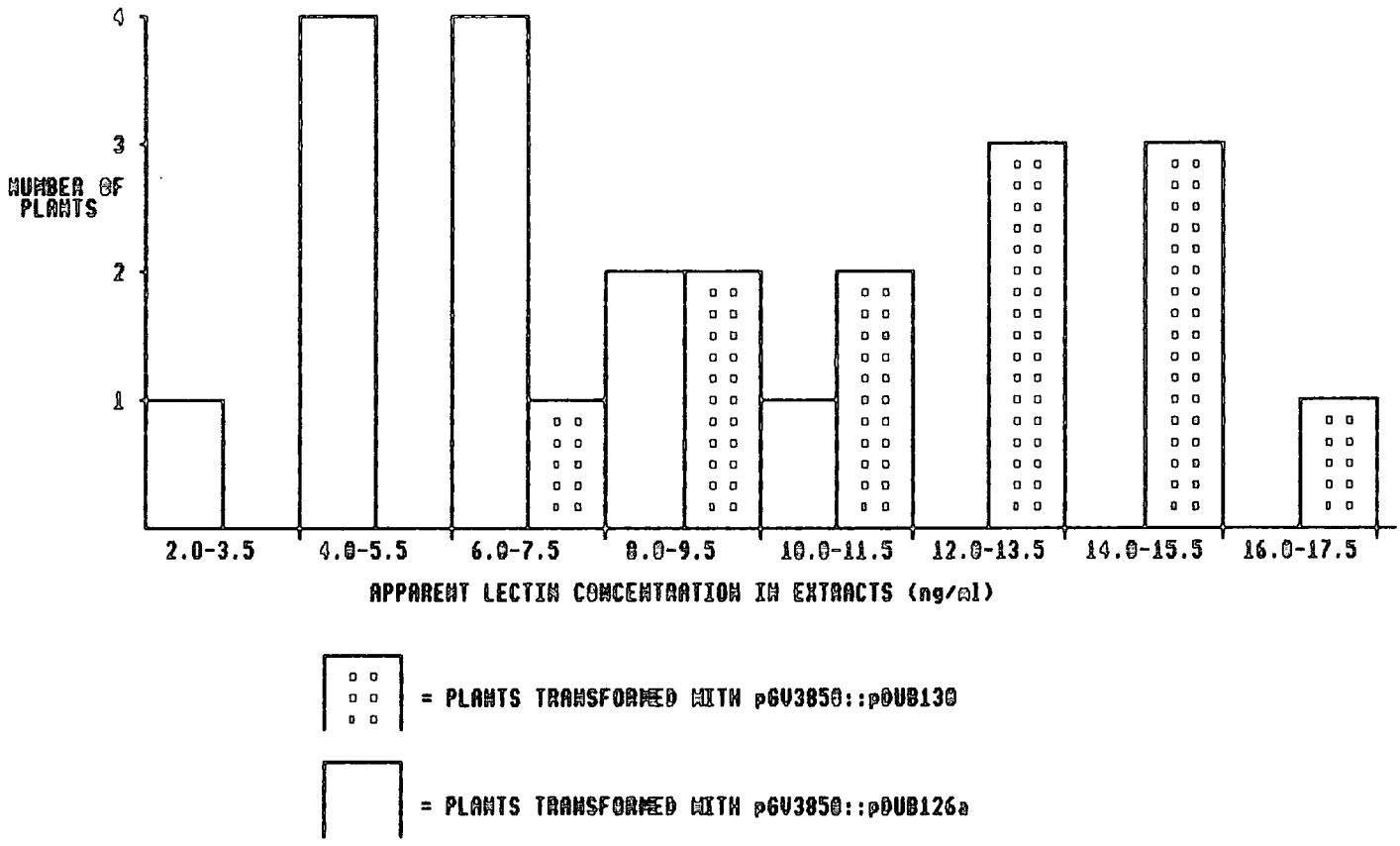
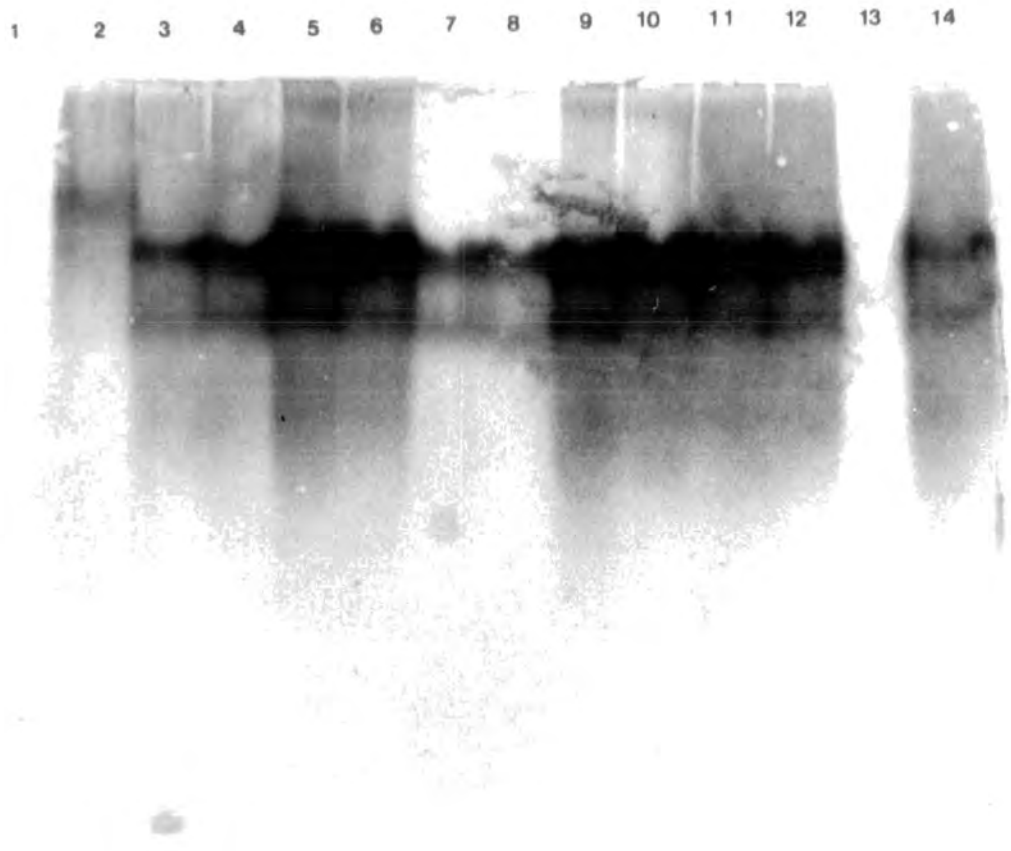


FIG 3.31



Screening of transgenic plants by non-denaturing western blotting and immunological detection. Lane 1, 200μg leaf protein from plant C1. Lane 2, 200ng purified lectin from pea cotyledon. Lanes 3-14, 200μg leaf protein from plants L1-L12 respectively.

3.5.7. SOUTHERN ANALYSIS OF T-DNA IN TRANSGENIC PLANTS

The probes and restriction enzymes used in the analysis of the structure of the integrated T-DNA of plants L1-L12 are shown in figure 3.32, along with the predicted sizes of bands of homology in unrearranged integrated T-DNA. The analysis used detects whether a 14.4kb central region of the T-DNA has been transferred using the pBR322 probe to detect the right and left pBR322 *Hind 3* fragments. The presence of an unrearranged CaMV-*LecA* gene is determined by detection of a 2.1kb *Eco R1/Hind 3* fragment using the *LecA* coding region probe and linkage between the two pBR322 homologous fragments and between the 2.1kb CaMV-*LecA* fragment and the right pBR322 *Hind 3* fragment is determined by the presence of the 6.5kb *Bam H1* fragment with homology to a 600bp *Hind 3/Pst 1* probe containing the Nos promoter and 300bp of the NPT coding region. This probe also has 300bp of homology to the Nos gene in *Hind 3* fragment 23 at the right border of the T-DNA and detects the presence of heterologous sized fragments occuring across the junction of the right T-DNA border with the plant DNA.

The complete analysis consisted of running six 0.7% agarose gels (2 gels for each digest and probe), each gel containing 8μg of digested DNA from plants transformed using pGV3850::pDUB130 (L1-L6 or L7-L12), DNA from one plant transformed with pGV3850::pDUB126a (C1 and C2 respectively), DNA from an untransformed tobacco plant and gene copy number reconstructions of 1, 2 and 5. Gene copy number reconstructions were calculated using a tetraploid *N. tabacum* genomic content of 1.5 x 10^{10}bp DNA (Bennet and Smith, 1976) and details of the constructs and amounts of DNA used are shown in table 3.11, along with amounts and activities of the probes used. Transfer, hybridisation and washing conditions used were as previously described.

Figures 3.33. 3.34 and 3.35 show the agarose gels and autoradiographs obtained for the complete analysis of plants L7-L12 inclusive. A summary of the results obtained in the analysis of all the transgenic plants, including genotypes determined in sections 3.5.5. and 3.5.6. is shown in table 3.12.

Table 3.11 Activity of probes and details of DNA used in gene copy no. reconstructions

Probe	Amount of probe used in hyb.	Probe activity (c.p.m./μg DNA)	DNA used in reconstruction	Size	Amount of DNA for 1 copy equiv.
pBR322	200ng	3.8×10^8	*Hind 3* cut pBR322	4.37kb	2.39pg
1.2kb *Sph 1/Eco R1* *LecA* coding region from pDUB80	200ng	2.1×10^8	as for probe	1.2kb	0.75pg
600bp *Hind 3/Pst 1* fragment from pNos-NPT	200ng	1.5×10^8	*Eco R1* cut pNos-NPT	4.05kb	2.16pg

The analysis using the pBR322 and *LecA* probes gave easily interpreted banding patterns but the analysis using the 600bp Nos-NPT probe gave high background levels. The expected 6.5kb *Bam H1* containing the Nos-NPT gene with full homology to the probe can be seen but the heterologously sized fragments spanning the T-DNA and plant DNA junctions cannot be seen clearly. These fragments were expected to show up as bands with a copy number equivalent of 0.5 as they have only 300bp of homology to the probe and the resultant intensities of the bands of hybridisation are not high enough to be detected above the high backgrounds.

The results show that 4 of the plants analysed (L1, L4, L12 and C1) contain a single copy of unrearranged T-DNA. Plants L3, L5, L7, L8, L9 and L10 all contain between 1 and 5 copies and plant L11 over 5 copies of T-DNA. Where multiple copies of T-DNA were transferred, some rearranged copies of T-DNA were present, as seen by the presence of aberrant sized bands with pBR322 homology or differences between copy number estimations of the two expected pBR322 bands (i.e. plants L5 and L10). Although it is possible that the plants with multiple copies of T-DNA do not contain any full length copies of the T-DNA, (i.e. all the delivered T-DNAs are rearranged but in the population of rearranged fragments expected sized fragments are still detected) this is not thought to be the case because no rearrangments in the fragments homologous with the *LecA* and Nos-NPT probes were detected, the amount of rearrangments detected using the pBR322 probe and the general agreement of the copy number equivalents is not consistent with a high level of rearrangments. Hence the majority of the T-DNAs present in these plants are thought to be full length.

Plants L6 and C2 have had extensive rearrangments within the T-DNA and do not contain any full length copies. Plant L6 contains an intact *LecA* gene, no detectable homology to pBR322 and a rearranged *Bam H1* Nos-NPT fragment 7.5 kb in size. These results suggest that the rearrangment has occurred adjacent to Nos-NPT gene and also to the left of the *LecA* gene. Plant C2 contains an

4.0kb *Bam H1* fragment homologous to the Nos-NPT probe and some rearranged pBR322 DNA.

In all the plants analysed except plant L11 the phenotype with respect to the expression of the CaMV-*LecA* gene corresponds to the presence or absence of the 2.1kb *Eco R1/Hind 3* fragment. Plant L11 contains multiple copies of T-DNA but expression of CaMV-*LecA* and Nos was not detected and it is possible that this plant is chimaeric.

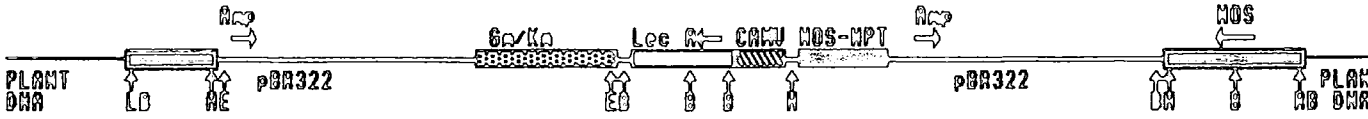
Table 3.12 Summary of results of Southern analysis of transgenic plants

Plant	Lectin expression	Nopaline synthase activity	Presence of predicted bands of homology to probes and estimated copy no.				Rearranged DNA with pBR322 homology
			LecA	Nos	pBR322		
					a	b	
L1	+	+	1	1	1	1	-
L2	+	+	2	2	2	2	+
L3	+	+	5	5	5	5	+
L4	+	+	1	1	1	1	-
L5	+	+	1	1	2	1	+
L6	+	-	1	-	-	-	-
L7	+	+	2	2	2	2	+
L8	+	+	2	2	2	2	+
L9	+	+	5	5	5	5	+
L10	+	+	3	3	3	2	+
L11	-	-	> 5	> 5	> 5	> 5	+
L12	+	+	1	1	1	1	-
C1	N/A	+	N/A	1	1	1	-
C2	N/A	+	N/A	-	-	-	+

Key: a = 6.0kb *Bam H1* fragment, b = 8.4kb *Bam H1* fragment.

FIGURE 3.32. STRATEGY USED FOR THE SOUTHERN ANALYSIS OF T-DNA IN TRANSGENIC PLANTS L1-L12

PREDICTED STRUCTURE OF FULL LENGTH T-DNA OF pGV3850::pDUB130 INTEGRATED IN PLANT DNA



PREDICTED SIZES OF DNA (kb) FRAGMENTS WITH HOMOLOGY TO pBR322 PROBE

DIGEST

Hind 3 — 8.4 — 6.0

PREDICTED SIZES (kb) OF DNA WITH HOMOLOGY TO 1.2kb Eco R1/Sph 1 FRAGMENT CONTAINING Lec A CODING REGION.

DIGEST

Eco R1/Hind 3 — 2.1

PREDICTED SIZES (kb) OF DNA WITH HOMOLOGY TO 600bp Hind 3/Pst 1 FRAGMENT FROM pNOS-NPT

DIGEST

Bam H1 — 6.5 — JUNCTION FRAGMENTS

KEY
E = Eco R1 H = Hind 3 B = Bam H1

SCALE

= 1kb

3.5.8. DETECTION OF LECTIN MESSAGE IN TRANSGENIC TOBACCO PLANTS

Total leaf RNA was prepared from plant L9, a transgenic plant expressing relatively high levels of lectin as determined by ELISA and western blotting (see sections 3.5.4.2 and 3.5.5), plant L6 a similarly defined low expresser and C1 a control plant regenerated using pGV3850::pDUB126a. Aliquots containing 10μg of each RNA were glyoxylated in duplicate along with aliquots containing 10μg of total RNA from developing pea cotyledons (18 days after fertilisation). After separation by agarose gel electrophoresis (see section 2.2.5.3) the gel was bisected, one half being stained with acridine orange (see figure 3.36.). The RNA in the remaining part of the gel was transferred to a nitrocellulose filter and hybridised with a 1.2kb *Eco R1/Sph 1* DNA fragment from pDUB80 containing the *LecA* coding region and and 3' untranslated sequence (specific activity = 2 x 10^8 c.p.m./μg DNA) using the hybridisation and washing conditions previously described in section 2.2.13. The resultant autoradiograph is shown in figure 3.37.

The RNA banding pattern obtained on the gel shows the presence of rRNAs and also the abundant RNAs coding for the major leaf proteins in the tobacco plants. The bands obtained are discrete and show no signs of degradation of the isolated RNA. The autoradiograph shows single bands of hybridisation of approximately 1.0 kilobases in size in both the pea cotyledon and the transgenic tobacco RNA, with the pea cotyledon band showing some smearing down the gel, indicating that the RNA is slightly degraded. Plant L9 RNA gives a strong band of hybridisation of comparable intensity to the pea cotyledon RNA and plant L6 gives a much fainter band of hybridisation which was only clearly visible with a more prolonged exposure (results not shown). The size of the lectin message in the transgenic plant leaves is slightly smaller than the message in the developing pea cotyledons. This result could be artefactual as the pea rRNAs can also be seen to have run slightly slower in the gel. Alternatively, the slight size difference could be due to differences in the extent of poly-adenylation of the RNAs.

3.5.9. FURTHER CHARACTERISATION OF LECTIN EXPRESSION IN TRANSGENIC TOBACCO PLANTS

3.5.9.1. SDS-PAGE AND WESTERN BLOTTING OF LECTIN IN TRANSGENIC PLANTS

Protein extracts from leaves of plants C1, L3, L9 and L12 (a control plant, two high lectin expressing plants and one low expressing plant respectively, as determined in sections 3.5.4.2 and 3.5.5) were prepared (using Tris-HCl pH6.8 extraction buffer) and duplicate samples containing 100μg of protein were separated by SDS-PAGE (17% gel) along with 500ng of purified pea cotyledon lectin. After electrophoresis the gel was bisected, one half stained and the proteins in the remaining half transferred to a nitrocellulose filter and the lectin detected immunologically. The stained gel and the processed filter are shown in figure 3.38.

The gel clearly shows the large (M_r 17000) β lectin subunit in plants L3 and L9 and a faint band can also be seen in plant L12. This subunit is also seen on the western blot but the α subunit (M_r 6000) is not detected, presumably because this subunit has not elicited an antigenic response in the rabbits and the antibody preparation does not contain IgG directed against this polypeptide. Larger lectin forms of approximately M_r 18000 and 25000 can also be seen in both the purified pea cotyledon lectin and in the transgenic plants, possibly representing the pre-processed forms. Some much larger lectin bands can also be seen in the pea cotyledon lectin preparation and these are possibly artefacts of the purification method or storage conditions.

The intensities of the β subunit bands indicate that plants L3 and L9 express the CaMV-Lec A gene at a higher level than plant L12, agreeing with the earlier indications of the relative levels of expression in the transgenic plants. The intensities of these bands in plants L3 and L9 are comparable to the intensity of the band in the pea cotyledon lectin, indicating that the level of expression in these plants is in the region of 0.5% of total soluble leaf protein.

3.5.9.2. LECTIN EXPRESSION IN DIFFERENT TISSUES OF TRANSGENIC PLANTS

Protein extracts from selfed seeds, unexpanded leaves on the elongated flower stem, fully expanded lower leaves, stems and roots of plants C2 and L9 (using 50mM Tris-HCl pH6.8 extraction buffer) were prepared and aliquots containing 100μg protein were separated by non-denaturing PAGE (11% gel) and SDS-PAGE (17% gel) along with purified pea cotyledon lectin. The separated proteins were transferred to nitrocellulose filters and the the lectin detected immunologically. The processed filters are shown in figures 3.39 and 3.40.

The banding pattern seen after separation of the extracts by non-denaturing PAGE shows that all tissues tested do contain pea lectin and that the majority of the lectin present in the leaves, stem and root consists of the second fastest migrating isolectin, the fastest migrating isolectin also

being present. All three isolectins are present in the seed, the two slower migrating bands being present in approximately equal amounts and in larger amounts than the fastest migrating band. The pea cotyledon lectin does not show up well on this blot but the slower migrating band can be seen.

The banding pattern seen after separation by SDS-PAGE shows that the β lectin subunit from all the transgenic plant tissues is the same size as that of the pea cotyledon lectin. The preprocessed forms of lectin can also be seen in the extracts as well as the high molecular weight artifactual bands in the pea cotyledon lectin (see previous section). Smaller bands seen in the seed extract of plant L9 are possibly lectin degradation products. The lanes containing the pea cotyledon lectin are equivalent to 0.1%, 0.5% and 1% protein content in the soluble extracts from the transgenic plants and a comparison of the band intensities give estimates of expression levels of greater than 1% in seeds and roots, and of approximately 0.5% in the other tissues. The young unexpanded leaf appears to contain more lectin than the expanded leaf.

3.5.9.3. HAEMAGGLUTINATION ACTIVITY OF LEAF EXTRACTS OF TRANSGENIC PLANTS

Protein extracts from expanded leaves of plants L9 and C1 were prepared and a haemagglutination assay conducted as described in section 2.2.25 and the results are shown in figure 3.41.

The end point of the assay in the control wells containing pea cotyledon lectin can be seen to be at approx. 0.75μg/ml. By comparison, the extract from the control plant has haemagglutination activity equivalent of 1.5μg/ml lectin/mg plant protein (the end point is row B, this dilution containing 500μg/ml plant protein). The equivalent value for the lectin expressing plant is 6μg lectin /mg of plant protein (end point is at row D, this dilution containing 125μg/ml plant protein). The increase of 4.5μg/mg is a minimum figure and indicates a lectin content at least 0.45% of total soluble leaf protein in plant L9. This figure is in agreement with the earlier estimations of lectin expression in plants L3 and L9 of approx. 0.5% of total soluble leaf protein (see sections 3.5.9.1 and 3.5.9.2.) and suggests that the lectin produced in the transgenic plants has a haemagglutination activity comparable to that of pea cotyledon lectin.

3.5.9.4. DETECTION OF LECTIN ON THE SURFACE OF ROOTS OF TRANSGENIC TOBACCO

In order to determine whether lectin was present on the surface of roots of lectin-expressing transgenic tobacco plants, roots tips of plants L3, L9 and C1 and C3 were incubated with rabbit red blood cells and examined microscopically (see section 2.2.26) to determine the extent of blood cell binding. Initially the root tips were taken from plants which were setting seed, and examinations of a large number of tips gave no indication of any red blood cell binding in the lectin expressing plants (L3 and L9) or in the control plants. The experiment was repeated on cloned plantlets of L3,

L9 and C1 on young roots in rooting agar (see section 3.5.10.) and again no increase in red blood cell binding to the lectin expressing plants could be detected in comparison to the controls.

The presence of lectin on the root surface of lectin expressing tobacco plants was also determined using fluorescence microscopy (see section 2.2.27) using the same plants described above. Numerous repeats of this experiment showed no increase in fluorescence in the roots of the transgenic lectin expressing plants, after processing using the anti-lectin antibodies, compared to the control plants or to lectin expressing plants when processed using the preimmune serum at both the concentrations of secondary antibody used.

3.5.9.5. SUBCELLULAR LOCALISATION OF PEA LECTIN IN TRANSGENIC TOBACCO ROOT

Root sections from plant L9 (a high lectin expressing plant) and plant C1 (a non-expressing control plant) were prepared and lectin detected by the method described in section 2.2.28.

Sections from both plants incubated with the rabbit pre-immune serum prior to incubation with the gold-labelled secondary antibodies had few gold particles bound when viewed under the EM. The particles that could be seen were widely distributed over the sections and did not appear to be associated with any one organelle (results not shown). When rabbit anti-lectin antibodies were used in the procedure, gold particles were seen associated with electron dense particles present within the vacuole in plant L9 but not in the control plant (C1), as shown in figure 3.42. The micrographs show only small amounts of background deposition of gold particles in the control plant section and in other parts of the cell in the section of the lectin expressing plant.

3.5.9.6. SCREENING OF LECTIN EXPRESSING TRANSGENIC TOBACCO PLANTS FOR RESISTANCE TO INFECTION BY *Meloidogyne incognita*

Disinfected leaf explants from plants L3, L9 and C1 (two transgenic tobacco plants expressing lectin at a relatively high level and one non-expressing control plant) were placed on shooting medium and the resultant shoots rooted using the methods described in section 2.2.17. The clonal plants were then screened for pea lectin expression using SDS-PAGE/western blotting as previously described. All of the clonal plants derrived from both L3 and L9 contained comparable levels of lectin to the original parent plants (approx. 0.5% of total soluble leaf protein). Ten clonal plants derived from each of L3, L9 and C1 were then infected with *M. incognita* as described in section 2.2.29.

After four weeks growth after infection, all the infected plants showed signs of severe infestation, being stunted and chlorotic in comparison to uninfected plants, with no apparent differences between the lectin-expressing and non-lectin expressing plants seen. Upon inspection, the roots of all the infected plants were heavily infested with nematodes and a large number of root-knots were

present. A typical infested primary root is shown in figure 3.43. Comparison of infected roots from the non-lectin expressing plants with the lectin-expressing plants showed no obvious differences in the level of root-knot formation between the two populations. Infested roots of one each of the L3, L9 and C1 clones are shown in figure 3.43.

4. DISCUSSION

4.1 CHARACTERISATION AND DEVELOPMENT OF THE pGV3850/pGV3851 BASED Ti PLASMID CLONING SYSTEM

4.1.1 CHOICE OF Ti PLASMID VECTOR SYSTEM

A homology-mediated *cis* Ti plasmid cloning system was chosen for use in this project because this laboratory wanted to gain experience in, and to have available, an easily manipulated Ti vector system of this type for delivery of genes to plants. The acceptor Ti plasmids pGV3850 and pGV3851 were chosen as the basis of this *cis* vector system because these acceptor Ti plasmids give a range of plant transformation techniques which can be employed, the disarmed pGV3850 acceptor plasmid being suitable for use in *in vitro* plant transformation techniques, enabling transgenic callus or regenerated plants to be recovered, whilst pGV3851 appeared to be suitable for use with *in vivo* plant transformation methods. These Ti vectors therefore give a large degree of flexibility with regard to plant transformation techniques but enable the same methods and integrating vector constructs to be used with both the disarmed and oncogenic acceptor plasmids. The availability of the oncogenic acceptor Ti plasmid was considered to be important during the conception of this project, because not only did this appear to give the option of using simple plant transformation methods which do not require any *in vitro* plant tissue culture techniques and therefore has merits in its own right, but also the *in vitro* tissue culture methods needed for use with disarmed Ti plasmid vectors had not been succesfully employed in this laboratory at that time. Hence the oncogenic Ti acceptor plasmid was available for immediate use to study plant gene function and expression and the disarmed vector (pGV3850) could be used later in the project when the relevant plant tissue culture methods became available (as indeed was the case).

4.1.2 DESIGN OF T-DNA INTEGRATING VECTORS FOR USE WITH THE pBR322 HOMOLOGY MEDIATED Ti VECTOR SYSTEM

A pBR322 based plasmid, pDUB114, was used as the starting point for the construction of suitable T-DNA integrating vectors for use in this Ti plasmid cloning system. This plasmid contains the Km/Gm resistance marker from the W-type plasmid Mini-Sa which has previously been used as a selective marker in *A. tumefaciens* (Leemans *et al.*, 1982; Shaw *et al.*, 1984a and 1984b). This gene has the advantage that the Gm resistance function is independent of any other antibiotic resistance genes in the *E. coli* mobilising strain GJ23. Zambryski *et al.* (1984) used a Km resistance marker on the T-DNA integrating plasmid for selection of cointegrates and (Timko *et al.*, 1985) used the Tc marker of pBR322 for the same purpose. However, the plasmids in the mobilising strain, pGJ28 and R64*drd11* contain Km/Nm and Tc resistance markers respectively, and selection of cointegrates using these markers could select for (albeit rare) events where these plasmids have interacted with

the genetic complement of the *A. tumefaciens*. By using the Gm/Km resistance marker and selecting cointegrates on the basis of Gm resistance, any such events involving the mobilising plasmids would not be directly selected for, and Sanchez-Serrano *et al.* (1987) have presumably used a spectinomycin resistance gene for the same reason. The Km/Gm gene has an added advantage over these other genes in that it gives rise to a higher level of Gm resistance in *A. tumefaciens* than in *E. coli* (section 3.1.2). Upon plating out the conjugation mix for selection of cointegrate-containing *A. tumefaciens* using kanamycin as the selective agent, the high mutation rates of the *E. coli* donor strain to rifampicin resistance made subculturing of the desired isolates difficult, as the colonies were 'swamped'by colonies of the mutant *E. coli* strain. Subsequent characterisation of isolates from this selection showed that the level of Gm resistance derived from the Km/Gm resistance is higher in the *A. tumefaciens* GV3101 than in the *E. coli* strain. Hence by selecting cointegrates on media containing gentamycin at a concentration of 5μg/ml or above, any donor *E. coli* which mutate to Rif resistance do not grow. An alternative to this selection procedure would be to select for cointegrate-containing *A. tumefaciens* on minimal media as the donor *E. coli* strain is a multiple auxotroph, but the disadvantage with this approach is that *A. tumefaciens* grow very slowly on minimal media (Dhaese *et al.*, 1979).

The dominant selectable marker gene for expression in plants used in the construction of the DNA integrating vectors was the Nos-NPT gene of Bevan (1984). This gene was used rather than other dominant selective markers available at the start of this project, such as chimaeric gene constructs containing coding regions for methotrexate or chloramphenicol resistance, because it has been reported that the NPT 2 gene, when used in conjunction with kanamycin as the selective agent, is superior to these other markers in terms of differential resistance between transformed and untransformed tissues and the lack of inhibition of normal plant development when transformed tobacco tissue is cultured on media containing kanamycin (Herrera-Estrella *et al.*, 1983; De Block *et al.*, 1984). The gene used is an improved version of earlier Nos-NPT constructs (Herrera-Estrella *et al.*, 1983; Bevan and Flavell, 1983; Frayley *et al.*, 1983) as an out of frame ATG translational start codon has been removed giving higher levels of expression of this gene. A Shine-Delgano sequence was also removed which was present between the transcriptional start and the (in frame) ATG codon, preventing the expression of this gene in bacteria, which occurred with the earlier constructs (Herrera-Estrella *et al.*, 1983). If the gene was expressed in *E. coli*, this would interfere with the selection for pGJ28 as the selection for this plasmid is also by means of a Km/Nm gene.

The initial T-DNA integrating plasmid constructed, pDUB116, contains only two restriction enzyme sites suitable for cloning genes for subsequent transfer to plants. Although this plasmid was succesfully used to transfer the SV40 enhancer to plants (section 3.2) the plasmid is difficult to use as

a cloning vehicle because of this lack of unique restriction enzyme sites and the lack of a simple system for detecting recombinant plasmids after cloning steps. The plasmids pDUB126a/b and pDUB127a were constructed to both increase the number of restriction enzyme sites and give a direct method of identifying recombinant plasmids using the *lac Z* insertional inactivation system. There is room for considerable improvement to these integrating vectors however, in terms of increasing the number of unique restriction enzyme sites and size reduction to give vectors which are more amenable to *in vitro* genetic manipulation.

4.1.3 FORMATION AND CHARACTERISATION OF COINTEGRATES BETWEEN T-DNA INTEGRATING PLASMIDS AND Ti ACCEPTOR PLASMIDS

As the terminology used in this work suggests, the frequencies of transmission/recombination giving rise to the expression of antibiotic resistance markers present on the integrating plasmids in *A. tumefaciens* are a function of two processes, namely the frequency of transfer of the T-DNA integrating plasmid to the acceptor strain of *A. tumefaciens*, and the frequency of recombination between the integrating and acceptor Ti plasmids within *A. tumefaciens*. Amongst the factors which may affect the transfer of the T-DNA integrating vectors from the *E. coli* donor strain to *A. tumefaciens* are the size of the plasmid and the number of donor/acceptor bacteria in the conjugation mix. The frequencies quoted in this work are the ratios of cointegrate-containing *A. tumefaciens*/acceptors and do not take into account the number of donors in the conjugation mix at the end of incubation. Because the two bacterial species grow during the relatively long co-incubation, small differences in the media may radically effect the ratio of donors to acceptors during the conjugation step with a concomitant affect on the frequency of transmission of plasmids. Factors which affect the frequency of homology-mediated cointegrate formation are the extent of the length of homology between the plasmids and, possibly, the nature of those sequences undergoing homologous recombination. The affect of the length of homologous sequences undergoing recombination has been assessed in both *E. coli* and in *Bacillus subtilis* with recombination frequencies increasing as a quadratic function of the increase in the length of the homologous sequence (Michel and Erhlich, 1984). It is also possible that DNA sequences analogous to the Chi sequences, which are known to promote homologous recombination in *E. coli* (Stahl, 1979), are involved in homologous recombination processes in *A. tumefaciens*, but the recombination systems in *A. tumefaciens* have not been investigated and any such involvement is unknown. Because of these numerous variables it is difficult to draw any conclusions with regard to factors involved in the process of homologous recombination from recombination frequencies obtained in this work, and in comparison to other published results, as the controls needed to fully characterise the system were not conducted. The frequencies obtained in this work are lower than those reported by Van Haute *et al.* (1983), where overall frequencies

of $6.7x10^{-6}$ were obtained, comprised of a transmission frequency of $4.5x10^{-3}$ and a recombination frequency of $1.5x10^{-3}$, for a 7.7kb plasmid with 3.3kb of homology between the integrating and acceptor plasmids. Overall frequencies obtained in this work are between $8.1x10^{-7}$ (section 3.1.2) and $1x10^{-6}$ (section 3.2.2) for integrating plasmids of 7.5-8.5kb in size with 4.0kb of homology, rising to a maximum of $2x10^{-6}$ for a 17.8kb plasmid (pGV0319) with 13.5kb of homology between plasmids (section 3.1.5), this latter figure possibly being higher due to the more extensive length of homology present. However, the frequencies of transmission/recombination obtained in this work were, in all cases, high enough to recover enough isolates for further characterisation of the cointegrate structures with a minimal number of bacterial platings being required.

The initial characterisation of pGV3850::pDUB116, pGV3851::pDUB116 (section 3.1.3) and pTiGE1 (section 3.1.5) by Southern blotting did not take into account that the integrating vector could be present in multiple copies within the cointegrate structures. It was assumed that because the recombination frequency between the plasmids is in the region of $1x10^{-3}$, the frequency of multiple integration events would be multiples of that frequency and hence extremely rare. The strategies used in the Southern analyses of these cointegrates used restriction enzymes which cleaved twice within pBR322 and the integrating vector DNA, and therefore liberated similar sized DNA fragments with homology to the probe from all copies of the integrating vector present, indicating that the integrating vectors were present and unrearranged within the cointegrates but giving no indication of the presence of multiple copies of the integrating vectors. In the analysis of pGV3850::pDUB116 and pGV3851::pDUB116, five isolates of each cointegrate were analysed and it should of been obvious (with hindsight) from the intensities of the bands of hybridisation on the autoradiograph (these results are not shown) that multiple copies of the integrating vector were present in some cases. However, the variations in yield of DNA from each isolate and the overexposure of the autoradiograph which 'saturated'the dose response of the autoradiographic film, conspired to prevent the differences in band intensities becoming apparent. The problem of multiple copies of the integrating vector being present within cointegrates only became apparent during the Southern analysis of cointegrates of pTiGE1 and integrating vectors containing the SV40 enhancer sequence (pDUB118a-pDUB119c). The strategy employed in the analysis used restriction enzymes which either cleaved outside pBR322 and the integrating vector (*Sma 1*) or only cleaved once within the integrating vector alone (*Eco R1*) giving rise to bands of hybridisation larger than expected in cases where multiple copies of the integrating vector were present. The analysis of these cointegrates indicated that pTiGE1 also contains multiple copies of pGV0319, as determined from the results of the Southern analysis of pTiGE1::pDUB119 (a full explanation of these results is given in section 3.2.2). Hence, of the six cointegrate structures characterised in this analysis (pTiGE1, and pTiGE1::pDUB118a-pDUB119c), four contained multiple copies of the respective integrating plasmids.

The high frequency of multiple cointegrate structures obtained may (in part) be due to the level of antibiotic selection used during their isolation. During the construction of pTiGE1, 100μg/ml carbenicillin was used to select for cointegrates between pGV3100 and pGV0319 as the presence of the ampicillin gene from pBR322 in *A. tumefaciens* GV3101 [pGV3850]/[pGV3851] was known to be capable of conferring this level of resistance at a copy number of one per Ti plasmid (Zambryski *et al.*, 1984). However, it is possible that multiple copies of the ampicillin resistance gene in the cointegrate gave higher levels of resistance resulting in faster growth of bacteria containing these multiple cointegrate structures. Hence it is possible that the initial selection of isolates was biased towards isolation of multiple-cointegrates as faster growing (larger) colonies were chosen for subsequent characterisation. This bias may have been enhanced as *A. tumefaciens* is known to be resistant to penicillins (discussed later) and the choice of small colonies was avoided as they may have represented selection 'escapes'. A similar argument applies to the choice of isolates containing pTiGE1::[pDUB118a-119c] cointegrates when selected on 10μg/ml gentamycin. However, once the possibility of this gene dosage/bias effect was realised steps were taken to avoid choosing large colonies, as in the selection of pTiGE2 on 100μg/ml carbenicillin, but three of the five isolates characterised contained multiple copies of the integrating plasmid (section 3.4.1). Also, in the selection of pGV3850::pDUB130 and pGV3850::pDUB126a cointegrates (section 3.5.2), the concentration of gentamycin used was reduced to 5μg/ml in order to minimalise this effect, but in each case, four of the five isolates contained multiple copies of the integrating vector. Although this effect of gene dosage and the level of antibiotic selection may account in part for the high level of multiple cointegrates obtained, these results indicate that multiple cointegrates do arise at a very high frequency.

This high frequency of multiple cointegrate formation could possibly be due to the mechanism of the recombination system of *A. tumefaciens*, with intermediates in the (initial) recombination event being highly recombinogenic with further copies of delivered integrating plasmid or the integrating plasmid being amplified as a result of the recombination event. An alternative possibility to these processes occurring during or soon after the initial recombination events is that multiple copies of the integrating plasmid arise by amplification during the subsequent growth of the bacteria and are due to inherent instability of the cointegrate structures. The evidence does not support this hypothesis however, as in only one case (see section 3.5.2) was evidence obtained indicating that a multiple cointegrate structure had arisen during the growth of the *Agrobacterium* strain, with the other single cointegrate structures all being stable. Hence the apparent low frequency of this type of event does not explain the very high frequency of multiple cointegrate formation, suggesting that they are formed during or soon after the initial recombination event.

In practical terms this means that if there is a specific requirement for cointegrates containing a single copy of cointegrating plasmid, a number of isolates must be screened by Southern analysis, (in this work five appeared to be sufficient, see sections 3.4.1 and 3.5.2), in order to identify these before a more thorough analysis is conducted to confirm the cointegrate structure in detail, which adds a time-consuming step to the overall cloning system. Strategies which can overcome this disadvantage are discussed in section 4.1.6.

4.1.4 ONCOGENIC Ti ACCEPTOR PLASMIDS

One of the main reasons for choosing the pGV3850/pGV3851 vector system for use in this project was the availability of pGV3851 as an oncogenic Ti acceptor plasmid for use in an *in vitro* transformation system. The *aux*$^-$ genotype of this plasmid gives rise to small shooty tumours, and although Zambryski *et al.* (1984) suggested that these tumours need to be further propagated *in vitro*, the results of Joos *et al.* (1983) and Van Montagu and Schell (1982) indicated that pTiC58 derivatives with this phenotype may give rise to tumours of sufficient size to enable routine assays for plant gene expression studies to be conducted without the need for *in vitro* propagation of callus. However, when pGV3851 and pGV3851::pDUB116 was tested for its ability to induce tumours on *K. diaigromontana* leaves and *N. tabacum* stems (section 3.1.4), tumours were rarely formed, and if formed were extremely slow growing, showing that this plasmid is not a suitable vector for use in a rapid *in vivo* transformation system. pGV3851 has since been succesfully used in gene expression studies (Van Den Broek *et al.*, 1985; Schreier *et al.*, 1985; Kaulen *et al.*, 1986) but in all cases tumours were cultured *in vitro* after induction on the plants.

A fully oncogenic plasmid with pBR322 homology (pTiGE1) was therefore constructed to replace pGV3851 (section 3.1.5). This acceptor Ti plasmid was designed before the problem of multiple cointegrate formation was fully appreciated. The T-DNA of this plasmid was thought to have the structure shown in figure 3.3, comprising two 13.5kb direct repeats both containing fully functional *aux* and *cyt* loci and separated by pBR322. It was reasoned that two copies of the *onc* genes would not affect the oncogenicity of the plasmid or the morphology of the resultant tumours as it has been shown that tumours containing tandem arrays of wild-type T-DNA have normal morphology. Additionally, the increased size of the T-DNA (approximately 41kb against the wild-type 23kb) was not thought to be a problem as it has been demonstrated that up to 80kb of T-DNA can be transferred to plants (David *et al.*, 1988; and references therein) and hence this vector, even after a further cointegration step with an integrating vector, would be well within this size. However, subsequent analysis of pTiGE1 showed that the construct was a multiple cointegrate between pGV3105 and pGV0319 and hence contained at least two copies of pBR322 homology, at

least three copies of the direct repeat containing the *onc* genes and therefore had a T-DNA of at least 58kb in size and possibly much larger. This may in itself not be a great problem, as the multiple copies of the *onc* genes did not subsequently appear to affect the morphology of callus produced *in vivo* and *in vitro* (see sections 3.15 and 3.2) and the vector did prove capable of transferring desired genes into plants in an *in vitro* transformation system (see section 3.2.5). However, it may be a problem in *in vivo* transformation systems where selection of the transformed material is by the oncogenic phenotype and the gene(s) of interest may be situated in the T-DNA at a site distal (and relatively unlinked) to some of the *onc* gene repeats; hence cotransformation of these genes cannot be assumed with any degree of confidence.

The same strategy used to construct pTiGE1 could have been repeated and isolates screened by Southern blotting to identify cointegrates containing single copies of pGV0319 and hence obtain the desired construct. However, the strategies for the Southern analysis and identification of multiple cointegrate structures is difficult in this instance. The two strategies available are to digest the integrated vector at a unique restriction enzyme site with multiple cointegrates being identified by a band of homology the size of the integrating vector, or to digest the T-DNA outside the area of direct repeats and identify multiple cointegrates by the increased size of homologous DNA fragment. The first of these strategies would have necessitated finding and mapping pGV0319 for a unique restriction enzyme site, as no suitable sites were shown on the restriction maps published at this time, and the second strategy is difficult as it would necessitate separating and estimating the size of DNA fragments in excess of 40kb on agarose gels.

In order to avoid these problems, pTiGE2 was constructed (section 3.4.1). This oncogenic acceptor plasmid was constructed using a similar strategy to the construction of pTiGE1, but used a smaller T-DNA fragment for homologous recombination, enabling isolates containing a single region of pBR322 homology to be identified by Southern blotting, and giving a smaller T-DNA. The resultant plasmid also contains a single *onc* gene locus and hence the plasmid more closely resembles wild-type pTiC58 than does pTiGE1, giving greater confidence of desired gene delivery to plants.

4.1.5 ANTIBIOTICS FOR THE CONTROL OF *A. tumefaciens* GROWTH IN PLANT TISSUE CULTURE

The resistance of *A. tumefaciens* GV3101 [pGV3850::pDUB116] to carbenicillin in plant tissue culture was to some extent expected (section 3.1.6.1), as this construct contains two copies (at least) of the β-lactamase (Amp^r) gene derived from pBR322, and may have contained more copies of this gene as the cointegrate was not analysed for the presence of multiple copies of the integrating vector. In addition to the β-lactamase genes on the Ti plasmid, *A. tumefaciens* C58

has been shown to possess chromosomally located β-lactamase activity, albeit more active against ampicillin than carbenicillin (Leemans *et al.*, 1981). However, the strain was tested for resistance against carbenicillin in the hope that high concentrations of the antibiotic would overcome the level of resistance derived from these genes. Carbenicillin proved to be ineffective against this strain at 1.5mg/ml, the maximum level used, as it has been shown that above this level carbenicillin is toxic to *N. tabacum* tissues (J.R. Ellis, personal communication). 1mg/ml carbenicillin has been used successfully against a C58 strain in tissue culture (Wullems *et al.*, 1981) but this strain did not contain any plasmid born β-lactamases and hence the increased level of resistance in the strain tested in this work is assumed to be derived from the plasmid-borne Amp^r genes. The high level of resistance of the strain used to the cephalosporin cefotaxime was not expected and the results of Zambryski *et al.* (1984), who reported that 0.5mg/ml was inhibitory to the growth of C58 containing pGV3850 derivatives, could not be repeated, The strain was found to be sensitive to 1.5mg/ml cefotaxime, but at this concentration the explants would not form callus or shoots, making it unusable in the plant transformation system. However, other workers have successfully used cefotaxime against C58 strains containing plasmid-borne Amp^r genes (Herrera-Estrella *et al.*, 1984; Timko *et al.*, 1985; Schreier *et al.*, 1985; Van den Broek *et al.*, 1985; Kaulen *at al.*, 1986) The increased resistance to cefotaxime found in this work could possibly be due to gene dosage effects with high copy numbers of the Amp^r of pBR322 being present in the isolate used in the plant transformation due to multiple copies of pDUB116 being present in the cointegrate. The Amp^r gene of pBR322 is a TEM-1 class β-lactamase derived from the plasmid R1 (Heffron *et al.*, 1975) which does hydrolyse cefotaxime, but at a much slower rate than carbenicillin (Brown and Reading, 1983). Assuming that the isolate used did contain a large number of these genes, the increased gene copy number may have resulted in increased expression of the β-lactamase giving rise to the increased resistance. However, other workers have also found that cefotaxime is ineffective against similar *A. tumefaciens* strains in tissue culture (various personal communications) and some workers have resorted to other antibiotics (see later), suggesting that they have also experienced difficulties in preventing growth of these C58 strains.

A. tumefaciens GV3101 [pGV3850::pDUB116] was therefore screened for sensitivity to a number of antibiotics to identify possible alternatives to cefotaxime (section 3.1.6.2) The antibiotics chosen were all inhibitory to bacterial cell wall synthesis, as it has been shown that antibiotics with other modes of action are toxic to plant tissues (George and Sherrington, 1984; and references therein). A pTiAch5 strain (LBA4404 [pAl4404, Bin19]) was used as a control strain as it is known that this strain is sensitive to both carbenicillin and cefotaxime in plant tissue culture media (Bevan 1984; J.R. Ellis, personal communication), and hence gives a point of reference with regard to relative resistance, as the results obtained in the screen are not directly comparable to resistance in plant

tissue culture media due to to pH, media constituent and inoculum size differences. The results show that the C58 strain is resistant to a wide range of penicillins and cephalosporins whereas the pTiAch5 strain is relatively sensitive. Resistance to some of the penicillins such as penicillin G, ampicillin and carbenicillin, and some cephalosporins such as cefoperazone, cefazolin and cefotaxime can be attributed to the TEM-1 plasmid born β-lactamase which is absent in the control strain. Resistance to some penicillins such as cloxacillin and ticarcillin, and cephalosporins such as cephalexin, cefotoxin and ceftazidine cannot be due to this gene however, as these antibiotics are not hydrolysed by this class of β-lactamase (Richmond and Sykes, 1973; Brown and Reading, 1983), and must therefore be due to the chromosomal β-lactamase gene. The spectrum of activity of this gene would indicate that it is a class 3 or 4 β-lactamase.

Of the five antibiotics which were active against the C58 strain, augmentin and timentin were non-inhibitory to the shooting of explants, and augmentin proved to be the most suitable for use in plant tissue culture as it did not inhibit the rooting of shoots at concentrations active against the *A. tumefaciens* strain (sections 3.1.6.2- 3.1.6.4). Both augmentin and timentin are cocktail antibiotics containing a penicillin (amoxicillin and ticarcillin respectively) and clavulanic acid, which is a powerful inhibitor of both plasmid-borne and chromosomal β-lactamases (Reading, 1982). The preparation of augmentin used contains 16.7% clavulanic acid (timentin; 5.2% potassium clavulanate), is available commercially and is relatively inexpensive. This antibiotic is now the preferred method of inhibiting the growth of all *Agrobacterium* strains in this laboratory and has been used successfully against both *A. tumefaciens* and *A. rhizogenes* strains, in the transformation of *N. plumbaginifolia*, oilseed rape, potato, *Lotus* and *Arabidopsis*, with concentrations of 200μg/ml being used routinely in the media.

This is the first report of the use of augmentin in plant tissue culture to inhibit the growth of *Agrobacterium* strains, and the antibiotic appears to be superior to other antibiotics used for this purpose in terms of bacteriostatic activity and lack of inhibition of plant tissue responses. Other antibiotics which have now been used against similar C58 strains are vancomycin (Matzke *et al.*, 1984) and mixtures of carbenicillin, vancomycin and streptomycin (de Frammond *et al.*, 1986), but at much higher concentrations than those at which augmentin has been shown to be effective in this work.

An alternative approach to using antibiotics against *A. tumefaciens* in plant tissue culture is to use lytic bacteriophages. This approach was attempted using six different phage isolates and was partially successful (results not shown). This strategy has the advantage that no overnight washing of infected explants is needed and, after further development, it is envisaged that this could be the

method of choice in preventing growth of *A. tumefaciens* in plant tissue culture.

4.1.6 FURTHER POSSIBLE IMPROVEMENTS TO THE HOMOLOGY MEDIATED *A. tumefaciens* VECTOR SYSTEM

In addition to constructing integrating vectors which are more amenable to *in vitro* genetic manipulation (see section 4.1.2), considerable room for improvements to the pBR322 homology-mediated vector system are possible. One improvement would be to transfer the Ti acceptor plasmids to different (compatible) *A. tumefaciens* host strains to give the potential to transform a wider range of host species with high efficiency. For instance, the efficiency of T-DNA delivery from *A. tumefaciens* GV3101 containing pTiC58 derivatives to *N. tabacum* cultivars is low compared to *A. tumefaciens* Ach5 based vector systems (Frayley *et al.*, 1986), the converse being the case with some varieties of potato (A. Hepher, personal communication). Hence by transferring the acceptor Ti plasmids to (cured) host strains with widely differing host ranges, it may be possible to produce host/vector combinations which enable the vector system to transfer genes efficiently to a wider range of plant species and varieties than possible at the present time.

Another major improvement to the vector system would be use a strategy similar to that employed in the SEV (split end vector) system of Frayley *et al.* (1985) where the right and left T-DNA borders are on the integrating and acceptor plasmids respectively, and the resultant cointegrate formed by homologous recombination contains the right and left borders flanking the genes for subsequent delivery to plants. Various variations on this theme could be used in the vector system employed in this work but the simplest would be to include a left border sequence adjacent to the gentamycin resistance gene in an integration vector such as pDUB126a. The resultant cointegrates (including multiple cointegrates) would then contain only one copy of the integration vector which is bordered by both right (essential) and left T-DNA border sequences, and only monomer copies of the integrating vector should be transferred to plants. hence removing the need to screen for monomeric cointegrate structures by Southern blotting.

4.2 THE INVESTIGATION OF THE FUNCTIONALITY OF THE SV40 ENHANCER IN PLANTS

This experiment was conceived because the findings of Neuhaus *et al.* (1984) indicated that the SV40 enhancer was functional in *Acetabularia*, suggesting that the enhancer element may have a very broad host range and be active in higher plants. The experiment follows the classical strategy for assessing enhancer function by placing the putative element 5' and 3' and in both orientations with respect to an assayable marker (the Nos-NPT gene) and determining whether gene expression

is increased.

The NPT 2 coding sequence was chosen as the assay of Reiss *et al.* (1984) enables the gene product to be quantified and was a considerable improvement over other assays that were available at this time, such as the nopaline assay as used by Shaw *et al.* (1984b), which rely on visual comparisons. To determine whether the NPT assay is suitable in a study of this type, the assay was characterised and found to give good linearity of dose response (section 3.2.3.1), and the gene product was found to be relatively stable within the plant extracts (section 3.2.3.2). The reproducibility of the assay with respect to clonal lines of callus tissue showed that the response does vary, the highest to lowest values obtained having a ratio of 1.6 (section 3.2.3.3). This variation could be due to inaccuracies in the assay (protein determinations or loading of the gel) or in variations within the clonal lines of callus, but the level of variation was deemed to be tolerable as the SV40 enhancer, if fully functional in plants, would be expected to enhance gene expession well above this background level of variation. The assay does have the drawback that no commercial preparation of NPT2 is available and therefore a value for the NPT2 content of plant extracts cannot be assigned to each sample. The NPT2 activity of each sample can therefore only be compared with other samples run in the same assay (gel) as variations in the activity of similar samples run in separate assays do occur (results not shown). This would be expected in a method of this type, due to the difficulties of reproducing exact conditions for each individual assay, and hence this limits the number of samples which can be compared to each other at any one time, due to the relatively small number of samples which can be run on a single polyacrylamide gel.

One major source of variation in gene expression in plants is variation due to the so called 'positional'effects where individual transgenic plant lines express integrated genes at different levels. Similar variation in gene expression has been found in transgenic mice (Lacy *et al.* 1983) and *Drosophila* (Hazelrigg *et al.*, 1984) but the molecular basis for this differential expression is not known. Differences in expression of up to 200-fold have been found in transgenic plants (Jones *et al.*, 1985; An, 1986). In order to average out this variation and attempt to obtain some measure of the underlying level of expression of the Nos-NPT gene in the *in vivo* transformation of *K. diaigromontana*, a pattern of inoculation was conducted in order to cancel any callussing response differences between leaves and plants whilst at the same time generating large amounts of callus material (section 3.2.4). By pooling and subsequent extraction of callus derived from the long inoculation sites of five leaves and hence containing callus derived from a considerable (but undefined) number of individual transformation events, the detected variation of NPT 2 gene expression due to positional effects would be expected to be averaged out. The residual level of variation remaining after this process was then to be determined using pooled callus samples containing a single gene

construct, and subsequent differences in detected NPT2 levels above the residual positional effect variation could have then been assigned to differences due to increased expression of the different gene constructs due to increased promoter strengths. However, the failure of the assay to detect any NPT2 activity in callus tissue derived from pTiGE1::pDUB116 or the constructs containing the SV40 enhancer, prevented any estimation of the residual variation due to positional effects and made any conclusions regarding the functionality of the SV40 enhancer impossible.

There are a number of possible reasons why the Nos-NPT gene is not expressed in the callus induced *in vivo* at levels detectable by the NPT assay. Selection for the callus is by means of phytohormone independent growth but may be heterologous and consist of both transformed and non-transformed cells, with the untransformed cells undergoing undifferentiated proliferation due to the production of phytohormones from the transformed cells within the callus. Evidence to suggest that this can occur has been obtained by copy number determinations of T-DNA genes in callus produced *in vivo* where copy numbers of less than one were found (Scoffl and Baumann, 1985; Goldsbrough *et al.*, 1986), and in tissue transformed by *A. rhizogenes*, where some cells showing the hairy root phenotype were found not to contain functional *aux* genes (Melchers and Hooykaas, 1987; and references therein). Also, because there is no direct selection of the Nos-NPT gene, some of the transformed cells may not contain the gene due to incomplete transfer or rearrangement of the T-DNA. Whilst this phenomenon has been shown to be relatively uncommon with pTiC58 based vector systems (see later), the relatively large distance between the T-DNA phytohormone genes and the Nos-NPT gene means that the genes are not closely linked, and may increase the possibility of only the phytohormone genes being transferred and integrated into the plant genome. These phenomena, potentially giving rise to low proportions of NPT2 expressing cells within the callus, coupled with the relatively low amount of protein extracted from the callus samples and subsequently assayed and the low basal level of expression from the Nos promoter (Saunders *et al.*, 1987), probably account for the lack of detectable NPT2 activity.

The lack of detectable NPT2 activity in callus indicates that the Nos-NPT gene is unsuitable as an assayable marker in *in vivo* transformation systems and it is notable that there have been no reports in the literature of its use in any such system. The NPT2 coding region may be suitable if expression was greatly increased using a more active promoter region such as the CaMV 35S promoter which has been estimated to give 110-fold higher levels of expression of the NPT2 protein than the nopaline synthase promoter (Sanders *et al.*, 1987). Alternatively, a much more sensitive gene product/assay combination such as the glucuronidase system (Jefferson *et al.*, 1987) could possible be compatible with gene expression studies using *in vivo* transformation systems. However, since this experiment was conceived other transformation systems have been developed which are

superior to the type of transformation system used in this work and for a gene expression study of this type a protoplast transient assay system (Fromm *et al.*, 1986; Okada *et al.*, 1986; Nagata *et al.*, 1987) may now be the method of choice. Transient assay systems are rapid (results can be obtained in 48h) and have no requirement for DNA to be integrated into the plant genome, which avoids the problems which are encountered due to positional variation of gene expression in other systems.

In order to obtain conclusive evidence for the functionality of the SV40 enhancer in plants using the existing constructs, an *in vitro* transformation system with selection for both phytohormone independent growth and selection for the Nos-NPT gene was used (section 3.3), ensuring that cells of all callus tissue recovered contained the Nos-NPT gene. However, by directly selecting for the marker gene to be assayed, an element of bias is introduced into the system as recovery of tissue expressing the Nos-NPT gene at a high level may be more efficient than those expressing at a low level. This bias would tend to exaggerate any differences in Nos-NPT gene expression due to the presence of a functional enhancer element. However, the Nos-NPT gene has been shown to confer resistance to *N. tabacum* of up to 600μg/ml kanamycin (Bevan, 1984) and another similar Nos-NPT construct has been shown to confer kanamycin resistance to even higher levels (LD_{50} 750-1000μg/ml) (Frayley *et al.*, 1985) and hence the level of selection used in this work (100μg/ml) is relatively low and any such bias should be minimal.

Quantitation of NPT2 expression in pooled callus samples induced *in vitro* from a single construct (section 3.3.1) showed that even after pooling approximately 25 individual (clonal) calli in each sample assayed, the NPT2 activity varied 16-fold between samples. This high level of residual variation due to the positional effects (10-fold after correcting for the non-reproducibility of the assay, see section 3.2.3.3), after averaging by pooling the samples, indicates that the differences of NPT2 expression between individual clonal callus lines is extremely high.

Comparison of NPT2 activity in callus induced by constructs containing the SV40 enhancer sequence with the control construct (section 3.3.2) showed that the presence of the SV40 enhancer does not appear to influence expression from the Nos promoter. Other evidence which corroborates this finding is that the presence of the SV40 enhancer did not affect the extent of formation or the morphology of callus in either the *in vivo* or *in vitro* callusing systems, as may be expected due to enhanced expression of the *onc* genes, and no increase in the amount of nopaline in the callus produced *in vivo* could be detected. Southern blotting of DNA from a number of plant species (tobacco, pea, soya, wheat, potato and petunia) with a probe containing the SV40 enhancer failed to detect any homologous sequences, in contrast to monkey cells (McCutchan and Singer, 1981), indicating that DNA elements with homology to the SV40 enhancer are not present, which infers

that any *trans* acting factors which could interact with the SV40 enhancer to increase expression are unlikely to be present. Goldsbrough *et al.* (1986) have also shown that the SV40 enhancer does not increase expression of maize zein genes in sunflower callus.

In the light of more recent analysis of the SV40 promoter region, some doubt can be cast on the finding that the SV40 enhancer is functional in *Acetabularia*. Neuhaus *et al.* (1984) used the SV40 early promoter for expression of the T-antigens and compared two constructs, one with the 72bp repeat enhancer region, and one where the enhancer had been deleted. However, deletion of the enhancer region also deletes sequences now known to reduce expression from the early promoter *in vivo* and *in vitro* (Rio and Tjian, 1984) and the decrease in gene expression obtained from the construct with the deletion may be due to reduction in expression from the promoter rather than from removal of the enhancer sequence.

A number of enhancer and enhancer-like elements which function in plants have now been identified. Duplication of a 250bp element upstream of the CaMV-35S promoter (-90 to -343) has been shown to result in a ten-fold increase in the level of NPT2 expression from the CaMV 35S promoter. The element also had an effect on the levels of expression from T-DNA genes 5 and 7, but had no appreciable affect on expression of the nopaline synthase gene (Kay *et al.*, 1987). This element, and a 176bp element from the 5′ region of the octopine synthase gene, have also been shown to restore expression to a truncated maize *Adh-1* promoter in tobacco. The elements worked in an orientation independent manner, but the expression of the reporter gene was still under environmental control of the 247bp *Adh-1* promoter (Ellis *et al.*, 1987). Several plant genes have been shown to possess enhancer-like elements in their 5′ regulatory regions. A 268bp fragment from the wheat *Cab-1* gene (-89 to -347) has been shown to confer orientation-independent light-inducible expression to a truncated CaMV-35S promoter (Nagy *et al.*, 1987) and a 247bp fragment from a pea *Cab* gene similarly increases expression from the nopaline synthase promoter, with tissue-specific expression also being demonstrated (Simpson *et al.*, 1986). Similar elements have also been shown to be present in the 5′ regions of pea ribulose-1,5-bisphosphate carboxylase small-subunit genes (Timko *et al.*, 1985; Fluhr *et al.*, 1986) and in light-inducible gene from potato (Stockhaus *et al.*, 1987). An enhancer-like sequence which can increase gene expression in regenerating protoplasts has also been isolated (Horth *et al.*, 1987).

4.3 REGENERATION OF TRANSGENIC *N. tabacum* PLANTS WITH IMPROVED AGRONOMIC POTENTIAL

4.3.1 DETERMINATION OF GENOTYPE AND PHENOTYPE OF REGENERATED PLANTS

Southern blot analysis of the T-DNA of the transgenic *N. tabacum* containing the CaMV 35S-*LecA* chimaeric gene indicated that the majority of the plants (11 of the 14 plants analysed, including the controls) contained full length T-DNA copies. Of these, only one plant contained a T-DNA copy number in excess of five (plant L11). Two plants (L6 and C2) did not contain any full length T-DNA copies and many of the plants also contained rearranged T-DNA in addition to the full length copies, as determined using the pBR322 probe. These results show a similar pattern of T-DNA copy number and rearrangements in transgenic plants to other published results using the pGV3850 vector system (Budar *et al.*, 1986; Chyi *et al.*, 1986; Czernilofsky *et al.*, 1986; Bytebier *et al.*, 1987; Sanchez-Serrano *et al.*, 1987).

Attempts to determine the lectin expressing phenotype of plants by ELISA of leaf extracts proved inconclusive and plants expressing pea lectin at a low level could not be assigned a definite phenotype (see later discussion). The lectin expressing phenotype was therefore determined using western blotting of nd-PAGE separated extracts, as this technique enables far more total protein to be immobilised onto a solid matrix and hence enabled plants expressing pea lectin at a low level to be identified. The results obtained from this screen and from the nopaline assay showed that all of the plants had phenotypes corresponding to the presence of the respective genes within the T-DNA with the exception of plant L11. This plant contained in excess of five T-DNA copies and yet no lectin was detected by western blotting and nopaline was also not apparent. However, when assayed using ELISA, this plant gave an apparent lectin content well above that detected in the control plants. One possible reason for this disparity is that different leaves were used for the DNA extraction and for each of the three assays and it is possible that the plant was chimaeral, having been regenerated from two or more individual cells. Periclinal chimaeras, in which the cells of the different apical layers of the tunica have different genotypes, have previously been observerved in tobacco plants regenerated from mixed callus cultures (Carlson and Chaleff, 1975). The different phenotypes found in individual leaves in this plant would indicate that the plant is a sectored periclinal (mericlinal) chimaeron, where the genotype of any individual leaf would be dependent upon its position within the developmental spiral of the plant (Stewart and Dermen, 1974).

4.3.2 EXPRESSION OF THE CaMV-*LecA* CHIMAERIC GENE IN TRANSGENIC TOBACCO PLANTS

Northern blotting of total transgenic leaf RNA (section 3.5.8) indicates that the chimaeric gene is correctly transcribed and polyadenylated, and is also stable within tobacco leaf tissue. The lectin message has now been quantified in the high expressing plant (L9) RNA preparation shown in the northern blot (figure 3.36 and 3.37) nd was found to be 0.03% of total RNA, compared to a maximum of 0.15% of total RNA in developing (16 days after flowering) pea cotyledons (A. Thompson, unpublished results).

Attempts to quantify the pea lectin content of transgenic tobacco leaves by the ELISA method employed (section 3.5.4) proved unsatisfactory. It was not possible to identify plants expressing lectin at low levels and the results obtained for the plants expressing lectin at a higher level were not reproducible using this method. The limit of detection of the assay was in the region of 1ng/ml lectin (as seen from the standard curve) and the total (saturated) binding microtitre plate wells was found to be approximately 1.5μg/ml of soluble leaf protein, as determined in the competition assay. The minimum level of expression which could be detected was therefore approximately 0.1% of total soluble protein, even when the maximum amount of extracted protein was assayed (by saturating the binding capacity of the wells with an excess of protein). Also, the maximum signal obtained, even for the highly expressing plants, is relatively low, introducing inaccuracies in the results due to the lack of precision of the plate reader at low optical densities. The other problem with the assay is the high and variable background signal obtained, due to β-galactosidase activity in the leaf extracts. β-galactosidase activity in *N. tabacum* has previously been demonstrated (Helmer *et al.*, 1984) and assays conducted which omitted the primary antibody showed that the high background is due to inherent enzymic activity and not to any cross-reacting proteins in the leaf extract (results not shown), evidence which is corroborated by the lack of cross-reacting bands on western blotting. Attempts to correct for the background, by running duplicate assays and treating each individual plant extract both with and without the primary antibodies, gave results which were not reproducible, due to the very low signals obtained (results not shown). Possible ways to reduce the problem of interfering enzymic activity would be to use commercially available secondary antibodies conjugated with other enzymes such as alkaline phosphatase, glucose oxidase or horse radish peroxidase, which may give lower backgrounds. Another stratagy would be use a 'sandwich'technique where the microtitre plate is pretreated with primary antibody prior to blocking. The antigen then binds to the primary antibody and is quantified by binding to a second antibody, conjugated with the desired enzyme. This type of method overcomes the difficulties associated with both interfering enzymic activities and with the low binding capacities of the plates (Staehelin *et al.*, 1981). Similarly,

radio immuno-assay techniques could also be used to overcome these problems (see later).

Although levels of lectin expression in the plants were not determined accurately, the ELISA assay did enable plants expressing lectin at a relatively high level to be identified and subsequent comparison of band intensities on the (nd-PAGE) western also indicated that these plants expressed lectin at a relatively high level. Western blotting of SDS-PAGE separated samples (sections 3.5.9.1 and 3.5.9.2) gave expression levels of approximately 0.5% of total soluble protein in expanded leaves, as estimated by comparing the intensities of the lectin β-subunit band with that of the controls of purified pea lectin.

Comparisons of the maximum amount of pea lectin expression from the CaMV-35S promoter in *N. tabacum* leaves found in this work to the maximum levels of expression found by other workers using different coding regions is complicated by several factors. Detected expression may vary due to positional effects and the size of the transgenic plant population studied, which may result in underestimations of the maximum possible levels of expression from the promoter. Variation may be due to differential transcription rates, RNA stability, or differences in translational initiation and elongation rates, and the stability of individual proteins, which in turn may be dependent on the subcellular site of deposition, may also effect the levels of protein accumulated. The stage of leaf development at which sampling has been conducted is anothervariable that may affect the estimated expression levels. The CaMV-35S promoter, although constitutive with regard to expression in different tissues, is expressed only during the S-phase of the cell cycle (Nagata *et al.*, 1987). However, the cell layers of tobacco leaves stop dividing at different stages of leaf development. The cells of the upper epidermis cease dividing when the leaf is 6-7cm long (approximately one fifth/ one sixth its final surface area) and continued increase in size is by cellular expansion. Cells destined for the spongy mesophyl layer terminate cell division after the the epidermal cells but before the pallisade cells, which stop division just before leaf expansion ceases (Wareing and Phillips, 1981). Also the protein content (% of dry weight) of leaves changes during leaf development and ageing (Vianna and Metivier, 1980). An indication that the stage of leaf development at sampling may indeed affect detected expression levels was obtained from western blotting, where the younger unexpanded leaf extract did appear to contain higher levels of lectin than the older leaf (section 3.5.9.2). Despite this large potential for variation in the level of accumulation of gene products, the level of pea lectin in transgenic tobacco leaves (0.5%) compares favourably with those of other gene product levels detected in *N. tabacum* leaves when expressed from the CaMV-35S promoter, with maximum levels (% of total soluble leaf protein) of 1% for the cowpea trypsin inhibitor (Hilder *et al.*, 1987), 0.4% (0.8% in tomato) for the alfalfa mosaic virus coat protein (Tumer *et al.*, 1987), and 0.1-0.16% for the tobacco mosaic virus coat protein (Bevan *et al.*, 1985; Abel *et al.*, 1986). One class of proteins which

has not been expressed to these relatively high levels is the *Bacillus thuringensis* crystal proteins, with maximum reported levels being in the order of 0.001% of total soluble leaf protein (Fischoff *et al.*, 1987; Barton *et al.*, 1987) but this is possibly due to instability of the mRNA which has been shown to be degraded in tobacco leaves (Barton *et al.*, 1987).

Conclusive evidence that both subunits of pea lectin are correctly processed in tobacco plants to give the same mature forms found in pea cotyledons could not be obtained by immunological detection in SDS-PAGE separated extracts due to the inability of the antibody preparation to bind to the α-subunit. This could possibly be due to the structure of the mature pea lectin, where the β-subunits are arranged as β-pleated sheets around the α-subunits and hence may mask the antigenic sites (Einspahl *et al.*, 1986). However, the results (sections 3.5.9.1 and 3.5.9.2) do show that the β subunit is present in all the tobacco tissues and co-migrates with the pea cotyledon lectin β subunit, indicating that removal of the signal peptide, cleavage of the prolectin and C-teminal processing to give a 179aa β subunit all occur correctly. Bands detected at approximately M_r25000, which are also present in the pea lectin preparation, and a band at M_r18000, which corresponds to the β-subunit prior to C-terminal processing, indicate that the β-subunit is processed in tobacco by a similar mechanism to that which occurs in the pea cotyledon. The distinct β-subunit bands and the lack of any smaller cross-reacting bands (with the exception of the tobacco seed extract) indicate that pea lectin is not significantly degraded in tobacco tissues.

Immunological detection of nd-PAGE separated extracts (sections 3.5.5 and 3.5.9.2) shows that the lectin in the transgenic tobacco plants co-migrates with some of the pea cotyledon isolectins. This indicates that the overall subunit structure in tobacco is the same as in pea ($\alpha_2\beta_2$) (although some of the isolectins in the lanes containing the pea lectin do not show up well on the photographs, these could be seen quite clearly on the freshly processed filters). The co-migration of the isolectins in tobacco with isolectins of the pea cotyledon lectin also suggests that the α-subunit is C-terminally processed correctly in tobacco plants in a similar manner as in pea cotyledons. Assuming that the pea isolectins migrate in the order (of increasing migration rate) 2 3 1 (i.e. net charges of +2, +1 and 0 respectively, see introduction) bands can be seen in the tobacco stem, leaves and roots which correspond to isolectins 1 and 3, and all three isolectin forms are present in the tobacco seeds. Whilst more work is needed to further characterise the isolectins expressed in transgenic tobacco (see section 4.3.4) these results strongly indicate that pea lectin expressed in all tobacco tissues is correctly processed.

The haemaggluttination activity of extracts of transgenic tobacco leaves (section 3.5.9.3) shows that the activity of the lectin is comparable with pea cotyledon lectin. Whilst correct pro-

cessing of pea lectin is not a pre-requisite for its activity (see section 1.6), the correlation between the amount of pea lectin present in transgenic tobacco leaves and the haemagglutination activity corroborates the findings that the lectin has the correct overall subunit structure and is stable in the transgenic plants.

A number of other proteins which accumulate in the seed storage bodies of legume seeds have now been expressed in transgenic plants, including the soybean seed lectin in tobacco (Okamuro *et al.*, 1986), the *Phaseolus vulgaris* lectin in tobacco (Sturm *et al.*, 1988), pea legumin in *N. plumbaginifolia* (Ellis *et al.*, 1988), a β-phaseolin gene tobacco (Sengupta-Gopalan *et al.*, 1985) and a soybean β-conglycinin gene in petunia (Beachy *et al.*, 1985). All of these genes were expressed from their own promoter and contained the signal peptides for transport of the gene product to the protein bodies. These proteins were all correctly processed to give subunit structures as found in the legume seeds, and some were also shown to be associated with the protein bodies in the transgenic plant seeds (Sturm *et al.*, 1988; Ellis *et al.*, 1987.). However, expression of these genes was limited to the seed and hence no information was obtained on the targeting and processing of these proteins in vegetative tissues. The immuno-localisation of the pea lectin in the vacuoles of root cells (section 3.5.9.5) is believed to be the first evidence that a signal peptide which normally targets a seed protein to protein bodies can target the protein to vacuoles in vegetative tissue. Correct processing of seed storage proteins in the heterologous tissues of transgenic plants may be dependent on such targeting, as a phaseolin gene expressed from a constitutive promoter in sunflower callus but which had part of the signal peptide removed was not processed correctly (Murai *et al.*, 1983). Another seed protein which contains a signal peptide which has been expressed in transgenic plants from a constitutive promoter is the cowpea trypsin inhibitor (Hilder *et al.*, 1987), but the subcellualar site of deposition in both the cowpea or the transgenic tobacco has not been determined (V. Hilder, personal communication).

Pea lectin could not be detected on the root surface of transgenic tobacco by fluorescence microscopy and by the red blood cell binding assay. This suggests that the mechanism for exporting the protein to an extracellular site is not present in tobacco.

4.3.3 SCREENING OF LECTIN-EXPRESSING TRANSGENIC PLANTS FOR RESISTANCE TO INFECTION BY *Meloidogyne incognita*

No differences in the level of infection by the root-knot nematode in expressing and nonexpressing plants were apparent (section 3.5.9.6). However, the method used was not entirely suitable for detecting increased resistance to these nematodes. The number of nematodes used in the challenge was far in excess of that which would normally be encountered in a field infestation and satu-

rated the roots with galls, making it impossible to detect decreased infection efficiencies. *Meloidogyne incognita* often infects resistant plants but then migrates out of resistant hosts, an effect which would also have been masked by the massive inoculum. Also, the reproductive rates of the nematode were not investigated, with the plants only being examined over a time period sufficient for less than one reproductive cycle. Resistant plants often restrict the reproductive capacity of the nematode and several cycles of infection should have been studied to assess the levels of resistance of the transgenic tobacco plants(Franklin, 1978).

4.3.4 CONTINUING WORK AND FUTURE PROSPECTS

The pea lectin gene has now been introduced into a major temperate crop plant under the control of both a constitutive and a tissue specific promoter, in a collaborative programmewith a major agrochemical company. Levels of expression (using a radioimmuno-assay) and further characterisation of the qualitative expression of this gene is currently in progress, including isoelectic focussing of isolectins and subcellular localisation in a number of transgenic tissues. These plants will undergo field trials in order to assess possible yeild penalties incurred, and will shortly be assayed for increased resistance to a range of insect pests. Potential resistance to nematode pests will also be assessed and this report is thought to be the first to suggest a mechanism for engineering nematode resistance. Work on the potential anti-fungal properties has been abandoned as pea lectin has not shown any any anti-fungal properties *in vitro* (A.M.R. Gatehouse, personal communication) and previous reports of anti-fungal properties of other lectin preparations has been shown to be due to contaminating chitinases (Schlumbaun *et al.*, 1986).

Another experiment which is being considered is to attempt to to manipulate the *Rhizobium* binding specificity of a suitable forage legume by the expression of pea lectin.

REFERENCES

Abel, P.P., Nelson, R.S., Barum, D., Hoffman, N., Rogers, S.G. Frayley R.T. and Beachy, N.R. (1986). Delay of desease resistance in transgenic plants that express the tabacco mosaic virus coat protein. *Science 232,* 738-743.

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W. and Gordon, M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme for cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA. 81,* 5994-5998.

An, G. (1985). High efficiency transformation of cultured tobacco cells. *Plant Phys. 79,* 568-570.

An, G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline promoters in transformed tabacco plants. *Plant Physiology 81,* 86-91.

An, G, Watson, B., Stachel, S., Gordon, M. and Nester, E. (1985). New cloning vehicles for the transformation of higher plants. *EMBO J. 4,* 277-284.

Banerji,J., Rusconi,S. and Shaffner,W. (1981). Expression of the rabbit β-globin gene is enhanced by remote SV40 DNA sequences. *Cell 27,* 299-308.

Banerji,J., Olsen,L. and Shaffner,W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobin heavy chain genes. *Cell 33.* 729-740.

Barkai-Golan, R., Mirelmar, D. and Sharon, N. (1978). Studies on growth inhibition by lectins of *Penecillia* and *Aspergilli. Arch. MiKrobiol 116,* 414-416.

Barry, G.F., Rogers. S.G., Fraley, R.T. and Brand, L. (1984). Identification of a cloned cytokinin biosynthetic gene. *Proc. Natl. Acad. Sci. USA. 81* 4776-4780.

Barton, K.A., Binns, A.N., Matzke, A.J. and Chilton, M-D. (1983). Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA and transmission of T-DNA to R1 progeny. *Cell 32,* 1033-1043.

Barton, K.A., Whitely, H.R. and Yang, N-S. (1987). *Bacillus thuringensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiology 85,* 1103-1109.

Baulcombe,D.C., Saunders,G.R., Bevan,M.W., Mayo,M.A. and Harris, D.B. (1986). Expression of biologically active satellite RNA from the genome of transformed plants. *Nature 321,* 446-449.

Beachy, R.N., Chen, Z.L., Horsch, R.B., Rogers. S.G., Hoffman, N.J. and Frayley, R.T. (1985).

Accumulation and assembly of soybean β-conglycinin in seeds of transformed petunia plants. *EMBO J. 4*, 3047-3053.

Beaty, J.S., Powell, G.K., Lica, L., Regier, D.A., Macdonald, E.M.S., Hommes, N.G. and Morris, R.O. (1986). *Tzs*, a nopaline Ti plasmid gene from *Agrobacterium tumefaciens* associated with *trans*-zeatin biosynthesis. *Mol. Gen. Genet. 203*, 274-280.

Beck, E., Ludwig, G., Auerswald, E., Reiss, B. and Schaller, H. (1982). Nucleotide sequence and exact localisation of the neomycin phosphotransferase from Tn5. *Gene 19,* 327-336.

Begbie, R. and King, T.P. (1985). The interaction of dietry lectin with porcine small intestine and the production of lectin specific antibodies. In : Bog-Hansen, T.C. and Boboritch, J. (Eds.) Lectins biology, biochemistry, clinical biochemistry. Vol 5, 15-27. de Gruyter and Co. New York. Berlin.

Bennet,M.D. and Smith,J.B. (1976). Nuclear DNA amounts in angiosperms. *Phil. Trans. Royal. Soc (Lon). B 274*, 227-276.

Benoist,C. and Chambon,P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. *Nature 290*, 304-310.

Bevan, M.W. and Chilton, M-D. (1982a). T-DNA of the *Agrobacterium* Ti and Ri plasmids. *Ann. Rev. Genet. 16*, 357-364.

Bevan, M. and Chilton, M-D. (1982b). Multiple transcripts of T-DNA detected in nopaline crown gall tumours. *J. Mol. Appl. Genet. 1*, 539-548.

Bevan, M. and Flavell, R.B. (1983). A chimaeric antibiotic resistance gene as a selectable marker for plant transformation. *Nature 304*, 184-187.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant trans formation. *Nucl. Acids. Res. 12*. 8711-8721.

Bevan, M., Mason, S.E. and Goelet, P. (1987). Expression of tabacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plant cells transformed by *Agrobacterium tumefaciens. EMBO J. 4*, 1921-1926.

Birnboim,H.C. and Doly,J. (1979). A rapid alkaline extraction procedure for screening recombinant DNA.*Nucl. Acids. Res. 7*, 1513-1523.

Bolivar,F., Rodriques,R., Greene,P., Betloch,M., Heynecker,H., Boyer,M., Crosa,J. and Falkow,S. (1977). Construction and characterisation of new cloning vehicles. A multipurpose cloning system. *Gene 2*, 95-103.

Boulter,D. (1986). In "Biomolecular Engineering in the European Community, Achievments in

the research programme (1982-1986)," final report. (715-725). E. Magnien (Ed). Martinus Nijkoff publishers.

Bradford,M. (1976) A rapid and sensitive method for the quantitation of micro-gram quantities of protein using the principle of dye binding. *Analyt. Biochem. 72*, 248-254.

Brown, A.G. and Reading, C. (1983). The β-lactam antibiotics. *Culture 4*. (Published by Oxoid Ltd. Basingstoke. UK.)

Buchmann, I., Marner, F-J., Schroder, G., Waffenschmidt, S. and Shroder, J. (1985). Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. *EMBO J. 4*, 853-859.

Budar, F., Thia Toong, L., Van Montagu, M. and Hernalsteens, J.P. (1986). *Agrobacterium*-mediated gene transfer results mainly in transgenic plants transmitting T-DNA as a single mendelian factor. *Genetics 114*, 303-313.

Bytebier, B., Doboek, F., De Greve, H., Van Montagu, M. and Hernalsteens, J.P. (1987). T-DNA organisation in tumour cultures and transgenic plants of the monocotyledon *Asparagus officinalis. Proc. Natl. Acad. Sci. USA. 84*, 5345-5349.

Carlson, P.S. and Chaleff, R.S. (1975). Heterologous associations of cells formed *in vitro*. In 'Genetic manipulation with plant material' 245-261. L. Ledoux (Ed). Plenum Press. New York.

Cereghini,S., Piette,J., Kryske,M.H. and Yaniv,M. (1985). Interaction of cellular factors with the SV40 and polyoma enhancers. In : Eukaryotic transcription, the role of *cis* and it trans acting elements in initiation. 78-85. *Current Communications in molecular biology.* Gluzman.Y. (Ed). Cold Spring Harbor. N.Y.

Chahal, V.P. and VilKhu, K.S. (1985). Transient appearance of lectin receptors in homologous and heterologous strains of *Rhizobium* capable of nodulating pea (*Pisum sativum L.*). *Microbiology 54*, 642-646,

Chyi. Y.S., Jorgensen, R.A., Goldstein, D., Tanksley, S.D. and Loaiza-Figueroa, F. (1986). Locations and stability of *Agrobacterium*-mediated insertions in the *Lycopersicon* genome. *Mol. Gen. Genet. 204*, 64-69.

Czernilofski, A.P., Hain, R., Herrera-Estrella, L., Lorz, H. Goyvaerts, E., Baker, B. and Schell, J. (1989). Fate of selectable marker DNA integrated into the genome of *N. tabacum. Gene 5*, 101-103.

Dagert.M. and Ehrlich.S.D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene 6*, 23-28.

David, C., Petit, A. and Tempe, J. (1988). T-DNA length variability in hairy roots: more than

50kb of pRi T-DNA can integrate into plant cells. *Plant Cell Rep. 7,* 92-95.

Deasey, M.C. and Matthysse, A.G. (1984). Interactions of wild-type and a cellulose-minus mutant of *Agrobacterium tumefaciens* with tobacco mesophyl and tobacco tissue culture cells. *Phytopathology 74.* 991-994.

De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J. and Zambryski, P. (1984). Expression of foreign genes in regenerated plants and in their progeny. EMBO J, 1681-1688.

De Block, M., Schell, J. and Van Montagu, M. (1985). Chloroplast transformation by *Agrobacterium tumefaciens. EMBO J. 4,* 1367-1372.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Rao Morra, N., Thompson, C., Van Montagu, M. and Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J. 6* 2513-2518.

De Framond, A.J., Barton, K.A. and Chilton, M-D. (1983). Mini-Ti: a new stratagy for plant genetic engineering. *Biotechnology 1,* 262-269.

De Frammond, A.J., Back, E.W., Chilton, W.S., Kayes, L. and Chilton, M-D. (1986). Two unlinked T-DNA's can transform the same tobacco plant and segregate in the F1 generation. *Mol. Gen. Genet. 202,* 125-131.

Depicker, A., De Wilde, M., De Vos, G., De Vos, R., Van Montegu, M. and Schell, J. (1980). Molecular cloning of overlapping segmentds of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. *Plasmid 3,* 193-211.sig

Dessaux, Y., Petit, A., Tempe, J., Demarez, M., Legrain, C. and Wiame, J.M. (1986). Argenine catabolism in *Agrobacterium* strains: role of the Ti plasmid. *J. Bacteriol. 166.* 44-50.

Dhaese,P., De Greve,H., Decraemer,H., Schell,J. and Van Montagu,M. (1979). Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmid of *Agrobacterium tumefaciens. Nucl. Acids. Res. 7.* 1837-1849.

Diaz,C.L., Van Spronsen,P.C., Bakhiusen,R., Logman,G.J., Lugenberg,E.J. and Kinge,J.W. (1986). Correlation between infection by *Rhizobium leguminosarum* and lectin on the surface of *Pisum sativum* L. roots. *Planta 168,* 350-359.

Diaz, C.L., Lems-Van Kan, P., Van der Shaal, I.A. and Kijne, J.W. (1984). Determination of pea (*Pisum sativum* L. root lectin using an enzyme linked immunoassay. *Planta 161,* 302-307.

Dretzen,G., Bellard,M., Sassone-Corsi. P.and Chambon,P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Analyt. Biochem. 112,*

295-298.

Einspahr, H., Parks, E.H., Suguna, K. and Subramanian, E. (1986). The crystal structure of pea lectin at 3.0 Angstrom resolution. *J. Biol. Chem. 261,* 16518-16527.

Ellis, J.G., Kerr, A., Petit, A. and Tempe, J. (1982). Conjugal transfer of nopaline and agropine Ti-plasmids. The role of agrocinipines. *Mol. Gen. Genet. 186,* 269-274.

Ellis, J.G., Llewellyn, D.J., Dennis, E.S. and Peacock, W.J. (1987). Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tabacco. *EMBO J. 6,* 11-16.

Ellis,J.R. Shirsat,A.H., Hepher,A., Yarwood,J.N., Gatehouse,J.A., Croy,R.R.C. and Boulter,D. (1988). Tissue specific expression of a Pea legumin gene in seeds of *Nicotiana plumbaginifolia. Plant Mol. Biol. 10,* 203-214.

Engvall,E. (1980). Enzyme immunoassay: ELISA and EMIT. *Meths. Enzym. 70,* 419-439.

Fischoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Frayley, R.T. (1987). Insect tolerant transgenic tomato plants. *Biotechnology 5,* 807-813.

Fluhr, R., Kuhlemeier, C.G., Nagy, F. and Chau, N-H. (1986). Organ specific and light induced expression of plant genes. *Science 232,* 1106-1112.

Fraley, R.T., Rogers, S., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, P.S., Bittner, M.L., Brand, L.A., Fink, C.C., Fry, J.S., Galluppi, G.R., Goldberg, B.S., Hoffman, N.L. and Woo, S.G. (1983). Expression of bacterial genes in plant cells. *Proc Natl. Acad. Sci. USA. 80,* 4803-4807.

Fraley, R.T. Rogers, S., Horsch, R.B., Eichholtz, D.A., Flick, J.S., Fink, C.L., Hoffman, N.L. and Sanders, P.R. (1985). The SEV system: A new Ti plasmid vector system for plant transformation. *Biotechnology 3,* 629-635.

Fraley, R.T., Rogers,S.G. and Horsch, R.B. (1986). Genetic transformation in higher plants. *Critical Rev. Plant. Sci. 4,* 1-45.

Franklin, M.T. (1978). *Meloidogyne.* In: Plant nematology. Southey, J.F. (Ed). 98-124. MAff/ADAS. HMSO GD01.

Fromm,M. and Berg,P. (1983). Simian virus 40 early- and late-region promoter functions are enhanced by the 72bp repeat inserted at distant locations and inverted orientations. *Mol. Cell Biol. 3,* 991-998.

Fry, J., Barnason, A. and Horsch, R. (1987). Transformation of *Brassica napus* with *Agrobac-*

terium based vectors. *Plant Cell Reports 6* 321-325.

Garfinkel, D.J. and Nester, E.W. (1980). *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine metabolism. *J. Bacteriology 144*, 732-743.

Garfinkel, D.J. Simpson, R.B., Ream, L.W., White, F.F., Gordon, M.P. and Nester, E.W. (1981). Genetic analysis of crown gall: fine structure map of the T-DNA by site directed mutagenesis. *Cell 27*, 143-153.

Gatehouse, A.M.R., Dewey, F.M., Dove, J., Fenton, K.A. and Pusztai, A. (1984). Effect of seed lectins from *Phaseolus vulgaris* on the development of Callosobruchus maculatus; mechanism of toxicity. *J. Sci. Food Agric. 35*, 373-380.

Gatehouse, J.A. and Boulter, D. (1980). Isolation and properties of a lectin from the roots of *Pisum sativum. Physiol. Plant 49*, 437-442.

Gatehouse, J.A., Evans, I.M., Croy, R.R.D. and Boulter, D. (1986). Differential expression of seed genes during legume seed development. *Phil. Trans. R. Soc. Lond. B 314*, 341-500.

Gatehouse, J.A., Bown, D., Evans, M, Gatehouse, L.N. and Croy, R.D. (1987). Sequence of the seed lectin gene from *Pisum sativum* L. *Nucleic Acids Res. 15*, 7642.

George,E. and Sherrington,P. (1984). Plant propagation by tissue culture. Eastern Press. Reading, Berks. UK.

Godson,N. and Vapnek,G. (1973). A simple method for preparing large amounts of ϕx174 RF1 supercoiled DNA. *Biochim. Biophys. Acta. 299*, 516-520.

Goldsbrough. P.B., Gelvin, S.B. and Larkins. B.A. (1986). Expression of maize zein genes in transformed sunflower cells. *Mol. Gen Genet. 202*, 374-381.

Graham,D. (1978). The isolation of high molecular weight DNA from organisms or large tissue masses. *Analyt. Biochem. 85*, 609-613.

Grunstein,M. and Hogness,D. (1975). Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA. 72*, 3961-3975.

Hain. R., Steinbiss. H.H. and Schell, J. (1984). Fusion of *Agrobacterium* and *E. coli* sphaeroplasts with *Nicotiana tabacum* protoplasts: direct gene transfer from microorganisms to higher plants. *Plant Cell Rep. 3*, 60-64.

Hall,T.C., Ma.Y., Buchbinder,B.U., Pyne,J.W., Sun,S.M. and Bliss,F.M. (1978). Messenger RNA for G1 protein of French bean seeds: Cell free translation and product character isation. *Proc. Natl. Acad. Sci. USA. 75*, 3196-3200.

Hamblin,J. and Kent,S.P. (1973). Possible role of phytohaemagglutinin in *Phaseolus vulgaris*. *Nature New Biol. 245*, 28-30.

Hames,D. (1981). An introduction to polyacrylamide electrophoresis. In "Gel electrophoresis of proteins: a practical approach" (Hames,D. and Rickwood,D. Eds) IRL Press Ltd, London. 1-99.

Haughn, G.W., Smith, J., Mazur, B. and Somerville, C. (1988). Transformation with a mutant acetolactate synthase gene renders tabacco resistant to sulphonyl urea herbicides. *Mol. Gen. Genet. 211*, 266-271.

Hazelrigg, T., Levis, R. and Rubin, G. (1984). Transformation of *white* locus DNA in *Drosophila*: dosage compensation and possition effects. *Cell 36,* 469-481.

Heffron, F., Sublett, R., Hedges, R.W., Jacob, A. and Falkow, S. (1975). Origin of the TEM-β lactamases found on plasmids. *J. Bacteriol. 122,* 250-256.

Heidecker,G. and Messing,J. (1986). Structural analysis of plant genes. *An. Rev. Plant Physiol. 37*, 439-466.

Helmer, G., Casadaban, M., Bevan, M., Keyes, L. and Chilton, M-D. (1984). A New chimaeric gene as a marker for plant transformsation: the expression of *Escherichia coli* β-galactosidase in sunflower and tobacco cells. *Biotechnology 3*, 520-526.

Hernalsteens, J-P., Van Vliet, F., DeBeuckeleer, M., Depicker, A., Engler, G., Lemmers, M., Holsters, M., Van Montagu, M. and Schell, J. (1980). The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign genes into plant cells. *Nature 289*, 654-656.

Herr,W. and Gluzman,Y. (1985). Duplications of a mutated Simian virus 40 enhancer restore its activity. *Nature 313*, 711-714.

Herr,W., Clarke,J. and Gluzman,Y. (1985). Sequence Duplications that reactivate mutated SV40 enhancer elements. In : Eukaryotic transcription, the role of *cis* and its trans acting elements in initiation. 27-32. *Current Communications in molecular biology.* Gluzman,Y. (Ed). Cold Spring Harbor. N.Y.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.P., Van Montagu, M. and Schell, J. (1983). Chimaeric genes as dominant selective markers in plant cells. *EMBO J. 2*, 987-995.

Herrera-Estrella, L., Van den Broek, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. (1984). Light inducable and chloroplast associated expression of a chimaeric gene introduced into *N. tabacum* using a Ti vector. *Nature 310,* 115-120.

Higgins, T.J.V., Chandler, P.M., Zurawaski, G., Button, S.C. and Spencer, D. (1983a). The synthesis and primary structure of pea seed lectin. *J. biol. Chem. 258*, 9544-9549.

Higgins, T.J.V., Chrispeels, M.J., Chandler, P.M. and Spencer, D. (1983b). Intracellular sites of synthesis and processing of lectin in pea cotyledons. *J. biol. Chem. 258*, 9550-9552.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F. and Boulter, D. (1987). A novel mechanism of insect resistance engineered into tobacco. *Nature 330*, 160-163.

Hille, J., Verheggen, F. Roelvink, P., Franssen, H., Van Kammen, A. and Zambel, P. (1986). Bleomycin resistance: a new dominant selective marker for plant cell transformation. *Plant Mol. Biology 7*, 171-176.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983). A binary vector stratagy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti plasmid. *Nature 303*, 179-180.

Holsters,B., Silva,B., Van Vliet,F., Genetello,M., De Block,M., Van Montagu,M. and Schell,J. (1980). The functional organisation of the nopaline *A.tumefaciens* plasmid pTiC58. *Plasmid 3*, 212-230.

Holsters, B., Villarroel, R., Gielen, J., Seurinck, J., De Greve, H., Van Montagu, M., and Schell, J. (1983). An analysis of the boundries of the octopine T_L-DNA in tumours induced by *Agrobacterium tumefaciens. Mol. Gen. Genet. 190*, 35-41.

Horsch.R.B., Fry,J.E., Hoffman,N.L., Eicholtz,D., Rogers,S.G. and Frayley,R.T. (1985). A simple method for transferring genes into plants. *Science 227*, 1129-1231.

Horth, M., Negrutin, I., Burny, A., Van Montagu, M. and Hererra-Estrella, L. (1987). Cloning of a *Nicotiana plumbaginofolia* protoplast specific enhancer-like sequence. *EMBO J. 6* 2525-2530.

Hosslet, M., Van Driessche, E. Van Poucke,M. and Kanarek, L. (1983). Purification and Characterisation of an endogenous root lectin from *Pisum sativum* L. In : Bog-Hansen, T.C. and Spengler, G.A. (Eds.) Lectins biology, biochemistry, clinical biochemistry. Vol 3, 549-558. de Gruyter and Co. New York. Berlin.

Jayaprakash, A., Jansson, H.B., Marban-Mendoza, N. and Zuckerman, B.M. (1984). *Caenorhabditis elegans*: Lectin-mediated modification of chemotaxis. *Experimental Parasitology 59*, 90-97.

Jefferson, R.A., Bevan, M. and Kavanagh, T. (1987). The use of the β-glucuronidase gene as a gene fusion marker for studies of foreign gene expression in higher plants. *Biochem. Soc. Trans. 15*, 17-18.

Johnson,D.A., Gautsch,J.W., Sportsman,J.R. and Elder,J.H. (1984). Improved technique utilising nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Analysis Techniques 1*, 3-8.

Jones, J.D.G., Dunsmuir, P., and Bedbroek, J. (1985). High level expression of integrated genes in regenerated transformed plants. *EMBO J 4*, 2411-2418.

Joos, H., Inze, D., Caplan, A. Sormann, M., Van Montagu, M., and Schell, J. (1983). Genetic analysis of T-DNA transcripts in nopaline crown galls. *Cell 32*, 1057-1067.

Kay, R., Chau, A., Doly, M. and McPherson, J. (1987). Duplication of 35S promoter sequences creates a strong enhancer for plant genes. *Science 236*, 1299-1302.

Kaulen, H., Schell, J. and Krenzaler, F. (1986). Light induced expression of the chimaeric chalcone synthase-NPT2 gene in tobacco cells. *EMBO J. 5*, 1-8.

Kijne, J.W., Van der Schall, I.A.M., Diaz, C.L. and Van Iren, F. (1983). Mannose specific lectins and the recognition of pea roots by *Rhizobium leguminosarum*. In : Bog-Hansen, T.C. and Spengler, G.A. (Eds.) Lectins biology, biochemistry, clinical biochemistry. Vol 3, 521-530. de Gruyter and Co. New York. Berlin.

Klee, H.J., Gordon, M.D. and Nester, E.W. (1982). Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid mutations affecting oncogenicity. *J. Bacteriology 150*, 327-331.

Klee, H., Horsch, R. and Rogers, S. (1987). *Agrobacterium* mediated plant transformation and its further applications to plant biology. *Ann. Rev. Plant Physiology 38*, 467-486.

Kyhse-Anderson.J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods 10*, 203-209.

Lacy E., Roberts, S., Burtenshaw, M.D. and Constantini, F.A. (1983). A foreign β-globin gene in transgenic mice: integration at abnormal chromosomal possitions and expression in appropriate tissues. *Cell 34*, 343-358.

Laemmli,U.K. (1970).Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Science 227*, 680-685.

Law, I.J. and Strijdom, B.W. (1984). Role of lectins in the specific recognition of *Rhizobium* by *Lotononis bainesii*. *Plant Physiology 74*, 779-785.

Leemans, J., Shaw, C.H., Deblaere, R., De Greve, H., Hernalsteens, J.P., Maes, M., Van Montagu, M. and Schell, J. (1981). Site-specific mutagenesis of *Agrobacterium tumefaciens* Ti plasmid and transfer of genes to plant cells. *J. Mol. Appl. Genetics 1*, 149-164.

Leemans,J., Langenakens,J., De Greve,H., Deblaere,R., Van Montagu,M. and Schell,J. (1982). Broad host range cloning vectors derived from the W-plasmid Sa. *Gene 19*, 361-364.

Leon,O.A.B. and Schilperoort,R.A. (1978). A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochim. et Biophys. Acta 527*, 497-500.

Leroux, B., Yanofski, M.F., Winans, S.C., Ward. J.E., Ziegler, S.F. and Nester, E.W. (1987). Characterisation of the *virA* of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. *EMBO J. 6*, 849-856.

Lis, H. and Sharon, N. (1986). Lectins as molecules and tools. *Annu. Rev. Biochem. 55*, 35-68.

Livingstone,D.M. (1974). Immunoaffinity chromatography. *Meth. Enzymol. 34*, 723-731.

Lusky,M., Berg,L., Weiler,H. and Botcham,M. (1983). Bovine papilloma virus contains an activator of gene expression at the distal end of the early transcription unit. *Mol. Cell Biol. 3*, 1108-1116.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982). Molecular Cloning-A Laboratory Manual. (Cold Spring Harbour Laboratory, New York).

Maliga,P., Brenovitz,S.Z. and Marton,L. (1978). Streptomycin resistant plants from callus culture.*Nature New. Biol. 244*, 29-30.

Marban-Mendoza, N., Jeyaprakash, A., Jansson, H.B., Damon, J.A. and Zuckerman, B.M. (1987). Control of root-knot nematodes on tomato by lectins. *J. Nematology 19*, 331-335.

Marton, L., Wullens, G.J., Molendijk, L. and Schilperoort, R.A. (1979). *in vitro* transformation of cultured cells of *N. tabacum* by *Agrobacterium tumefaciens. Nature 277*, 129-131.

Matthysse. A.G. (1986). Initial interations of *Agrobacterium tumefaciens* with plant host cells. *Crit. Rev. Microbiology 13*, 281-307.

Matthysse, A.G. (1987). Characterisation of non attaching mutants of *Agrobacterium tumefaciens*. *J. Bacteriology 169*,313-323.

Matzke, M.A., Susani, M., Binns, A., Lewis, E.D., Rubenstein, I. and Matzke, A.J.M. (1984). Transcription of a zein gene introduced into sunflower using a Ti-plasmid vector. *EMBO J. 3*, 1525-1531.

McCutchan, T.F. and Singer, M.F. (1981). DNA sequences similar to those around the simian virus 40 origin of replication are present in the monkey genome. *Proc. Natl. Acad. Sci. USA 78*, 95-99.

McMasters,G.K. and Carmichael,G.G. (1977). Analysis of single- and double-stranded nucleic

acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA. 74*, 4835-4838.

Melchers, L.S. and Hooykas, P.J.J. (1987). Virulence of *Agrobacterium.* In 'Oxford surveys of plant molecular and cell biology'Vol. 4. 167-220. Miflin, B.J (Ed). Oxford Un. Press.

Messens, E., Lenaerts, A., Van Montagu, M. and Hedges, R.W. (1985). Genetic basis for opine secretion from crown gall tumour cells. *Mol. Gen. Genet. 199*, 344-348.

Michel, B. and Ehrlich,S.D. (1984). Recombination efficiency is a quadratic function of the length of homology during protoplast transformation of *Bacillus subtillis* and *Escherichia coli* competent cells. *EMBO J. 3,* 2879-2844.

Miller,H.J. (1972). Experiments in molecular genetics. Cold Spring Harbour, New York.

Mirelmar, D., Galun, E. and Sharon, N. (1978). Inhibition of fungal growth by wheat germ agglutinin. *Nature 256*, 414-416.

Murai, N., Sutton, D.W., Murray, M.G., Slighton, J.L., Merlo, D.J., Reichert, N.A., Sengupta-Gopalan, C.. Stock, C.A., Barker, R.F., Kemp. J.D. and Hall, T.C. (1984). Phaseolin gene from bean is expressed after transfer to sunflower via Tumour-inducing plasmid vectors. *Science 222*, 476-482.

Murashige,T. and Skooge,F. (1962). A revised medium for rapid growth and assay with Tobacco tissue cultures. *Physiologica Pl. 15*, 473-497.

Nagata, T., Okada. K., Kawazu, T. and Takeba, I. (1987). Cauliflower mosaic virus promoter directs S-phase specific expression in plant cells. *Mol. Gen. Genet. 207,* 242-244.

Nagy, F., Boultry, M., Hsu, M-Y., Wong, M. and Chua, N-M. (1987). The 5'-proximal region of the Cab-1 gene contains a 268bp enhancer like sequence for phytochrome response. *EMBO J.* 2537-2542.

Neuhaus, G., Neuhaus-Url, G.. Gruss, P. and Shweiger, H-G. (1984). Enhancer-controlled expression of the Simian virus 40 T-antigen in the green algae *Acetabularia. EMBO J. 3.* 2169-2172.

Okada, K.. Takebe. I. and Nagata. T. (1986). Expression and integration of genes introduced into highly synchronised plant protoplasts. *Mol. Gen. Genet. 205*, 398-403.

Okamura, J.K., Jofuku, K.D. and Goldberg, R.B. (1986). Soybean seed lectin gene and flanking nonseed protein genes are developmentally regulated in transformed tobacco plants. *Proc. Natl. Acad. Sci. USA 83*, 8240-8244.

Ondek,B., Shepard,A. and Herr.W. (1987). Descrete elements within the SV40 enhancer region

display different cell-specific enhancer activities. *EMBO J. 6*, 1017-1025.

Osborne,T.F., Arridon,D.N., Tyau,E.S., Dunsworth-Browne,M and Berk,A. (1984). Transcriptional control region within the protein coding portion of Adenovirus E1A genes. *Mol. Cell Biol. 4*, 1293-1299.

Ow, D.W., Wood, K.V., Deluca, M, de Wet, J.R. and Helinski, D.S. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science 234*, 856-859.

Peerbolte, R., Ruigrok, P., Wullens, G. and Schilperoort, R. (1987). T-DNA rearrangements due to tissue culture: somaclonal variation in crown gall tissues. *Plant Mol. Biol. 9*, 51-58.

Reading, C. (1982). In: Augmentin. Leigh, D.A. and Robinson, O.P.W. (Eds). 5-21. Excepta Medica. Amsterdam.

Reiss,B., Sprengel,R., Will,H. and Schaller,H. (1984). A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. *Gene 30*, 211-218.

Richmond, M.H. and Sykes R.B. (1973). The β-lactamases of Gram-negative bacteria and their possible phisiological roles. *Adv. Microbial Physiol. 9*, 31-88.

Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase 1. *J. Molec. Biol. 113*, 237-251.

Riker, A.K. (1930). Studies on infectious hairy root of nursery apple trees. *J. Agr. Res. 41*. 507-540.

Rini, J.M., Hofman, T. and Carver, J.P. (1987). Amino acid sequence differences in the alpha chains of pea seed isolectins: C-terminal processing. *Biochem. Cell Biol. 65*, 388-344.

Rio,D.C. and Tjian,N. 1984. Multiple control elements involved in the initiation of late transcription. *J. Mol. Appl. Gen. 2*, 423-435.

Robbins,P. and Botcham,M. (1985). Promoter specificity of the SV40 enhancer : Activation by T antigen. In : Eukaryotic transcription, the role of *cis* and its*trans* acting elements in initiation. 41-47. *Current Communications in molecular biology.* Gluzman,Y. (Ed). Cold Spring Harbor. N.Y.

Rogowski, P., Close, T.J. and Kado, C.I. (1987). Dual regulation of virulence genes of *Agrobacterium* plasmid pTiC58. In 'Molecular genetics of plant-microbe interactions.'Verma, D.P.S. and Brisson, N. (Eds). M. Nijhoff publishers, Dordrecht. 14-19.

Sanchez-Serrano, J.J., Keil, M., O'connor, A. Schell, J. and Willmitzer, L. (1987). Wound induced expression of a potato inhibitor II gene in transgenic tabacco. *EMBO J. 6*, 303-306.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Frayley R.T. (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. *Nucl. Acids Res. 15*, 1543-1558.

Schell, J., Van Montagu, M., De Beuckeleer, M., De Block, M., Depicker, A., De Wilde, M., Engler, G., Genetello, C., Hernalsteens, J.P., Holsters, M., Seurinck, J., Silver, B., Van Vliet, F. and Villarroel, R. (1979). Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host. *Proc. Roy. Soc. London B 204*, 251-266.

Schell, J. and Van Montagu, M. (1983). The Ti plasmids as natural and as practical gene vectors for plants. *Biotechnology 1*, 175.

Schlumbaum, A., Mauch, F., Vogeli, U. and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature 324*, 365-367.

Schoffl, F. and Baumann, G. (1985). Thermo-induced transcripts of a heat shock protein after transfer into sunflower using a Ti plasmid vector. *EMBO J. 4*, 1119-1124.

Schreier, P.H., Seftor, E., Schell, J. and Bohnert, H.J. (1985). The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein in plant cells. *EMBO J. 4*, 25-32.

Sengupta-Gopalan, C., Reichert, N.A., Barker, R.F., Hall, T.C. and Kemp, J.D. (1985). Developmentally regulated expression of the bean β-phaseolin gene in tobacco seeds. *Proc. Natl. Acad. Sci. USA 82*, 3320-3324.

Shaffner,W. (1985). Enhancer elements. In: Eukaryotic transcription: The role of *cis* and *trans* acting elements in initiation. *Curent communications in molecular biology*. 1-18. Gluzman,Y. (Ed). Cold Spring Harbor N.Y.

Shah, D.M., Horsch, R.B., Klee, H.J., Kishore, G.M., Winter, G.A., Tumer, N.E., Hironaka, C.M., Sander, P.R., Gasser, C.S. Aykent, S., Siegel, N.R., Rogers, S.G. and Fraley, R. (1986). Engineering Herbicide tolerance in plants. *Science 233*, 478-481.

Sharon,N. and Lis,H. (1972). Lectins: Cell-agglutinating and sugar-specific proteins. *Science 177*, 949-959.

Shaw, C.H., Watson, M.D., Carter, G.H., and Shaw, C.H. (1984a). The right hand copy of the nopaline Ti-plasmid 25bp repeat is required for tumour formation. *Nucl. Acids Res. 12*, 6031-6041.

Shaw, C.H. Carter, G.H., Watson, M.D. and Shaw, C.H. (1984b). A functional map of the nopaline synthase promoter. *Nucl. Acids Res. 12*, 7831-7846.

Shaw, C.H., Ashby, A.M., Brown, A., Royal, C., Loake, G.J. and Shaw, C. H. (1988). *virA* and *virG* are the Ti-plasmid functions required for chemotaxis of *Agrobacterium tumefaciens* towards acetosyringone. *Molecular Microbiology 2*, in press.

Shepard, J., Biclney, D., and Shanhin, E. (1980). Potato protoplasts in crop improvement. *Science 208*, 17-24.

Simpson, J., Schell, J., Van Montagu, M. and Hererra-Estrella. L. (1986). Light inducible and tissue specific pea *lhcp* gene expression containing enhancer-like and silencer-like properties. *Nature 323*, 551-554.

Sin-Cheong, H., Shahnaz, H., Wang, J.L. and Shindler, W. (1986). Endogenous lectins from soybean cells: Isolation of a protein immunologically cross reactive with seed soybean agglutinin and analysis of its role in binding *Rhizobium japonicum. J. Cell Biol. 103*, 1043-1054.

Smith, E.F. and Townsend, C.O. (1907). A plant tumour of bacterial origin. *Science 25*, 671-673.

Southern,E. (1955). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Molec. Biol 98*, 503-517.

Stachel, S.E., Messens, E., Van Montagu, M. and Zambryski, P.C. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens. Nature 318*, 624-629.

Stachel, S.E., Timmerman, B., and Zambryski, P. (1986). Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature 322*, 706-712.

Stachel, S.E. and Nester, E.W. (1986). The genetic and transcriptional organisation of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens. EMBO J. 5*, 1445-1454.

Stachel, S.E., Timmerman, B., and Zambryski, P. (1987). Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple T-strand molecules from the pTiA6 T-region: requirements for 5′ *virD* gene products. *EMBO J. 6*, 857-863.

Staehelin, T., Stahli, C., Hobbs, D.S. and Pestka, S. (1981). A rapid quantitative assay of high sensitivity for human leukocyte interferon with monoclonal antibodies. *Methods in Enzymology 79*, 589-595.

Stahl, F.W. (1979). Specialised sites in general recombination. *Ann. Rev. Genet. 13*, 7-24.

Stewart, R.N. and Dermen, H. (1970). Determination of number and mitotic activity of shoot apical initial cell by analysis of mericlinal chimaeras. *Amer. J. Bot. 57,* 816-826.

Stockhaus, J., Eckes, P., Rochasosa, M., Schell, J. and Willmitzer, L. (1987). Analysis of *cis*-active sequences involved in the leaf specific expression of a potato gene in transgenic plants. *Proc. Natl. Acad. Sci. USA. 84,* 7943-7947.

Stubbs, M.E., Carver, J.P. and Dunn, R.J. (1986). Production of pea lectin in *Escherichia coli. J. Biol. Chem. 261,* 6141-6144.

Sturm, A., Voelker, T.A., Herman, E.M. and Chrispeels, M.J. (1988). Correct processing of signal peptide, correct glycosylation, golgi processing and targeting to protein bodies of a vacuolar protein phytohaemagglutinin in transgenic tobacco. *Planta 175,* 170-183.

Tempe, J., and Goldmann, A. (1982). Occurrence and biosynthesis of opines. In 'Molecular biology of plant tumours.'Khal, G. and Schell, J. (Eds). Academic Press. New York. 427-449.

Tepfer, D.A. (1984). Transformation of several species of higher plant by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. *Cell 37,* 959-967.

Thomas,P.S. and Farquar,M.N. (1978). Specific Measurement of DNA in nuclei and nucleic acids using diaminobenzoic acid. *Analyt. Biochem. 89,* 35-44.

Thomas,P.S. (1980). Hybridisation of denatured RNA and DNA fragments transferred to nitocellulose. *Proc. Natl. Acad. Sci. USA. 77,* 5201-5205.

Thomashow, M.F., Hugly, S., Buchholtzs, W.G. and Thomashow, L.S. (1986). Molecular basis of the auxin-independent phenotype of crown gall tumour tissues. *Science 231,* 616-618.

Timko, M.P., Kausch, A.P., Castreana, C., Fasler, J., Hererra-Estrella, L., Van den Broeck, G., Van Montagu, M. and Cashmore, A.R. (1985). Light regulation of plant gene expression by an upstream enhancer-like element. *Nature 313,* 579-582.

Tooze,J. (1981). (Ed.) Molecular biology of tumour viruses. (2^{nd} Edn.) DNA Tumour viruses. Cold Spring Harbor. N.Y.

Towbin,H., Staehelin,T. and Gordon,J. (1979). Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA 76,* 4350-4354.

Trowbridge,I.S. (1974). Isolation and characterisation of a mitogenic lectin from *Pisum sativum. J. Biol. Chem. 249,* 6004-6012.

Truchet, G.L., Sherwood, J.E., Prankratz, H.S. and Dazzo, F.B. (1986). Clover root exudate contains a particulate form of lectin, trifoliin A, which binds to *Rhizobium trifolii*. *Phisiol. Plant 66*, 575-582.

Tumer, N.E., O'Connell, K.M., Nelson, R.S., Sanders, P.R., Beachy, N.R., Frayley, R.T. and Shah, D.M. (1987). Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *EMBO J. 6,* 1181-1188.

Vanden-Bosch,K.A. (1986). Light and electron microscopic visualisation of uricase by immuno-gold labelling of sections of resin-embedded soybean nodules. *J. Microscopy 143 (2)*, 187-197.

Van den Broek, G., Timko, M., Kausch, A.P. and Cashmore, A.R. (1985). Targetting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate cardoxylase. *Nature 313,* 358-363.

Van den Elzen, P., Lee, K.Y., Townsend, J. and Bedbrook, J. (1985). Simple binary vectors for DNA transfer to plants. *Plant Mol. Biol. 5*, 149-154.

Van Haaren, M.J.J., Pronk, J.T., Schilperoort, R.A., and Hooykaas, P.J.J. (1986). T-region transfer from *Agrobacterium tumefaciens* plant cells: functional characterisation of border repeats. In 'Recognition in microbe-plant symbiotic and pathogenic interactions'. Lugtenberg, B. (Ed). NATO ISI series H, Cell biology, Vol. 4, 203-214.

Van Haaren, M.J.J., Pronk, J.T., Schilperoort, R.A., and Hooykaas, P.J.J. (1987). Functional analysis of the *Agrobacterium tumefaciens* octopine Ti plasmid left and right T-region border fragments. *Plant. Mol. Biol*, 95-104.

Van Haute,E.. Joos,H., Maes,M., Warren,G., Van Montagu,M., and Schell,J. (1983). Intergenic transfer and exchange recombination of restriction fragments cloned in pBR322: a novel stratagy for the reversed genetics of the Ti-plasmid of *A. tumefaciens*. *EMBO J. 2*, 411-417.

Van Larebeke, N., Genetello, C., Hernalsteens, J.P., Depicker, A., Zaenen, I., Messens, E., Van Montagu, M. and Schell, J. (1977). Transfer of Ti plasmids between *Agrobacterium* strains by mobilisation with the plasmid RP4. *Mol. Gen. Genet. 152*, 119-124.

Van Montagu, M. and Schell, J. (1982). The Ti plasmids of *Agrobacterium*. Curr. Topics Microbiol. Immunol. 96, 237-54.

Vervliet,G., Holsters,M., Teuchy,H., Van Montagu,M. and Schell,J. (1975). Characterisation of different plaque-forming and defective phages in *Agrobacterium* strains. *J. Gen. Virol. 26*, 33-48.

Vianna, A.M. and Metivier, J. (1980). Changes in the levels of total soluble proteins and sugar

during leaf ontogeny in *Stevia rebaudiana*. *Ann. Bot. 45*, 469-474.

Wallroth, M., Gerats, A.G.M., Rogers,S.G., Fraley, R.T. and Horsch, R.B. (1986). Chromasomal localisation of foreign genes in *Petunia hybrida*. Mol. Gen. Genet. 202, 6-15.

Wang, K., Stachel, S.E., Timmerman, B., Van Montagu, M., and Zambryski, P.C. (1987). Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium vir*-gene expression. *Science 235*, 587-591.

Warren,C.J., Twigg,A. and Sherrat,D.J. (1978). ColE1 mobility and relaxation complex. *Nature 274*, 259-261.

Wartell,R.M. and Reznikoff,W.S. (1980). Cloning DNA restriction fragments with protruding, single stranded ends. *Gene 9*, 307-319.

Wareing, P.F. and Phillips, I.D.J. (1981). Growth and differentiation in plants. Third Edn. Pergamon Press.

Willmitzer, L., Sanchez-Serrano, J., Buschfeld, E. and Schell, J. (1983). Size, location and polarity of T-DNA encoded transcripts in nopaline crown gall tumours; common transcripts in octopine and nopaline tumours. *Cell 32*, 1045-1056.

Winans, S.C., Ebert, P.R., Stachel, S.E., Gordon, M.P. and Nester, E.W. (1986). A gene essential fo *Agrobacterium* virulence is homologous to a family of possitive regulatory loci. *Proc. Natl. Acad. Sci. USA. 83*, 8278-8282.

Wullems, G.J., Molendijk, L., Ooms, G. and Shilperoort, R.A. (1981). Differential expression of crown gall tumour markers in transformants obtained after *in vitro Agrobacterium tumefaciens* induced transformation of regenerating protoplasts derived from *N. tabacum* cultivar White Barley. *Proc. Natl. Acad. Sci. USA 78*, 4344-4348.

Yadav, N.S., Vanderlayden, J., Bennett,D.R., Barnes, W.M., and Chilton. M-D. (1982). Short direct repeats flank the T-DNA on a nopaline Ti plamid. *Proc. Natl. Acad. Sci. USA. 79*, 6322-6326.

Yanish-Perron,C., Vieira,J. and Messing,J. (1985). Improved M13 cloning vectors and host strains. Nucleotide sequence of the M13 mp18 and pUC19 vectors. *Gene 33*, 103-119.

Yanofski, M.F., Porter, S.G., Young, C., Albright, L.M., Gordon, M.P., and Nester, E.W. (1986). The *virD*-operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell 47*, 471-477.

Zambryski, P., Depicker, A. Kruger, K. and Goodman, H.M. (1982). Tumour induction by *Agrobacterium tumefaciens*: analysis of the boundries of the T-DNA. *J. Mol. Appl. Genet. 1*, 361-370.

Zambryski,P., Joos,H., Genetello,C., Leemans,J., Van Montagu,M. and Schell,J. (1983). Ti-plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J. 2*, 2143-2150.

Zambryski,P., Herrara-Estrella,L., De Block,M., Van Montagu,M. and Schell,J. (1984). The use of the Ti-plasmid of *Agrobacterium* to study the transfer and expression of foreign DNA in plant cells: New vectors and methods. In "Genetic engineering; principles and methods. Vol 6. (Hollaender,A and Setlow,J. Eds). Plenum Press,NY.