Studies on plant gene transfer systems

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TO MY PARENTS.

STUDIES ON PLANT GENE TRANSFER SYSTEMS

by

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ABSTRACT

A number of methods for the transfer of genes to plants are assessed in this work. The potential of *Agrobacterium tumefaciens* infection of germinating pollen tubes *in vitro* as a method of gene transfer was investigated and evidence presented for an essential pre-requisite of infection, that of attachment of Agrobacteria to the pollen tube wall, with both a dicot and a monocot species. In addition, the possibility of direct uptake of DNA molecules by germinating pollen tubes was suggested by *in vitro* uptake studies. Microinjection of DNA molecules into the loculus of ovaries, with the aim of facilitating *in vivo* DNA uptake by the male and/or female gametes, was investigated with *Salpiglossis sinuata* ovaries. Evidence was presented for the persistence of DNA molecules in the ovary loculus and gene transfer using a non-oncogenic *Agrobacterium* plasmid vector was attempted and the resulting progeny were screened for transformation.

The techniques of *Agrobacterium*-infection of leaf discs and direct DNA uptake by protoplasts were applied to *Nicotiana tabacum*. DNA transformation vectors containing a kanamycin resistance marker gene and a chimeric pea seed storage protein gene were constructed for use in this study. The seed-specific promoter of the pea legumin A gene was replaced with the nopaline synthase promoter that is expressed in a more constitutive manner in plant tissues. Kanamycin resistant transgenic plants were regenerated following both transformation techniques and the presence and structure of inserted foreign DNA was determined by Southern blot hybridizations. The transmission of kanamycin resistance to transformant progeny after self-fertilization demonstrated characteristic Mendelian inheritance.

The expression of the inserted legumin gene in leaf tissue of a number of *Agrobacterium*-derived transformants was assessed by Northern blot hybridization; legumin transcripts were detected, although the protein immuno-detection procedures of Western blotting and ELISA did not detect legumin in the seeds or leaf tissue of transgenic plants derived from either transformation technique.

CONTENTS

List of Figures

List of Tables

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Memorandum

Part of the work described in this thesis has been previously presented in the following publication.

Hepher, A., Sherman, A., Gates, P. and Boulter, D. (1985). Micro-injection of gene vectors into pollen and ovaries as a potential means of transforming whole plants. In: *Experimental Manipulation of Ovule Tissues*, eds. G.P. Chapman, S.H. Mantell and R.W. Daniels. Longman.

Abbreviations

Abbreviations were those used according to the Biochemical Society instructions to Authors, *Biochem. J.* (1982) **209**, 1-27, with any additions given below:

ATP	-	adenosine 5'-triphosphate
BAP	-	6-benzylamino purine
BSA	-	bovine serum albumin
cDNA	-	complementary DNA
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetra-acetic acid
EGTA	-	ethylene glycol bis (-amino-ethyl ether)-N,N,N^1-tetra-acetic acid
ELISA	-	enzyme-linked immunosorbent assay
EtBr	-	ethidium bromide
HSDNA	-	herring sperm DNA
kbp	-	kilobase pairs
kb	-	kilobases
NAA	-	naphthalene acetic acid
dNTP	-	deoxynucleotide triphosphate
OD	-	optical density
PAGE	-	polyacrylamide gel electrophoresis
PEG	-	polyethylene glycol
SDS	-	sodium dodecyl sulphate
Tris	-	tris(hydroxymethyl) aminomethane
uv	-	ultra-violet
X-Gal	-	5-dibromo-4-chloro-3-indoylgalactosidase
5'	-	5' terminal phosphate of DNA or RNA molecule
3'	-	3' terminal phosphate of DNA or RNA molecule

CHAPTER 1.

INTRODUCTION.

Introduction

1.1 General Introduction

Traditional plant breeding methods exploit the natural genetic variation found in plant species to create new genetic permutations; the recombination of desired inherited characteristics is achieved by performing crosses between varieties of interest and successively selecting progeny that show the desired traits. The crop varieties in use today derived from such breeding programmes often have improved characteristics such as better crop yield and quality, disease resistance and climate tolerance. However, further quantitative and qualitative improvements are frequently highly desirable.

The ability to isolate, characterize even manipulate specific genes responsible for traits that may be advantageous to crop plants, has been made possible by the advent of molecular biological and recombinant DNA technologies. The successful introduction, stable incorporation and efficient expression of isolated genes in a plant genome of another species offers enormous potential for the transfer of characteristics among plant species, even between widely divergent species, not possible using conventional breeding methods.

An assessment of potential methods of achieving such a transfer of plant genes, and an analysis of the expression of a transferred gene, forms the basis of this work.

1.2 Plant Genetic Manipulation

The genetic manipulation of eukaryotic genes was made possible by the development in the late 1960's and early 1970's of recombinant DNA technology, following the advancement of knowledge concerning the molecular biology of bacterial genetics. The discovery of restriction endonucleases and their ability to specifically cleave DNA (for example, Eco RI, Mertz and Davis, 1972) and the subsequent use of the enzyme DNA ligase to recombine DNA molecules *in vitro* to

form biologically functional bacterial plasmids which could be introduced into a bacterial host by transformation procedures (Cohen *et al.*, 1973), together with the use of powerful screening techniques to select for specific (recombinant) DNA sequences, led to the development of bacterial plasmid vectors that permitted the cloning of DNA sequences and genes from both prokaryotic and eukaryotic origin (see Old and Primrose, 1981).

The possibility of cloning and manipulating DNA sequences made possible the isolation of eukaryotic genes and the formation of complementary DNA (cDNA) and genomic libraries of plant and animal genes (Dahl *et al.*, 1981), genes from many species have been isolated (for example, a comprehensive list of cloned eukaryotic genes up to 1981 was published by Davies (1981)). In more recent years, a large number of plant genes have been identified and their DNA sequence published. Many of these sequences can be obtained from the computer gene data storage facility "Genbank". An analysis of gene codon usage in plant genes by Murray *et al.* in 1988 listed 53 monocot and 154 dicot plant genes, for which DNA sequence data was available. The availability of isolated genes and the ability to perform DNA sequence analysis and recombine elements of the gene allowed the molecular biology of plant and animal genes to be studied in detail, and to elucidate possible functions of DNA sequence elements found conserved in certain genes. The goals of genetic manipulation and genetic engineering, particularly as applied to plant systems are: (i) to identify and isolate genes and regulatory elements of interest to the improvement of plant species; (ii) to be able to introduce novel and potentially useful gene products to a plant (for example, disease resistance); (iii) to alter gene products already present in the plant so as to improve their properties (for example, nutritional or functional characteristics); and/or (iv) to alter the expression of existing gene products, either by an increase or decrease in expression levels. In order to achieve these goals, a thorough

knowledge of the molecular biology of plant genes and the regulation and control mechanisms that operate is essential.

The development of efficient gene transfer procedures applicable to a wide range of plants is essential for the further investigation of the process of plant gene expression control and regulation in heterologous and homologous species. Thus, plant genes can be isolated and modified and the effects of these modificiations on their expression can be studied in transformed plants. The use of modifying enzymes, synthetic oligonucleotides or site-specific mutagenesis are now routine methods for altering the gene coding regions by addition, substitution or deletion of DNA sequences. The addition or removal of enhancer or silencer elements or the use of appropriate tissue-specific promoters will allow the directed expression of genes introduced into plants and thus permit greater scope in the genetic manipulation of plant genes.

1.3 Gene Transfer Techniques

1.3.1 Plant breeding programmes

Traditional plant breeding techniques involve the cross-fertilization of two parent plants of the same or related species and the selection of progeny exhibiting desired inherited characteristics resulting from an exchange of genetic material. In the course of such crosses undesirable traits may also be acquired and these are removed by further crosses and the use of selection procedures that favour more desirable gene combinations. Thus, natural genetic variation is exploited by directed sexual crossing and subsequent artificial selection in the production of improved commercial crop varieties. Modern cultivated varieties of many crop species originated from such intra-specific gene transfer programmes, with desired traits being introduced from wild varieties of the species. However, intraspecific genetic crossing has the limitation that only the existing genetic variability found

in that particular species can be utilized; often the incorporation of beneficial traits of other species is desired. While such interspecific genetic transfer has occurred naturally in some related species, for example *Zea mays* and *Z. mexicana* (Heisser, 1973), the natural barriers to sexual exchange of genes between species have to be overcome for the vast majority of species; after all genetic isolation generally defines a species.

In this century, many crop varieties have been improved by the interspecific transfer of genes from related non-cultivated species. Examples of traits transferred to agronomically important crop species, such as oat, sugarbeet, cotton, tomato and rice, are given by Goodman *et al.* (1987); perhaps one of the most important advances made by such breeding techniques has been the improvement of wheat varieties. In the 1930's, two disease resistance traits (stem rust and loose smut) were transferred to the bread wheat *Triticum aestivum* from *T. tauschii* by McFadden (see Goodman *et al.*, 1987), and many other resistance genes have since been transferred to wheat varieties by interspecific crossing.

More ambitious crosses have been used in plant breeding, with the transfer of genes between species of different genera. The novel cereal Triticale was developed by intergeneric crossing of wheat (*Triticum*) and rye (*Secale*). Examples of intergeneric transfer of specific traits include the transfer to wheat crop varieties of resistance to leaf rust and of high kernel protein from wild grass species (*Aegilops* sp.) (Goodman *et al.*, 1987).

The crossing of plant species, particularly of different genera, is clearly not as simple as the above description implies. Often, there are specific biological mechanisms which prevent the formation of interspecific hybrids. The advent of *in vitro* culture and cytogenic manipulation techniques has allowed the circumvention of some of these mechanisms. For example, a hybrid zygote may not be capable of normal development due to genetic incompatibility with the

ovule. This incompatibility can be circumvented in certain cases, for example, treatment of wheat kernels with the plant hormone gibberelic acid allows the initial development of an otherwise incompatible wheat/barley embryo. Subsequent *in vitro* culture of the embryo during its development facilitates the production of the hybrid plant (Hart *et al.*, 1980). Promoting recombination between donor and recipient genomes can be achieved by the use of irradiation techniques, this may be especially necessary in crosses between unrelated species where natural recombination may not take place. Non-sexual methods of transferring genes such as somatic cell fusion, have also been studied as a method of overcoming the difficulties of incompatibility in sexual crosses. Genetic transfer can be achieved by the fusion of protoplasts of two species, subsequent callus production and hybrid plant regeneration. However, this cell fusion technique as with sexual crosses has drawbacks, such as potential genetic instability of the introduced chromosomes and hybrid sterility. Successive back-crossing remains a requirement to produce stable incorporation of the desired genes.

Clearly then, many plant breeding programmes have resulted in the production of improved crop varieties containing novel genes. However, even when crosses are possible between the species of interest, the selection procedures involving numerous back-crosses that are necessary to fix a desired trait in a genome, are very time-consuming and problems, such as the linkage of unwanted genes to the desired genes, may prove an impediment to the production of a useful crop variety.

The transfer of genes by the direct manipulation of DNA has been made possible by the advent of recombinant DNA methodologies. The ability to identify and clone genes of interest, and the development of techniques that permit the introduction of these genes in host plants has, in principle, enormous

advantages over the traditional plant breeding strategies. The two main advantages are firstly, that the introduction of specific genes governing a particular trait is much more precise than methods relying on recombination events between entire genomes. The problem of co-inheritance of undesirable genes is thus overcome by only introducing the genes of interest. Indeed, genetic elements involved in the control of the introduced genes if identified can potentially be modified to influence its expression in the new host plant. Secondly, the genes that can be introduced to plants can be obtained from a much greater array of genetic sources than available by traditional breeding. Thus, genes can be derived from widely divergent plant species, and even from animal, bacterial or viral genomes. Plant breeding will continue to be used as a method of crop improvement but genetic engineering techniques can be used as important tools to produce beneficial changes that are difficult or even impossible using conventional methods and could potentially achieve results in a much shorter time period.

1.3.2 *Agrobacterium* Infection

Agrobacterium tumefaciens is a gram-negative soil bacterium that naturally infects a wide range of dicotyledonous plant species, causing a neoplastic disease known as 'Crown Gall' (Bevan and Chilton, 1982; Holsters *et al.*, 1982; Nester *et al.*, 1984). On infection, the bacterium transfers a set of genes to the plant cell that directs the production of phytohormones (auxins and cytokinins) that stimulate the plant cells to proliferate forming a tumorous growth (Barry *et al.*, 1984; Schroder *et al.*, 1984). Also transferred are genes that mediate the synthesis of novel metabolites called opines that the Agrobacteria specifically catabolize allowing the bacteria to proliferate (Schell *et al.*, 1979; Tempe and Goldman, 1982; Kemp, 1982). Indeed, Agrobacteria are classified by the opine type they produce; the two major classes are the octopine and nopaline producing strains.

The transferred genes are contained on a well-defined section of the large (~200 kbp) *Agrobacterium* Ti (tumour inducing)-plasmid termed the T-DNA (for Transfer-DNA). On infection, the T-DNA is transferred from the Ti-plasmid to the plant cell nucleus where it becomes integrated into the plant genomic DNA (Zambryski *et al.*, 1989). Expression of the T-DNA genes is then mediated by the plant cell.

The T-DNA is flanked by a 25 bp sequence motif that is directly repeated at both ends, these are known as the left and right T-DNA borders as they specify the termini of the integrated DNA in the plant genome (Barker *et al.*, 1984; Slightom *et al.*, 1985). It has been found that the right border sequence is essential for the transfer of the T-DNA, in contrast to the left border, the absence of which does not prevent integration (Joos *et al.*, 1983; Shaw *et al.*, 1984b; Wang *et al.*, 1984; Horsch and Klee, 1986) and that the transfer appears to proceed in an oriented fashion from the right to left border (Wang *et al.*, 1984; Peralta and Ream, 1985).

The transfer and integration of T-DNA is mediated by a group of genes resident on the Ti-plasmid. These genes are clustered in an ~40 kbp region known as the *vir* region (for virulence) (Garfinkel and Nester, 1980; Ooms *et al.*, 1980; Klee *et al.*, 1983) that is not part of the transferred portion of Ti-plasmid DNA. Genetic analysis has revealed that the *vir* genes are organized at six loci, namely *vir* A to E and *vir* G (Stachel and Nester, 1986). Specific activation of these *vir* genes by plant wound substances (Stachel *et al.*, 1985; Yanofsky *et al.*, 1986) results in the transfer of the T-DNA. It is believed a *vir* D encoded site-specific endonuclease nicks the borders of the T-DNA (Alt-Moerbe *et al.*, 1986; Yanofsky *et al.*, 1986; Yamamoto *et al.*, 1987; Wang *et al.*, 1987) creating a single-stranded linear, free T-DNA intermediate (the lower strand of the T-DNA) (Stachel *et al.*, 1986; 1987). Circular T-DNA intermediate molecules have also been implicated in

the transfer process (Koukolikova-Nicola *et al.*, 1985). The exact molecular mechanisms that act in T-DNA transfer are not yet fully known but it is believed that other *vir* gene products mediate the transfer and integration of these T-strands from the bacterium into the plant genome (see Zambryski *et al.*, 1989).

The T-DNA transfer constitutes a natural genetic engineering system, certain features of which are amenable to manipuplation to provide a convenient and efficient plant gene transfer system. It was found that the oncogenic genes contained within the T-DNA borders could be removed without impeding the transfer process creating so-called "disarmed" strains of *Agrobacterium* (Zambryski *et al.*, 1983). This is important as it is necessary to introduce genes into plant cells and subsequently obtain normal differentiating tissues without abnormal cell proliferation. Importantly, it was found that any DNA sequence introduced between the T-DNA borders was faithfully integrated into the plant genome upon transfer (Hernalsteens *et al.*, 1980; Holsters *et al.*, 1982).

Early work in assessing the feasibility of using the Ti-plasmid of *Agrobacterium* as a gene transfer vector used oncogenic Ti-plasmids with genes inserted into the T-DNA. Hernalsteens *et al.* (1980) demonstrated the integration of bacterial transposon Tn7 DNA in tobacco tumour tissue using a Ti-plasmid containing the Tn7 DNA in the nopaline synthase gene of the T-DNA. However, the use of intact T-DNA meant that regeneration of plants from the tumour tissue was not possible. Barton *et al.* (1983) reported the insertion of a yeast alcohol dehydrogenase gene into a site of the T-DNA of a Ti-plasmid that inactivated one of the oncogenic loci. Healthy tobacco plants containing intact T-DNA copies were regenerated from tumour tissue derived from infected tobacco stem segements. However, Zambryski *et al.* (1983) removed all the T-DNA sequence except the borders and nopaline synthase gene (*nos*) and introduced a T-DNA specific marker (pBR322 plasmid DNA) to create a non-oncogenic Ti-plasmid vector (pGV3850).

Use of this vector, enabled the regeneration of plants following T-DNA transfer, the *nos* gene provided a marker for the presence of the T-DNA in transformed tissues.

So the T-DNA was demonstrated to be a useful tool in the transfer of genes to plants. With the ability to culture transformed tissue and regenerate plants, the *Agrobacterium* mediated gene transfer procedure offered great potential for the genetic manipulation of plant species that were susceptible to *Agrobacterium* infection.

1.3.3. Direct uptake of DNA molecules

Interest has been focussed on alternative gene transfer techniques that do not depend on the specific plant/bacterial interaction of the *Agrobacterium*-infection process, in order to develop efficient transformation systems for species that do not fall within the *Agrobacterium* host range.

The uptake of naked DNA molecules by plant cells was studied as a method of introducing genes before the advent of the *Agrobacterium*-mediated gene transfer techniques. Incubation of germinating pollen and seeds with DNA solutions (Hess, 1969; Ledoux *et al.*, 1974) and more recently cultured cells and protoplasts (Davey *et al.*, 1980; Krens *et al.*, 1982) was attempted in an effort to demonstrate uptake of DNA.

Early work on the uptake of DNA by pollen grains and pollen tubes has been reviewed by Hess (1987). Generally, these experiments were inconclusive as they relied on examination of phenotypic markers such as flower colour, following treatment of pollen of a mutant type with wild-type total genomic DNA (Hess, 1980), and this evidence was open to different interpretations. Similarly, studies to demonstrate DNA uptake were proved inconclusive, as autoradiographic analysis of seeds and pollen incubated with radio-labelled DNA gave equivocal results, with

the possibility that the DNA was merely adsorbed to the cell wall or represented degraded nucleotides (Hess *et al.*, 1974). The development of techniques such as Southern blotting and nick-translation in the mid-1970's, led to the possibility of more convincing results as molecular genetic evidence could be presented for the incorporation of exogenously applied DNA into plant genomes.

The transformation of animal cells was achieved in the 1970's by chromosome mediated gene transfer (McBridge and Ozer, 1973) and by purified-DNA mediated gene transfer (Bacchetti and Graham, 1977; Wigler *et al.*, 1977). The most notable achievement was the development of the calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973) which was shown to enhance transformation efficiencies. The success of many of the animal cell transformations performed was due in part to the availability of good transformant selection procedures. By the late 1970's, the direct uptake of DNA technique had been used to transfer a number of genes to cultured mammalian cells (Wigler *et al.*, 1978; Graf *et al.*, 1979). High efficiency transformation was also achieved using direct microinjection of purified DNA into the nucleus of animal cells (Capecchi, 1980; Wagner *et al.*, 1981; Rubin and Spradling, 1982).

With the demonstration that purified genes could be delivered to animal cells and that these foreign genes subsequently became integrated in the genome and expressed, similar direct gene transfer techniques seemed to offer potential in plant cell transformation. Direct uptake by plant cells is prevented by the presence of the plant cell wall. Its removal to produce cell protoplasts allows exogenous DNA to interact directly with the cell membrane.

The initial direct gene transfer to protoplasts were carried out in the early 1980's (Davey *et al.*, 1980; Krens *et al.*, 1982) and followed the development of efficient protoplasting methodologies, and more particularly the availability of

suitable selectable marker genes under the control of regulation signals capable of functioning in the plant cell, thus allowing the expression of the transferred genes.

1.4 Seed storage proteins

Seeds are important nutritionally as a rich source of protein with seed storage proteins forming a high proportion of this protein content. In legumes for example, the seed storage proteins can represent up to 70%-80% of the total seed protein content (Shewry *et al.*, 1981). The protein content of food consumed by the world's human population is mostly derived from storage proteins of cereal and legume seeds whether directly or via animals fed on foodstuffs containing such proteins (Shewry *et al.*, 1981; Payne *et al.*, 1983; Croy and Gatehouse, 1985). Therefore, the seed storage proteins are agriculturally and economically important.

Plant breeding programmes have attempted to increase the efficiency of many crop species by selecting varieties with improved yields of higher quality, with desired traits such as resistance to various diseases and tolerance to particular climates. In the case of seed storage proteins, improvements to the nutritional quality of the proteins would be of great benefit as they are not considered to be good nutritional proteins, as they tend to lack certain essential amino acids. Their role in the seed as a source of nitrogen with the consequent high levels of amide amino acids, tends to lead to deficiencies in certain other amino acids, notably lysine and tryptophan in cereal seeds and methionine and cysteine in legume seeds (Payne, 1983; Higgins, 1984). Additionally, seed proteins tend to be poorly digestible and many are toxic or anti-metabolic proteins such as the lectins and protease inhibitors (Gatehouse, 1984; Pusztai *et al.*, 1983).

Potential applications for genetic manipulation and gene transfer techniques to the improvement of seed storage proteins might involve modification of the proteins by coding sequence alterations, perhaps to include a higher proportion of

the less-abundant amino acids or the addition of new genes to confer novel qualities, such as resistance to particular diseases.

1.4.1. Seed storage proteins of *Pisum sativum* L. (Pea)

The seed storage proteins of pea have been been extensively reviewed in recent years (Gatehouse *et al.*, 1984; Casey and Domoney, 1984; Croy and Gatehouse, 1985); however, a brief description of these proteins is given below.

There are two immunologically distinct classes of seed storage protein in pea, namely the 11S legumin (Croy *et al.*, 1980a) and 7S vicilin globulins (Croy *et al.*, 1980b), together making up 60-70% of the total seed protein content (Croy and Gatehouse, 1985). A third class of proteins related both immunologically and in amino acid sequence to the vicilins, is convicilin (Croy *et al.*, 1980c).

Pea vicilin is a protein of M_r 145,000-170,000 and is initially synthesised as precursor polypeptides of M_r 47,000 and M_r 50,000, which may subsequently be processed. Considerable charge and size heterogeneity has been observed with the vicilin subunits, with various polypeptides of between M_r 12,500 and M_r 33,000 visible following electrophoresis under denaturing conditions (Higgins and Spencer, 1981; Croy and Gatehouse, 1985). The proposed structure of vicilin is however, a trimer of M_r 50,000 subunits held together by non-covalent forces (Gatehouse *et al.*, 1981). Some vicilins are glycosylated. Convicilin is found as M_r 70.,000 subunits assembled as a M_r 210,000-280,000 protein (Croy *et al.*, 1980c).

Pea legumin is a protein of M_r 380,000-410,000 consisting of six ~M_r 60,000 subunits. Each subunit is made up of an acidic (α) polypeptide of ~M_r 38 ,000 and a basic (β) polypeptide of ~M_r 21,000, linked by a di-sulphide bond. These two polypeptides are initially synthesised as one ~M_r 60,000 polypeptide which is subsequently cleaved. α -polypeptides of pea legumin exhibit considerable size and charge heterogeneity whereas β -polypeptides are more consistent in size but

also vary in charge. A total of 22 α - and 11 β -polypeptides have been identified. A detailed classification of these major and minor legumin polypeptide types has been developed (see Casey, 1979; Matta *et al.*, 1981). Legumin proteins, in contrast to some vicilin proteins, are not glycosylated (Casey, 1979).

The genes encoding the seed storage proteins are developmentally regulated, that is the level of expression of the genes varies according to the developmental stage of the seed. The accumulation pattern of vicilin and legumin proteins differ in the developing pea seed. It has been found that vicilin accumulates faster than legumin reaching a maximum level at about 14 days after flowering (d.a.f.), whereas although legumin accumulates at a lower rate, it continues to be synthesised up to 20 d.a.f. (Boulter, 1981; Boulter *et al.*, 1987). The pea seed reaches maturity at approximately 24 d.a.f.

The developmental regulation of the expression of the legumin genes is described by Boulter *et al* (1987). Briefly, the level of legumin mRNA dramatically increases from low levels at 7 d.a.f. to a maximum at 18 d.a.f. after which the levels fall. Translational control is implicated in the later stages of seed maturation as legumin mRNA levels peak at 18 d.a.f. and polysomal associated mRNA at 22 d.a.f., but the rate of legumin protein synthesis is constant throughout this period.

The pea legumin protein once synthesised is extensively co- and post-translationally modified. The legumin is synthesised on the rough endoplasmic reticulum, then transported to and deposited in the protein bodies via the golgi apparatus (Harris, 1979; 1986).

1.4.2 Legumin A gene

Pea legumin, in common with many seed storage proteins, is coded for by a multigene family (Boulter *et al.*, 1987; Levasseur, 1988). The genes that make up

this gene family can be divided into two types according to the subunits they produce. One group, the "A-type" legumin genes, encode the major legumin subunit pairs and comprise five genes designated *leg* A to E (legumin D is a pseudogene). The second group, the "B-type" legumin genes, of *leg* J to L, encode the minor legumins. A third sub-family of *leg* M and X encoding minor legumins is also thought to exist.

The *leg* A gene was used as the gene to be transferred to tobacco plants in this study as it had been well characterized and sequenced (Lycett *et al.*, 1984). It is known that this gene is transcriptionally active and has all its transcriptional control sequences as the genomic clone demonstrated perfect homology with complementary DNA (cDNA) sequences. Recently, it has been demonstrated that the *leg* A gene under the control of its own promoter, is expressed in the seed tissues of *Nicotiana plumbaginifolia* (Ellis *et al.*, 1988; Shirsat *et al.*, 1989).

From the genomic DNA sequence, the predicted protein sequence contains a 21 amino-acid signal peptide followed by an α -legumin polypeptide of 36.44 kd and a β -polypeptide of 20.19 kd. Examination of the coding sequence reveals that the *leg* A gene exhibits many common features of plant genes (Messing, 1983). The gene has three introns which have the typical intron/exon boundary nucleotides of GT/AG. The 5'-flanking sequences contain the promoter motifs of a 'TATA' and a 'CAAT' box. There is also homology to the plant 'AGGA' box. The 3'-flanking sequence contains multiple polyadenylation signals of 'AATAAA' (Lycett *et al.*, 1984).

1.5 Strategies used in this study

The construction of transformation vectors used in this study was directed towards producing two constructs, one for use in *Agrobacterium*-mediated transformation and the second for use with direct gene transfer to protoplasts. In

addition to an antibiotic resistance marker gene, necessary to allow selection of transformed plant cells, the constructs also contained an unselected gene in order to assess its co-transformation with the marker gene and to study its expression in the transformed plants.

The unselected gene chosen was one of the genes coding for the A-type legumin of pea (Pisum sativum L.) (see Section 1.5.3). This legA gene is a member of the multi-gene family that codes for legumin, a major seed storage protein, and is expressed only in the seed tissues of pea. The 5'-flanking region of this gene has been shown to contain sequences that control its seed-specific expression (Gatehouse et al., 1986; Boulter et al., 1987; Ellis et al., 1988; Shirsat et al., 1989). The aim in this study was to produce a chimeric legumin gene for use in gene transfer experiments, whereby the 'seed-specific' promoter of *leg*A was replaced with a promoter known to be expressed in a more constitutive manner in plant tissues.

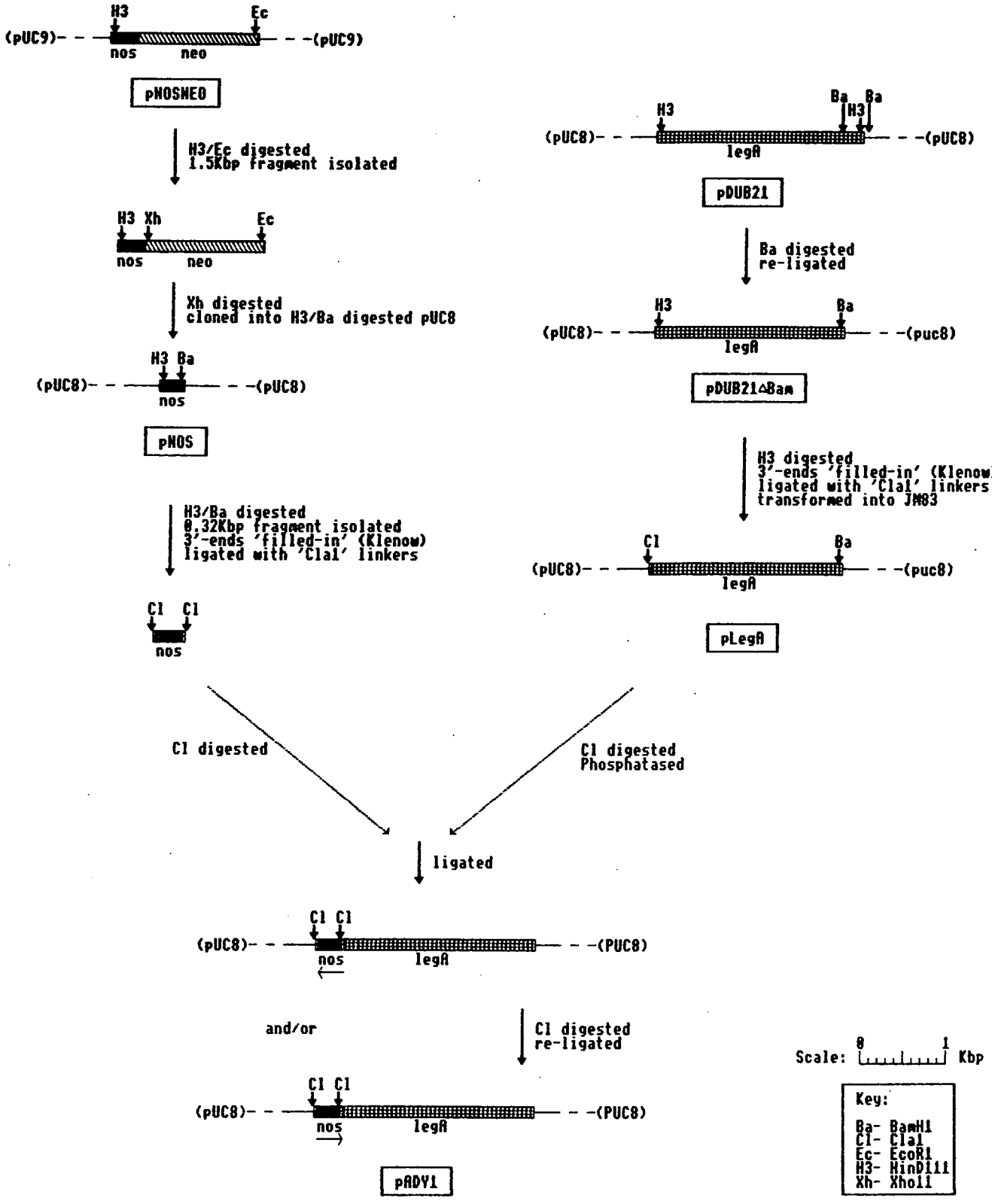
The promoter chosen for this purpose was that of the nopaline synthase gene (nos). This gene is present on the T-DNA segment of the Ti-plasmid of *Agrobacterium tumefaciens* (see Section 1.4.2), which is transformed and integrated into the plant genomic DNA on *Agrobacterium* infection (Thomashaw *et al.*, 1980; Nester *et al.*, 1984). The *nos* gene is constitutively expressed in the plant cells into which it is transferred (Wullems *et al.*, 1981). The *nos* promoter has been demonstrated to contain all sequences necessary to direct the constitutive expression of chimeric foreign genes in plants (Herrera-Estrella *et al.*, 1983b; Bevan *et al.*, 1983b; Fraley *et al.*, 1983).

The complete nucleotide sequence of the *leg*A gene has been determined (Lycett *et al.*, 1984). The sequence was analysed in an attempt to devise a construction strategy to remove the *leg*A 5'-flanking region and hence the putative 'seed-specific' control sequences, whilst retaining the translation start codon and an

FIGURE 1.

Construction of pADY1.

Strategy for the construction of the chimeric *nos/legA* gene.


H3
Ec
(pUC9)- -
-(pUC9)
nos
neo
pNOSNEO
H3/Ec digested
1.5Kbp fragment isolated
H3 Xh
Ec
nos
neo
Xh digested
cloned into H3/Ba digested pUC8
H3 Ba
(pUC8)- -
-(pUC8)
nos
pNOS
H3/Ba digested
0.32Kbp fragment isolated
3'-ends 'filled-in' (Klenow)
ligated with 'ClaI' linkers
Cl Cl
nos
Cl digested
Cl digested
Phosphatased
ligated
Cl Cl
(pUC8)- -
-(PUC8)
nos
legA
and/or
Cl digested
re-ligated
Cl Cl
(pUC8)- -
-(PUC8)
nos
legA
pADV1
H3
Ba Ba
H3
(pUC8)- -
-(pUC8)
legA
pDUB21
Ba digested
re-ligated
H3
Ba
(pUC8)- -
-(puc8)
legA
pDUB21ΔBam
H3 digested
3'-ends 'filled-in' (Klenow)
ligated with 'ClaI' linkers
transformed into JM83
Cl
Ba
(pUC8)- -
-(puc8)
legA
pLegA
Scale:
0
1
Kbp
Key:
Ba- BamHI
Cl- ClaI
Ec- EcoRI
H3- HinDIII
Xh- XhoII

intact coding sequence. Since no suitable restriction sites were found immediately 5' of the 'ATG' start codon an alternative strategies was devised (see Fig. 1), whereby a *leg*A genomic clone containing the coding and 3'-flanking sequences minus the start codon and next 4 bp was used.

The *nos* promoter used to produce the chimeric legumin gene was obtained as a fragment from the chimeric neomycin phosphotransferase gene - p*NosNeo* (Bevan, 1984). In this gene the *nos* promoter is coupled to the *neo* coding region and *nos* 3'-flanking region. The DNA sequence of the *nos* promoter fragment has already been determined (Bevan *et al.*, 1983a).

The fusion of the *nos* promoter to the *leg*A coding region was achieved by the use of a synthetic oligonucleotide linker molecule. Neither the promoter nor coding sequence used contained a start codon, so the sequence of the linker molecule was chosen in order to include the sequence 'ATG' in such a position as to be "in-phase" with the *leg*A reading frame when finally ligated.

The construct used in the study of *Agrobacterium*-mediated plant transformation was cloned into the 'binary vector' Bin 19 (Bevan, 1984). In the Ti-plasmid binary vector system, the virulence (*vir*) region and the T-DNA sequence (see Section 1.3.2) are contained on separate plasmids within an *Agrobacterium tumefaciens* host; the *vir* region acting *in trans* to transfer the T-DNA to the plant cell upon infection (Hoekema *et al.*, 1983; De Framond *et al.*, 1983). The plasmid containing the T-DNA is capable of replication in *Escherichia coli*; this allows the insertion of desired sequences into the T-DNA to be carried out in an *E. coli* host with subsequent transfer of the genetically manipulated T-DNA plasmid to the *vir* region-containing *Agrobacterium* host. This system was chosen as it is simpler than the technique that relies upon reciprocal recombination between an intermediate vector containing a manipulated T-DNA region and the resident Ti-plasmid in an *Agrobacterium* host (Van Montagu *et al.*, 1980; Leemans *et al.*, 1981; Shaw,

FIGURE 2.

Construction of pADY2.

Strategy for the insertion of the chimeric *nos/legA* gene into the binary vector plasmid Bin 19.

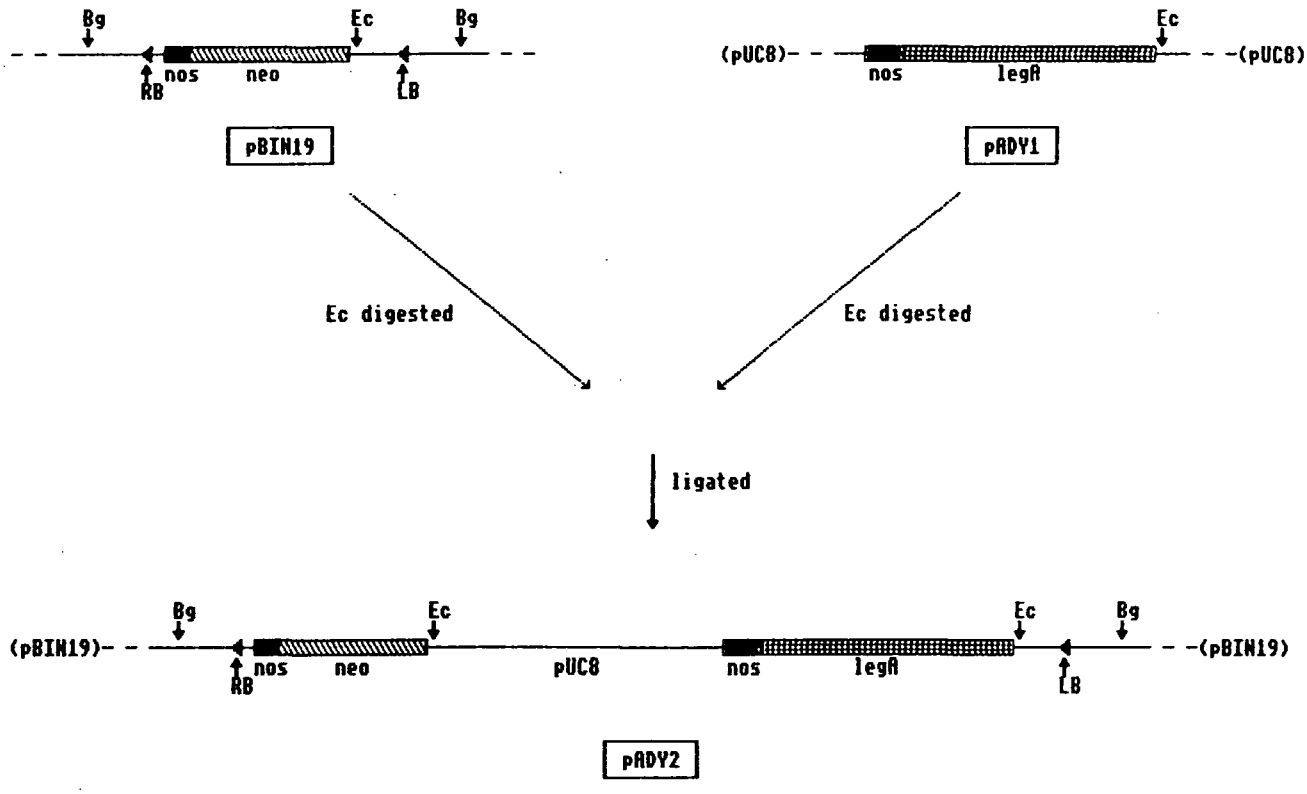
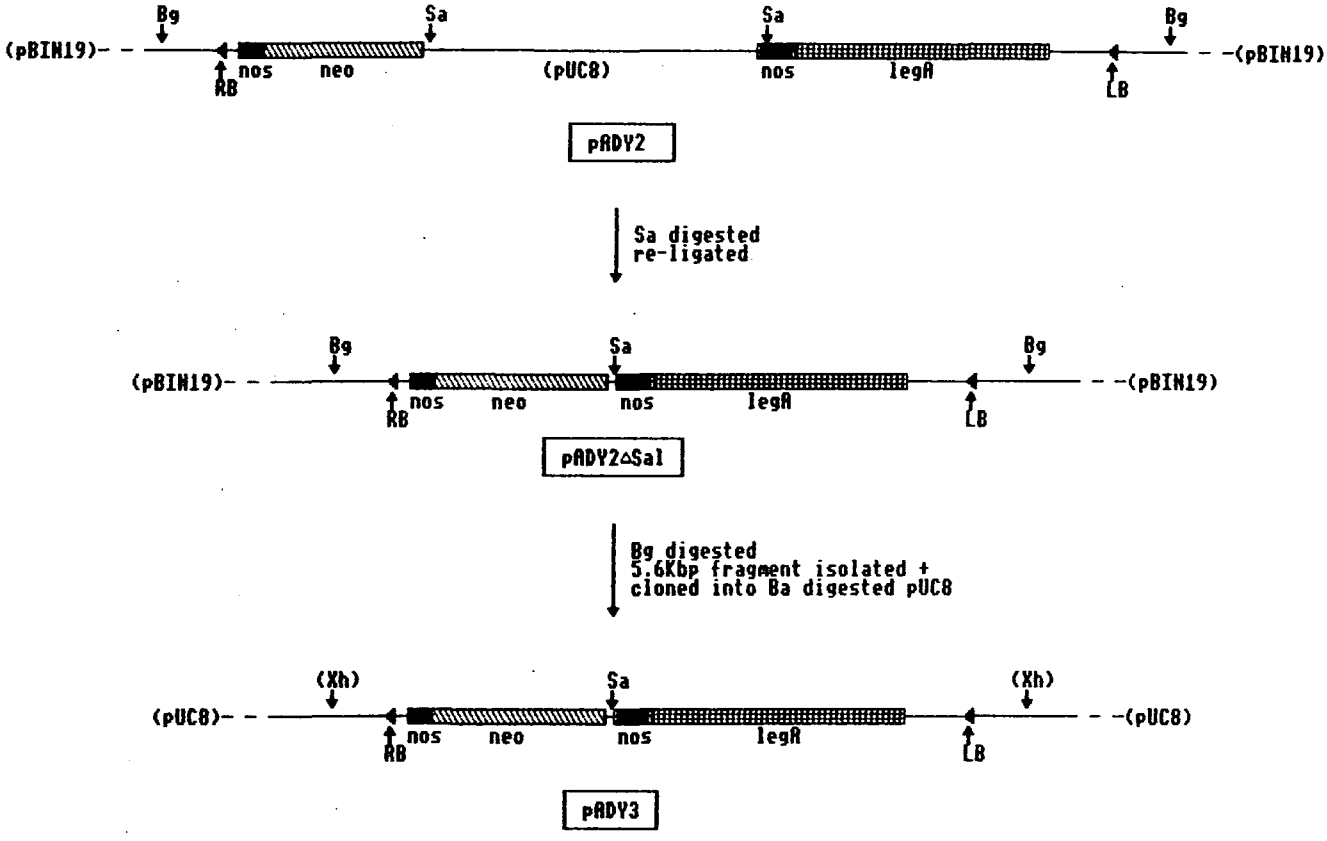

Bg
Ec
Bg
RB
nos
neo
LB
pBIN19
(pUC8)-
Ec
nos
legA
-(pUC8)
pADY1
Ec digested
Ec digested
ligated
(pBIN19)-
Bg
Ec
Ec
Bg
RB
nos
neo
pUC8
nos
legA
LB
-(pBIN19)
pADY2
Scale:
0
1
Kbp
Key:
Bg- BglII LB- T-DNA left border
Ec- EcoRI RB- T-DNA right border

FIGURE 3.

Construction of pADY3.

Strategy for the removal of the chimeric *nos/neo* and *nos/legA* genes from pADY2 and their insertion into the plasmid vector pUC8.


Bg
Sa
Sa
Bg
(pBIN19)
nos
neo
(pUC8)
nos
legA
RB
LB
(pBIN19)
pADV2
Sa digested
re-ligated
Bg
Sa
Bg
(pBIN19)
nos
neo
nos
legA
RB
LB
(pBIN19)
pADV2ΔSal
Bg digested
5.6Kbp fragment isolated +
cloned into Ba digested pUC8
(Xh)
Sa
(Xh)
(pUC8)
nos
neo
nos
legA
RB
LB
(pUC8)
pADV3
Scale:
0
1
Kbp
Key
Ba- BamHI
Bg- BglII
Sa- SalI
Xh- XhoI
LB- T-DNA left border
RB- T-DNA right border

1984), and thus more efficient; typical frequencies of formation of the desired recombined Ti-plasmid in *Agrobacterium* are 10^{-5} compared to 10^{-1} for transformation with the binary vector (Klee *et al.*, 1987). The T-DNA of Bin 19 consists of a multiple cloning site, interrupting a β -galactosidase gene, and chimeric *nos/neo* gene conferring resistance to kanamycin in transformed plants, between the left and right 25 bp T-DNA border repeat sequences. The construction strategy was to insert the chimeric legumin gene into the T-DNA region of Bin 19 (see Fig. 2), the pUC8 vector used in the construct of the chimeric legumin gene was also inserted (see Section 4.3.2).

The construct used for the study of direct gene transfer to protoplasts was required to be a simple vector containing only the selective marker and chimeric legumin genes. The strategy devised (Fig. 3) was based on the Bin 19/legumin construct in that the *nos/neo* and *nos/leg* genes were excised and transferred to the plasmid cloning vector pUC8.

1.6 Aims and objectives of this study

The aim of this study was to evaluate a number of gene transfer techniques that have potential application in the genetic manipulation of plant species. At the beginning of the study (1983), plant gene transfer techniques were in their infancy; initially novel methods such as direct uptake of DNA molecules by pollen tubes and microinjection into ovaries, were assessed as alternatives to the then developing *Agrobacterium* infection technique. However, with the advent of non-oncogenic *Agrobacterium*-mediated gene transfer and direct uptake of exogenously applied DNA by protoplasts, a strategy was devised to transfer a gene construct into tobacco plants using both techniques. In so doing, a comparison of the two methods was made possible, particularly an assessment of the relative efficiencies of the methods, with reference to factors such as the transformation frequency and

structural integrity of the introduced foreign genes. The expression of the foreign gene was studied in the tobacco transformants obtained. As the gene chosen was a developmentally regulated, tissue-specific gene, its expression when directed by a constitutive promoter, in the heterologous environment of the tobacco leaf tissue, was of particular interest.

CHAPTER 2.

MATERIALS AND METHODS.

Materials and Methods

2.1 Materials

2.1.1 Chemicals and biological reagents

All reagents, except those listed below, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of analytical grade or the best available.

Acridine orange acrylamide, bis-acrylamide, adenosine 5'-triphosphate (ATP), antibiotics used for selection (ampicillin, kanamycin, rifampicin and streptomycin), bovine serum albumin (BSA), dithiothreitol (DTT), ethidium bromide (EtBr), ethylene glycol bis (∝-amino-ethyl ether)-N,N,N'-tetra-acetic acid (EGTA), herring sperm DNA (HsDNA), egg white lysozyme, pronase P and protease K, RNase A, sodium dodecyl sulphate (SDS) and spermidine, were from Sigma Chemical Co., Poole, Dorset, U.K.

'Repelcote' was from Hopkins and Williams, Romford, U.K.

Ficol 400, Sephadex G-50, 'Cla I oligomeric linker' molecules and oligonucleotide primer molecules were from Pharmacia PL Biochemicals Inc., Pharmacia (GB) Ltd., Milton Keynes, Bucks, U.K.

α ^{32}P-dCTP, γ ^{32}P-ATP and 'Nick-translation kit' were from Amersham International plc, White Lion Road, Amersham, Bucks., U.K.

'Liquiscint' scintillation fluid was from National Diagnostics (U.K.) Ltd., 45 Long Plough, Aston Clinton, Bucks, U.K.

Glass-fibre filters (GF/C) and 3 MM paper were from Whatman Ltd., Maidstone, Kent, U.K.

Dialysis tubing (size 1-8/32") was from Medicell International Ltd., 239 Liverpool Road, London, U.K.

Fuji RX-100 X-ray film was from Fuji Ltd., Swindon, Wilts., U.K.

Dupont 'lightning plus cronex' intensifying screens were from E.I. Dupont de Nemours and Company (Inc.), Photoproducts Department, Wedgewood Way, Stevenage, Herts., U.K.

BBL trypticase peptone was from Becton Dickinson and Company, Cockeysville, Md., U.S.A.

Yeast extract was from Sterilin Ltd., Teddington, U.K.

Cellulase and Macerozyme (Onozuka R-10) were from Yakult Honsha Co. Ltd., Minato-Ku, Tokyo, 105-Japan.

MS medium was from Flow Laboratories, Woodcock Hill Ind. Est., Harefield Road, Rickmansworth, Herts., U.K.

"Timentin" (77.9% Ticarcillin, 5.2% Potassium Clavulanate) was obtained from Beecham Pharmaceuticals, Brentford, Middx., U.K.

Restriction endonucleases were from Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, Northumberland, U.K. or Boehringer Corporation (London) Ltd., (BCL), Lewes, E. Sussex, U.K.

Tris (hydroxymethyl) aminomethane (Tris), 'Klenow' fragment, T4 DNA ligase, T4 polynucleotide kinase, and 5-Dibromo-4-Chloro-3-indoylgalactoside (X-Gal) were from BCL (London) Ltd.

Agarose, low melting point agarose, deoxy and dideoxynucleoside triphosphates and DNA sequencing kits were from Bethesda Research Laboratories (U.K.) Ltd., (BRL), Cambridge, U.K.

High gelling temperature agarose and 'Gelbond' were from ICN Biomedicals Ltd., High Wycombe, Bucks., U.K.

2.1.2 Bacterial strains and plasmids

The genotype, source and/or reference for each of the bacterial strains and plasmids used is shown in Table 1.

Table 1.

Bacterial strain	Genetic character	Reference or source
JM83	ara$^-$, (lac-proAB), rps L (= str A) 080, lac$^-$ Z M15	Bethesda Research Laboratories (BRL)
JM101	lac pro, sup$^-$ E, thi$^-$, F'tra D36, pro$^-$ AB, lac$^-$ I, Z M15.	Dr. J. Messing or BRL
K514	(R$^-$ deriv. C600) sup$^-$ 2, ton$^-$ A21 thr$^-$ 1, leu$^-$8, thi$^-$ 7, lac$^-$ Yl, R^-_k, M^+_k.	
LBA4404	contains Ti-plasmid pAL4404 strep.^{r}, Rif^r.	Ooms *et al.* (1982)
Plasmid		
pBR322	Tet^r, Amp^r	Bolivar *et al.* (1977)
pUC8/pUC9	Amp^r, Lac$^-$ Z	Vieira & Messing (1982)
pDUB21	Hind III fragment of Leg A gene in pUC8	Lycett *et al.* (1984)
BIN 6	contains *nos/neo* and *nos* gene between T-DNA borders. Kan^r	Bevan (1984)
BIN 19	contains *nos/neo* gene between T-DNA borders. Kan^r	Bevan (1984)
pNosNeo	*nos/neo* gene in pUC9	Bevan (1984)
pRK2013	Kan^r	Ditta *et al.* (1980)
pGV3850	Ti-plasmid with pBR322 and *nos* gene between T-DNA borders. Kan^r	Zambryski *et al.* (1983)
pGV0601	Hind III 'fragment 23' of Ti-plasmid C58 in pBR322. Tet^r Amp^r	Zambryski *et al.* (1983)

2.1.3 Growth media for bacteria

The composition of media used for the growth of bacteria is given in Table 2.

Table 2. Composition of growth media

Medium	Composition (1 L)
L-broth	10 g trypticase 5 g yeast extract 5 g NaCl
L-agar	As L-broth + 10 g agar
YT-broth	8 g trypticase 5 g yeast extract 5 g NaCl
YT-agar	As YT-broth + 15 g agar
YEB-broth	5 g beef extract 1 g yeast extract 5 g bacto-peptone 5 g sucrose 2 ml 1 M $MgSO_4$
YEB-agar	As YEB-broth + 15 g agar
Minimal agar	20 g agar (autoclaved in 788 ml water then the following sterile stocks added) 10 ml glucose 1 ml 1 M $MgSO_4$ 1 ml thiamine (10 mg/ml) 200 ml 5 x 'Min A' salts* *(for 1 L) 52.5 g K_2HPO_4 22.5 g KH_2PO_4 1.0 g $(NH_4)_2 SO_4$ 2.5 g Na Citrate-$2H_2O$

2.1.4 Plant material

Salpiglossis sinuata 'Triumph' mixed var. seeds and *Hippeastrum vittatum* were obtained from Sutton Seeds Ltd., Hele Road, Torquay, Devon, U.K. *Vicia faba* seeds were a gift from Dr. J.A. Gatehouse.

Nicotiana tabacum cv. Petit Havana SRI seeds were a gift from Dr. R. Shillito, Friedrich Miescher Institut, Basel, Switzerland.

The plants were grown in Fisons Universal compost obtained from FAC (Fisons), Bishop Meadow Road, Loughborough, Leics., U.K. and/or compost mixed with 'Perlite' obtained from Tilcon Ltd., Knaresborough, N. Yorks., U.K.

2.2 Methods

2.2.1 Biochemical techniques

2.2.1.1 Reagents and equipment

All glassware and plastic-ware used in handling nucleic acid samples was sterilised by autoclaving before use. Plastic eppendorf tubes and Corex glass tubes were siliconised by coating with 'Repelcote', dried, rinsed in distilled water and autoclaved.

Centrifugation of eppendorf tubes (12,000 g) was performed using an MSE Micro-Centaur micro-centrifuge. Medium speed centrifugation of samples in Corex glass tubes, 100 ml and 250 ml samples was performed using a Sorval RC-5B centrifuge. An MSE-18 centrifuge was also used for 100 ml samples. Low-speed spins (~500 rpm were performed using an MSE Mistral 4L centrifuge. A Sorval OTD-65 ultracentrifuge with a VTi-50 vertical rotor was used for caesium chloride gradient centrifugation.

2.2.1.2 Phenol extraction of DNA samples

Protein was removed from DNA samples by two sequential extractions with phenol/chloroform (1:1 w/v) (henceforth referred to as 'phenol'). An equal volume of phenol was added to the DNA solution, mixed by vortexing, and the phases separated by centrifugation at 12,000 g for 3 mins. The aqueous phase was transferred to a fresh tube and extracted with phenol once more. In cases where a small amount of DNA was present, the first phenol phase was 'back-extracted' with an equal volume of

TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) (or water) and the resulting second aqueous phase combined with the first. Traces of phenol in the final aqueous phase were removed by two extractions with equal volumes of chloroform/iso-amyl alcohol (24:1 v/v). DNA was finally recovered from the aqueous phase by ethanol precipitation.

2.2.1.3 Ethanol precipitation of DNA

DNA was precipitated by the addition of 0.1 volumes 3M NaAc pH 4.8 and 2-3 volumes of absolute ethanol (-20°C), mixed by gentle inversion and then placed at -20°C for between 30 mins and overnight. The precipitated DNA was recovered by centrifugation at 12,000 g for 15 mins, washed twice in 80% ethanol (-20°C), dried under vacuum for 5-10 mins and resuspended in a small volume of sterile distilled water or TE buffer (Section 2.2.1.2).

2.2.1.4 Spectrophotometric analysis of nucleic acid solutions

The concentration of nucleic acid and presence of contaminants in DNA and RNA preparations were assessed by recording their absorbance spectra from 320 nm to 200 nm in quartz glass cuvettes using a Pye Unicam SP8-150 uv/vis spectrophotometer.

The OD_{260} values of solutions of 1 µg/ml DNA and of 1 µg/ml RNA are 0.02 and 0.024 respectively. Pure DNA and RNA solutions have OD_{260}/OD_{280} ratios of 1.8 and 2.0 respectively and OD_{320} values of zero. Analysis of the absorbance spectra gave an indication of the presence of protein, phenolic and other contaminants in nucleic acid preparations.

2.2.1.5 Storage of bacteria

Bacterial colonies were stored at 4°C for up to 8 weeks on inverted agar plates sealed with 'Nescofilm' (Nippon Shoji Kaisha Ltd., Osaka, Japan). Long-term storage

was at -80°C in a 40% glycerol solution. A bacterial lawn, prepared from a single colony, was transferred by sterile loop to a small glass vial containing 1 ml sterile L-broth (see Section 2.1.3), and uniformly resuspended 1 ml sterile 80% glycerol was added and then mixed by vortexing.

2.2.2 Isolation of plasmid DNA

2.2.2.1 'Mini-preparation' of *E. coli* plasmid DNA

The alkaline lysis method of Birnboim and Doly (1979) modified as described by Delauney (1984) was used.

RNA contamination present in the DNA preparations was removed by treatment with RNase during restriction endonuclease digestion (Section 2.2.5.1).

2.2.2.2 Large-scale preparation of *E. coli* plasmid DNA

The 'SDS lysis' method of Katz *et al.* (1977) was used as described by Waterhouse (1985) with the following modifications.

The plasmid bearing strain was grown at 37°C in 1 litre L-broth supplemented with the appropriate antibiotics to an OD_{650} of 0.9. Chloramphenicol (200 µg/ml) (Clewell, 1972) was then added to amplify the plasmid and the cultures grown at 37°C for a further 24 hr. The cells were harvested by centrifugation and resuspended in 15% (w/v) sucrose in 50 mM Tris/HCl, 50 mM EDTA pH 8.0. After the addition of 4 ml of lysozyme (10 mg/ml in 15% sucrose/Tris/EDTA) (0°C for 10 min), 12 ml of 5 M potassium acetate and 6 ml of 10% (w/v) SDS were added and the sample incubated at 0°C for 1 hr. The lysate was cleared by centrifugation and the DNA precipitated by NaCl and polyethylene glycol (PEG) treatment overnight, as described by Waterhouse (1985). The DNA was resuspended in 10 ml of TE buffer (Section 2.2.1.2) and then purified by caesium chloride density gradient centrifugation.

Ethidium bromide (EtBr) (10 mg/ml stock) was added to 300 µg/ml and solid CsCl was added to 48.4% (w/w) and the sample centrifuged at 44,000 rpm for 24 hr at 15°C (Sorvall OTD-65 ultracentrifuge with VTi-50 vertical rotor). The banded plasmid DNA was removed with a wide bore hypodermic needle inserted through the side of the centrifuge tube and re-purified on a second CsCl gradient, as described above. EtBr was removed by repeated iso-amyl alcohol extraction and then the DNA was dialysed extensively against TE buffer. Finally, the DNA was ethanol precipitated (section 2.2.1.3) and re-dissolved in 200 µl of TE buffer.

2.2.2.3 'Mini-preparation' of *Agrobacterium* plasmid DNA

A 10 ml L-broth *Agrobacterium* culture was grown under appropriate antibiotic selection at 28°C overnight. The cells were harvested by centrifugation (7000 g for 10 mins), resuspended in 400 µl of lysis buffer (1 mg/ml protease K in 50 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0, 0.8% (w/v) SDS) and incubated at 37°C for 1 hr. The high molecular weight chromosomal DNA was sheared by being sucked up and down in a siliconized glass pasteur pipette three times. The sample was phenol/chloroform extracted twice and the DNA ethanol precipitated, and finally re-dissolved in 100 µl TE buffer.

2.2.2.4 Large-scale preparation of *Agrobacterium* plasmid DNA

The method of Currier and Nester (1976) as modified by A. Hepburn (pers. comm.) was used.

Two 500 ml YEB cultures were grown at 28°C on a fast orbital shaker to an OD_{600} of 0.6 (approx. 16 hr). The cells were harvested by centrifugation (7000 g, for 10 mins), washed in 200 ml of 50 mM Tris-HCl, 20 mM EDTA pH 8.0 (TE·8), resuspended in 160 ml of TE·8 and transferred to a 500 ml Schott bottle (Gallenkamp, Loughborough, U.K.). The cells were lysed by addition of 20 ml pronase solution (5

mg/ml pronase in TE·8) and 20 ml of 10% (w/v) SDS (in TE·8) followed by incubation at 37°C for 60 mins with occasional gentle swirling. The DNA was denatured by addition of 3 M NaOH until the pH of the lysate was 12.3 (measured by pH meter probe). After gentle mixing for 10 mins the pH was adjusted to 8.5 with 2 M Tris-HCl pH 7.0. After a further 5 mins mixing, 24 ml of 5 M NaCl and an equal volume of phenol (equilibrated with 3% (w/v) NaCl) was added and mixed by two quick inversions followed by gentle mixing for 10 mins. The solution was poured into glass centrifuge bottles (MSE) and centrifuged (500 g, for 45 mins). The aqueous phase was transferred (using wide bore pipette to prevent shearing) to four centrifuge tubes and to each was added 7.5 ml of 3 M NaAc pH 4.8, 15 ml of 5 M NaCl and 40 ml of absolute ethanol. The DNA was precipitated overnight at -20°C, recovered by centrifugation and resuspended in 5 ml of TE (10 mM Tris HCl, 1 mM EDTA pH 8.0) overnight. The DNA was purified by CsCl gradient centrifugation (Section 2.2.2.2), dialysed against TE, ethanol precipitated and finally re-dissolved in 150 µl TE buffer.

2.2.3 Isolation of plant genomic DNA

2.2.3.1 *Nicotiana* leaf tissue DNA

The method for the purification of DNA from *Nicotiana* leaf material was adapted from Graham (1978). All operations were performed in a 'cold room' at 4°C. 2.0 g-4.0 g (2 or 3 fully expanded leaves) leaf tissue was harvested, immediately frozen in liquid N_2, ground to a fine powder in a pre-cooled mortar and pestle and transferred to a corex tube. 2.5 ml of 'homogenizing buffer' (H.B. - 0.1 M NaCl, 0.025 M EDTA pH 8.0, 2% (w/v) SDS, 0.1% (v/v) diethyl pyrocarbonate) was added and mixed by vortexing. 0.7 ml of 6 M sodium perchlorate, 2.1 ml of phenol and 2.1 ml of chloroform/octanol (99:1 (v/v)) was added, mixed and shaken gently for 50 mins on an orbital shaker at 4°C. The phases were separated by centrifugation and the aqueous phase extracted with chloroform/octanol (99:1 (v/v)). The DNA was ethanol

10 min. After phenol/chloroform extraction, the DNA was ethanol precipitated and finally re-dissolved in 30 µl of RB.

2.2.4 Isolation of total RNA from *Nicotiana* leaf tissue

The 'hot SDS' method of Hall *et al.* (1978) was used, modified as outlined below.

2 g of leaf tissue was harvested, frozen in liquid N_2 and ground to a fine powder in a pre-cooled mortar and pestle. 25 µl of 1 M DTT and 10 ml of hot (100°C) SDS/borate buffer (0.2 M sodium borate, 1% (w/v) SDS, 30 mM EGTA, pH 9.0) was added and mixed. The homogenate was transferred to a corex tube, vortexed for 30 secs, then incubated at 37°C for 1 hr with 2 mg protease K. 0.8 ml of 2 M KCl (ice cold) was added and the extract was placed on ice for 10 min then centrifuged at 12000 g for 10 min. Solid LiCl was added to the supernatant (to a final concentration of 85 mg/ml) and dissolved by gentle mixing. After overnight incubation on ice, the precipitated RNA was pelleted (12,000 g for 10 min), washed twice with 2 M LiCl and re-dissolved in 1.6 ml of H_2O. 0.4 ml of 0.2 M potassium acetate pH 5.5 was added and the sample placed on ice for 1 hr. After a clearing spin (12,000 g for 10 min) RNA was recovered from the supernatant by ethanol precipitation (section 2.2.1.3) and re-dissolved in 0.5 ml of H_2O. The preparation was phenol extracted (section 2.2.1.2), the RNA ethanol precipitated and finally re-dissolved in 100 µl of H_2O and stored in liquid N_2.

The concentration and purity of RNA was assessed by spectrophotometric analysis (section 2.2.1.4) and the integrity of the RNA was assessed visually after glyoxalation and agarose gel electrophoresis (section 2.2.7.3).

2.2.5 Enzymatic reactions used in the manipulation of DNA

2.2.5.1 Restriction endonuclease digestion

The restriction endonuclease enzymes used to digest DNA samples were incubated with the DNA in one of the three buffers recommended by Maniatis (1982) with the addition of BSA to a final concentration of 0.1 µg/ml (BRL recommendation), and at the temperatures recommended by the manufacturer. Most enzymes were supplied at a concentration of 5 units/µl and so 5 units were used to digest up to 1 µg of plasmid DNA for 2 hr. The amount of enzyme used and the incubation time was increased in genomic DNA digestion reactions (typically 5-10 units per µg for 4-5 hr was used). Digests of 'mini-prep' plasmid DNA included RNase at 50 µg/ml (pre-boiled for 15 mins to inactivate contaminating DNases) and 5 mM spermidine.

Digests involving more than one enzyme were either performed in the same reaction, if the specified enzyme buffers were compatible, or the buffer components were altered after the first enzyme digest to provide the requirements for the second.

2.2.5.2 Ligation of DNA molecules

DNA fragments with 'cohesive' or 'blunt' termini were covalently ligated using T4 DNA ligase (1-3 units) in 'Ligation buffer' (6 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM ATP). The reaction volume was typically 10 µl and incubation was at room temperature for 2 hr or at 15°C for 16-20 hr.

2.2.5.3 'Filling-in' recessed 3' DNA termini

ds-DNA fragments with recessed 3' termini were 'filled-in' using the 'Klenow' fragment of DNA polymerase I. 0.5 µg DNA was treated with 5 units of 'Klenow' enzyme in 'Klenow reaction buffer' (supplied by the manufacturer) in the presence of 0.2 mM dATP, dTTP, dCTP and dGTP, and the reaction incubated at room temperature for 45 mins, followed by heat inactivation of the enzyme at 70°C for 5 mins.

2.2.5.4 Phosphorylation of 'linker' DNA molecules

Oligomeric 'linker' molecules containing the required restriction site sequences were supplied in an unphosphorylated form and were phosphorylated by a kinase reaction before use. 2 µg (300 pmoles) of the linker oligonucleotide was phosphorylated for 90 mins with 4.5 units of polynucleotide kinase in 'kinase buffer' (66 mM Tris-HCl pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM DTT, 0.2 µg/ul BSA), including 1 mM γ ^{32}-PATP (10 µCi) in 10 µl at 37°C. Incorporation of the radiolabelled phosphate allowed analysis of subsequent ligation and restriction reactions with the 'linkers' by PAGE and autoradiography.

2.2.5.5 5'-dephosphorylation of DNA by alkaline phosphatase

The 5'-terminal phosphate groups were removed from DNA fragments, to prevent self-ligation in subsequent ligation reactions, by treatment with alkaline phosphatase. The enzyme (10 u) was added to a completed restriction endonuclease digest and incubated at 37°C for 1 hr (followed by heat inactivation at 70°C for 3 mins).

2.2.5.6 S1 nuclease digestion of plasmid DNA

The 'supercoiled' (SC) form of plasmid DNA was reduced to the 'open-circle' (OC) form by treatment with S1 nuclease. The nicking of SC forms to produce oc forms is a feature of single-strand specific nucleases such as S1 nuclease (Wiegand *et al.*, 1975). 1.0 µg of plasmid DNA was incubated with 30 u of S1 nuclease in 'S1 buffer' (20 mM sodium acetate pH 4.5, 2 mM $ZnCl_2$, 60 mM NaCl) in a total volume of 20 µl, at 37°C for 30 mins.

2.2.6 Transformation of *E. coli* cells by plasmid DNA

E. coli JM83 and K514 cells were rendered 'competent' to take-up DNA by treatment with calcium chloride, using a procedure based on that of Dagert and Ehrlich (1979). A 50 ml YT-broth culture was grown to an OD_{600} of 0.2 and the cells harvested by centrifugation (7000 g). The pellet was resuspended in 40 ml ice-cold 0.1 M $CaCl_2$ and kept on ice for 1 hr, then re-centrifuged, gently resuspended in 1 ml of the above $CaCl_2$ solution and stored on ice overnight.

A 100 µl aliquot of competent cells was transformed with 10 µl (~0.1 µg) DNA solution. The DNA was added to the cells, gently mixed and kept on ice for 30 mins. The cells were then heat-shocked at 37°C for 3 mins, followed by the addition of 1 ml L-broth and incubation at 37°C for 1 hr to allow the expression of plasmid encoded antibiotic resistance. 100 µl aliquots of this transformation mixture were spread onto agar plates containing appropriate antibiotics to select for transformants (ampicillin 50 µg/ml and/or kanamycin 25 µg/ml). In the case of transformations with the vector pUC8, where DNA had been ligated into the multiple cloning site, recombinants were identified as white colonies on antibiotic selection plates containing X-Gal (40 µg/ml) (re-ligated vectors with no insert gave blue colonies).

2.2.7 Size fractionation of nucleic acid molecules by electrophoresis

2.2.7.1 Polyacrylamide gel electrophoresis of DNA molecules

Polyacrylamide gels were used for separation of oligomeric 'linker' DNA molecules and for DNA sequencing (see Section 2.2.1.4). Gels were prepared as described by Delauney (1984).

2.2.7.2 Agarose gel electrophoresis of DNA molecules

Agarose gels (0.6-0.8%) were used to size fractionate DNA fragments of 0.1 - 20 kb. The agarose was dissolved in 200 ml of electrophoresis buffer (40 mM Tris-acetate

pH 7.7, 2 mM EDTA) by boiling, EtBr was added to 1 μg/ml and the gel poured into a perspex mould secured to a horizontal glass plate by silicon vacuum grease. When set, the gel was submerged in the above buffer in an electrophoresis tank and the DNA samples, mixed with 0.5 volume 'agarose beads' (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 30% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 0.2% (w/v) agarose - autoclaved then extruded through a fine-needled syringe when cool), and loaded into the wells. The gel was run at 30 V for 16-20 hr (or 120 V for 3-5 hr) and the DNA bands visualised using short-wave uv illumination. Photographs were taken using a red/orange filter (Kodak 23A Wrattan) and polaroid type 667 (3000 ASA) film (f16 for 4-5 secs)

2.2.7.3 Agarose gel electrophoresis of RNA molecules

Agarose gel electrophoresis of RNA samples was performed using the method of McMaster and Carmichael (1977). The RNA was denatured by glyoxalation, run on agarose gels and visualized by acridine orange staining.

10 μg of RNA was glyoxalated by incubation at 50°C for 1 h in 1 M glyoxal (de-ionized with 'amberlite' resin (BDH Ltd.) under N_2 and stored at -20°C), 50% (w/v) re-distilled dimethyl sulphoxide (DMSO) and 10 mM NaH_2PO_4 pH 6.8 in a total reaction volume of 40 μl. 5 μl of 'agarose beads' (in 10 mM NaH_2PO_4 pH 6.8 - see Section 2.2.7.2) were added and the sample stored on ice before being loaded onto the gel.

1.5% high gelling temperature agarose gels were prepared on the hydrophyllic side of a sheet of 'gelbond' placed between a horizontal glass-plate and perspex mould surround (see Section 2.2.7.2). The gels, attached to the 'gelbond', were submerged in running buffer (10 mM NaH_2PO_4 pH 6.8) and the samples loaded. the gels were run at 100 v for 3-4 h with slow stirring and re-circulation of the buffer.

Gels to be stained were gently rocked in freshly prepared acridine orange (30 μg/ml in running buffer) for 5 mins (dark, 4°C), then destained in running buffer for 15-20 h (dark, 4°C). The RNA bands were visualized using transmitted UV light (254 nm) and the gel photographed (Section 2.2.7.2).

2.2.8 Recovery of DNA fragments from agarose gels

Two methods were used, both yielded DNA that could be efficiently restriction endonuclease digested, ligated or ^{32}-P labelled.

2.2.8.1 Glass-fibre disc method

This was a modification of the method of Yang *et al.* (1979) and is described by Sawyer (1986). Basically, an agarose gel slice containing the DNA band was dissolved in 6M sodium perchlorate and passed through a glass-fibre disc (Whatman GF/C). The DNA retained on the disc was washed with sodium perchlorate washing solution (6M $NaClO_4$, 100 mM Tris-HCl pH 7.4), then ethanol precipitated. After the filter was air-dried, the DNA was redissolved in TE buffer and then eluted by centrifugation into an eppendorf tube.

2.2.8.2 'Freeze-squeeze' method

In this method, based on that of Tautz and Renz (1983), a gel slice, containing the desired DNA band, was equilibrated for 15 mins in 1 ml of 0.3 M NaAc,1 mM EDTA, blotted on 3 MM paper and transferred to a 0.75 ml eppendorf tube plugged with siliconised glass wool and pierced at the base. This tube was then frozen at -80°C for 15 mins and the DNA spun from the gel slice (12,000 g, 15 mins) into a 1.5 ml eppendorf tube. 0.03 volumes of 1 M $MgCl_2$, 10% (v/v) acetic acid was added and the DNA was ethanol precipitated twice at -20°C for 1 hr and finally dried and re-dissolved in a small volume of sterile water.

2.2.9 Transfer of nucleic acids from agarose gels to nitrocellulose filters

2.2.9.1 Transfer of DNA by 'Southern blotting'

The 'Southern blotting' procedure (Southern, 1975) was used as described by Waterhouse (1985). Briefly, the agarose gel was denatured in 1.5 M NaCl, 0.5 M NaOH, 1 mM EDTA for 40 min (with one change) and then neutralized in 3 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA pH 7.0 for 1 h (with two changes). After a brief wash in 20 x SSC (3M NaCl, 0.3 M tri-sodium citrate pH 7.0) the gel was placed on a wick of Whatman 3 MM paper in a reservoir of 20 x SSC and a nitrocellulose sheet placed on the gel, then 3 MM paper and a stack of absorbant towels and a weight were placed on top. Capillary action drew 20 x SSC through the gel, eluting the DNA onto the nitrocellulose filter. When blotting was complete (~20 h), the filter was removed and baked at 80°C under vacuum for $2^1/_2$ h.

2.2.9.2 Transfer of RNA by 'Northern blotting'

The blotting procedure for transferring RNA from agarose gels to nitrocellulose filters was the same as for 'Southern blotting', except that the gel was not denatured or neutralized but placed directly on the blotting apparatus. Gels to be blotted were not stained with acridine orange (Section 2.2.7.3) as this is thought to interfere with transfer and subsequent hybridization.

2.2.10 Transfer of bacterial colonies to nitrocellulose filters

The transfer of DNA from bacterial colonies to nitrocellulose filters was achieved by an *in situ* lysis method, which was a modification of that of Grunstein and Hogness (1975). A circular (7.5 cm diameter) nitrocellulose filter was placed on the surface on an agar plate and the colonies lifted off by careful removal of the filter. The filter was allowed to air-dry, then was placed on 3 MM paper soaked in

denaturing solution (Section 2.2.9) for 15 mins. This was repeated with neutralising solution (Section 2.2.9) and 4 x SSC (Section 2.2.9), air-drying between each step. Finally, the filter was baked at 80°C under vacuum for 2 hr. The original 'master' plate was incubated overnight to re-grow the colonies then stored at 4°C.

In cases where there were many colonies on a plate, they were transferred by sterile toothpick to a fresh selective plate in a grid pattern and in a duplicate pattern on a nitrocellulose filter placed on a similar plate. Both were incubated until the colonies were visible then the nitrocellulose filter was processed as above and the master stored at 4°C.

2.2.11 ^{32}P Labelling of DNA molecules

2.2.11.1 'Nick-translation' method

A nick-translation kit was used according to the manufacturer's instructions (Amersham International Ltd.). 0.5-1.0 µg of DNA was labelled to a specific activity of 10^7-10^8 cpm per µg.

Unincorporated radionucleotide was separated from labelled DNA by passage through a 5 ml sephadex G-50 (superfine) column, equilibrated and eluted in 'column buffer' (150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.1% SDS). 400 µl fractions were collected and 1 µl of each added to 4 ml of scintillation fluid ('Liquiscint') for counting using a Packard PL-tri-carb scintillation counter. Fractions containing the first peak of radioactivity were pooled for use as labelled probe.

2.2.11.2 'Random primer' method

In this technique, DNA restriction endonuclease fragments were heat denatured and radiolabelled with the 'Klenow' fragment of *E. coli* DNA polymerase I synthesizing complementary DNA in the presence of α ^{32}P-dCTP using random hexameric oligonucleotides (P.L. Biochemicals No. 2166) as primers.

The method of Feinberg and Vogelstein (1984) was used, except that the DNA fragments to be labelled were isolated from agarose gels by the 'freeze-squeeze' technique (Section 2.2.8.2) and resuspended in 10 ul H_2O for the labelling reaction. The labelled DNA fragments were purified by passage through a sephadex G-50 column (Section 2.2.11.1). Specific activities of up to 10^9 cpm/µg were obtained using 100 ng of DNA fragment per reaction.

2.2.12 Hybridization of ^{32}P labelled DNA probes to filters

2.2.12.1 'Southern blot' hybridization

This technique was used to detect specific DNA sequences bound to nitrocellulose filters by hybridization with radio-labelled probes. The filters were incubated in heat-sealed polythene bags at 65°C in a shaking waterbath throughout. They were incubated first in 'pre-hybridization' solution (5 x Denhardts solution - 0.1% (w/v) each Ficoll 400, PVP and BSA - in 5 x SSC with 100-200 µg/ml denatured herring sperm DNA) at 0.25-0.5 ml per cm^2 filter for 1 hr and then the solution was replaced with 'hybridization' solution (1 x Denhardts solution in 5 x SSC with 100 µg/ml denatured herring sperm DNA) at 0.1-0.25 ml per cm^2 filter. The labelled probe was denatured by boiling for 6 mins and then added to the hybridization solution. Incubation was for 16-20 hr.

Non-specifically hybridized probe was removed by the following washing procedure. The hybridization solution was poured from the bag (and stored at -20°C for possible future use) and replaced with wash solutions at 0.5 ml per cm^2 filter, in the following order:- (a) 1 x 30 mins in 2 x SSC; (b) 2 x 15 mins in 1 x SSC; (c) 2 x 15 mins in 0.1 x SSC, all incubations at 65°C. This high stringency wash procedure was used for all filters. After the final wash, the filter was air-dried and prepared for autoradiography.

2.2.12.2 'Northern blot' hybridization

Northern blots were probed with radio-labelled DNA probes using essentially the same hybridization and washing procedures as detailed above for Southern blots, with the following exceptions:

All solutions included 0.1% (w/v) SDS; pre-hybridization (0.1 ml per cm^2 filter) was performed for 16-20 hr and hybridization (0.03 ml per cm^2 filter) for 48 hr.

2.2.13 Autoradiography

This technique was used to detect the position of the hybridized probe on the filter blots. In a dark-room using a safety lamp, pre-flashed X-ray film and an intensifying screen were placed over the filter and these were sandwiched between two glass plates held together with elastic bands. This was then wrapped in three black plastic bags, and placed at -80°C for exposure times of between 1 hr and 6 wks, depending on the amount of radioactivity on the filter. The film was developed in Kodak X-Omat developer for 6 mins, fixed in Kodak fixer for $2^1/_2$ mins (both at room temperature), washed in running water and then allowed to air-dry.

2.2.14 DNA sequencing

M13 cloning in preparation for DNA sequencing and sequencing using the dideoxynucleotide chain termination method of Sanger *et al.* (1977), was performed according to the protocols described in the instruction booklet supplied with the BRL sequencing kit. The sequencing reactions, polyacrylamide gel electrophoresis and subsequent autoradiography were performed by Mr. P. Preston.

The DNA sequences were read from the autoradiographs and the complementary strand sequences obtained using a programme on a BBC microcomputer.

2.2.15 Pollen germination

2.2.15.1 Attachment of *Agrobacterium* to pollen tubes

A 10 ml L-broth culture of *Agrobacterium tumefaciens* strain C58 harbouring pGV3850 was grown to saturation (28°C, 24 hr). The cells were harvested by centrifugation (7,000 g, 10 min) and resuspended in 2 ml of 'Germination Medium' (GM-'Brewbakers medium' (Brewbaker and Kwack, 1963), containing 10% (w/v) sucrose). Pollen grains of *Hippeastrum vittatum* were germinated at room temperature overnight in a drop of this bacterial suspension on a glass microscope slide. Slides were placed in a petri-dish sealed with "Nescofilm" to maintain humidity. Pollen of *Vicia faba* was also germinated under the same conditions except the GM contained 20% (w/v) sucrose.

2.2.15.2 Incubation of pollen tubes with ^{3}H-labelled plasmid DNA

Pollen grains of *Vicia faba* were imbibed in a drop of GM containing 20% (w/v) sucrose (Section 2.2.15.1) on a microscope slide. When the pollen began to germinate the medium was drawn-off using a micro-capillary and replaced with a drop of the above medium containing 0.5 µg of ^{3}H-labelled pBR322 plasmid DNA (labelled to 10^{6} cpm/µg by 'Nick-translation' (Section 2.2.11.1) using ^{3}H-dCTP). After incubation at room temperature for 90 min, the pollen tubes were washed five times with the GM sucrose medium. The washings were retained for scintillation counting (Section 2.2.11.1). The pollen tubes were incubated for a further 90 min in GM sucrose medium (without labelled DNA) and after this 'cold chase' the medium was removed and the pollen tubes fixed in 2% (v/v) gluteraldehyde, 20% (w/v) sucrose, washed three times with sterile distilled water, once with 10% (w/v) trichloroacetic acid and then allowed to air-dry. The slide was dipped in photographic emulsion in complete darkness (Kodak "striping film"), air-dried, then placed at -80°C for two weeks. The micro-autoradiograph was developed and fixed as described in Section 2.2.13.

2.2.16 Microinjection of DNA into ovaries of *Salpiglossis*

2.2.16.1 Preparation for microinjection

Glass microcapillaries for the microinjection work were produced using a 'two stage puller' apparatus, then broken to give an external tip diameter of 25 μm, as described by Hepher *et al.* (1985). Microinjections were performed under sterile conditions, viewed under a dissecting microscope and using a microcapillary holder attached to a micromanipulator.

Ovaries of *Salpiglossis sinuata* were excised from the plants by cutting the pedicel, then the corolla was removed. The cut edge was sealed with paraffin wax and then the ovary was surface-sterilized as described by Hepher *et al.* (1985) and placed in 0.7% water agar for injection.

Microinjections were also performed using ovaries which were not excised from the plant; the corolla was removed and the ovary immersed in the sterilizing solutions (Section 2.2.16.1). Microinjection was performed as above, except the plant was placed adjacent to the microinjection apparatus and the ovary positioned for injection.

For experiments requiring the pre-pollination of ovaries, pollen was applied to the stigma 14 to 15 hr before microinjection, in order to ensure the DNA was introduced into the ovary immediately prior to the pollen tubes reaching the base of the style and entering the locular cavity (Hepher and Boulter, 1987) (see Fig. 4a).

2.2.16.2 Ovary microinjection

A microcapillary was used to make a hole in the neck of the ovary wall, then the DNA solution was microinjected into the locular cavity at the base of the ovary until the injectant exuded from the first hole (Fig. 4b). Each ovary was microinjected twice, once into each loculus. The holes were sealed with drops of paraffin wax, then re-cut and the ovary cultured on the agar medium of Nitsch (1951) at 25°C in a

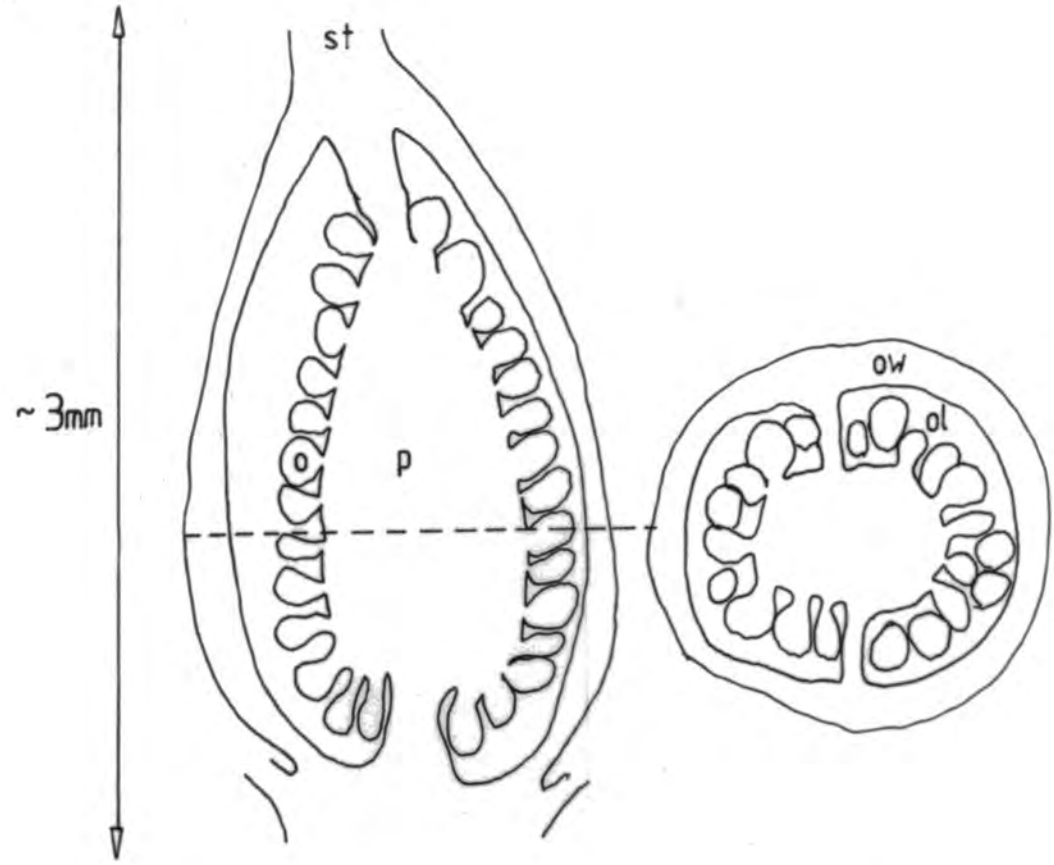
FIGURE 4.

A. Line drawing of longitudinal (LS) and transverse (TS) sections through an ovary of *Salpiglossis sinuata.*

st	-	style
p	-	placenta
o	-	ovule
ow	-	ovary wall
ol	-	ovary loculus

B. Injection of dye into the loculus of a *Salpiglossis sinuata* ovary. The dye can be seen exuding from a hole pierced in the neck of the ovary.

A.



B.

"Nescofilm" sealed petri-dish. In the case of ovaries microinjected whilst still attached to the plant, after sealing the holes as above, the plant was returned to the growth room (18 hr photoperiod, 70% relative humidity, 25°C).

Where DNA analyses of the injected ovaries were required (Section 2.2.3.3), the ovaries were washed four times in sterile distilled water, then the style and pedicel were removed and the ovaries were frozen in liquid N_2 and stored at -80°C until required.

2.2.16.3 Nopaline assay

The expression of the nopaline synthase gene in *Salpiglossis sinuata* tissue was assayed using the 'microscale' method of Otten and Schilperoort (1978).

Mature seeds, collected from pGV3850 microinjected *Salpiglossis* ovaries, were germinated and ~100 mg of leaf tissue was harvested from each of the seedlings to be tested. The tissue was incubated overnight at 25°C in buffer containing arginine (Otten and Schilperoort, 1978), then ground in a small eppendorf tube using a stainless steel rod and a little acid-washed sand. After centrifugation (12,000 g, 3 min) the supernatant was transferred to a fresh tube, freeze-dried overnight and resuspended in 10 µl H_2O.

The 10 µl samples were 'spotted' onto Whatman 3MM paper with samples of authentic nopaline and octopine, to provide standards and electrophoresed for $1^1/_2$ hr, as described by Otten and Schilperoort (1978). The electrophoretograms were air-dried then stained by spraying with phenanthrenequinone. Nopaline positive spots were identified as fluorescent yellow/green spots under uv illumination.

2.2.17 Transferring plasmids to *Agrobacterium*

The cloning of plasmid constructs was performed in *E. coli* strains, the transfer of a construct to *Agrobacterium* was achieved by a tri-parental mating, utilizing the

plasmid pRK2013 in *E. coli* HB101 to mobilize the plasmid construct from the host *E. coli* strain to the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983).

The antibiotic resistance characteristics of each strain was checked on selective plates, then single colonies were used to inoculate 10 ml YEB-broth cultures grown at 37°C overnight (*E. coli* strains) or 28°C for 2 days (*Agrobacterium* strain). 200 µl of each strain culture was mixed in a 1.5 ml eppendorf tube and 150 µl of this 'mating mixture' was spread onto L-agar plates and incubated at 28°C for 16-20 hr. The bacterial lawn produced was suspended in 5 ml 'suspension buffer' (50 mM $MgCl_2$, 10 mMTris/HCl pH 7.5) and serial dilutions made to 10^{-6}. 100 µl of each dilution was spread on minimal agar plates containing 50 µg/ml kanamycin and 100 µg/ml Rifampicin and incubated at 28°C for 4 days to select for the desired *Agrobacterium* transconjugants. Well isolated colonies were grown up on fresh selective plates for analysis and storage.

2.2.18 Infection of *Nicotiana* leaf pieces with *Agrobacterium*

Agrobacterium infection of leaf pieces was performed using the method of Horsch *et al.* (1985) modified as described below. Manipulations were performed under sterile conditions in a laminar flow cabinet.

The *Agrobacterium* strain was grown to saturation at 28°C (24 h) in 50 ml of L-broth medium, supplemented with 20 mg/ml kanamycin, and the cells harvested by centrifugation (7,000 g, 10 min), washed three times in 2 mM $MgSO_4$, then resuspended (10^9 cells/ml) in MS medium (Murashige and Skoog, 1962), supplemented with 1% (w/v) sucrose (MSIS).

A fully-expanded *Nicotiana tabacum* SRI leaf was excised from the plant and surface-sterilized (70% (v/v) ethanol for 2 min then 20% (v/v) "Chloros" (sodium hypochlorite solution - Imperial Chemical Industries PLC) for 15 min. After three washes in sterile distilled water the leaf was cut into pieces (approx. 5 mm x 5 mm)

and immersed in the bacterial suspension for 10 min. The leaf pieces were transferred to 'shooting medium' (MSIS + 2 mg/l kinetin, 0.2 mg/l naphthalene acetic acid (NAA), 0.8% agar) and cultured for 48 h at 25°C, washed in MSIS + 200 µg/ml Timentin (Tim) for 15-20 h, briefly washed in MSIS, blotted dry and cultured on shooting medium + 200 µg/ml Kan and 100 µg/ml Tim in a 25°C growth room (16 h day, 14 K lux). The leaf pieces were transferred to fresh medium after 4 weeks.

Shootlets were excised from the leaf edge and transferred to 1/2 MS medium + 0.5% (w/v) sucrose (1/2 MS 1/2 S) + 200 µg/ml Kan and 100 µg/ml Tim. Surviving shootlets were transferred to 1/4 MS + 100 µg/ml Kan + 50 µg/ml Tim in sterilin pots and when roots had developed, they were transferred to 1/2 MS + 50 µg/ml Tim in 'Beatson' glass jars. When the plantlets were a few cm high they were planted into compost/'perlite' (1:1) mixture and placed in a growth room (25°C 16 h day - covered with a clear polythene bag for the first week). When well established, the plants were potted-up in compost.

2.2.19 Direct gene transfer to plant protoplasts

This technique involved the treatment of isolated leaf protoplasts with naked DNA. All manipulations involving protoplast suspensions were performed under sterile conditions in a laminar flow cabinet using autoclaved or filter-sterilized (millipore, 0.45 µm pore size) solutions.

2.2.19.1 Protoplast isolation

Young expanding leaves of *Nicotiana tabacum* SR1 were excised from the plant and surface sterilized (Section 2.2.18). After three washes in sterile distilled water, the lower epidermis was removed (using fine forceps) and the leaf floated exposed side down for 1 hr on 'pre-plasmolysis' solution, CPW (Reinert and Yeoman, 1982) containing 10% (w/v) mannitol (CPW10M). This solution was replaced with CPW10M

containing 0.4% (w/v), cellulase 0.1% (w/v) macerozyme (Onozuka R-10) and incubated at 28°C for 16-20 hr in the dark to digest the cell walls. The protoplasts were liberated by gentle agitation and after the leaf debris was removed the protoplasts were pelleted by centrifugation (50 g for 10 mins), resuspended in 10 ml CPW 20% (w/v) sucrose, overlaid with 2 ml CPW10M, and re-centrifuged (50 g for 10 mins). Intact protoplasts banded at the interface and were carefully drawn-off and washed three times in CPW10M. The number of protoplasts isolated was determined using a 0.2 mm haemocytometer (Hawksley and Sons Ltd., Lancing, England, U.K.) then the suspension divided into aliquots containing 10^6 protoplasts. After centrifugation (50 g for 10 mins) the protoplast aliquots were resuspended in 1 ml T_0 (Caboche, 1980). Five to six young leaves yielded approximately 6 x 10^6 intact protoplasts.

2.2.19.2 Protoplast transformation and culture.

Transformation of the isolated protoplasts was performed essentially as described by Krens *et al.* (1982), but with the addition of a 'heat shock' step (Potrykus *et al.*, 1985). Freshly isolated protoplast aliquots (10^6 protoplasts in 1 ml T_0) were 'heat shocked' at 45°C for 5 mins (with occasional gentle swirling), followed by rapid cooling in ice for 10 secs, then to each was added, in order: (i) 20 µl 2.5 µg/µl HsDNA (prepared according to Lorz *et al.*, 1985); (ii) 20 µl 0.5 µg/µl plasmid DNA; (iii) 0.5 ml F medium (Krens *et al.*, 1982) containing polyethylene glycol (40% (w/v) PEG4000) (FPEG), with gentle swirling after each addition. This transformation mixture was incubated at room temperature for 30 mins then 10 ml F medium was added in 2 ml steps every 5 mins. The protoplasts were then pelleted (50 g for 10 mins), resuspended in 1 ml T_0, transferred to a 4.5 cm petri-dish and solidifed with the addition of 4 ml To containing 1% (w/v) sea-plaque agarose (dissolved by autoclaving and kept at 45°C until use). This solidification step was based on that of Shillito *et al.* (1983).

2.2.19.3 Regeneration of plants from transformed protoplasts

The protoplasts set in agarose were incubated in the dark at 25°C. After one week the protoplasts were examined under a microscope to confirm protoplast division had begun. Then the agarose was cut into quarters and transferred to a 'Beatson' jar containing 50 ml of 'A-medium' (Caboche, 1980) supplemented with 100 µg/ml kanamycin and 8% (w/v) mannitol (AKan 8 M). This "bead culture" (Shillito *et al.*, 1983) was swirled gently on a gyrating shaker (60 rpm, 0.6 cm throw) under low light conditions (2.5 Klux, 16 h day, 25°C). After one week, 25 ml of the solution was drawn-off and replaced with 25 ml of AKan 4 M (to give a 6% (w/v) mannitol concentration). This was repeated with AKan (without mannitol) every week thereafter, to further reduce the osmoticum (to 3%, 1.5% etc.).

Kanamycin resistant transformed protoplasts survived this selection and continued to divide forming green resistant colonies that could be seen against a background of dead or dying sensitive colonies (brown). When these resistant colonies or "micro-calli" reached 3 or 4 mm in diameter (4 to 6 weeks after transformation), they were transferred to "Protoplast shooting medium" (PSM - MS medium, 2% (w/v) sucrose, 1 µg/ml 6-benzylamino purine (BAP), 0.1 µg/ml naphthaleneacetic acid (NAA), 0.8% agar, pH 5.7), supplemented with 100 µg/ml kanamycin (PSMKan) and incubated in a growth room (14 Klux, 16 h day, 25°C). After 3 to 4 weeks shooting areas of the calli were transferred to fresh PSMKan plates and sections of callus with no shoots were transferred to "protoplast callussing medium" (PCM - as PSM, except 0.2 µg/ml BAP and 2.0 µg/ml NAA) supplemented with 100 µg/ml kanamycin, to provide more callus material if needed.

When the shootlets were approximately 1 cm high (3 to 5 weeks), they were excised (care was taken to remove all callus from the stem) and transferred to 'sterilin' pots containing "rooting medium" (1/2 MS medium, 0.5% (w/v) sucrose, 0.8% agar, pH 5.7) supplemented with 100 µg/ml kanamycin. After 3 to 5 weeks, shootlets with well

established root systems were potted into compost and grown as described in Section 2.2.18.

2.2.20 Protein analysis of transformed plants

2.2.20.1 Preparation of crude protein extracts of *Nicotiana* leaf tissue

Nicotiana tabacum leaf tissue was harvested, immediately frozen in liquid N_2, then ground to a fine powder in a pre-cooled pestle and mortar. A 1.5 ml eppendorf tube was filled with the frozen leaf powder (~800 mg), then 1.0 ml of 20 mM sodium borate pH 8.0 was added, vortexed for 1 min then incubated at 4°C on a rotary wheel for 45 min. After centrifugation (12,000 g, 2 min) the supernatant was transferred to a fresh tube, freeze-dried overnight, then resuspended in 150 µl of the above borate extraction buffer. the samples were stored at -20°C.

2.2.20.2 Total protein concentration estimation

The total protein concentration in each sample was estimated using the Bradford 'protein-dye binding' 'microprotein assay' method (Bradford, 1976). 10 µl of the protein sample was added to a test-tube, followed by 1 ml of 'protein reagent' (0.01% (w/v) Kenacid blue R, 4.7% (v/v) ethanol, 8.5% (v/v) phosphoric acid). After thorough mixing and incubation at room temperature for 10 mins, the absorbance at 595 nm was measured (against a borate extraction buffer/protein reagent blank). the concentration of total protein was estimated from a standard curve constructed using known protein (bovine serum albumin) standard concentrations of 1 µg to 100 µg in 10 µl of borate extraction buffer.

2.2.20.3 SDS-PAGE analysis of protein samples

Fractionation of denatured protein samples was performed on SDS-polyacrylamide slab gels using a discontinuous buffer system (Laemmli, 1970). The

gels were prepared and run as described by Delauney (1984). Briefly, the gels consisted of a 3% (w/v) acrylamide 'stacking' gel above a 17% (w/v) acrylamide 'resolving' gel, and were run in 192 mM glycine, 26 mM Tris base, 0.1% (w/v) SDS.

The protein samples were mixed with an equal volume of 2x sample buffer (1x = 20 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol) and incubated at 70°C for 3 mins. After centrifugation (12,000 g, 2 min) to remove insoluble material, the samples were loaded onto the gels, with the prior addition of a few drops of tracker dye (1% (w/v) bromophenol blue in ethanol) to the top buffer reservoir. 1 µl of β - mercaptoethanol was added to each well then the gels were run at 8 mM for 16-20 hrs until the tracker dye reached the base of the gel.

After electrophoresis, the proteins were visualized by staining or transferred to nitrocellulose filters by electroblotting in preparation for immunodetection of specific polypeptides (Section 2.2.20.4). The gels were stained by soaking for 16 to 20 hrs in kenacid blue stain (0.05% (w/v) kenacid blue R, 50% (v/v) methanol, 7% (v/v) acetic acid), then the protein bands were revealed by destaining the gel in several changes of the above methanol/acetic acid solution minus dye.

2.2.20.4 Transfer of proteins to nitrocellulose filters - 'Western' blotting

17% SDS-PAGE gels were run (Section 2.2.20.3) and the fractionated proteins transferred to nitrocellulose filters by electroblotting (Towbin *et al.*, 1979). A Sartorius semi-dry electroblotter (SM 17556) was used according to the manufacturers instructions. Briefly, the following were layered in the order given onto the lower anodic graphite plate (16 cm x 16 cm); 2 sheets of Whatman 3 MM paper soaked in Anode buffer 1 (0.3 M Tris base, 20% (w/v) methanol, 0.1% (w/v) SDS); 1 sheet 3 MM paper soaked in Anode buffer 2 (25 mM Tris base, 20% (v/v) methanol, 0.1% (w/v) SDS); a nitrocellulose sheet soaked in distilled water; the protein gel; 1 sheet of 3 MM paper soaked in cathode buffer (25 mM Tris base, 20% (v/v) methanol, 0.1% (w/v)

SDS 40 mM 6-amino N-hexanoic acid); 1 sheet of cellophane soaked in distilled water; 2 sheets of 3 MM paper soaked in cathode buffer. Finally, the cathodic graphite plate was placed on top. The apparatus was run at 0.2 A for $1^1/2$ hr at room temperature. The blot was then removed, air-dried and stored at room temperature until use.

The electroblotted gel was subsequently stained (Section 2.2.20.3) to confirm the uniform transfer of proteins to the nitrocellulose.

2.2.20.5 Immunodetection of immobilised proteins on 'Western' blots

The presence of pea legumin protein on 'Western' blots was detected using an immunodetection procedure involving anti-pea legumin primary antibodies with radio-labelled secondary antibodies allowing detection by autoradiograph.

The following incubations were performed in a sealed polythene bag in a shaking waterbath at 40°C. Firstly, the 'Western' blot filter was incubated for 90 min in 100 ml 'Blotto' (5% (w/v) non-fat dried milk in TS buffer (20 mM Tris, 0.9% (w/v) NaCl, pH 7.2)), to block the free binding sites on the filter. Following three 10 min washes in 100 ml of 'Blotto', the blot was incubated for $2^1/2$ h with 50 ml of 'Blotto', including 50 ul of rabbit anti-pea legumin IgG (primary antibodies). After three 'Blotto' washes as above, the blot was incubated for $2^1/2$ h in 50 ml of 'Blotto', including 40 µCi iodinated (^{125}I) donkey anti-rabbit IgG (secondary antibodies). Finally, after a further three 'Blotto' washes as above, and one 10 min wash in 100 ml of TS buffer, the filter was air-dried and autoradiographed (Section 2.2.13).

2.2.20.6 Enzyme linked immunosorbent assay (ELISA)

The presence of pea legumin in seeds and leaf samples of transformed plants was assayed using the immunodetection technique of ELISA. The ELISA assays were performed in collaboration with Dr. A.S. Kang.

Nicotiana tabacum leaf discs (~5 mm in diameter) or seeds (20 per extraction) were ground in a 1 ml eppendorf tube with a little acid washed sand and 220 µl extraction buffer (0.2 M Tris-NCl, 0.3M NaCl, pH 9.5), after a clearing spin, aliquots of the supernatant were transferred to the ELISA assay plates.

50 µl of leaf or seed protein extract was diluted with 150 µl extraction buffer and incubated at 4°C for 18 h. on a microtitration plate (Nunc Immunoplate 1, Gibco Europe). After washing with PBST (Phosphate buffered saline, pH 7.4 containing 0.05% Tween 20) three times, then water three times, non-specific protein binding sites were blocked with bovine serum albumin (BSA - 1% in PBS - 300 µl/well) at room temperature for 2 h. After washing as above, rabbit anti-pea legumin IgG antibodies were added to the wells (diluted 1:100 in PBST, 0.1% BSA - 200 µl/well) and incubated at room temperature for 2 h. After washing as above, goat anti-rabbit IgG antibodies coupled to horse radish peroxidase was added (diluted 1:1000 in PBST, 0.1% BBA - 200 µl/well) and incubated at 35°C for 1 h. Following washing, the substrate ABTS (2,2'-azino-bis (3-ethylbenzthiazoline sulphonic acid) (55 mg in 0.012% H_2O_2 in 0.05M NaH_2PO_4, 0.25 M citric acid) was added (200 µl/well) and incubated at room temperature. The peroxicase activity was inhibited after ~30 min. by the addition of 50 µl 1MNaF. the optical density at 414 nm was determined for each sample well using a Titretek Multiscan MCC plate reader (Flow Laboratories).

The results were ocmpared to a standard curve constructed using untransformed plant leaf and seed samples prepared with added purified legumin (0-100 ng/ml legumin loaded into well) and assayed as above.

2.2.21 Testing the kanamycin resistance of transformed plant progeny

Approximately 100 seeds of self-pollinated transformed *Nicotiana tabacum* SRI plants were plated-out in a grid pattern on 1/2 MS medium, 0.8% agar plates supplemented with kanamycin and incubated in a growth room (25°C, 16 h day). After 14 days the plates were examined, and the number of kanamycin resistant and sensitive seedlings was recorded. Kanamycin sensitive seedlings were dead or had bleached leaves at this stage, whereas the resistant seedlings had healthy green leaves.

2.2.22 Checking kanamycin resistance of transformed plant leaves

When fully mature, each transformed plant was checked for the continued presence of the kanamycin resistance gene by incubating leaf pieces on callussing medium.

A single leaf was harvested from each plant, surface-sterilized and cut into pieces as described in Section 2.2.18. Two leaf pieces from each plant were incubated on PCMKan (Section 2.2.19.3).

2.2.23 'Gene copy equivalent' calculation in genomic DNA hybridization

'Gene copy equivalents' were used to provide an estimate of the number of copies of a particular gene in a genome. Genomic DNA digests were run on agarose gels along with plasmid DNA fragments containing the gene sequence to be used as probe in subsequent Southern blotting hybridizations. The amount of plasmid DNA equivalent to one gene copy per genome was calculated as below:

$$\text{1 gene copy equivalent} = \frac{\text{plasmid fragment size (bp)} \times \text{amount of genomic DNA per track (g)}}{\text{plant genome size (bp)}}$$

Restriction endonuclease digested plasmid DNA was diluted until the appropriate amount of fragment corresponding to one gene copy per haploid genome was present in 1 µl TE and then multiples of this were loaded onto the gel to provide a range of gene copies from 1-5. After hybridization to the gene sequence probe, the intensity of hybridized bands in the genomic tracks was compared with those of the 'gene copy' range; equivalent intensities gave an estimate of the gene copy number in that genome.

CHAPTER 3.

RESULTS.

Results

3.1 Pollen Germination

3.1.1 Attachment of *Agrobacterium* to pollen tubes of *Hippeastrum* and *Vicia*

Pollen grains of the monocot *Hippeastrum vittatum* (Amaryllis lily) and the dicot *Vicia faba* (Broad bean) were germinated in the presence of *Agrobacterium tumefaciens* strain C58 as described in Section 2.2.15.1. The aim was to observe the germinated pollen tubes after a period of incubation with *Agrobacterium* to assess whether the vegetative bacteria become attached to the pollen tube wall. The results of the *Hippeastrum* incubation were of particular interest as attachment of *Agrobacterium* has been demonstrated to the cell walls of some monocot species but not others (see Discussion).

Figure 5 a, b and Figure 6 a, b show the results obtained with *H. vittatum* and *V. faba* respectively.

In the case of *Hippeastrum*, large numbers of *Agrobacteria* (pink-stained Gram negative bacteria) were seen at the pollen tube surface, particularly in the region of the tip. The bacteria adhered to the pollen tube wall in the form of a thin layer of bacteria in some regions, for example the "upper" surface in Fig. 5a and the "lower" surface in Fig. 5b, but as aggregates of large numbers of bacteria in others, for example the tip region of the pollen tube shown in Fig. 5a and the "upper" surface and "lower" tip region of the pollen tube shown in Fig. 5b. The dark stained region inside the pollen tube is the contracted pollen tube cytoplasm resulting from plasmolysis during the preparative procedures.

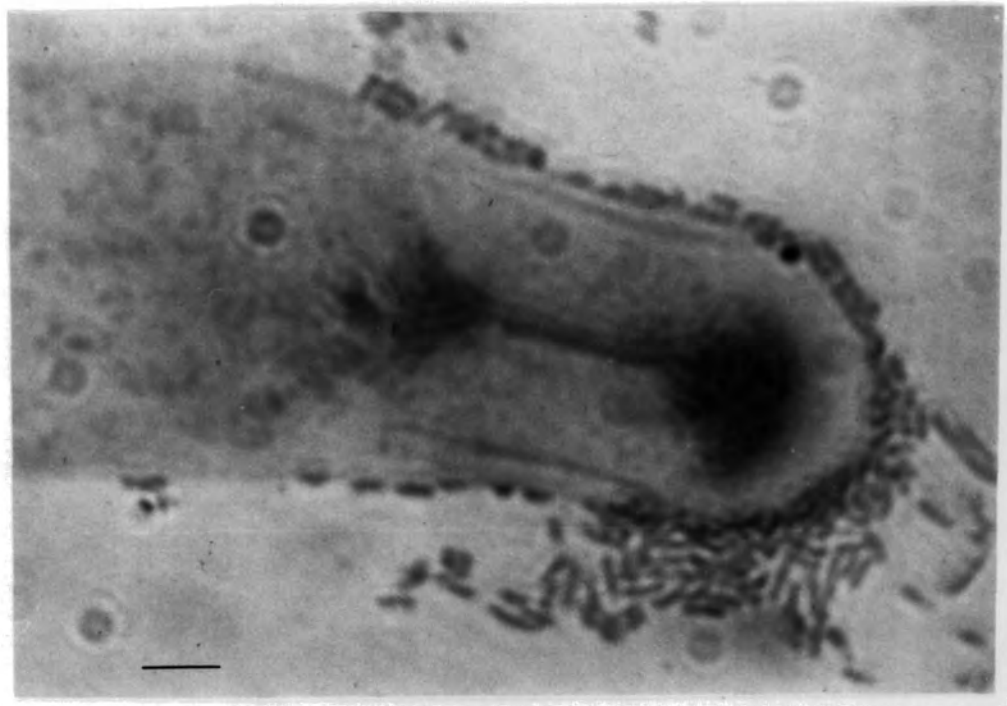
In the *Vicia* pollen incubations (which were not Gram stained) many *Agrobacteria* were seen as large aggregates around the pollen tube. Fig. 6 clearly shows large numbers of bacteria surrounding the pollen tube, especially at the point of emergence of the tube from the pollen grain and at the tube tip. In Fig. 6b, a large aggregate of bacteria can be seen surrounding the tube tip and at the

FIGURE 5.

Attachment of *Agrobacterium tumefaciens* to pollen tubes of *Hippeastrum vittatum.*

Gram stained pollen tubes viewed under transmission light microscope. Scale bars represent 2 μm.

A.



B.

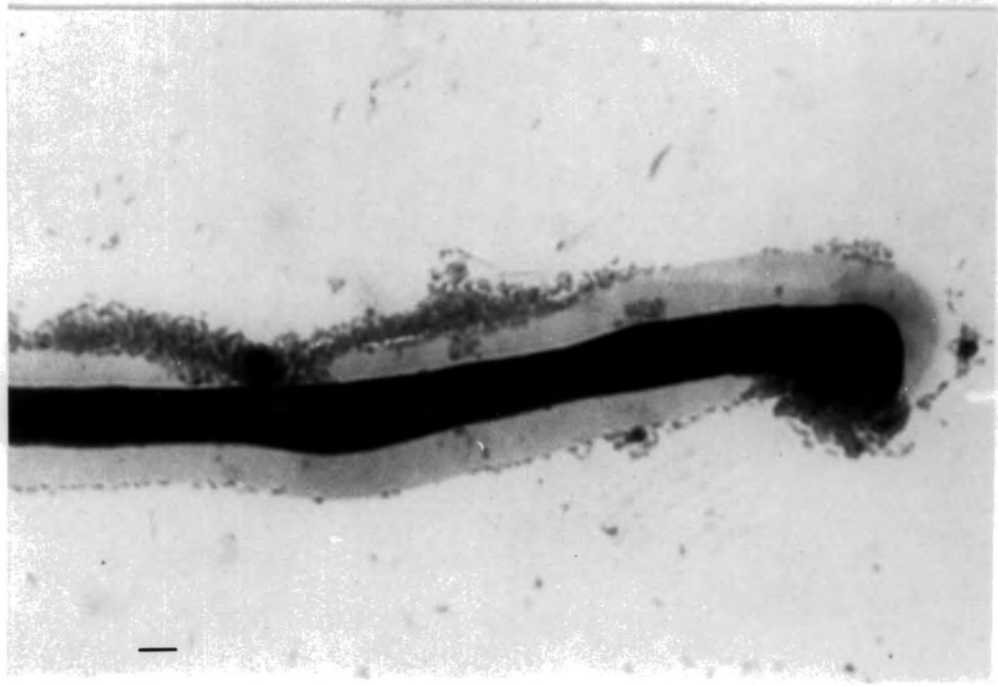


FIGURE 6.

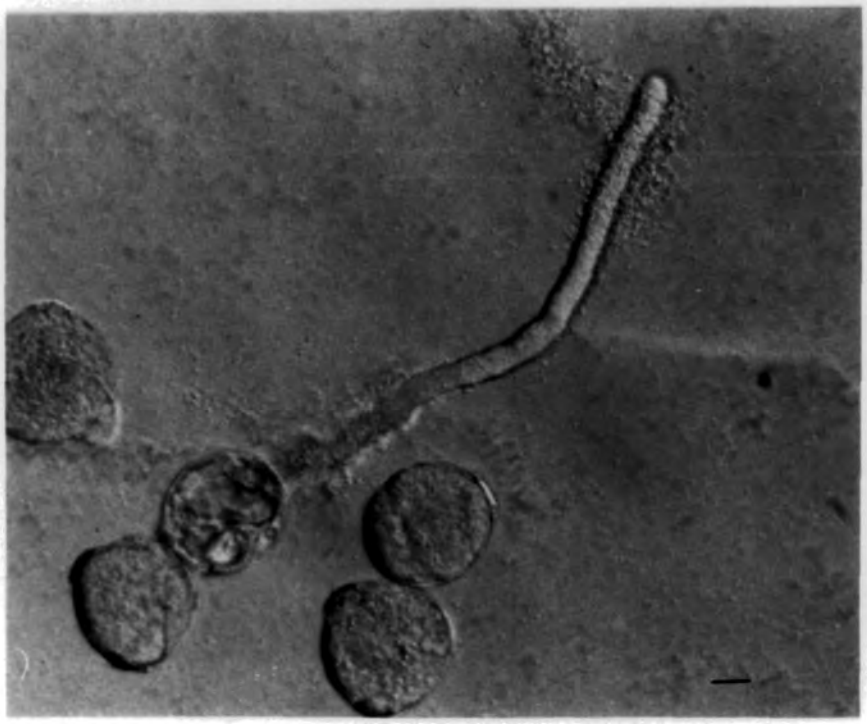
Attachment of *Agrobacterium tumefaciens* to pollen tubes of *Vicia faba*.

pollen tubes viewed under transmission light microscope (Nomarski optics).

Scale bars represent 10 um.

A.

B.



point of emergence but with less adherence to the middle section of the pollen tube.

It was apparent that few bacteria were seen associated with ungerminated pollen grains or on the surface of the slide, indicating that the washing procedures were successful in removing unattached bacteria. The results also indicate that this virulent strain of *Agrobacteria* can become attached to the pollen tube wall of the two species studied.

Such attachment is an important initial step in the process of infection and transformation by *Agrobacterium* and so the results demonstrate that *Agrobacterium* infection of germinating pollen tubes is feasible.

3.1.2 Germination of pollen tubes of *Vicia faba* in the presence of ^{3}H-pBR322 plasmid DNA

The possibility of direct uptake of DNA molecules by pollen tubes was investigated by the germination of pollen grains of *Vicia faba* in a medium containing radio-labelled pBR322 plasmid molecule (Section 2.2.15.2). The aim was to demonstrate the presence of the labelled DNA within the pollen tubes after such incubation.

Figs. 7 a, b and c show the results of the micro-autoradiography of a number of *Vicia* pollen tubes germinated in the presence of radio-labelled DNA. Blackening of the photographic film caused by silver grains, can be seen above the pollen tubes indicating the presence of the radio-labelled DNA in these areas; this is particularly evident at the pollen tube tips. The labelled DNA is associated with the pollen tubes, whether adhering to the outside of the pollen tube cell wall or actually present within the pollen tube following uptake.

In the procedure used, the pollen tubes were extensively washed after incubation with the labelled DNA. The scintillation counts of the final wash solution showed that the radioactivity was almost at background level (results not

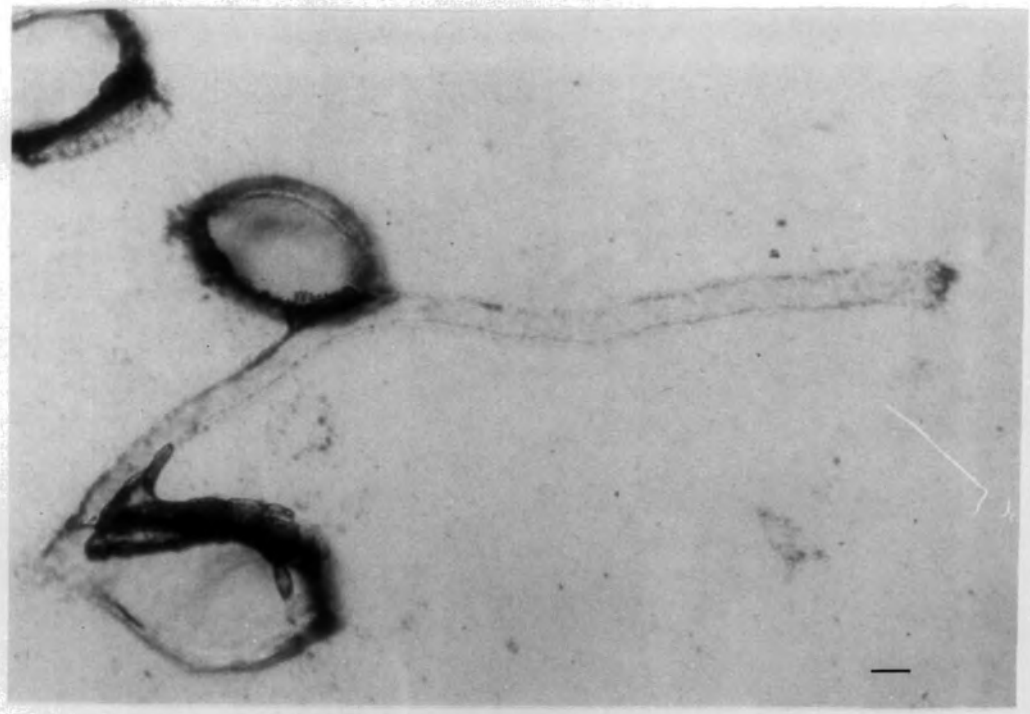
FIGURE 7.

Direct uptake of DNA molecules by pollen tubes.

Microautoradiographs of *Vicia faba* pollen tubes after germination in the presence of radio-labelled pBR322 plasmid DNA.

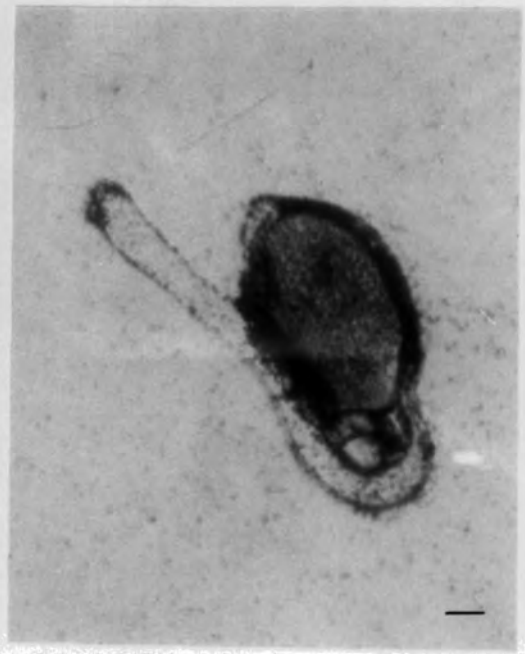
Scale bars represent 10 um.

A.



B.

C.



presented), indicating the removal of virtually all the free labelled DNA from the samples. The pollen tubes were incubated for a further period in medium without label, to allow the pollen tubes to continue growth in the absence of label. The growth of pollen tubes is restricted to the tip so that the tube extends from the tip and not from elongation of the already formed tube wall (see Discussion). Thus, if the labelled DNA was merely adsorbed to the pollen tube wall, little or no label would be expected in the growing-tip region after growth in the second incubation medium. The conclusion was, therefore, that the results were consistent with the presence of the labelled DNA within the pollen tubes, suggesting that there had been uptake of naked DNA molecules from the medium.

3.2 Micro-injection of DNA into *Salpiglossis* ovaries

3.2.1 Confirmation of the integrity of pGV3850 DNA in the plasmid preparation

In preparation for micro-injection of Ti plasmid, the integrity of the plasmid molecules in the preparations of the Ti-plasmid pGV3850 was checked by 'Southern' blot analysis of restriction endonuclease digests. This was needed as the plasmid is very large (~200 kbp) and, therefore, susceptible to shearing.

pGV3850 DNA was prepared using the large-scale method (Section 2.2.2.4) and was digested with Hind III and EcoRI and run on an agarose gel with Hind III digested pGV0601 (Fig. 8a). A Southern blot of this gel was probed with ^{32}P-labelled 'Hind III fragment 23' (3.2 kbp Hind III insert of pGV0601), a fragment contained in pGV3850. The probe hybridized to a 3.2 kbp band of the Hind III digest and a 12.6 kbp band in the EcoRI digest (Fig. 8b), as expected.

3.2.2 Efficiency and reproducibility of the ovary DNA extraction method

The DNA extraction method detailed in Section 2.2.3.3 was performed with fifteen ovaries of *Salpiglossis sinuata.* 0.01 µg of undigested pBR322 DNA was

FIGURE 8.

Southern blot analysis of pGV3850 plasmid DNA.

A. Restriction digests of pGV3850 plasmid DNA. (3 ug/ml).

Tracks

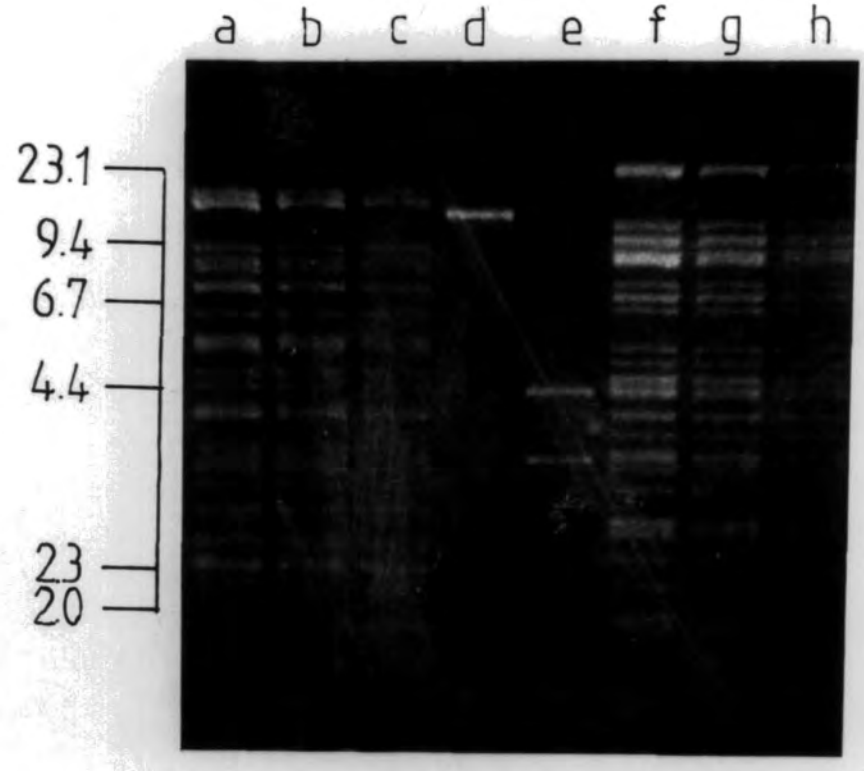
a	3 µl	pGV3850	EcoRI	
b	2 µl	"		
c	1 µl	"		
d	(linearized plasmid DNA marker)			
e		pGV0601	Hind III	(0.09 µg 3.2 kbp insert)
f	3 µl	pGV3850	Hind III	
g	2 µl	"		
h	1 µl	"		

Numbers on the left are size markers (kbp).

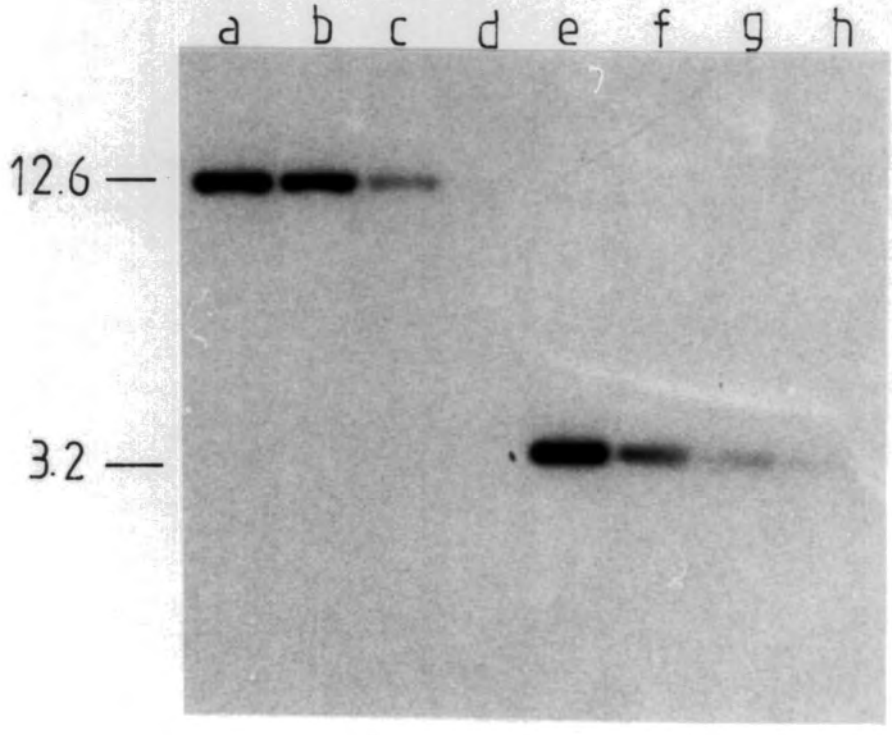
B. Autoradiograph of a blot of the gel above hybridized to 'pGV3850 Hind III fragment 23' isolated as the 3.2 kbp Hind III insert fragment of pGV0601.

Tracks are as in A.

A.



B.



added to each eppendorf tube prior to the grinding step, except in one case when it was added afterwards. The extracts were run on an agarose gel and a Southern blot of the gel was probed with ^{32}P-labelled pBR322. Figure 9 shows the results of this hybridization. Both 'supercoiled' (SC) and 'open-circle' (OC) forms of the plasmid were present in the extracts, with proportionately more OC forms than in the plasmid controls. This was most probably due to mechanical damage to the plasmids during the extraction procedure (a single 'nick' in one DNA strand of SC plasmid would convert it to the OC form). The intensities of the bands in eleven of the fourteen samples were similar and comparable to the bands of the 0.01 µg pBR322 track. Two of the extracts gave bands that were less intense and one showed no bands at all. The sample that had the DNA added after grinding was more intense than the others, indicating a small loss of DNA during the grinding step.

3.2.3 Persistance of pBR322 plasmid DNA microinjected into *Salpiglossis* ovaries

Firstly, in order to assess in which order the pBR322 plasmid forms migrated in an agarose gel, untreated (supercoiled) pBR322 was run with Hind III digested (linearized) and S1 nuclease treated (single-strand 'nicks' introduced converting the 'supercoiled' (SC) forms to 'open-circle' (OC) forms - Section 2.2.5.6). The gel (Fig. 10a) showed that the uppermost band increased and the lowest band decreased in intensity after S1 nuclease treatment, which indicated the former to be the OC form and the latter to be the SC form. The linearized plasmid migrated to just below the OC band.

The level of DNase activity in the ovaries of *Salpiglossis sinuata* was assessed by microinjection of 0.15 µl of 0.5 µg/µl pBR322 plasmid DNA into each loculus (0.3 µl total) of fourteen ovaries (Section 2.2.16). At time intervals up to 24 hr, the ovaries were then frozen in liquid N_2 and the DNA extracted (Section

FIGURE 9.

Efficiency and reproducibility of ovary DNA extraction procedure.

Autoradiograph of a blot of a gel containing *Salpiglossis sinuata* ovary DNA preparations extracted after the addition of 0.01 µg pBR322 plasmid DNA. The blot was hybridized to pBR322 plasmid DNA.

Track	
a	0.01 µg pBR322
b	ovary DNA (0.01 µg pBR322 added after grinding)
c to q	ovary DNA (0.01 µg pBR322 added prior to grinding)

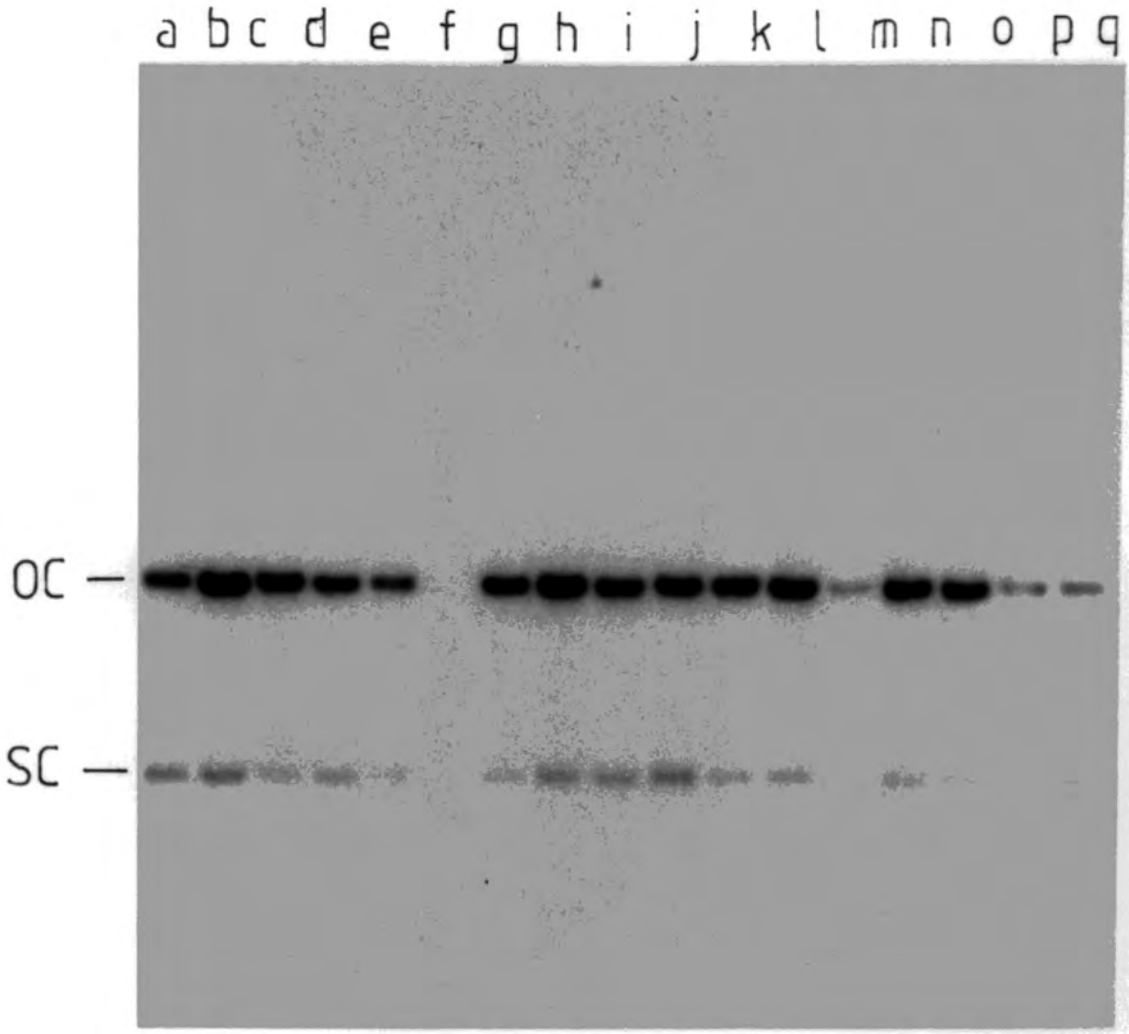

a b c d e f g h i j k l m n o p q
OC
SC

FIGURE 10.

Persistence of pBR322 plasmid DNA microinjected into *Salpiglossis sinuata* ovaries.

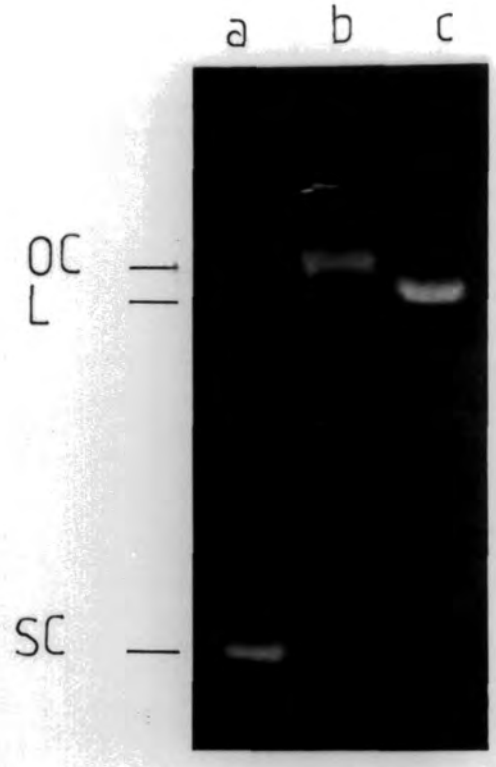
A. Migration of pBR322 plasmid forms in an agarose gel.

Track		
a	1.0 µg pBR322	untreated (supercoiled -SC)
b	1.0 µg pBR322	S_1 nuclease treated (open circular - OC)
c	1.0 µg pBR322	Hind III digested (linear - L)

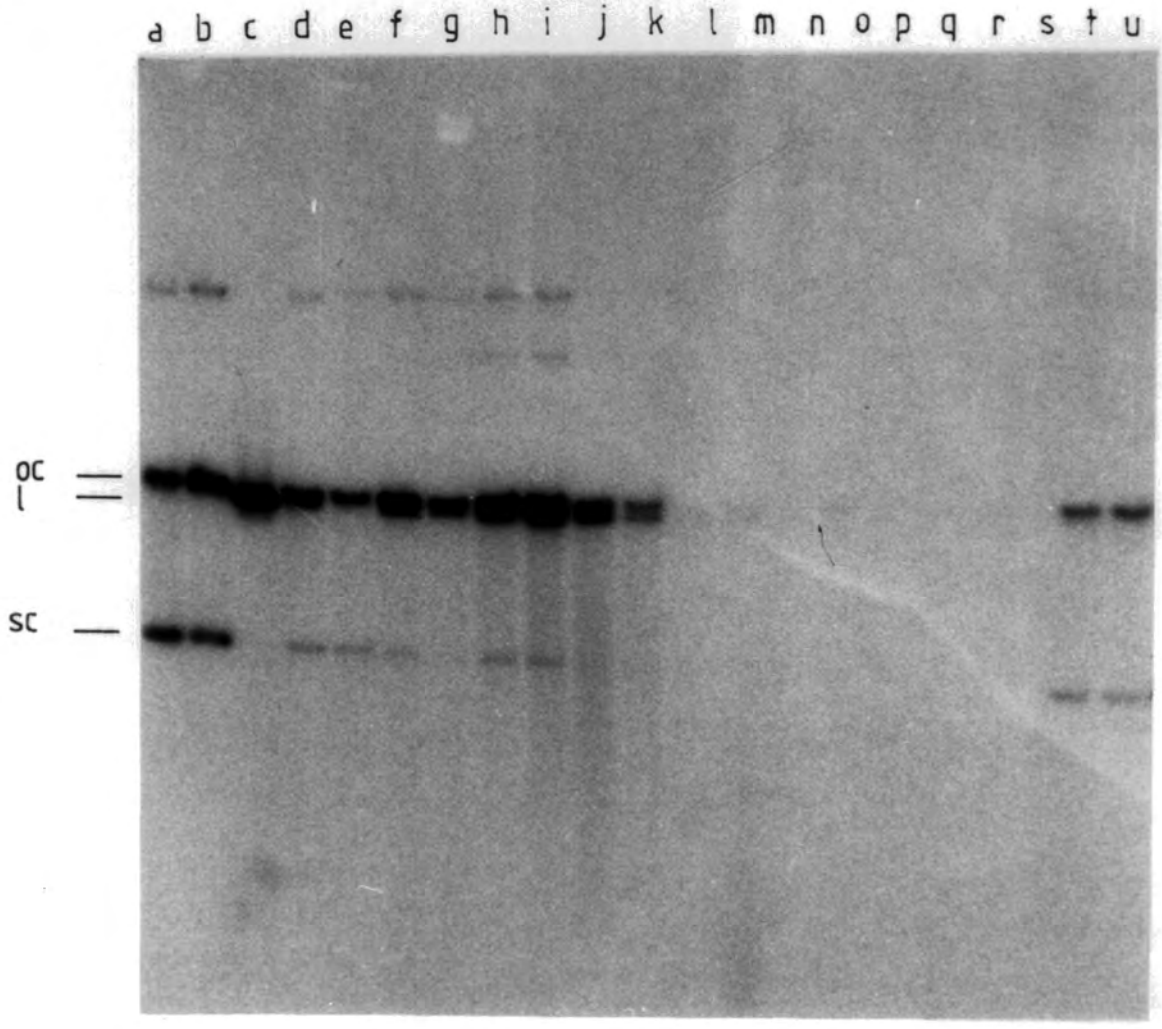
B. Persistence of pBR322 plasmid DNA microinjected into *Salpiglossis* ovaries. Autoradiograph of a blot of a gel containing ovary DNA preparations extracted at time intervals after microinjection of pBR322 plasmid DNA. The blot was hybridized to pBR322 plasmid DNA.

Track			
a	0.1 µg pBR322	l	6 hr
b	0.1 µg pBR322 S_1 nuclease	m	"
c	0.1 µg pBR322 Hind III	n	12 hr
d	0 hr	o	"
e	"	p	24 hr
f	1 hr	q	"
g	"	r	uninjected ovary
h	2 hr	s	"
i	"	t	uninjected ovary + 0.1 µg pBR322
j	3 hr		
k	"	u	"

A.



B.



2.2.3.3). Uninjected ovaries, with and without the addition of pBR322 DNA during the extraction procedure, were used as controls.

One-third of each ovary extract was run on an agarose gel, Southern blotted and probed with ^{32}P-labelled pBR322. Fig. 10b shows the results of this hybridization with bands indicating the persistance of the SC form in the ovary up to 2 hr and the appearance of the linear form at 1 hr. After 6 hr very much lower levels of DNA were present, although even at 24 hr, a longer exposure of the autoradiograph (not presented) revealed faint bands corresponding to the OC and linear forms. There was no hybridization to the uninjected ovaries without added DNA.

3.2.4 Persistance of pGV3850 plasmid DNA microinjected into *Salpiglossis* ovaries

0.15 µl of 0.45 µg/µl pGV3850 plasmid DNA (Section 3.2.1) was microinjected into each loculus of twenty *Salpiglossis sinuata* ovaries (Section 2.2.16); the injection included 0.8 µg/ml herring sperm DNA (HSDNA) for ten of the ovaries. Two ovaries from each treatment were frozen in liquid N_2 at time intervals up to 6 hr after injection. DNA was extracted from the ovaries (Section 2.2.3.3), digested with EcoRI and run on two agarose gels (one gel containing the replicates of the other) with EcoRI digested pGV3850. EcoRI digestion excised a 12.6 kbp fragment from pGV3850 containing the right T-DNA border and nopaline synthase gene. Southern blots of the gels were probed with ^{32}P-labelled T-DNA right border sequences (3.2 kbp Hind III fragment of pGV0601). Figure 11 a and b show the results of this hybridization.

The probe hybridized to a band of 12.6 kbp. The bands were distinct although there was some degradation of the fragment, even at time 0 hr. The introduced DNA persisted for up to 4 hr in the ovary with (tracks g to i; Fig. 11a) or without (tracks a to d, Fig. 11b) HS DNA. One set of replicates showed bands

FIGURE 11.

Persistence of pGV3850 plasmid DNA microinjected into *Salpiglossis sinuata* ovaries.

A. Autoradiography of a blot of a gel containing *Salpiglossis sinuata* ovary DNA preparations extracted at time intervals after microinjection of pGV3850 plasmid DNA with or without HSDNA. The blot was hybridized to a 3.2 kbp Hind III fragment at pGV0601.

Track

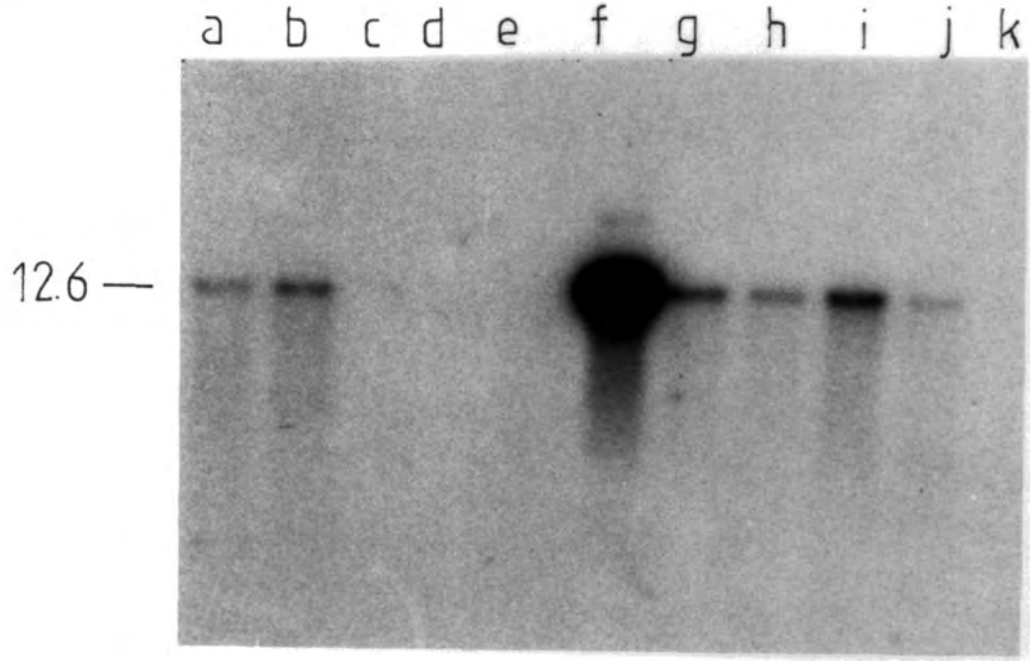
a	0 hr	-HSDNA	g	0 hr	+HSDNA
b	1 hr	"	h	1 hr	"
c	2 hr	"	i	2 hr	"
d	4 hr	"	j	4 hr	"
e	6 hr	"	k	6 hr	"
f	pGV3850 EcoRI (0.02 µg 3.2 kbp fragment)				

The number on the left is the size (kbp).

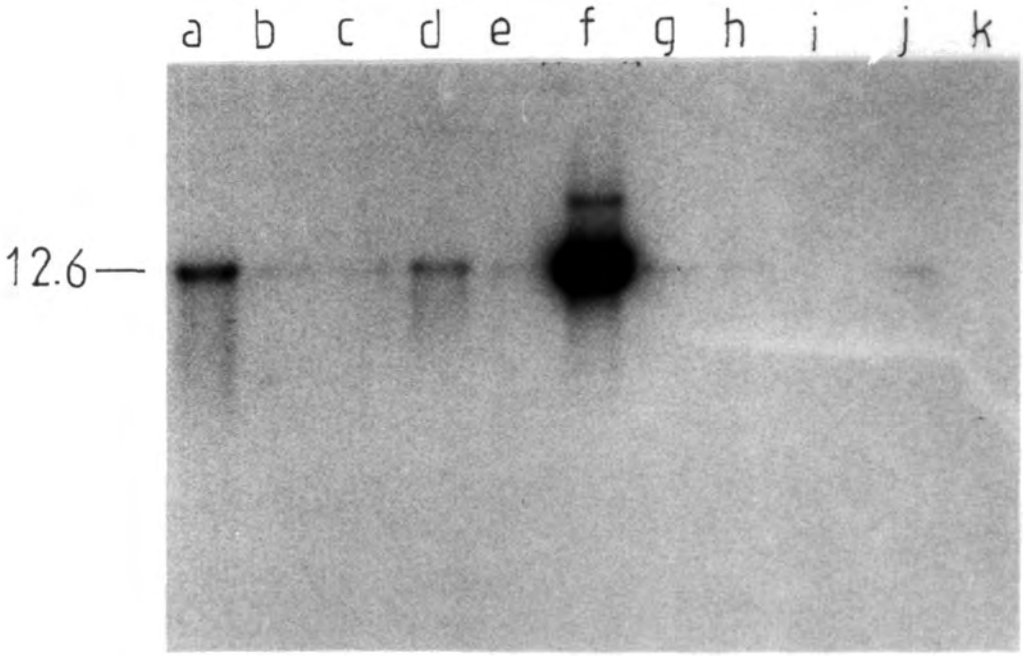
B. Autoradiograph of a blot of a gel containing replicates of the above samples.

Tracks are as described above.

A.



B.



at greater intensity with added HSDNA (Fig. 11a, tracks a to k compared to tracks a to e), whereas the other set showed greater intensities without added HSDNA (Fig. 11b, tracks a to e compared to tracks g to k), indicating that added HSDNA did not appear to increase the persistance of the plasmid DNA.

3.2.5 Analysis of putative transformed *Salpiglossis* plants derived from microinjected ovaries

Fifty ovaries of *Salpiglossis sinuata* were microinjected still attached to the plant, with the non-oncogenic Ti-plasmid pGV3850 between 14 hr and 15 hr after pollination; using the techniques described in Section 2.2.16. Approximately 0.4 µl of a 0.35 µg/µl pGV3850 plasmid preparation was microinjected into each loculus (~0.6 µg per ovary). After injection the plants were returned to the growth room to allow maturation of the seeds.

3.2.5.1 Nopaline assay screening

Mature seeds were collected from the treated plants and germinated to allow screening for the presence of the introduced DNA. Approximately 250 seedlings were screened for the expression of the nopaline synthase gene contained within the right and left T-DNA borders of pGV3850 transferred DNA fragment, using the 'nopaline assay' methods. The assay was performed using tissue from the best seedlings and control seedlings, from uninjected ovaries, and nopaline and octopine standards (Section 2.2.16.3).

Examination of the electrophoretograms under uv illumination showed that none of the microinjection-derived seedlings assayed, gave fluorescent 'spots' corresponding to the nopaline standards (results not presented). However, several samples gave a fluorescent spot which migrated between the nopaline and octopine standards, although with a blue fluorescence compared to the yellow/green fluorescence of the standards. (This was not shown by the control seedlings

assayed.) It was found that seedlings exhibiting this property did so consistently when the assay was repeated with fresh extracts. If nopaline was present in these samples then it may have been masked by this blue fluorescent compound due to its position on the electrophoretogram.

3.2.5.2 'Southern' blotting of putative transformants

As an alternative to the NOS assays to assess transformation, the genomic DNA of a number of the plants producing this compound was examined by 'Southern' blot hybridization, probing with a DNA sequence that would be present in a pGV3850 transformed genome. Seven microinjected ovary-derived seedlings that exhibited the electrophoretic property detailed above, were grown to maturity. Genomic DNA was prepared from leaf tissue of each of these plants and an untransformed control (Section 2.2.3.2). 10 µg of each DNA sample was digested with Hind III and EcoRI, run on an agarose gel and 'Southern' blotted. The blot was probed with the T-DNA right border "23" fragment (^{32}P-labelled 3-2 kbp HindIII fragment of pGV0601), a fragment containing the nopaline synthase gene and T-DNA right border sequence that would be present in a pGV3850 transformed genome.

No hybridization was found to any of the genomic DNA samples (results not presented). As a high specific activity probe (>10^8 cpm/µg) was used and the autoradiograph was exposed for 8 weeks, this result suggested that the transferred section of pGV3850 DNA was not present in the genomic DNA of these ovary microinjection-derived seedlings. The result indicated that these plants did not contain T-DNA sequence and so had not been transformed. The compound that reacted with the assay stain was not therefore produced as a result of transformation.

3.3 Fusion of the nopaline synthase promoter to the coding and 3'-flanking regions of legumin gene A

The strategy employed to fuse the promoter sequences of the nopaline synthase (*nos*) gene to the legumin gene A (*legA*) coding and 3'-flanking sequences, is given in Fig. 1. The plasmid pDUB21 contains a 2.4 kbp Hind III fragment of a pea (*Pisum sativum* L.) genomic DNA clone (Lycett *et al.*, 1984). This fragment contains the entire *legA* coding sequence and 3'-flanking region with the exception of the first 7 bp (the 'ATG' start-codon and the next 4 bp). The strategy was to join the *nos* promoter to the 5'-end of the *legA* gene using a linker molecule that would provide an 'ATG' start-codon 'in-phase' with the *legA* codons. The linker molecule used contained a restriction endonuclease site for Cla I and the sequence 'ATG' in such a position as to be 'in-phase' when ligated to the 'filled-in' 3'-end of the *legA* gene.

The steps involved in this construction are detailed below.

3.3.1 Preparation of the *legA* gene

The strategy adopted required a unique Hind III site at the 5'-end of the *legA* coding region in pDUB21. This was achieved by the removal of a 230 bp BamHI fragment containing the second Hind III site. pDUB21 was digested with BamHI, then the products were re-ligated, transformed into *E. coli* JM83 and plated-out on YT Amp X-Gal plates. DNA was prepared from six of the transformants, digested with Hind III and run on an agarose gel. Five of the six gave a single 4.9 kbp band, indicating the successful removal of the Bam HI fragment (results not presented). These deletion clones were designated pDUB21 Δ Bam.

3.3.2 Preparation of the *nos* promoter

pNosNeo was double-digested with Eco RI and Hind III to produce the 2.7 kbp pUC9 vector and the 1.7 kbp insert containing the chimeric nopaline synthase/neomycin phosphotransferase gene (*nos/neo*). The digest was run on an agarose gel and the 1.7 kbp fragment isolated and digested with Xho II to cleave the *nos* promoter from the *neo* coding sequence. The products of this digestion were ligated with Hind III/Bam HI double-digested pUC8, transformed into *E. coli* JM83 and plated-out on YT Amp X-Gal plates, in order to clone the Hind III/Xho II fragment containing the *nos* promoter. The sequence of the Xho II site used in this case was such that when ligated to the Bam HI cut pUC8 the resulting combined site could be subsequently cut with Bam HI. DNA was prepared from six transformants, double-digested with Hind III and Bam HI and run on an agarose gel. Five of the clones gave fragment sizes of 2.7 kbp and 0.32 kbp, indicating the successful insertion of the promoter fragment (results not presented). These clones were designated pNos.

3.3.3 Addition of 'linkers' to the *nos* promoter and *legA* gene fragments

3.3.3.1 *LegA* gene fragment

0.5 µg of pDUB21 Δ Bam was linearized by digestion with Hind III and after purification by phenol extraction and ethanol precipitation (Sections 2.2.1.2 and 2.2.1.3), the 3'-recessed termini were 'filled-in' (Section 2.2.5.3). This 'blunt-ended' fragment was ligated with 1 µg of decameric 'linker' molecules (containing the sequence of the Cla I restriction endonuclease site and an 'in-frame' 'ATG' start codon, see Fig. 1) that had been radio-labelled by phosphorylation with γ ^{32}P-ATP (Section 2.2.5.4). This ligation mix was transformed into *E. coli* JM83 yielding five transformants. An aliquot of the ligation mix, together with a similar aliquot digested with Cla I, were run on a 10% polyacrylamide gel (Section

2.2.7.1). An autoradiograph of the gel (Fig. 12a) shows the radio-labelled linkers ligated to form a 'step-ladder' of oligomeric forms which were digested to the monomeric form with Cla I. DNA was prepared from the transformants (Section 2.2.2.1) and was digested with Hind III, Cla I and double-digested with Bam HI/Cla I. Successful addition of one or more 'Cla I' linker molecules onto the linearized pDUB21 Δ Bam and subsequent re-circularization of the plasmid would result in a single 4.9 kbp fragment with Cla I digestion, fragments of 2.7 kbp and 2.2 kbp with the Bam HI/Cla I double-digestion and no digestion with Hind III. Three of the five clones gave these results (results not presented) and were designated pLegA.

3.3.3.2 *Nos* promoter fragment

pNos was double-digested with Hind III/Bam HI and the 0.32 kbp *nos* promoter fragment was isolated. 0.25 μg of this fragment was blunt-ended and ligated to 'Cla I linker' molecules as described above (except the ligation mix was not transformed into *E. coli*). An aliquot of the ligation reaction and a similar one digested with Cla I were analysed by polyacrylamide gel electrophoresis as described above (Fig. 12b). The DNA fragment was small enough to migrate into the gel and a labelled band corresponding to this fragment, can be seen on the autoradiograph, indicating the successful ligation of radio-labelled linkers onto the fragment.

3.3.4 Fusion of the *nos* promoter with the *legA* gene

Fusion was achieved by the insertion of the 'Cla I-linkered' *nos* promoter fragment into the Cla I site of plegA.

FIGURE 12.

PAGE analysis of linker/ligation reactions.

A. Autoradiograph of a polyacrylamide gel containing ^{32}p-ATP end-labelled Cla I linkers ligated to the 5'-end of the *leg*A gene.

7.5 pmoles of Cla I linker molecules in each track.

Track

a ligation mixture (Cla I linkers + linearized (Hind III) pDUB21Δ Bam)

b Cla I restriction digest of ligation mixture.

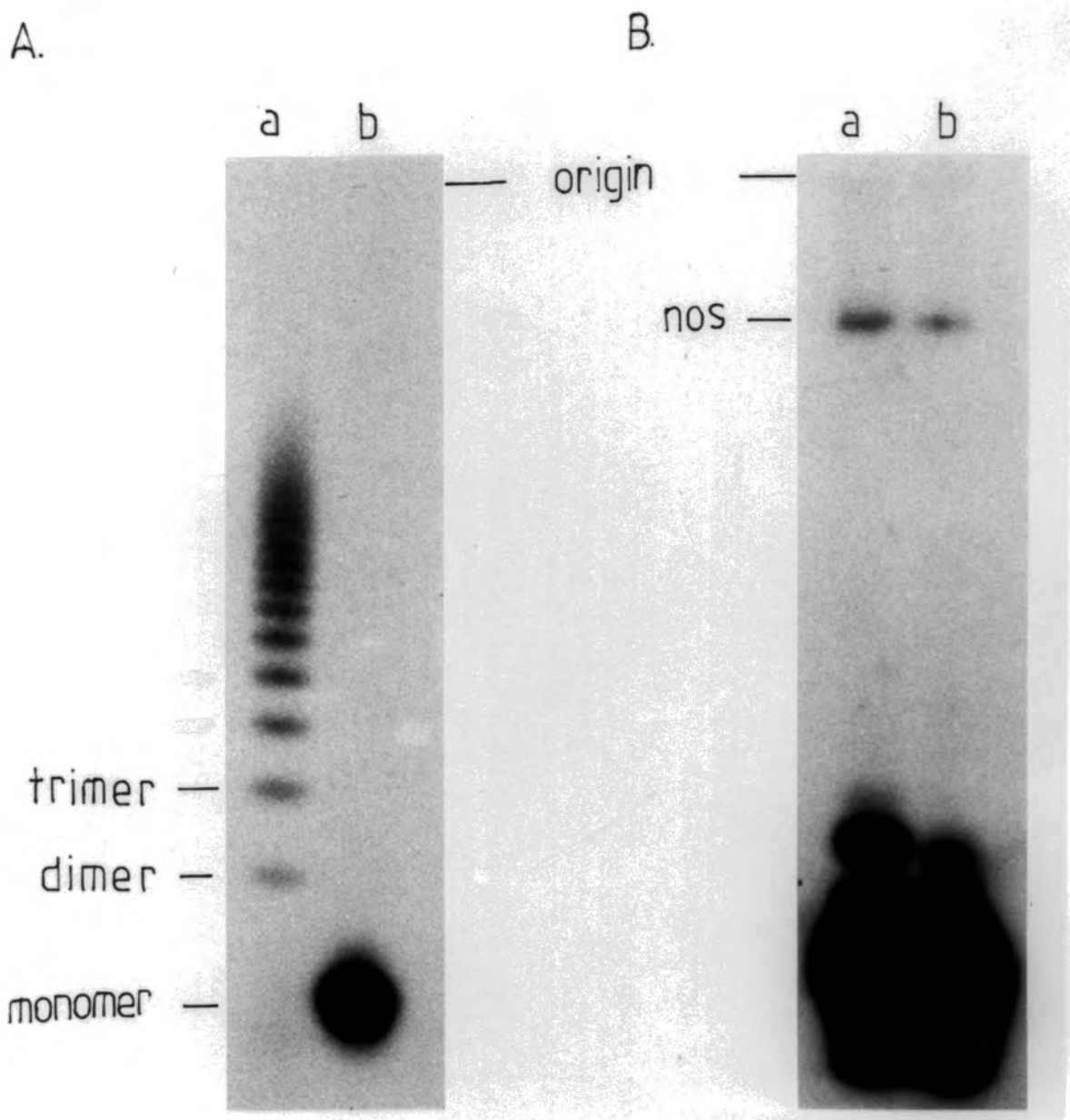
B. Autoradiograph of a polyacrylamide gel containing the ^{32}p-ATP end-labelled Cla I linkers ligated to the *nos* promoter fragment.

7.5 pmoles Cla I linker molecules in each track.

Track

a ligation mixture (Cla I linkers + 0.32 kbp pNos promoter fragment)

b Cla I restriction digest of ligation mixture


A.
a
b
origin
trimer
dimer
monomer
B.
a
b
origin
nos

pLegA was linearized by digestion with Cla I and then treated with alkaline phosphatase (Section 2.2.5.5) to remove 5'-phosphate groups to prevent self-ligation in subsequent ligation reactions.

The *nos* promoter fragment was prepared for cloning by digestion of an aliquot of the linker-ligation reaction with Cla I, followed by heat inactivation of the enzyme.

0.25 µg of the phosphatased pLegA Cla I fragment was ligated with the Cla I digested *nos* fragment/linker-ligation reaction (0.025 µg of *nos* fragment). The ligation mix was transformed into *E. coli* JM83 and plated-out on YT Amp X-Gal plates (Section 2.2.6), which resulted in 429 white and 13 blue colonies in total. (No colonies were obtained with a control transformation with a ligated aliquot of the phosphatased pLegA Cla I fragment.)

The transformants were screened for the insertion of the *nos* promoter fragment by transfer of the colonies to nitrocellulose filters followed by *in situ* lysis (Section 2.2.10) and hybridization (Section 2.2.12) to ^{32}P-labelled *nos* promoter fragment (0.32 kbp Bam HI/Hind III fragment of pNos). The autoradiograph of the probed filters (results not presented) showed hybridization to seven colonies.

These seven 'positives' were identified on the master plates and were designated pNosLeg1 to 7. DNA was prepared from each colony (Section 2.2.2.1) and digested with Cla I and Bam HI. One of the six clones, pNosLeg4, gave fragment sizes of 4.9 kbp and 0.33 kbp with Cla I and 2.7 kbp and 2.5 kbp with Bam HI (results not presented), indicating that the *nos* promoter fragment had been inserted but in the 'wrong' orientation with respect to the *legA* coding sequence.

3.3.5 Reversing the orientation of the *nos* promoter in pNosLeg4

Reversing the orientation of the 0.33 kbp Cla I insert was achieved by the digestion of pNosLeg4 with Cla I, re-ligation of the products and transformation

FIGURE 13.

Restriction digests of pADY1.

10 µl mini-prep. samples of pADY1 DNA was digested with the restriction enzymes given below.

Track		
a	pADY 1	Bam HI
b	"	Cla I
c	(pBR322 Alu I)	
d	pADY1	Eco RI
e	"	Xho I
f	"	Eco RI/Xho I

Numbers on the left are size markers (kbp).

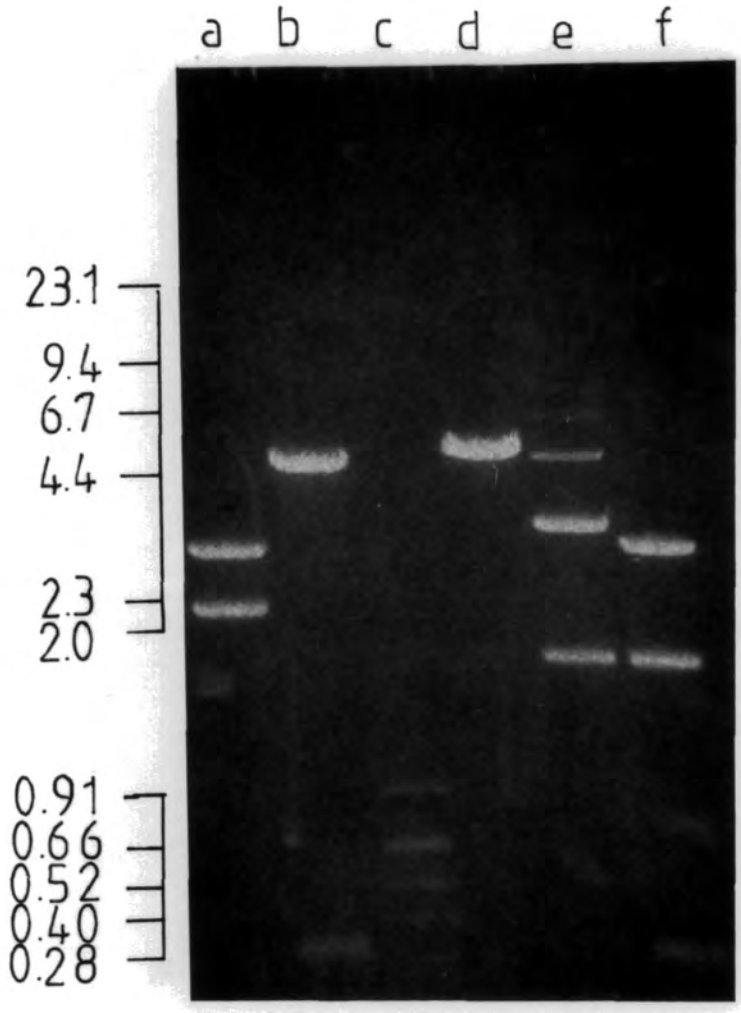
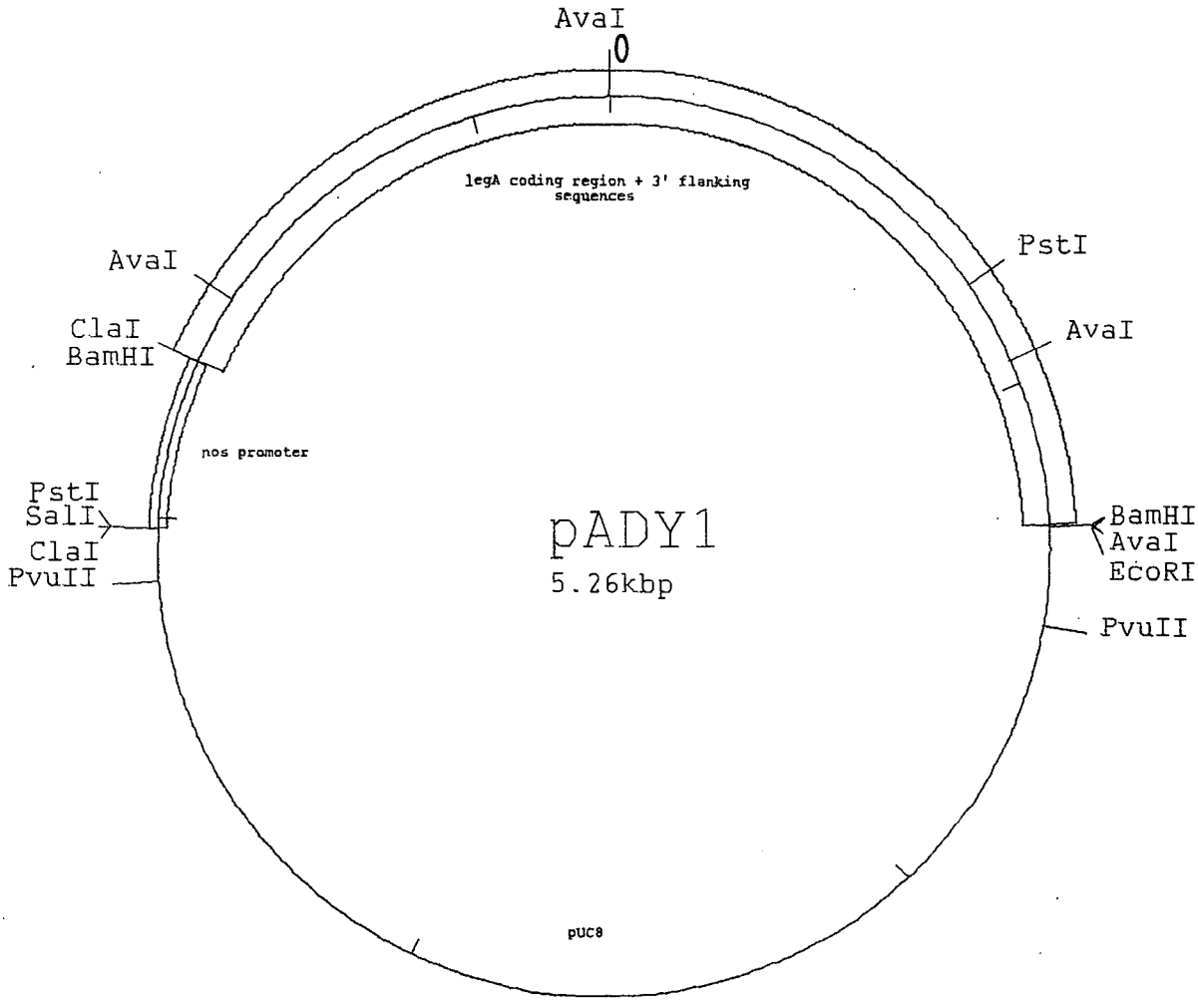

a
b
c
d
e
f
23.1
9.4
6.7
4.4
2.3
2.0
0.91
0.66
0.52
0.40
0.28

FIGURE 14.

Restriction map of pADY1.



Internal divisions denote 1 kbp (from 0).

into *E. coli* JM83, selecting for transformants containing the 0.33 kbp insert by *in situ* colony hybridization then screening these for the desired insert orientation by restriction endonuclease analysis.

The transformation resulted in 609 white colonies on YT-Amp-X-Gal plates and after transfer to nitrocellulose filters and hybridization with ^{32}P-labelled 0.32 kbp *nos* promoter fragment (re-used from the previous section), 83 "positives" were detected (results not presented). The colonies corresponding to the positive signals were identified on the master plates and sixteen were chosen for restriction endonuclease analysis. DNA was prepared from each, digested with Bam HI and run on an agarose gel. Only one clone gave fragment sizes expected for the correct orientation of the insert (3.0 kbp and 2.2 kbp). This clone was further analysed by digestion with other restriction endonucleases (Fig. 13). The sizes of the fragments obtained were as follows. 4.9 kbp and 0.33 kbp with Cla I, 5.2 kbp with EcoRI, 3.4 kbp, 1.8 kbp and faint unrestricted bands with Xho I and 3.1 kbp, 1.8 kbp and 0.33 kbp with EcoRI/Xho I. This analysis confirmed that this clone contained the promoter fragment correctly aligned with the *legA* coding sequence and was designated pADY1. A restriction map of pADY1 is shown in Fig. 14.

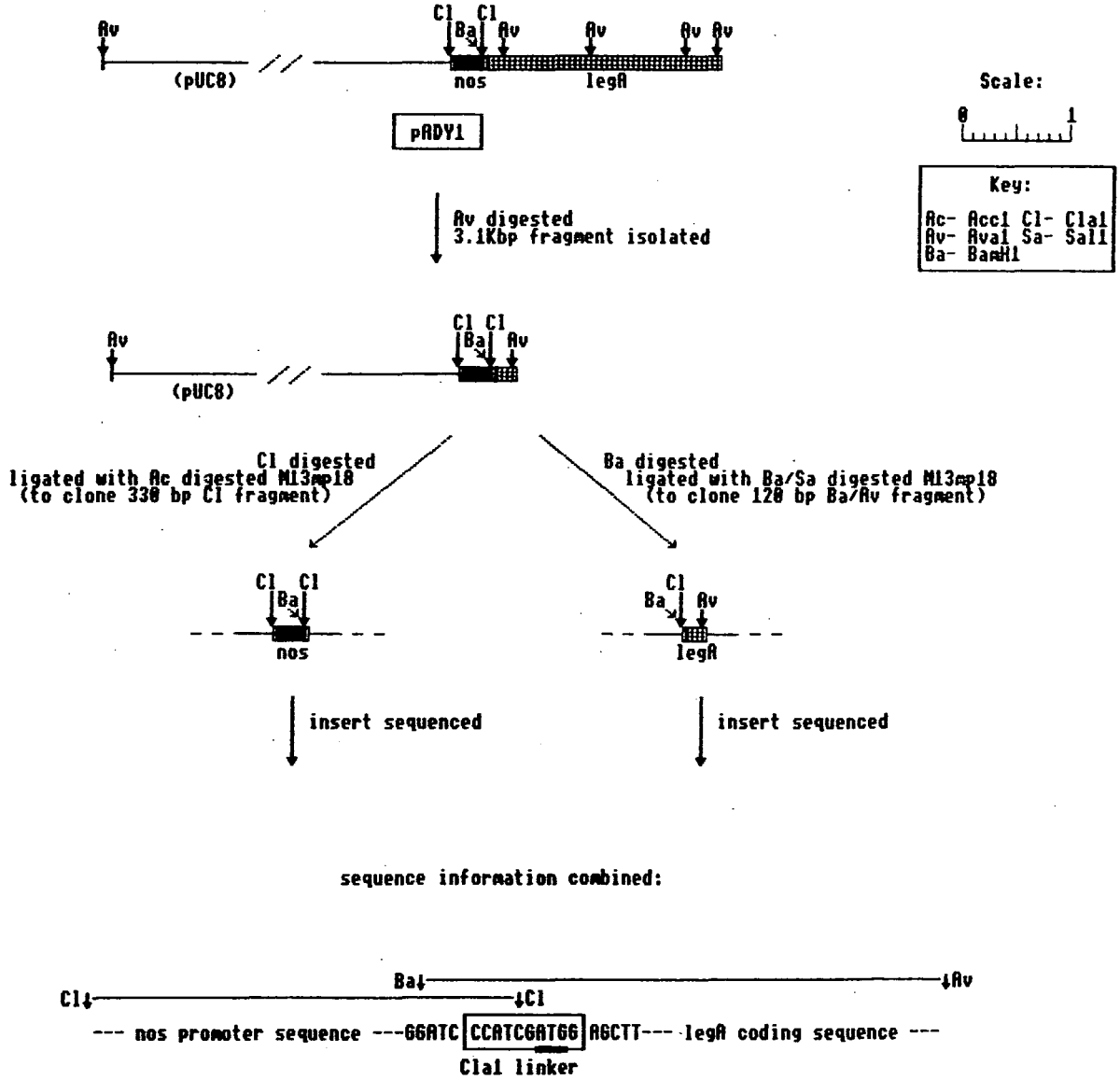
3.3.6 DNA sequencing of the *nos/legA* fusion region

The fusion of the *nos* promoter to the *legA* coding region was checked by DNA sequencing to confirm that the linker molecule used had placed an 'ATG' start codon 'in-phase' with the codons of the *legA* gene. Due to the lack of suitable restriction sites, two overlapping fragments were sequenced. The strategy is outlined in Fig. 15.

pADY1 was digested with Ava I and a 3.1 kbp fragment containing the region of interest was isolated. This fragment was digested with Cla I and the products ligated with the sequencing vector M13 mp18 digested with AccI (to clone

FIGURE 15.

Strategy for DNA sequencing of the fusion region between the *nos* promoter and legA coding region in pADY1.



sequence information combined:

FIGURE 16.

DNA sequence of the *nos/legA* fusion region.

A. DNA sequence of M13 mp 18 containing 330 bp Cla I fragment of pADY1.

B. DNA sequence of M13 mp 18 containing 120 bp Bam HI/Ava I fragment of pADY1.

C. Combined DNA sequence surrounding the Cla I linker.

A.

```
        <..mp18:
GGATCCTCTAGAGTCGATGGAGCTTGGCTGCAGGTCGACGGATCATGAGCGGAGAATTAA          60
          Acc1^[Cla1]                          :nos prom. seq.->

GGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGTTTGGAACTG          120

ACAGAACCGCAACGTTGAAGGAGCCACTCAGCCGCGGGTTTCTGGAGTTTAATGAGCTAA          180

GCACATACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCTAAGGTCACTATCAGCTAG          240
                                                  "CAAT"

CAAATATTTCTTGTCAAAAATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAAT          300
                                          "TATA"

      *5'-end of mRNA                                    :mp18..>
TAGAGTCTCATATTCACTCTCAATCCAAATAATCTGCACCGGATCCCATCGACCTGCAGG          360
                     <- nos prom. seq.:        [Cla1]^Acc1
```

B.

```
<..mp18:    [  Cla 1 ]
ACCCGGGGATCCCATCGATGGAGCTTCTTGCACTTTCTCTTTCATTCTGTTTTCTACTTT          60
  BamH1^          ---
                  <M  E  L  L  A  L  S  L  S  F  C  F  L  L

TGGGTGGCTGTTTTGCTTTGAGAGAACAGCCACAGCAAAATGAGTGCCAGCTAGAACGCC          120

L  G  G  C  F  A  L  R  E  Q  P  Q  Q  N  E  C  Q  L  E  R

          :mp18..>
TCGATGCCCTCGACCTGCAG                                                   140
          ^Sal1
L  D  A
```

C.

```
                    [ "Cla1" ]
AATAATCTGCACCGGATCCCATCGATGGAGCTTCTTGCACTTTCTCTTT
                           ---
<- nos prom. seq.:           : legA coding seq. ->
```

the 338 bp Cla I *nos* fragment). The 3.1 kbp Ava I fragment was also double-digested with Bam HI and Xho I and the products ligated with M13 mp18 double-digested with Bam HI and Sal I (to clone the 122 bp Bam HI/Xho I *leg* fragment).

The two ligation reactions were transformed into *E. coli* JM101 and transformants identified and their inserted fragments sequenced (Section 2.2.14). The sequence of both fragments is presented in Fig. 16.

3.4 Insertion of the chimeric legumin gene into the *Agrobacterium* 'binary vector' Bin 19

The chimeric legumin gene was inserted into the multiple cloning site of Bin 19, as outlined in Fig. 2. This construction and its transfer to an *Agrobacterium* host strain is described below.

3.4.1 Cloning of pADY1 into Bin 19

pADY1 containing the *nos/leg* chimeric gene was linearized with EcoRI and ligated with EcoRI digested Bin 19. The ligation products were transformed into *E. coli* K514 and plated-out on YT Kan X-Gal plates. The transformants were initially screened by transfer to nitrocellulose filters (Section 2.2.10) and hybridization to a ^{32}P-labelled *nos/legA* gene probe (2.7 kbp EcoRI/Pvu II fragment of pADY1). A number of 'positives' were identified (results not presented) and 35 chosen for analysis.

Each was picked from the master plate to both YT Amp and YT Kan plates. Sixteen clones grew on both, DNA was prepared from each and digested with EcoRI. Three of the sixteen gave fragment sizes of ~11.5 kbp and 5.2 kbp, but also a fragment of ~16 kbp (results not presented). Further analysis by Pst I digestion was performed; two clones gave aberrant fragment sizes but one gave sizes of ~5.2 kbp (x2), 2.7 kbp, 2.0 kbp and 1.3 kbp, as expected for the insertion of pADY1, but an additional ~3.9 kbp band was present (results not presented).

FIGURE 17.

Restriction digests of pADY2.

Track		
a	pADY2	Pst I
b	"	Sal I
c	"	Bam HI
d	"	Eco RI
e	"	Hind III
f	"	unrestricted

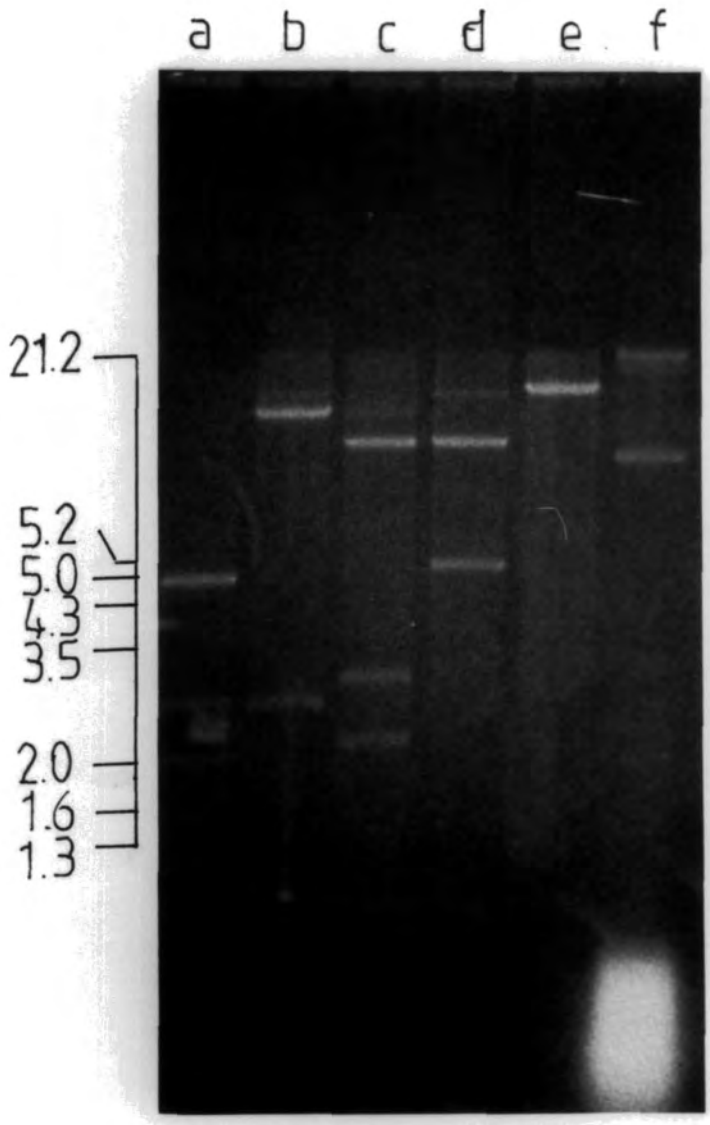
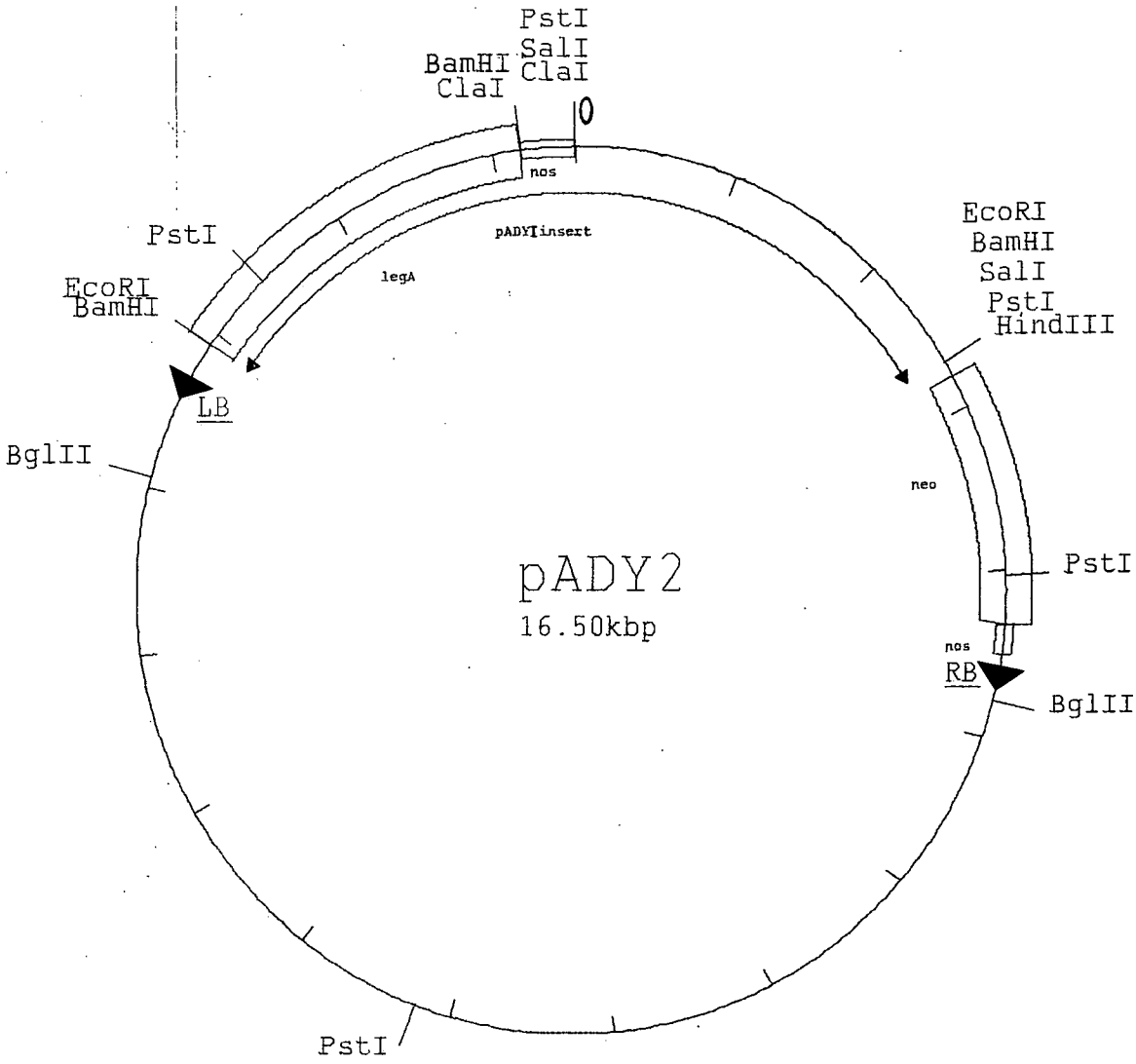

a
b
c
d
e
f
21.2
5.2
5.0
4.3
3.5
2.0
1.6
1.3

FIGURE 18.

Restriction map of pADY2.



Internal divisions denote 1 kbp (from 0).

As it was possible that the additional bands found in the above analyses were due to contamination by another clone in the colony, the original colony of the latter mentioned clone above was streaked for single colonies on YT Amp X-Gal plates. DNA was prepared from twelve well-isolated colonies and analysed by digestion with Pst I. One of the twelve gave the above expected Pst I fragment sizes. This clone was further analysed by digestion with other restriction endonucleases, the fragment sizes obtained were as follows (Fig. 17): ~14 kbp and 2.6 kbp (faint ~16 kbp) with Sal I, ~11.5 kbp, 3.0 kbp and 2.2 kbp (faint ~14 kbp) with Bam HI, ~11.5 kbp and 5.2 kbp (faint ~16 kbp) with EcoRI and ~16 kbp with Hind III. These sizes were as expected for the insertion of pADY1 in Bin 19; the clone was designated pADY2. A restriction map of pADY2 is shown in Fig. 18.

3.4.2 Analysis of pADY2 after transfer to *Agrobacterium*

The transfer of pADY2 from its *E. coli* host to *Agrobacterium* was achieved by a tri-parental mating (Section 2.2.17). Transconjugant colonies were only visible on the 10^{-1} dilution, selection plate and one was chosen for analysis.

The colony was grown-up on a fresh selective plate and DNA prepared by the small-scale method (Section 2.2.2.3). This DNA was analysed by digestion with Bam HI, EcoRI and Bgl II/Sal I double-digestion. The digested DNA was run on an agarose gel (with pADY1 and Bin 19 controls to demonstrate hybridization to the correct fragments) (Fig. 19a). As the DNA preparations contained *Agrobacterium* chromosomal DNA, no distinct staining bands were visible so the DNA was transferred from the gel to a nitrocellulose filter by 'Southern blotting' (Section 2.2.9) and probed with specific sequences as detailed below .

The filter was cut into three sections, the first with the three *Agrobacterium* DNA digests, pADY1 and Bin 19 digested with Bam HI, was probed with ^{32}P-labelled pADY1 Bam HI 2.2 kbp fragment (containing the *legA* coding and 3'-

flanking sequences). The second, with the three *Agrobacterium* DNA digests, pADY1 digested with Pst I and Bin 19 double-digested with Pst I/Hind III was probed with pNosNeo Pst I/Eco RI 1.1 kbp fragment (containing the *neo* coding sequence). the third with the three *Agrobacterium* DNA digests, pADY1 digested with Cla I and Bin 19 double-digested with Bgl II/Pst I, was probed with pADY1 Cla I 0.33 kbp fragment (containing the *nos* promoter sequence).

After hybridization and washes (Section 2.2.12), the filters were autoradiographed. These results are presented in Fig. 19b. The control bands hybridized as expected; the 'leg' probe hybridized strongly to the pADY1 Bam HI 2.2 kbp (faintly to the remaining 3.0 kbp fragment) but not to Bin 19 Bam HI at all. The 'neo' probe hybridized strongly to the Bin 19 Pst I/Hind III 1.3 kbp band (although faintly to the 3.2 kbp band of pADY1 Pst I). The 'nos' probe hybridized strongly to both pADY1 Cla I 0.33 kbp fragment and Bin 19 Bgl II/Pst I 0.66 kbp fragment as expected, although the other pADY1 Cla I fragment (4.9 kbp) did also hybridize.

The results of the hybridization to the LBA4404/pADY2 Eco RI digests were as expected, with hybridization to the 5.2 kbp fragment with the 'leg' probe, to the 11.5 kbp fragment with the 'neo' probe and both these fragments with the 'nos' probe. Similarly, the Bam HI digest gave expected results, with the 'leg' probe hybridizing to the 2.2 kbp fragment, the 'neo' probe hybridizing to the 11.5 kbp fragment and the 'nos' probe hybridizing to the 11.5 kbp and 3.0 kbp fragments.

The Bgl II/Sal I double digest gave the expected 3.4 kbp band hybridized with the 'leg' probe, but the 'neo' probe hybridized only faintly to the expected 2.0 kbp fragment but more strongly to an ~4.8 kbp band. Similarly, with the 'nos' probe, the expected hybridization was obtained to the 3.45 kbp fragment and to the 2.0 kbp band (although faint) but there was an additional ~4.8 kbp band.

FIGURE 19.

Southern blot analysis of pADY2 plasmid DNA isolated from *Agrobacterium tumefaciens* LBA4404/pADY2.

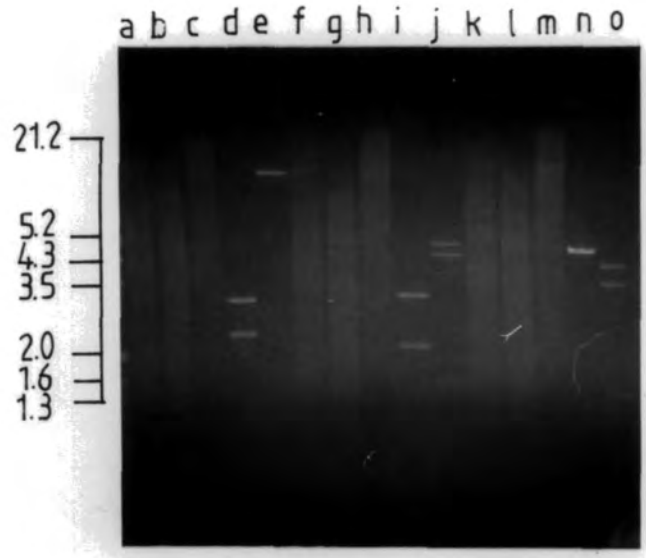
A. Restriction digests of *Agrobacterium tumefaciens* strain LBA4404 harbouring pADY2.

Track		
a	LBA4404/pADY2	Eco RI
b	"	Bgl II/Sal I
c	"	Bam HI
d	pADY1	Bam HI
e	Bin 19	Bam HI
f	LBA4404/pADY2	Eco RI
g	"	Bgl II/Sal I
h	"	Bam HI
i	pADY1	Pst I
j	Bin 19	Pst I/Hind III
k	LBA4404/pADY2	Eco RI
l	"	Bgl II/Sal I
m	"	Bam HI
n	pADY1	Cla I
o	Bin 19	Bgl II/Pst I

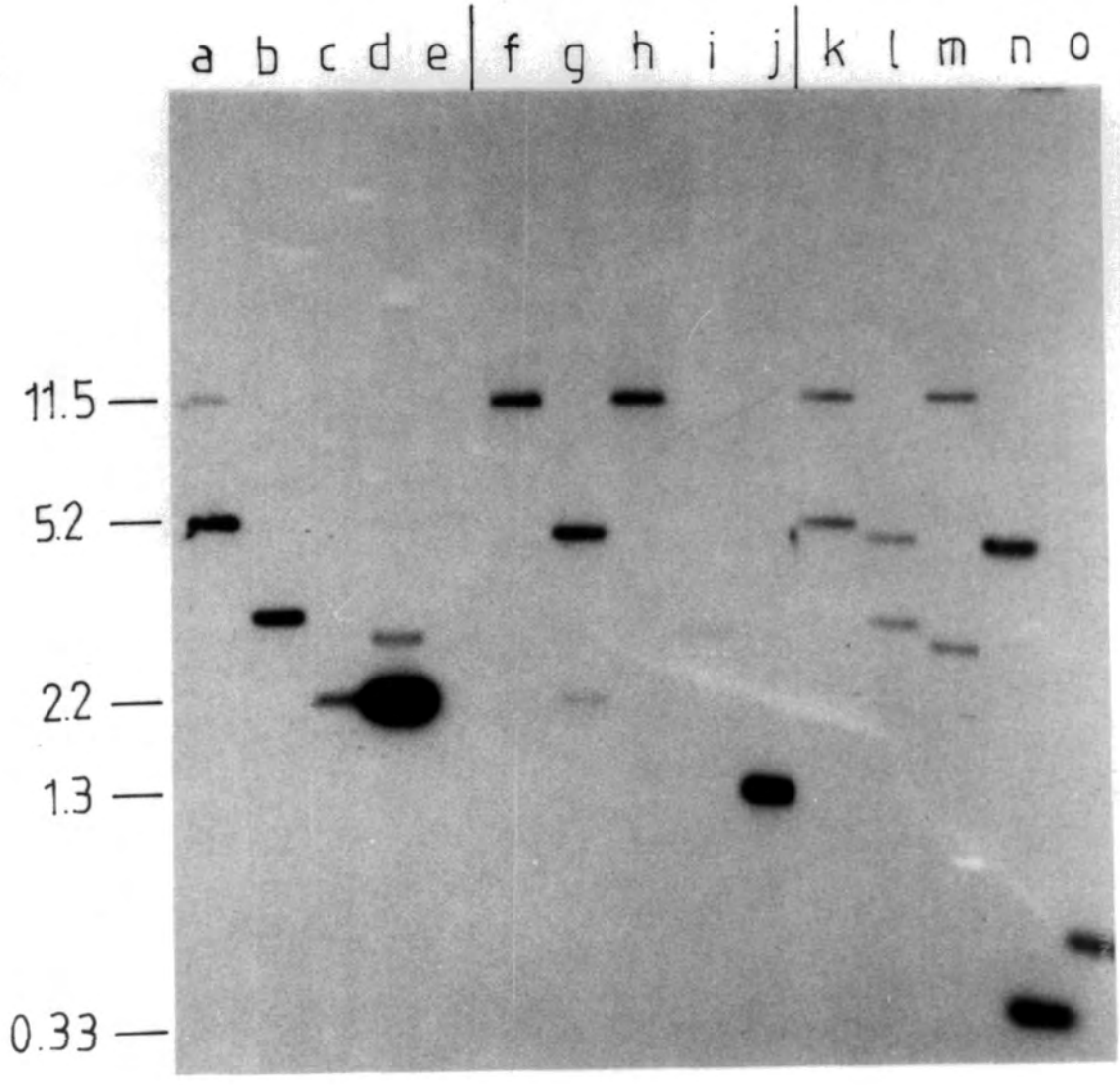
B. Autoradiograph of a blot of the gel above hybridized to:

tracks a-e	2.2 kbp	Bam Hi fragment of pADY1 (Legumin gene)
tracks f-j	1.1 kbp	Pst I/Eco RI fragment of pNosNeo (*Neo* gene)
tracks k-o	0.33 kbp	Cla I fragment of pADY1 (*Nos* promoter)

A.


a b c d e f g h i j k l m n o
21.2
5.2
4.3
3.5
2.0
1.6
1.3

B.


a b c d e f g h i j k l m n o
11.5
5.2
2.2
1.3
0.33

3.5 Transfer of the chimeric legumin and neomycin phosphotransferase genes from pADY2 to pUC8

For protoplast transformation, it was necessary for the chimeric legumin and neomycin phosphotransferase genes to be contained on as small a fragment or plasmid as possible, as the size of the DNA molecules used in the 'direct gene transfer' method was thought to affect the efficiency of transformation (see Discussion). For this reason, the pUC8 sequence between the two chimeric genes in pADY2 was removed by deletion of a 2.7 kbp Sal I fragment and a fragment containing the two genes was transferred to the plasmid vector pUC8. This strategy is outlined in Fig. 3. The construction is described below.

3.5.1 Removal of a Sal I fragment from pADY2

pADY2 was digested with Sal I and run on an agarose gel. The ~14 kbp band was isolated from the gel, re-ligated and transformed into *E. coli* K514 and transformants selected on YT Kan plates. Thirty-six transformants were obtained and DNA was prepared from eight of these and analysed by Eco RI digestion. Five of the eight clones resulted in a single fragment of approximately 14 kbp , indicating successful deletion of the 2.7 kbp Sal I fragment. One of the five clones was chosen at random and further analysed by digestion with Bam HI and Pst I which resulted in the expected fragment sizes (results not presented) (11.6 kbp and 2.2 kbp with Bam HI instead of 11.3 kbp, 3.0 kbp and 2.2 kbp as for pADY2; 5.2 kbp x 2, 2.0 kbp and 1.3 kbp with Pst I instead of 5.2 kbp x 2, 2.7 kbp, 2.0 kbp and 1.3 kbp as for pADY2). This clone was designated pADY2 Δ Sal.

3.5.2 Cloning of legumin and neomycin phosphotransferase genes into pUC8

pADY2 Δ Sal was digested with Bgl II and run on an agarose gel. The 5.5 kbp fragment (containing *nos/leg* and *nos/neo* chimeric genes) was isolated from

FIGURE 20.

Restriction digests of pADY3.

Tracks

a	pADY3	Pst I
b	"	Eco RI
c	"	Sal I
d	"	Eco RI/Hind III

Numbers on the left are size markers (kbp).

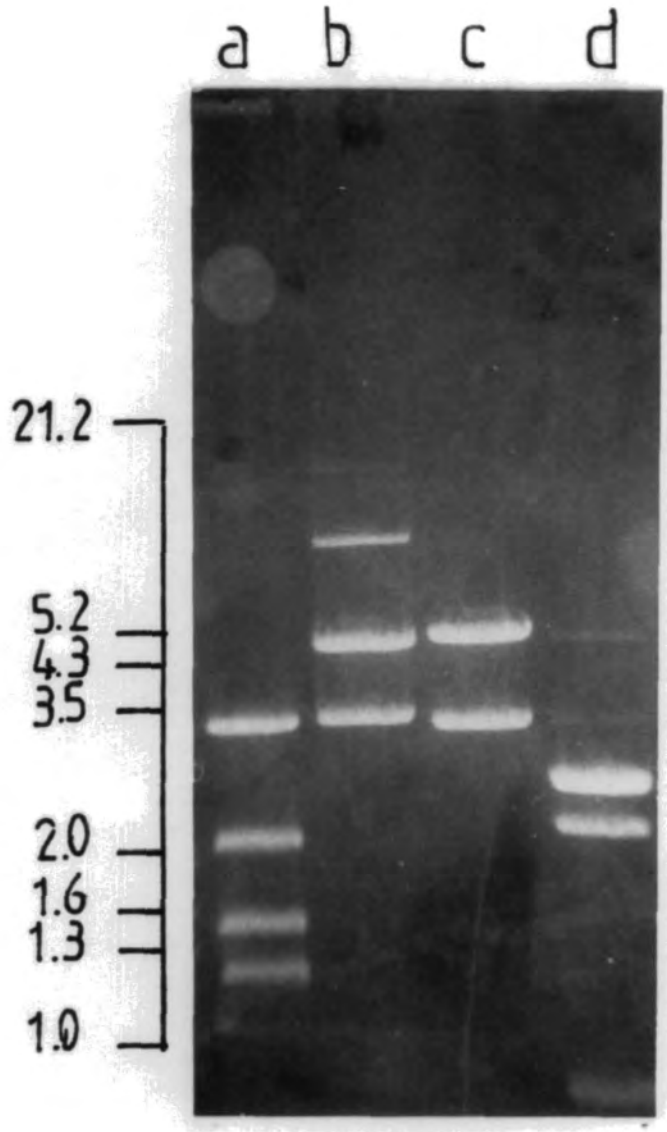
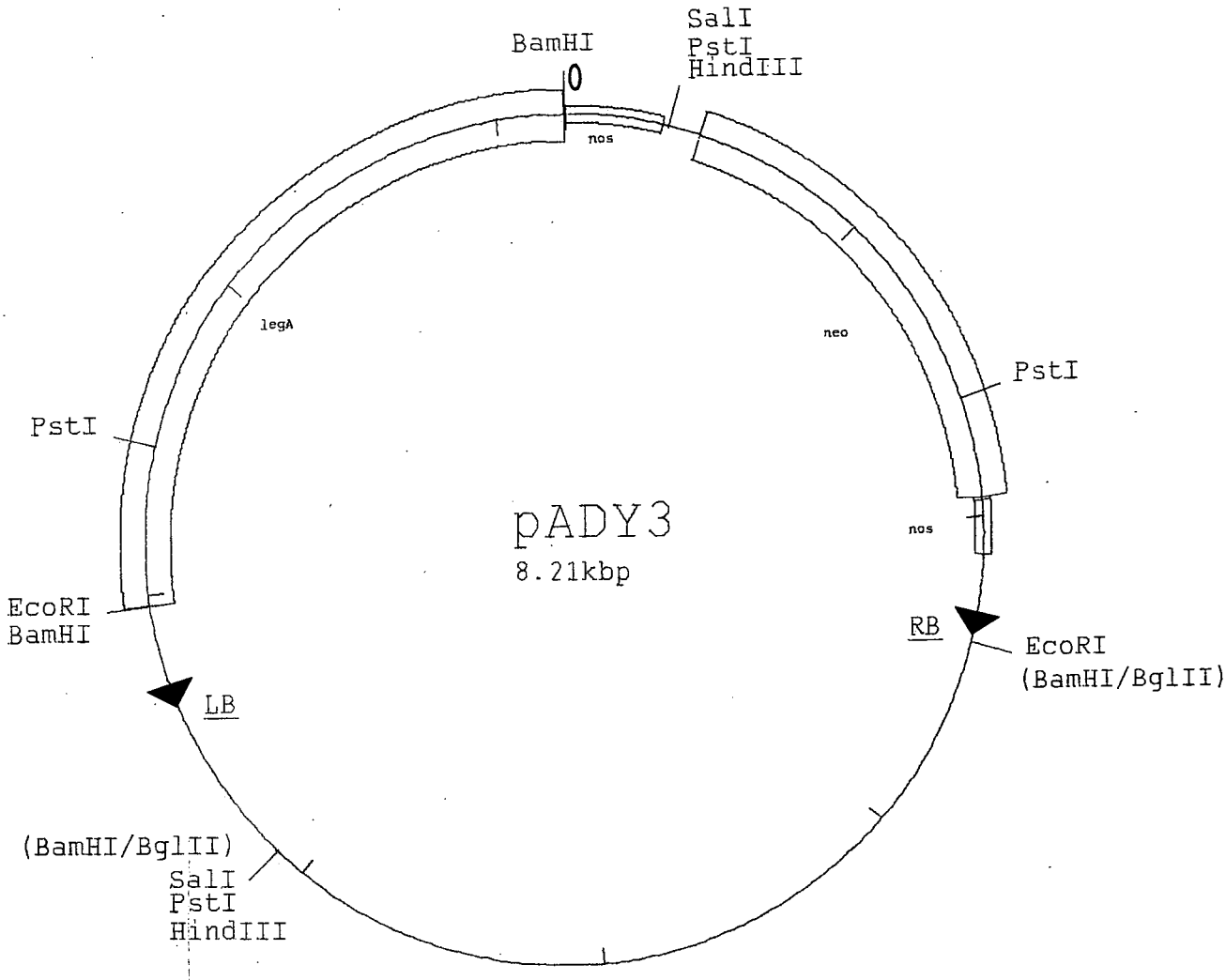

a
b
c
d
21.2
5.2
4.3
3.5
2.0
1.6
1.3
1.0

FIGURE 21.

Restriction map of pADY3.



Internal divisions denote 1 kbp (from 0).

the gel and ligated with Bam HI digested pUC8. The ligation products were transformed into *E. coli* JM83 and transformants selected on YT Amp X-Gal plates. Twelve white colonies were obtained and DNA was prepared from these and analysed by digestion with Eco RI. Three of the twelve gave fragment sizes of 4.6 kbp and 3.6 kbp, indicating one orientation of the 5.5 kbp insert and five gave fragment sizes of 7.3 kbp and 0.9 kbp, indicating the reverse orientation. (The remaining four gave aberrant sizes). A clone with the first orientation mentioned above was selected and DNA prepared using the large-scale method (Section 2.2.2.2). The DNA was further analysed by digestion with Pst I, Eco RI, Sal I and Eco RI/Hind III double-digestion. The digests were run on an agarose gel and the fragment sizes obtained were as expected (Fig. 20); namely 3.5 kbp, 2.0 kbp, 1.4 kbp and 1.3 kbp with Pst I; 4.6 kbp and 3.6 kbp with Eco RI; 4.8 kbp and 3.4 kbp with Sal I; 2.7 kbp, 2.6 kbp, 2.0 kbp and 0.9 kbp with Eco RI/Hind III.

This clone was designated pADY3. A restriction map of pADY3 is shown in Fig. 21.

3.6 Transformation of *Nicotiana* leaf pieces with *Agrobacterium* harbouring pADY2

Nicotiana tabacum SR1 leaf pieces were infected with the *Agrobacterium* strain containing pADY2 and transferred to selective shoot medium, as described in Section 2.2.18. The leaf piece edges callussed and produced shootlets (Fig. 22) which were excised and transferred to rooting medium. Bacterial contamination did not carry through the tissue culture steps, indicating the *Agrobacterium* were killed by the antibiotic Timentin.

Ten of the rooted shootlets produced were transferred to compost and grown as described in Section 2.2.18. These transformed plants, designated T300 to T309, were phenotypically normal and developed to full maturity. The flowers of each plant were self-pollinated; cross-pollination was prevented by "bagging" the flower

FIGURE 22.

Regeneration of *Nicotiana tabacum* SRI plants after *Agrobacterium tumefaciens* infection of leaf pieces. Shoots can be seen developing from infected leaf pieces.

clusters with clear polythene bags and ties. At intervals, fully expanded leaves were harvested from mature plants (maximum of two at a time) and the plants allowed at least a week to recover before further removal.

3.7 'Direct gene transfer' to *Nicotiana tabacum* protoplasts

Nicotiana tabacum SR1 protoplasts were isolated and transformed (as described in Section 2.2.19) with either pNosNeo, pADY3 (both undigested) or the 5.5 kbp EcoRI fragment excised from pADY3. Transformation with no plasmid DNA (i.e. carrier DNA only) was performed as a control. This set of four treatments was repeated a further three times, using a fresh protoplast isolation for each set.

One week after transformation, protoplast division was seen when the 'solidified' cultures were viewed under the microscope. It was found that up to 90% had entered division, with up to 30% at the 8-cell stage. Fig. 23a shows *N. tabacum* leaf protoplasts after isolation and Fig. 23b isolated protoplasts after several divisions during regeneration,

Regeneration of the protoplasts was continued under kanamycin selection in "bead culture" (Section 2.2.19.3). Some of these cultures became contaminated with bacteria and/or fungi after a number of changes of the kanamycin medium. It was found that once contaminated, any resistant colonies present did not subsequently survive if plated on PSMKan, so these cultures were discarded. Fig. 23c shows kanamycin resistant microcalli developing in "bead culture".

The total numbers of kanamycin resistant colonies obtained from each transformation is given in Table 3 below.

Table 3. Numbers of kanamycin resistant colonies obtained from each transformation

DNA treatment in transformation	No. of kanamycin resistant colonies obtained A	B	C	D
HSDNA (only)	0	0	0	0
" + pNosNeo	21	31	6	13
" + pADY3	-	-	7	1
" +pADY3 Eco fragment	7	-	13	4

(- = "bead culture" discarded due to contamination).

Shootlets were regenerated from the kanamycin resistant colonies on PSMKan plates and a number of these were transferred to 'rooting medium'. Some shootlets took only two or three weeks to develop a root system whereas, others failed to produce roots after many weeks in culture. Shootlets that did produce roots were potted into compost and mature plants obtained.

Two pNosNeo derived transformed plants (designated T360 and T361) and seven pADY3 EcoRI fragment derived transformed plants (designated T370 to T376) were obtained. All the transformed plants developed normally and had the appearance of control untransformed plants. The flowers of each plant were "bagged" (with clear polythene bags and ties) to ensure self-pollination and mature seeds were collected. Fully expanded leaves were excised from mature plants as described in Section 3.6.

FIGURE 23.

Regeneration of *Nicotiana tabacum* plants after direct gene transfer to protoplasts.

A. Isolated *Nicotiana tabacum* SRI leaf protoplasts.

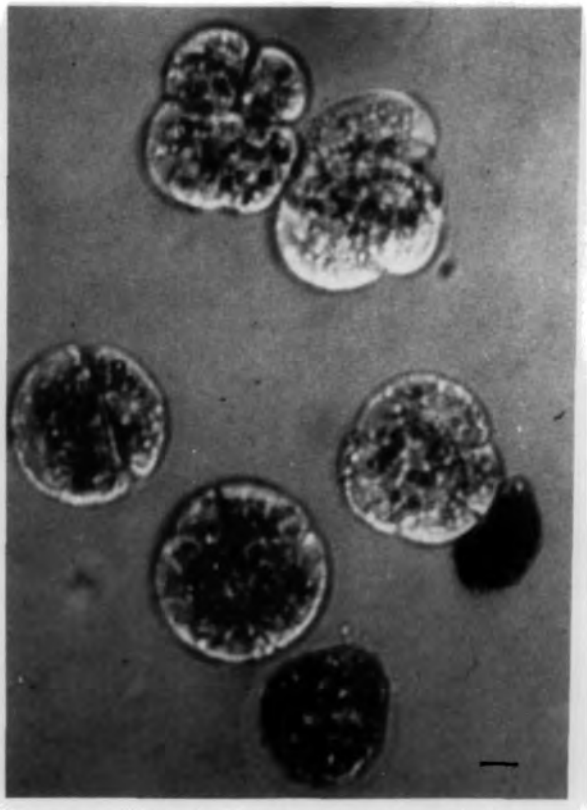
B. *N. tabacum* protoplasts 7 days after isolation.

C. pADY3 transformed *N. tabacum* protoplasts in 'bead culture' under kanamycin selection (100 µg/ml). Resistant microcalli can be seen against a background of dead, untransformed protoplasts.

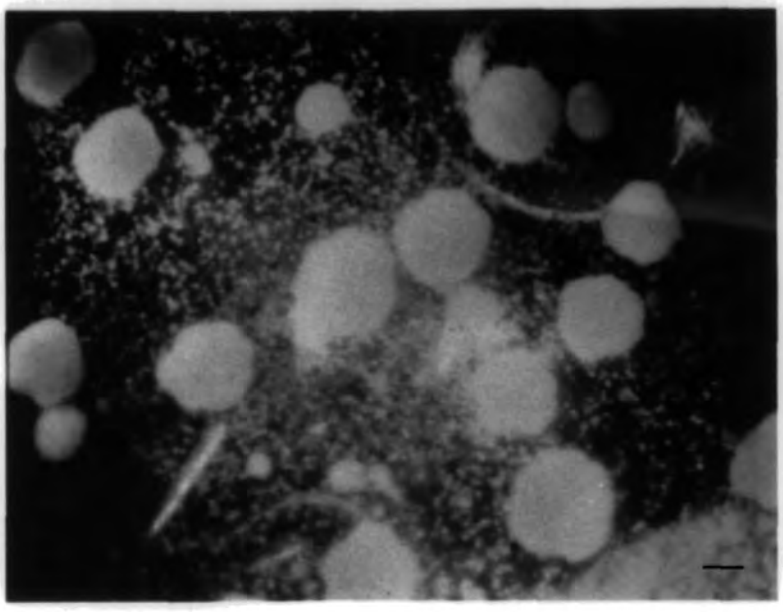
Scale bars represent:	10 um in A and B
	1 mm in C

A.

B.



C.



3.8 Analysis of transformed plants

3.8.1 Transformed plant genomic DNA analysis

3.8.1.1 *Agrobacterium*-mediated transformed plants

Genomic DNA was prepared from leaf tissue of ten *Agrobacterium*-transformed *Nicotiana tabacum* plants (T300 to T309, Section 3.6) and an untransformed 'control' plant. Bam HI restriction was performed to excise the 2.2 kbp *legA* gene sequence as a single fragment from the inserted DNA. This restriction was also expected to produce a 3.0 kbp fragment containing pUC8 sequence and the *nos* promoter of the chimeric legumin gene and a fragment of unknown size consisting of the right-border insertion fragment containing the *nos/neo* gene and plant genomic DNA, assuming a single intact insertion had occurred (see Fig. 24).

The Bam HI digests of each transformed plant DNA and control DNA were divided between three agarose gels. After electrophoresis the gels were 'Southern' blotted. Fig. 25 shows one of the gels, the other two were identical. Each blot was probed with one of three probes; these probes together with the expected hybridizing fragments, are shown in Fig. 24.

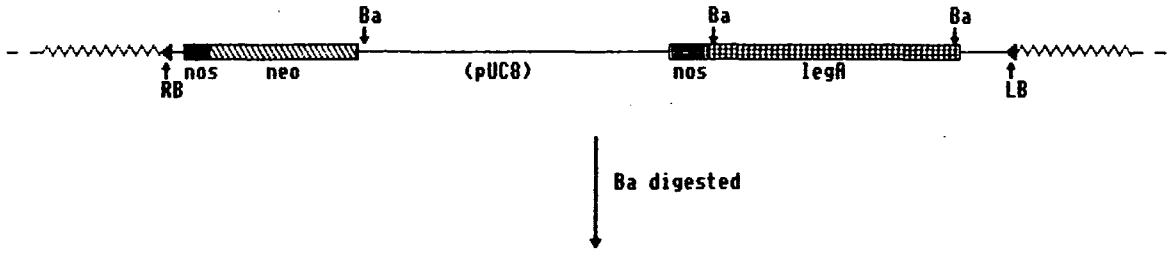
Fig. 26 shows the results obtained when the *legA* gene probe (2.2 kbp Bam HI fragment of pADY1) was hybridized to the appropriate Southern blot. There was slight hybridization to a 1.5 kbp fragment in the SR1 'control' digest (this band did not appear in the other tracks). Seven of the transformed plant digests showed hybridization to a single 2.2 kbp fragment as expected, three of these, T301, T303 and T309) showed a weaker hybridization signal than the other four (T300 and T305 to T307). T302 showed slight hybridization to an approx. 30 kbp fragment. T304 and T308 showed no hybridization at all.

Fig. 27 shows the results obtained when the *neo* gene probe (1.1 kbp Pst I/EcoRI fragment of p*NosNeo*) was hybridized to the appropriate Southern blot.

FIGURE 24.

Expected hybridizing fragments in "Southern" blot analysis of *Agrobacterium tumefaciens* LBA4404/pADY2 transformed *Nicotiana tabacum* regenerants.

A single intact pADV2 insert:



			Probes expected to hybridize:
A	nos neo	>1.7 Kbp	Nos, Neo
B	(pUC8) nos	3.0 Kbp	Nos
C	legA	2.2 Kbp	Leg
D		>0.4 Kbp	—

Probes:

nos promoter	"Nos"	0.33 Kbp ClaI fragment of pADV1	(▬)
neo gene	"Neo"	1.05 Kbp PstI/EcoRI fragment of pMOSNEO	(▬)
leg gene	"Leg"	2.22 Kbp BamHI fragment of pADV1	(▬)

Key:

Ba- BamHI
LB- T-DNA left border
RB- T-DNA right border
∿∿∿ - plant genomic DNA

Scale:

0 1 Kbp

FIGURE 25.

Restriction digests of DNA prepared from leaf tissue of *Nicotiana tabacum* transformants derived from *Agrobacterium tumefaciens* infection.

10 µg genomic DNA per track.

Tracks

a	(untransformed control) SRI	Bam HI
b	T300	Bam HI
c	T301	"
d	T302	"
e	T303	"
f	T304	"
g	T305	"
h	T306	"
i	T307	"
j	T308	"
k	T309	"

The numbers on the left are size markers (kbp).

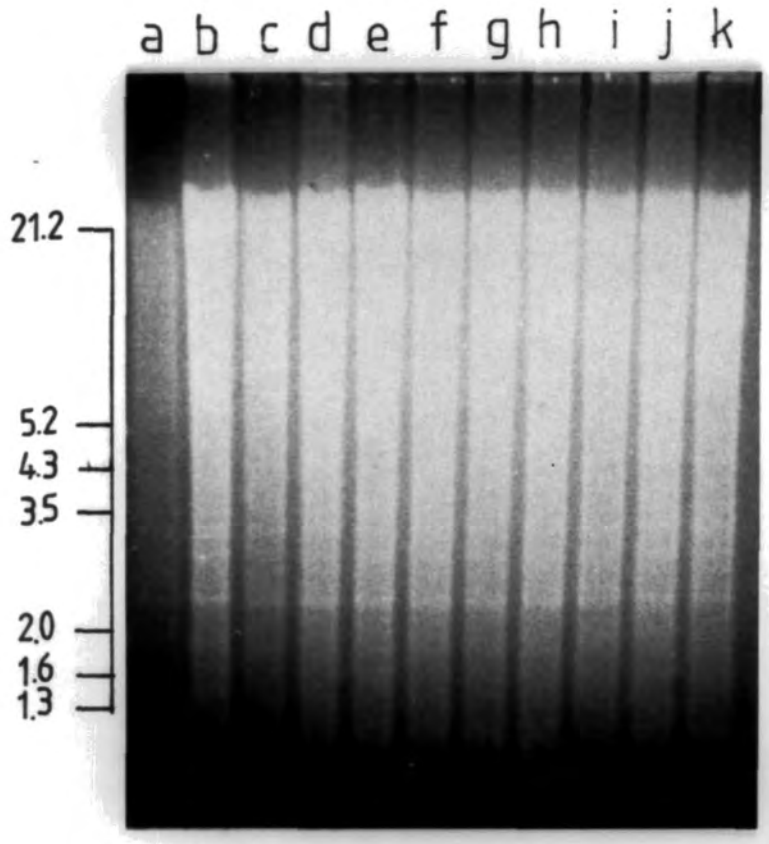

a b c d e f g h i j k
21.2
5.2
4.3
3.5
2.0
1.6
1.3

FIGURE 26.

Autoradiograph of a blot of the gel in Fig. 25 hybridized to the 2.2 kbp Bam HI fragment of pADY1 (legumin A coding region and 3'-flanking sequences).

Track		
a	(untransformed control)	
	SRI	Bam HI
b	T300	"
c	T301	"
d	T302	"
e	T303	"
f	T304	"
g	T305	"
h	T306	"
i	T307	"
j	T308	"
k	T309	"

The numbers on the left are sizes in kbp.

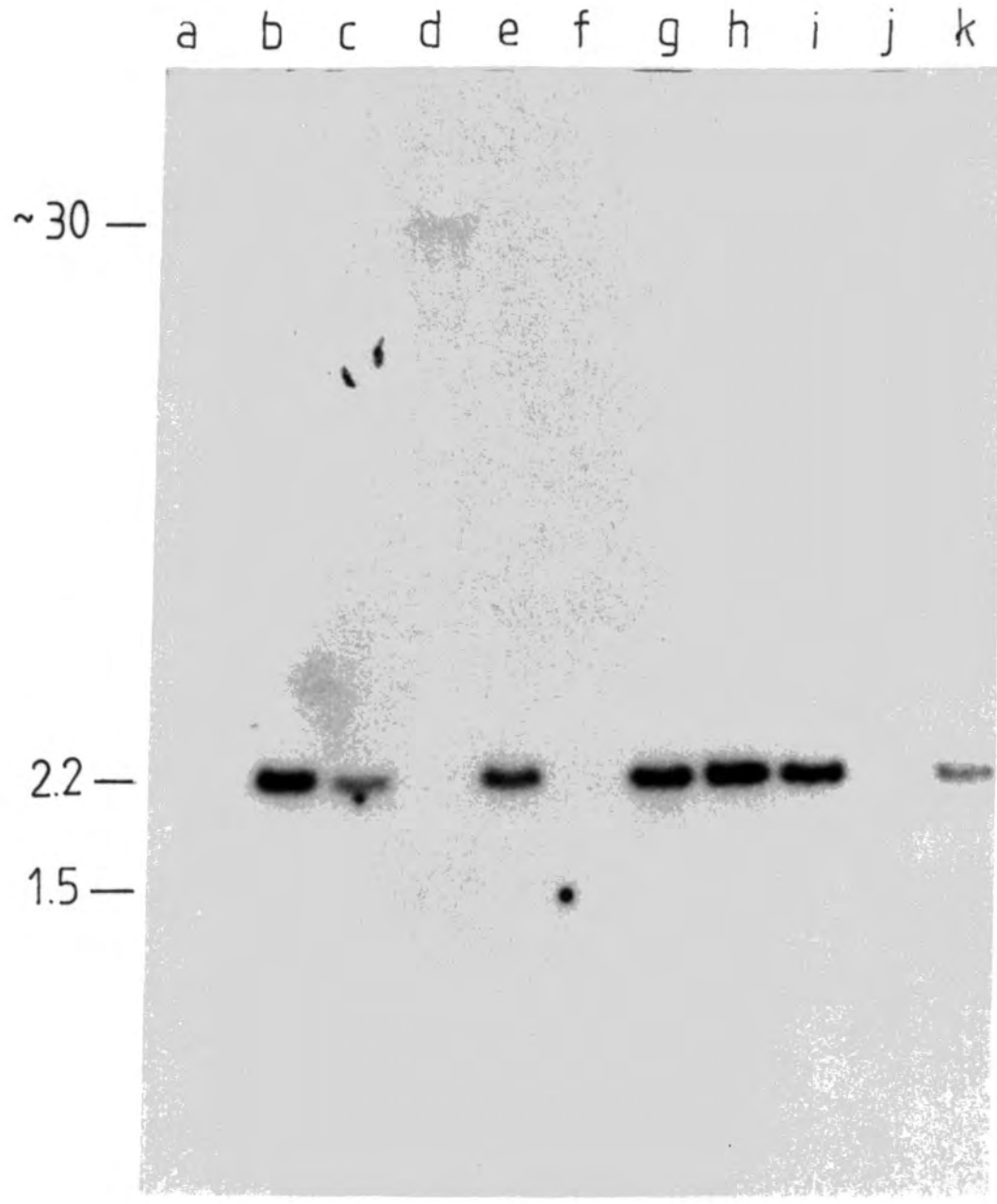

a b c d e f g h i j k
~30
2.2
1.5

FIGURE 27.

Autoradiograph of a blot of a gel containing replica samples of those in Fig. 25 hybridized to the 1.1 kbp Pst I/Eco RI fragment of pNosNeo (*Neo* gene).

Track

a	(untransformed control)	
	SRI	Bam HI
b	T300	"
c	T301	"
d	T302	"
e	T303	"
f	T304	"
g	T305	"
h	T306	"
i	T307	"
j	T308	"
k	T309	"

Numbers on the left are sizes in kbp.

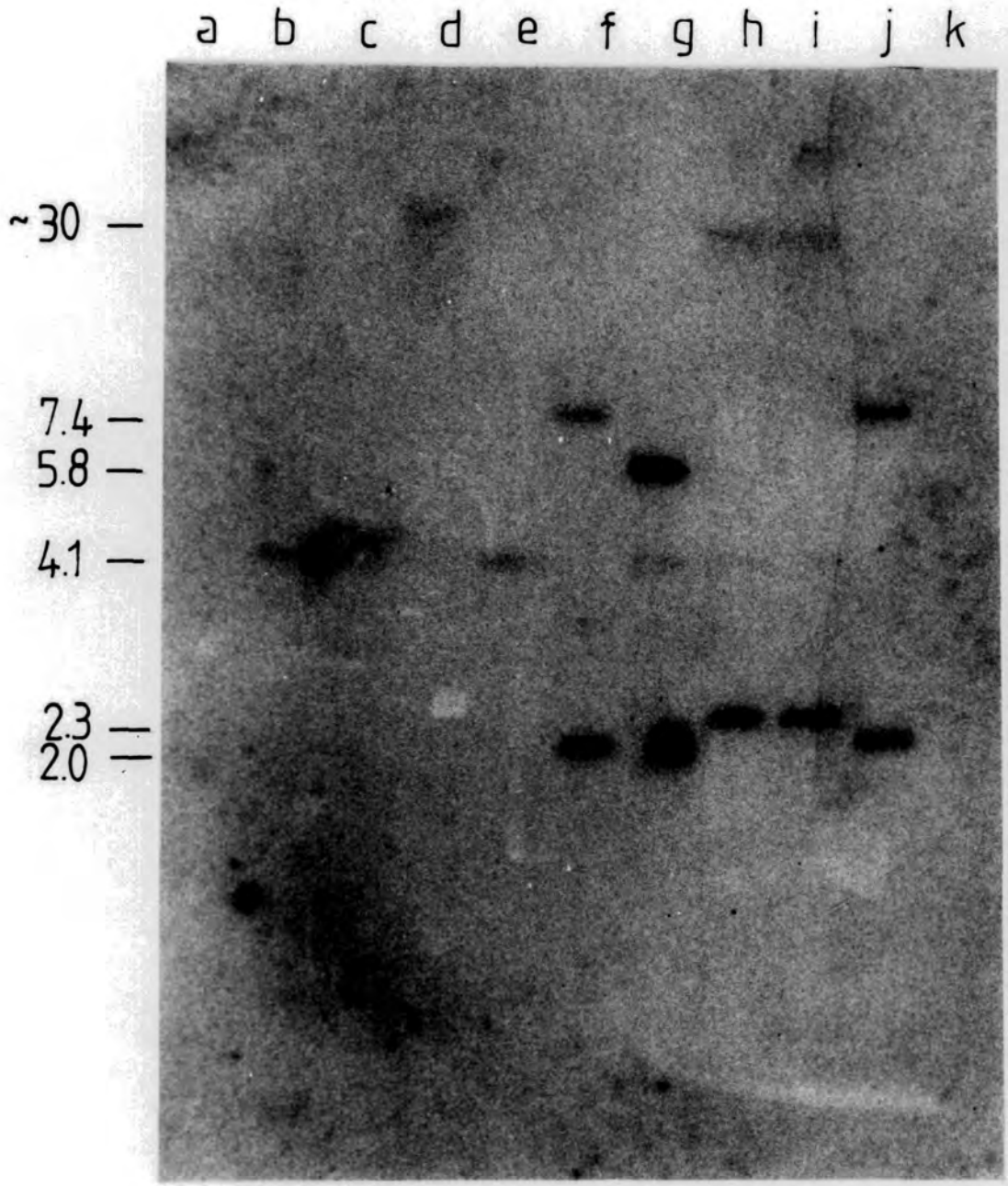

a b c d e f g h i j k
~30
7.4
5.8
4.1
2.3
2.0

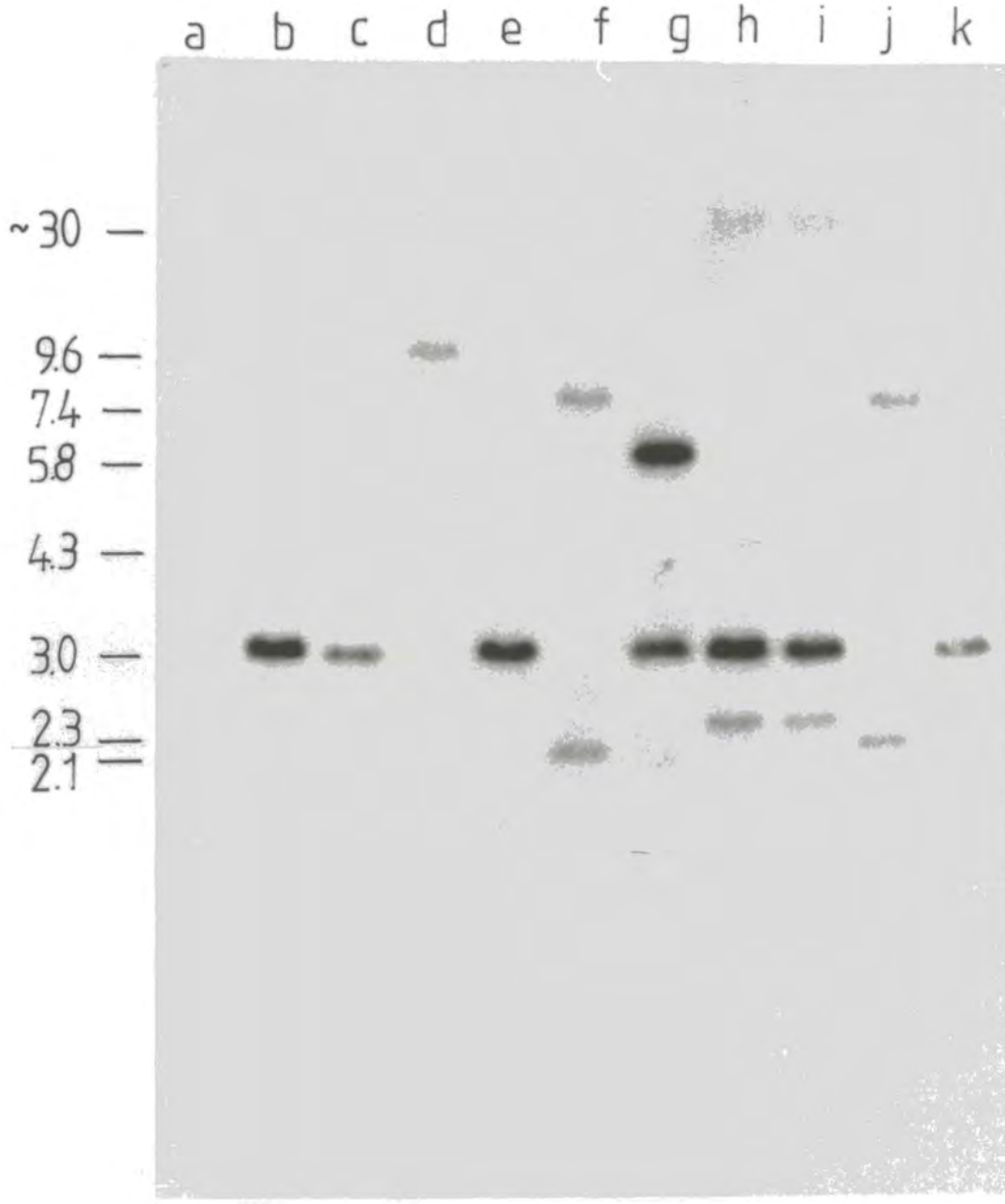
FIGURE 28.

Autoradiograph of a blot of a gel containing replica samples of those in Fig. 25 hybridized to a 0.33 kbp Cla I fragment of pADY1 (*nos* promotor).

Track

a	(untransformed control)	
	SRI	Bam HI
b	T300	"
c	T301	"
d	T302	"
e	T303	"
f	T304	"
g	T305	"
h	T306	"
i	T307	"
j	T308	"
k	T309	"

Numbers on the left are sizes in kbp.


a b c d e f g h i j k
~30
9.6
7.4
5.8
4.3
3.0
2.3
2.1

The probe did not hybridize to the 'control' SR1 digest. The expected hybridization pattern for intact inserts was one or more bands of at least 1.7 kbp, depending on the site(s) of insertion in the plant genome (the Bam HI restriction would produce a fragment containing 1.7 kbp of transferred DNA, plus plant genomic DNA to the next Bam HI site). As expected all bands were indeed greater than 1.7 kbp; these bands are detailed below. There was hybridization to a single 4.1 kbp fragment of T300 and T303. T302 showed a faint band of approximately 30 kbp. T304 and T308 gave an identical hybridization pattern with bands of 7.4 kbp and 2.0 kbp. T305 showed a strongly hybridizing band of 5.8 kbp and a faint 4.1 kbp band. T306 and T307 gave an identical hybridization pattern with slight hybridization to an approximately 30 kbp and 4.1 kbp bands and stronger hybridization to a 2.3 kbp band. T309 gave no bands and T301 may have had an approximately 4.3 kbp band but this was masked by a region of non-specific hybridization.

Fig. 28 shows the results obtained when the *nos* promoter probe (0.33 kbp Cla I fragment of pADY1) was hybridized to the appropriate Southern blot. This hybridization was expected to produce bands at 3.0 kbp (the fragment containing the *nos* promoter of the chimeric legumin gene), together with the fragment containing the *nos/neo* gene, the size of which would be identical to that found in the *neo* probe hybridization. More than one insert would be apparent as a stronger hybridizing 3.0 kbp band and more than one additional band. The band sizes detailed below should, therefore, be compared with those obtained with the *neo* probe. T300, T301, T303 and T309 gave the expected band of 3.0 kbp (T300 and T303 were more intense than the other two). However, no additional bands were present. T302 gave a 9.6 kbp band and a very faint approximately 30 kbp band. T304 and T308 gave an identical hybridization pattern with bands of 7.4 kbp and 2.1 kbp. T305 showed strongly hybridized bands of 5.8 kbp and 3.0 kbp, which

were expected as both hybridized to the *neo* probe but faint additional bands of 2.2 kbp and 2.0 kbp were present. T306 and T307 gave an identical hybridization pattern with a faint band of approximately 30 kbp, 4.3 kbp and 2.3 kbp and a strongly hybridized 3.0 kbp band. This 2.3 kbp band also hybridized to the *neo* probe (the 4.3 kbp band in this case is possibly the same as the *neo* hybridizing 4.1 kbp band).

3.8.1.2 'Direct gene transfer' derived transformed plants

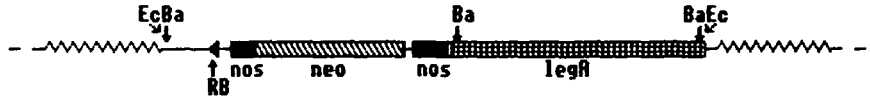
Genomic DNA was prepared from leaf tissue of transformed *Nicotiana tabacum* plants derived by direct gene transfer (Section 3.7), seven transformed with the pADY3 EcoRI fragment (T370 to T376), two transformed with p*NosNeo* (T360 and T361) and one untransformed 'control' plant. The DNA samples were digested with Bam HI to excise the 2.2 kbp fragment containing the *legA* gene. This digest would also produce a 2.4 kbp fragment containing the *nos/neo* gene and the chimeric legumin gene *nos* promoter (see Fig. 29) if the EcoRI fragment of pADY3 was inserted intact into the plant genome. More than one insert, if entirely intact, would produce the same bands but more strongly hybridizing due to increased copy number. However, degradation of the transferred DNA termini may result in the loss of Bam HI sites adjacent to the EcoRI sites and in this event larger fragments would be expected of a size depending on the plant genomic Bam HI sites at the particular point of insertion. The blots were hybridized with either the *legA* gene probe, or the *neo* gene probe, previously described (Section 3.8.1.1).

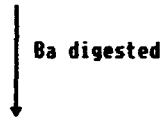
Fig. 30 shows the gel containing Bam HI restriction digests of pADY3 transformed *N. tabacum* transformants and Fig. 31 shows the results of hybridization to a blot of this gel of the *legA* gene probe. There was a very faint band of 1.1 kbp in the SRI 'control' digest, this band did not appear in the other

FIGURE 29.

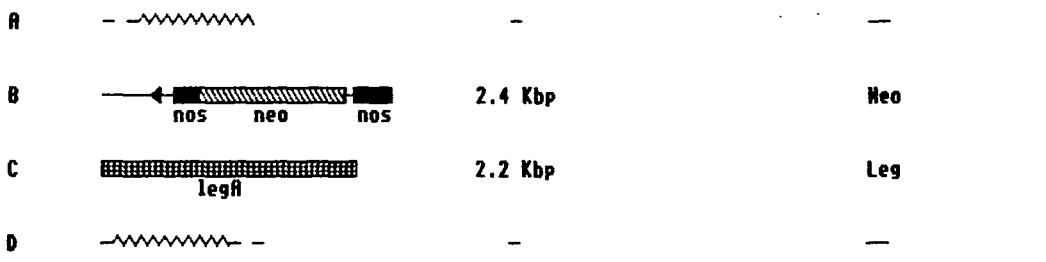
Expected hybridizing fragments in "Southern" blot analyses of 'direct gene transfer' derived *Nicotiana tabacum* transformants.

A single intact pADV3 insert:





Probes expected to hybridize:



Probes:			
neo gene :	"Neo"	1.05 Kbp Pst1/EcoR1 fragment of pNOSNEO	( )
leg gene :	"Leg"	2.22 Kbp BamH1 fragment of pADV1	( )

Key:	
Ba- BamH1	RB - T-DNA right border
Ec- EcoR1	∿∿∿ - plant genomic DNA

Scale:

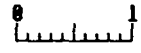


FIGURE 30.

Restriction digests of DNA prepared from leaf tissue of *Nicotiana tabacum* transformants derived by 'direct gene transfer'.

10 µg genomic DNA per track.

Track

a	(untransformed control) SRI	Bam HI	
b	T360	Bam HI	(pNosNeo transformed)
c	T361	"	"
d	T370	"	(pADY3 transformed)
e	T371	"	"
f	T372	"	"
g	T373	"	"
h	T374	"	"
i	T375	"	"
j	T376	"	"

Numbers on the left are size markers (kbp)

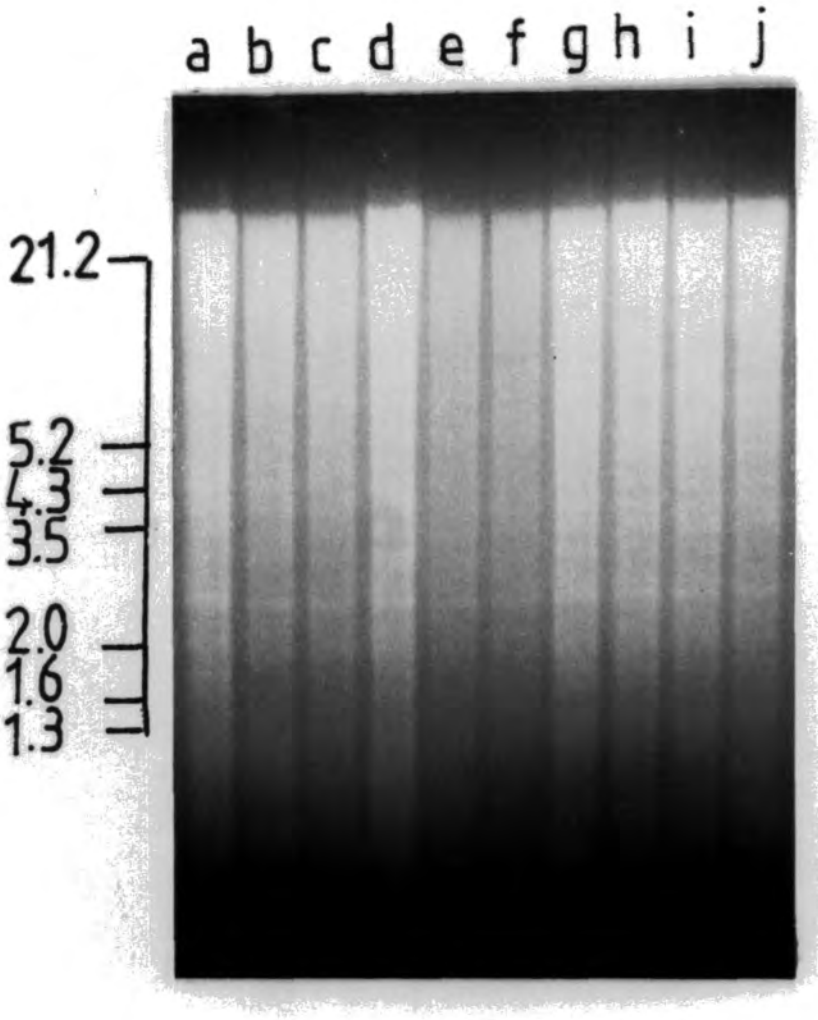

a b c d e f g h i j
21.2
5.2
4.3
3.5
2.0
1.6
1.3

FIGURE 31.

Autoradiograph of a blot of the gel shown in Fig. 30 hybridized to the 2.2 kbp Bam HI fragment of pADY1 (legumin A coding region and 3'-flanking sequences).

Track			
a	(untransformed control) SRI	Bam HI	
b	T360	Bam HI	(pNosNeo transformed)
c	T361	"	"
d	T370	"	(pADY3 transformed)
e	T371	"	"
f	T372	"	"
g	T373	"	"
h	T374	"	"
i	T375	"	"
j	T376	"	"

Numbers on the left are sizes in kbp.

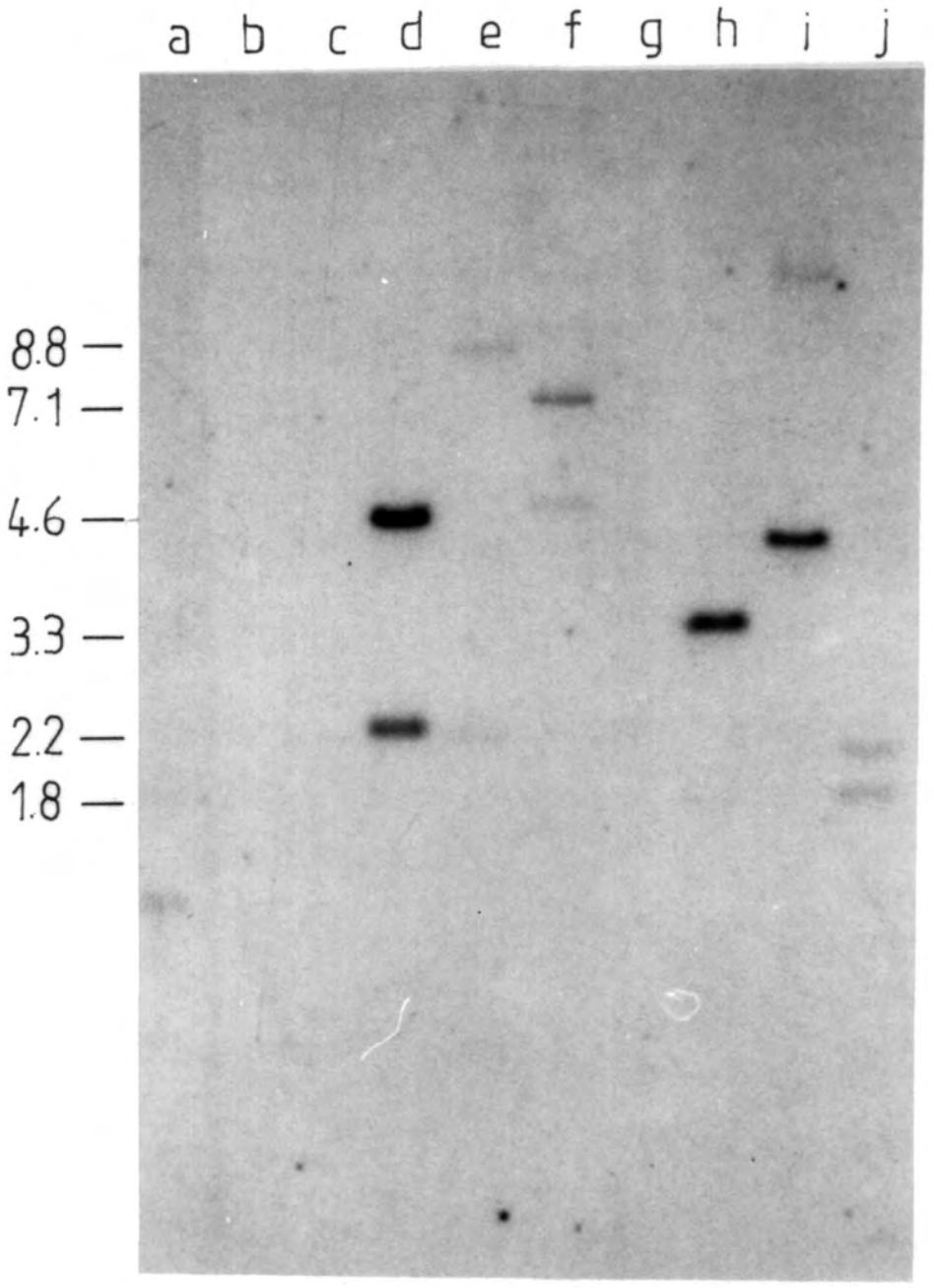
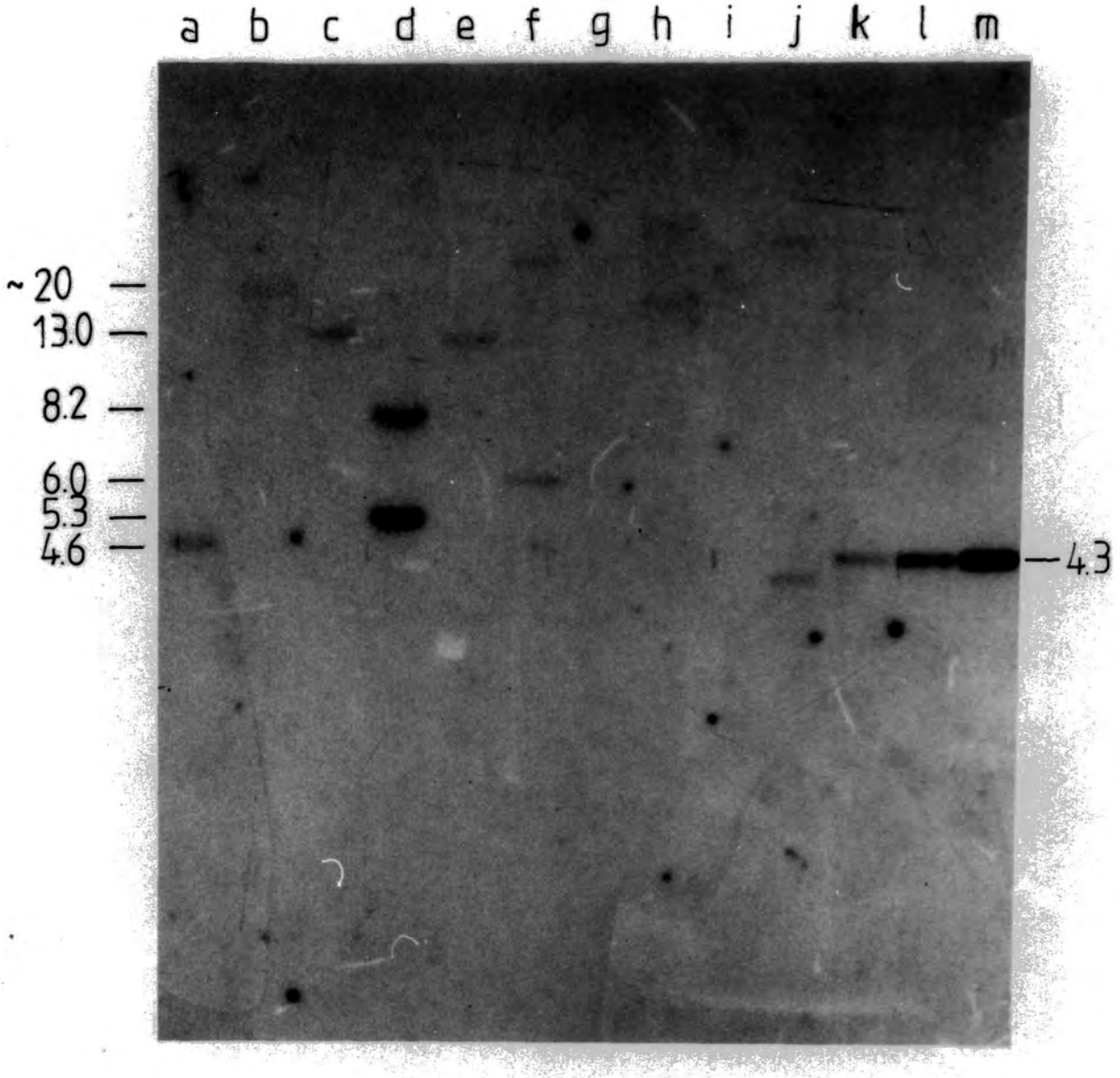

a b c d e f g h i j
8.8
7.1
4.6
3.3
2.2
1.8

FIGURE 32.

Autoradiograph of a blot of a gel containing replica samples of those in Fig. 30 hybridized to the 1.1 kbp Pst I/Eco RI fragment of pNosNeo (*neo* gene).

Track			
a	(untransformed control) SRI	Bam HI	
b	T360	Bam HI	(pNosNeo transformed)
c	T361	"	"
d	T370	"	(pADY3 transformed)
e	T371	"	"
f	T372	"	"
g	T373	"	"
h	T374	"	"
i	T375	"	"
j	T376	"	"
k	1 gene copy equivalent		
l	2 gene copy equivalent		
m	5 gene copy equivalent		

Numbers on the left are sizes in kbp.


a b c d e f g h i j k l m
~ 20
13.0
8.2
6.0
5.3
4.6
4.3

digests. T370 showed strongly hybridized bands of the expected 2.2 kbp with an additional band of 4.6 kbp. T371 showed very faint hybridization to the expected 2.2 kbp, with an additional band of 8.8 kbp. T372 gave a band of 7.1 kbp and a faint band of 4.7 kbp. T374 gave one band of 3.3 kbp. T375 gave a faint band of 1.5 kbp and a stronger band of 4.3 kbp. T376 gave two bands, the expected 2.2 kbp, with an additional band of 1.8 kbp. There was no hybridization to T373, T360 and T361.

Fig. 32 shows the results of hybridization to the *neo* gene probe. None of the bands obtained were of the expected 2.4 kbp, all were larger. The probe hybridized to a 4.6 kbp fragment of the SR1 'control' digest, this band did not appear in any of the other digests. T360 and T361 showed faint bands of appproximately 20 kbp and 13 kbp, respectively. T370 gave a band of 8.2 kbp and a more strongly hybridized band of 5.3 kbp. Faint hybridization was observed to T371 (13 kbp), T372 (approx. 20 kbp and 6.0 kbp), T374 (approx. 20 kbp and 17 kbp) and T376 (4.1 kbp). No hybridization was observed to T373 or T375.

3.8.2. Confirming kanamycin resistance of transformed plant leaves

Leaf tissue of fully mature transformed *Nicotiana* plants was analysed for the presence and expression of the introduced *nos/neo* gene by testing the ability to form callus under kanamycin selection (Section 2.2.22).

Leaf pieces were tested from each of the transformed plants used in this study (T300 to T309 and T370 to T376). All produced callus in the presence of kanamycin, 'control' untransformed *Nicotiana tabacum* SR1 leaf pieces did not (results not presented). This indicated that the inserted kanamycin resistance gene present during plant regeneration was stabily maintained during maturation of the plants and was subsequently capable of expression in the mature leaf tissue.

3.8.3. Transmission of kanamycin resistance to the progeny of transformed plants

Seeds collected from self-pollinated transformed plants derived from both '*Agrobacterium*-infection' and 'direct gene transfer' methods were germinated in the presence of kanamycin (Section 2.2). Table 4 shows the numbers of sensitive and resistant seedlings for each plant tested, together with the expected numbers based on a 3:1 Mendelian segregation ratio.

Table 4. Kanamycin resistance of transformed plant progeny

Parent plant	No. of seeds plated-out	No. of germinated seeds	Sensitive*	Resistant*
SR1	100	85	85	0
T301	92	80	23 (20)	57 (60)
T304	109	102	25 (25)	77 (75)
T305	100	93	25 (23)	68 (69)
T306	101	94	23 (23)	71 (69)
T309	89	82	20 (20)	62 (60)
T371	104	101	19 (25)	82 (75)
T373	102	99	20 (25)	79 (75)
T374	99	91	23 (23)	68 (69)
T375	103	90	25 (23)	65 (69)

*The numbers in brackets are the expected numbers if a 3:1 Mendelian ratio was observed in each case. The results do not differ significantly from the expected ratio at the 5% level (Chi-squared test).

All SR1 seedlings that germinated subsequently became bleached and died. The progeny of all the transformed plants tested showed an approximate 3:1 ratio of resistant to sensitive phenotype (T371 and T373 were close to 4:1, however). One of the plates was photographed. Fig. 33 shows the results obtained with seeds of T306, the other transformed plant seedlings were identical in appearance.

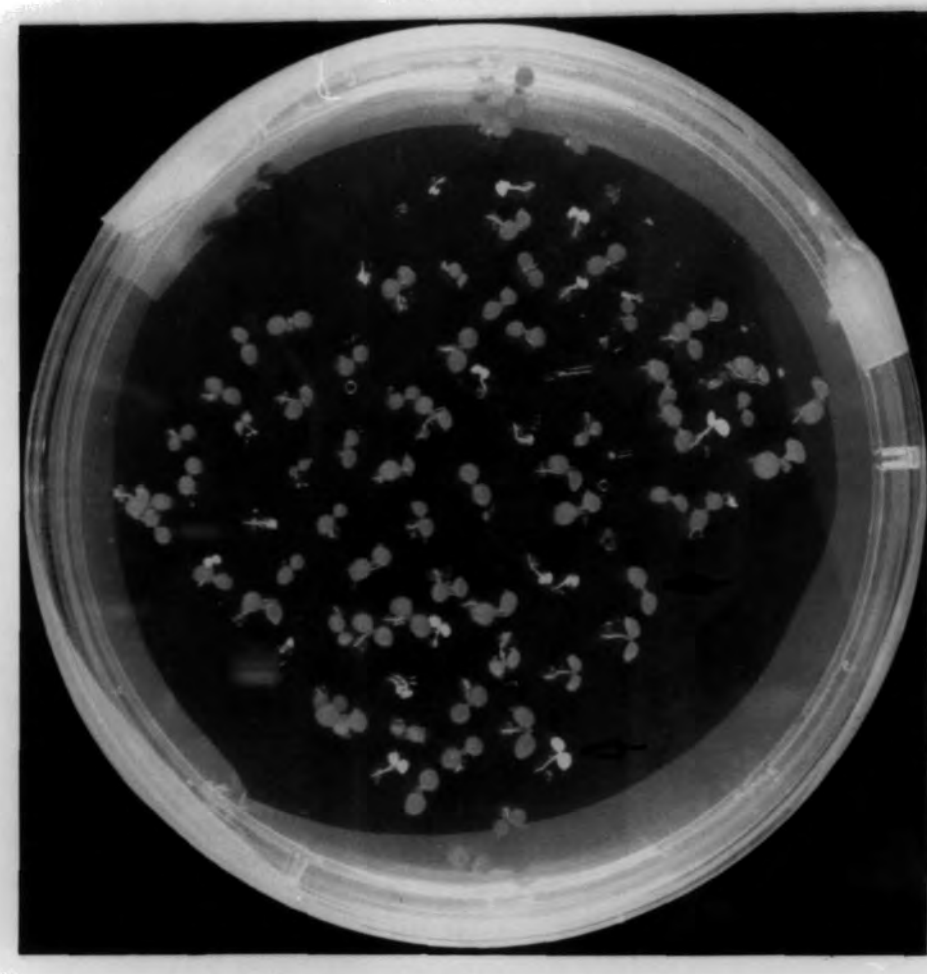
FIGURE 33.

Transmission of kanamycin resistance gene to F1 progeny of *Nicotiana tabacum* transformants.

Photograph of seeds derived from self-fertilized transformant T376, germinated on 1/2 MS plates containing 100 µg/ml kanamycin (14 days after sowing).

Open arrow indicates a kanamycin sensitive seedling.

Closed " " " " resistant seedling.



3.9 'Northern' blot analysis of RNA from transformed plants

Total RNA was prepared from leaf tissue of the seven *Agrobacterium*-transformed *Nicotiana tabacum* plants that showed hybridization to the legumin gene probe in 'Southern' blot analyses (Section 3.8.1.1), together with one untransformed plant to provide a control. 10 µg of each RNA sample were glyoxalated and run on agarose gels (Section 2.2.7.3) with DNA size markers and 0.8 µg of pea cotyledon (15 days after flowering) total RNA. The gels were 'Northern' blotted (Section 2.2.9.2) and the blots hybridized with a ^{32}P-labelled *leg*A gene probe (2.2 kbp Bam HI fragment of pADY1) (Section 2.2.12.2). the results of these hybridizations are given in Fig. 34a and 34b.

A number of the RNA samples were also glyoxyalated, run on an agarose gel and visualized by acridine orange staining to assess the integrity of the RNA in the preparations (Fig. 35). The major RNA species can be seen as distinct bands indicating that there was not extensive non-specific degration of the RNA in the samples used (specific cleavage of chloroplast rRNA was observed).

Non-specific hybridization of the nitrocellulose filters was found despite a high stringency washing procedure. However, the probe hybridized to give a single very faint but distinct band in the tracks of the transformed plants T300, T305, T306, T307 and T309 in Fig. 34a, and T305 and T306 in Fig. 34b, corresponding approximately to the position of the legumin mRNA band of the pea cotyledon sample. In comparison, there is clearly no hybridization to the SR1 untransformed control samples in Fig. 34 a and b. These results indicate the presence of legumin transcripts in the leaf tissue of the above detailed transformed plants.

DNA size markers run on the gel used in Fig. 34a were used to estimate the size of the hybridizing bands obtained (as glyoxalated RNA and DNA molecules of the same size migrate at approximately the same rate in agarose gels (McMaster and

FIGURE 34.

Northern blot of total RNA prepared from leaf tissue of *Nicotiana tabacum* transformants.

A. Autoradiograph of a Northern blot of a gel containing total leaf RNA of *Agrobacterium* transformed plants hybridized to the 2.2 kbp Bam HI fragment of pADY1 (Legumin A gene).

10 µg total RNA per track.

Track

a T309

b T307

c T306

d T305

e T303

f T301

g T300

h SRI

i (space)

j 0.8 µg total RNA
pea cotyledon 15 d.a.f.

k [shorter exposure of track j]

The number on the right indicates the size in kb (derived from DNA size markers run on gel).

B. Repeat Northern blot.

Tracks are as described above.

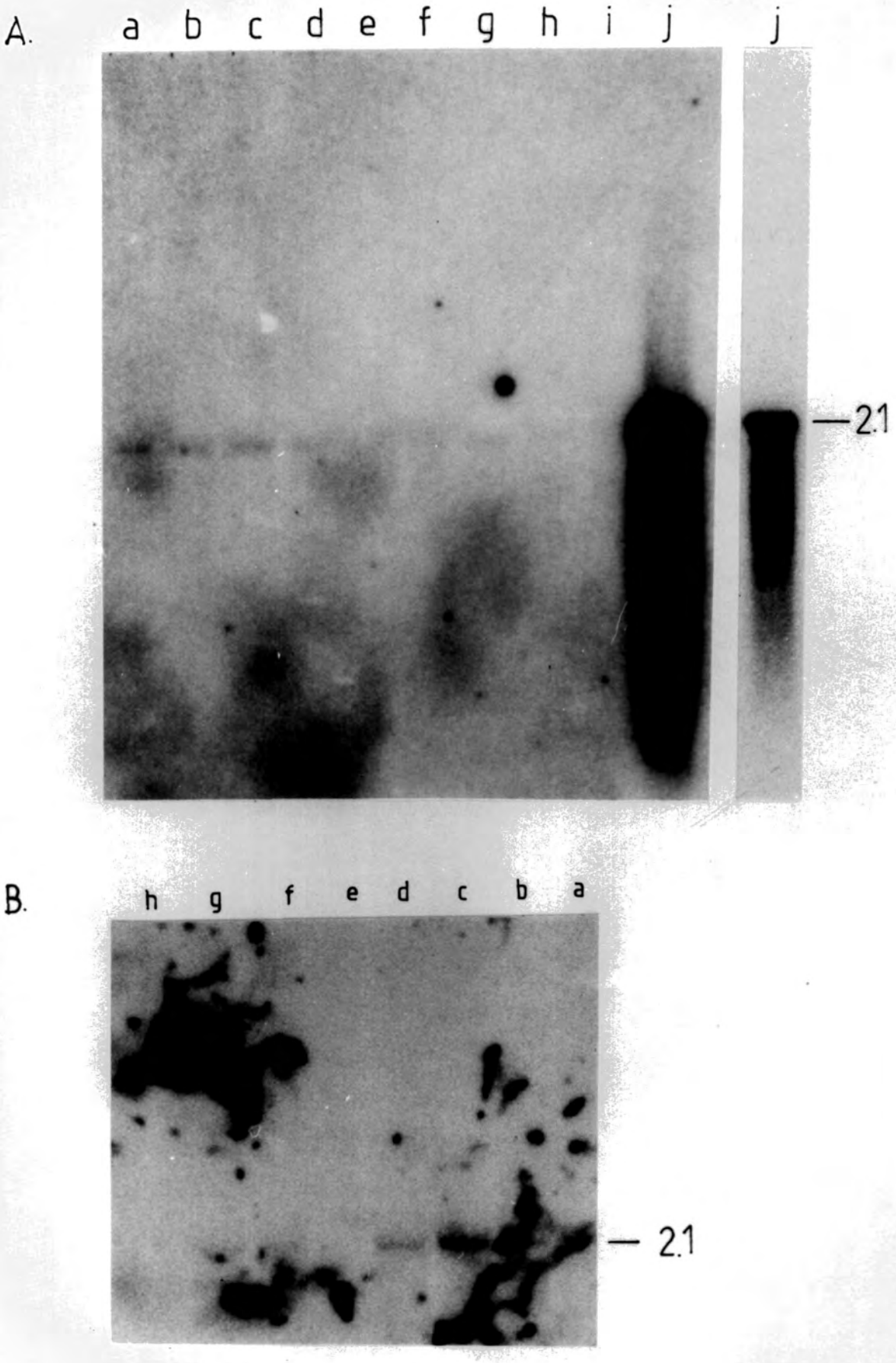

A.
a b c d e f g h i j
j
2.1
B.
h g f e d c b a
2.1

FIGURE 35.

Integrity of total RNA preparation.

Total RNA prepared from *Nicotiana tabacum* leaf tissue, run on a glyoxal gel; 10 µg total RNA per track.

Track

a SRI (untransformed control)

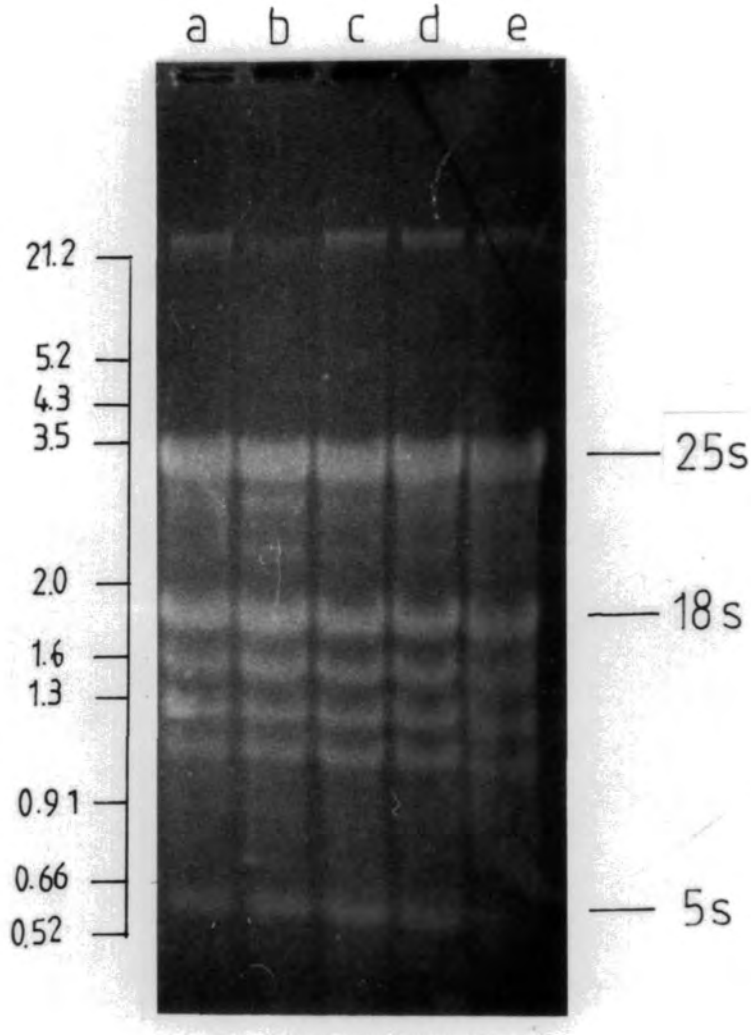
b T300

c T305

d T307

e T309

Numbers on the left are DNA size markers (kbp).


a
b
c
d
e
21.2
5.2
4.3
3.5
2.0
1.6
1.3
0.91
0.66
0.52
25s
18s
5s

Carmichael, 1977), the distance migrated by the hybridizing RNA bands could be compared with those of the DNA size markers). The pea cotyledon legumin mRNA was estimated at ~2000 bases in length and the transformed plant bands were judged to have migrated slightly further in the gel, indicating a smaller size, estimated at ~1850 bases in each case.

3.10 Analysis of Transformed Plant Proteins

3.10.1 SDS-PAGE analysis of transformed *Nicotiana* leaf tissue protein extracts

Crude protein extracts were prepared from leaf tissue of 11 transformed *Nicotiana tabacum* plants; six *Agrobacterium* infection derived plants (those demonstrating the presence of the intact chimeric legumin gene in 'Southern' blotting analyses (Section 3.8.1.1.), five plants derived from the protoplast transformation series and one untransformed 'control' SRI plant, according to the method described in Section 2.2.20.1.

25 µl of each protein sample (75 µg total protein, as estimated by the Bradford method - Section 2.2.20.2) was added to SDS-sample buffer, heat treated, then loaded onto a 17% SDS-PAGE gel (Section 2.2.20.3), together with purified pea legumin protein as a standard (20 µl of a 1 µg/µl solution). After electrophoresis, the gel was stained to reveal the protein bands.

Figure 36 shows the kenacid blue stained gel. Unfortunately, despite loading apparently equivalent total protein amounts per sample according to concentration estimates, there is clearly a difference in the amounts in each sample. However, protein banding patterns are visible in each case and so a comparison of the major protein bands in the transformed and the untransformed control tracks can be made.

The two pea legumin subunits can be seen in the legumin standard track; the upper ~40,000 M_r acidic polypeptide and the lower ~20,000 M_r basic

FIGURE 36.

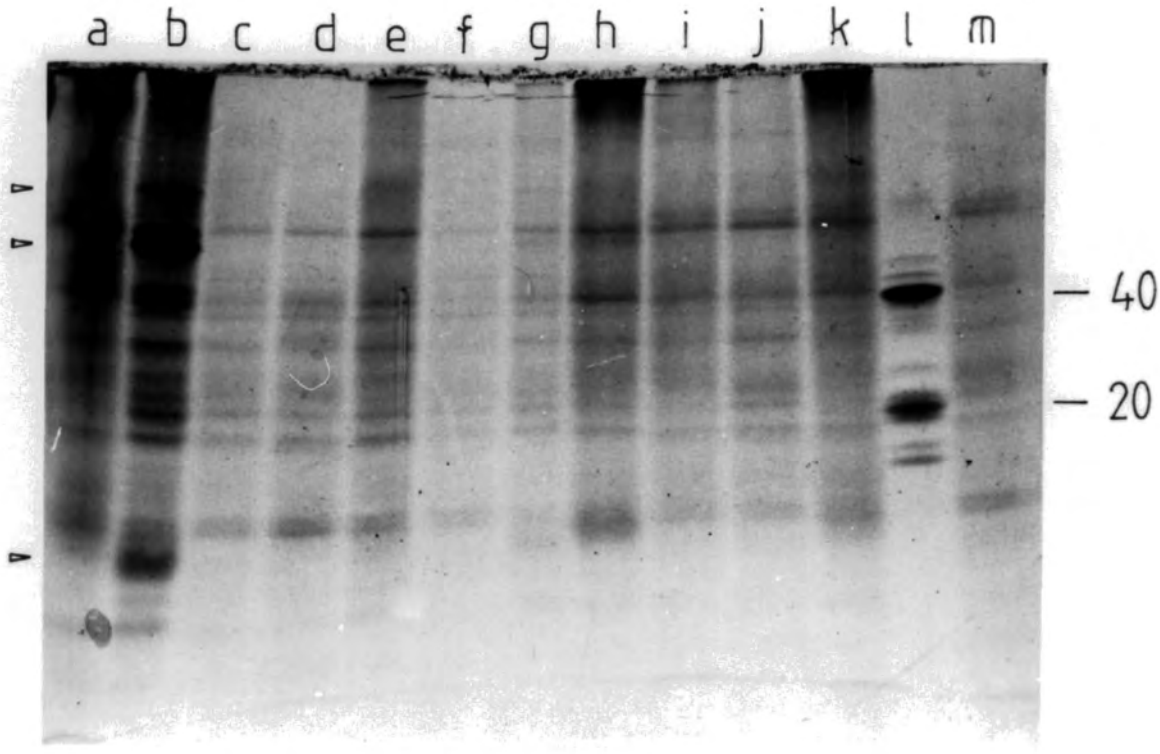
SDS-PAGE analysis of total protein extracts of leaf tissue of *Nicotiana tabacum* transformants.

7 5 µg total protein per track.

Track	
a	T303
b	T300
c	T305
d	T306
e	T307
f	T309
g	T370
h	T371
i	T373
j	T374
k	T375
l	20 µg Legumin A standard
m	SRI (untransformed control)

Numbers on the right indicate size in M_r x 10^{-3} of legumin A subunits.

Arrows on the left refer to bands of track b.


a b c d e f g h i j k l m
40
20

polypeptide (Derbyshire *et al.*, 1976). By comparison with the adjacent untransformed control plant tracks, it can be seen that the presence of these subunits in the transformed plant samples, if in sufficient amount to produce a stained band, would be visible as no major untransformed plant protein bands migrate to these positions.

In fact, none of the eleven transformed plant samples had visible protein bands that co-migrated with the legumin subunit standards. Although many of the samples gave faint banding patterns, it is possible to conclude that in the transformed plants, the introduced legumin gène has not been expressed to the extent of producing levels of legumin protein similar to the naturally occurring major protein bands.

There are however, certain differences between some of the transformed plant protein banding patterns and the untransformed control. The most striking of these is the two additional bands in T300 (track 2). In this sample, there is a heavily stained high molecular weight band (in fact, the most heavily stained band of the sample) and a low molecular weight band in positions where there are no visible bands in the untransformed control.

In addition, many of the transformed plant samples, namely T370, T371, T300, T305, T306, T307 and T309, appear to have lost one of the high molecular weight bands (see Fig. 36). This band is clearly visible in the SRI control track and T373, T374 and T375 tracks.

3.10.2 Immunodetection of legumin in transformed plant protein samples

Total protein samples prepared from leaf tissue of transformed *Nicotiana tabacum* plants together with that of an untransformed control plant and pea legumin protein standards, were electrophoresed on a polyacrtylamide gel under reducing conditions exactly as described in the previous Section. After

electrophoresis the separated proteins were transferred to a nitrocellulose filter by electroblotting (see Section 2.2.20.4) and the presence of legumin protein in the transformed plant sample was subsequently examined by immunodetection using affinity purified, polyclonal antibodies raised against pea legumin (Section 2.2.20.5).

The autoradiograph of the 'Western' blot showed the two pea legumin subunits of 40,000 m_r and 20,000 M_r as distinct bands; however, no bands were visible in any of the protein sample tracks despite prolonged exposure of the autoradiograph (~8 weeks). This result was obtained in two repeat blots (results not presented). Transfer of the proteins to the nitrocellulose was complete as the gels were stained with kenacid blue after the electroblotting procedure and were found not to contain protein bands, in contrast to replica gels run at the same time, and not blotted, that gave protein bands after staining.

3.10.3 Detection of legumin by ELISA

The presence of pea legumin in the leaf and seed tissues of transformants T300 to T309, T370, T371, T373 to T375 and T361, was assayed using the ELISA immunodetection technique (Section 2.2.20.6). T361, derived from pNosNeo transformed protoplasts, was used as it provided a pea legumin$^-$ control.

The transformed tissue samples were analysed together with pea legumin standards to provide a calibration curve. The legumin standards were stepwise dilutions of 100 ng/ml (50, 25 etc. to 0.75). It was found in the ELISA assays that there was no significant difference between the OD of the standards at concentrations less than 12.5 ng/ml. So using this method it was not possible to detect less than ~2 ng legumin protein per well.

With regard to the detection of pea legumin in the transformed plant tissues, both the leaf and seed samples of each of the transformed plants gave OD readings that were not significantly higher than that of the T361 legumin$^-$ control,

and furthermore, these OD results gave legumin concentration values in the <12.5 ng/ml range (results not presented). The ELISA assays were performed three times in total (with 4 replicates of each transformant sample per assay) and this result was found on each occasion. It was therefore concluded that if legumin protein was present in the transgenic plant tissues of the leaves and/or the seeds, it was not in amounts sufficient to be detected with the ELISA protocol employed.

CHAPTER 4.

DISCUSSION.

Discussion

4.1 Pollen Transformation

4.1.1 *Agrobacterium*-mediated transformation of pollen tubes

The first stage of the infection process of *Agrobacterium tumefaciens* is the attachment of the bacterium to the plant cell surface, it has been shown that this attachment is a pre-requisite for the formation of the crown gall tumour (Lippincott and Lippincott, 1969; Glogowski and Galsky, 1978). The possibility of *Agrobacterium* infection of germinating pollen tubes as a method of plant transformation was therefore initially investigated by assessing the ability of *Agrobacterium* to become attached to the pollen tube surface. The results of co-culturing germinating pollen tubes of both a dicotyledonous and a monocotyledonous species with *Agrobacterium* are given in Section 3.1.1. Bacterial attachment was demonstrated to the pollen tubes of both species studied and a feature of the attachment was the formation of large aggregates of bacteria, particularly in the region of the pollen tube tip. These aggregates persisted despite thorough washing procedures, demonstrating that the bacteria were firmly bound to the pollen tube wall.

Bacterial aggregation has previously been observed in studies of *Agrobacterium* infection (Ohyama *et al.*, 1979; Matthysse *et al.*, 1981; Pueppke, 1984). Matthysse *et al* (1981) investigated the aggregates formed in the infection of tissue-cultured carrot cells and reported that the bacteria were held together by a network of cellulose fibrils. Further studies showed that these cellulose fibrils were produced by the bacteria in response to attachment to the plant cell wall as bacterial mutants unable to produce cellulose were found attached individually but not clumped together (Matthysse, 1983). These mutant bacteria were still virulent however, indicating that the aggregation was not essential for the transformation process. It was further shown that attached cellulose mutants could be washed from the plant cell surface, whereas wild-type cellulose fibril producing bacteria

could not (Matthysse, 1983), suggesting that these fibrils play a major role in the anchorage of the bacteria to the plant cell. The aggregates are formed as other bacteria become entrapped in the fibril network and bacterial multiplication occurs within the aggregate. This is clearly an advantage to the infection process as many *Agrobacterium* cells are clustered on the plant cell surface, increasing the possibility of infection in that region.

The attachment and formation of aggregates on pollen tubes was observed for both the dicot *Vicia* and the monocot *Hippeastrum.* This finding is of interest as some studies have suggested that *Agrobacterium* cannot attach to the cell walls of monocots (Lippincott and Lippincott, 1978). The attachment of *Agrobacterium* to the plant cell surface is thought to occur at specific "attachment sites" on the plant cell wall (Lippincott and Lippincott, 1969). These sites were postulated when it was found that the prior innoculation of Pinto bean leaves with an avirulent strain of *Agrobacterium* prevented infection by a subsequently inoculated virulent strain. Studies involving incubation of a virulent *Agrobacterium* strain with preparations of cell walls of dicot and monocot species followed by a quantitative assessment of infectivity of the treated cultures by inoculation of Pinto bean leaves (Lippincott and Heberlein, 1965) showed that the presence of the cell walls of the dicots, pear, tobacco and tomato, in the *Agrobacterium* culture inhibited subsequent infection by the bacteria, whereas such inhibition did not occur after incubation with monocot cell walls of barley, corn and Asparagus (Lippincott and Lippincott, 1978). It was suggested that this inhibition arose due to the adherence of the *Agrobacterium* to the specific attachment sites on the cell walls of the dicot species, but that attachment was not possible to the monocot species due to the absence of such sites. However, other studies have shown attachment to the cell walls of certain monocot species, for example *Zea mays* and *Triticum monococcum* (Ohyama *et al.*, 1979), *Asparagus officinalis* (Draper *et al.*, 1983), and *Narcissus* cv. 'paperwhite' (Hooykaas-

van Slogteren *et al.*, 1984). Interestingly, Ohyama *et al.* (1979) observed attachment to specific areas of the cell walls of both dicot and monocot species, but that there were fewer sites on monocot cell walls.

The difference in attachment sites between monocots and dicots is perhaps due to differences in their cell wall constituents. The binding is thought to result from an interaction of the lipopolysaccharide component of the *Agrobacterium* surface (Whatley *et al.*, 1976; Banerjee *et al.*, 1981) with a component of the plant cell wall, polygalacturonic acid, a component of dicot cell walls has been implicated (Lippincott and Lippincott, 1980), although contrary evidence to both components has been presented (Matthysse *et al.*, 1982; Pueppke and Berry, 1983). The observed attachment to the pollen tubes of *Vicia* and *Hippeastrum* suggest that their cell walls contain the necessary components.

The first step in the infection process of *Agrobacterium*, namely the binding of the bacteria to the plant cell wall, has been demonstrated with pollen tubes. This attachment is mediated by two bacterial chromosome genes (*chv* A and *chv* B), which are constitutively expressed (Douglas *et al.*, 1985). However, the next step in the infection process, the transfer of T-DNA to the plant cell, is mediated by the regulated genes of the virulence *vir* region of the Ti-plasmid (Stachel and Nester, 1986; Stachel and Zambryski, 1986). The expression of the six *vir* genes is induced by certain chemicals produced by wounded or actively growing plant cells and tissues (Stachel *et al.*, 1985, 1986; Bolton *et al.*, 1986). For example, acetosyringone has been identified as such a signal molecule in *Nicotiana tabacum* (Stachel *et al.*, 1985). This and related phenolic compounds that induce gene expression, are involved in biochemical pathways that produce components of the plant cell walls, e.g. lignin (Grisebach, 1981).

Do pollen tubes produce the necessary signals for *vir* gene induction? Growing pollen tubes are metabolically active and continually synthesize a cell

wall at the growing tip. It is likely therefore, that the chemical signals for *vir* induction are produced by the developing pollen tubes of dicot species, as they are for example by cell wall regenerating protoplasts of *Nicotiana tabacum* (Marton *et al.*, 1979; Krens *et al.*, 1985). There are conflicting reports about the presence of *vir* inducing signals in monocot species. Usami *et al.* (1987) were unable to detect *vir* gene expression when seedlings of a number of monocot species were co-cultivated with *Agrobacterium*, whereas such induction occurred with dicot seedlings, although more recently, it has been reported that wheat and oat contain substances that induce *vir* gene expression (Usami *et al.*, 1988). The substances were extracted from seed tissues and the root/shoot transition region, but were not the same as the phenolic inducers found in dicots. Hooykaas-van Slogteren *et al.* (1984) have reported the presence of T-DNA encoded octopines in wound sites of *Narcissus* and Hernalsteens *et al.* (1984) demonstrated the formation of nopaline producing tumours on *Agrobacterium* infected stems of *Asparagus officinalis*, indicating the successful activation of the *vir* region in monocots. (Although the opines assayed were absent in the untreated control tissue of the above examples, it must be noted that some non-transformed plants have the ability to synthesize opines (Christou *et al.*, 1986).)

So it is possible that the cell wall synthesizing pollen tubes of monocot, as well as dicot, species could induce *vir* gene expression and so initiate the T-DNA transfer process. Interestingly, *Hippeastrum*, the monocot used in this study, belongs to the amaryllidaceae, the same family as *Narcissus* and so may indeed be capable of *vir* gene induction.

If the pollen tubes do excrete *vir* gene inducing chemicals, then it is likely these would be present in the highest concentrations at the pollen tube tip, the region of cell wall synthesis. In view of recent reports that *Agrobacterium* move up an Acetosynringone concentration gradient (Ashby *et al.*, 1987; Parke *et al.*, 1987),

it is interesting to speculate that the high concentration of *Agrobacterium* found in the tip region in this study, is a result of such an 'attraction' to this area.

An attempt at investigating *vir* gene induction during incubation of *Agrobacterium* with pollen tubes was made by germinating *Nicotiana alata* pollen in the presence of *Agrobacterium* harbouring the Ti-plasmid Bin 6 (Bevan, 1984), after which the pollen tubes were preferentially lysed and the pollen tube debris and bacteria were pelleted by centrifugation. The supernatant containing the pollen tube extract, was taken and the DNA isolated, restricted with Hind III and analysed on agarose gels alongside control treatments (*Agrobacterium* culture medium without pollen tubes similarly treated). 'Southern' blots of the gels were probed with a radiolabelled T-DNA right border fragment ('Hind III 23'), a fragment that would be transferred during transformation. Despite confirmation that the lysis treatment disrupted the pollen tubes (visual microscopic examination) but not the bacteria (as judged by colony counts of bacterial platings before and after lysis treatment), there was hybridization to the pBin 6 fragment in the control tracks comparable with that of the pollen co-cultivation treatments. Therefore, it was not possible to confirm that T-DNA transfer had taken place by these methods.

If pollen tubes produce *vir* inducing factors or indeed if such factors can be added to the incubation medium to promote the T-DNA transfer to the pollen tube, as has been demonstrated with the transformation of the monocotyledonous *Dioscorea bulbifera* with Agrobacteria preincubated with dicotyledonous wound substances (Schafer *et al.*, 1987), then the system can be considered as a possible method of plant transformation; pollination performed with pollen tubes that had been co-cultured with *Agrobacterium* could result in the transfer of T-DNA to embryos via the pollen tube on fertilization.

The incorporation of T-DNA in the genome of progeny plants would be dependent on the T-DNA becoming integrated into the genomic DNA either of the pollen or the fertilized egg cell. A pollen grain has two nucleii, the 'generative' and the 'vegetative'; however, the 'generative' nucleus divides mitotically to give two male gametes, the sperm nucleii. In some plant species, e.g. the Gramineae, this division takes place before pollen germination, whereas in others it divides in the pollen tube. On fertilization, one male gamete fuses with the egg cell to form the zygote which develops into the embryo, the second male gamete fuses with the two polar nucleii to form the endosperm (Fahn, 1974). Clearly then, if the T-DNA becomes integrated into the DNA of vegetative nucleus or the sperm nucleus that fuses to form the endosperm, the embryo will not have been transformed; only integration into the DNA of the generative nucleus before it divides or if division has taken place, the sperm nucleus that fuses with the egg cell can produce a transformed embryo.

Integration need not necessarily take place before fertilization. It is possible that T-DNA introduced into the pollen tube cytoplasm does not become integrated but is transferred to the embryo sac on fertilization and only then becomes integrated into the genome of the zygote.

Whatever the route of T-DNA integration, it seems feasible and of great value to use this method to obtain transformed plants. Hess (1987) has investigated this possibility in experiments involving the co-culture of *Agrobacterium* with pollen tubes of *Petunia hybrida*. The mature seeds of plants pollinated with pollen tubes co-cultured with *Agrobacterium tumefaciens* strain C58, were germinated and the young plantlets obtained were wounded to elicit callus formation. The callus formed was cultured on hormone-free media. In contrast to the controls, some of the progeny derived from the co-cultured pollen resulted in callus that exhibited nopaline synthase activity and could be repeatedly sub-cultured on the hormone-

free media. Similar co-culture experiments were performed with an *Agrobacterium* strain containing a neomycin phosphotransferase gene within the T-DNA borders, conferring resistance to kanamycin. Resulting seeds were germinated on media containing kanamycin. It was reported that a number of the co-culture derived progeny grew better than the controls as judged by the appearance of the primary leaves (Hess, 1987). Clearly then, there is no genetic proof that any transformation event has taken place in these experiments; however, these preliminary experiments are sufficiently promising to warrant further investigation of the callus material and seedlings obtained (e.g. 'Southern' blotting analyses of the putative transformants) and to encourage further research in this method of transformation.

4.1.2 Direct Uptake of DNA by Pollen Tubes

Agrobacterium-mediated transformation of plants via the pollen tube relies upon the ability of the bacteria to successfully infect the pollen tube; however, another potential method of pollen transformation does not depend on this specific bacterial/plant cell interaction, namely the direct uptake of 'naked' DNA molecules of germinating pollen tubes.

The transformation of bacteria by direct uptake of exogenous DNA is a well known phenomenon and is routinely used in genetic research (e.g. in plasmid cloning); bacterial cells incubated with calcium ions and after a heat-shock treatment become competent to take up DNA molecules (Daghert and Ehrlich, 1979). Yeast cells can also be transformed by naked DNA, for example after polyethyleneglycol treatment (Hinnen *et al.*, 1978) and mammalian cells in culture are also capable of direct uptake of naked DNA (Graham and van der Eb, 1973; Pellicer *et al.*, 1980). The barrier to such uptake in plant cells is their cell walls; this barrier can be removed by enzymatic digestion to produce protoplasts and

successful direct uptake of exogenous DNA has been demonstrated with such protoplasts and is a very useful technique, as will be discussed later (see Section 4.5). However, the pollen tube itself may have the potential for DNA uptake due to the nature of the cell wall in certain areas.

It is believed that there is a "pore" near the pollen tube tip at germination that lacks a cell wall (Picton and Steer, 1982). The extending pollen tube also has a "growth zone" at the very tip where new cell membrane and wall are being formed. This zone, the tip-most 4 μm in *Nicotiana alata* (Cresti *et al.*, 1985) and Easter Lilly (Rosen, 1971), has only a very thin pectocellulosic cell wall (Cresti *et al.*, 1985) and at the very tip where the wall is forming, may in places be momentarily incomplete.

To demonstrate the uptake of DNA by pollen tubes, pollen grains of *Vicia faba* were germinated in a medium containing radiolabelled plasmid DNA (Section 3.1.2). The results obtained were consistent with the presence of radiolabelled DNA within the pollen tubes. The microautoradiographs showed that the labelled DNA was associated with the pollen tubes and concentrated in particular areas, notably in the tip region. The possibility that the DNA molecules were merely adsorbed to the pollen tube wall was recognised and the experimental procedure was designed to include a second incubation or "cold-chase" (Section 2.2.15.3) to allow the pollen tubes to grow in the absence of label. The region formed during the "cold-chase" incubation would be expected to show virtually no label if the DNA was adsorbed to the pollen tube wall. This pattern was not found; the presence of the labelled DNA in the pollen tube tip region suggested that the DNA had been taken-up during the first incubation and was thus present in the cytoplasm of the pollen tube formed during the second incubation. The apparent concentration of label at the pollen tube tip suggested the accumulation of introduced molecules at the tip as the tube extended. (The possibility that the

pollen tubes did not extend significantly during the second period of incubation was discounted as *Vicia* pollen, being binucleate, would be expected to grow *in vitro* for up to 5 hr (Mulcahy and Mulcahy, 1985), each incubation was for $1^1/_2$ hr.)

Similar uptake results were obtained by Hess *et al.* (1974) using pollen of *Nicotiana glauca* after incubation with radiolabelled bacterial DNA and microautoradiography. In their experimental protocol, the pollen tubes were DNase treated to remove superficially adhered DNA.

It is possible that the labelled DNA was digested by nuclease present in the medium (it is known that the pollen tubes of some species produce nucleases (Matousek and Tupy, 1983; van der Westhuizen, 1987)) and that it was uptake of single labelled nucleotides that was observed. This was thought unlikely, for example Hess *et al.* (1987) were able to recover intact plasmid DNA after incubation with *Nicotiana glauca* pollen tubes.

These results suggest then that direct uptake of DNA by pollen tubes is possible; however, more convincing evidence would, of course, be the demonstration of genetic and phenotypic transformation by this method. Dieter Hess has recently reviewed the work on pollen-mediated plant transformation (Hess, 1987) and describes a number of experiments involving the pollination of plants with exogenous DNA treated pollen.

Anthocyanin synthesis in *Petunia hybrida*, showing phenotypically as a red pigmentation of the flower, was shown to be partially corrected (after self-pollination) with pollen germinated in medium containing 'wild-type' red-flowered *Petunia* DNA (Hess, 1980). this partial correction of the mutation was observed as an accumulation of the red pigment around the corollar tube in 0.09% of the derived plants (full red coloration of the flower was not obtained). Control plants, derived from pollen treated with homologous 'mutant' DNA, did not develop such anthocyanin production. Furthermore, the plants were shown to be heterozygous

for the introduced anthocyanin gene by Mendelian segregation of the acquired trait to the next generation (Hess, 1980). Interspecific transfer was also demonstrated when in similar exogenous DNA treatment and pollination experiments, *Petunia* anthocyanin synthesis was partially restored in some progeny plants derived from the mutant line self-pollinated with pollen treated with DNA of an anthocyanin producing line of *Nemesia strumosa* (Hess, 1980).

De Wett *et al.* (1985) working with maize (*Zea mays*) demonstrated the transfer of genes governing cob colouration and resistance to the disease common rust, after self-pollination of a white cobbed, rust susceptible maize line with pollen germinated with total DNA of a red cobbed, rust resistant line. 1.1% of the seedlings obtained were red cobbed, and 2.7% of these were also rust resistant, 0.03% of the seedlings were rust resistant. All transformed plants grew more vigorously than the controls. Red cob and rust resistance was not found in any of the control pollination progeny, including those derived from homologous DNA treated pollen. Additionally, a maize line was self-pollinated with pollen treated with the total DNA of *Tripsicum dactyloides* and some of the progeny plants obtained exhibited the phenotypic characteristics of a cytogenetically produced hybrid of the two species (De Wett, 1985).

Ohta (1986) also demonstrated genetic transformation of maize using this pollen/DNA treatment. High-efficiency transformation of the endosperm was found (up to 9.29% per ear when pollen grains were mixed with DNA and immediately applied to the silks), observing phenotypic markers such as aleurone colour and waxy or non-waxy starch. The cobs obtained from treated plants showed combination of these phenotypes different from either the selfed donor or recipient or the naturally produced F1 hybrid, although there was no control experiment of pollen incubated with homologous DNA. Kernels from cobs exhibiting a transformed endosperm phenotype were harvested, grown-up, test-

crossed with the recipient line and the kernels of this next generation were examined. The appearance of transformed endosperm in this generation indicated the transformation of some of the original embryos, although at a much lower frequency (Ohta, 1986). This work demonstrates that exogenous DNA transferred to the embryo sac by pollen can result in the transformation of the embryo and/or endosperm following double-fertilization and that it is possible for genes introduced into the embryo to the stably maintained and expressed in the next generation.

It is also worthy of note that in many of the control experiments, the treatment of pollen with 'homologous' DNA resulted in some cases in progeny developing aberrent characteristics or morphology. For example, smaller mature maize plants than normal were obtained by De Wett (1985). This indicates the possibility of unspecific DNA effects in experimental progeny plants.

There have thus been a number of reports of the transfer of genes by the treatment of germinating pollen with exogenous DNA, implying the uptake of DNA by the pollen grain or developing pollen tube. However, in these examples it is the phenotypic trait that has been studied; it may be possible that the appearance of a certain characteristic in experimental progeny is not due to the exogenous DNA. For example, it has been suggested by Sanford *et al.* (1985) that the red pigmentation in the "transformed" *Petunia* of Hess (1980) could be due to a naturally occurring "red blush phenomenon", although this possibility is discounted by Hess (1987). Clearly then, molecular genetic evidence, such as DNA analysis of putative "transformed" progeny by 'Southern' blotting would provide more direct evidence for transfer of genetic material by this method. Such analyses would also indicate the nature of the incorporated DNA, for example the size of fragments inserted or whether the insertion is by homologous recombination with the genome

or random insertion at non-homologous sites. This would require, of course, the study of known genes and the existence of suitable gene probes.

Evidence supporting the uptake of exogenous DNA by pollen tubes has been presented; it was found that the results of the incubation of *Vicia* pollen tubes in raidolabelled plasmid DNA were consistent with the uptake of the DNA. The reported transfer of phenotypic characteristics to progeny plants following pollination with exogenous DNA treated pollen, provides further evidence for such direct uptake. However, further study of this technique as a method of plant transformation was not carried out in favour of the investigation of a technique based on the application of DNA to pollen tubes *in vivo* by the introduction of DNA into pollinated ovaries. Such a method was judged to be more likely to yield transformants due to the advantages of the technique discussed in the following seciton.

4.2 Transformation by Ovary Microinjection

In the previous discussion of pollen tube uptake of exogenous DNA, experiments were described in which the DNA was applied to the pollen tube *in vitro*, that is, the pollen was germinated in a solution of the donor DNA before application to the recipient plant. However, another possible method of exogenous DNA uptake is the exposure of the growing pollen tubes to DNA solutions *in vivo*, by the introduction of the DNA into the ovary itself following pollination. Microinjection offers an excellent method of such introduction as the DNA can be delivered accurately to the required compartments with minimal disruption to the ovary. The injection procedure was used to introduce DNA directly into the locular cavities of the ovary.

A major advantage of this intra-ovarian injection of foreign DNA technique over *in vitro* exogenous DNA treatment of pollen tubes is that the natural

process of pollination is utilized, the germination and manipulation of fragile pollen tubes and application of pollen/DNA mixtures to the style is avoided. Germination efficiency and pollen tube growth is often reduced *in vitro* in comparison to the *in vivo* situation (Mulcahy and Mulcahy, 1985). The use of pre-germinated pollen for pollination often results in poorer seed set. For example, De Wett *et al.* (1985) reported that in *Zea mays* no seed set was observed in 50% of the ears pollinated with pre-germinated pollen, and those that did produce seeds, produced under 10% of the number observed following normal self-pollination. More efficient seed set in transformation experiments allows for the production of greater numbers of progeny plants per treatment, thereby increasing the numbers available for screening.

The uptake of exogenous DNA by the pollen tube has been mentioned as a route for transformation following introduction of DNA into the ovary. However, the egg cell, zygote and early embryonic cells are also potential targets for DNA uptake as they lack cell walls. These cells are active in DNA replication and recombination. It seems feasible that 'foreign' DNA if present in the egg cell would similarly be subject to the naturally occurring replication and recombination processes so, whether the foreign DNA is introduced to the egg cell through direct uptake by the egg cell itself or through delivery by the pollen tube on fusion with the egg cell or indeed through direct uptake by the zygote or early embryonic cells, the introduction of DNA into the ovary and its consequent availability for uptake seems to offer potential as a method for plant transformation.

Evidence supporting the feasibility of this technique has been reported by Zhou *et al.* (1983; 1985). They demonstrated phenotypic alterations in the progeny of cotton plants following microinjection of donor DNA into the ovary placenta 24 hr after self-pollination. The progeny acquired certain donor characteristics, for

example anther colouration, whereas control injections with homologous DNA or buffer produced no phenotypic alterations.

In this study, the possibility of such uptake of exogenous DNA by either the male or female gametophyte or embryo *in situ*, was investigated by the microinjection of the non-oncogenic Ti-plasmid pGV 3850 into the locular cavities of ovaries of *Salpiglossis sinuata*. Prior to microinjection, the ovaries to be used were checked by microscopic examination of the stigma to ensure that no previous pollination had occurred. Earlier pollination may have resulted in seed set before the introduction of the DNA and hence reduce the likelihood of transformants being obtained. The ovaries were then self-pollinated. It is known that after germination on the stigma, the pollen tubes grow down the central pollen-transmitting tissue of the style at a constant rate with the majority of tubes reaching the base of the style ~16 hr after germination (Hepher and Boulter, 1987). Performing the microinjections at 15 hr to 18 hr post-pollination ensured that the DNA was only introduced into the ovary during or immediately preceeding the emergence of the pollen tubes into the loculus. This was important in order to minimize any nuclease degradation of the DNA in the locular cavity prior to the presence of the pollen tubes and to maximize the period of interaction of DNA with the pollen tubes.

The pollen tubes emerge from the style into the loculus via a small channel at the neck of the placenta and grow onto the placental surface amongst the ovules (see Fig. 4a). Fertilization involves the tubes growing away from the placenta wall and entering the micropyle of the ovule (Hepher and Boulter, 1987). An important consideration was the distribution of the DNA once microinjected into the ovary as the method relied upon the pollen tubes growing in the presence of the introduced DNA. It was shown by injection of dye (see Fig. 4b) and subsequent visual analysis of sections, that injected material was distributed about the locular cavity

and was present on the ovules and placenta wall (Hepher *et al.*, 1985). It was concluded therefore, that introduced DNA would behave similarly and thus come into direct contact with the pollen tubes on the placenta wall and, of course, the ovules themselves.

In similar experiments, it was found that seed set was not affected by the injection of dye (results not presented) demonstrating that the efficiency of fertilization was not adversely affected by the introduction of injectant into the ovary in this way.

4.2.1 Persistence of exogenous DNA microinjected into the ovary loculus

Preliminary experiments were performed in order to assess the degree of degradation of DNA molecules introduced into the locular cavity of *Salpiglossis* ovaries; clearly, significant nuclease activity in the ovary would rapidly degrade any introduced foreign DNA and hence reduce the chances of uptake by the pollen tubes of DNA fragments large enough to contain functional genes.

The nuclease activity in the ovary loculi was assessed by microinjection of DNA into the ovary and subsequent isolation of DNA after specific incubation time periods. The integrity of the isolated DNA was determined by 'Southern' blot hybridization analyses. The isolation method used in these analyses was judged to be reproducibly efficient as the amounts of plasmid DNA extracted from microinjected ovaries were equivalent to the amount introduced in 78% of ovaries tested (Section 3.2.2).

The plasmid pBR 322 was microinjected into the loculus of a number of ovaries to provide a time course of incubation periods. After isolation of the DNA and 'Southern' hybridization analysis (Section 3.2.3) it was found that a small proportion of the introduced plasmid DNA remained in the ovary for up to 24 hr. Although nuclease activity was shown to be present as judged by the increase in

the relative amounts of open circular and linear form with time, undegraded DNA remained present for many hours. For example, after three hours in the ovary loculus, the introduced DNA was demonstrated to be present in significant amounts as open circular and linear molecules and these linear forms were predominantly undegraded. The closed covalent circular form was also shown to be present for up to 2 hr.

These initial results demonstrated the possibility of prolonged incubation of the pollen tubes in the presence of significnt amounts of undegraded DNA in the ovary loculus. However, the pBR 322 plasmid used is 4.3 kbp in size, relatively small compared to the Ti-plasmid that was to be used in the ovary transformation experiments. The persistence of the ~200 kbp pGV 3850 plasmid was therefore examined in a similar way, in order to confirm that the larger plasmid was likewise not significantly degraded.

The integrity of the pGV 3850 plasmid after isolation from *Agrobacterium* was first confirmed in order that any degradation of the plasmid molecules in the DNA preparation used could be discounted in the analysis of the post-ovary microinjection results. Clear evidence was produced by 'Southern' hybridization that the 12.6 kbp Eco RI 'fragment 10' and the 3.2 kbp Hind III 'fragment 23' (Depicker *et al.*, 1980) of pGV 3850 were present in an undegraded form (Section 3.2.1), indicating that the plasmid preparation was suitable for use in this work.

Analysis of the DNA isolated from pGV 3850 microinjected ovaries by Eco RI digestion followed by 'Southern' hybridization to a specific pGV 3850 region, revealed that the hybridizing 12.6 kbp Eco RI fragment of the introduced plasmid remained largely intact for up to 6 hr in the ovary loculus; although there was some nuclease activity even when the DNA was extracted virtually immediately after microinjection (Section 3.2.4). The inclusion of 'carrier' DNA at 0.8 µg/ul in the microinjected DNA solution did not appear to reduce the amount of

degradation observed. It was thought possible that carrier DNA, in this case herring sperm DNA, would provide additional substrate for the ovary nucleases and consequently reduce the level of nuclease degradation of the plasmid molecules.

The microinjection of such large DNA molecules could in itself result in the loss of integrity of the plasmids due to the shearing of the molecules during the ejection through the microcapillary. This was largely avoided by the use of relatively large bore microcapillaries (25 μm), although some shearing of the plasmid molecule might still be expected, thus, accounting for the observed degradation in 'time zero' samples.

The DNA persistence experiments detailed above were carried out using unpollinated ovaries and so demonstrate the level of ovary nuclease activity. The persistence of pBR 322 DNA was also compared between unpollinated and pollinated ovaries (Results not presented) and it was found that the *Salpiglossis* pollen tubes did not produce amounts of nucleases sufficient to significantly degrade the introduced DNA.

With evidence that introduced DNA molecules remained largely undegraded for significant periods of time in the locular cavity of *Salpiglossis* ovaries, the assessment of intra-ovarian microinjection of foreign DNA as a method of plant transformation was feasible.

4.2.2. Analysis of the progeny derived from microinjected ovaries.

The initial screening of the progeny derived from pGV3850 microinjected ovaries relied upon detection of the expression of the T-DNA encoded nopaline synthase (*Nos*) gene. This gene, present within the right and left T-DNA borders, is transferred to the plant genomic DNA on successful T-DNA integration and is constitutively expressed in plant tissues. The assay for the expression of this gene

is a well established technique and has been used as a screening assay in a number of transformation studies (e.g. Barton *et al.*, 1983). The activity of the enzyme in putative transformants is detected by assaying for the production of nopaline in the plant tissues (Section 2.2.16.3).

Progeny of the microinjected ovaries were assayed and no unequivocal nopaline "positives" were detected in any of the seedlings tested. However, many demonstrated the presence of a compound that reacted with the assay stain to produce a fluorescent spot (Section 3.2.5). The nature of this compound was unknown, it was not seen in control (non-microinjected derived) seedlings and appeared as a result of incubation with arginine. The difference in colouration of the fluorescence and its migration in comparison to the authentic nopaline standard, indicated that it was not nopaline. However, the reaction with the stain suggested that it was a related compound. As the presence of this fluorescence may have masked a nopaline "positive" spot, seven plants exhibiting this property were examined further by 'Southern' hybridization to a *nos* gene probe.

It was found that the *nos* gene sequence was not present in the genomic DNA of the plant tissue tested. The probe used also contained the T-DNA right border sequence, known to be required for T-DNA transfer and integration (Yadav *et al.*, 1982; Shaw *et al.*, 1984; Zambryski *et al.*, 1989). Therefore, it was concluded that these progeny plants were not transformed. It follows that the production of the compound that gave rise to the aberrant spots in the nopaline assay was not as a result of the presence of the *nos* gene or as a non-specific effect of a transformation event. It is possible that the *Salpiglossis* tissue itself is capable of producing opine-like compounds. Other species (for example, tobacco) have been demonstrated to produce such opine-like compounds, for example (Christou *et al.*, 1986; Rogers *et al.*, 1986; Yang *et al.*, 1987). The absence of this compound in the controls could be a reflection of the relatively smaller numbers of plants used for

the controls if the production is only detectable in a small proportion of individual plants tested.

So, of the progeny derived from the intra-ovarian microinjections, none were demonstrated to have been transformed. This, however, does not necessarily indicate that no transformation events had occurred. Fifty ovaries were microinjected and with approximately 350 ovules per ovary (Hepher and Boulter, 1987) up to 17,500 seeds were produced. Clearly, the 250 plants that were screened for nopaline production represented only a fraction of the total progeny (1.4%) and the 7 that were analysed by DNA hybridization an even smaller proportion. These results highlight the need for the use of selectable markers in these transformation studies. At the time that this work was carried out, suitable selectable markers such as genes coding for enzymes that produce resistance to antibiotics in transformed plant tissue, were not available. For example, the neomycin phosphotransferase gene coupled to a promoter constitutively expressed in plant tissues, renders transformed cells resistant to the antibiotic kanamycin (de Block *et al.*, 1984; Bevan, 1984). The use of such selectable genes allows the screening of thousands of progeny simply by seed germination on media containing the appropriate antibiotic. Resistant seedlings can be selected and then further analysed by, for example, a nopaline synthesis assay if appropriate or DNA hybridization studies.

The potential of this technique has been demonstrated already in cotton. Zhou (1985) reported the transfer of wilt resistance to a wilt susceptible strain of cotton, following microinjection of the recipient plants ovary placenta with total DNA of a wilt resistant strain. It was also reported that the acquired resistance trait was stably inherited (Zhou, 1985), although the genetics of wilt resistance is unknown.

Molecular genetic evidence for the incorporation of microinjected DNA into genomic DNA has also been reported. A repetitive DNA sequence from a species of cotton (*Gossypium babadense*) was cloned into an M13 vector (of Vierra and Messing, 1982) and microinjected into self-pollinated ovaries of *G. babadense*, with the aim of promoting integration by homologous recombination. The resulting seeds from these injected ovaries were harvested and the DNA isolated and analysed by 'Southern' hybridization to M13 DNA sequence probes. Hybridization was found to genomic DNA of some of the progeny, whereas none was found to control seeds derived from uninjected ovaries, indicating the presence of M13 sequences in "transformed" seeds. However, the number of transformants per treatment and evidence of stable inheritance was not given (Zhou, 1985).

De la Pena *et al.* (1987) showed the potential of microinjection in plant transformation when they microinjected naked plasmid DNA into developing floral tillers of rye and subsequently demonstrated the incorporation and expression of the foreign DNA in progeny plants. They postulated the DNA was taken up by the germ cells, following transport in the plant vascular system.

These results are encouraging and suggest that the intra-ovarian microinjection technique has potential as a method of plant transformation. The use of suitable selectable marker genes in DNA constructs used in transformation studies, coupled with detailed molecular genetic analyses of any transformed progeny obtained, will make a more thorough assessment of the technique possible.

The advent of other transformation techniques, such as *Agrobacterium*-mediated infection of leaf discs and 'direct gene transfer' to protoplasts (discussed in detail in Sections 4.4 and 4.5, respectively) has attracted much attention and many instances of successful transformation have been reported. However, there are a number of advantages to the intra-ovarian microinjection technique. The technical ease of the method, simple one-step injections and above all the absence

of tissue culture propagation, thus avoiding possible somaclonal variation in the progeny (Shepherd *et al.*, 1980; Larkin *et al.*, 1984; Peerbolte *et al.*, 1987b), are important advantages. A further major advantage of this technique is that it may prove to be applicable to a wide range of species. *Agrobacterium*-infection is restricted in the main to dicotyledonous species (DeCleene and DeLey, 1976), although there are isolated examples of the transformation of some monocotyledonous species (Hernalsteens *et al.*, 1984; Hooykaas-van Slogteren *et al.*, 1984) and the 'direct gene transfer' to protoplasts requires efficient isolation of protoplasts and plant regeneration systems for each species. Many economically important crop plants belong to the Gramineae, and although there have been reports of successful protoplast transformation, for example in *Triticum monoccocum* (Lorz *et al.*, 1985), efficient plant regeneration has still to be achieved in this important family. Use of this system of uptake of DNA by pollen tubes or egg cells with subsequent 'natural' plant generation may overcome the limitations of the *Agrobacterium* host range or the difficulties encountered in producing protoplasts and regenerated plants in some species.

4.3 Construction of the DNA vectors used in the *Agrobacterium*-mediated and 'direct gene transfer' transformation studies

4.3.1 Construction of a chimeric legumin gene

The genomic clone pDUB21 (Lycett *et al.*, 1984) contained the *legA* coding and 3'-flanking sequences minus the start codon and the next 4 bp on a 2.4 kbp HindIII fragment (see Fig. 1). It was necessary to remove the 3'-terminal HindIII site in order to facilitate subsequent construction steps. The removal of a 230 bp BamHI fragment from pDUB21 achieved this (Section 3.3.1). In doing so, 220 bp were deleted from the 3'-end of the *legA* sequence. However, over 400 bp of 3'-flanking sequence remained. The *legA* gene contains three polyadenylation signals positioned within the first 200 kbp of the 3'-flanking region (Lycett *et al.*, 1984)

and so this deletion was judged unlikely to have removed any necessary expression signals. The 3'-flanking regions of a number of plant genes have been reported to be under 400 bp in length (Messing *et al.*, 1983). The *legA* gene containing 3'-flanking sequence to this BamHI site has, for example, since been demonstrated to be successfully expressed in *Nicotiana plumbaginifolia* following transformation (Ellis *et al.*, 1988; Shirsat *et al.*, 1988). This confirms that the removal of the BamHI to HindIII fragment would not be expected to adversely affect the level of expression.

The *nos* promoter was excised from pNosNeo as a 320 bp Hind III/Xho II fragment that successfully cloned in pUC8 (Section 3.3.2). The fusion of this *nos* fragment to the *legA* coding region was achieved with the use of a synthetic linker molecule. Neither the promoter nor coding sequence used contained a start codon, so the sequence of the linker molecule was chosen in order to include the sequence 'ATG' in such a position as to be "in-phase" with the *legA* reading frame when finally ligated. The "Cla I linker" used, fulfilled this requirement. In addition, it provided a restriction enzyme site not present on either fragment or the pUC8 vector, which was useful in subsequent construction steps and restriction enzyme analyses.

pDUB21 Δ Bam was linearized using the Hind III site at the 5'-end of the *legA* coding sequence and subsequently blunt-ended (by 'filling-in' the 3'-recessed termini). The addition of linker molecules onto this linear fragment was followed by transformation (Section 3.3.3.1). Only five transformants were obtained and restriction enzyme analysis revealed that three of these had been successfully blunt-ended (demonstrated by the loss of the Hind III site) and that one or more linker molecules were present in the position required (demonstrated by the appearance of a Cla I site). The fact that more than one linker may have been

inserted was not important at this stage as subsequent restriction and ligation steps removed the excess.

The *nos* promoter fragment, excised from pNos, was also ligated to the linker molecules (Section 3.3.3.2) and successful attachment of Cla I linkers was revealed by PAGE and subsequent autoradiography (Fig. 12b). This PAGE analysis was made possible with the use of radiolabelled linkers. The efficient ligation and restriction of the linker molecules can be seen in Fig. 12a for example. In Fig. 12b the absence of higher polymer forms indicated that the ligation was obviously less efficient. However, the *nos* fragment has clearly been linkered due to its appearance in the gel as a labelled band. This band is less intense in the restricted aliquot as the fragment had many linker molecules attached in the ligated aliquot but these will have been removed by the restriction to leave the fragment with a single linker at each terminus.

The fusion of the *nos* promoter to the *legA* gene was achieved by the insertion of the linkered promoter fragment into the linker-derived Cla I site of pLegA (Section 3.3.4). As the linkered *nos* promoter fragment was not purified prior to ligation with the phosphatased pLegA ClaI fragment, linker molecule concatamers were present in the ligation reaction and hence many of the clones obtained had only linker molecules "inserted". Those with the linkered promoter fragment "insert" were selected by colony hybridization to the *nos* promoter sequence. In fact, less than 2% of the clones obtained from the ligation contained the promoter sequence. This fragment could have been inserted in either orientation. Restriction endonuclease analysis of the clones identified as containing the *nos* promoter fragment (Section 3.3.4) revealed that all had the fragment in the 'wrong' orientation. A simple restriction/re-ligation procedure was performed (Section 3.3.5) to excise and re-insert the promoter fragment, and a clone was obtained that contained the promoter in the desired orientation. A low

frequency of re-insertion of the fragment was observed, however colony-hybridization to the *nos* promoter sequence and subsequent restriction analyses of the selected clones resulted in the identification of a clone containing the promoter in the correct orientation.

The DNA sequence of *nos/legA* fusion region was of particular importance in this study. It was necessary to demonstrate that the 'ATG' was indeed 'in-phase' with the coding region and that the integrity of the *nos* promoter and the *legA* coding sequences had been conserved through the construction steps. In particular, it was important to demonstrate the presence of only one linker molecule between the two fragments. More than one 'ATG' start codon may have had an adverse affect on the level of expression of the chimeric gene by reducing translational efficiency (Kozak, 1983; Bevan, 1984).

The DNA sequence of the fusion region was therefore determined. Two overlapping fragments were sequenced to provide the sequence of the entire *nos* promoter fragment, the initial *legA* coding region and the fusion region (Fig. 15). Suitable restriction enzyme sites were not available to produce a single fragment containing the entire region. The derived sequences overlapped at the site of the linker; however, the existence of more than one linker would have been revealed by the sequence of the Bam HI/AvaI fragment (Fig. 16).

So, the derived composite sequence (Fig. 16) demonstrated the existence of only one 'ATG' start codon and that it was 'in-phase' with the *legA* codons. It also confirmed the integrity of the initial *legA* sequence and the full *nos* promoter sequence (Bevan *et al.*, 1983a); additionally revealing a Bam HI linker molecule and a section of M13 mp7 sequence at the 5'-end of the promoter fragment (Fig. 16) introduced during the construction of pNosNeo (Bevan *et al.*, 1983a).

4.3.2 *Agrobacterium*-mediated gene transfer construct

In the construction of pADY2, the chimeric legumin gene (*nos/leg*) contained on pADY1 (Section 3.3.) was inserted into the multiple cloning site of the T-DNA region of Bin 19. This was achieved by linearization of pADY1 with EcoRI and subsequent insertion into the unique EcoRI site of Bin 19 (Section 3.4.1). The transformants were initially screened by colony hybridization to a *nos/legA* gene probe. This was necessary as the host *E. coli* strain used (K514) contains an intact β -galactosidase gene (i.e. is not a *lac* Z mutant but a *lac* Y-permease - mutant) and so the usual selection of white recombinant colonies on X-Gal substrate was not possible as recombinant colonies rapidly became blue. The K514 strain was used as it was found to give significantly higher transformation efficiencies with large plasmids such as Bin 19 compared with the *lac* Z mutant strain JM83.

Following hybridization, "positive" colonies were identified and the antibiotic resistance characteristics checked to confirm the presence of both Bin 19 encoded Kan^R and pADY1 encoded Amp^R in the recombinants. Analysis of the DNA of a number of recombinants revealed one clone that gave restriction fragments of the predicted size after digestion with PstI; however, the presence of an additional 3.9 kbp band indicated a contaminant. After purification of the clone by re-streaking for single colonies, a clone was identified that gave the predicted PstI fragments. The faint bands in this latter analysis (Fig. 17) were due to partial digestion and represent the linearized recombinant plasmid.

Other cloning strategies were attempted in order to insert only the chimeric legumin gene itself and not the 2.7 kbp of pUC8 sequence of pADY1 (for example by insertion of the 2.8 kbp Pvu II fragment of pADY1 into the SmaI site of Bin 19); however, the desired recombinants were not obtained. The existence of the pUC8 ampicillin resistance gene in the construct meant that an alteration in the

Agrobacterium-infection procedure was necessary. Carbenicilin is the standard antibiotic used to kill the Agrobacteria during the infection procedure; however, the pUC8 ampicillin gene confers resistance to carbenicilin and so an alternative antibiotic, 'Timentin', was used for this purpose (see Section 4.4.).

The pADY2 construct was transferred from its *E. coli* host to *Agrobacterium tumefaciens* by a tri-parental mating. Analysis of the DNA of one of the transconjugant colonies obtained by restriction endonuclease digestion and Southern blot hybridization to specific probes, confirmed the integrity of the pADY2 plasmid after transfer (Section 3.4.2). The faintly hybridized bands of the 'control' tracks (Fig.19) can be explained by the presence of contaminating pUC8 sequence in the probe preparations as each was isolated from a pUC8 vector (for example, the faint hybridization of the 'neo' probe to the 3.2 kbp (pUC8 containing), Pst1 fragment of pADY1. The Bam HI and EcoRI digests of the *Agrobacterium* DNA gave bands of the predicted size following hybridization to each of the three probes. The Bgl II/Sal I double-digest resulted in the expected bands after hybridization to the '*leg*' probe but the '*nos*' and '*neo*' hybridized to a band of 4.8 kbp in addition to the expected bands. This was thought due to partial digestion at the Sal I site 3' of the *nos/neo* gene (Fig. 18). (The addition of the 2.7 kbp and 2.15 kbp fragments would give a partial fragment of 4.85 kbp that would hybridize to the *nos* and *neo* probes used.)

4.3.3 'Direct gene transfer' construct

The construct that was used in the study of 'direct gene transfer' to protoplasts was derived from the plasmid pADY2. The use of pADY2 itself in this transformation study was considered, but it was thought that the relatively large size of the plasmid (16.5 kbp) may have adversely affected the efficiency of transformation. Protoplast transformation has been reported with plasmids of

comparable size; for example, pCT1T3 at 16.3 kbp (Uchimiya *et al.*, 1986a) and pCT2T3 at 18.3 kbp (Uchimiya *et al.*, 1986b). However, in order to increase the transformation efficiency in this study, it was thought desirable to use a smaller construct, so a simple construct strategy was devised that retained the plant selectable marker and legumin genes of pADY2, whilst eliminating unnecessary sequences and replacing the large Bin 19 vector sequences with the much smaller vector pUC8. This strategy, described in Section 3.5, resulted in the construct pADY3 which was 8.2 kbp in size. An additional advantage of the use of a smaller plasmid was that it could be isolated more efficiently from the bacterial host. Plasmid yields of Bin19 constructs such as pADY2 were significantly lower than pUC8 constructs such as pADY1. This was important as large amounts of purified plasmid DNA was required for the protoplast transformation experiments.

Another factor that was thought to possibly affect the efficiency of transformation of protoplasts was the form of the DNA molecule used, that is whether linear, supercoiled or open-circular. Work on direct DNA uptake by mammalian cells has suggested that linear molecules transform more efficiently than circular ones (Folger *et al.*, 1982). In this study, transformations were performed with both unrestricted plasmid preparations and linear DNA fragments. It was important therefore, that the two genes could be easily isolated from pADY3 on a single restriction fragment. The 5.5 kbp EcoRI fragment was used.

In mammalian cell transformation, evidence has been presented suggesting that the presence of restriction endonuclease enzymes on transforming DNA molecules may reduce the transformation efficiency 10 to 50 fold (Gusew *et al.*, 1987). During the preparation of the linear pADY3 DNA used for transformation, purification by thorough phenol extraction and ethanol precipitation was performed. This procedure would have removed the EcoRI enzyme from the pADY3 DNA fragment termini before transformation.

4.4 *Agrobacterium*-mediated plant transformation

The method used for the study of *Agrobacterium*-mediated gene transfer was that of *Agrobacterium*-infection of leaf "discs" (Horsch *et al.*, 1985); although referred to here as leaf-pieces because in the protocol used the leaf sections were cut squares not "punched" discs. This has become an established transformation technique in recent years and has been employed by many workers to obtain transgenic plants using a number of species; for example, tobacco (Horsch *et al.*, 1985; Keith *et al.*, 1986; Spielmann *et al.*, 1986), petunia (Horsch *et al.*, 1985; Beachy *et al.*, 1985; Wallroth *et al.*, 1986) and tomato (Horsch *et al.*, 1985). Alternative plant tissues can be used in *Agrobacterium* infection protocols; the choice of explant is largely dependent on the regeneration capability of the plant species to be transformed. Some species can be more efficiently regenerated from *Agrobacterium*-infected stem sections; for example *Brassica napus* (Klee *et al.*, 1987), stem sections have also been used as an alternative to leaf pieces in tobacco (Barton *et al.*, 1983) and tomato (Chyi *et al.*, 1986) transformation.

Cocultivation of *Agrobacterium* with regenerating protoplasts is another technique and indeed was the method used in early work to obtain transgenic plants (Marton *et al.*, 1979; Fraley *et al.*, 1983; Horsch *et al.*, 1984; De Block *et al.*, 1984). This method is clearly more appliable to plant species that can be efficiently regenerated from protoplasts such as tobacco or petunia, and has the advantage of generating large numbers of independently transformed plants. The disadvantages of the use of protoplasts is that the isolation procedure and subsequent regeneration of plantlets is time-consuming and involves many tissue-culture manipulations. Many important species have yet to be efficiently regenerated from protoplasts.

The use of *Nicotiana tabaccum* as the model plant system in this study of *Agrobacterium* transformation, meant that either the protoplast or one of the tissue explant techniques could be employed. The leaf piece procedure was favoured as it was considered that suitable numbers of transgenic plants could be obtained relatively quickly and efficiently.

In the *Agrobacterium* infection of leaf piece procedure, following the co-culturing of leaf pieces with Agrobacteria, the bacteria are killed by the inclusion of certain antibiotics in the media to prevent bacterial contamination of the callus and regenerating plantlets in the subsequent tissue-culture steps. Carbenicillin is routinely used for this purpose (for example, Horsch *et al.*, 1985; de Framond *et al.*, 1986). However, the use of the construct pADY2 in this study led to the need for an alternative antibiotic as the pUC8 derived ampicillin resistance gene (encoding β -lactamase) present in pADY2, also conferred resistance to carbenicillin. Cefotaxime has been used in place of carbenicillin in some protocols (for example, Spielmann and Simpson, 1986; Thornburg *et al.*, 1987). However, in this case "timentin" was used. This antibiotic consists of two components, clavulanic acid, which inhibits β -lactamase activity, and the antibiotic ticarcillin (a "pencillin" consisting of a β -lactam nucleus with a semi-synthetic side-chain). The inhibition of the ampicillin resistance gene product allows the ticarcillin to act on the Agrobacteria.

The use of this novel antibiotic was judged to be successful as the Agrobacteria were killed, bacterial contamination did not carry through the various tissue culture steps and, importantly, the growth of callus and regeneration of plantlets was not adversely affected by the antibiotic, demonstrating that there was no toxic effect on the tobacco tissues (Section 3.6).

Timentin has also been used with similar success in leaf-piece transformations by Edwards (1988). This antibiotic has been shown therefore, to

be a useful alternative to carbenicillin in *Agrobacterium* transformation protocols, where the construct used contains an ampicillin resistance gene.

In this study, the *Agrobacterium tumefaciens* transformation techniques was used to successfully produce transgenic tobacco plants. Following the transfer of genes coding for antibiotic resistance in the development and assessment of the *Agrobacterium* transformation techniques as a method of producing transgenic plants (see references at the beginning of this Section), attention has turned to the transfer of more agriculturally useful genes. The enormous potential of this transformation technique for crop plant improvement, has been demonstrated with the use of *A. tumefaciens* to produce transformed plants resistant to insect attack or tolerant of particular herbicides.

The broad-spectrum herbicide glyphosate inhibits an enzyme (5-enolpyruvylshikimate-3-phosphate synthase-EPSP) in the biosynthesis of aromatic amino acids. Comai *et al.* (1985) isolated a mutant gene coding for EPSP synthase from the bacterium *Salmonella typhimurium*, that was resistant to glyphosate inhibition and transferred it to tobacco by *Agrobacterium* infection of leaf-discs. The expression of the introduced gene in transgenic tobacco plants was found to enhance glyphosate tolerance. More recently, Shah *et al.* (1986) isolated the EPSP synthase gene from a *Petunia* line that was resistant to glyphosate and replaced its promoter with the Cauliflower Mosaic Virus 35S promoter and transferred this chimeric gene to *Petunia* using an *Agrobacterium* leaf disc transformation protocol. The 35S promoter directed high level expression of the transferred gene in transgenic *Petunia* plants, the over-production of the enzyme conferred glyphosate tolerance to these plants.

The introduction of resistance to insect pests and specific diseases are also highly desirable goals in a plant genetic manipulation. The application of the *Agrobacterium* transformation method has resulted in the production of resistant

transgenic plants. For example, Vaeck *et al* (1987) transferred a modified toxin gene, bt2, isolated from the bacterium *Bacillus thuringiensis* that codes for a specific insecticidal protein and introduced it into tobacco. The transgenic tobacco plants produced the bt2 toxin and were found to be protected from tobacco hornworm larvae feeding damage as the larvae were rapidly killed.

Protection from a viral infection has also been attained by the transfer of a gene coding for a tobacco mosaic virus coat protein to tobacco and tomato plants with the use of *A. tumefaciens* (Abel *et al.*, 1986). Up to 60% of the transgenic plants expressing the coat protein gene failed to develop symptoms following inoculation with the virus.

So, the application of the *Agrobacterium* transformation technique to agriculturally important plant species has great potential as greater numbers of genes, such as those conferring resistance to herbicides and protection from insect attack are isolated and modified for use in transgenic plant protocols.

4.5 Plant Transformation by 'Direct Gene Transfer' to Protoplasts

The natural gene vector system of *Agrobacterium tumefaciens* has been adapted for use as a reproducible and efficient method of transferring genes to plants. However, one major limitation to its use is that the host-range of the bacterium is largely restricted to the dicotyledonous species (DeCleene and DeLey, 1976; Nester *et al.*, 1984). As previously discussed (see Section 4.1.1.), there has been some indication of *Agrobacterium*-infection of certain members of the monocotyledonous families Liliaceae and Amaryllidaceae, but the evidence was restricted to the apparent detection of opine synthesis and no proof of integration of T-DNA has been presented. Many of the world's most economically important crop species are monocotyledons, in particular members of the Gramineae, such as wheat, maize and rice. Therefore, an efficient transformation system for these

species would be of enormous value. Alternative methods of introducing genetic material to plants have been studied that do not involve the specific bacterial/plant interaction of the *Agrobacterium*-infection system in an attempt to achieve an efficient transformation system applicable to the monocotyledonous species.

The cell wall is the major barrier to the entry of DNA molecules into plant cells; its enzymatic removal to produce protoplasts, permits the use of techniques that act on the cell membrane. A number of methods of introducing DNA to protoplasts have been investigated, for example fusion with liposome encapsulated DNA (Rollo *et al.*, 1981; Ohgawara *et al.*, 1983; Deshayes *et al.*, 1985; Rosenberg *et al.*, 1988), fusion with *Escherichia coli* or *Agrobacterium tumefaciens* spheroplasts (Hasezawa *et al.*, 1981; Hain *et al.*, 1984; Tanaka *et al.*, 1984; Harding and Cocking, 1986) and direct microinjection into the protoplast cytoplasm or nucleus (Crossway *et al.*, 1986; Reich *et al.*, 1986). However, the technically simpler and more efficient method was found to be the chemically-stimulated uptake of naked DNA molecules by protoplasts, that is, with the use of fusogens, such as polyvinyl alcohol (Hain *et al.*, 1985), poly-L-ornithine (Draper *et al.*, 1982), or polyethylene glycol. (PEG) (Krens *et al.*, 1982; Paskowski *et al.*, 1984), chemicals known to facilitate the uptake of DNA by protoplasts. The use of PEG-induced DNA uptake has been shown to be more efficient than poly-L-ornithine in transformation studies with *Nicotiana* and *Petunia* (Krens *et al.*, 1982; Draper *et al.*, 1982), and in an evaluation performed by Freeman *et al.* (1984) the PEG-induced 'direct gene transfer' technique was judged to be superior to the two fusion methods given above.

The protocol used for the preparation of mesophyll protoplasts of *Nicotiana tabacum* was developed from a number of published methods (see references in Section 2.2.14.1), with factors such as the concentration of cell wall digesting

enzymes and type of media used adjusted to optimize the efficiency of isolation. The resulting procedure was found to be reproducibly efficient, yielding numbers of healthy viable protoplasts per isolation run, sufficient for each set of transformations.

The transformation procedure included the use of PEG and a heat-shock step. These are believed to facilitate the uptake of DNA molecules by protoplasts due to their effects on the permeability of the cell membrane. Care was taken over the order of addition of the components of the transformation solution (in particular the addition of the transforming DNA to the protoplasts before the PEG solution) and the step-wise addition of F-medium, as these were thought to be important in optimizing the transformation efficiency (Krens *et al.*, 1982).

The inclusion of carrier DNA, in this case HSDNA, in the transformation solution, is believed to enhance the uptake of DNA molecules by protoplasts and follows the observation that increased transformation efficiencies are found with the use of such carrier DNA in the transfection of cultured animal cells (Graham and van der Eb, 1973). The type of carrier DNA used does not appear to be critical, both herring sperm DNA (e.g. Lorz *et al.*, 1985) and calf thymus DNA (e.g. Peerbolte *et al.*, 1985) have been used successfully in protoplast transformations.

An efficient transformation protocol requires a good selection procedure. The use of the bacterial neomycin phosphotransferase gene under the control of expression signals capable of constitutive expression in plant cells has enabled the successful selection of transformants using kanamycin in a number of plant transformation studies (Bevan, 1984; Fraley *et al.*, 1983), including direct gene transfer (Paszkowski *et al.*, 1984; Hain *et al.*, 1985). In this 'direct gene transfer' study no resistant colonies were obtained from the transformations with carrier DNA only (Section 3.7, Table 3), demonstrating that the use of kanamycin at a concentration of 100 µg/ml in the 'bead culture' plant regeneration system,

provided a tight selection procedure, thus affording confidence that resistant colonies obtained following treatment of protoplasts with pNosNeo or pADY3 DNA (containing the kanamycin resistance marker) were true transformants.

The frequency of transformation after incubation of the protoplasts with pNosNeo and pADY3 was of the order of 1 per 10^5 treated protoplasts (1 to 31 per 10^6). It was not possible to assess the effect of the size of plasmid molecules (i.e. a difference between the frequencies of transformation with the 5.4 kbp pNosNeo and the 8.2 kbp pADY3 plasmids), or the form of the transforming DNA (i.e. linear or circular pADY3), due to the small number of transformation results, particularly as no results were obtained from three treatments due to bead-culture contamination. However, any effect did not cause the frequencies to vary by more than one order of magnitude. The size of the transforming DNA may well not effect the transformation efficiency as the original direct gene transfer experiments were performed using the large ~200 kbp Ti-plasmids (Davey *et al.*, 1980; Krens *et al.*, 1982) and comparable frequencies of transformation were obtained (1 per 10^5 to 10^6 treated protoplasts).

The transformation efficiency obtained in this study, compares with those obtained by other workers with *Nicotiana tabacum* protoplasts using a similar PEG-induced direct gene transfer protocol, for example Uchimiya *et al.* (1986). Paszkowski and Saul (1986) using the same technique without a heat-shock step, obtained a lower frequency of 1 per 10^6 treated protoplasts, reflecting the increase in efficiency possible with such heat treatment.

In comparing quoted transformation frequencies, it is important to differentiate between those expressed in terms of number of protoplasts treated (as in this study) and those obtained from the number of viable protoplast-derived colonies. The latter takes into account the number of protoplasts that do not survive the transformation and/or regeneration procedure, quite apart from the

antibiotic selection (the 'plating efficiency'). The plating efficiency for *Nicotiana tabacum* has, for example, been found to be between 10% (Paszkowski and Saul, 1986) and 50% (Krens *et al.*, 1982). The plating efficiency varies markedly between species, for example 1% for *Lolium multiflorum* (Italian Rye grass) (Potrykus *et al.*, 1985b) and as low as 0.1% for *Oryza sativa* (Rice) (Uchimiya *et al.*, 1986b).

Optimization of the PEG-induced direct gene transfer technique has resulted in reported transformation frequencies in the order of 10^{-5}. Greater transformation efficiency would be advantageous in the routine recovery of transgenic plants and frequencies of up to 10^{-2} have been achieved with the addition of a further treatment, that is the application of a high voltage electric pulse to the protoplasts by means of a discharged capacitor during incubation with the transforming DNA (Shillito *et al.*, 1985). This process, known as 'electric field mediated gene transfer' or 'electroporation', acts by inducing a reversible permeability change in the protoplast cell membrane (Kinosita and Tsong, 1977; Zimmermann, 1982), facilitating the uptake of large macromolecules such as DNA. Electroporation has been used in the transformation of animal cells and transformation frequencies up to 10^{-4} have been achieved with, for example, mouse cells (Neumann *et al.*, 1982) and human cells (Potter *et al.*, 1984). The amplitude and duration of the electric pulse have been found to be important in determining the transformation efficiency; two types of pulse have been employed, high voltage for a short time period (for example, 8,000 v/cm for 15 μsec - Neumann *et al.*, 1982) or a lower voltage for a longer time (for example, 112 v/cm for 60 msec - Potter *et al.*, 1984).

Riggs and Bates (1986) achieved stable transformation of tobacco following electroporation of protoplasts, with the maximum transformation frequency of 2.2 x 10^{-4} treated protoplasts using a 2,000 v/cm 250 μsec pulse. Larger and longer pulses reduced the efficiency. In this case, the protoplasts were electroporated

without the inclusion of other transformation enhancing treatments. With the use of PEG in the electroporation medium, prior heat-shock treatment, and optimization of the electric pulse, Shillito *et al.* (1985) were able to improve the transformation frequency of tobacco protoplasts to 10^{-2}.

High transformation frequencies have been shown to be possible using the direct gene transfer technique without electroporation. Negrutiu *et al.* (1987a) report an average transformation rate with *Nicotiana tabacum* protoplasts of 3.8% of the protoplast-derived colonies using $MgCl_2$ and PEG treatment, demonstrating that electroporation is not an absolute requirement in achieving high efficiency transformation.

Application of the direct gene transfer technique to species of the monocot family Gramineae, has resulted in reports of stable transformation. Potrykus *et al.* (1985b), using PEG-induced uptake with heat-shock treatment, transferred the neomycin phosphotransferase (*neo*) gene under the control of the Cauliflower Mosaic Virus 35s (CaMV 35s) promotor and 3'-expression signals to protoplasts prepared from cell suspension culture of *Lolium multiflorum* (Italian rye grass). One in 4 x 10^3 of the protoplast-derived colonies exhibited *neo* activity and transferred DNA was detected by Southern blot analysis of the callus genomic DNA. The transformed callus was incapable of regeneration to plants, however. The antibiotic G418 was used to select for transformants in this case as non-transformed protoplast colonies showed some tolerance to kanamycin. This natural kanamycin resistance has been observed in some species of the Gramineae (Vasil, 1987) and alternative selection antibiotics have been investigated, although kanamycin selection has been effective in studies such as those given below (Hauptmann *et al.*, 1988).

Lorz *et al.* (1985) in a similar study with protoplasts derived from cultured cells of wheat (*Triticum monococcum*) detected *neo* activity in kanamycin resistant colonies following the PEG-induced uptake of the *neo* gene under the control of

nos promoter and 3'-expression signals. the frequency of transformation was 1 in 5 x 10^5 protoplast-derived colonies; again, plant regeneration was not possible.

Transformation of the important cereal species rice (*Oryza sativa*) was reported by Uchimiya *et al.* (1986b) using PEG-induced uptake. The *neo* gene, under the control of *nos* 5'- and CaMV 35s 3'-expression signals, was expressed in 2.3% of protoplast-derived colonies, although only 0.01% of treated protoplasts survived the transformation and subsequent culture procedures.

The electroporation technique was applied to the protoplasts of a maize (*Zea mays*) cell suspension culture by Fromm *et al.* (1986). The chimeric *neo* gene (CaMV 35s promoter/*nos* 3' expression signals) was transferred to 1 in 10^4 treated protoplasts using electroporation alone (without PEG or heat-shock treatment), with up to 1% of the dividing protoplasts giving kanamycin resistant colonies. the presence of the transferred DNA was confirmed by Southern blot analysis.

In the above examples of transformation, it was not possible to regenerate plants from transformed callus. Species of the Gramineae have proved recalcitrant to such regeneration (Vasil, 1987) and much work is needed in the investigation of optimal protoplast and callus culture conditions for the efficient regeneration of plants in this family. Some success has already been achieved, for example with the efficient regeneration of rice plants from protoplasts (Fujimura *et al.*, 1985; Cocking and Davey, 1987; see also, Marx, 1987).

Recently, Rhodes *et al.* (1988) have succeeded in regenerating transformed maize plants from electroporated protoplasts of an embryogenic cell suspension culture. The *neo* gene (coupled to the CaMV promoter) was shown to be present (by Southern blot analysis) and expressed (*neo* activity detected) in 1% of the protoplast-derived kanamycin resistant colonies. It was noted that heat-shock treatment increased this frequency by a factor of three. Eighteen percent of the transformed calli regenerated plants using maize "feeder cells" in the post-

transformation culture to provide as yet unidentified required nutrients to greatly increase the plating efficiency. The mature maize plants were phenotypically normal except that they did not produce viable pollen. The reason for this was unclear, but was thought possibly related to the cell line as regenerants from non-transformed plants derived from old suspension cultures are affected by genetic changes that cause sterility.

In addition to its applicability to species from which protoplasts and regenerated plants can be efficiently produced, the direct gene transfer technique also has the advantage that vector construction can be simplified. There is, for example, no need for specific integrative sequences to be included in the construct, such as the T-DNA border sequences required in *Agrobacterium* infection. The direct gene transfer construct requires only a suitable selectable marker gene in addition to the gene of interest to be transferred. Indeed, the selectable marker need not necessarily be present on the same plasmid as other transforming genes. It has been demonstrated that DNA sequences on separate plasmids, mixed in the transformation solution, can be transferred and integrated into the genomic DNA of a single protoplast. This 'co-transformation' of selectable and non-selectable genes has the advantage of obviating the need to produce a single construct containing the desired DNA sequences to be transferred. Two genes transferred separately in this manner are not both necessarily faithfully integrated. Such co-transformation is not a rare event however; for example, it has been reported (Schocher *et al.*, 1986) that 25-50% of selected tobacco protoplast-derived clones also contained an intact copy of a co-transformed non-selected gene (up to 80% contained partial copies).

Evidence for the direct uptake of DNA by protoplasts of monocot and dicot species and the regeneration of stably transformed plants has been presented. The optimization of protocols in some species, notably tobacco, has resulted in

transformation efficiencies using this technique comparable with those found with *Agrobacterium* mediated transformation of protoplasts. With further optimization of the transformation procedures and the development of more efficient plant regeneration protocols, especially for gramineaceous species, direct gene transfer offers tremendous potential for plant transformation being a relatively simple and efficient technique.

4.6 Structure and integrity of foreign DNA in the genome of transformed plants

4.6.1. *Agrobacterium*-mediated transformed plants

Analysis of the results of the hybridization of the three DNA probes to 'Southern' blots of restriction endonuclease digested genomic DNA of the *Agrobacterium*-transformed *Nicotiana tabacum* plants and comparison with the predicted hybridization patterns for intact T-DNA inserts, revealed that in the majority of cases, the T-DNA was not inserted as a single entirely intact copy. Some alteration in T-DNA integrity, either sequence deletions or rearrangements, was indicated in some transformants. An outline of the interpretation of the 'Southern' hybridization results is given below.

Firstly, with regard to the hybridization to control untransformed *N. tabacum* genomic DNA. The slight hybridization to a 1.1 kbp fragment of the control DNA by the legumin gene sequence probe was not thought significant as none of the ten transformants showed hybridization to a fragment of similar size, indicating that this hybridization was not due to sequence homology with tobacco genomic DNA. The exact origin of this band is not known, but the possibility of contaminating legumin sequence in this particular sample cannot be overlooked. There was no hybridization to the control DNA with either the *nos* or *neo* sequence probes.

The results of hybridization with the *leg* probe demonstrated that seven of the ten transformed plants contained the entire 2.2 kbp of *leg*A coding sequence in an unaltered form. Furthermore, a comparison with the *nos* probe results revealed that the predicted 3.0 kbp fragment containing the chimeric legumin gene promoter was present in these seven transformants so, where integration of this unselected gene occurred, it was in a complete and unaltered form.

Identical hybridization patterns were obtained for T304 and T308 in addition to T306 and T307. The results obtained for the latter transformants were consistent with two T-DNA insertion points; one giving a T-DNA right border/genomic DNA insertion fragment of 2.3 kbp and the second of 4.3 kbp. The hybridization pattern obtained with both T304 and T308 suggest in each case two incomplete inserts with genomic insertion fragments of 7.4 kbp and 2.0 kbp. In both 'pairs' of transformants, two presumed independent transformation events leading to identical insertion points in the transformed genome, suggesting that these points are favoured sites of integration. Favoured insertion sites have not been reported in the *Nicotiana tabacum* genome in the numerous accounts of *Agrobacterium*-mediated transformation. While it is feasible that such favoured sites exist in the tobacco genome, it is perhaps more likely that these transformants were not, in fact, derived from independent transformation events. In the plant regeneration procedure, the greatest care was taken to excise developing shoots from separate areas of the *Agrobacterium*-infected leaf-piece edge; however, it is not inconceivable that two shoots could inadvertently have been taken that had arisen from the same callus mass and thus be derived from a single transformation event.

A 4.1 kbp Bam HI fragment that hybridized to the *neo* probe, was present in five of the ten transformants and was only detected in transformants that demonstrated the presence of intact chimeric legumin gene. It was not detected in

untransformed plant genomic DNA. The origin of this 4.1 kbp Bam HI restriction fragment is not clear; its size cannot be predicted from the structure of the transforming pADY2 T-DNA and it was not detected in the Southern blot analysis of the pADY2 construct with the same *neo* sequence probe. There is no corresponding 4.1 kbp fragment found hybridizing to the *nos* probe, indicating that this fragment does not contain *nos* promoter sequence. The formation of repeated T-DNA structures in either a direct or inverted array does not predict a 4.1 kbp fragment with such hybridization properties. It would seem then, that a specific rearrangement has occurred in the structure of the T-DNA copies containing the legumin gene. In transformants T300 and T303 (possibly T301 too) no T-DNA right-border insertion fragments, in addition to this 4.1 kbp band, were detected with the *neo* probe, suggesting that this rearrangement placed a Bam HI site close to the T-DNA right border so that only internal T-DNA fragments were detected in the transformed plant genomic DNA hybridizations.

The hybridization patterns obtained with the *neo* probe demonstrated that *neo* gene sequences were present in all the transformed plant genomes, detected as one or more distinct restriction fragments, except in T301, T302 and T309. In the case of T301, it is possible that a 4.1-4.3 kbp band is masked by a region of non-specific hybridization. T302 shows hybridization to an approximately 30 kbp fragment, suggesting either a large genomic insertion fragment of this size or, more likely, a partial digestion of this sample. T309 (and possibly T301) however, would appear, from these results, not to contain any *neo* gene sequence, suggesting that the selective marker gene was not present. This conflicts with the observation that leaf-pieces harvested from this plant were shown to be resistant to kanamycin in contrast to untransformed control leaf tissue (Section 3.8.3) and that T309 progeny gave rise to kanamycin resistant progeny in a Mendelian ratio indicating the presence of kanamycin resistance as a single dominant trait (Section 3.8.4). The

selection of *Nicotiana tabacum* transformants using kanamycin is regarded as a strong selection procedure with no reports of untransformed regenerating tobacco plants surviving selection at 100 µg/ml (the level used in this study). Undoubtedly then, the *neo* gene was present in the T309 genome during the selection process and in the mature plant tissues. The failure to detect gene sequences in the particular leaf sample used may have been due to a technical reason, for example an inefficient transfer of the genomic DNA to the filter or a weak hybridization signal not being detected against a high background of non-specific hybridization. It is possible that the leaf from which the genomic DNA was prepared did not contain the inserted foreign DNA in contrast to other regions of the plant. Such a 'chimera', where one or more sections of a plant differ in genetic origin from the others, has been suggested in previous transformed plant analyses. Edwards (1988) described an *Agrobacteriuim*-transformed *Nicotiana tabacum* plant in which the structure of the inserted DNA is different in genomic DNA samples prepared from separate leaves. Such a chimera may have resulted from the formation of a shoot from a mixed callus in the tissue-culture (Carlson and Chaleff, 1975; Stewart, 1978).

Evidence obtained from Southern blot analyses of *Agrobacterium*-derived transformants has suggested that the type of Ti-plasmid used may have an effect on the structure of the integrated T-DNA. In particular the use of derivatives of the Ti-plasmid C58 (for example, pGV3850) has been noted to result in a high proportion, (up to 50% of integration events) of inverted repeat structures if more than one copy of the T-DNA is integrated with the Left and Right borders equally favoured as the point of inversion (Jorgenson *et al.*, 1987; Jones *et al.*, 1987). Binary vector plasmids in the bacterial host LBA4404 appear to result in a much lower frequency of inverted repeats (Spielmann and Simpson, 1986; Jorgensen *et al.*, 1987). The results presented here using a Bin 19-based binary vector in

LBA4404 lend support to these findings as they did not indicate inverted repeat structures about the right border in any of the transformants, due to the absence of a 3.4 kbp *neo* and *nos* probe hybridizing Bam HI restriction fragment, or direct repeat structures about the right border due to the absence of a 1.7 kbp *neo* and *nos* hybridizing fragment. However, the presence of inverted or direct repeats about the left border in this case, would not have been detected by hybridization to Bam HI restriction digests, as the Bam HI site immediately adjacent to the left border prohibited the formation of a 'double-sized' fragment containing the border.

In the analyses described here, the insertion of the T-DNA into the tobacco genome was subject to certain unpredictable structural alterations. However, from the number of right-border insertion fragments (*neo* probe) and the number of corresponding *nos* promoter fragments, it was concluded that no more than two T-DNA inserts were present in the transformed genomes. Such low copy number insertion has been found in the majority of the genomic DNA analyses of *Agrobacterium* transformed plants (Lemmers *et al.*, 1980). Generally, copy numbers up to six have been cited, although higher numbers have been reported, for example, approximately 20 (Barton *et al.*, 1983). This is so for species other than tobacco, for example, petunia (Nagy *et al.*, 1985; Wallroth *et al.*, 1986) and sunflower (Goldsborough *et al.*, 1986).

The method of *Agrobacterium*-infection seems not to affect the number of T-DNA inserts in the transformed genome as low copy numbers have been reported with the leaf-disc system (Spielmann and Simpson, 1986; Baumann *et al.*, 1987) and co-cultivation with protoplasts (Nagy *et al.*, 1985; Jones *et al.*, 1985; 1987; Sengupta-Gopolan, 1985).

The results supported the hypothesis that T-DNA transfer occurs in an orientated way that is initiated at the right border and proceeds towards the left border (Wang *et al.*, 1984; Peralta *et al.*, 1985); if the reverse were the case, then

all the transformants would be expected to contain the legumin gene as the selection required an intact kanamycin resistant gene which would be at the right border end of the integrated sequence.

It is difficult to determine the exact structure of the foreign DNA inserted into the genomes of the transformants from the analysis of the results of those particular restriction endonuclease digests. Use of additional restriction enzymes (perhaps Pst I) with the *leg nos* and *neo* sequence probes would complement these Bam HI results and help to elucidate the nature of a proportion of the rearrangements that have taken place during the insertion of the T-DNA molecules.

4.6.2 Direct gene transfer derived transformants

The Southern blot analyses of the *Nicotiana* transformants derived from the direct gene transfer experiments consisted of hybridization to the *leg* and *neo* sequence probes only. Use of the *nos* probe with these Bam HI digests was expected to merely replicate the results obtained with the *neo* probe (see Fig. 29). The use of gene copy reconstruction allowed an estimate of the copy number of the *neo* hybridizing fragments. Gene copy numbers with the *leg* probe results were based on the relative intensities of the bands.

A faintly hybridizing band of 1.6 kbp was detected in the untransformed SR1 control track. As with the *Agrobacterium* transformant results, this band was not detected in any of the transformed plant samples or in the pNosNeo transformants. The band was of a different size to that detected in the *Agrobacterium* hybridization. This hybridization was not thought to be due to sequence homology with the tobacco genome as both hybridizations involved the same tobacco species and the same probe sequence.

The main feature of these results was that in general the sizes of the hybridizing fragments were not those predicted and in many cases were much

larger. Indeed, none of the restriction fragments hybridizing to the *neo* probe corresponded to those expected. Three transformants, namely T370, T371 and T376, gave the predicted *leg* probe hybridizing fragments of 2.2 kbp, suggesting that these transformants contained intact copies of the legumin gene sequence. However, the *neo* probe results for these transformants do not give the expected 2.4 kbp fragment containing the chimeric legumin genes *nos* promoter sequence and so the exact structure of this gene in these transformants cannot be identified.

The transformants were derived from protoplasts treated with linear pADY3 DNA molecules with Bam HI sites immediately adjacent to the terminal Eco RI sites (Section 3.5) . The hybridization results with both probes, showing fragments much larger than predicted, are consistent with the loss of these Bam HI sites in the transforming DNA. All of the *neo* hybridizing bands were larger than the predicted 2.4 kbp, suggesting the loss of the Bam HI site close to this end of the transforming DNA in the inserted foreign DNA of all the transformants. Loss of integrity of the termini of the linear DNA molecule, possibly by nuclease degradation or a rearrangement event, may well have removed the Bam HI sites. The loss of integrity of the ends of linear transforming DNA molecules has previously been shown for example. Riggs and Bates (1986) report the loss or modification of terminal Eco RI sites of linear DNA in the genomes of tobacco transformants following direct gene transfer to protoplasts using electroporation.

If such a loss of the terminal Bam HI sites occurred, then the large hybridization fragments obtained could either be the genomic insertion fragments or possibly concatamers of the truncated *leg* or *neo* gene sequences.

A comparison of the gene copy equivalent bands with the hybridized fragments of the transformants in the *neo* sequence probe hybridization, indicated gene copy number of between 1 and 3, certainly none of the bands were as intense as the 5 gene copy equivalent band.

The uptake of isolated DNA by plant protoplasts has been found to frequently exhibit unpredicted hybridization patterns, suggestive of complex sequence modifications and rearrangements (Hain *et al.*, 1985; Krens, 1985). Reported copy number of inserted genes are frequently high, for example ~10 per genome (Uchimiya *et al.*, 1986a), 10-25 per genome (Czernilofsky *et al.*, 1986a, b), following direct gene transfer to tobacco protoplasts. Often the foreign DNA is found inserted as tandem repeat structures, either head-to-head or head-to-tail (Deshayes *et al.*, 1985; Hain *et al.*, 1985; Czernilofsky *et al.*, 1986a). It is thought that linear (or circular) DNA molecules introduced into the plant cell are subject to recombination and replication processes before chromosomal integration takes place (Riggs and Bates, 1986). For example, Wirtz *et al.* (1987) showed homologous recombination between two co-transformed plasmid vectors occurred prior to integration. It has been proposed that multiplication of a "precursor" DNA molecule that has been modified by recombinational events during passage through the cell gives rise to concatamers that are subsequently integrated into the chromosome (Czernilofsky *et al.*, 1986a).

DNA sequence rearrangement, deletion, insertion and point-mutation have also been detected, following mammalian cell transformation with naked DNA (Folger *et al.*, 1982; Calos *et al.*, 1983; Miller *et al.*, 1984; Hauser *et al.*, 1987, and references therein). Similarly, concatemerization is often reported (Perucho *et al.*, 1980; Scangos and Ruddle, 1981; De Jonge and Bootsma, 1984). It would seem then that such unpredictable rearrangement of naked DNA molecules is a feature of direct gene uptake into eukaryotic cells.

The nature of the integration of foreign DNA into the protoplast genome in the 'direct gene transfer' transformants reported here, was presumed to be non-homologous or illegitimate recombination, as no chromosomal DNA homologous sequences were present on the pADY3 transforming DNA. It was not thought that

the right border repeat sequence present on the pADY3 linear fragment used for these transformations constituted a preferred sequence for integration as previous analyses of transformed genomes, following uptake of isolated DNA have shown that neither T-DNA border is used as a site of integration (Krens *et al.*, 1985; Crossway *et al.*, 1986). Integration by non-homologous recombination has previously been demonstrated in plant protoplast transformation involving direct uptake of DNA (Paszkowski *et al.*, 1984; Deshayes *et al.*, 1985; Hain *et al.*, 1985; Krens *et al.*, 1985; Shillito *et al.*, 1985; Peerbolte, 1985; Riggs and Bates, 1986; Jongsma *et al.*, 1987). It has been proposed that the inclusion of homologous DNA sequences in transformation vectors may increase the frequency of integration by promoting homologous recombination such as is found, for example, with yeast transformation (Hinnen *et al.*, 1978). Recently, a comparison of the transformation rate of tobacco protoplasts by electroporation with a plasmid vector containing a *nos/neo* gene and the same vector additionally containing a 1.2 kbp section of 5'-sequence of the *N. tabacum* chromosomal ribulose 1,5-bisphosphate carboxylase gene, showed that the presence of the homologous sequence had no influence on the transformation efficiency (Lurquin and Paszty, 1988), suggesting that homologous recombination did not constitute a major mechanism of integration. Similarly, in many mammalian transformation studies, it has been found that integration of foreign DNA takes place by non-homologous recombination (Pellicer *et al.*, 1980), even when homologous sequences are present in the transforming DNA. (For example, ribosomal DNA included in the vector used in mouse cell transformation (Steele *et al.*, 1984) and the use of a β-globin gene in a transgenic mice study (Lacy *et al.*, 1983)). In contrast to these findings, Smithies *et al.* (1985) demonstrated the targeting of DNA sequences into the human chromosomal β-globin locus by homologous recombination and Lin *et al.* (1985) showed the reconstruction of a functional thymidine kinase gene (tk) in mutant tk-

mouse cells transformed with plasmid containing completely functional tk gene sequences. These latter results indicate that targeted gene insertion utilising DNA sequence homology is possible.

The apparent absence of any hybridizing bands for T373 and the absence of *neo* hybridizing bands for T375, suggests that the selective marker gene was absent from these transformants. However, the progeny of these plants demonstrated kanamycin resistance and leaf-pieces of the mature transformants produced callus under kanamycin selection. The explanation offered for transformant T309 that exhibited similar characteristics (see previous section), may also apply here.

The control transformants, T360 and T361, showed only the presence of *neo* hybridizing sequence, as expected. These transformants were derived from protoplasts incubated with circular DNA molecules. Although the exact nature of the integration events cannot be ascertained from these results, the relatively large hybridizing fragments obtained (20 kbp and 13 kbp respectively) are perhaps due to concatamerization. pNosNeo contains only one Bam HI site; it is thought that circular DNA is subject to linearization once introduced into the plant cell and thereafter, modified by the recombination and replication processes that are implicated with introduced linear DNA, so it is possible that the modified plasmid did not contain Bam HI sites.

In general, the intensity of the hybridizing bands was low, this was despite the use of high specific activity probes (above 10^8 cpm/μg) and autoradiographic exposure times of many weeks. The tobacco genome is relatively large at 1.4×10^{10} bp compared to, for example, tomato (7.15×10^8 bp; Young *et al.*, 1988), maize (3×10^9 bp; Helentjaris *et al.*, 1988), or *Arabidopsis thaliana* (7×10^7 bp; Chang *et al.*, 1988). Consequently detection of low copy number sequences is difficult with the amount of genomic DNA used per sample track.

4.6.3 Transmission of foreign DNA to transformed plant progeny

The integration of foreign DNA sequences has been demonstrated in the transformants derived from the *Agrobacterium* and direct gene transfer techniques. However, it is vital that once integrated, the foreign DNA is stable in the genome and is faithfully transmitted to the progeny of the transformants, that is, the inserted DNA is both mitotically and meiotically stable. This is especially important in genetic engineering of commercially important plant species as clearly the introduced genes must remain present and intact through many generations.

Evidence for the mitotic stability of the inserted DNA in the transformants described in this study, is found in the successful regeneration of all leaf pieces on kanamycin-containing media. This, of course, only tests for the presence of the *nos/neo* gene in the mature tissues of the plants and does not indicate whether the *leg* gene, if present, is also conserved. The kanamycin selection allowed only those cells that expressed the resistance gene to proliferate, but as all leaf-pieces tested produced callus and the gene was present in the gametes (see next paragraph), it can be seen that the introduced DNA was not lost during the development of the plant. In general, a high degree of mitotic stability is found in transformants (for example by comparison of Southern blots of different genomic DNA samples individual plants) (Negrutiu *et al.*, 1987).

Evidence for the meiotic stability of the introduced gene was provided by the demonstration of kanamycin resistance in the progeny derived from self-fertilized transformants of both *Agrobacterium*-transformed and direct DNA uptake transformed tobacco plants. In the majority of cases, the ratio of resistant to sensitive phenotype was 3:1, indicating that the trait was transmitted in a Mendelian fashion. Such Mendelian inheritance of introduced genes has previously been observed in the majority of transformants produced using the transformation techniques of *Agrobacterium* infection (DeBlock *et al.*, 1984; Horsch *et al.*, 1984;

Budar *et al.*, 1986; Wallroth *et al.*, 1986; Muller *et al.*, 1987; Peerbolte *et al.*, 1987a), and direct gene transfer (Potrykus *et al.*, 1985; Hain *et al.*, 1985; Peerbolte, 1985). The Mendelian characteristics of the inheritance of the transforming DNA sequences suggests that integration of foreign DNA takes place at a single chromosomal locus, even if multiple copies of the introduced gene are present. Mouras *et al.* (1987) demonstrated by *in situ* hybridization to metaphase chromosomes of tobacco transformants containing 3-5 copies of the kanamycin-resistant gene that the hybridization of the foreign gene probe was localized at a single point on only one pair of homologous chromosomes with two independent transformants having the inserted genes on different chromosomes.

Additional genetic crosses, for example a cross with untransformed SR1 plants, would confirm the number of genetic loci at which the kanamycin resistance genes are located and would complement the gene copy number data derived from the molecular genetic analyses.

4.7 Expression of the legumin gene in transformed plants

4.7.1 Transcription and post-transcriptional processing

Despite a high background of non-specific hybridization, legumin mRNA was detected in five of the seven transformants (see Section 3.9) that contained the intact chimeric legumin gene. No legumin hybridization was detected to the untransformed tobacco control sample. The presence of the legumin transcripts in the transformed tobacco leaves indicated that the *nos* promoter used had directed the transcription of this foreign gene in a tissue environment in which the native legumin gene is not ordinarily expressed. The Northern blot analysis was performed only on leaf tissue RNA, it could be inferred that the transcription of the chimeric gene if constitutive would also be found in the other plant tissues.

The level of transcription appears to be low as the hybridizing bands are barely visible on the autoradiographs, despite the use of high specific activity probes (up to 8 x 10^8 cpm/μg) and long exposure times. The pea cotyledon sample (Fig. 34a) was estimated to represent ~500 pg leg mRNA; the *leg* message was taken to be between 2% and 7% of the poly A^+ fraction (Gatehouse *et al.*, 1986), which is 1% of the total pea cotyledon RNA (Evans *et al.*, 1979). If the hybridizing bands of the tobacco transformants are assumed to be of the order of one hundredth the intensity of the pea mRNA standard, then they represent ~5 pg per 10 μg total tobacco leaf RNA. (This estimate of 5 pg per track seems sensible when compared with the observation that ~1 pg *leg* mRNA is the limit of detection by this Northern blot hybridization (Gatehouse *et al.*, 1982; Evans, I.M., personal communication). It would seem then that the transcription of the chimeric legumin gene in the heterologous environment of the tobacco leaf tissue is of the order of 10^3 times less efficient than in its native seed environment under the control of its tissue-specific promoter. However, the degree of hybridization in the Northern blots indicates the steady-state level of messenger RNA, the stability of the message and the post-transcriptional processing may affect this level and hence this estimate of transcriptional activity. For example, a high transcription rate may be masked by relatively short-lived transcripts. Messenger RNA levels of chimeric genes driven by the nopaline synthase promoter, have in general been reported as representing 0.001% of the total mRNA in transformants (Jones *et al.*, 1985), so the levels estimated in this study appear to be low. Between transformant variation in the *leg* mRNA levels is also apparent. For example, T306 gave a band 2 or 3 times as intense as T305 (Fig. 34a), suggesting a higher level. Such differences may be due to so-called "position effects", that is the location of insertion of the foreign DNA in the genome. More efficient transcription may be possible at some

chromosomal location than at others (Jones *et al.*, 1985; Nagy *et al.*, 1985; Gelvin, 1987).

The legumin transcripts of the transformants appeared to have migrated further in the gel than the pea standard legumin message, suggesting a size reduction of ~150 bases. The high intensity of the pea standard track makes determination of the position of the message band difficult but accurate measurements of these bands and the same filter exposed for a much shorter time (Fig. 34a) confirmed that the transcripts in the transformants were smaller. The *leg* A coding region used was not altered from the naturally occurring gene, apart that is for the first few base-pairs due to the fusion with the *nos* promoter. This resulted in the net loss of 3 bpr, clearly not sufficient to account for the size reduction observed.

It would seem that the post-transcriptional process of intron-removal occurred, as a failure to remove the three introns would have resulted in a larger transcript by some 275 bpr (Lycett *et al.*, 1984). The legumin introns have been shown to be correctly excised from transcripts of the *leg* A gene in transformed tobacco seeds (Ellis *et al.*, 1988) and from other pea genes in transformed tobacco leaf tissue (for example, the ribulose 1,5-biphosphate carboxylase gene (Nagy *et al.*, 1985)). The introns conform to the 3' and 5' intron boundary "GT/AG" splice rule of Breathnach *et al.* (1978) and are in general agreement with the concensus intron/exon junction sequence of plant genes (Slightom *et al.*, 1983; Lycett *et al.*, 1984).

The loss of 150 bpr may have been due to the absence of the poly-A tail. Over 100 Adenosine bases are added to the 3'-terminus of the transcript. The addition of this poly-A tail is prompted by the polyadenylation signal motif 'AAUAAA'. The chimeric legumin gene used here contained an unaltered 3'-non-translated region which therefore had the naturally occurring legumin

polyadenylation signals (Lycett *et al.*, 1983). The results of the Southern blot analyses (Section 4.6.1) indicated that the legumin DNA sequence was unaltered in these transformants, so these signals were present in the integrated genes. It is unclear why the polyadenylation was not initiated or completed, many other transcripts have been correctly polyadenylated in a heterologous environment. For example, a phaseolin gene of *Phaseolus vulgaris* was polyadenylated when expressed in sunflower tissues (Murai *et al.*, 1983) and tobacco seed (Sengupta-Gopolan *et al.*, 1985).

4.7.2. Translation and detection of legumin protein

Before the analysis of the detection of legumin protein in the tissues of transformed plants, it is necessary to consider whether the translation of the introduced chimeric gene may have been affected by the DNA sequence changes introduced during its formation.

The construction strategy employed has, by necessity, altered the DNA sequence around the translation start codon in the chimeric gene from that of the original *leg*A gene. Analysis of the final DNA sequence of the *nos/leg* fusion region, shows that whereas the naturally occurring N-terminal *leg*A peptide begins "Met, Ala, Lys, Leu....", the chimeric *leg*A sequence predicts "Met, Glu, Leu .....". There has been an overall deletion of one amino acid caused by the replacement of alanine and lysine with glutamic acid. The N-terminal of the peptide contains a "leader" or "signal" sequence (Blobel and Dobberstein, 1975; Kreil, 1981; Verner and Schatz, 1988), that is required for the transport of the polypeptide across a membrane. This "signal" directs the co-translational secretion of the legumin polypeptide into the endoplasmic reticulum (Higgins and Spencer, 1981; Spencer, 1984; Croy and Gatehouse, 1985). Signal peptides contain a high proportion of hydrophobic amino acids; however, there is a variation seen in the type of amino

acid residues found in the peptide (von Heijne, 1985), and it was thought that the substitution of a neutrally charged (Ala) and positively charged (Lys) amino acids with a negatively charged hydrophilic (Glu) amino acid, was unlikely to have an adverse affect on the signal. The reduction in length of the signal peptide by one residue (from 21 to 20 amino acids) was similarly thought to have little effect, as other storage protein gene signals are of different lengths, for example, maize zein genes at 21 and 20 residues (Messing *et al.*, 1983) and pea vicilin (50 K Da) at 27 residues (Spencer, 1984). In general, eukaryotic signal peptides range from 15 to 30 residues in length (von Heijne, 1985).

The use of a synthetic linker molecule and a slightly truncated *leg*A coding sequence, resulted in an alteration in the DNA sequence flanking the initiation codon in the chimeric *leg*A gene. It has been shown that the sequences surrounding the initiation codon are not random in eukaryotic genes; a study of eukaryotic mRNA sequence has shown, for example, that almost all functional AUG triplets have a purine (usually A) 3 bases downstream and are followed by a purine (usually G) (Kozak, 1981; 1986). However, Lutcke *et al.* (1987) have suggested that in plants the nucleotide in position -3 (relative to the A of the AUG) is less important but that consensus is found for positions +4 and +5. They proposed the plant consensus sequence 5'-AACAAUGGC-3'. Examination of the sequences of the *nos*, *leg*A and *nos/leg*A chimeric gene reveals that, whilst in common with the *nos* and *leg*A genes and in agreement with this plant consensus, the chimeric gene has a G at position +4, there is an A at position +5 (compared with C in the others). It should also be noted that the bases downstream of the ATG in *leg*A (CTTC ATG GC) differ entirely from this consensus.

It would seem then that the composition of the bases flanking the initiation codon in the chimeric gene do not differ dramatically from other plant sequences

and so the translational efficiency of the message was not expected to have been adversely affected.

The SDS-PAGE analysis of transformed *Nicotiana* leaf tissue protein extracts (Section 3.10.1) did not reveal legumin protein subunits with any of the transformants, although this is perhaps not surprising as a high level of expression would be required to produce a protein level compatible with that of endogenous proteins. The differences in protein banding patterns between the transformants, most notably T300, and the control sample, were most likely attributable to the effects of the insertion of foreign DNA into the genome. The random insertion of DNA may have resulted in the over-expression of an endogenous gene due, for example, to the alteration of regulatory sequences.

It was also not possible to detect legumin protein in Western blots of transformed plant protein samples (Section 3.10.2). It was shown that the proteins had been transferred to the nitrocellulose; however, it is possible that the detection procedure was not fully optimised. For example, the polyclonal pea legumin antibodies used were thought not to be particularly efficient (A. Kang, personal communication); indeed, their use has since been replaced with the more efficient monoclonal pea legumin antibodies (for example, Shirsat *et al.*, 1989). These antibodies were also used in the ELISA assays.

The inability to detect legumin protein in the leaves and seeds of the transgenic plants produced in this study, did not necessarily indicate that legumin protein was not produced in the transformants, the immunodetection procedures employed formed a preliminary analysis in that the protocols were not fully optimized.

There are a number of possible reasons why the failure to detect legumin in the transformed tissues was perhaps not surprising. Firstly, with regard to the ELISA procedure itself, binding studies performed by Edwards (1988) using the

microtitre plates used in this study with *Nicotiana tabacum* leaf protein samples, showed that the maximum binding capacity of the wells was ~1.5 µg/ml; that is, 300 ng when 200 µl protein sample is loaded. The results obtained for the legumin standards using the ELISA procedure showed that values below ~10 ng/ml legumin were not detectable (2 ng per well for the 200 µl sample). Therefore, an expression level producing a legumin protein content of greater than ~0.6% of the total soluble protein was required to give detectable quantities of legumin. This compared with, for example, an estimated expression level of 1% of the total soluble seed proteins obtained with the pea legumin A gene, under the control of its own promoter sequences in transgenic *Nicotiana plumbaginifolia* seeds (Ellis *et al.*, 1988).

The *nos* promoter, although containing all the sequences necessary for constitutive expression (An *et al.*, 1986), is now recognised as not being a particularly efficient promoter. It has recently been demonstrated to be significantly less efficient than the promter of the Cauliflower Mosaic Virus 35S subunit (CaMV 35S) (Odell *et al.*, 1985). A comparison of the level of expression in transgenic *Petunia* plants containing the *nos* and CaMV 35S promoters linked to a common reporter gene (*neo*) (Sanders *et al.*, 1987), showed that the *neo* transcript levels were 30-fold lower in transformants with the *neo* gene under the control of *nos* promoter and leader sequences than with those of CaMV 35S and that the *neo* enzyme activity was 110-fold lower. Other studies have yielded similar results. For example, Harpster *et al.* (1988) showed that in transformed tobacco callus tissue, the *nos* promoter was 20-25 times less efficient than the CaMV 35S promoter. Recently, a comparison of the *nos* and CaMV 35S promoters using a β-galactosidase reporter gene in transgenic tobacco plants, showed that the *nos* promoter gave barely detectable expression in transformed leaf tissue, whereas the CaMV 35S

promoter construct gave significantly higher levels of expression (Teeri *et al.*, 1989).

Edwards (1988) using a chimeric pea lectin gene fused to the CaMV 35S promoter demonstrated a maximum expression level of 0.8% of total soluble protein in leaf samples of *Nicotiana tabacum* transformants. In view of the relative efficiencies of the *nos* and CaMV 35S promoters, an expression level of significantly less than 0.8% might be anticipated with the use of the *nos* promoter.

Another factor that may have contributed to a lower level of expression of the chimeric legumin gene, is suggested by recent evidence that the *nos* promoter is not entirely constitutive in that it has been seen to produce various levels of expression in different transformant tissues. For example, higher levels of expression have been demonstrated in root tissue than in the leaf tissue of transformants (Simpson *et al.*, 1985; Teeri *et al.*, 1986). A study of the organ-specific and developmental regulation of the *nos* promoter in transgenic *Nicotiana tabacum* plants (An *et al.*, 1988) revealed that the promoter was highly active in lower parts of the transformants, but that this decreased in the upper (younger) parts and that this gradient of expression was found until the flowering stage when the promoter strength decreased significantly. Many of the leaf samples of the transformants in this study were taken at or after the time of flowering when the plant had become established and so, these samples may be expected to demonstrate low expression levels.

So, if the legumin protein was produced, it was expected to be at barely detectable levels. A further possibility for the inability to detect legumin protein in the leaves of the transformants, was that if produced the legumin might be subjected to degradation. Legumin, as a seed storage protein, is a substrate for proteolytic enzymes. In the leaf tissue environment it may well be rapidly degraded. Other proteins produced in transformants may not be subject to such

degradtion and may therefore be more easily detected. For example, the chimeric pea lectin gene studied by Edwards (1988) produced detectable quantities of lectin in transgenic tobacco leaf tissue; this protein is naturally resistant to proteolytic degradation. It is possible that the signal sequence present at the N-terminal of the legumin protein, that in the pea seed environment directs its secretion into the protein bodies, may in the leaf tissue direct its secretion into the cell vacuole where it would be rapidly broken down. The legumin protein might be expected to accumulate with less degradation in the more 'natural' environment of the tobacco seed tissue. However, foreign seed storage proteins produced in transgenic tobacco seeds have been shown to be subject to degradation (for example, β -phaseolin of french bean - Sengupta-Gopalan *et al.*, 1985).

No legumin protein was detected by the ELISA procedure employed here. The use of immuno detection protocols of greater efficiency, capable of detecting much lower levels of expression, would be of great value in more accurately assessing the presence of legumin in these transformed tissues. Consideration of time at the end of this project prevented improvements, such as the full optimization of the protocols by, for example, alteration of the antibody dilutions or protein concentrations, to be fully investigated. An improvement would be the use of the more efficient monoclonal pea legumin antibody in place of the polyclonal antibody used in this study. Such an antibody is now available and has recently been used successfully to detect pea legumin in seed tissues of transgenic tobacco plants (Shirsat *et al.*, 1989).

The study of the expression of seed storage protein genes in transgenic plants has hitherto generally been confined to the investigation of the regulation of expression of a gene under the control of its own expression signals in transformants. These gene transfers demonstrated in many cases the ability of the transferred genes to be correctly expressed in the heterologous environment of a

different plant species. The intact pea legumin A gene transferred to *Nicotiana plumbaginifolia* showed correct tissue-specific expression (Ellis *et al.*, 1988; Shirsat *et al.*, 1989), as did the legumin B gene of *Vicia faba* in transgenic tobacco (Baumlein *et al.*, 1988). The developmentally regulated expression of β-phaseolin, a major storage protein of *Phaseolus vulgaris* (French Bean), was shown in transgenic tobacco seeds; the protein produced was found only in the embryonic tissues and was correctly processed (Sengupta-Gopalan *et al.*, 1985; Greenwood and Chrispeels, 1985). Studies involving the transfer of genes to petunia, showed that a β-conglycinin gene of *Glycine max* (soybean) (Beachy *et al.*, 1985; Bray *et al.*, 1987) and a Zein gene of *Zea mays* (maize) (Williamson *et al.*, 1988) were correctly expressed; they demonstrated tissue-specific expression and that the polypeptides produced were correctly assembled into multi-meric proteins. The correct expression of the tuber-specific storage protein patatin of potato has also been shown in transgenic tobacco plants (Rosahl *et al.*, 1987). The situation may not necessarily be as clear-cut as the above examples imply. The correct expression is not invariably found with gene transfers of this nature. For example, Weng *et al.* (1988) in investigating the expression of a maize seed storage protein gene in transgenic petunia plants, found that the expression was not entirely restricted to the seeds. They suggested the monocot regulation signals were not readily recognized in the dicot environment; indeed, Keith and Chua (1986) have suggested that the processing of monocot pre-mRNA in tobacco transformants is less efficient than those of dicots.

Studies with transgenic tobacco and petunia plants have also shown that in addition to tissue-specificity, gene expression can be correctly induced in a heterologous environment. For example, the photo-regulation of a pea ribulose 1,5-bisphosphate carboxylase gene (rbcS) in petunia and tobacco transformants has been demonstrated (Nagy *et al.*, 1985), as has the thermal inducibility of a soybean

heat shock gene in tobacco transformants (Baumann *et al.*, 1987). Indeed, the light-regulated and organ-specific expression of a monocot gene, that encoding the major chlorophyll a/b binding protein (Cab) of wheat, has been demonstrated in transgenic tobacco plants (Lamppa *et al.*, 1985), showing that it is possible for monocot gene to be transferred and successfully expressed in dicot plant species.

The use of reporter genes such as chloramphenicol acetyltransferase (CAT) or Neo fused to promoter sequences of plant genes in transgenic plant studies, has proved a useful technique in investigating the sequences responsible for tissue-specific and/or inducible expression. For example, with wound-inducible expression of a potato inhibitor gene (Thornburg *et al.*, 1987), light-inducible expression of the rbcS gene of pea (Herrera-Estrella *et al.*, 1984) and the Cab gene of wheat (Simpson *et al.*, 1985) and petunia (Jones *et al.*, 1985).

4.8 Comparison of transformation methods studied

The evidence presented here and that obtained in numerous published plant transformation studies has indicated that *Agrobacterium*-infection has been developed so that it is now an efficient and reproducible technique for achieving gene transfer. For those plant species that are susceptible to *Agrobacterium* infection, this technique currently constitutes the method of choice as the transferred genes are rarely extensively rearranged and in general are faithfully integrated into the plant genome in a predictable manner. Once integrated the transferred gene is stabily maintained and transmitted to the progeny. With the availability of transformation vectors such as the binary vectors with selectable marker genes and restriction endonuclease site arrays for foreign gene insertion, gene transfer to plants has now become a matter of routine using this technique. However, the major disadvantage of this method is the limitation imposed by the restricted number of plant species that can be infected by *Agrobacterium.* A

number of commercially important plant species, most notably those of the Gramineae, are not susceptible to *Agrobacterium*-infection and so alternative transformation techniques are necessary in these cases.

The direct gene transfer technique offers a valuable alternative to *Agrobacterium*-infection as it does not rely on the bacterial/plant interaction. In early studies the frequency of transformation was low, but with increased efficiency attained by the use of such techniques as electroporation, reasonably efficiency has been achieved with many plant species, in some cases transformation rates have equalled those achieved with comparable transformation with *Agrobacterium*. An important advantage of this method over that of *Agrobacterium*-infection is that transformation vector construction can be simplified in that co-transformation of genes is possible; indeed, with transformation using linear DNA fragments, only the genes of interest need be transferred obviating the need for complex vector construction. A disadvantage of the technique is the requirement for efficient protoplasting and plant regeneration protocols from transformed protoplasts. When such protocols are available and gene transfer is achieved, it is found that while in common with *Agrobacterium*-derived transformants, the inserted genes are stably maintained and inherited, the inserted DNA is often subject to structural rearrangement, which is extensive in some cases. However, despite these shortcomings, the directed gene transfer technique has been successfully applied in the production of many transgenic plants, including those of crop species such as rice, that are not amenable to the *Agrobacterium* technique.

The techniques of microinjection, to enable the direct uptake of DNA by pollen tubes or eggs *in vivo*, were demonstrated to have potential as means of achieving gene transfer without the need for tissue culture plant regeneration. Further work in these areas of gene transfer may result in the establishment of alternative techniques for the production of transformants of species for which

other techniques prove difficult. Research into other possible methods of gene transfer has already resulted in the emergence of a technique that has tremendous potential. The bombardment of plant cells with microprojectiles coated with DNA solutions has been demonstrated to result in stable genetic transformation of plant cells (Klein *et al.*, 1987). The main advantage of this technique as an alternative plant gene transfer system, is that intact cells can be used, eliminating the requirement for protoplast generation (Klein *et al.*, 1988a; 1988b).

The transformation techniques of *Agrobacterium*-infection and direct gene transfer to protoplasts are currently well established methods and offer tremendous potential for the transfer of genes between widely divergent plant species. Many genes have already been transferred and their expression in homologous and heterologous genetic environment studied. Plant genetic transformation has now become not only a technique for the transfer of desirable genes to plants, but also a valuable analytical technique, in that the expression of plant genes can be studied following the genetic manipulation of their DNA sequence. Regulatory sequences such as enhancer or silencer elements or promoter sequence motifs, can be deleted or inserted into genes and the effects on the expression studied in transgenic plant tissues (Schell *et al.*, 1987).

The future development of plant gene transfer methods that allow reproducibly efficient plant transformation and directed gene expression, will allow a much greater understanding of the molecular biology of the regulation of plant gene expression and in so doing provide valuable techniques for the genetic improvement of plant species.

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