DNA INSERTION MUTAGENESIS IN HIGHER PLANTS

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To my wife and daughter for their inspiration and motivation,
and my parents for many years of encouragement and support.

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ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	1-Naphthaleneacetic acid
BAP	6-Benzylaminopurine
IAA	Indole-3-acetic acid
Kn	Kinetin
Km^r	Kanamycin resistant
Km^s	Kanamycin sensitive
NPT-II	Neomycin phosphotransferase-II
npt-II	Neomycin phosphotransferase-II gene coding region
nos	Nopaline synthase promoter
CTAB	Cetyltrimethylammonium bromide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
Cx	Cefotaxime
EMS	Ethylmethane sulfonate
ENU	N-ethyl-N-nitrosourea
kb	kilobase pair
bp	base pair
MS	Media based on that of Murashige and Skoog (1962)
NR^-	Nitrate reductase defficient mutant
vir	virulence genes (Ti and Ri plasmid)
onc	Oncogenicity genes (Ti and Ri plasmid)
Mo-co	Molybdenum cofactor (Nitrate reductase)
nia and *cnx*	Classes of nitrate reductase difficient mutant
XDH	Xanthine dehydrogenase
NADH	Nicotinamide adenine dinucleotide (reduced form)
S	Sedimentation coefficient
F_1, F_2,	1st and 2nd filial generations
R_o, R_1, R_2	Regenerated plant, 1st and 2nd filial generations

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

Plant transformation technology broadens the possibilities of transferring genes between unrelated organisms (between plants, and from other organisms to plants) and also creating novel genetic information by specific alteration of genes prior to transfer. The development of this technology may allow in the future the directed and highly specific manipulation of genetic material to both improve and speed up plant breeding programmes. However, applications of these techniques are often impeded by the lack of understanding of a genotype at the molecular level, including even the gene products involved in the expression of a particular phenotype, as well as the unknown identity and character of the genes for most complex plant traits. Making the link between phenotype and genotype is thus a problem in many higher organisms. The isolation of specific mutants is often of paramount importance in understanding complex genetic traits. However, defining a genotype at the biochemical or gene expression level is often extremely difficult using conventional, molecular biology and recombinant DNA technology.

Genetic techniques for gene recognition include mutagenesis or 'tagging' interesting loci with defined nucleic acid sequences, and subsequent cloning of affected genes. Thus, a second use for gene transfer technology may be to help to identify previously uncharacterised genes for use in plant breeding based on their mutagenesis by DNA insertion. Such

techniques have been successfully applied, particularly in prokaryotes, to create useful mutants in many experiments aimed at understanding the genetic regulation of an interesting phenotype. Initially, available techniques only allowed the random mutagenesis of a population of organisms, followed by screening and selection for mutants with a characteristic phenotype. The mutated site was then localised by complementation and recombinational analysis with other mutants, or by physical mapping. The cloning of genes mutated by insertion of specific DNA fragments becomes possible with the advent of techniques such as, the specific cleavage and enzymatic manipulation of DNA.

1.1 Mutagenesis in plants

The effort of increasing crop quality, productivity and reliability by developing new and improved cultivars is a difficult task faced by plant breeders. Ways are continually being sought to bring together desired genes and then select for desirable phenotypes. To date, the generation of variability and genetic recombination, coupled with selection processes, still remain the essence of plant breeding. Variability can arise as a result of mutation, which is defined as any heritable alteration in the genetic material, including changes in the number of chromosomes, changes in the gross structure of the chromosomes and changes within the genes themselves.

Aneuploid and polyploid individuals are the result of a change in chromosome number by the loss or acquisition of one or more chromosomes or complete sets of chromosomes respectively. Many polyploid plants are larger and more vigorous than the corresponding diploids, but they sometimes have reduced fertility. Structural changes of the chromosomes are caused by chromosome breakage which results in rearrangements including deletion or deficiency, duplication, inversion and translocation. Point mutations cause changes within the gene and can be classified as either base substitution mutations due to the replacement or substitution of one base pair for another or frameshift mutations, due to the addition or deletion of one or two base pairs from the DNA. The occurrence of these mutations can be due to spontaneous mutations which arise naturally in populations as a response to enviromental stresses, and are acted on by natural selection. Continuous selection may eventually produce a new variety which is better adapted to the environment. This process can be speeded up by artificial mutagenesis using chemical and physical mutagens.

Figures 1.1 and 1.2 show how mutagens can be applied to mutagenise different stages of development of either vegetatively (asexually), or sexually propagated plants. A widespread interest in the potential of artificially-induced mutations in plant breeding developed after the discovery of effective mutagenic agents. Using intact plants, mutagen

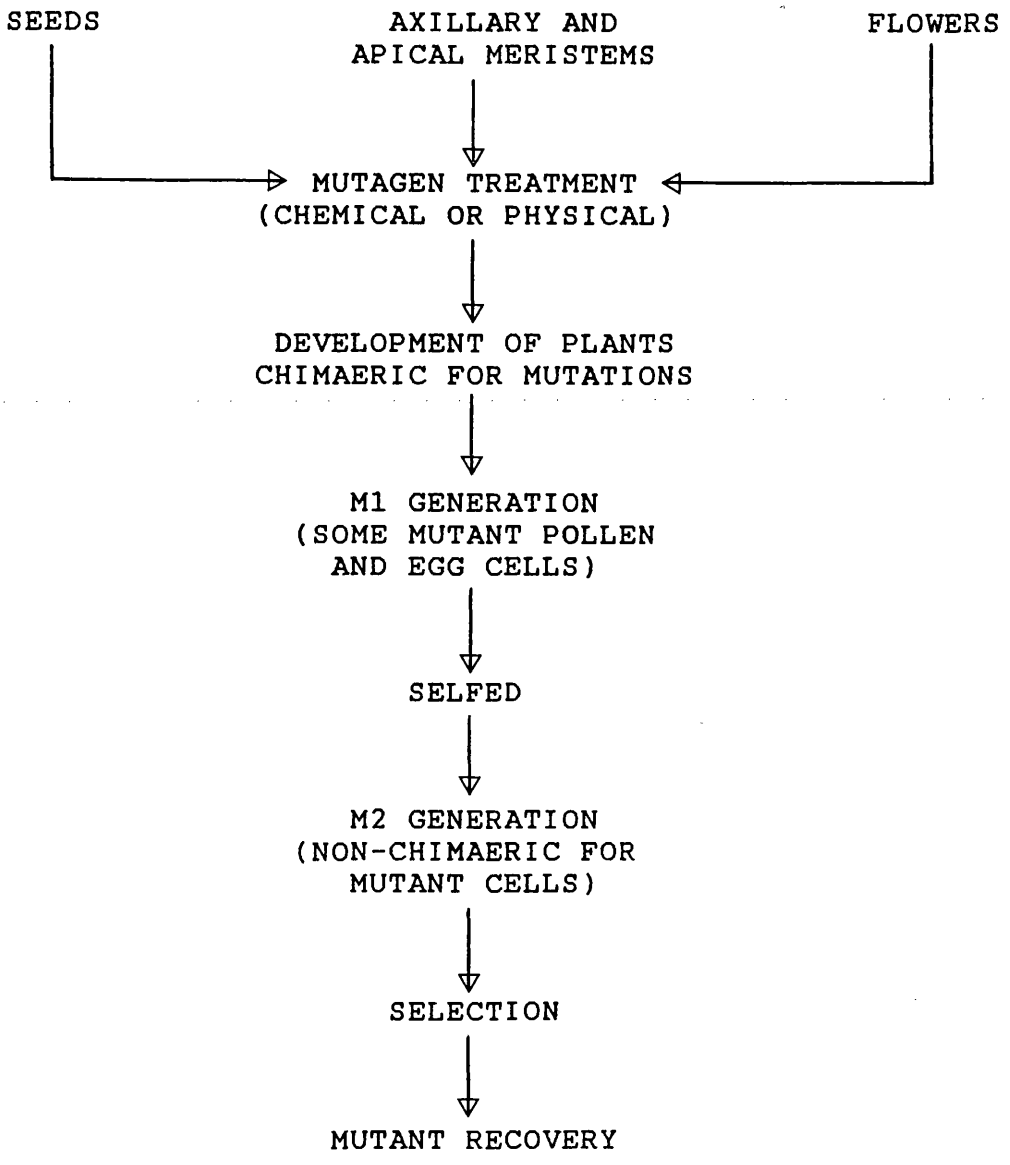


Fig. 1.1 RECOVERY OF MUTANTS AFTER *IN VIVO* MUTAGENESIS OF WHOLE PLANT ORGANS.

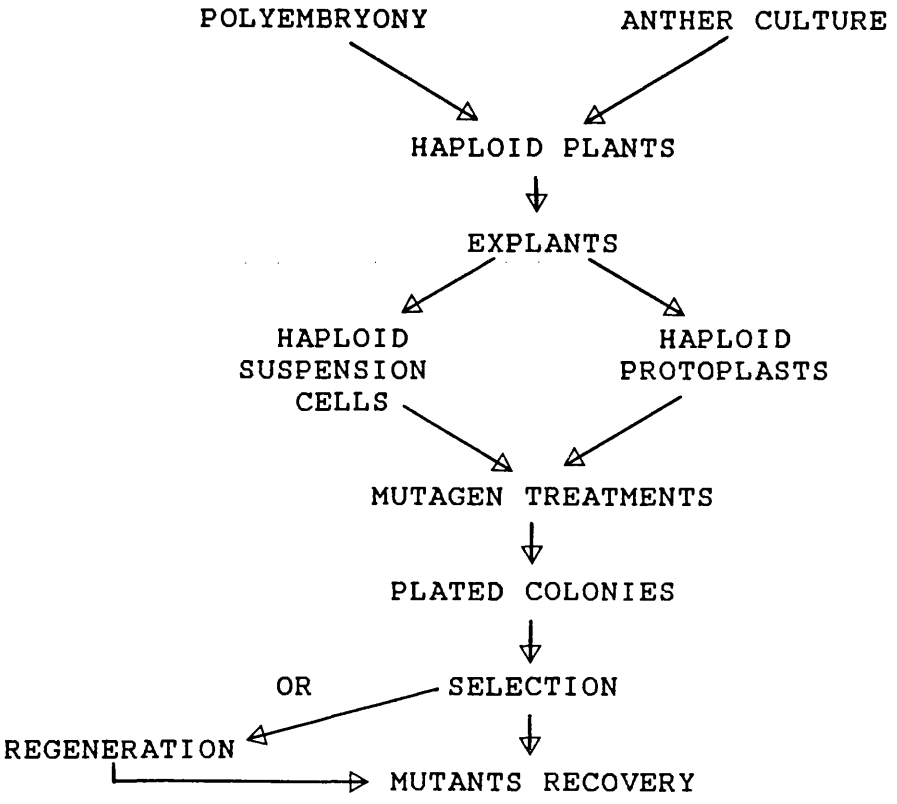


Fig. 1.2 MUTANT RECOVERY FOLLOWING *IN VITRO* MUTAGENESIS OF CULTURED PLANT CELLS.

treatment is applied to meristematic tissues, such as axillary and apical meristems, or seeds, followed by the regeneration of a whole plant. The plant (M_1) is selfed and seeds are collected and germinated. Selection for mutated phenotypes induced by the mutagen treatment is applied on the second mutant generation which is the M_2 progeny (Fig. 1.1).

In the case of plant cell cultures, selection can be applied immediately after the treatment of large numbers of plated cells or colonies (Fig. 1.2). Although direct selection in cell suspension cultures is possible, it often results in extensive 'cross feeding', as cells are bathed in liquid medium. Selection in liquid medium may produce a more heterogeneous cell population, because identification of individual colonies is not possible. Hence, plating techniques which immobilise and separate cell clones have been used most frequently. These reduce 'cross feeding' of beneficial or toxic compounds and it is also possible to retrieve more than one variant colony from a single selection plate (reviews, Flick, 1983; Maliga, 1984).

The use of mutagens, physical or chemical, increases the frequency of mutation to a useful experimentation level. Chemical mutagens such as ethylmethane sulfonate (EMS), sodium azide and nitrosoguanidine, or physical mutagens such as the ionising radiaton of gamma and X-rays, as well as UV-light, are amongst the most commonly used agents for this purpose because of their effectivenes. There have been no data reported showing

the superiority of physical over chemical mutagens or vice versa, thus the choice of using one type may be just a matter of convenience. Using a physical mutagen eliminates the need to manipulate mutagenised cells to remove mutagen, and it is easier to calibrate the optimum dosage needed for mutation induction. However, specialised equipment is needed and, besides being very expensive, it is not readily available. When using chemical mutagens, extensive washing is needed to remove traces of the mutagen from the treated cells. This may prove deleterious if fragile cells such as protoplasts are involved. It also presents problems in disposing of waste and decontaminating glasswares.

Mutagen treatment causes modifications of nuclear or organellar DNA which often results in genotypic or phenotypic changes in the organism. If the mutagenic treatment applied to an organism is sufficiently high it may prove lethal, or if it survives, there may be severe damage. With a lower sub-lethal dose there may be no visible damage but the organism may be sterile, either permanently or temporarily. Treatment with mutagens, physical or chemical, induces both point mutations and chromosome rearrangements. In the case of a physical mutagen, the frequency of each of these aberrations, is directly proportional to the radiation dose but independent of the way the dose is administered. The effects are cumulative; a low intensity of irradiation given for a long period of time (chronic irradiation), a high intensity given for a short

period of time (acute irradiation) or two or more small doses given several hours apart (called dose fractionation) all produce the same number of mutations (Smith-Keary, 1975). Thus, by considering the maximum effect with minimum damage, the optimum dosage level of the mutagens is achieved.

Plant mutagenesis has produced numerous types of mutants which are useful in the study of plant physiology, biochemistry, molecular biology and genetics. Mutations can be classified according to their most conspicuous effect on the phenotype as; visible mutations, where the effect of the mutation is seen in the phenotype of the organism; biochemical mutations, leading to the loss of a specific biochemical function resulting in inhibition of certain biochemical pathway; lethal mutations, inevitably causing death of the organism at certain stage in development; and conditional lethal mutations which are, viable under one set of conditions but lethal under other conditions. The type of mutations which may be isolated and selected often depends on the mutagenesis procedures.

1.2 Traditional mutagenesis *in vivo*

The more traditional procedures in plant mutagenesis involve treatment of seeds, meristems or seedlings with mutagens to generate new variants (Fig. 1.1). Mutagen treatment applied to multicellular tissues such as these, often forms a sector of mutated cells in organs subsequently

developing after mutagenesis. Most of the tissues derived from this sector will be mutant and the organism will have a mixture of cells of different genotypes and will therefore be chimaeric. For example, irradiation of a seedling can produce a flower with coloured petals on one side and white petals on the other. Unless the organisms can be vegetatively propagated such mutations cannot be maintained. Without the production of an easily recognisable phenotype, the induced mutations will not be obvious. If a mutation capable of immediate expression is induced during the early divisions of the embryo however, a large mutated sector is formed and can be accurately scored for the presence of the mutation. Thus, generally seeds are used as the target for mutagenesis for their obvious advantage of being easy to handle in large quantity, as well as a higher probability of mutagenising tissues that give rise to gametic cells.

Seeds are treated either by soaking in a solution of chemical mutagen, or by irradiating dry or imbibed seeds with ionising radiations. The mutagenised seeds (M_1) are allowed to germinate and develop into mature plants. Unless the mutant allele induced in the mutagenised seeds has a dominant expression it cannot be generally detected in the somatic cells in which it occurs because a large sector of the M_1 plant is chimaeric for any induced mutation. Its effect can only be observed in the later segregating generations, providing the mutation is present in tissues giving rise to gametic cells.

Thus, the segregation of the recessive mutants in the mutagenised seeds will be seen only in the second mutant generation (M_2).

A wide variety of mutant forms have been recovered from segregating populations of treated material. However, the mutant phenotypes that can be screened and selected by traditional mutagenesis on major crop plants have so far been mainly restricted to ones that are easily visible. Some of the most important visible mutations have been selected at the seedling or mature plant level in the more important crop plants such as barley, wheat, maize and rice (Oono, 1981; Wych and Rasmussen, 1983). They include floral development (early flowering), plant height and vigour, awn and tiller number, maturity date, grain weight, shape, size and contents, and many other characteristics that are agronomically important. In barley for example, some of the more beneficial mutations that have been produced by radiation treatment include early ripening, stiff straw, increased diastatic activity, larger grains and disease resistance (Williams, 1964). Working with a model system such as *Arabidopsis thaliana*, on the other hand, enables selection of wide varieties of mutations. The small size of its seeds (which can be produced in vast quantity) makes manipulation similar to those used for the mutagenesis of microbes possible. Chemicals toxic to plants have been used successfully to isolate mutations in genes coding for enzymes involved in various biochemical pathways (Rédei and Acedo,

1976; Braaksma and Feenstra, 1982; Carrol *et al.*, 1985). Many useful mutants which appear to specifically affect photosynthesis or photorespiratory carbon metabolism have also been identified and isolated in higher plants such as *Arabidopsis* (review by Somerville, 1984). For example, mutations in the acetolactate synthase (ALS) gene of *Arabidopsis* have been selected in germinating populations consisting of hundreds of thousands of seeds (Haughn and Somerville, 1986). Chlorsulfuron, a single semidominant allele, is a potent herbicide that inhibits ALS activity (Chaleff and Mauvais, 1984). By selecting about 300,000 M_2 seeds in herbicide-containing medium, four chlorsulfuron resistant mutants were obtained. Genetic analysis showed that the resistance is caused by mutations in the ALS gene.

However, it is doubful whether any of the mutant forms isolated using these techniques are unique in the sense that they could not be isolated from natural populations. Furthermore, the effort involved in isolating artificially-produced quantitative mutants which occur at low frequencies is considerably greater than that needed for the screening of natural populations. A series of crosses, carried through several generations is required to prove the genetic inheritance of the observed phenotype before it can be considered as a mutation. As the effect of mutagen treatment is random, there may occur a multiple induction of mutations. Induced beneficial mutations may be accompanied by loss of

other important traits already present in the treated material or may be accompanied by some undesired mutations. These will complicate the selection process and consume a lot of time as laborious crosses are necessary to rescue and bring together the more useful traits. The use of chemical and physical mutagens do not aid in any way the identification and isolation of the nucleotide sequences of the mutated gene. Thus, even though agronomically useful mutants have been induced in most cases, it is not possible to clone easily the genes responsible.

1.3 Mutagenesis of cultured cells

Isolation of mutants from tissue cultures of higher plants began only in the late sixties. Novel phenotypes have been observed in many monocot and dicot species when plants are regenerated from tissue culture, particularly from protoplast cultures (reviews by Scowcroft *et al.*, 1983; Evans and Sharp, 1986; Webb, 1988). These variant characters are transmitted sexually or through vegetative propagules. Since organ primordia arise from a single totipotent cell, whole variant individuals are found. Thus, unlike the M_1 plants generated by traditional mutagenesis, these variants are not chimaeric; the phenotypic characteristic of the initial mutant cell being expressed by the whole plant regenerated from it. These altered phenotypes, termed somaclonal variants, are thought to result from spontaneous and random changes in the cell genomic DNA during tissue culture. They are known to include gross

chromosomal rearrangement such as reciprocal and non-reciprocal translocations, inversions, duplications and deletions (McCoy *et al.*, 1982), or karyotypic changes resulting in polyploidy and aneuploidy.

Molecular analyses of the eukaryotic genome have revealed that structural integrity of a gene and its expression can be modified by an abundance of mechanisms including, gene amplification and depletion as a result of unequal exchange, internal gene rearrangements, altered methylation patterns, gene conversion and DNA transposition (Dover, 1982; Walbot and Cullis, 1985; Brettel *et al.*, 1986). It is thus likely that these mechanisms will also operate to give rise to somaclonal variants. To give one example, somaclonal variation in tomato (*Lycopresicon esculentum*) has yielded several single gene mutations which are dominant, semidominant or recessive (Evans and Sharp, 1982). These include recessive mutations for male sterility, jointless pedicel, several fruit colours, lethal chlorophyll deficiency, virescence, and mottled leaf appearance, a semidominant mutation controlling isozymes for alcohol dehydrogenase, and dominant mutations controlling fruit ripening, growth habit, and disease resistance.

An increase in genetic variability may be induced in large and homogeneous populations of plant cells by exposing cultures to physical or chemical mutagenic agents. The different types of materials which can be mutagenised in this way include, embryoids or plantlets originating from tissue

culture procedures (including anther culture), callus or cell suspension cultures and isolated cells and protoplasts. However, it is advantageous to be able to mutagenise a haploid single cell system (cell suspension cultures or protoplast) to avoid the problem of chimaeric cell colonies and to facilitate isolation of recessive mutants. Following mutagen treatment, traces of mutagen are removed by washing and then selection can be applied immediately on plated cells. Although in most cases cell suspensions consist of cell aggregates of various sizes, through sterile filtration the size can be limited. Careful plating of the cells from a suspension will ensure that the microcolonies derived will be clonal. In the case of protoplasts cultures however, each colony is almost guaranteed to be clonally derived. Thus, the problem of chimaerism is effectively eliminated. In addition, organ primordia, formed through organogenesis or embryogenesis, usually arise from single cells. The treated cells can therefore, be induced to regenerate mutant plantlets, which can then be selected for the desired phenotype. However, regeneration from protoplasts is very difficult and has only been achieved with very limited success especially with the more important crop plants.

This strategy of mutant isolation is helped greatly by the ability to produce haploid materials through anther culture in some plant species. Haploid materials simplify the recovery of fertile recessive mutants through doubling of chromosomes using chemicals such as colchicine, saving the time

consumed in performing crosses by traditional means. The large number of single cells produced through tissue culture enable manipulation of large cell populations for mutagenesis and mass-screening in a limited space. Mutations which otherwise will be lethal to the whole plant, can be selected by tissue culture techniques. Moreover, generation of single cells which are totipotent also promises a great potential of regenerating mutant plants arising from single mutated cells.

One of the first mutant cell lines isolated were leaky auxotrophic mutants selected by Carlson, (1970) using 5-bromodeoxyuridine-induced suicide of mutagen-treated amphihaploid *Nicotiana tabacum* cells. General interest was stimulated and resulted in the selection of a wide spectrum of mutants which included, disease resistance, nitrate metabolism, amino acid metabolism, carbon metabolism, antibiotic resistance and herbicide resistance. Some examples of mutants isolated from cell cultures are shown in Table 1.1. Progress in the field has been reviewed several times (Flick, 1983; Maliga, 1984; King, 1984; Chaleff, 1983, 1986; Daub, 1986). In some cases there were reports that mutagen treatment was not necessary for the induction of mutants (Heimer and Filner, 1970; Maliga *et al.*, 1973a; 1973b). Mutation frequencies ranging from 10^{-5} to 10^{-8} (Maliga, 1980) were often estimated, and varied depending on the species and types of mutation. The frequency could be increased to 10^{-3} by mutagen treatment (Bourgin, 1983; Maliga, 1983) although this may not always be

TABLE 1.1

Amino Acid and Amino Acid Analog Resistant Cell Lines

Selective Agent	Species	Mutagen	References
S-(aminoethyl)-L-cysteine	*Arabidopsis thaliana*	EMS	Negrutiu *et al.*, 1978
	Hordeum vulgare	Azide	Bright *et al.*, 1983
Lysine plus threonine	*Zea mays*	Azide	Hibbert and Green, 1982
Valine	*Nicotiana plumbaginifolia*	Gamma rays	Nielsen *et al.*, 1985
4-methyl-tryptophan	*Peganum harmala*	None	Berlin *et al.*, 1987
Antibiotic Resistance			
Chloramphenicol	*Nicotiana sylvestris*	None	Dix, 1980a
Cycloheximide	*Daucus carota*	None	Lazar *et al.*, 1981
Streptomycin	*Nicotiana sylvestris*	None	Maliga, 1981
Agriculturally Useful Mutants			
Cold Tolerance			
	Daucus carota	EMS	Templeton-Somers *et al.*, 1981
	Nicotiana sylvestris	None	Dix, 1977
Herbicide Resistance			
Chlosulfuron-resistant	*Linum usitatissimum*	None	Jordan and McHughen, 1987.
	Arabidopsis thaliana	None	Haughn and Somerville, 1986.

Paraquat	*Nicotiana tabacum*	X-ray	Miller and Hughes, 1980.
Thiocarbamate	*Nicotiana tabacum*	None	Flashman, 1985.

Pathotoxin Resistance

Fusarium oxysporum	*Lycopersicon esculentum*	None	Miller *et al.*, 1985.
Phytophthora infestans	Potato	None	Wenzel, 1985.

Salt Tolerance

	Linum usitatissimum	None	McHughen and Swartz, 1984.

Auxotrophic Mutants

Nitrate reductase-less	*Nicotiana plumbaginifolia*	UV/Gamma	Gabard *et al.*, 1987.
	Nicotiana tabacum	ENU	Evola, 1983.

EMS = Ethylmethane sulfonate, ENU = N-ethyl-N-nitrosourea,

UV = ultraviolet rays

possible.

The mutants which can be isolated through tissue culture techniques are classified into (1) *auxotrophic* mutants which lack the ability to synthesise the necessary compounds needed for growth, and thus require nutritional supplements for normal growth, (2) *resistance* mutants which resist specific drugs, antimetabolites, or abnormal environmental or nutritional conditions that are normally inhibitory, and (3) *autotrophic* mutants which no longer require a compound which is normally required, and are thus able to grow in deficient media and are capable of synthesising some substance normally required by wild-type lines.

Mutants can be isolated by direct selection, indirect (enrichment) procedures, or by individually testing single-cell-derived colonies (mass screening). The majority of the variants have been isolated by direct selection, in which the new cell types have a selective advantage over the rest of the population because of their tolerance to the toxic compound. In the indirect approach, the wild-type cells are selectively killed because they are metabolically active under conditions (minimal medium, certain temperature) restrictive to the growth of the mutants (auxotrophic, temperature sensitive). The mutants (survivors) are then recovered under conditions allowing normal growth (supplemented medium, permissive temperature). Carlson (1970), and Malmberg (1979), isolated auxotrophic mutants from *Nicotiana tabacum* cells by BUdR

enrichment. Other enrichment procedures employing FUdR (Aviv and Galun, 1976), and arsenate (Polacco, 1979; Horsch and King, 1983) have also been reported. Individual testing has also been used extensively to recover auxotrophic lines from protoplast-derived colonies (King, 1984).

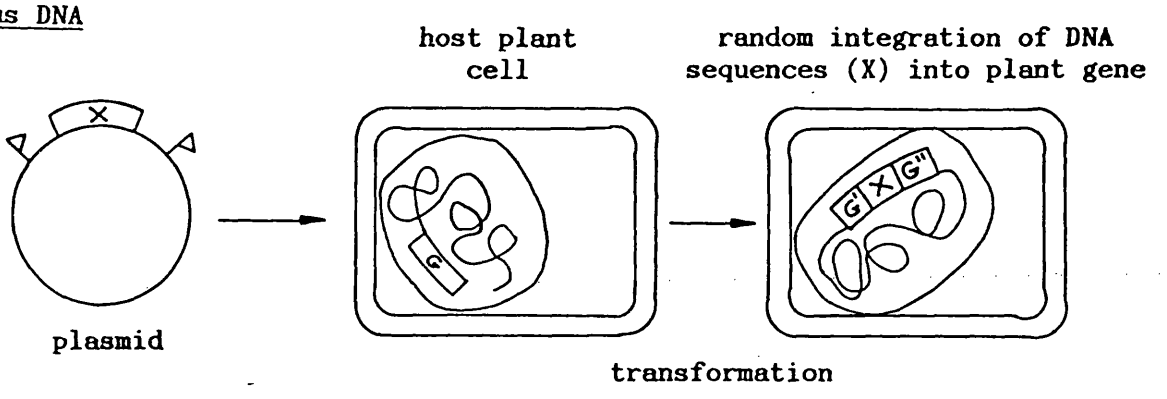
Although chemical and physical mutagens have been widely and succesfully used in mutagenesis for a long time, the techniques have several drawbacks. At the higher dosages of mutagen which are required to obtain a usable mutation frequency, the treated cells are either killed, or a lot of undesired mutations are induced. Hence, many additional mutations may be present in the selected cell line. Secondary, unselected mutations may cause effects such as reduced viability, interference with plant regeneration, pollen sterility, or innumerable other effects that could interfere with plant regeneration and/or genetic analysis of the mutant cell line. Full and partial sterility are also the most common effects of mutagen treatments. The random effects of mutagen treatment often mutate more than one gene, thus making it very difficult to prove linkage between any particular altered gene and the aberrant phenotype(s). Without the presence of a recognisable marker at the affected gene, and also not knowing the translation product of the mutant gene, the gene responsible for the phenotypic change cannot be easily identified or cloned.

1.4 DNA insertion mutagenesis

Besides relying on the capacity of mutagenic agents to achieve mutagenesis, random mutations can also be created in the plant genome through insertion of DNA sequences. By inserting a defined piece of DNA into the recipient plant nuclear genome (Fig. 1.3) a single mutation can be created in a cell line by disruption of a gene (Fig. 1.4). It should be possible to create only a small number of insertions at a few sites which can easily be recognised by Southern blotting (Southern, 1975), provided a hybridisation probe for the inserted DNA is available. The DNA insertion mutagenesis scheme is best illustrated in a strategy adopted to clone genes in maize (Fig. 1.5). A well characterised transposable element is inserted into the maize genome to induce a mutant allele of the desired gene. The transposable element acts as a marker that localised the affected gene. Thus, by using a molecular probe specific to its sequence, the gene sequence of the mutant allele flanking the inserted element can be isolated. Subsequently, segments of the mutant gene can be used to clone the wild-type gene.

Since only a small number of easily recognised inserts or mutants are created, linkage between the insert and induced mutation is easily proven. Mutagenesis in this way usually involves less genome damage. In addition, cloning of the desired gene is also possible even though the gene product is unknown. These features represent the main advantages of

Exogenous DNA



Transposable elements

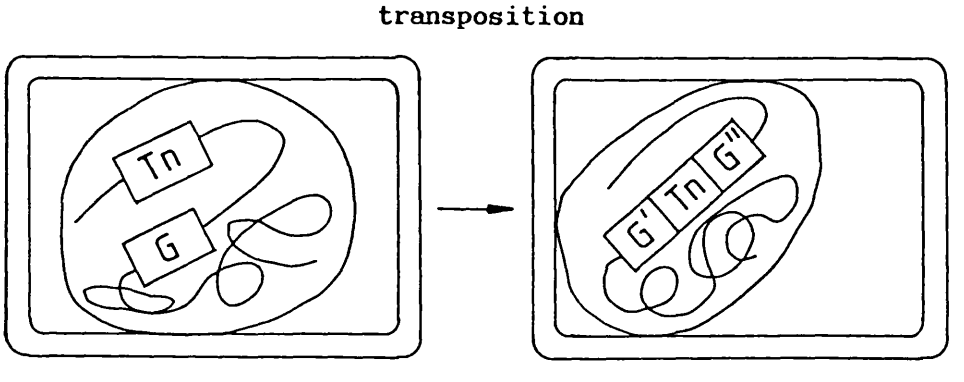
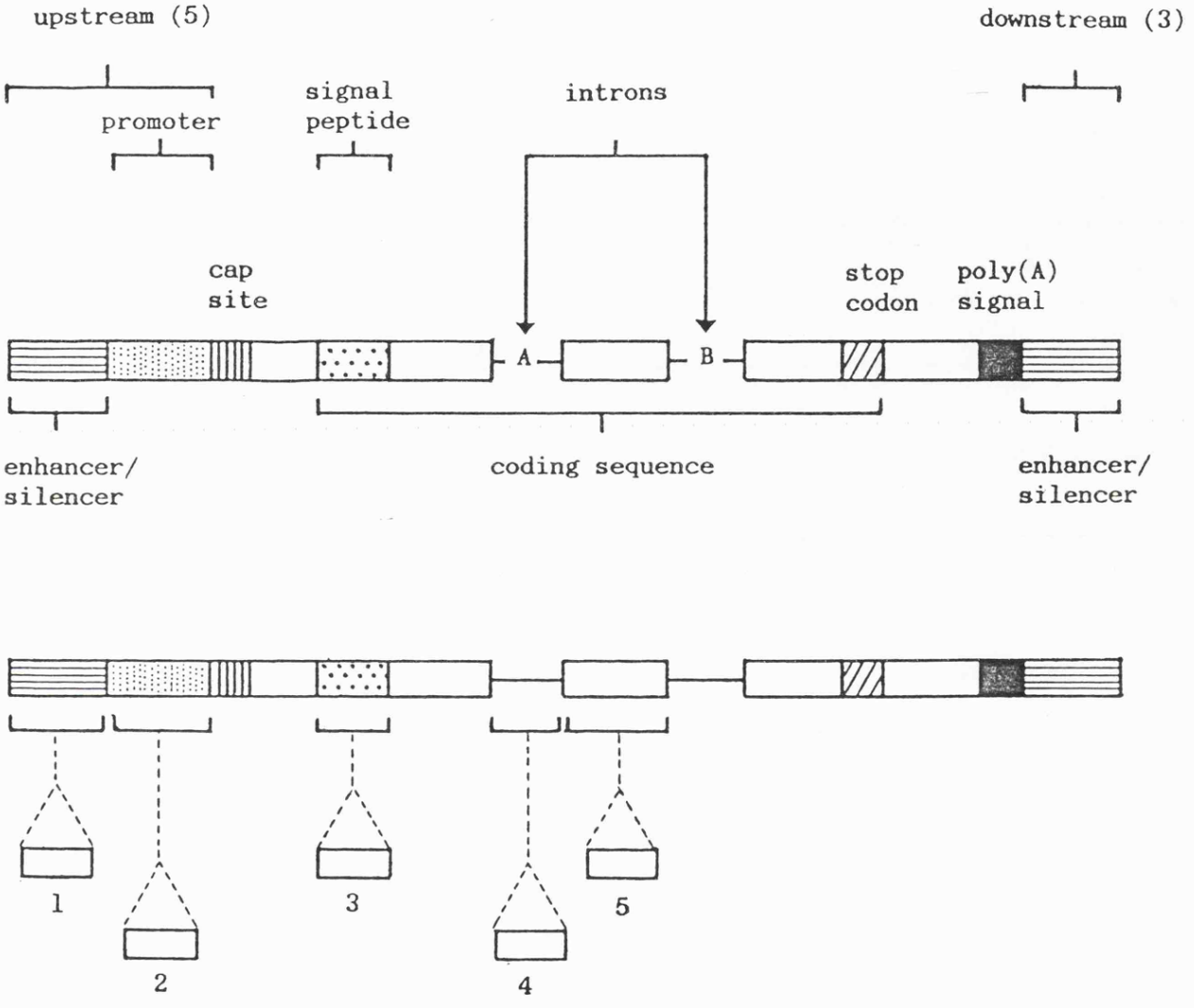


Fig 1.3 Methods for insertion mutagenesis

Fig 1.4 Gene disruption by DNA insertion



1) Disruption of enhancer/silencer region may reduce or increase gene expression. Possibility of producing dominant mutation.
2) Insertion in promoter region may inhibit transcription thereby producing a null mutation.
3) Signal/transit peptide disruption will effect compartmentalisation of the gene product and therefore the effect is unpredictable, but in any case is unlikely to elevate gene expression.
4) Insertion within an intron may effect splicing which may reduce gene expression.
5) Insertion into the coding region should destroy the gene and produce a null mutation.

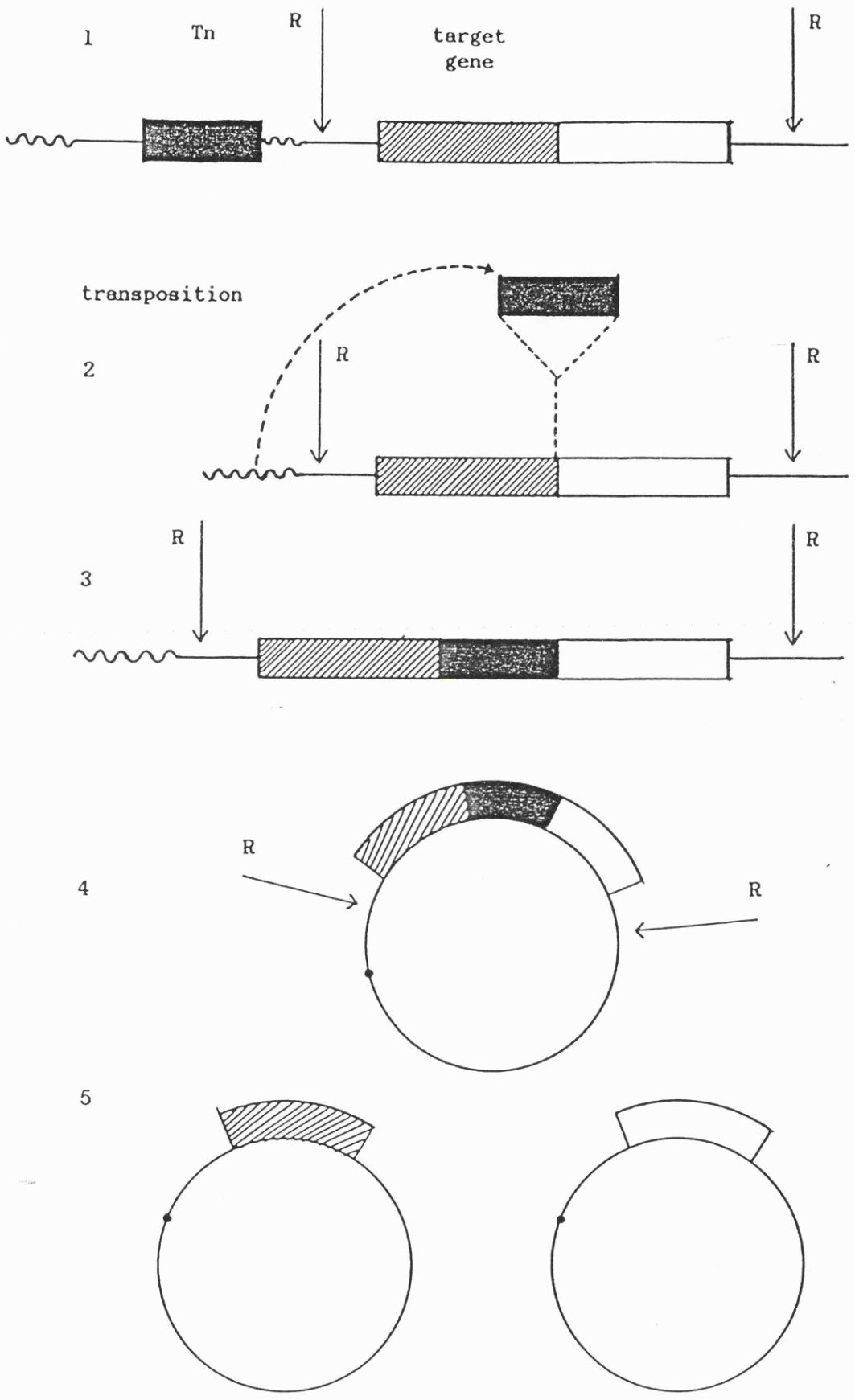


Fig 1.5 Strategy for gene cloning using transposable elements
1) Define a genetically characterised line with Tn near to target gene.
2) Transposition
3) Construct genomic library of mutated plant.
4) Screen for clones with homology to Tn.
5) Sub-clone flanking plant gene and use as probe to clone wild-type gene.
R - unique restriction site

using a random DNA insertion technique for mutagenesis as opposed to traditional mutagenesis methods. In the latter techniques, cloning of a mutant gene is not as easy unless the biochemistry of the mutant phenotype is well characterised and the gene products are known.

Two main features on which DNA insertion mutagenesis in higher plant is dependent upon are therefore firstly, the availability of methods for random transfer and integration of an exogenous DNA into large numbers of cells, and secondly the control of activity of transposable elements.

1.4.1 Mutagenesis by random insertion of exogenous DNA

Several factors are important in determining the suitability of a method for transferring exogenous DNA into a host plant genome for insertion mutagenesis. The transfer system has to;

i) insert the DNA randomly into the host genome,

ii) be applicable to a wide host range and able to transform at high frequency a system allowing a large number of target cells to be mutagenised,

iii) transfer only one or a few inserts to facilitate the cloning of the mutated locus.

The transferred DNA insert must be a known sequence, carry a marker for transformant selection, and have useful restriction sites for cloning purposes.

The uptake of exogenous DNA into the host plant nucleus has been used for years as a tool to study plant virus

infection, replication and expression. The more current methods which have proved useful involve DNA mediated gene transfer into plant protoplasts and *Agrobacterium*-mediated DNA transfer. The former methods can be classified into five groups;

1. Liposome-mediated uptake
2. Fusion of bacterial spheroplasts
3. Chemically-stimulated endocytosis (PEG 6000)
4. Electroporation
5. Microinjection

The first two techniques have met with varying degrees of success, involving more work than the later techniques and are relatively inefficient (Deshayes *et al.*, 1985; Hain *et al.*, 1984).

The most recent techniques reported for transformation are macroinjection and microprojectiles (de la Pena *et al.*, 1987; Klein *et al.*, 1987). These techniques promise a more universal application and a possibility of transforming intact plant tisues. Although they may be useful in the future, at present their applications are still limited. Therefore, the only feasible options available are PEG uptake, electroporation and microinjection. However, transformation strategies based on PEG uptake and electroporation require a good protoplast system in the species concerned. This presents a major limitation as protoplast isolation has been successful only in a few plant species.

After tedious isolation procedures it can be very difficult to get them to divide. Furthermore, regeneration from protoplasts has proved to be very difficult even though some successes have been achieved.

The feasibility of plant cell transformation by direct microinjection of foreign DNA was shown by Crossway *et al.* (1985). Unlike the techniques described above, microinjection does not rely on the permeability of the cell membrane. Thus, removal of the cell wall is not required. This brings about a prospect of microinjecting cells capable of embryogenesis such as pollen, embryogenic suspension cultured cells or isolated ovules. However, there are physical problems such as difficulty in penetrating the cell wall, visualisation of the nuclei to inject DNA into, and getting access to totipotent cells in *planta*. Tissue culture associated problems of the target cells have to be overcome as well as the possibilities of chimaerism if multicellular units are injected. Microinjection of single cells on the other hand, requires high skill in manipulating the plant cells in order to get access to the nucleus. This in turn will not only reduces the speed of performing the transformation procedures but also the number of cells that can be handled. For these reasons, the use of microinjection for insertion mutagenesis is also precluded.

1.4.1.1 Transformation by direct gene transfer

The DNA mediated gene transfer techniques such as the PEG-mediated endocytosis and electroporation have been used to achieve efficient uptake of small plasmids with multicloning sites and dominant selectable markers into protoplasts (Paszkowski *et al.*, 1984; Hain *et al.*, 1985; Shillito *et al.*, 1985). Transformation frequencies of up to 4.6% have been achieved using a system based on the synergistic interaction of PEG and electroporation (Negrutiu *et al.*, 1987). The transformation efficiencies could also be improved 3 to 5 fold by using synchronised protoplasts at S-phase and mitosis (Meyer *et al.*, 1985; Okada *et al.*, 1986). These techniques are generally performed either to obtain stable transformed cell lines, or to examine the transient expression of a gene in order to test out a new constructs. The techniques are applicable to transform both monocot and dicot protoplasts (Fromm *et al.*, 1985, 1986).

PEG uptake and electroporation are however not very suitable for use in DNA insertion mutagenesis studies. Firstly, the limitations of protoplast culture systems pose problems as discussed earlier in this section. The viability of the protoplasts is often reduced after treatment (Jongsma *et al.*, 1987; Negrutiu *et al.*, 1987). The probability of creating the desired mutant is very slim due to the very low transformation frequency and it is rather expensive to make plasmids. As with whole Ti plasmid-mediated transformation, the

25 bp repeat sequences placed into the small DNA mediated gene transfer vector are not required for integration and any site on the vector can be used in the recombination process. This results in transformants with multiple integrations, and a variety of modifications to the inserted vector sequences to give inserts including single plasmid copies, fragments of plasmid, concatemers and cotransfer of carrier DNA (Riggs and Bates, 1986; Jongsma *et al.*, 1987). Even though the insertion is random, the unpredictability of the insert organisation prevents easy cloning of the affected gene. Furthermore, the presence of multiple insertions in a single genome makes linkage to the mutant very difficult to prove and the cloning of the desired mutant allele very difficult.

1.4.1.2 Transformation mediated by *Agrobacterium*

Agrobacterium is a member of family Rhizobiaceae and is traditionally divided into a number of species based on plant pathogenicity. The virulent species of *Agrobacterium tumefaciens* which commonly induce undifferentiated tumour formation (the crown gall disease), and *Agrobacterium rhizogenes* which induces 'hairy root' proliferation on most plants (it infects at wound sites), are two important ones. Crown galls and 'hairy root' tumours can synthesise novel metabolites termed opines (Guyon *et al.*, 1980; Petit *et al.*, 1983; Gheysen *et al.*, 1985; Stachel and Zambryski, 1986). Most strains infect a wide variety of dicotyledonous plants. Infection is not limited to wounded cells of intact whole

plants, but also a variety of target cells in culture, such as large organ explants, callus, protoplasts and cells in suspension derived from susceptible plants. Cells are thought only to be competent for transformation during a short period of time, probably representing the S-phase of the cell cycle when genome replication is underway (Meyer *et al.*, 1985).

In both cases, large plasmids, the Ti (tumour inducing) and Ri (root inducing) are responsible for the pathogenicity of the oncogenic bacteria. The Ti plasmid of *Agrobacterium tumefaciens* carries the genetic information for tumour formation, synthesis of opines in crown gall cells, catabolism of opines by the bacterium, and for conjugation (Fig. 1.6). Ti plasmids are grouped into several types depending on the type of opine produced in the tumours. The opines synthesised are used as a source of carbon and nitrogen by the *Agrobacterium*.

Upon infection, a segment of the plasmid is transferred, integrated, and maintained in the nuclear genome of the host plant (Fig. 1.7) where it is expressed (Chilton *et al.*, 1977; Thomashow *et al.*, 1980; Willmitzer *et al.*, 1982; reviews by Nester *et al.*, 1984; Gheysen *et al.*, 1985; Stachel and Zambryski, 1986). This piece of foreign DNA becomes part of the plant genome and is called the transferred DNA or T-DNA (Fig. 1.7). The T-DNA is stable within the plant genome and no major rearrangements of the sequence take place during tumour establishment. The site of integration of the T-DNA into plant

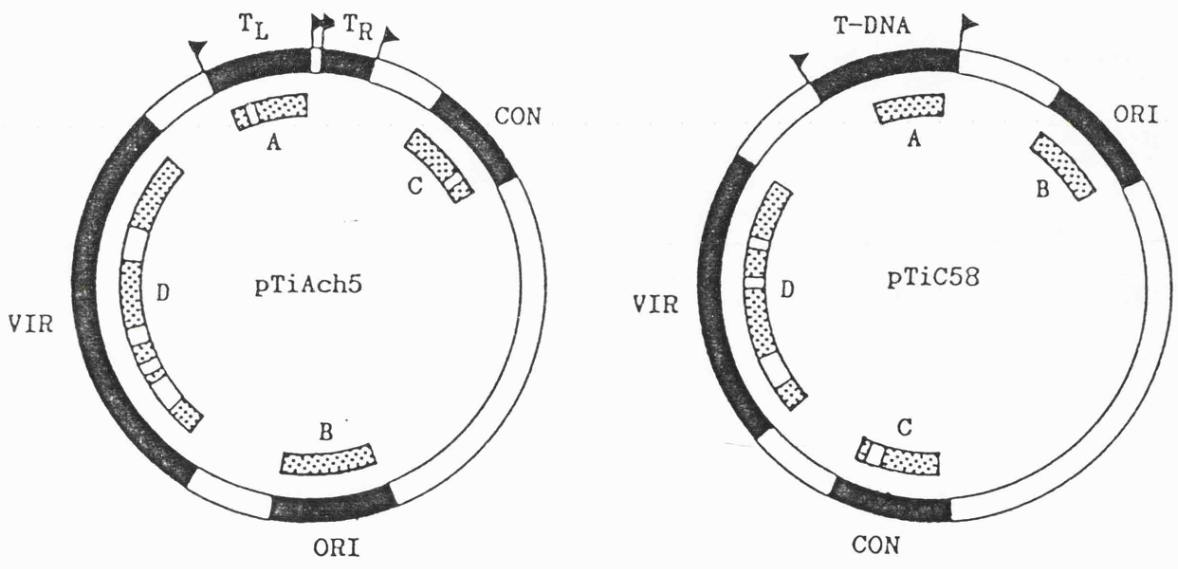


Fig 1.6 Map of an octopine type (pTiAch5) and a nopaline type (pTiC58) Ti-plasmid showing the relative positions and sizes of the major functional regions ▬▬▬. Shaded areas ▒▒▒▒ indicate the regions of extensive homology between the two plasmids. ▶ -25 bp direct repeats; CON - regions encoding conjugation functions; ORI - region encoding replication functions and origin of replication; VIR - virulence region; T-DNA, T_L, T_R - regions containing T(Transfered)-DNA

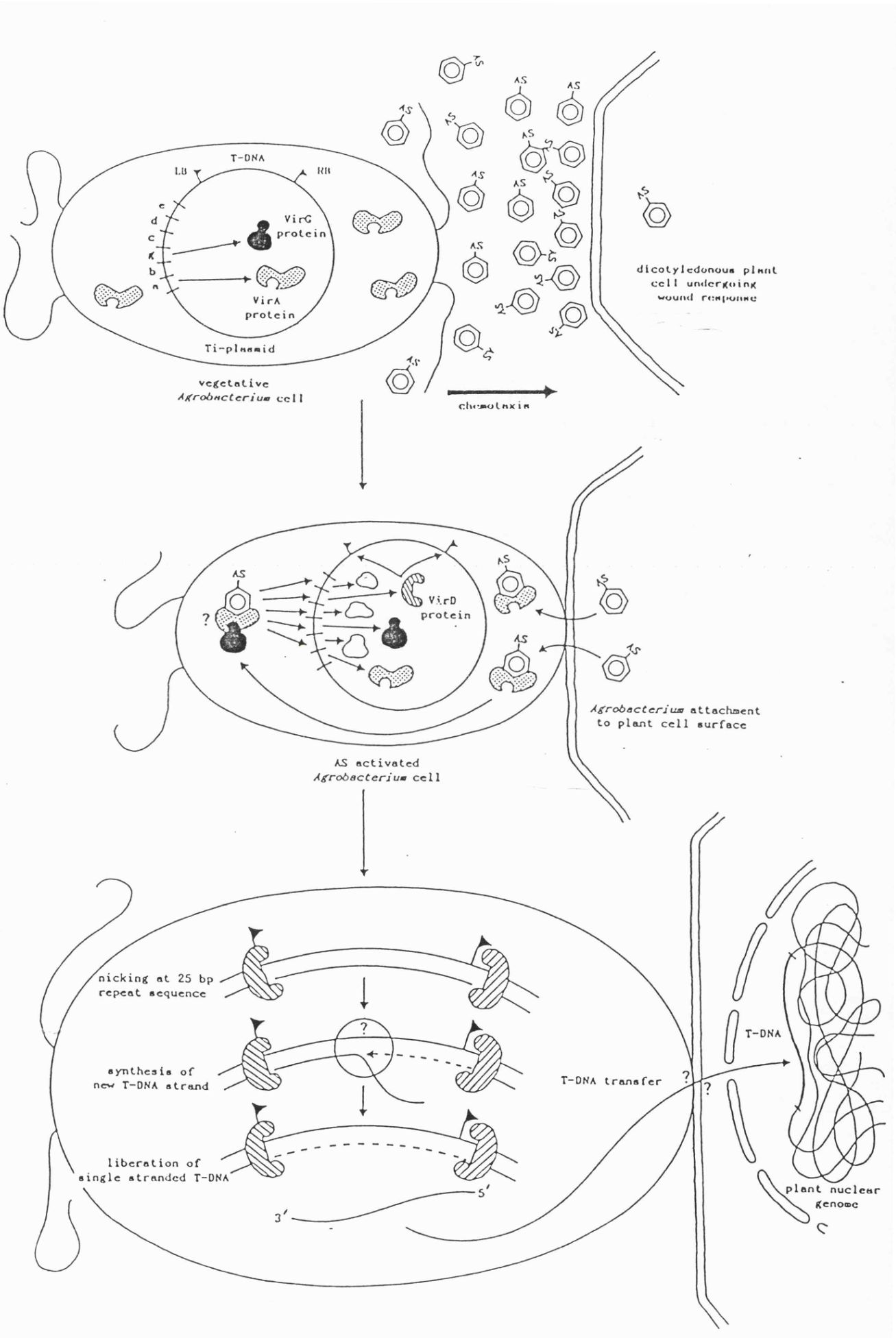
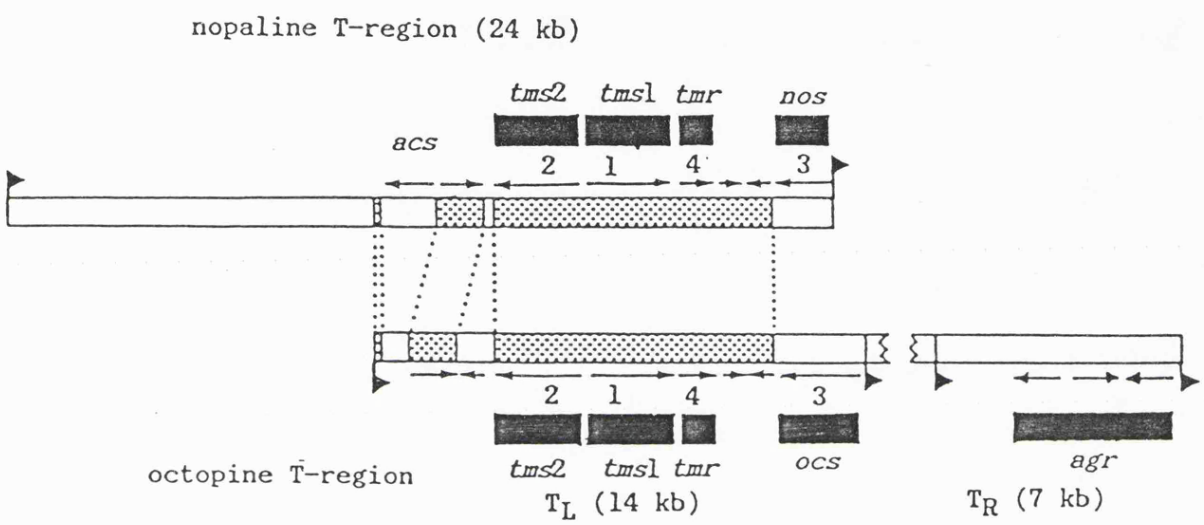


Fig 1.7 Mechanism of T-DNA transfer

DNA is apparently random. After *Agrobacterium tumefaciens* transformation, the majority of transformants contain foreign DNA at a single locus, even when more than one copy was integrated. This T-DNA is often inherited as a single Mendelian dominant trait (Otten *et al.*, 1981; de Block *et al.*, 1984; Horsch *et al.*, 1984).

The enzymes for opine synthesis, the oncogenes (onc-genes) which are responsible for the tumorous growth or oncogenic state of the crown gall cells, are transcribed in the T-DNA (Fig. 1.8; Inzé *et al.*, 1984; Akiyoshi *et al.*, 1984). The oncogenes consist of *tms1*, *tms2*, and *tmr* loci whose gene products override the normal regulation of plant metabolic pathways involved in the synthesis of phytohormones. The *tms1* and *tms2* loci are involved with the unregulated synthesis of auxins, and cause 'shooty mutants' when mutated. The *tmr* locus encodes an enzyme involved in the synthesis of cytokinin, and when mutated results in root-forming tumours on some species ('rooty mutants'). However, these genes are not required for the transfer of the T-DNA to the plant cell, nor its stable maintenance in the plant genome.

The T-DNA is flanked on the Ti plasmid by 25 bp direct repeats acting as borders. The endpoints of integrated T-DNA in the plant genome are found close to these sequences. They are both polar and *cis*-acting, and essential for transfer (Leemans *et al.*, 1982; Wang *et al.*, 1984). The *vir*-region of the Ti plasmid, which has been mapped a short distance away



Maps of a nopaline- and octopine-type T-region showing the major functional domains ▬, the regions of homology ▩ and transcripts ⟶. Products of transcription which have been identified to date are:

transcript	enzyme
3 (*nos*)	nopaline synthase
3 (*ocs*)	octopine synthase
acs	agrocinopine synthase
1 (*tms1*)	tryptophan mono-oxygenase
2 (*tms2*)	aminohydrolase
4 (*tmr*)	DNA transferase
agr	agropine synthase

Fig 1.8 T-DNA gene expression

from the T-region, is not transferred into the plant genome, but is essential for the transfer process and tumour formation (De Frammond *et al.*, 1983; Hoekema *et al.*, 1983; Stachel *et al.*, 1985). It contains at least seven operons of which some (*virA*, *virB*, *virD*, *virG*) are absolutely essential for tumour induction, whereas others (*virC*, *virE*, *virF*) are only necessary for tumour induction on certain plant species (Hooykas *et al.*, 1984; Lundquist *et al.*, 1983; Stachel and Nester, 1986; Yanofsky and Nester, 1986). The T-region transfer process is activated via the induction of expression of the *vir* genes by plant signal molecules secreted by wounded plant cells. A number of phenolic compounds including acetosyringone and hydroxyacetosyringone have been identified as the active compounds (Stachel *et al.*, 1986; Ashby *et al.*, 1987). The induction is followed by the appearance of single stranded nicks within the 25 bp border sequences which flank the T-DNA (Stachel and Zambryski, 1986) and the appearance of a single stranded linear molecule which corresponds to the T-DNA. The *vir* gene products thus act *in trans* to mobilise the T-region from the bacterial Ti plasmid (De Frammond *et al.*, 1983).

The oncogenic transformants, 'hairy roots' and crown gall tumours, are phenotypically recognised, can be selected by their ability to grow on medium lacking phytohormones, and easily screened by the opines produced. The oncogenic cells of the crown gall are incapable of regenerating whole transformed plants. 'Hairy root' tumours which are of clonal origin (Petit

et al., 1986), on the other hand, are able to regenerate shoots which in some cases have developed into mature plants (Tepfer, 1984; Jensen *et al.*, 1986; Trulson *et al.*, 1986; Shahin *et al.*, 1986). Unfortunately, such transformed plants often exhibit a degree of morphological abnormality (Tepfer, 1984) such as leaf wrinkling, reduced apical dominance, squat growth habit, variable fertility and loss of positive geotropism in much of the root system.

By taking into account the characteristics of the T-DNA process, vectors have been constructed in which the oncogenes were removed and only the 25 bp repeat sequences and occcasionally the opine synthase gene retained. Bacterial antibiotic resistance and herbicide resistance genes have been placed under the control of T-DNA promoters and polyadenylation signals and inserted between the 25 bp repeat sequences. These resistance genes act as markers for transformed cell selection. These non-oncogenic vectors can be divided into two types, the *cis*- or *trans*-acting vectors. In the *cis*-acting vectors, the genes to be transferred are inserted between the border sequences of the T-DNA by homologous recombination. The *vir* regions are carried in the same vector. *Trans*-acting or 'binary' vectors consist of two plasmids, one contains the *vir* genes and the other carries the genes to be transferred flanked by the border sequences. Details of specific vectors will be described in a later chapter.

A variety of useful Ti-plasmid-derived vectors and

selectable markers have been constructed which allow for efficient plant transformation (Zambryski *et al.*, 1983; Fraley *et al.*, 1985; Debleare et al., 1985). Although there is very little homology between the T-DNA regions of the Ti and Ri plasmids, the significant sequence homology between the DNA from the virulence regions of the two plasmids (Huffman *et al.*, 1984; Jouanin, 1984) suggests that they are functionally similar. Thus, the binary vectors based on Ti plasmids are able to function in both *Agrobacterium tumefaciens* and can also be mobilised by *Agrobacterium rhizogenes* into the plant genome (Van den Elzen *et al.*, 1985; Simpson *et al.*, 1986; Shahin *et al.*, 1986; Hamill *et al.*, 1987).

These vectors are not only useful in delivering known agronomically important genes into host plant genome, but also for the insertion of known DNA sequences for creating mutations in the recipient genomes. The general strategy for isolating mutants by *Agrobacterium*-mediated gene transfer would be to first infect susceptible and competent target cells with the *Agrobacterium* strain carrying the gene of interest and a dominant selectable marker. This would be followed by removal of the *Agrobacterium* by treating the infected cells with an antibiotic which kills the *Agrobacterium*. The presence of dominant selectable marker facilitates easy recognition and selection of transformants. The T-DNA sequences in the affected gene become a marker for the isolation and cloning of the resultant mutant flanking gene sequences.

1.4.1.3 Mutation using transposable elements

Transposons (Tns) have been exploited to generate mutants in several prokaryotes. They are mobile genetic elements often carrying antiobiotic resistance genes, that are able to insert themselves into large numbers of different sites in prokaryotic genomes (Kleckner *et al.*, 1977). When insertion occurs within a structural gene or elsewhere in an operon, recognisable mutations are often induced and the phenotype is completely linked to antibiotic-resistance carried by the Tn in genetic crosses. Interrupted genes often suffer complete loss of function and reversion only occurs by a precise, or nearly precise excision with concomitant loss of antibiotic resistance at that site. Transposons are also associated with other kinds of recombination events, notably deletions and inversions. These properties enable transposons to be used in prokaryotes as *in vivo* mutagenesis agents to induce mutations at random sites in a population of cells to produce mutants with defined physiological defects.

Although transposons can be found at a large number of sites on the bacterial chromosomes, their distribution is not random. They display some degree of specificity for sites of insertion resulting in unequal probability of yielding mutations in all genes. A 'hot spot' (a site preferred by a transposon for insertion) with up to 21 Tn5 insertions has been identified in the tet gene of pBR322 (Berg et al., 1983). Other workers have also reported 'hot spots' for Tn1, Tn7 and

Tn 10 insertions (Krishnapillai *et al.*, 1981; Caruso and Shapiro, 1982). The frequency of recovery of transposon insertions in a chromosome is very low. Rella *et al.*, (1985) only recovered Tn5 insertion mutants at ~5 x 10^{-7}/donor. Besides these, there is also a problem of curing transposon-mediated cointegrates to leave a single copy of the transposon in the mutated gene (Poole and Hancock, 1986; Haas *et al.*, 1981).

Despite these drawbacks, transposon mutagenesis has been proved possible and useful in prokaryotes. In 1981, Ruvkun and Ausubel proposed a site-directed mutagenesis technique using a method which allows transposon insertions in cloned DNA fragments. Wild-type genomic DNA sequences may then be replaced by homologous recombination and the mutated phenotypes examined. Following these same principles, Garfinkel *et al.* (1981) were able to isolate and characterise a large collection of mutations within a relatively small portion of an *Agrobacterium* Ti plasmid and obtained a detailed functional map of the T-DNA. Overlapping restriction fragments of the T-DNA were cloned into *Escherichia coli* and mutagenised by transposon Tn3 or Tn5. The locations of the transposon insertions in the T-DNA were determined by restriction enzyme analysis/mapping. By homologous recombination, the transposon insertions were introduced back into the Ti plasmid, and recombinant strains were tested for altered tumour phenotypes. Characterisation of the transposon insertions generated,

defined 4 phenotypic loci in the T-DNA.

Random insertion has also produced other mutants in prokaryotes. For example transposon Tn5 which has a kanamycin-neomycin resistance gene (*npt-II*), has been shown to induce relatively stable auxotrophic mutants which require cysteine and methionine for growth, also indoleacetic acid-negative and indoleacetic acid-overproducers mutants in *Azospirillum lipoferum* (Abdel-Salam and Klingmüller, 1986). Mutants with altered indoleacetic acid biosynthesis pathway and completely defective in nitrogen fixation have been isolated from Tn5 mutagenised *Azospirillum brasilense* (Singh and Klingmüller, 1986). In this case, it should be noted that Tn5 is working in a transgenic environment.

Based on the same principles, the transposon insertion mutagenesis technique could be extended to eukaryotes, particularly plant species. Here, DNA insertion could be achieved using a transposable element found in plants, which is a DNA sequence which has the ability to insert and excise itself into or from any site in a plant genome. These elements were first recognised in maize through genetic studies by McClintock (1946). Since then, a number of these elements have been identified and cloned from maize (Fedoroff *et al.*, 1983; Müller-Neumann *et al.*, 1984; Barker *et al.*, 1984), *Antirrhinum majus* (Upadhyaya *et al.*, 1985; Sommer *et al.*, 1985a, b) and *Glycine max* (Vodkin *et al.*, 1983). These elements have been classified into two types; the autonomous

element, which can transpose by itself, and the non-autonomous element, which is stable until an autonomous element is introduced into the genome.

Plant transposable elements characterised so far have a length of 1.4 to 17 kb. Two structural features seem to be characteristic of these elements in plants: the terminal sequences consist of either direct perfect or inverted repeats, and a flanking short perfect base duplication of the recipient DNA of characteristic length upon the element insertion (review refs. Nevers *et al.*, 1985; Döring, 1985). Upon excision, the duplication remains behind as a 'footprint', though often in mutated form due to base substitutions or deletions. This suggests a subtle difference between the insertion and the excision mechanism. Based on the two characteristic features, most transposable elements so far isolated can be categorised into two groups. In one group, which share some similarity between their termini, are the *Ac/Ds* system in maize (Döring *et al.*, 1984; Fedoroff *et al.*, 1983b; Pohlman *et al.*, 1984) and the *Tam3* element of *Antirrhinum majus* (Sommer *et al.*, 1985b). These elements have similar inverted termini (7 out of 11 nucleotides are identical) and create duplications of 8 nucleotides upon insertion. The *En* (*Spm*) system of maize (Schwarz-Sommer *et al.*, 1984; Pereira *et al.*, 1985), *Tam1* and *Tam2* elements of *Antirrhinum majus* (Bonas *et al.*, 1984; Upadhyaya *et al.*, 1985) and *Tgm1* element of *Glycine max* (Vodkin *et al.*, 1983) form the second group. These elements generate

a 3 bp duplication upon integration and have the terminal five nucleotides (CACTA) of their inverted repeats in common.

There are other transposable elements which do not fall into any of the two groups above. These include the *Mu1* element (Robertson, 1980), which has terminal inverted repeats of 213 and 215 nucleotides and creates a duplication of 9 nucleotides upon insertion (Barker *et al.*, 1984), and the *Tz86* and *Bs1* elements which create a duplication of 10 nucleotides and 6 nucleotides respectively on insertion (Dellaporta *et al.*, 1984; Johns *et al.*, 1985).

Transposable elements are able to move from one site within the chromosome to other sites on the same chromosome or they can subsequently be found on a non-homologous chromosome. When they move to a new location, they are absent from the previous, or donor, location. They can move in one direction initially and at subsequent transpositions, move in the reverse direction. Movements of the transposable element cause chromosome breakage. For example, a chromosome 9 breakage at *Ds* resulted in the formation of an acentric-dicentric chromosome pair and loss of some of the alleles as well as the element carried (Fedoroff, 1983). A model for insertions and excisions has been proposed from the structure of footprints (Saedler and Nevers, 1985). It is primarily based on two characteristic features of transposition in plants. (i) Transposition is not replicative but occurs via excision and reintegrations of one and the same element. (ii) Excision is

usually imprecise and associated with additions, deletions or inversions within the region of the target site duplication immediately adjacent to the element. Transposase, an element-specific enzyme or enzyme complex thought to be encoded by an autonomous element, recognises and binds to the ends of the integrated transposable element and draw them together in a semi-stable DNA/protein complex. Staggered nicks are then set at the ends of the duplicated sequences flanking the element. This happens during the excision process as well as upon insertion of a transposable element. The protruding single-stranded ends created at the excision site, and the ends of the excised transposon, could be used as substrates for exonuclease, DNA polymerase-catalysed fill-in reactions, and template switches of the polymerase. However, an excision event of *Tam3* of *Antirrhinum majus* created a slightly more complicated footprint, which requires additional explanation (Sommer *et al.*, 1985b). The altered target site duplications may be explained by assuming that the polymerase slips or 'stutters' along the template.

Members of different transposable element families have been shown to differ not only in the types of genetic rearrangements caused by these elements but also the frequency and developmental timing of their transposition. Transposition of most elements occurs during both somatic and meiotic development. In some cases the events take place predominantly at specific stages of the plant development. The Mu1 element

for example, transposes in germ line tissue, primarily late in development, meiotically or post-meiotically (Robertson, 1980). The transposition of *Ac* from the *P* locus of maize has been shown to occur after replication of the donor locus but only into a replicating target site (Chen *et al.*, 1987).

The ability of transposable elements to cycle between active and inactive cycles has been observed in the *Spm* system (McClintock, 1958), the *Dt* system (Doerschug, 1973), the *Ac* element (McClintock, 1963, 1964), and the *Mu1* element (Chandler and Walbot, 1986). The function of the silent transposable element can be activated, in maize for example, after exposing the plants to genomic stress. Genomic stress may be a chromosome break after exposure to ionising radiation or after formation of a dicentric chromosome under certain circumstances. Another type of genomic stress may be the infection of maize plants with viruses such as the barley mosaic virus (BSMV) or wheat streak mosaic virus (WSMV). The loss of activity in the inactive phase has been correlated with extensive DNA modifications to the elements (Chandler *et al.*, 1986; Chomet *et al.*, 1987). Modifications appeared to involve cytosine methylation of the DNA sequences which affect the sensitivity of some restriction enzymes to their target sites. Chomet *et al.* (1987) suggested that the loss of *Ac* activity was not due to gross structural arrangements in the element as previously shown for other functional changes of an *Ac* to a *Ds* element (Fedoroff *et al.*, 1983), but due to the DNA

modification itself. However, whether this modification is a consequence of the DNA changes due to genomic stress, and reflects the state of expression of the element or, it directly regulates the expression of the element itself is not known. The copy number of the transposable element is also thought to be involved in the regulatory mechanism of transposition.

Generally, the insertion of transposable elements into genes can cause two types of mutations. A null mutation, which is often recessive, is caused by a complete loss of function of the gene affected by the inserted sequences. Mutations that completely block expression of the *pallida* (*pal*) gene which encodes a product required for synthesis of the red anthocyanin flower pigment in *Antirrhinum majus*, result in ivory flowers. The pal^{rec} allele which gives ivory flowers with red spots or sectors is produced by insertion of the *Tam3* transposable element at the *pal* locus (Sommer *et al.*, 1985b; Martin *et al.*, 1985).

Another consequence of DNA insertion is either an increase or decrease in the affected genome expression. An insertion in the intron of the gene affects the mRNA processing, thus the expression of the gene. The insertion of the *Mu1* element in the first intron of maize alcohol dehydrogenase-1 (*Adh1*) caused reduced levels of messenger RNA (Bennetzen *et al.*, 1984). If the insertions occur in the 5' region of the promoter gene, the mutants can be dominant, semi-dominant or recessive (Carpenter *et al.*, 1987).

These mutations can revert to wild-type by excision of the element. In some, but not all cases, the reversion at one locus is accompanied by a mutation at another locus, indicating transposition of the element. The versatility of a transposable element as an instrument for insertion mutagenesis is best described in maize. Some of the numerous maize genes which have been mutated by transposable element insertion are shown in Table 1.2.

The vast collection of unstable mutations caused by insertion elements, and the identification of transposable element system in maize far outnumbers the identification of transposable element systems in other eukaryotes. This is because of several factors: i) the array of unstable mutations readily visible in the maize kernel; ii) somatic mutations associated with mobility of transposable elements can be isolated in germinal progeny, since plants do not segregate germ line tissue early in development. If an event occurs in meristematic cells during plant development that develop into gametophytic tissue (pollen and ovules), then sectors of mutant tissue will eventually lead to mutant progeny; iii) cultural practices such as inbreeding and selection may propagate these types of instabilities; and iv) the concentrated genetic research on maize controlling elements has greatly contributed to the identification of these elements.

The greatest advantage of maize as a genetic system is that the effects of these transposable elements in maize are

TABLE 1.2

Genes	Transposable element	Reference
alcohol dehydrogenase (*Adh1*)	*Mu1*	Bennetzen *et al.*, 1984
c locus anthocyanin biosynthesis	*En1*	Paz-Ares *et al.*, 1986
a1 locus anthocyanin biosynthesis	*En1* and *Mu1*	O'Reilly *et al.*, 1985
c2 locus chalcone synthase	*En*(*Spm*)	Wienand *et al.*, 1986
bronze (*bz*) locus UDPglucose-flavonol glucosyltransferase	*Ac*	Fedoroff *et al.*, 1984
pa1 locus anthocyanin biosynthesis	*Tam3*	Martin *et al.*, 1985

usually observed in the endosperm and kernel characteristics. Many thousands of kernels can be obtained with relative ease; these kernels are large and seed characters can easily be scored. The embryo and endosperm are of the same genotype. During the reproductive process, double fertilisation takes place. Two sperm cells migrate down the pollen tube and are deposited within the embryo sac which contains an haploid unfertilised egg cell at the base and two haploid primary endosperm nuclei in the central cell. One of the sperm cells fuses with the egg cell and produces diploid zygote which develops into the embryo. The second sperm cell fuses with the two primary endosperm nuclei of the central cell and gives the triploid primary endosperm nucleus. Because the embryo sac develops from a single haploid, postmeiotic cell, as does the pollen grain, the sperm cells contain sister haploid nuclei, as do the egg cell and the central cell of the embryo sac. Thus, the embryo that develops from the fertilised egg and the endosperm that develops from the central cell normally have the same genetic constitution, except that the endosperm contains two copies of the maternal haploid genome represented in the embryo sac.

Mutations affecting either anthocyanin production in the aleurone and pericarp cells, or the quality of starch and protein synthesis in the endosperm have received the most attention, since these are highly visible and developmentally dispensable in the mature plant. The formation of pigments in

maize kernels illustrates the interaction of several genes in the simplest possible way. Generally, the genes involved in anthocyanin biosynthesis have been especially useful in the genetic study of unstable mutations because alterations in the pigmentation pattern of the kernel can reflect subtle changes in the patterns of anthocyanin gene expression caused by controlling element movement.

Despite all the above advantageous features there are limitations in the use of transposable elements as mutagens in their natural host. As the transposable elements are present at many copies in their natural host genome (Sommer *et al.*, 1985; Chandler *et al.*, 1986) the main difficulty in an insertion mutagenesis programme will be to identify the particular copy inserted in the gene to be isolated. In several cases, this difficulty has been circumvented by the use of partial probes hybridising to only a few copies (Fedoroff *et al.*, 1984; Paz-Ares *et al.*, 1986; Wienand *et al.*, 1986) or by tagging of the same gene with two transposable elements in different mutants (O'Reilly *et al.*, 1985). In case of the *Ds* element, it is even more difficult as there are many copies with variable structures of the *Ac/Ds* family present in the maize genome (Geiser *et al.*, 1983; Fedoroff *et al.*, 1983b; Döring and Starlinger, 1986). In addition, there are *Ds* elements (*Ds1*) that do not hybridise to the autonomous *Ac* element (Sutton *et al.*, 1984).

The mutant alleles so far isolated are limited to

phenotypes which are easily selectable by their shades of pigmentation or kernel morphology. They include genes involved in anthocyanin biosynthesis in maize and *Antirrhinum majus* (O'Reilly *et al.*, 1985; Upadhyaya *et al.*, 1985; Martin *et al.*, 1985) and the *shrunken* and *waxy* locus of maize (Werr *et al.*, 1985). The only mutant which was not isolated by visible selection is the alcohol dehydrogenase mutant (*Adh1* gene) which can be classified by histochemical staining of pollen for ADH activity (Taylor and Walbot, 1985) or resistance to allyl alcohol.

Since several transposable elements have been isolated and sequenced, an alternative approach to mutagenesis by transposable element could be to move the element into other systems than their natural host. This requires a stable integration of the element into a suitable transgenic plant. Transfer can be mediated by an *Agrobacterium* vector system as described in the earlier section (1.4.1.2). High frequency and random insertion of only one or two copies of an element in a transgenic host should simplify the cloning of affected genes. Also, the repeated induction of transposition in the transgenic plant or cells in culture might eliminate the need for constant transformation to create new insertion mutants. The growing evidence that the underlying molecular mechanism of transposition is the modification of the DNA may also provide a means of regulating the events. Transposable elements which are in an inactive state could be induced to transpose by

introducing stress such as radiation or 5-azacytosine treatment which acts to demethylate modified DNA. Growth under low temperature increases frequency of *Tam3* excision at both *pal* and *niv* loci by about 1000 times (Carpenter *et al.*, 1987). In comparison, only a six fold increase was shown by the *Tam1* element (Harrison and Carpenter, 1973). This may reflect the altered levels or efficiencies of transposases or repressors of transposition.

Besides inducing and regulating transposition, one other problem foreseen in the approach is the assurance that the transposable element inserts and excises, without causing major deletions in itself or the adjacent sequences in a transgenic plant, at high frequency. The element *Tam3* excision has been shown to be imprecise, causing deletions or rearrangements at the ends of the element (Sommer *et al.*, 1985; Coen *et al.*, 1986). In the case of *Ds* elements, major structural rearrangements occur, which introduces potential difficulties in recognising active loci and thus problems in mutant isolation in transgenic plants. Very recently the *Ac* element has been shown to transpose in transgenic tobacco and carrot albeit at low frequency (Baker *et al.*, 1986, 1987; Van Sluys *et al.*, 1987).

1.5 Practicalities of mutagenesis and gene cloning by random insertion of DNA

The concept of using either T-DNA or transposable elements as mutagens in an insertional inactivation scheme has several advantages over the use of chemical or physical mutagens. Firstly, since the inserted DNA sequences are known and in some cases carry a dominant marker for easy selection of the transformed line, depending on the mutation, the resultant change of phenotype can hopefully be selected from the transformed recipient plant cells. Sequences in the vicinity of the inserted element can be easily cloned and hopefully the wild-type gene cloned using these sequences as probes. The cloning of agronomically important genes which are associated with, or closely linked to, the inserted gene is thus possible using standard recombinant DNA technology. However, it should be noted also that only a few mutants/genomes will be generated and so linkage can easily be verified by Southern blotting.

General problem for insertion mutagenesis application is posed by the large genome size of most plants, especially the more important crop plants. A high percentage of the genome consists of repetitive DNA. Therefore, many T-DNA insertion events will not disrupt genes, making it an inefficient method of producing mutations.

1.5.1 Problems using exogenous DNA

The requirements for this approach to work have been described in an earlier section (1.4.1). Most of these

requirements could easily be fulfilled by the *Agrobacterium*-mediated transformation system. T-DNA transfers have been shown to be random and only one or a few such transfers occur per transformed cell (Horsch *et al.*, 1985; Deak *et al.*, 1986; Guerche *et al.*, 1987). The infrequency of the T-DNA transfer however could pose a problem. While it is preferable to have few inserts so that the affected gene can easily be cloned, it means that the chances of disrupting the desired gene is very low. Thus, a transformation system is needed where a very high transformation frequency can be obtained in a large population of cells. Selection can then be performed on the transformed cell population and regeneration of mutated plant be induced therefrom.

1.5.2 Problems of mutation by transposable elements

The strategies of gene mutation using transposable elements as described earlier require the use of a cloned element whose DNA sequence is known. It should be able to excise and insert precisely without causing major deletions or rearrangements and also be able to insert randomly, without preference to any particular loci, at high frequency. Some form of regulation of transposition is also required. The Robertson's *Mutator* element, *Mu1*, which will be described in greater detail in a later chapter, was chosen for studies described in this thesis mainly for its higher background level of mutation than standard inbred lines of maize (Robertson,

1978). Robertson's Mutator lines exhibit a 10-fold higher frequency of mutation than four of the classic controlling element systems including *Ac/Ds* (Robertson and Mascia, 1981). In maize, many of the recessive mutants induced by this element are somatically and germinally unstable (Robertson, 1981; Freeling 1984). However, its transposition in other species has not been unequivocally shown (Lillis *et al.*, 1985).

Using a Ti plasmid-derived vector with a dominant selectable marker, the transposable element can be transferred and integrated into the recipient plant genome without rearrangement. However, once stably integrated, there is still an uncertainty concerning whether the transposable element will transpose randomly in a transgenic host and perform as required. Transposition may be limited to various sites within a chromosome. Transposition may have to be induced by the various means of induction described earlier. The possibilities of 'hot spots' for integration present in the genome cannot be ruled out. In fact, observation of sequence homologies at sites of *Tam3* integration at the *pal* and *niv* loci by Coen *et al.* (1986), support the probability that *Tam3* integration may not be random but has preferred 'hot spots'. As in their natural host, the copy number of the transposable element may build up in the transgenic plant and complicate mutant isolation. In the present study it is hoped to examine all these questions.

1.6 Choice of plant system for insertion mutagenesis

One way of getting around many of the problems outlined above is to use a plant with a simple small genome. It is beneficial if such plants respond to tissue culture, are capable of generating haploids and yielding large numbers of single haploid cells able to divide, form callus and eventually regenerate plants. One such plant is flax (*Linum usitatissimum* L.), a flowering, dicotyledenous plant of family *Linaceae* with a great economic value. Flax satisfies most of the criteria needed as an experimental system for insertion mutagenesis. The genus Linum comprises many species which are grouped into homostyled and heterostyled categories. The former which self-fertilises easily is preferred for genetic research. After fertilisation, the ovary develops into a fruit called a capsule that matures about two weeks after pollination. Each capsule contains up to 10 seeds. *Linum usitatissimum* L. cv Antares and RA91 usually start to give mature seeds in about 2.5-4 months and since the apical meristem branches out and continually produces flowers, seeds are produced continuously for several months. *Linum usitatissimum* L. is in the homostyled group and has a great number of different forms. The large number of hereditarily distinguishable forms include several physiological and morphological variants, oil content of the seed, ability to resist frost and diseases and many others (Tammes, 1928; Green, 1986; Gaudreault *et al.*, 1986).

Certain flax varieties have been shown to produce stable heritable changes induced by environmental conditions which breed true, irrespective of the subsequent environment (Durrant, 1971). A number of characters for these environmentally induced lines, which are termed genotrophs, are shown to vary during the induction (Durrant, 1971; McLellan and Durrant, 1973; Evans *et al.*, 1966; Evans, 1968; Cullis and Kolodynska, 1975; Timmis and Ingle, 1973, 1975; Cullis, 1975. 1976; Gaudreault and Tyson, 1986). The genotrophs differ from one another and the original line from which they were derived in a number of these characteristics including the total nuclear DNA amount (Evans 1968), the number of genes coding for the 18S and 25S ribosomal RNAs (rDNA) (Cullis 1976), and the number of genes coding for the 5S RNA (Goldsborough *et al.*, 1981). Some of these characters have been shown to reverse through the next few generations under subsequently altered environments (Joarder *et al.*, 1975) thus giving rise to nuclear reversion lines. The induction of these characters can either be independent or they may be associated with one another.

DNA variations have been observed between genotrophs, as well as between flax cells in culture, including *Agrobacterium* derived callus (Hepburn *et al.*, 1983), and of plants regenerated from callus compared to the plant from which they were derived (Cullis and Cleary, 1986a). This variation can occur within a single generation, without any change in chromosome number. In most cases there is a

reduction in the proportion of certain repeated sequence families in the callus DNA (Cullis and Cleary, 1986a, b). Variation of each repetitive sequence occurs independently and, within a family of sequences, particular subsets could be differentially affected. The extent of variation differed according to the genotype. It was also observed that the same repetitive sequence varied in tissue culture in a similar way to that seen in different genotrophs (Cullis and Cleary, 1986a, b). A major part of the differences observed in the genotrophs occurred in the highly repetitive sequence. Thus, the flax genome can be considered as being categorised into two groups of sequences, one of which is variable and the other is constant (Cullis, 1984). Changes in the variable component which includes the ribosomal DNA (Cullis, 1979), 5S DNA (Goldsborough *et al.*, 1981) and also a number of characterised highly repetitive sequences (Cullis and Cleary, 1986a), as a response to environmental 'stress' result in the phenotypic variations subsequently observed. The possible mechanisms of generating these variations include unequal recombination, amplification, deletions, and transposition (Cullis and Cleary, 1986b). However, a cloned fragment from the flax genome which has been characterised to have a number of properties of a transposable element, has not been demonstrated to play any role in the formation of the genotrophs (Cullis, 1983). The ability of the genome to respond to environmental stimuli appears to be genotype dependent. However, the genetic basis for the ability

to respond is not known. Therefore, depending on the environmental stimuli and the genotype, foreign DNA inserted in the flax genome may be subjected to similar changes as observed if insertion occurs in the variable component. The plasticity of the flax genome may also be an important factor in transposition of an integrated transposable element if an appopriate inducing stimulus is applied.

Haploid materials are easily available from some flax varieties through polyembryony (Rajhathy, 1976). These varieties produce twin seedlings at a rate as high as 30%. Flax also shows good response to tissue culture manipulations. Plantlet regeneration has been obtained from several types of explant (Gamborg and Shyluk, 1976; Murray et al., 1977; Rybczynski, 1975; Barakat and Cocking, 1983)). Crown gall induction by wild type Ti plasmids of *Agrobacterium tumefaciens* has been shown to be possible by Hepburn et al., (1983).

The flax genome has been substantially characterised and members of all the highly repetitive sequence families in the genome cloned (Cullis 1981; Cullis and Cleary 1986a, b). DNA reassociation studies estimated that the flax genome consists of fold back (1%), repetitive sequences (55%) and single copy sequences (44%). The repeated and single copy sequences are arranged in a long period of interspersion patterns. The genome of flax is small compared with most plants (1.4 pg/2C nucleus) with a chromosome number of 2n=2x=30 (Bennet and Smith, 1976). The haploid genome size of flax is

estimated to be equivalent to 7.0×10^5 kb. Assuming only one insertion per cell in a gene of average size 1.0 kb about 7.0×10^5 inserts are needed to cover the whole of the haploid genome. Since only about 44% of the genome is unique sequences then with only 3.92×10^5 inserts, some are likely to affect an active gene. Thus at least 4×10^5 independently-transformed haploid cells are required to isolate one particular mutant. As described above, by whichever means mutants are induced, a large population of cells or other explants are needed.

1.7 Summary of objectives

This project is concerned with the application of a random DNA insertion approach to plant cell mutagenesis. The overall aim is to develop a model system for the isolation and cloning of agronomically important genes for use in the breeding of important crop species. To approach this, four main strategies were adopted;

1) The development of an efficient transformation system as a means of integrating foreign genes, that will induce mutations, into the flax genome. Two systems were developed. Firstly a method was developed to stably integrate transposable elements into totipotent flax cells which can regenerate plants. Thus, transposition can hopefully be induced throughout the plant development to create mutations. Secondly, a technique was developed to randomly insert T-DNA into large cell populations.

2) To develop a technique of generating large populations of

haploid cells which are competent for transformation by the above systems.

3) To examine the behaviour of transposable elements in transgenic flax in order to determine if its transposition can be induced or regulated.

4) To use the developed systems to isolate mutant cell lines by DNA insertion *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Plant materials

2.1.1 Sterile seedlings

Commercial flax seeds (*Linum usitatissimum* L. cv. Antares; Sharpes Seeds) were surface sterilized by immersion for 20 min in 10% commercial bleach followed by rigorous washing with sterile distilled water. Sterile seeds were then placed in a Kilner jar, on sterile vermiculite wetted with a modified Hoagland's solution (Johnson *et al.*, 1957; Appendix 2) and germinated at 27°C with a 16 hr photoperiod. After two days, 1.0-2.0 mm sections of hypocotyls were excised and used in the transformation experiments. Other explants were taken from the cotyledons and root-tips for shoot regeneration experiments.

2.1.2 Mature plants

Seeds of the high-twinning line of *Linum usitatissimum* L. cv. RA91 were obtained from CSIRO, Australia. Seeds were germinated on filter paper wetted with Hoagland's solution in a 90 mm Petri dish for 2-3 days. Twin seedlings were separated and the haploid members were separately grown from the diploid members. All plants were grown in a vermiculite:compost mixture (50:50) in a 'Saxil' controlled environment cabinet at 27°C with a 16 hr photoperiod. When appropriate, the plants were selfed and seeds collected after the plants had been dried out. Haploid plants were used as a

source of explants for transformation experiments, cell suspension initiation and root tip squashes.

2.2 Flax Tissue Culture

2.2.1 Callus and cell suspension cultures

2.2.1.1 Diploid suspension cell cultures

Callus was induced from cotyledons taken from 2 day old seedlings incubated on agar-solidified MSP1 medium comprising of MS basic salts and vitamins (Flow-labs; Appendix 1; Murashige and Skoog, 1962) containing 2.0 mg/l naphthaleneacetic acid (NAA), 0.5 mg/l benzylaminopurine (BAP) and 3% sucrose. Suspension cultures were started by transferring 2-4 g of callus into 80 ml of MS liquid medium containing different levels of auxins and cytokinins in a 250 ml conical flask. The cultures were agitated on a rotary shaker at 27°C. The growth of freshly initiated cultures from callus in a range of hormone levels was observed after 2 and 4 weeks in culture. Suspension cultures which were not composed of single cells or small colonies of cells were subcultured into a fresh medium containing different auxin and cytokinin concentrations after about 4 weeks. The viability of the cells was assessed by staining with 0.01% (w/v) fluorescein diacetate (section 2.2.1.3).

2.2.1.2 Haploid cell suspension cultures

Haploid cell suspension cultures were initiated from friable callus derived from leaf explants taken from mature

haploid plants. Expanding leaves were taken from one month old haploid plants , sterilised as described for seedlings (2.1.1), and cut into 1.0-3.0 mm strips. Initially callus was induced on MSD4x2 medium, comprised of MS basic salts and vitamins (Flow-labs) supplemented with 1.0 mg/l benzylaminopurine (BAP) and 0.1 mg/l napthaleneacetic acid (NAA) and 3% sucrose, for about two weeks. Then friable callus was obtained by subculturing the primary callus into the same medium or on MSP1 medium. Suspension cultures were initiated in liquid medium based on MS salts supplemented with 0.1 mg/l 2,4-Dichlorophenoxyacetic acid. The suspension cells were subcultured every 7-10 days. The suspension consisted mainly of single cells and small aggregates of cells. The viability of the suspension cells was determined as above.

2.2.1.3 Determination of cell viability

The viability of cells in suspension cultures was estimated using the technique developed by Widholm (1972). A 0.5% stock solution of fluorescein diacetate was prepared in 100% acetone and stored at -20°C. 0.5 ml of this was added to 25 ml of culture medium on ice. This solution was freshly made up when needed and replaced every 30 min. One drop was added to 2-3 drops of cell suspension on a microscope slide and the proportion of fluorescent cells determined using a Nikon 'Diaphot-TMD' inverted microscope fitted with an epi-fluorescence attachment and excitation filter block B.

2.2.2 Shoot regeneration

Explants were taken from different parts of 2 day old aseptically grown flax seedlings and used in all experiments. The hypocotyls and root-tips were cut into 1.0-2.0 mm sections. The cotyledons were cut transversely into 1.0-2.0 mm sections. All the explant sections were placed on agar-solidified medium containing different levels of auxins and cytokinins. All the media were MS-based, with 3% sucrose and 0.8% agar at pH 5.7-5.8. Generally four explants were placed in each 50 mm Sterilin Petri dish containing 5.0 ml of medium. The 'Nescofilm'-sealed Petri dishes were placed in a culture room at 27°C with a 16 hr photoperiod. Regenerated shoots were transferred into 40 ml of agar-solidified MS medium containing 0.05 mg/l kinetin in 175 ml 'Powder Round' jars for shoot elongation.

2.2.3 Vegetative propagation

2.2.3.1 Mature plants

Vigorously-growing plants were selected as donors at a pre-flowering stage when the axillary buds were just about to appear on the internodes. The stems were surface sterilised for 20 min in 10% commercial bleach and rinsed thoroughly in sterile distilled water. The upper and lower 3.0 cm ends of the stems were discarded and 1.5-2.0 cm segments of internodes, were excised. The basal end of the internode segment was placed into an MS agar-solidified medium (Appendix 1) containing different levels of auxins and cytokinins. Three to

four internode segments were placed in each 175 ml 'Powder round' jar filled with 40 ml of medium. The 'Nescofilm'-sealed 'Powder round' jars were placed in a culture room at 27°C with a 16 hr photoperiod.

2.2.3.2 Regenerated shoots

Sterile regenerated shoots were used to provide axillary bud explants for propagation as described above for mature plants.

2.3 Cytology

The following procedure was used to prepare slides for chromosome counts from root tips, callus and suspension cells. It was preceded by a pretreatment in which the root tips and cells were incubated in the presence of 0.8% hydroxyquinoline for 1-4 hr at room temperature. Cultured cell samples were transferred to 10 ml centrifuge tubes. This allowed the solutions used in the subsequent stages to be rapidly decanted after a 2 min spin at 2000-3000 x *g*. Root tip samples were placed in a small glass vial throughout the treatments. The material was fixed in 3:1 alcohol:acetic acid for at least 4 hr, but after this could be kept in the fridge (4°C) for weeks. The resuspended cells and root tips were then hydrolysed by incubating in 5 N HCl for 1-8 min at room temperature, depending on the sample. The acid was decanted after a rapid spin in case of the cultured cell samples (total time for removal = 2 min) and the cells resuspended in aceto-

orcein solution and kept at room temperature for a minimum of 2 hr. The cells were then washed 3 times (5 min per wash) in double distilled water before being resuspended in 25% acetic acid. Root tips were transferred into 70% alcohol after hydrolysis.

Squash preparations were prepared by placing a small number of cells in 25% acetic acid, or a root tip, in a drop of stain on a glass slide. Using fine needles, and working under a dissecting microscope, about 2 mm was cut off from the tip of the root and the rest was discarded. The tissue was then teased into very small groups of cells using a pair of fine needles. A cover-slip was mounted over the material and tapped with a glass rod having a blunt end, taking care not to move the cover-slip. The glass slide was then heated gently by holding it a few centimeters above an alcohol lamp for about 5 sec. The slide was placed between two sheets of blotting paper and firm vertical pressure was applied with a finger over the area of the cover-slip. Lateral movement must be avoided for this will cause the material to 'roll-up'. If air bubbles were present after pressing, a little drop of stain or 45% acetic acid was run under the cover-slip. Pressure helps to flatten the cells, spread the chromosomes and differentiate the stain. It also helps to stick the cells to the glass. The preparation was now ready for examination under the microscope.

2.4 Plant cell transformation procedures

2.4.1 Bacterial cultures

Agrobacteria for transformation were prepared by growing a single colony, picked from a selection plate, overnight at 27°C (on a rotary shaker) in 5.0 ml of nutrient broth (see below) containing a suitable antibiotic for selection.

Oxoid nutrient broth

Ingredients in 1.0 l	gm
'Lab-Lemco' Beef Extract	1.0
Yeast Extract (Oxoid L20)	2.0
Peptone (Oxoid L37)	5.0
Sodium Chloride	5.0
pH 7.4	

Dissolve 13gm of Oxoid nutrient broth in 1.0l of distilled water.

Agrobacterium strains	Antibiotics (µg/ml)
C58 (pGV3850::1103)	100 Carbenicillin
LBA4404 (pAL4404)	50 Rifampicin
LBA4404 (pBin19::Mu1)	50 Kanamycin
A4	none
A4 (pMuNK14)	5 Tetracycline
9402 (pDLT)	5 Tetracycline

2.4.2 Transformation of flax explants

The hypocotyl sections from flax seedlings or leaf sections from mature haploid flax plants were soaked in a 1/50

or 1/10 dilution of the overnight *Agrobacterium* culture in MS-based liquid medium, with or without hormones, for 1 hr or more at room temperature. Then explants were then removed to the corresponding agar-solidified medium and placed at 27°C in the dark overnight. Explants were then transferred onto appropriate media containing in addition cefotaxime and solidified with 0.8% agar. Selection of transformed tissues was applied immediately after co-cultivation, or two weeks after inoculation, on medium containing kanamycin. The cultures were incubated at 27°C under a 16 hr photoperiod. Control (uninoculated) explants were treated in the same way.

2.4.3 Mass transformation of flax cells

2.4.3.1 Feeder plate preparation

20 ml of cell suspension was poured into a sterile plastic Universal and allowed to settle for 5 min. When the larger cell clumps had settled, about 2 ml of the fine cell suspension was pipetted out using a sterile Pasteur pipette into a 90 mm Petri dish. 15 ml of molten MSD4x2 medium cooled to 40°C was then poured into the Petri dish and the cell suspension was dispersed by gently swirling the plate. The media was then allowed to solidify for 20 min. A piece of sterile 90 mm filter paper (guard disk) was then placed on the surface of the solid agar followed by two pieces of 50 mm filter papers, which had been cut into two halves, to act as transfer disks.

2.4.3.2 Seeding of transfer disks

A fine suspension culture in a 250 ml flask was allowed to settle for 5 min. This allowed larger clumps of cells to settle down. The supernatant, which consist of mostly single cells and smaller cell clumps, was transferred into a sterile plastic Universal using a sterile Pasteur pipette. To determine the number of single cells or cell clumps, 1 ml of the supernatant was pipetted into a Sedwick/Rafter counting chamber and the number of plating units in 25 squares were counted at random. This number was multiplied by 40 to give the plating units/ml. The concentration of plating units was adjusted to 1000 units/ml, by diluting with liquid medium or concentrating by centrifugation. Each transfer disk in a feeder plate was then seeded with 500 plating units using a Gilson P1000, taking care to cover as much of the transfer disk surface as possible. The plates were sealed with Nescofilm and incubated at 27°C for 5-7 days.

2.4.3.3 Co-cultivation with *Agrobacterium*

After about 1-2 weeks of incubation, micro-colonies grown from cell suspension cultures on the transfer disks, were inoculated with 1/10 dilution of an overnight *Agrobacterium* culture. Using a Gilson P200 micropipette, 200 µl of the diluted *Agrobacterium* culture was spread evenly on the micro-colonies. The inoculated transfer disks were left on 'feeder plates' for another 5-6 days. Micro-colonies were then picked up and transferred onto selection medium containing kanamycin.

Putative transformed callus lines were grown in fresh medium with cefotaxime and kanamycin. These transformed callus lines were continually maintained in the same medium. Control (uninoculated) explants were treated in the same way.

2.4.3.4 Selection, propagation and rooting of transformed shoots

Regenerated shoots of 1.0-2.0 cm in height were separated from hypocotyl explants and proliferating callus and then subcultured onto propagation + selection medium comprising of MS basic salts with 0.05 mg/l kinetin, 200 µg/ml cefotaxime, 100 µg/ml kanamycin, 3% sucrose and solidified with 0.8% agar. When 4.0-5.0 cm in length, putatively transformed shoots were transferred to root induction medium consisting of agar-solidified half-strength B5 medium (Gamborg *et al.*, 1968; Appendix 1) without phytohormones and containing kanamycin (100 µg/ml).

2.5 Detection of nopaline in transformed tissues

Transformed callus and shoots selected in kanamycin containing medium were screened for nopaline synthase activity. The nopaline synthase assay was done according to protocols developed by Dr. A. Depicker (personal communication to Dr. J. Draper). Samples (approximately 200 mg) were taken aseptically from transformed callus and shoots, and untransformed tissues as control. They were placed in an Eppendorf tube and incubated overnight at 27°C in 500 µl of MSP1 medium

supplemented 10 mM arginine, and 10 mM α-ketoglutaric acid. The incubation medium was then poured through a sieve and the tissues rinsed with distilled water several times and blotted dry with tissue paper. Approximately 200 mg of tissue was homogenized with a plastic stirring rod (Sarstedt) in an Eppendorf tube (Sarstedt) and centrifuged for 2 min in a minicentrifuge (MSE).

The crude extracts were subjected to paper electrophoresis as follows: An opine electropherogram paper was made by cutting out a 20 x 23 cm piece of 3MM chromatography paper (Whatman) and a fold made 3.5 cm from each end. Using a soft pencil, sample spots were marked at 1 cm intervals, 1 cm away from one of the folds. All this was performed wearing disposable gloves and on a piece of new 'Benchcote' to avoid contaminating the electropherogram with amino-acids found in perspiration.

5 µl of the tissue extracts were taken up by capillarity into a 5 µl 'microcap'. Under a stream of warm air from a hair dryer the sample spots were loaded by touching the electropherogram several times with the 'microcap' until all the extract was drawn out by capillarity attraction. The spots were made as small as possible. When the spots had dried, 1 µl of standard nopaline or arginine solutions (1 mg/ml) was spotted onto appropriate marked spots.

Wearing gloves and working in a fume hood on a piece of 'Benchcote', the electropherogram was dampened (using

cotton wool), with electrophoresis buffer (comprising Formic Acid: Acetic Acid : Water, 5:15:80), avoiding a 1 cm strip along the origin where the samples had been spotted. The electropherogram was quickly placed into a flat-bed electrophoresis tank (Shandon) containing 500 ml of buffer, with the origin at the anode (+ve) side. The electropherogram was only in contact with the buffer at both folded ends, which were 2/3 immersed. Electrophoresis was carried out at 400 volts for 45 min.

Following electrophoresis, the electropherogram was hung origin upwards and dried under warm air in the fume hood for 20 min. Once dried it was developed by smoothly pulling it through the staining reagent (2 mg of phenanthrenequinone in 10 ml of 100% ethanol mixed before use with 1 g of NaOH in 10 ml of 60% ethanol), origin end first, and then hung up to dry in the fume hood (origin upwards) under a stream of hot air for 20 min.

The stained electropherogram was then viewed under U.V illumination and photographed on the Polaroid Land Camera. Wearing a pair of goggles, any green spots visible under U.V illumination were marked with pencil. The mobility of opines in samples was compared to standard tracks.

2.6. NPT-II assays

A radioactive assay for NPT-II in extracts from plant cells was performed on crude tissue extract fractionated by

non-denaturing polyacrylamide gel electrophoresis, using methods developed by Reiss *et al.*, (1985).

2.6.1 Polyacrylamide gel preparation

Gel plates were assembled using a slab gel apparatus (Studier type). Glass plates were cleaned with detergent then with ethanol/acetone mix (1:1) and allowed to dry. The glass gel plates were assembled with 1.0 mm spacers, held together with metal fold-back clips, and sealed around the bottom and side edges with hot (95°C) 1% high gelling temperature agarose (Seakem).

The 10% resolving gel was prepared by mixing the following in order, with TEMED (Sigma) and freshly prepared ammonium persulphate added after degassing.

13.4 ml 30:0.8% acrylamide/bisacrylamide (BDH)

14.8 ml 1M Tris-HCl pH 8.8

7.2 ml Distilled water

Degass for 1 min

20.0 µl TEMED

2.0 ml 1.5% ammonium persulphate

The solution was poured into the assembled glass plates to a depth of 14 cm and overlaid with 1-2 ml of propanol:water (1:1) using a Pasteur pipette. The resolving gel was allowed to polymerise for 30-60 min as indicated by a sharp line between the gel and the overlay. When polymerised, the propanol:water mixture was tipped off and the gel was carefully rinsed with distilled water. The water was then

poured away and the plates were blotted dry with filter paper.

The stacking gel was prepared by mixing the following;

2.5 ml 30:0.8% acrylamide/bisacrylamide (BDH)

2.5 ml 1M Tris-HCl pH 6.8

13.8 ml distilled water

Degass for 1 min

5.0 µl TEMED

1.0 ml 1.5% ammonium persulphate

The solution was poured into the glass plates and the well former was inserted. When the gel had polymerised, indicated by a distinct interface on the surface after about 15 min, the well former was removed and the wells rinsed with 1x Reservoir Buffer (dilution of 5x Reservoir Buffer which consists of: 139.6 g of glycine and 15.15 g of Tris Base in 1.0 l of distilled water, pH 8.3).

The bottom spacer was removed and the plates lowered at an angle into the bottom reservoir filled with 1x Reservoir Buffer and trapped air was allowed to run from between the plates. The plates were rested on the supports in the bottom reservoir and secured to the top reservoir with fold back metal clips. The top reservoir was filled with 1x Reservoir Buffer. The assembled electrophoresis apparatus was then equilibrated to 4°C in a cold room for about 30 min.

2.6.2 Radioactive *in situ* assay for electrophoresed NPT-II

Plant samples were extracted by crushing 100 mg of transformed or control tissues in Eppendorf tubes (Sarstedt) containing 20 µl of Buffer A (see below), with a disposable plastic rod (Sarstedt). Buffer A:

10% glycerol
40.0 mM EDTA
150.0 mM NaCl
100.0 mM NH_4Cl
10.0 mM Tris-HCl pH 7.5
2.31 mg/ml DTT (Sigma)
0.12 mg/ml leupeptin (Sigma)
0.21 mg/ml trypsin inhibitor (Sigma)

Crystalline phenylmethylsulfonyl fluoride (PMSF) is added immediately before use to a concentration of 100 µg/ml.
The tubes were then centrifuged in a microcentrifuge (MSE) for 3 min and the supernatant transferred into new tubes. 1.0 µl of a bromophenol blue solution (0.01% in 25% glycerol) was added and mixed into the supernatant.

Using an automatic pipette (Gilson P200), the wells were loaded with 40 µl of transformed or control tissue extract. A bacterial NPT-II preparation was loaded into one lane as a positive control. The 10% non-denaturing polyacrylamide gel was then electrophoresed at 30 volts for 16 h using an LKB power pack, with the negative terminal (black) connected to the top and the positive (red) to the bottom

reservoir. When the bromophenol blue marker dye reached the bottom of the gel, the power was turned off and the gel plates were disassembled.

The bottom and sides of the gel were trimmed by chopping them with a scalpel to an exact size of 13 cm long and 11 cm wide, and the gel was placed into a plastic sandwich box (Steward Plastics), rinsed in distilled water and equilibrated against 100 ml of Buffer B (see below) for 10-15 min at 4°C.

Buffer B:

67.0 mM Tri-malate pH 7.1

42.0 mM $MgCl_2$

400.0 mM NH_4Cl

When equilibrated, the gel was transferred onto a clean 11x13 cm glass plate which has been edged with a paper sticky tape former and frosted in a freezer for a few minutes. Working behind a radiation screen and in a plastic disposable tray, a 1.5 mm thick layer of 1% low gelling temperature (LGT) agarose (Seakem) was poured onto the non-denaturing gel. The LGT agarose gel contained the following;

100 µCi (^{32}P) ATP (3000 Ci/mmol) (Amersham)

40 µl kanamycin sulphate (25 mg/ml) (Sigma)

12 ml Buffer B

12 ml LGT agarose (2% in distilled water) (Seakem)

The gel was incubated for 30 min at room temperature. The sticky paper tape former was cut at the corners and flattened. A sheet of Whatman P81 paper cut to size was placed

on top of the gel followed by a stack of 12 pieces of 13x11 cm 3MM papers (Whatman) and a 1 kg weight.

After 1-3 h, the P81 paper was removed from the sandwich and any adhering agarose was removed. Any phosphorylated proteins which may cling to the paper were removed by incubation at 60°C in a protease solution (1 mg/ml trypsin in 1% SDS). After 30 min the paper was washed 5 times in hot (80-90°C) 10 mM phosphate buffer (pH 7.5) for 5 min each wash, and then dried using a heat lamp or hot air blower. The paper was exposed to X-ray film 'Hyperfilm-MP' (Amersham), after wrapping it in 'Saranwrap', for an appropriate time, depending on the signal.

2.6.3 Preparation of bacterial NPT-II

NPT-II can be prepared from a strain of *E. coli* containing a multicopy plasmid harbouring the transposon Tn5. Since the expression levels are extremely high in such strains, it is sufficient for most purposes to simply sonicate an exponentially growing culture of the bacteria and use the crude extract. All manipulations were performed at 4°C.

20 ml of bacteria were grown to a density of $4x10^8$ cells/ml and harvested by centrifugation (7000 rpm). The cells were resuspended in 500 µl of 10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 25 mM NH_4Cl, 0.6 mM ß-mercaptoethanol (or 2.0 mM DTT), and then sonicated on ice 3x, 5 sec bursts with 2-4 min cooling periods on ice. This was followed by centrifugation (16000 rpm) to remove debris. The extract was then removed into an

Eppendorf tube (Sarstedt) and stored at -20°C until required.

2.7 Genomic DNA isolation from plant tissues and callus

DNA was isolated from freeze dried callus tissues or leaves from mature plants by the CTAB procedures outlined by Lichstenstein and Draper (1985). 0.1 g of freeze dried callus or 0.07 g of freeze dried leaf material was ground into fine powder with 0.1 g of alumina (Sigma, type 305) in a small mortar and pestle (9 cm diameter). The powdered plant tissue was dispersed in 600 µl of 1x CTAB extraction buffer (see below) in a sterile Eppendorf tube (Sarstedt) with a plastic stirring rod (Sarstedt). x 1 CTAB Extraction Buffer;

50.0 mM Tris-HCl pH 8

0.7 M NaCl

10.0 mM EDTA

1.0% (w/v) CTAB (Sigma H-5882)

1.0% (v/v) 2-mercaptoethanol (2ME).

The extract was then incubated for 10-20 min at 56°C with occasional shaking to keep the extract mixed, followed by 600 µl of chloroform:octanol (24:1) and emulsified by shaking. The chloroform:octanol solution was used to denature and precipitate protein from the extract. The emulsion was centrifuged for 2 min in a microcentrifuge (MSE) and the top aqueous phase removed to a clean Eppendorf tube (Sarstedt). The denatured protein interface in the original tube was washed with 100 µl of 1x CTAB extraction buffer and the tube was

recentrifuged and the aqueous phase pooled in the clean tube.

To the pooled aqueous phase, 1/10 volume (~70 µl) of 10% CTAB was added and the chloroform:octanol extraction was repeated. Avoiding debris, the aqueous phase was removed into a siliconised 'Pyrex' centrifuge tube such as a 16 x 125 mm Corning tube and precipitated out with an equal volume (600 µl) of CTAB precipitation buffer (see below) at room temperature for 20 min. x 1 CTAB Precipitation Buffer;

50.0 mM Tris-HCl pH 8

10.0 mM EDTA

1.0% (w/v) CTAB

The precipitate was centrifuged for 15 min at 2000 *g* in a bench-top centrifuge. The supernatant then poured off and the drained nucleic acid/CTAB pellet was redissolved in 400 µl of 1 M NaCl, heating to 56°C if required. The nucleic acid/CTAB solution was removed to a clean Eppendorf tube (Sarstedt) when the pellet was fully dissolved and two volumes (800 µl) of absolute ethanol was added, mixed and the tube placed at -70°C (dry ice/ethanol) or -20°C overnight.

The precipitated nucleic acid was centrifuged in a microcentrifuge for 2 min and the pellet washed with 1 ml 65% ethanol for 1 min twice, followed by x2 washes with 85% ethanol. The pellet was then dried in a vacuum desiccator and redissolved in 85 µl of sterile distilled water and stored at -20°C. The DNA was quantified by the diphenylamine assay and used for Southern blot analysis.

2.8 Isolation of total nucleic acids from *A. tumefaciens*

The procedure was modified from Dhaese *et al.* (1979). A single colony of *Agrobacterium* picked from a selection plate was grown overnight in 5 ml of nutrient broth (section 2.4.1) containing selective antibiotics at 27°C in orbital shaker. 1.5 ml of the culture was placed in an Eppendorf tube (Sarstedt) and centrifuged in a microcentrifuge for 5 min. The supernatant was removed and the cells resuspended in 380 µl TE (10.0 mM Tris-HCl pH 7.6, 1.0 mM EDTA pH 7.6) and 25 µl of 25% SDS. 100 µl of 'Pronase' solution (5 mg/ml Pronase [Sigma] in TE, predigested for 2 h at 37°C) was added to the mixture and the cells lysed at 37°C for 30 min or more. Then 50 µl of 5M NaCl was added and the lysate incubated at 68°C for 30 min.

Nucleic acids were extracted with 500 µl of phenol/chloroform (see below) and the chromosomal DNA in the bacterial lysate was sheared by passing the mixture 5x through a blue pipette tip on a Gilson P1000.

(Phenol/chloroform (1:1): Phenol/chloroform is a mixture of phenol, equilibriated with 10 mM Tris-HCl pH 7.4; 1.0 mM EDTA pH 8.0, containing 0.1% hydroxyquinoline and 0.2% ß-mercaptoethanol. The chloroform used is a 24:1 (v/v) mixture of chloroform and isoamyl alcohol).

The aqueous phase was separated from the organic phase by centrifugation in a microcentrifuge (MSE) for 5 min. The aqueous phase was then phenol/chloroform extracted 3x, retaining each time the upper aqueous phase. Nucleic acids

were precipitated from the aqueous phase with 2 volumes of absolute ethanol chilled to -70°C in a dry ice bath. The precipitate was collected by centrifugation for 10 min. The nucleic acid pellet was then washed in 70% ethanol, vacuum dried and dissolved in 50 ul of sterile distilled water.

2.9 Determination of DNA concentration in crude nucleic acid preparations using the diphenylamine reagent

The folowing components were added to an Eppendorf tube in order, in a fume hood;

10 µl of sample DNA solution,

140 µl of distilled water,

150 µl 3 N perchloric acid (49.2 ml of perchloric acid (BDH) made up to 200 ml with distilled water),

180 µl of diphenylamine solution (8 g of diphenylamine (Analar grade; BDH) added to 20 µl of paraldehyde (Analar grade; BDH) in 198 ml of glacial acetic acid (Analar grade; BDH). This reagent can be used in a quantitative colourimetric assay specific for DNA in crude extracts of a plant tissues.

The solutions were mixed by inversion and incubated for 16-20 h in the dark at 30°C. It was important to rigidly stick to the same time and temperature of incubation, otherwise a calibration curve had to be constructed using standard DNA samples each time the assay was performed. The optical densities of the DNA solutions were read off a spectrophometer set at 600 nm, and the zero setting was occasionally checked against a solution of distilled water plus diphenylamine reagents. A calibration curve of DNA versus $O.D._{600}$ was

constructed using samples of pure herring sperm DNA, and the concentration of each plant DNA sample was read off the graph. There is a linear relationship between $O.D._{600}$ and DNA concentrations between the limits of 5-75 µg/ml.

2.10 DNA restriction and analysis by Southern blotting

2.10.1 Restriction enzyme digestion of DNA

Digestions were performed on 5 µg of sample DNA (estimated by diphenylamine assays), using the appropriate restriction enzymes by following the procedures recommended by the manufacturer (Bethesda Research Laboratory). Generally 3 µl of restriction enzyme and 3 µl of restriction enzyme buffer were added to 5 µg of sample DNA and the volume was made up to 30 µl with distilled water. The reaction mixture was incubated at 37°C for 4-18 h.

5 µl of gel loading buffer (50% glycerol, 0.25 M EDTA, 0.01% bromophenol blue pH 8.0) was added to the reaction mixture and mixed well.

2.10.2 Fractionation of DNA by agarose gel electrophoresis

0.8 g of high gelling temperature agarose (Seakem) was added to 100 ml TAE/EtBr electrophoresis buffer (89.0 mM Tris-OH, 89.0 mM boric acid, 2.5 mM EDTA pH 8.3, 5.0 µg/ml ethidium bromide), dissolved completely by boiling and then cooled 70°C.

The gel plate was prepared by sealing both ends of a clean 'Perspex' gel plate with tape, and the well former was

positioned into the appropriate slots. The cooled molten agarose was poured into the assembled gel apparatus, and allowed to solidify. When the gel had set, the well former and the tape at the ends were carefully removed. The gel was then submerged in the electrophoresis tank containing the TAE/EtBr electrophoresis buffer. The tank was then connected to a powerpack (LKB or Kinghill).

The restricted DNA samples, positive bacterial plasmid reconstruction control, negative control and the molecular weight-marker DNA were loaded into appropriate wells. The gel was then electrophoresed, the origin at the negative, at 100 volts for about 3 h until the bromophenol blue had migrated to the bottom of the gel. DNA was viewed with a UV transilluminator and photographed using a Polaroid Land Camera fitted with an orange filter. The migration of the size markers was noted.

2.10.3 Southern blotting of fractionated restriction fragments

The fractionated DNA was transferred to a nitrocellulose filter by a 'blotting' procedure first described by Southern (1975). The gel was depurinated by shaking slowly on a rocker platform in 250 ml of 0.25 M HCl in a sandwich box for 7 min. The acid treatment breaks down larger DNA fragments to increase the efficiency of the transfer. The depurinating solution was decanted and the gel rinsed with distilled water.

The DNA in the gel was then denatured *in situ* by treatment with a strong alkaline solution (0.5 M NaOH, 1.5 M NaCl), for 30 min-2 h on the rocker platform. After the DNA had been denatured the gel was rinsed with distilled water and neutralised for 30 min-2 h in 250 ml of the neutralising solution (3.0 M NaCl, 0.5 M Tris-HCl pH 7.4).

The gel was washed in distilled water again and blotted onto nitrocellulose filter as follows. An 18x27 cm sponge was placed in a blotting tray filled with 20x SSC (3.0 M NaCl, 0.3 M Na citrate pH 7.0) to 0.5 cm below the top of the sponge. A double layer of 3MM paper (Whatman), cut to size, was placed on top of the sponge and allowed to soak up the 20x SSC.

A piece of 'Saranwrap' with a gel-sized hole cut out in the centre, was overlaid around the filter paper. Avoiding trapping any air bubbles, the gel was placed in the hole, over the filter paper. A piece of nylon-based hybridisation filter ('Hybond-N', Amersham) which had been cut to gel size was wetted with 3xSSC, and placed on the gel without trapping any air bubbles between the filter and the gel. The side of the 'Hybond-N' filter which was in contact with the gel was initially marked 'DNA side' with pencil. 4 sheets of gel-sized 3MM paper (Whatman) were placed on top of the 'Hybond-N' filter, followed by about a 10 cm layer of paper towels and a rigid plastic box lid. The stack of paper towels was compressed by placing a 500 ml medical flat bottle, filled with tap water, on its side in the center of the plastic box lid. The blotting set-up (Fig.

2.1) was left overnight for the single-stranded DNA to be transferred and immobilised on the 'Hybond-N' filter.

The 'Hybond-N' filter was then carefully removed, rinsed briefly in 3xSSC and blotted dry between 3MM paper (Whatman) followed by baking in an oven at 65°C for 10-15 min. The DNA side of the filter was then UV irradiated for 4 sec on a transilluminator. The filter was then either stored between a folded piece of 3MM paper (Whatman) in a dry environment or used straight away in the DNA hybridisation process.

2.11 Hybridisation of Southern blotted DNA

2.11.1 Prehybridisation

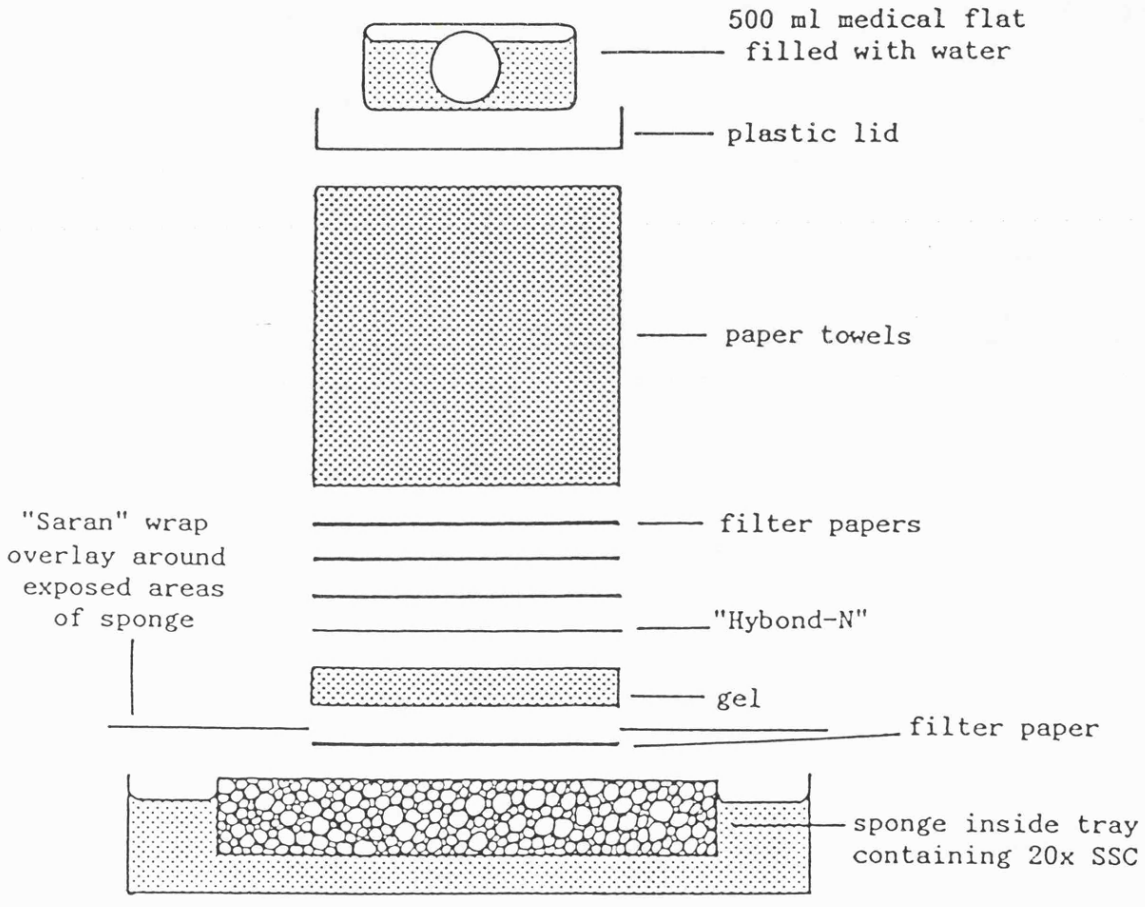
The dried and irradiated 'Hybond-N' filter was placed in a plastic hybridisation box containing 40 ml of Pre/Hybridisation solution (1.5x SSPE, 0.5% dried milk, 1.0% SDS, 6.0% PEG, 0.1-0.5 mg/ml herring sperm DNA), which has been preheated in a 65°C shaking waterbath (Gallenkamp). The lid of the box was put on to prevent evaporation, and the box slowly shaken in the waterbath for at least 4 h.

20x SSPE (3.0 M NaCl, 0.2 M $NaH_2PO_4.2H_2O$, 0.02 M EDTA).

2.11.2 Hybridisation

The Pre/Hybridisation solution was replaced with 40 ml of preheated hybridisation solution. A hybridisation probe which has been radiolabelled by an oligolabelling technique (see section 2.12) was denatured in a boiling water bath for 5 min and cooled on ice. The denatured hybridisation probe was added

Fig 2.1 Southern blot set-up



to the hybridisation solution and the box sealed with a tape. Hybridisation was performed overnight in a shaking water bath at 65°C.

The hybridisation solution was poured away into a radioactive sink, and the filter was rinsed twice with preheated (65°C) Wash Solution A (3x SSC, 0.1% SDS). This was followed by two 30 min washes in a shaking waterbath at 65°C using Wash Solution A.

The filter was then washed stringently 4x for 15 min in Wash Solution B (0.5x SSC, 0.1% SDS). A hand-held radiation monitor was used to follow the removal of radioactivity. After allowing the filter to dry between 3MM paper (Whatman), it was wrapped in a layer of 'Saranwrap' and exposed to X-ray film ('Hyperfilm-MP', Amersham) in an X-ray cassette fitted with intensifying screens at -70°C.

2.12 Oligolabelling technique for preparation of hybridisation probe

2.12.1 Labelling probe DNA

DNA probes used to detect the presence of sequences in a Southern blot were radiolabelled by the method of Feinberg and Vogelstein (1984). The probe to be used was boiled for 5 min in a water bath and cooled to 37°C in a water bath for 5-10 min. A standard oligo-labelling reaction for 2-6 filters was prepared by mixing the following solutions in an Eppendorf tube:

Component	Volume (µl)
Oligo-labelling Buffer (OLB)	3.0
BSA	0.6
^{32}P dCTP	1.5
DNA polymerase 'Klenow' fragment	0.6

(see Appendix 3 for solutions of 'oligo-labelling' reaction mixture)

The solutions were mixed, centrifuged if necessary, and 10 ng of the probe DNA was added. The volume of the reaction mixture was made up to 15 µl with sterile distilled water and mixed well by shaking and then centrifuged in a microcentrifuge. The Eppendorf tube was then placed in a lead pot and incubated at room temperature for 5-6 h.

The reaction mixture was diluted with Pre/Hybridisation solution to measure the incorporation of radioactivity into probe (see 2.12.2). The oligolabelling reaction mixture was boiled for 10 min, cooled briefly on ice and added to hybridisation solution (see section 2.11.2).

2.12.2 Measurement of radioactive incorporation in probe DNA

Oligolabelling reaction mixtures which had been incubated for 6 h at room temperature were diluted to 200 µl with Pre/Hybridisation solution. Using a Gilson P20 pipette, 1 µl of the diluted mixtures was spotted onto a 2.5 cm GF/C glass fibre filter disc (Whatman) and placed inside a plastic scintillation vial. This will give the total radioactivity in the sample.

Another 1 µl of the diluted reaction mixture was aliquoted into 500 µl of a herring sperm DNA solution (stock of 0.5 mg/ml) in an Eppendorf tube (Sarstedt). The mixture was precipitated with 125 µl of 50% TCA (trichloroacetic acid) on ice for 5 min.

The precipitate was collected on a 2.5 cm GF/C glass fibre filter disc (Whatman) and washed with 5 ml of 10% TCA followed by 5 ml 100% ethanol by vacuum filtration. The filter was then placed in a plastic scintillation vial. This sample corresponds to incorporated radioactivity.

Both scintillation vials were filled up with 5 ml of a toluene based scintillation liquid and the activity counted using an LKB 'Beta Rack' liquid scintillation counter. The percentage of radioactivity incorporated into the sample was then calculated. A probe of specific activity 10^9 cpm/µg of DNA was prepared routinely.

2.13 Removal of probe and re-use of DNA blots (Hybond-N filter membrane)

The procedures recommended by the manufacturer (Amersham International plc) were followed closely with slight modification. Blots were incubated at 45°C for 30 min in 0.4M NaOH in a hybridisation box under continuous agitation in a shaking water-bath. The NaOH solution was then replaced with 0.1x SSC, 0.1% SDS and 0.2M Tris-HCl pH 7.5 solution and the blots shaken for 30 min at 45°C. The blots were then rinsed

several times with distilled water heated to 45°C and the activity monitored with the GM-counter. If the activity was still high the whole process could be repeated. After removal of the probe, the filter membrane was wrapped in 'Saranwrap' and autoradiographed to ensure that removal of the probe had been successful.

CHAPTER 3

FLAX TISSUE CULTURE SYSTEMS

3.1 INTRODUCTION

3.1.1 Generation of haploid flax materials

Since most insertion mutations should characteristically produce a recessive phenotype, it is necessary to mutagenise a large population of haploid cells to reduce the complications of 'masking' by dominant alleles, which would occur at higher ploidy levels. Haploid tissues or cells are most often produced through tissue culture techniques such as the induction of androgenesis in cultured anthers (Guha and Maheshwari, 1964; reviews, Bajaj, 1983; Dunwell, 1985), culture of isolated pollen, or chromosome elimination through hybrid embryo culture (Reinert and Bajaj, 1977; Nitsch, 1977; Jensen, 1977). During normal plant development, pollen grains containing the male gametophyte are produced in relatively large numbers. Gametophyte development during *in vivo* fertilisation leads to the production of generative and vegetative nuclei in the growing pollen tubes. In androgenesis, either the generative or vegetative nuclei in appropriate pollen grains divide by mitosis repeatedly to produce a haploid embryoid (Fig. 3.1). Beside this technique, spontaneous haploid plants can be obtained by natural means through polyembryony in some species, for example flax.

Polyembryony in flax (*Linum usitatissimum* L.) was first reported by Kappert, (1933) who noted the occurence of haploid-diploid twin seedlings arising from the same seed at very low but repeatable frequencies. The frequency of twinning

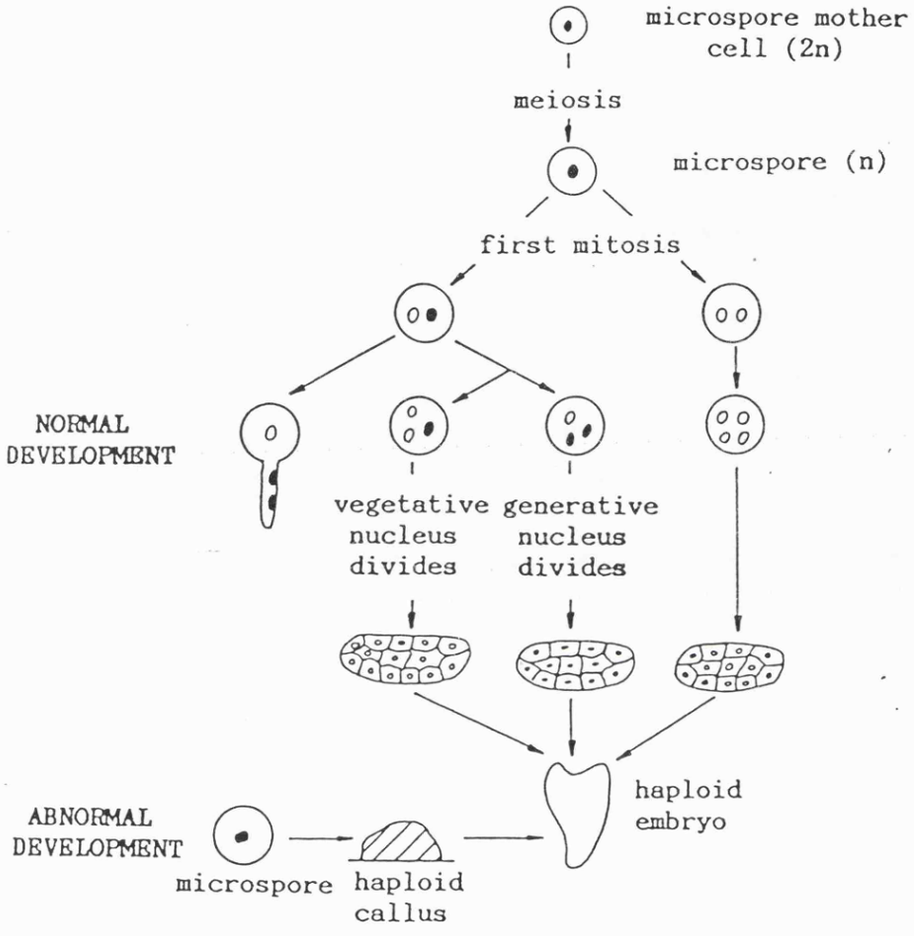


Fig 3.1 Normal and abnormal patterns of pollen development. Development of pollen *in vivo* leads to the production of two generative nuclei and the formation of the pollen tube. Haploid embryoids are produced by repeated division of either the generative or vegetative nucleus in each pollen grain, or by repeated division of similar nuclei produced after the first mitosis. Identical nuclei may also fuse and produce diploid embryoids.

has, since then, been increased to a level of over 30% (Rajhathy, 1976; Green and Salisbury, 1983). Studies on the inheritance of twins in flax have indicated the complex nature of the haploid-diploid twinning trait. It was first postulated that the trait is controlled by four recessive genes (Kappert, 1933). Later studies by Green and Salisbury, (1983) indicated that the occurrence of polyembryonic seeds in flax (*Linum usitatissimum*) is controlled by genes having predominantly additive effects. This observation is in agreement with the conclusion of Plessers (1965), who attributed the high twinning frequency in his materials to the cumulative action of a large number of genes. As to the origin of the haploid embryo, Kappert, (1933) and others suggested a synergid in the embryo sac, whereas Thompson (1977) concluded that the first cell of the haploid embryo was a product of maternal meiosis, presumably produced by apogamy, and the F2 twins obtained from crosses were hybrids. The progenitors of the twin embryo according to Murray, (1985) are two megaspore nuclei rather than one. The frequency of polyembryony in some of Kappert's selections was found to be influenced by factors such as the position of the capsules, soil moisture, potassium and nitrogen supply (Rajhathy, 1976). Generally, a high frequency of twins can be obtained by crossing high twinning with low twinning lines. The twins can be grown in an environmentally controlled growth cabinet (Murray, 1980a), or in a greenhouse (Rajhathy, 1976). The haploid member of the twins can be easily

identified at the seedling, as well as the mature plant stage of development. Besides chromosome counts, the haploids usually show some morphological differences from the diploids. Upon germination, twinning seeds produce two radicals, and about 85% of the haploid members tend to be conspicuously thinner (Rajhathy, 1976). Haploid plants usually, but not necessarily, grow slower, their leaves may be smaller, more lanceolate and remain shorter at maturity. They have smaller flowers and haploidy is clearly indicated by the poorly-developed non-dehiscent anthers.

The easy availability of haploid plants could make the manipulation of millions of haploid cells for mass transformation feasible. Mass selection of transformed haploid cells containing random T-DNA or transposable element inserts offers the possibility of the production of haploid mutants and the immediate phenotypic expression of mutations. Doubling of the chromosome complement of the haploid tissue leads, after regeneration, directly to a completely homozygous and fertile plant. Thus, all of the culture techniques have to be developed with specific varieties capable of twinning.

3.1.2 Flax tissue culture as a prerequisite for transformation

The success of *in vitro* insertion mutagenesis approaches to gene cloning requires the capacity to transform cultured cells of a given species, and to regenerate mature

plants from these cells. The regeneration of shoots from single transformed cells will not only produce a wholly mutated plantlet which can be screened for the presence of the integrated DNA sequences, but also produces seeds for genetic analysis, or mutant selection.

Previous studies have shown that flax responds well in a variety of tissue culture manipulations (Gamborg and Shyluk, 1976; Murray *et al.*, 1977; Barakat and Cocking, 1983; McHughen and Swartz, 1984; Pretová, 1986). Callus is readily induced on explants such as hypocotyls, cotyledons and roots taken from 2-5 day old seedlings. These explants have also been used as a source of protoplasts (Gamborg and Shyluk, 1976; Barakat and Cocking, 1983). However, the yield and division rate was too low to be useful for mutagenesis purposes. Protoplasts isolated from hypocotyls were found to divide once or twice, but then deteriorated after about 10 days of culture. Callus was induced from some protoplasts and microcolonies subsequently regenerated roots, but only at low frequency (Gamborg and Shyluk, 1976). The first report of plant regeneration from flax protoplasts was by Barakat and Cocking (1983). Despite the low yield and low growth rate of the protoplast cultures both root and cotyledon protoplasts were successfully induced to form callus and regenerate shoots.

The capacity of flax hypocotyl explants (*Linum usitatissimum* L., cv. Bison) to differentiate and give rise to buds and shoots *in vivo*, after removal of shoots and cotyledons

from the seedlings, was first recognised by Link and Eggers (1946). This unusual developmental characteristic was then exploited to induce morphogenesis and shoot regeneration *in vitro* on hypocotyl explants, by Gamborg and Shyluk, (1976). It was found that adding a cytokinin such as zeatin, benzyladenine or isopentenyladenosine promoted shoot formation; while auxins such as naphthaleneacetic acid or indoleacetic acid repressed shoot development and enhanced callus and root formation. Depending on the level of cytokinin and auxin in the medium, up to nine shoots were obtained from a single explant. Sebánek *et al.* (1979) found that prior to the regeneration of adventitious buds and shoots on the hypocotyl after shoot and cotyledon removal from the seedlings, the level of endogenous auxin in the hypocotyl had decreased and endogenous giberellin levels increased. This explains the inhibiting effects of exogenous auxin and promoting effects of exogenous giberellin upon the hypocotyl regeneration. A complete plant was obtained by rooting the regenerated shoot on 1/2 B5 medium with indoleacetic acid (Gamborg and Shyluk, 1976).

Studies on *in vivo* morphogenesis in hypocotyls have indicated that the buds and shoots originated from the epidermal cells (Link and Eggers, 1946; Sqalli and Chlyah, 1985). Nuclear activation and first mitosis in epidermal cells of hypocotyl segments cultured *in vitro* first appeared after 4-8 h of culture (Sqalli and Chlyah, 1985). However, the percentage of cells in mitosis or already divided, in relation

to the total number of epidermal cells, remained low (1.9%) after 72 h. In 22 division centers, only 7 were found to participate in vegetative bud formation. Since the response does not seem to be variety specific (Link and Eggers, 1946; Gamborg and Shyluk, 1976; Sqalli and Chlyah, 1985), this observation promises an efficient and simple method of producing a great number of transformed shoots if cells within the hypocotyl tissues responding to shoot induction media can be transformed. The number of transformed shoots would depend on the availability of seeds and number of competent cells in the explants. Hypocotyls from the high twinning lines of flax provide an easily obtainable source of haploid plant material for transformation and mutagenesis studies without getting involved in difficult anther culture techniques to generate haploid material as in other species.

Following regeneration, a simple and efficient technique is needed to clonally propagate transformed plantlets. It is often very difficult to obtain fertile mature transformed plants. Thus, propagating them aseptically will ensure a supply of transformed materials for analysis, in case the plants grown in soil are attacked by pathogens. Furthermore, for mutagenesis studies a lot of seed is needed for selection of any desired mutant phenotype. Clonal propagation provides a means of getting more plants and consequently, more seeds. It has been proposed that the shoot regeneration technique described above might be used for the

vegetative propagation of a particular line of flax. However, buds and shoot formation were often observed only in hypocotyl segments. An alternative way would be to regenerate shoots from mature stem explants (Murray *et al.*, 1977). However the problem with this technique was that, depending on their site of origin, shoots regenerated from mature stem explants were shown to be of increased ploidy level. Variability in morphogenetic expression was also observed in different stem segments (Chlyah *et al.*, 1980). Depending on the age and physiological state of the donor plant, as well as the site in the donor plant providing the explant, and with no external supply of regulatory substances, vegetative buds alone, callus alone, or buds and callus can be obtained. Normal shoots with no visible axillary buds and a few roots were obtained when explants were taken from a young plant. Explants taken from the upper part of an older plant produced a lot of callus. Shoots originating from calli were infrequent and had the doubled chromosome complement of the donor (Murray *et al.*, 1977).

By taking all of these observations into consideration, it was thought that clonal propagation could quite easily be done by using internodal cuttings of mature plant stems to directly induce the axillary buds to grow without a callus phase under aseptic conditions. Haploid material might also be aseptically propagated using the same method and be used in the future to isolate single cells for

transformation experiments. Aseptically propagated transformed flax plants can be induced to form roots and then grown to maturity in a vermiculite:compost mixture in a pot and to set seeds in a growth cabinet. By this method a lot of seeds could be produced from clonal lines for genetic analysis and mutant screening.

A large population of single cells is required to ensure the isolation of a desired mutant which occurs at a very low frequency. Large cell populations will also facilitate mass transformation and selection of mutants. Undifferentiated, friable callus derived from haploid materials, agitated in a liquid medium may produce a lot of single haploid cells or small colonies of haploid cells in suspension for transformation purposes. Since the ploidy levels of cells in culture will always tend to increase through time (Bayliss, 1980; Chaleff, 1981), it will be important to monitor both callus and suspension by chromosome counts. To facilitate the easy selection of transformed microcolonies and to achieve an optimum growth of the cells the plating density must be determined prior to transformation procedures. High frequency of transformation is often achieved by plating on a feeder layer (Horsch and Jones, 1980; Scott and Draper, 1987). It is of great advantage as well in the isolation of auxotrophic mutants if single cell clones can be plated out and recovered from low inoculum densities.

This chapter describes the development of flax tissue

culture systems and propagation of a high-twinning variety of flax which is thought to be useful for studies on cell mutagenesis.

3.2 RESULTS AND DISCUSSION

3.2.1 Generation of haploid material

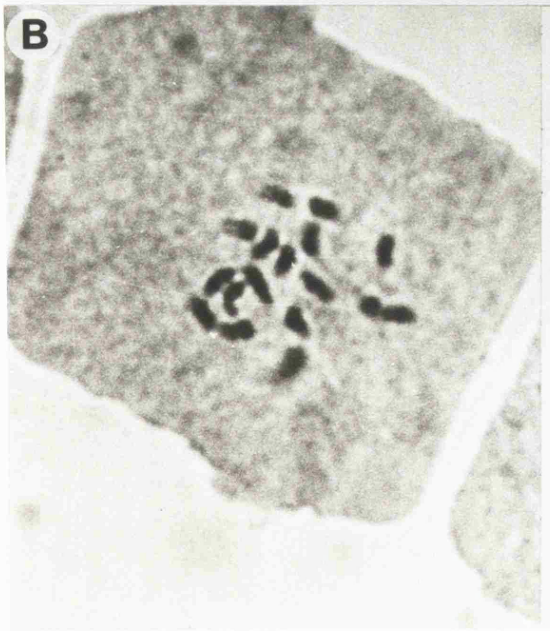
Production of haploid materials is made simple by the polyembryonic nature of the flax variety RA91. Haploid flax plants (2n=x=15) can be phenotypically identified and isolated from the haploid-diploid twin seedlings as described earlier (Rajhathy, 1976; Murray, 1980a, b). Seed germination usually occured within 24 hr of incubation on wet filter paper. Observations made on twin seedlings and mature haploid plants were similar to descriptions by other workers (Green and Salisbury, 1983). After 3-4 days of germination, the haploid members of the twin seedlings usually had a thinner tap root than the diploid members (Fig. 3.2A). The twin seedlings were separated and individually planted in a vermiculite:compost mixture. Chromosome counts from root-tip squashes were performed on putative haploid mature plants to determine their ploidy level (Fig. 3.2B and C). The results usually confirmed the haploid chromosome complement. The haploid plants usually grew slower, their leaves were smaller, more lanceolate, their stems thinner and they remained shorter than diploid plants. The mature plants started to flower after about 2.5 months and the haploids could then be definitely identified when the first flower blossomed. The flowers were smaller in size and had poorly-developed, shrivelled non-dehiscent anthers. The mature diploid plants were selfed and allowed to set seeds. Each fertile flower developed into a capsule which could

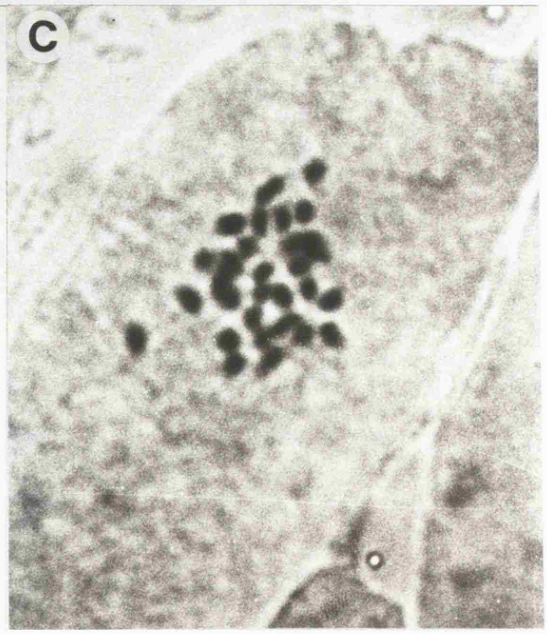
Fig. 3.2 Polyembryony in flax seeds

A) Twinning seeds of *Linum usitatissimum* L. c.v. RA91 germinated for 4 days on filter paper wetted with Hoagland's solution; note the haploid member of each twin has a thinner, slower-growing tap root.

B) Chromosomes of haploid flax (2n=x=15) and C) diploid flax (2n=2x=30) from root tips of mature plants.


A


B


C

produce about 10 seeds. Well over 100 seeds could be produced by each plant. By germinating the seeds of selfed mature diploid RA91 plants on wet filter paper the twinning frequency was estimated at about 25-30%. This unique system thus provides a constant source of haploid seedlings or mature explants for tissue culture work without the problem of inducing haploid plants via anther or pollen culture.

3.2.2 Shoot regeneration from flax explants

In this experiment, an attempt was made to regenerate shoots from seedling explants, hoping that the system could eventually be used to regenerate large numbers of transformed plants.

Shoot regeneration was found to be dependent on the source of explants and medium used. It was observed that root-tip and cotyledon sections from 2 day old seedlings could not be induced to regenerate shoots on any of the media tested (Table 3.1). However, callus was readily induced on all of the cultured explants except for the hypocotyl sections on medium 3. High auxin and low cytokinin concentrations generally induced root formation, via a callus phase, on hypocotyl and cotyledon sections. Callus was formed first at the wounded surface of the explants within 2 weeks of culture; any roots subsequently started to form only in the fourth week.

A small amount of callus was induced at both cut ends of hypocotyl sections in medium 3. However, on this medium,

TABLE 3.1

RESPONSE OF DIFFERENT SEEDLING EXPLANTS ON MS-BASED MEDIA CONTAINING DIFFERENT HORMONE LEVELS AFTER 3 WEEKS IN CULTURE

Hormone levels (mg/l)	Explants (1.0-2.0 mm)		
	Root-tips	Hypocotyls	Cotyledons
1. 2.0 2,4-D	A	A, R	F, R
2. 2.0 NAA, 0.5 BAP	A	A, R	A
3. 0.1 NAA, 1.0 BAP	A	F, S	A, R

Abbr. 2,4-D = 2,4-Dichlorophenoxyacetic acid, NAA = 1-Naphthaleneacetic acid, A = abundant callus, R = roots, F = few calli, S = shoots

TABLE 3.2

CLONAL PROPAGATION OF FLAX (*Linum usitatissimum* L. cv Antares) INTERNODE SEGMENTS ON MS-BASED MEDIA

Hormone levels (mg/l)	Response after 20 days in culture
1. 0	S (elongated), R
2. 1.0 NAA	S (elongated), R
3. 0.1 NAA, 1.0 BAP	S (short), A (greenish)
4. 2.0 NAA, 0.5 BAP	A (greenish)
5. 0.05 Kn	S (elongated), R (few)
6. 8.0 IAA, 2.56 Kn	S (short), A (greenish)

Abbr. NAA = 1-Naphthaleneacetic acid, BAP = 6-Benzylaminopurine, Kn = Kinetin, IAA = Indole-3-acetic acid, S = axillary shoots, A = abundant callus at the base, R = roots appear at the base

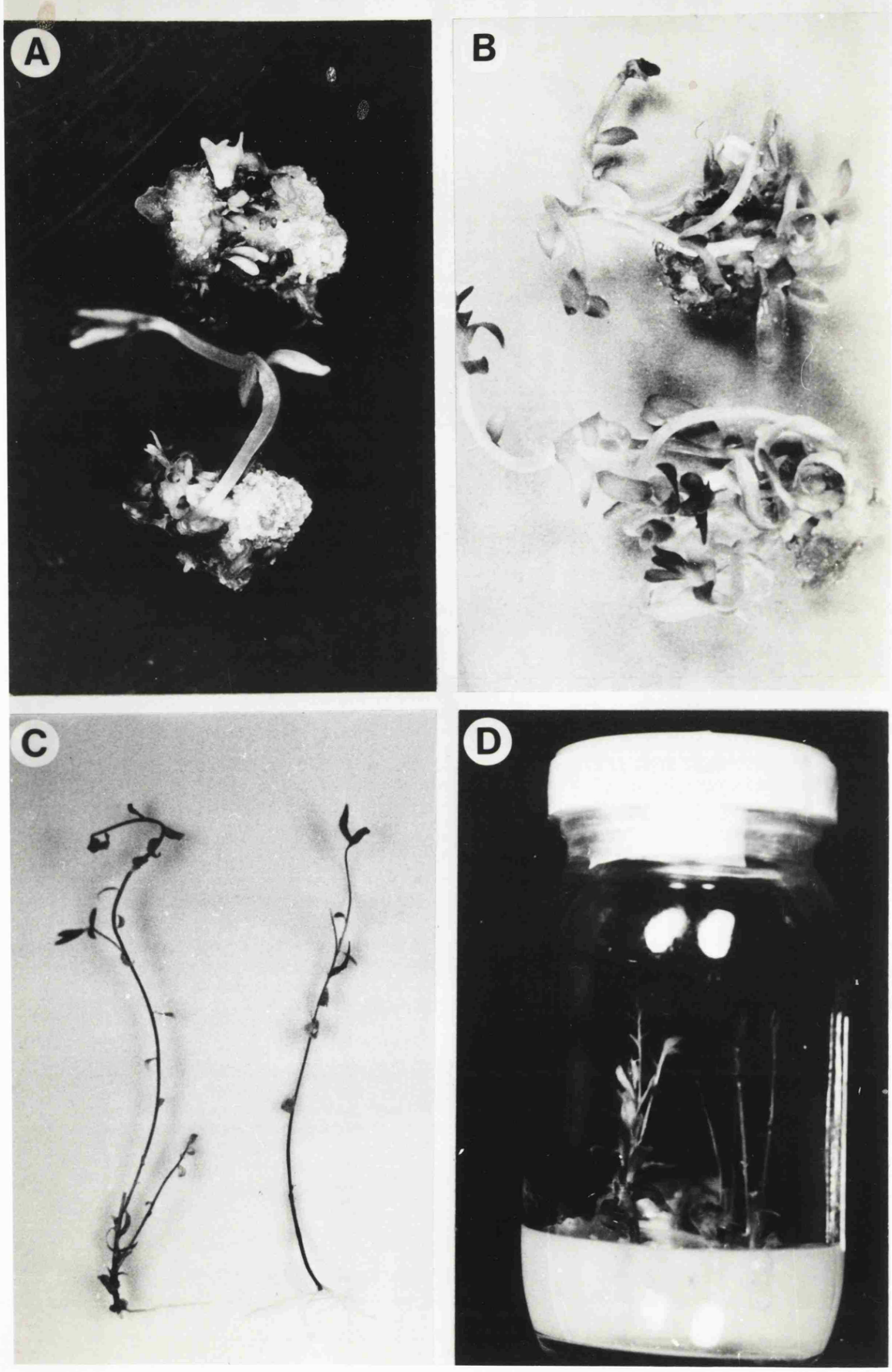
Fig. 3.3 Flax tissue culture

A) Hypocotyl segments of 2 day old flax seedlings after 2 weeks on regeneration medium (medium 3); leaf primordia and shoots have regenerated directly from the explant whilst callus has formed at both cut ends.

B) Development of leaf primordia and shoots is more dominant than formation of callus at cut ends after 3 weeks of culture on regeneration medium.

C) Rooted axillary shoots ready for transfer to soil; note the small leaves and elongated internodes.

D) Clonally propagated axillary shoots from internodal cuttings.


A
B
C
D

shorter shoots. With the exception of medium 5, the tip of the propagated shoots in the other media usually withered, turned yellow and died after more than 20 days in culture. Often this led to a loss of apical dominance and enhanced development of lateral buds from the lower leaf axils. Hence, medium 5 which contains a low cytokinin level which stimulates axillary shoot formation directly is preferred. This seemed to eliminate the problems of dimorphism observed in regenerated shoots described earlier (Chlyah *et al.*, 1980). Hopefully this technique will not bring about an increase in ploidy level of the regenerated axillary shoots, as observed in regenerated shoots from stem explants (Murray *et al.*, 1977), as they arise directly from the axillary buds without going through a callus phase. It may be necessary in the future to check the ploidy level of the regenerated axillary shoots by chromosome counting for example. At present, internode cuttings seems a useful method of continually propagating the regenerated shoot.

The leaves of the propagated shoots grown in culture were very small and the stems were usually thin and soft (Fig. 3.3D). The internodes were observed to be longer compared to plants of the same age grown in soil in the growth cabinet. Rooting was not very successful from the propagated shoots on any of the media used for propagation. Although a few roots were formed by some of the shoots, it was not until callus had been induced. These roots were not extensively developed enough to support further growth of the shoots. It was found

that an extensive root system could be induced in B5 or half-strength B5 medium (Gamborg *et al.*, 1968, see Appendix 1) with or without auxin supplement (Barakat and Cocking, 1976). A complete plantlet could then be transferred into a vermiculite:compost mixture and grown in the growth cabinet to maturity.

3.2.4 Callus and cell suspension culture

3.2.4.1 Determination of medium for flax cell suspension culture

Initial experiments were carried out using callus from cotyledon sections taken from 2 day old seedlings. The cell suspensions grown in media containing low auxin and high cytokinin levels, or vice versa, were highly heterogeneous and composed of a wide range of aggregate sizes. A high proportion of the aggregates in the freshly-initiated suspension from callus were rather large, formed as a result of breaking-up of the callus clumps. More cells were observed to be dissociated from callus in medium containing high auxin and low cytokinin levels; for example medium 1 in Table 3.3. Subculture into the same medium generated finer cell suspensions together with compact clumps of callus which eventually developed into roots if not subcultured within two weeks. Although a high number of expanded single cells and small colonies of cells were generated after a few passages, fluorescein diacetate staining showed them to have a low viability. It is believed that these

TABLE 3.3

RESPONSE OF FLAX SUSPENSION CULTURE TO DIFFERENT MS-BASED MEDIA CONTAINING DIFFERENT HORMONE LEVELS

Medium (mg/l)	Observations
1. MSP1 (2.0 NAA, 0.5 BAP)	Large cell aggregates, debris, few expanded single cells. Increased callus mass, produce more single cells after a few sub-cultures. Tend to form roots after a few weeks in culture.
2. MSD4x2 (0.1 NAA, 1,0 BAP)	Large cell aggregates, no single cells. No change after two sub-cultures. Reduced viability.
3. MS (1.0 2,4-D)	A lot of single cells, but roots formed after less than two weeks in culture. Reduced viability after subculture.
4. MS (0.1 2,4-D)	Single cells dissociated readily from callus pieces. Culture remained mainly as small cell aggregates and formed roots after more than two weeks in culture. Increased callus mass and friability after a few sub-cultures.

were, in the majority, dying cells which had been sloughed off the clumps of callus.

A medium containing a high level of 2,4-dichlorophenoxyacetic acid (1.0 mg/l) was employed to initiate suspensions from fresh callus and produced large numbers of free, single cells. However, the suspension formed roots after about 2 weeks and subculture in the same medium induced aggregation, but no cell division or expansion was observed. Fluorescein diacetate staining showed a declining percentage of viable cells to non-viable cells in such suspensions.

Lower concentration levels of 2,4-dichlorophenoxyacetic acid produced lots of single cells which generated a very friable suspension after a few subcultures and reduced the speed at which roots were formed (Fig. 3.4A and B). This friable suspension was mainly composed of small cell aggregates which had a reasonable viability (Fig. 3.4C and D). This state of growth in suspension could be maintained only if the culture was regularly subcultured after 7-10 days.

In general, a higher auxin level will increase dissociation of cells from the initial callus pieces. Similar observations were noted by Ibrahim (1971) and Gamborg and Shyluk, (1976), who initiated cell suspensions in medium containing high levels of indoleacetic acid and 2,4-dichlorophenoxyacetic acid, respectively. In these experiments, however, high auxin levels also increased the tendency of the dividing cells to differentiate into roots.

Fig. 3.4 Flax cell suspension culture

Cell suspensions initiated from friable callus derived from 2 day old cotyledons on MS medium supplemented with auxins and cytokinins

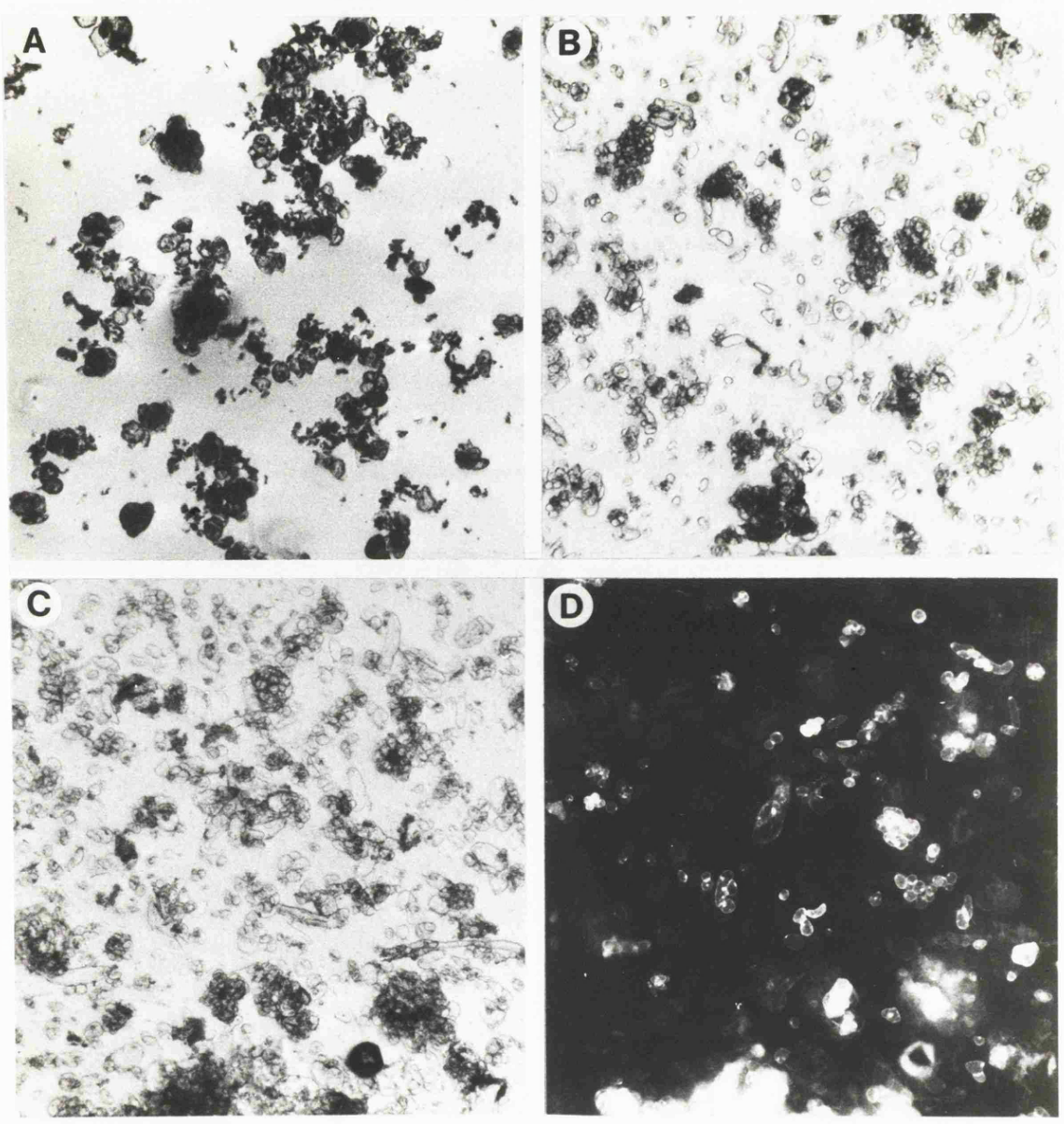
A) Appearance of culture after 3 weeks.

B) Appearance of culture after 5 weeks; note the small cell aggregates in the suspension.

C) Non-stained cells in a 5 week old suspension.

D) Same field as (C); cells stained with fluorescein diacetate and visualised under UV light. Note that only cell aggregates are viable.

(x200)


A
B
C
D

The speed by which this is achieved depends on the levels of the auxin. It was also observed, that following root formation, the cells in the suspension stopped dividing and continued differentiating into more roots. Thus, it was necessary to find the optimum level of auxin that will dissociate the cells from the callus explants in the inoculum, as well as support cell divisions without forming roots, after short periods of culture. Table 3.3 shows that the MS-based medium containing 0.1 mg/l 2,4-D gives a reasonably good cell suspension for the purpose of this study.

3.2.4.2 Initiation of haploid suspension cultures

Callus was initiated from haploid leaf sections on MSD4x2 medium for 2-4 weeks and then subcultured onto the same or MSP1 medium until highly friable tissue was obtained. The callus was always of varying consistency, but a high proportion was sufficiently friable for the initiation of suspension cultures. The ploidy of callus initiated from leaf sections of haploid plants was examined after 2-4, weeks before being subcultured. Squash preparations were made of actively growing callus pieces, pretreated with 0.08% hydroxyquinoline and stained with aceto-orcein. Of the dividing cells counted, it was found that about 70% were still haploid (Table 3.4).

In order to ensure a largely haploid inoculum for the suspension cultures, these were established from freshly initiated callus, either removed directly from the haploid explant, or after one or two subcultures in MSD4x2 or MSP1

TABLE 3.4

DETERMINATION OF PLOIDY LEVELS OF ROOT TIPS,

CALLUS AND SUSPENSION CULTURES DERIVED FROM HAPLOID EXPLANTS

Materials	Diploid cells	Haploid cells	Total number of cells examined
Root tips		20	20
Callus (3-4 weeks old)	8	22	30
Cell suspension (3 months old)	36	15	51

medium. Suspension cultures were routinely initiated by breaking up 2-4 g of sufficiently friable callus, and dispersing it in 50 ml of liquid medium in a 250 ml Erlenmeyer flask. The cultures were incubated at 27°C on rotary shaker for 14-28 days initially, depending on how rapidly the callus became dispersed and started to grow. Subsequent subcultures were routinely performed every 7-10 days by transferring 40-50 ml of the cultures into 40-50 ml of fresh medium. The cell aggregation in the suspension cultures decreased over the first three to four passages until the cultures consisted of a range of aggregate sizes. The cultures did not generally become any more finely dispersed after further subculture. The speed with which the different levels of aggregation were achieved depended on the friability of the callus used for the initiation of the suspension cultures.

The ploidy levels of the suspension cultures were also examined. As a prelude to the use of such suspension cultures for mutagenesis experiments it was considered important that the ploidy levels and viability of long established cultures be known. Cells from suspension cultures initiated from haploid callus and maintained in suspension after more than 3 months were used. Ploidy percentage was estimated by chromosome counting. About 30% of the cells in the suspension were found still to be haploid and the rest were either diploid or polyploid (Table 3.4).

3.2.5 Summary

The tissue culture techniques developed could be refined through time. A good tissue culture background had been set up at the end of the first year, enabling the techniques to be used in transformation procedures for mutagenesis studies. The unique polyembryonic nature of flax, coupled with an efficient clonal propagation technique, promise an easy and continuous availability of haploid material which is important if recessive mutants were to be recovered. A good cell suspension consisting of mainly viable single cells and small cell aggregates could be generated in a reasonable period of time. Cytology results show that the ploidy level is quite stable and that a large percentage of the cells in suspension are still haploid. Therefore, haploid cell suspensions provide a large haploid cell population which can be manipulated easily for mass transformation and selection for any desired mutant phenotype. In the future it is hoped that embryogenesis can be induced from these cells. Mutant selection could then be performed on large numbers of clonal embryoids and subsequently plants with the desired phenotype regenerated. At present however, regeneration can be induced from hypocotyl segments. Thus, provided the cells giving rise to shoots formation are competent and accessible to *Agrobacterium* transformation should be possible. It has been shown that shoot regeneration from hypocotyl is not variety specific, thus enabling the procedures to be applied to hypocotyls from a high-twinning variety.

CHAPTER 4

GENETIC TRANSFORMATION OF FLAX

4.1 INTRODUCTION

4.1.1 Gene transfer in plants

Central to the aim of plant genetic manipulation using recombination DNA technology is the successful accomplishment of gene transfer. The experimental strategies attempting to transfer genetic information into plant cells may depend on the cellular target (tissue explants, cells or protoplasts in culture), the totipotency of the treated materials and the susceptibility of the plant species to the transfer technique itself. Successful transfer of genetic information into plant cells would result in the introduction of specific genes into the genome, and the transmission and expression of the transferred genes in subsequent cell generations, regenerated plants and their progeny.

Uptake of exogenous DNA can be mediated by viruses, *Agrobacterium* transformation of tissue explants and cells, microinjection or the use of DNA coated microprojectiles (Horsch *et al.*, 1985; Wullems *et al.*, 1986; Guerche *et al.*, 1987; Crossway et al., 1985, 1986; Klein et al., 1987). Protoplasts, which lack a cell wall, can be transformed by the above methods, and in addition, by direct plasmid uptake mediated by polyethylene glycol and electroporation (Draper *et al.*, 1982; Shillito *et al.*, 1985; Fromm *et al.*, 1986; Negrutiu et al., 1987), and also by fusion with plasmid-filled spheroplasts or liposomes (Hain *et al.*, 1984; Deshayes *et al.*, 1985). Except for microinjection and microprojectiles which

promise to be universal, there is no single mechanism of transferring DNA into any plant variety which can be applied to all types of explants in culture, or to intact plants. Work in this thesis therefore concentrated on the use of an *Agrobacterium*-based transformation system.

4.1.2 Transformation mediated by *Agrobacterium*

Although crown gall tumours induced by wild type *Agrobacterium* are both easily recognised and established *in vitro*, their oncogenic nature presents a great disadvantage in their use as a transformation system. This is because oncogenic cells within crown galls are incapable of regenerating whole plants. Furthermore, non-transformed cells are also found in the crown gall tumours, supported by 'cross-feeding' of phytohormones from the tumour cells (Sacristan and Melchers, 1977; Ooms *et al.*, 1982). Therefore, the need arises for design of vectors based on the Ti plasmid that can eliminate the above problems.

Our understanding of how *Agrobacterium* causes crown gall disease, the elucidation of T-DNA's role and of the various *cis*- and *trans*-acting factors necessary for the transformation event to occur has permitted intelligent design of plant transformation systems (see Chapter 1). Molecular analysis has shown that only the 25 bp border regions and the *vir* genes are required for transfer of the T-DNA into host plant genomes (reviews, Gheysen *et al.*, 1985; Stachel *et al.*,

1986). Therefore, by deleting the *onc* genes (oncogenic genes) which are responsible for the neoplastic nature of the transformed cells, non-oncogenic vectors are created which allow the regeneration of normal fertile plants from transformed cells. Two types of non-oncogenic vectors are currently being used, *cis* and *trans*. A hybrid gene comprising a plant promoter fused to a neomycin phosphotransferase coding region from Tn5 provides an excellent selective marker (kanamycin resistance) for transformation events (Herrera-Estrella et al., 1983a, b; Bevan, 1984; Fraley, et al., 1985). This hybrid gene can be transferred into the plant genome by inserting it in between the T-DNA border regions of the two types of vector.

The *cis* acting vectors are often referred to as cointegrative vectors and the *vir* genes are carried on the same replicon as the T-DNA region. These are modified Ti plasmids with the *onc* genes replaced by a region with homology to part of a small *E. coli* cloning vector (intermediate vector). The intermediate vector often contains a selectable marker gene that will function in plant cells and unique sites for the insertion of foreign DNA. Cointegration of the intermediate vector into the *vir* helper Ti plasmid occurs in *Agrobacterium* by recombination between the regions of homology (Fig. 4.1A). An example of a *cis*-acting non-oncogenic vector is pGV3850 (Zambryski *et al.*, 1983). It is derived from a nopaline-type Ti plasmid (C58), in which the *onc* genes have been deleted and

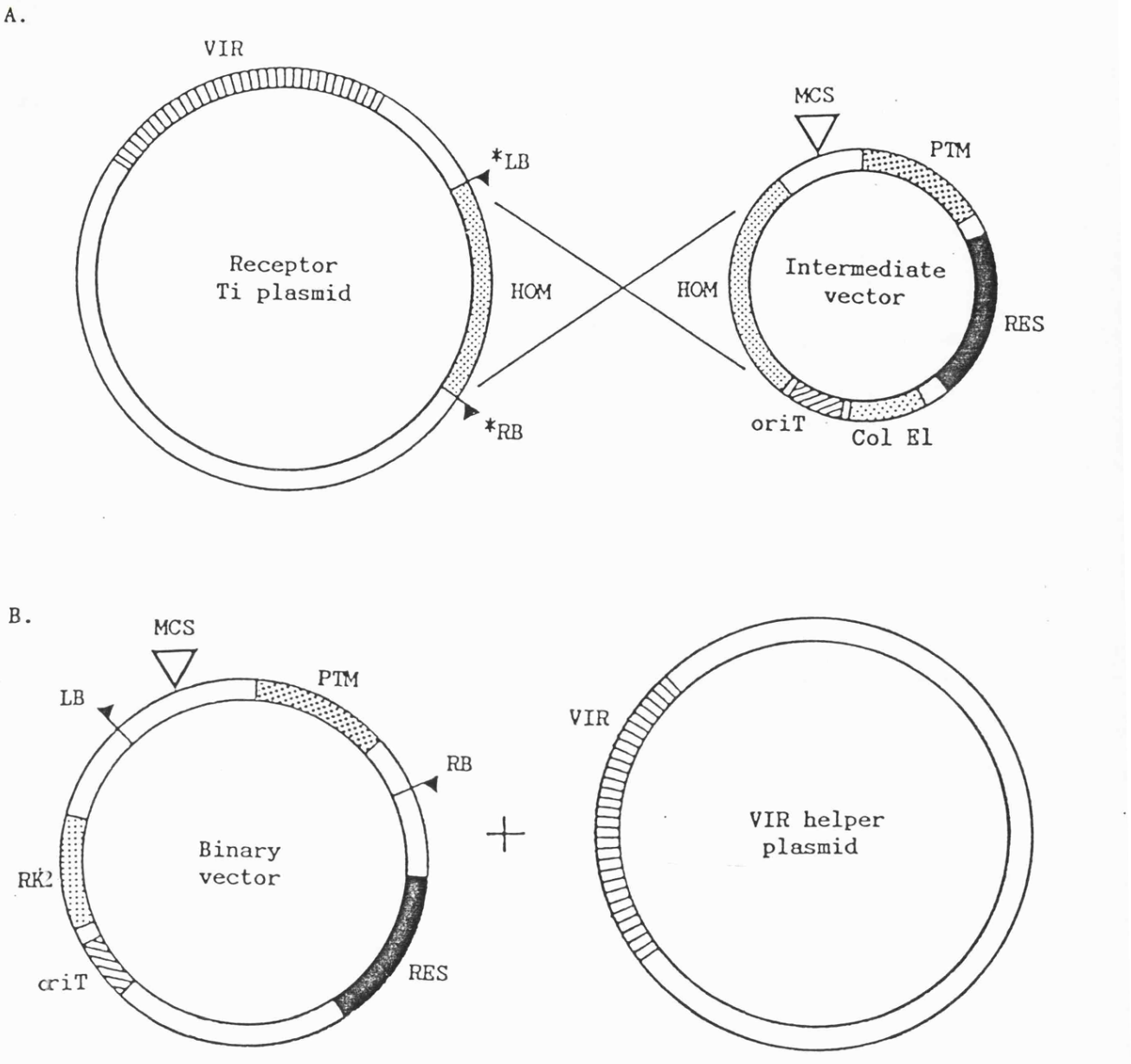


Fig 4.1 Schematic diagram of generalized cointegrative (A) and binary (B) vector systems. VIR - virulence region; HOM - homologous regions within which recombination may occur for cointegration. LB, RB - left and right borders (* either LB or RB or both may be present on the intermediate vector, see text); MCS - multicloning site; PTM - plant transformation marker; RES - antibiotic resistance marker to select for presence of vector sequences in bacterial host; oriT - origin of transfer and bom site for conjunctive mobilization of vectors; Col E1 - origin of replication; RK2 - wide host range origin of replication.

replaced by pBR322 (Fig. 4.2). Any foreign DNA sequence cloned in pBR322 can be introduced into pGV3850 by a two step process of transfer into *Agrobacterium* via conjugation, followed by recombination. The hybrid nos-*npt-II* genes in an intermediate plasmid pGV1103 has been cointegrated into pGV3850 for use in the transformation procedure described in this chapter. Thus, transformation events can be screened for by nopaline synthase activity, and selected for by expression of kanamycin resistance.

The *trans* vectors are commonly called binary vectors. Virulence functions are supplied by a *vir* helper plasmid, which is often a wild type octopine or nopaline-type Ti plasmid lacking the T-DNA and 25 bp repeat sequences. The binary vector is based on a wide host range plasmid which contains the T-DNA border sequences flanking a multicloning site and markers for direct selection of transformed plant cells. The plasmid can be manipulated in *E.coli* and transferred via conjugation to an *Agrobacterium* strain harbouring a *vir* helper plasmid (Fig. 4.1B). The binary vector pBin19 is based on the wide host range replicon of pRK252 (Bevan, 1984). This vector contains a kanamycin resistance marker for direct selection in bacteria as well as a *nos-npt-II* gene for expression in plants. It contains a multiple cloning region within the alpha complementary region of ß-galactosidase, flanked by T-DNA border sequences (Fig. 4.3). The vector can be mobilised into *Agrobacterium* by conjugation using the helper functions of pRK2013. The *Agrobacterium* host LBA4404, contains a Ti plasmid

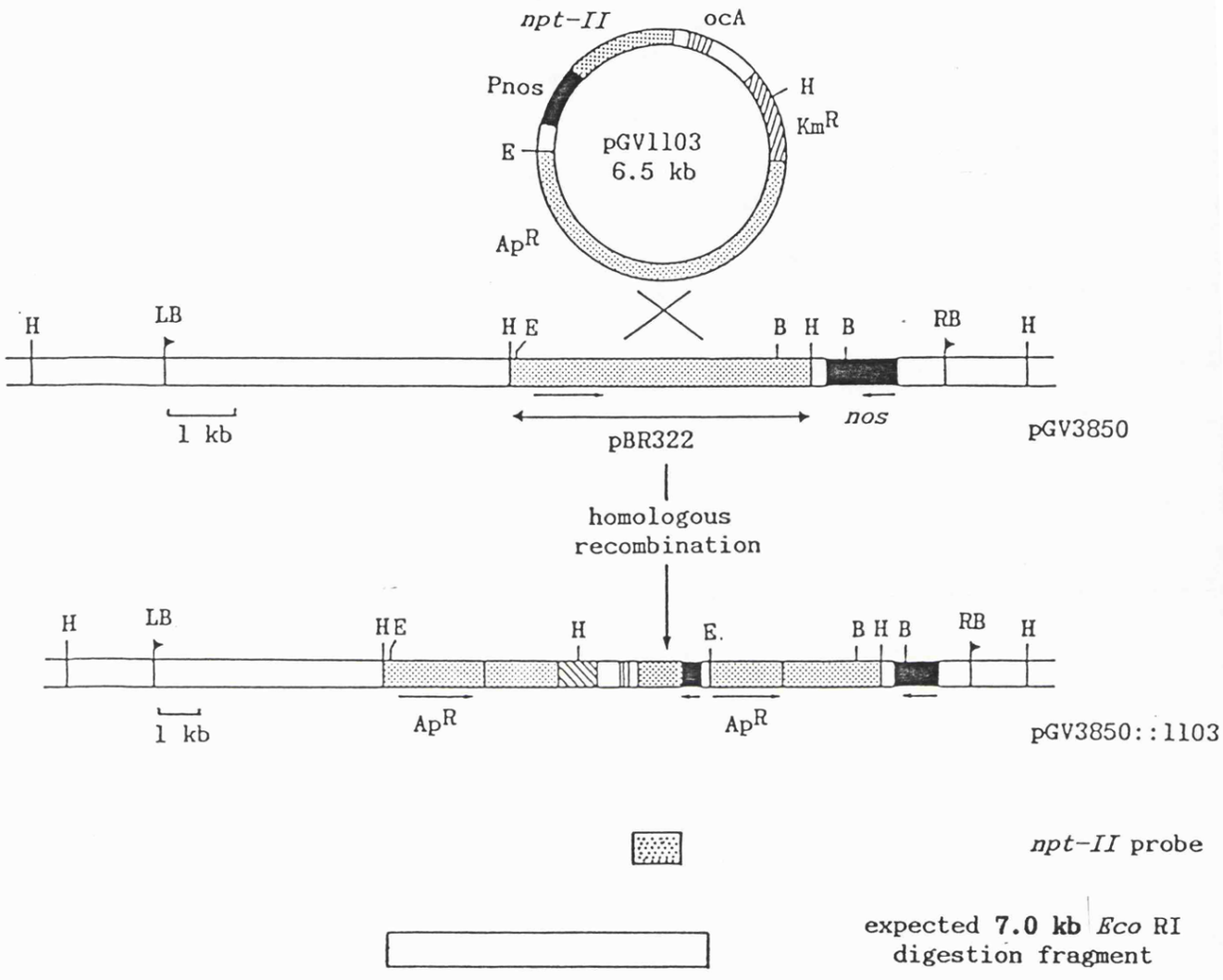


Fig 4.2

Maps showing the structure of the plant transformation vector pGV3850 and the intermediate vector pLGV*neo*1103, and a possible outcome of cointegration of pLGV*neo*1103 into pGV3850. LB, RB - left and right borders; *nos* - nopaline synthase promoter; *npt-II* - the neomycin phosphotransferase II coding sequence from Tn5; ocA - octopine synthase polyadenylation site; ▨ - pBR322-derived regions of homology between the plant transformation and intermediate vectors; E - *Eco* Rl; H - *Hind* III; B - *Bam* Hl

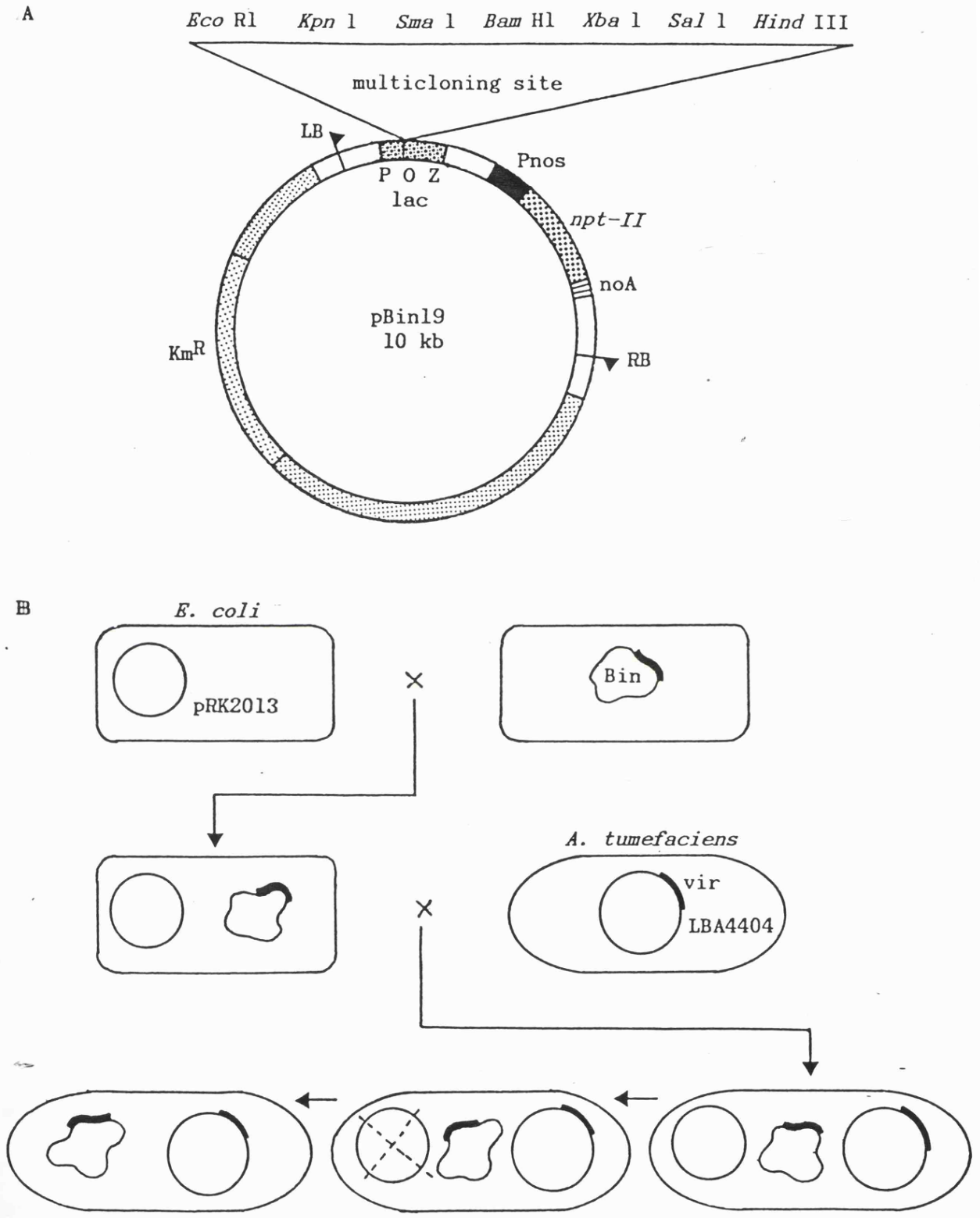


Fig 4.3 A. Map of the binary vector pBinl9. LB, RB - left and right borders; lac - α-complementary region of lac operon; Pnos - nopaline synthase gene promoter; *npt-II* - neomycin phosphotransferase II coding sequence from Tn5; noA - nopaline synthase gene polyadenylation site; ▨ - pRK2-derived sequences containing the RK2 wide host-range origin of replication.

B. Schematic representation of mobilization of pBinl9 into *A. tumefaciens* LBA4404 using the helper plasmid pRK2013, and subsequent elimination of the helper plasmid in *Agrobacterium*.

(pAL4404) from which the T-DNA and 25 bp repeat sequence have been deleted, but which contains an intact vir region.

4.1.3 General transformation procedures

A good response to all of the basic tissue culture techniques is essential for the development of a transformation system using *Agrobacterium*. As described in Chapter 1, *Agrobacterium* infection requires wounding of competent cells in susceptible plants. To date, essentially two basic approaches, differing in explant size, have been used to obtain transgenic plants; large explants (eg. leaf disks) and the co-cultivation of isolated cells (eg. protoplasts, cell suspensions) with *Agrobacterium* (Marton *et al.*, 1979; Pollock *et al.*, 1985; Horsch *et al.*, 1985; McCormick *et al.*, 1986; An, 1985; Scott and Draper, 1987).

Heterogeneous explants such as leaf disks often contain undetermined cells that are capable of switching to different pathways of development, depending on the environment imposed on them. Wounding stimulates these cells to undergo rapid cell division making them competent for transformation. Upon incubation on regeneration medium, these transformed cells are capable of regenerating adventitious shoots. Small pieces (circular or square) are cut out from leaves of *in vitro* propagated or greenhouse-grown plants. These are inoculated with a Ti plasmid derived vector and then incubated on an appropriate medium that induces rapid callus formation and adventitious shoot regeneration. An antibiotic can be applied

immediately following inoculation, or at a later stage, to kill the agrobacteria. When shoots have regenerated, they can be selected or screened to determine if they are transformed. This is a simple technique that can be used to regenerate transformed shoots.

Tobacco leaf lamina slices for example do not contain quiescent buds or pre-formed organ primordia, and therefore the majority of adventitious shoots forming at the cut edge originate from dedifferentiated cells which are susceptible to transformation by *Agrobacterium*. Subsequently, transformed shoots may be regenerated. However, the leaf disk method is not applicable to all species as it depends entirely on the ability to regenerate shoots efficiently from dedifferentiated cells at the wound site. For example in *Arabidopsis*, only about 7% of the leaf disks gave fertile transformed plants (Lloyd, *et al.*, 1986). The technique also has proved difficult to adapt for potato (Shahin and Simpson, 1986).

Single cell systems such as protoplasts and cell suspensions have also been shown to be competent for transformation by *Agrobacterium* (Pollock *et al.*, 1985; Meyer *et al.*, 1985; Scott and Draper, 1987). It was first shown that conversion of quiescent leaf mesophyll cells into protoplasts, followed by cell wall regeneration and cell division, mimicked the wound response of intact plants which is neccessary for infection of *Agrobacterium* (Mårton *et al.*, 1979). Subsequently the use of protoplasts was found unnecessary as dividing

suspension culture cells were also competent (Scott and Draper, 1987). Suspension cultures may contain from a few to several hundred cells and can be handled using techniques similar to those used with bacteria.

Co-cultivation methods involve incubation of agrobacteria together with large populations of dedifferentiated plant cells in a growth medium, often for several days. Large numbers of clonally derived transformants can be obtained by careful manipulation of the culture conditions, plating density and selection procedure. Transformed colonies can be selected on the basis of hormone independent growth when oncogenic vectors are used, or on the basis of resistance towards antibiotics when using non-oncogenic vectors having dominant selectable markers, such as genes encoding kanamycin resistance. Such systems are of practical use when mass transformation procedures are required. It is especially useful in the mutagenesis of plant genomes using the T-DNA as a random insertion mutagenic agent.

These two techniques can be adapted for application to other explants which are capable of regeneration. Depending on the plant species, large explants such as hypocotyls, stems or callus pieces could be use as target cells in the transformation system. Successful recovery of fertile transformed plants will depend on the cell competence and accessibility of the *Agrobacterium* to the totipotent cells.

An alternative transformation system utilises

Agrobacterium rhizogenes. As described in Chapter 1, the 'hairy roots' induced are clonal in origin (Petit *et al.*, 1986) and in some cases can be regenerated into mature plants (Tepfer, 1984; Trulson *et al.*, 1986; Shahin *et al.*, 1986). Therefore, without involving the generation of cell suspensions or protoplasts, which are often very difficult, a large number of clonal transformants in the shape of 'hairy roots' can be obtained. Hairy roots induced on inoculated large explants such as leaf pieces will be clonally derived and can be excised and selected for any desired mutant phenotype.

4.1.4 Transformation of flax

Flax protoplast systems are not sufficiently developed to be of use for the transformation of haploid flax lines (Chapter 3, section 3.1.2). However, the good response of flax hypocotyl sections to tissue culture, in particular the ability to regenerate shoots, may indicate this tissue to be a source of useful explants for transformation. This system may possibly be directly applied in insertion mutagenesis experiments, if it is possible to regenerate a lot of independently transformed haploid plants. A transformation system is also required for the introduction of transposable elements into flax for transposition induced insertion mutation studies (Chapter 5).

The flax cell suspension cultures described in Chapter 3 provide material for the mass transformation of small

explants. This is a very important factor in insertion mutagenesis studies. Mutations normally occur at a very low frequency. In protoplast cultures in which most of the cells are haploid the induced frequencies are 10^{-4}-10^{-5}, and in suspension cells 10^{-6}-10^{-7} (Maliga, 1980). Thus, in order to pick up a single desired mutant, insertion has to occur randomly at very high frequency in a large cell population. Millions of haploid single cells or small cell clumps in suspension provide convenient targets for mass transformation by *Agrobacterium* and random insertion of T-DNA. The stressing of large populations of independently-derived transformation events may allow the selection of mutant cell colonies, or shoots, at a reasonable frequency.

The possibility of transforming both systems using *cis*- and *trans*-acting *Agrobacterium* derived vectors is examined in the following experiments. These transformation systems were developed with the intention of applying them in later mutagenesis experiments, in attempts to isolate genes of interest, and for the delivery of exogenous transposable elements into the flax genome.

4.2 RESULTS AND DISCUSSION

4.2.1 Transformation of flax hypocotyl segments

The suitability of explants for efficient shoot regeneration in flax, and the type of cells which are competent for transformation with *Agrobacterium* have been described in detail in previous chapters. Large explants such as hypocotyls, often contain a heterogeneous cell population. Some of these may respond more readily to certain media by undergoing division and/or direct organogenesis, producing adventitious shoots. Earlier studies have shown that wounding of hypocotyls stimulated cells, particularly around the cut area, to dedifferentiate and undergo division to form an undifferentiated callus on appropriate hormone-containing media (Chapter 3). It has also been shown that cell division occured within 72 h of explanting (Sqalli and Chlyah, 1985) and thus cells will be competent for transformation at some time prior to this event. In addition, wounding may also provide limited bacterial access to totipotent cells which give rise to shoots via a callus phase.

Hepburn *et al.*, (1983), have shown that flax cells are competent for transformation by *Agrobacterium*. Inoculation of flax epicotyls with a nopaline type vector (C58) produced flax tumour lines. Molecular analysis of the transferred T-DNA indicated predominantly normal insertions although several abnormal forms existed. In Chapter 3 it was found that hypocotyl explants were very responsive to certain media and

very readily regenerated shoots. This property of totipotency and cell competence would appear ideal for the development of a transformation system in which whole and fertile transformed plants can be generated. In preliminary experiments, hypocotyl sections of 1.0-2.0 mm were excised from 2 day old sterile flax seedlings (*Linum usitatissimum* L. cv. Antares) and inoculated with an overnight culture of *Agrobacterium*. The *Agrobacterium* strain used in all experiments was C58C1 (pGV3850:1103) (Hain *et al.*, 1985). This strain harbours a modified version of the disarmed Ti-plasmid vector pGV3850 (Zambryski *et al.*, 1983) containing a chimaeric *nos-npt-II* gene, to allow selection of kanamycin resistant transformed plant cells, and a functional nopaline synthase gene (Fig. 4.2).

Following *Agrobacterium* inoculations the explants were transferred onto 5 ml of either medium A (MSD4x2 + 200 µg/ml cefotaxime), or medium B (MSD4x2 + 200 µg/ml cefotaxime + 100 µg/ml kanamycin), both solidified with 0.8% agar, and contained in 50 mm Petri dishes. Four explants were placed in each dish and incubated at 27°C with a 16 h photoperiod. Control (uninoculated) explants were treated in the same way.

The different responses shown by the hypocotyl explants under different culture and inoculation regimes are summarised in Table 4.1.

On MSD4x2 and medium A, hypocotyl sections expanded considerably during the first two weeks of culture. This was followed by the appearance of swellings on the surface near to

TABLE 4.1

RESPONSE OF *Agrobacterium*-INOCULATED AND NON-INOCULATED HYPOCOTYL EXPLANTS IN DIFFERENT MEDIA

Treatment	Medium	No. of explants	No. of explants with shoots
inoculated	medium A	70	70
inoculated	medium B	60	5
non-inoculated	medium A	30	30
non-inoculated	medium B	40	0
non-inoculated	MSD4x2	20	20

one end and generation of compact callus at both cut ends (Fig. 4.4A). The swellings subsequently developed into leaf primordia, eventually producing buds and shoots, during which phase the callus growth at both ends was reduced (Fig. 4.4B). After 3-4 weeks of culture on medium A the hypocotyl sections were full of leaf primordia with several dominant shoots growing out (Fig. 4.4C). The response of explants was markedly polar, so that primordia were formed only at the cotyledon end of the initial explant. Similar observations were noted by Gamborg and Shyluk (1976) upon incubation of hypocotyl sections taken from 5 day old seedlings after removal of the main shoot by cutting just below the cotyledons. Buds were again produced at greater frequency nearer the cut ends, suggesting an inherent hormonal gradient within the hypocotyl tissue cells. The presence of the antibiotic cefotaxime in the medium, or following inoculation of explants with *Agrobacterium*, did not affect the shoot regeneration capacity of the hypocotyl sections (Table 4.1; Fig. 4.4A-C). However, shoot regeneration from control (uninoculated) hypocotyl sections was completely inhibited in the presence of kanamycin.

Initial experiments concentrated on producing shoots from inoculated explants without selection pressure. Once these shoots were large enough to handle easily, they were cut at the base and transferred into propagation and selection medium. After about 4 weeks in this medium, these shoots elongated and began to bleach at the basal end and none were

Fig. 4.4 Transformation of flax hypocotyl explants

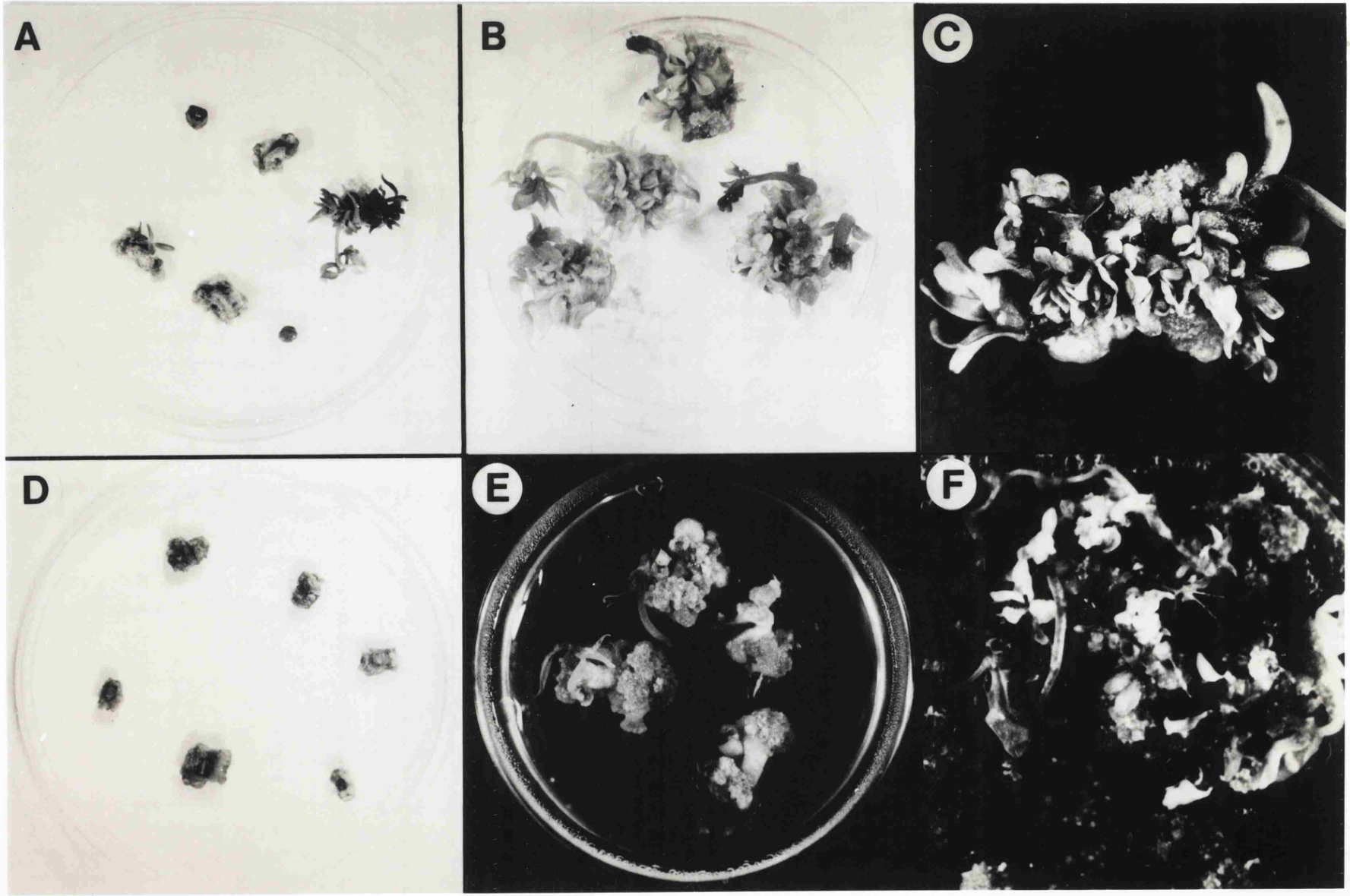
A) Development of shoot primordia on explants inoculated with *A. tumefaciens* strain C58 (pGV3850::1103) after 2 weeks on non-selective medium (medium A).

B) Growth of adventitious shoots on inoculated explants after 3 weeks on non-selective medium.

C) Close up of shoots and secondary shoot primordia developing in vicinity of primary primordia after 3 weeks culture of inoculated explants on non-selective medium (medium A).

D) and E) show callus formation after two weeks, and shoot regeneration after 3 weeks on inoculated 2 day old flax hypocotyl explants placed directly on medium B.

F) Shoots regenerating from Km^r callus after subculture on medium B.


A
B
C
D
E
F

found to be kanamycin resistant. The leaf primordia, subsequent buds and shoots formed are thought to be wholly derived directly from totipotent epidermal cells (Link and Eggers, 1946; Sebánek *et al.*, 1979) without dedifferentiation. These cells are covered in cutin and possibly not accessible to agrobacteria without gross damage. Furthermore, it is very likely that cell dedifferentiation is required for transformation and this may explain why none of the shoots regenerating directly from inoculated explants were found to be transformed.

Although most of these shoots arose by direct primordia formation from cells in the original explant tissue, which may not be accessible for transformation by *Agrobacterium*, it is often the case that callus formed on explants undergoing large amounts of shoot primordia development is also capable of regeneration, especially when still attached to the original explant. Such callus, which grows out of the cut ends of the hypocotyl, is accessible to the bacteria and thus, easily transformed. Since it has been previously 'primed' by growth at the wound sites on regenerating explants, it may continue to regenerate after excision and transfer to fresh regeneration medium. Therefore, transformed shoots may possibly be regenerated from this transformed callus. Two different approaches were then used in an attempt to produce transformed shoots, differing only in the timing of application of selection pressure following

inoculation of the explants with agrobacteria.

Method 1: Inoculated explants were plated originally on regeneration medium lacking kanamycin (medium A). After 3-4 weeks, and following the removal of well developed shoots, hypocotyl sections, which still contained leaf primordia and some callus, were cut into small pieces and placed on medium B for the selection of transformed cells and regeneration of further shoots. Callus formation resumed and some green 2° primordia developed in the vicinity of the bleached 1° primordia after 3-4 weeks on selection medium. Once subcultured onto fresh medium B, green Km^r callus proliferated from the region of these 2° leaf primordia and shoots were subsequently regenerated from the callus. In one experiment, eighteen independent lines of green, kanamycin resistant callus were isolated and three of them regenerated shoots. 2° primordia never arose from parts of the original explant which failed to produce 1° primordia during the initial phase of culture on medium A.

Method 2: Inoculated explants were cultured directly on regeneration medium containing kanamycin (medium B). After 2 weeks all the hypocotyl sections, enlarged and then bleached on medium B (Fig. 4.4D). By this time primordia and callus had also formed on some of the original explant tissues and at the cut ends of some of the hypocotyl sections respectively. Green callus developing at the cut ends of some of the bleached

hypocotyl sections soon regenerated green 2°Y shoot primordia(Fig. 4.4E). Some of these calli later bleached while others remained green and continued to grow rapidly upon subculture to fresh selection medium. Regeneration of Km^r shoots (Fig. 4.4F) proved to be possible only from green callus produced on explants which had initially showed signs of shoot primordia formation. In one experiment, twenty independent callus lines growing on kanamycin were isolated and two of them regenerated Km^r shoots.

Results obtained from both methods indicated that transformed shoots were recovered only from green callus proliferated from explants which had produced 1°Y primordia. Thus, a likely sequence of events leading to the production of transformed shoots would appear to be that cells at the cut end of the explant, in the region of developing shoot primordia, pass through a period of dedifferentiation during which time they are permissive to transformation by *Agrobacterium*. These cells are thus, 'primed' by growth at wound sites on regenerating explants. Subsequently these transformed cells establish Km^r callus which is competent for shoot primordia induction and the subsequent regeneration of Km^r plantlets. It may be significant in this respect that cefotaxime had to be maintained in the medium for several subcultures before all agrobacteria were finally eliminated and it may be these persistent bacteria which actually accomplish transformation of the newly permissive cells.

Several simple and efficient assays can be performed to confirm that the kanamycin resistant lines have been transformed. The presence of an active nopaline synthase gene in the transformants can be confirmed by screening for nopaline using a simple paper electrophoretic assay. An *in situ* assay for neomycin phosphotransferase-II (NPT-II), which uses fractionation by non-denaturing polyacrylamide gel electrophoresis (Reiss *et al.*, 1984) can be used to detect the expression of the selectable marker gene in transformed flax lines. Molecular analysis by Southern blotting (Southern, 1975) will confirm the presence of the above genes in the transformant and determine the copy number and structural organisation of the T-DNA in the transformed flax genome.

4.2.2 Selection, propagation and rooting of transformed shoots

Shoots regenerated from Km^r callus which grew rapidly and remained green on selection and propagation medium were transferred into 1/2 B5 + Km (100 µg/ml) for root induction and further selection of transformants. Cefotaxime was omitted since this tended to slow down root development. Most of these Km^r callus-derived shoots formed roots, whilst all shoots derived from the control hypocotyl sections failed to root and bleached in the presence of kanamycin. Generally, differentiated structures such as shoots often tend to be naturally less sensitive to the selection agent, kanamycin,

than callus. Thus, depending on the level of antibiotic employed, non-transformed shoots are sometimes able to survive the selection process (Horsch *et al.*, 1985; McCormick *et al.*, 1986) and these are often referred to as 'escapes'. However, since roots are generally more sensitive to antibiotics, the ability of a shoot to root on selection medium containing high levels of kanamycin gives a very strong indication of its transformed nature. Selection for transformed shoots on this basis is often preferred to a simple selection based on bleaching of sensitive cells.

Thus, in summary the recovery of transformed kanamycin resistant shoots were shown to be successful only via callus phase, during which the 'primed' cells were undergoing de-differentiation. They formed roots in the presence of kanamycin, unlike those shoots regenerated directly on the hypocotyl explants which either bleached on selection medium or unable to form rootson selection rooting medium.

4.2.3 Nopaline synthase activity in the Km^r flax tissues

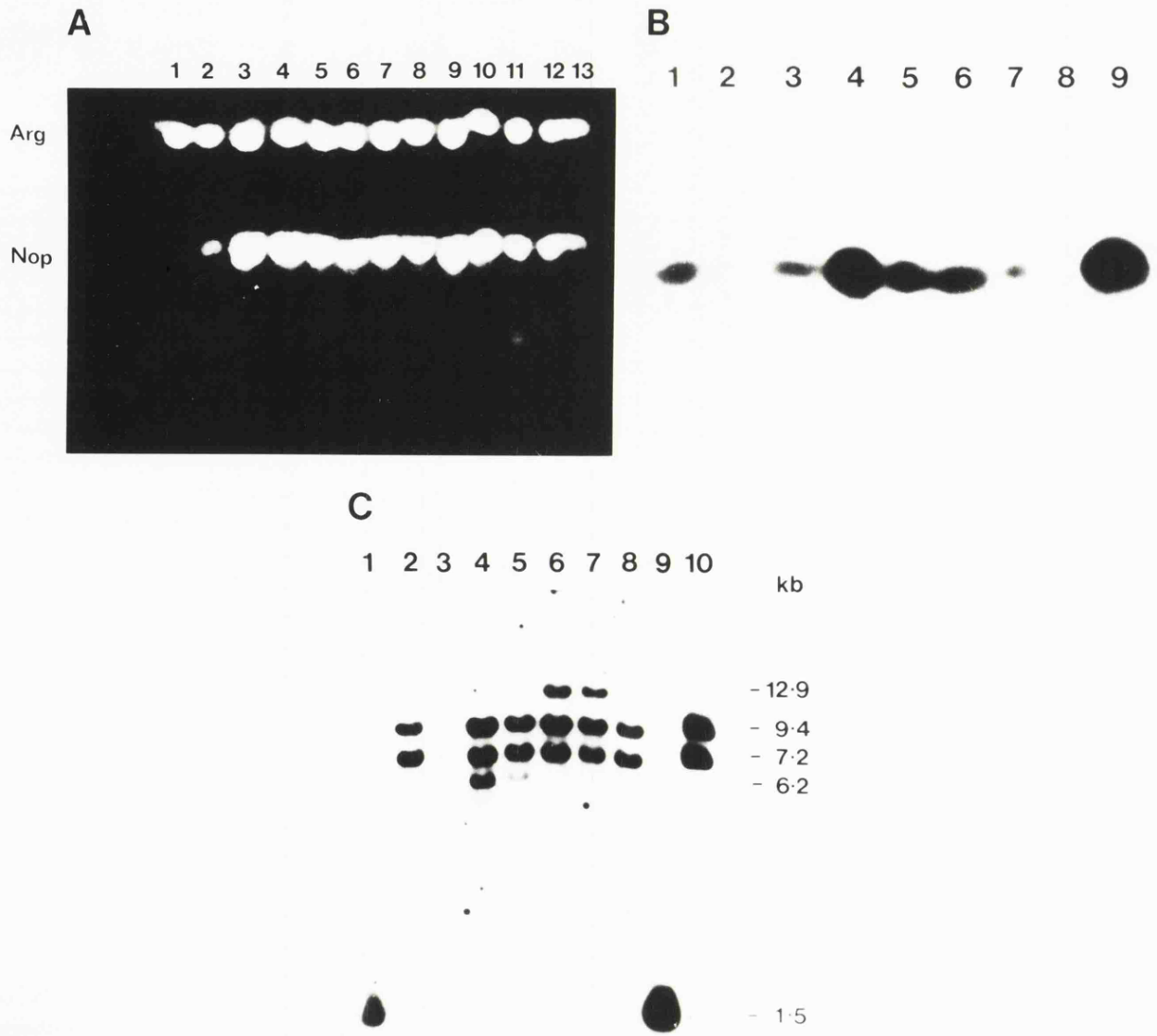
Seven independent lines of kanamycin resistant callus, obtained by Method 2, were assayed for nopaline synthase. Three different shoots regenerated from two of the callus lines were also screened for nopaline synthase activity. Nopaline was detected in all of the kanamycin resistant callus lines (Fig. 4.5A, lanes 3-9) and regenerated shoots (Fig. 4.5A, lanes 10-12). It is interesting to note that the amount of nopaline in these tissues was elevated as compared to

Fig. 4.5 Molecular analysis of Km^r flax tissues

A) Detection of nopaline in Km^r calli and shoots. Lane 1, control callus; lane 2, control callus plus standard nopaline; lanes 3-9, kanamycin resistant calli; lanes 10-12, regenerated Km^r shoots; lane 13, standard nopaline and arginine.

B) Detection of NPT-II activity in kanamycin resistant calli. Lanes 1-7, independent kanamycin resistant calli; lane 8, control, kanamycin sensitive callus; lane 9, bacterial NPT-II.

C) Southern blot analysis of transformed callus. Lanes 1 and 9, 1x and 5x copy number reconstructions respectively of the *npt-II* gene; lanes 2-8, *Eco* RI restricted DNA from independent Km^r calli (lines 1-7 respectively); lane 10, *Eco* RI-digested DNA from *Agrobacterium* strain C58 (pGV3850::1103).


A
1 2 3 4 5 6 7 8 9 10 11 12 13
Arg
Nop
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9 10
kb
12·9
9·4
7·2
6·2
1·5

transformed tobacco tissue produced using the same vector (personal communication, Dr. Roderick J. Scott).

4.2.4 NPT-II activity in transformed flax tissues

All of the Km^r callus lines contained NPT-II activity (Fig. 4.5B, lanes 1-7) which comigrated with authentic *E. coli* NPT-II (lane 9). This activity was not present in the protein extract of control callus (Fig. 4.5B, lane 8). Therefore, the transformants may be kanamycin resistant due to expression of the *npt-II* gene which is not present in the control wild type flax callus. It is interesting to note that the amount of NPT-II activity varied between the different callus lines. These variations may be simply due to the age of the callus tissues rather than gene expression differences between the callus lines. Alternatively, position effect may be the cause of the differences.

4.2.5 T-DNA analysis in transformed flax tissues

DNA was extracted from transformed callus lines and digested with restriction enzyme *Eco* RI to release a fragment containing the *npt-II* gene. After running the digested DNA on an agarose gel, it was blotted onto a hybridisation filter by Southern blotting (Southern, 1975). The filter was then probed with sequences homologous to the *npt-II* gene. The *npt-II* probe consisted of an ~1.0 kb *Sal I/Hind* III fragment of the *npt-II* gene from Tn5 (Fig. 4.2). *Eco* RI restricted DNA of all of the transformed callus lines contained fragments which hybridized

to the neomycin phosphotransferase gene probe (Fig 4.5C, lanes 2-8). Two hybridising fragments of 9.4 and 7.2 kb are common to all the lines and to the vector DNA within *Agrobacterium* (Fig 4.5C, lane 10). Lines 4 and 5 and 6 and 7 contain an extra restriction fragment with homology to *npt-II*. From the bacterial reconstruction, it is estimated that there are from <1 to 3 copies of the *npt-II* gene integrated in the flax genome.

The results of the T-DNA analysis were complicated by the fact that rearrangements of the T-DNA region appeared to have occurred in the *Agrobacterium* strain, as we would have expected an *Eco* RI digestion of pGV3850::1103 (Hain *et al.*, 1985) to release a single internal fragment of 7 kb containing the *npt-II* gene (Fig. 4.2). This was shown to be the case when the same vector was used to transform carrot cell suspensions (Scott and Draper, 1987). The cointegration of plasmids into pGV3850 results in the duplication of pBR322 sequences and it is likely that recombination between these homologous regions was responsible for the rearrangement of vector sequences. Although aberrant fragments were obtained in both vector and transformants, the results show that normal T-DNA transfer has occurred. It is also interesting to note that lines containing high copy number of T-DNA showed a strong NPT-II activity.

4.2.6 Transformation of flax suspension cultured cells

In order to accomodate mass selection of mutagenised cells a different approach has to be considered. Studies in other systems have shown the possibility of transforming cells in suspension at a reasonably high frequency (An, 1985; Pollock *et al.*, 1985; Scott and Draper, 1987). Flax cell suspensions can be initiated by shaking friable callus in liquid medium for 10-14 days. Friable callus was obtained from cotyledons of 2 day old flax seedlings, or from leaf sections of mature plants incubated on solid medium for 4-8 weeks (3.2.4; Ibrahim, 1971; Gamborg and Shyluk, 1976). A fine suspension of rapidly dividing cells was chosen and the viability determined by fluorescein diacetate staining. In all the experiments inoculations were performed using a binary vector, pBin19, carrying the transposable element *Mu1* in the multi-cloning site (see Chapter 5) and a neomycin phosphotransferase II gene which allows the selection of kanamycin resistant transformants. The binary vector acts in *trans* with plasmid LBA4404 which carries the *vir* functions (Fig. 4.3). The strain was always grown under selection (50 µg/ml kanamycin). Two methods of inoculation were tested.

Method 1: 5.0 ml of an overnight culture of *Agrobacterium tumefaciens* was added into 100 ml of cell suspension culture in a 250 ml flask. The flask was swirled to mix the culture and then left standing at room temperature for 1-2 hr to let all the cells settle to the bottom of the flask. Most of the

liquid medium was decanted leaving a high density of inoculated cells in suspension. The inoculated cell suspension was swirled again and then the larger cell aggregates were allowed to settle down for a few minutes. About 2.0 ml was then aliquoted using a Gilson P5000, from the upper layer of the suspension which contained smaller cell aggregates, leaving behind the larger cell clumps. This was used to seed a 'transfer disk' on MSD4x2 medium 'feeder plates'. The culture was incubated at 27°C with a 16 hr photoperiod for 7 days, after which a lot of yellowish micro-colonies were observed. When most of the colonies had grown to an average size of about 5.0 mm in diameter, the transfer disks were moved onto MSD4x2 medium containing 200 µg/ml cefotaxime. These colonies formed a mass of callus covering most of the surface of the transfer disk. The callus remained yellowish with occasional green spots. After allowing the green areas to develop further, they were individually picked up and transferred onto fresh medium with cefotaxime and kanamycin in 140 mm Sterilin Petri dishes. Within two weeks, these colonies developed into compact green clumps or friable green or yellowish callus. Later, approximately 45% of the colonies either turned brown or bleached. Meanwhile, the calli which remained green on selection developed into a mass of green kanamycin resistant calli after being individually subcultured into separate 50 mm dishes containing the selection medium. A total of 123 independent Km^r colonies were isolated and

maintained on this selection medium. In the control experiment (non-inoculated) a lot of yellowish callus was formed about a week after plating on a 'transfer disk' in a 'feeder plate'. Upon selection with kanamycin the callus turned brown and died.

Method 2: Small cell aggregates aliquoted from suspension culture were plated at a density of 200-500 units per plate and grown for 2-3 weeks on 'feeder plates'. The developing micro-colonies were inoculated with 1/10 dilution of an overnight *Agrobacterium* culture. 200 µl of the diluted *Agrobacterium* culture was spread evenly on the micro-colonies on the transfer disk using a P200 Gilson. After 5-6 days, the micro-colonies were transferred onto either medium A (MSD4x2 + 200 µg/ml cefotaxime), or medium B (MSD4x2 + 200 µg/ml cefotaxime + 100 µg/ml kanamycin), both solidified with 0.8% agar and contained in 90 mm Petri dishes. Control micro-colonies (LBA4404 inoculated and uninoculated) were treated in the same way.

After about two weeks both control and inoculated microcolonies grew into clumps of green calli of about 3-5 mm diameter on medium without kanamycin (Fig. 4.6A and C). Regardless of the treatment given, there was nearly 100% growth of the plated microcolonies (Table 4.2). Green kanamycin resistant colonies from inoculated tissue remained green on kanamycin-containing medium after about 3-4 weeks under selection (Fig. 4.6D). In some instances green spots were initially observed on bleached colonies and later grew out as

TABLE 4.2

Treatment	No. of colonies (- kanamycin)	No. of colonies (+ kanamycin)
LBA4404	25 (25)	0 (60)[method 2]
	none	none [method 1]
non-inoculated	30 (30)	0 (40)[method 2]
	45 (45)	0 (110)[method 1]
LBA4404:pBin19:	25 (27)	84 (162)[method 2]
Mu1	none	123 (247)[method 1]

Numbers in paranthesis represent number of microcolonies plated.

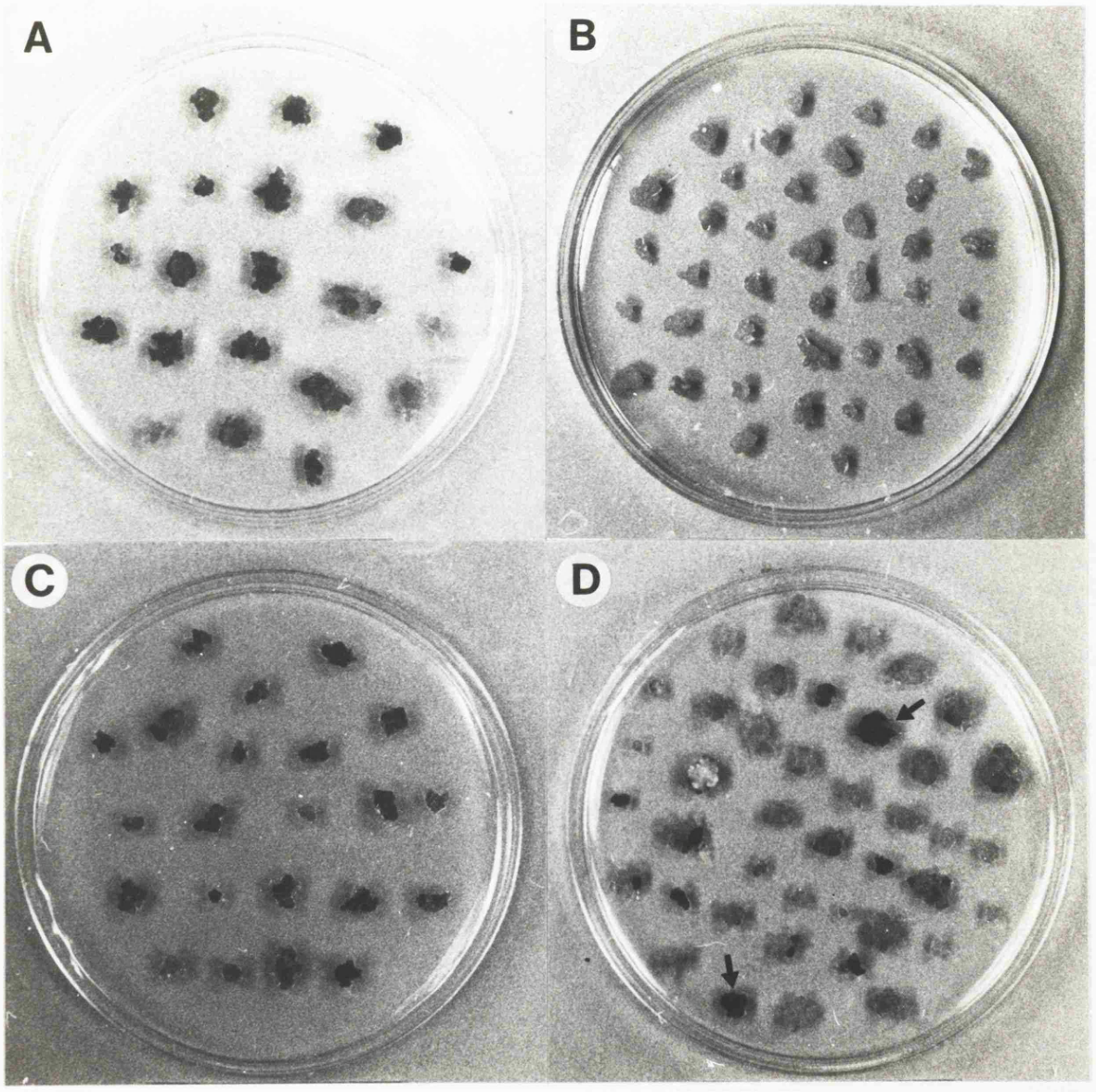
Fig. 4.6 Colonies from transformation of flax suspension cultured cells

A) Control (uninoculated) colonies after 3 weeks growth on non-selective medium (medium A).

B) Control colonies after 3 weeks growth on medium containing kanamycin.

C) Colonies derived from suspension cultured cells inoculated with LBA4404 (pBin19::*Mu1*) after 3 weeks growth on non-selective medium (medium A).

D) Colonies derived from inoculated cell suspensions after 3 weeks growth on medium containing kanamycin (medium B). Arrows point at Km^r colonies.


A
B
C
D

masses of green calli after further subcultures on kanamycin-containing medium. The controls (non-inoculated and LBA4404 inoculated) showed some initial growth on kanamycin containing medium but later bleached (Fig. 4.6B).

There was no significant difference in the response observed between the two methods. A transformation frequency of about 50% was obtained using both methods (Table 4.2).

4.2.7 NPT-II activity in transformed colonies

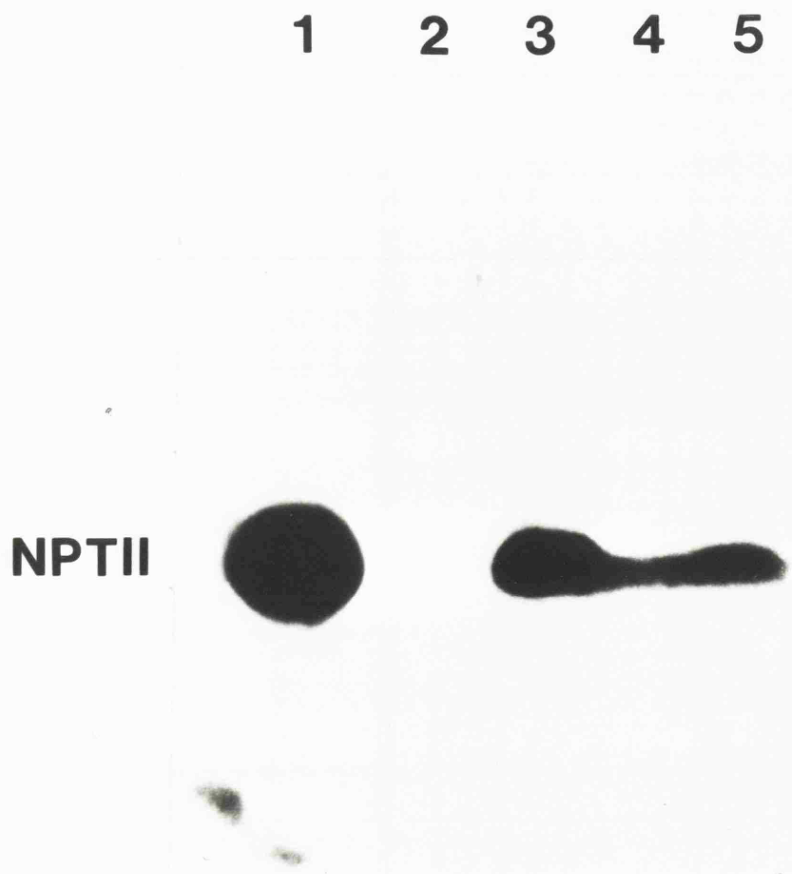
All of the Km^r colonies assayed contained NPT-II activity (Fig. 4.7, lanes 3-5) which comigrated with authentic *E. coli* NPT-II (lane 1). The control callus protein extract did not show any activity (lane 2). As in previous experiments, there are differences in the amount of NPT-II activity between each line. In some lines the gene was highly expressed. Detection of NPT-II activity indicates that the gene has been succesfully transferred from the transforming vector into the flax cell genome.

4.2.8 Summary

Results from both transformation systems have shown that flax cells are competent for transformation by *cis* or *trans*-acting non-oncogenic vectors. Under suitable conditions *cis*- and *trans*-acting, non-oncogenic Ti-plasmid vectors have been shown to be capable of transforming flax hypocotyl explants and cells in suspension respectively using kanamycin resistance as a marker. Analysis of control materials showed

Fig. 4.7 NPT-II activity in colonies derived from transformed suspension cultured cells

Lane 1, control NPT-II extract from *E. coli*; lane 2, control Km^s callus extract; lanes 3-5, NPT-II activity in extracts from transformed Km^r colonies.


1 2 3 4 5
NPTII

that kanamycin is effective as a selectable marker. No signs of spontaneous kanamycin resistant phenotypes were detected from non-transformed tissue. In the first system, the morphogenic potential of the hypocotyl was not affected by transformation procedures. The transformation system for flax hypocotyls is simple, reliable, and can produce reasonable numbers of transformed shoots. Although the transformed suspension cells did not develop shoots on the regeneration medium, the technique itself provides a means of mass transformation for mutagenesis studies. In most cases, the T-DNA is co-linear with vector sequences. Border analysis suggest that most transformants have low number of inserts which integrated into the genome randomly. The presence of nopaline in the co-integrative vector transformed callus and expression of the *npt-II* gene in the *cis*- and *trans*-acting vector-transformed callus lines further prove the integration of the T-DNA in the flax genome.

Future studies may be undertaken to induce embryogenesis of flax suspension cell culture. Through embryogenesis, high numbers of clonally derived transformed embryoids can be regenerated into plantlets (Scott and Draper, 1987). Mutant plants may then be recovered after selection for the desired phenotype. Both transformation systems developed here have properties that make them attractive for use in mutagenesis studies.

CHAPTER 5

BEHAVIOUR OF *Mu1* IN TRANSGENIC FLAX CELLS

5.1 INTRODUCTION

5.1.1 General features of transposition

The most important property of transposable elements (Tn) in view of insertion mutagenesis studies is their ability to create mutations upon insertion or excision from a gene. The high frequency at which they move and consequently produce mutations in their natural host, obviates the need for continuous transformation in an insertion mutagenesis programme if Tn's can be induced to behave in a similar manner in transgenic plants. These insertions and excisions within the genome, a phenomenon known as transposition, not only cause insertion mutations but also a variety of chromosomal rearrangements, including deletions, duplications, inversions, and translocations (McClintock, 1956b,1965). One type of mutation induced by this event are null mutations, which result from the disruption of a gene when an element inserts into the structural gene, or in their vicinity. Another consequence of the insertion of a transposable element can be the increase or decrease of the activity of the affected gene. The most conspicuous aspect of the mutations caused by transposable elements is their instability, which usually involves the full, or partial reversion to the wild type form. Germinal reversion to the wild type phenotype occurs at frequencies an order of magnitude higher than those of point mutations. Phenotypic reversion occurs when the element excises from the gene. A stable recessive phenotype can also be produced if the excision

is imprecise and destroys gene function.

5.1.2 Choice of suitable transposable elements for insertion mutagenesis

The transposable elements differ not only in their structural properties but also in their ability and frequency of transposition. The different categories of elements have been described in Chapter 1. For use in insertion mutagenesis, the transposable element has to have several important features;

i) it should have been cloned and have well characterised structural properties with the full nucleotide sequence known,

ii) it requires useful restriction sites to facilitate its manipulation and to aid cloning of flanking regions for mutant gene isolation (see Fig. 1.5),

iii) it must be possible to integrate it stably into transgenic plants (other than its natural host),

iv) it should have a high frequency of transposition, which can be regulated.

In 1984 when the project was initiated the properties of the transposable element *Mu1* described below, made it a reasonably good candidate for the above purpose.

5.1.3 *Mutator system*

Characteristics of *Mu1*, namely alteration of gene expression, inverted terminal repeat sequences, and target site

DNA duplications, strongly indicate that *Mu1* is an active transposable element in the Robertson's *Mutator* system. *Mutator* lines in maize were identified by Robertson (1978), as lines which were capable of generating recessive mutants at many loci at rates 20- to 50-fold above spontaneous levels; many of them somatically and germinally unstable (Robertson, 1978, 1981; Freeling, 1984). The transmission of the mutator characteristic does not follow a simple Mendellian pattern, and the mutation rate was found to be stable for at least 2 generations. New mutant alleles are readily recovered from *Mutator* lines at a frequency of 10^{-4} to 10^{-5}, and many of these mutations have somatically unstable phenotypes characteristic of insertion elements (Robertson, 1978). Mutation frequencies as high as 7.54×10^{-5} and as low as 4.0×10^{-6} were observed for 5 loci including *y1*, *yg2*, *bz1*, *sh2* and *wx* (Robertson, 1985).

5.1.3.1 Structural properties of transposable element *Mu1*

This *Mutator* activity was associated with the insertion of a 1.4 kb element, *Mu1* (Strommer *et al.*, 1982; Bennetzen *et al.*, 1984, 1987a), or a larger but homologous 1.7 kb element, *Mu1.7* (Taylor *et al.*, 1986). The ~1.4 kb element was cloned from *Mutator*-induced mutations at an unstable allele of the alcohol dehydrogenase gene *Adh1* (Freeling , 1982; Bennetzen *et al.*, 1984) and *A1* (O'Reilly *et al.*, 1985).

Fig. 5.1 shows a composite diagram of several

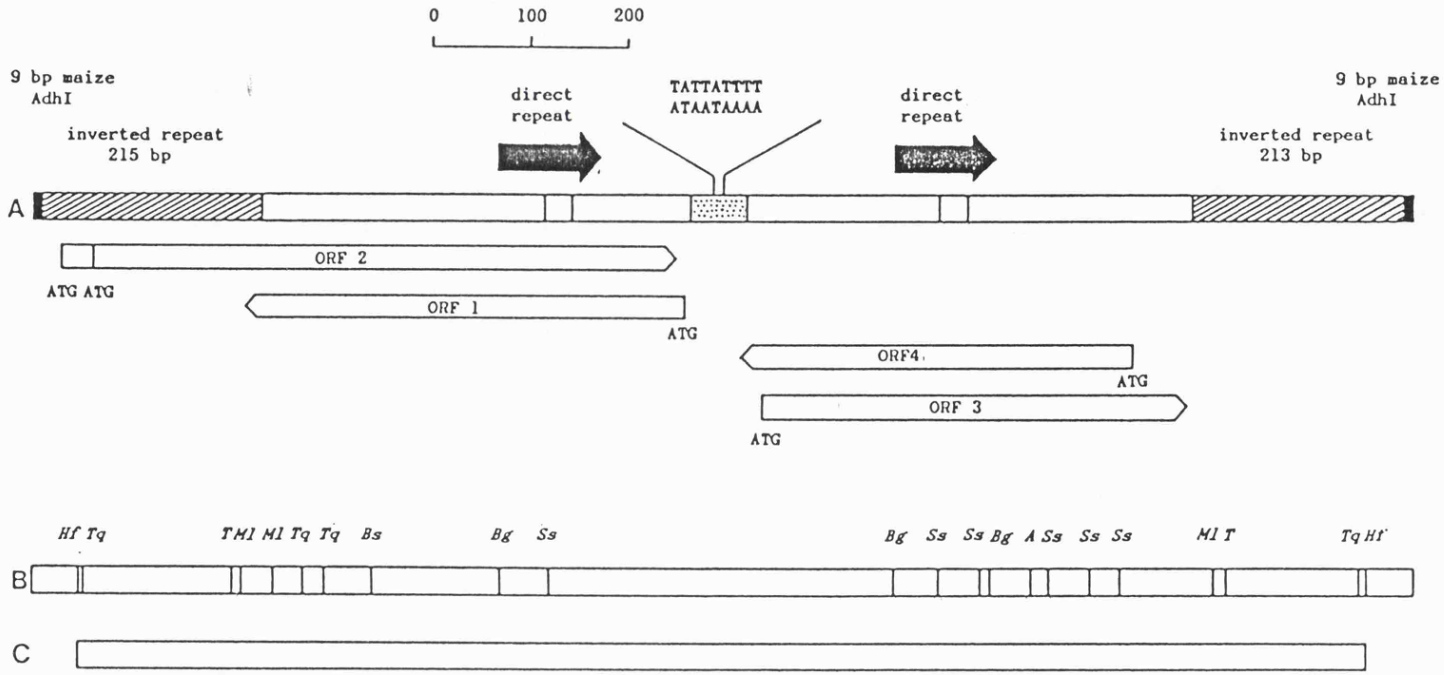


Fig 5.1
A. Composite diagram of several features found in the MuI sequence, showing regions of inverted repeats, direct repeats, the 4 open reading frames (ORF), maize AdhI sequences and the low GC region (possible eukaryotic promoter signal.
B. Restriction map of Mul. *A Ava* I, *Bg Bgl* I, *Bs Bst* NI, *Hf Hinf* I, *Ml Mlu* I, *Ss Sst* II, *T Tth* I, *Tq Taq* I.
C. 1.3 kb *Hinf* I fragment used as probe in all experiments.

important features found in the nucleotide sequence of the *Mu1* element. The transposable element, *Mu1* has a length of 1367 nucleotides and creates a duplication of nine nucleotides upon insertion (Bennetzen *et al.*, 1984; Barker *et al.*, 1984). The *Mu1* is delineated by long terminal inverted repeats of 213 and 215 nucleotides showing 95% homology which matches perfectly over the terminal 28 bp. The terminal inverted repeats contain recognition sites for the restriction endonucleases *Hinf* I, *Taq* I, *Tth 111* I, and *Mlu* I. The left-hand inverted repeat is defined by the presence of a site for *Mst* I. Use of these sites allows separation of *Mu1*-like sequences from other genomic DNA fragments on Southern blots.

Within the element are two long internal direct repeats of 104 bp showing 96% homology. This is a unique feature of *Mu1* when compared with other characterised eukaryotic transposable elements. These direct repeats contain sites for restriction endonucleases *Sst* II and *Bgl* I which allows isolation of the central portion of *Mu1*. There are four open reading frames (ORFs) found in the element, each beginning with an ATG codon, and having a GC content which is strikingly dissimilar from that of the whole maize genome. The open reading frames, numbered 1 to 4, extend for 402, 621, 435, and 483 bp and potentially encode polypeptides of 13.6, 21.2, 15.7, and 17.0 kD respectively. Polypeptides 2 and 3 may have similar compositions owing to the fact that they are in the same reading frame with respect to the placement of the

long internal direct repeat, giving them an area of homology.

The average GC content of *Mu1*, 67%, is very different from that of the maize genome as a whole. Within the long terminal inverted repeats, the GC content averages 51.5%, which is comparable to maize DNA. The GC contents of the regions containing ORFs 1 and 2 and ORFs 3 and 4 average 66% and 70% respectively. Near the centers of the ORFs are several short, approximately 20 bp, regions containing 90% GC. These very highly GC-rich regions correspond with positions in the 104 bp long internal direct repeats which themselves average 79% GC and which contain dense clusters of GC-rich restriction sites.

Fig. 5.1 shows that the *Mu1* element can be divided symmetrically into two halves by the central 50 bp area of low GC content which contains the only sequence (TATTATTTTC) resembling the 'TAATA' Goldberg-Hogness eukaryotic promoter signal. The four ORFs do not cross this region. Thus, *Mu1* can be divided into two halves which range from 26% to 52% GC at their ends, reach 90% GC near their centers, and which contain one long inverted terminal repeat copy, one long direct internal repeat copy, and one pair of ORFs.

There are no sequences with perfect homology to the eukaryotic promoter CCAAT sequence (Hefron *et al.*, 1979) in *Mu1*, but several with partial homology occur. The sequence GCAAT present in ORF1, may be involved in its transcription. The only sequence which resembles the poly(A) addition sequence AATAAA found in animal systems (Benoist *et al.*, 1980) is the

AATAA sequence found close to the termination codon of ORF 3. Short inverted repeats might constitute recognition and regulatory sites on DNA. The secondary structure of *Mu1* contains a number of short stem loops which correlates to the positions of open reading frames. The correlation however, appears to be related to the GC-richness of the central portions of the open reading frames rather than to the placement of regulatory signals in the DNA.

Another class of elements homologous to *Mu1* observed in most *Mutator* lines is an ~1.7 kb size class (Barker *et al.*, 1984) termed *Mu1.7* (Walbot *et al.*, 1985). It is not known whether *Mu1*, *Mu1.7*, or both encode trans-acting factors required for their transposition, or if other elements or factors are required for their mobilisation. The 1.7 kb element is structurally similar, shares terminal inverted repeats and internal direct repeats with the *Mu1*, but has an extra 2 open reading frames (ORFs). The other 4 ORFs are identical to those present in *Mu1* (Taylor and Walbot, 1987). It also contains an additional 385 bp of unique DNA, as well as changes in a few restriction sites relative to *Mu1*. A portion of the extra DNA in *Mu1.7* is part of a truncated direct repeat of *Mu1*. It is located immediately adjacent to the external terminus of the right direct repeat. The first 34 bp are identical to the 34 bp adjacent to the internal terminus of the direct repeat in the left portion of both *Mu1.7* and *Mu1*. These observations suggest that either *Mu1* is derived from *Mu1.7*, or

that both are derived from the same ancestor (Taylor and Walbot, 1987).

5.1.3.2 *Mu1* transposition

Cross-hybridising elements of a similar size and structure to *Mu1* are found in a high copy number (10 to 70 copies) in all *Mutator* lines, but are only present at 0 to 2 copies in other non-*Mutator* lines (Bennetzen, 1984; Bennetzen *et al.*, 1987a). However, sequences homologous to the terminal repeat are present at ~40 copies in the non-*Mutator* lines (Chandler *et al.*, 1986). There is known to be some correlation between the *Mutator* activity and the copy number of intact *Mu* sequences. In active *Mutator* lines, these *Mu* elements transpose very frequently, with an average rate of 0.5 to 1 transposition event per element per generation (Alleman and Freeling, 1986). Transposition events which generate multiple new chromosomal locations in the outcross progeny (Alleman and Freeling, 1986), are not however, accompanied by equally high germinal reversion frequencies (Bennetzen *et al.*, 1987a). This suggests that *Mu1* may commonly transpose via a DNA replicative process. However, little is known about the mechanism of transposition, and no mRNA with *Mu1* homology or transposase proteins have been identified yet.

About 40% of mutants induced by *Mu* are unstable and have mutable patterns, generally small and relatively uniform somatic sectors in either the leaf, endosperm or aleurone layer, that indicate a specific and late developmental timing

of reversion events (Robertson, 1985; Walbot *et al.*, 1985; Bennetzen, 1985). *Mu*-induced mutation frequencies were also shown to vary with respect to loci and, for some loci, may depend on other factors such as the sex of the *Mu* parent or the previous crossing history of the *Mu* parent. The *waxy* locus, for example, has a higher mutation frequency in *Mu* plants crossed as males than when *Mu* plants function as females (Robertson, 1985).

Regulation of *Mutator* activity has also been indicated by the concomitant loss of high mutagenic potential and *Mu* element transpositional activity by either outcrossing (crossing a *Mutator* plant to a non-*Mutator* plant), or intercrossing (crossing of a *Mutator* plant to a *Mutator* plant) (Robertson, 1983, 1986; Bennetzen *et al.*, 1987a). In such outcrosses, about 10% of outcross progeny had lost *Mutator* activity (Robertson, 1978, 1986). Mutagenically active *Mutator* plants decrease their *Mu* element copy number by 20%-30% on outcross to non-*Mutator* lines. The progeny which had lost *Mutator* activity often retained a high copy number (10-50 per diploid genome) and surprisingly contain relatively undermodified *Mu* elements (Bennetzen *et al.*, 1987a). These results, and the presence of some lines with equal copy numbers but showing contrasting *Mutator* activity, indicate that there is only a weak correlation between *Mutator* activity loss and copy number, and also suggest that regulatory factors other than DNA modification effect transposition. Bennetzen *et al.*

(1987a), suggest that the *Mutator* activity loss may be due to loss of a positive regulatory factor encoded by a subset of *Mu1*-like elements.

Strikingly different results were obtained in intercrossing. Repetitive intercrossing of *Mutator* lines, eventually result in 100% loss of *Mutator* activity of the offspring (Robertson, 1986; Bennetzen *et al.*, 1987a). Analysis of *Mu1* elements in intercrossed lines which have lost *Mutator* activity, has revealed the presence of modified elements (Bennetzen, 1985) that have lost the ability to transpose. Modification is indicated by resistance to digestion of the genomic DNA by some restriction enzyme isoschizomers, suggesting cytosine methylation (Chandler and Walbot, 1986; Bennetzen, 1987). Genetic evidence also suggests that the modification system once established, is active in subsequent generations because these interbred-loss lines do not seem to recover *Mutator* activity, even after three generations of outcrossing. The loss of *Mutator* activity in intercrossed *Mutator* lines progeny is accompanied by an increase in copy number (40-50 per diploid genome) which levels off after the third and fourth generations (Robertson, 1986; Bennetzen *et al.*, 1987a). This correlation might imply a self-encoded or self-activated negative regulator of *Mu1* transposition. However, some other factor(s) such as the backgrounds of *Mutator* lines, may have an influence on this inhibition since intercrossing of *Mutator* in some lines can lead to high copy

numbers of *Mu1*-like elements without loss of *Mutator* mutagenic activity (Bennetzen *et al.*, 1987a).

5.1.4 Detection of movement of *Mu1* in transgenic plants

In maize, movement of transposable elements is easily detected through reversion of certain unstable alleles which are known to be induced by transposable element insertion. The use of easily-visualised endosperm markers, allowed the transposable element insertion and excision to be scored (Chandler and Walbot, 1986; Robertson, 1985). The absence of such alleles, and the unknown mutant phenotypes induced by the transposable element insertion in a transgenic plant genome, necessitate the design of a gene construct that can be used as a phenotypic reporter assay for excision of the element. The expression of the gene in the construct should result in a dominant and selectable phenotype upon excision of the element. In many transformation systems including flax (see Chapter 4), the chimaeric nopaline synthase-neomycin phosphotransferase II (*nos-npt-II*) gene has been used successfully as a selectable marker gene to recover Km^r transformants. A phenotypic assay to monitor *Mu1* excision in transgenic plants can be designed using the *nos-npt-II* chimaeric gene construct by the following logic. Insertion of *Mu1* sequences into the untranslated leader sequence will split the structural gene from the promoter and prevent the expression of the Km^r phenotype. After its introduction into transgenic plant cells, *Mu1* can excise, thus

restoring the *npt-II* gene expression and consequently produce kanamycin resistant cells. Such a construct was developed by Simon Bright and Robert Simpson (pMuNK14) and was used in this study (see section 5.2.2). A similar construct has been designed more recently with the transposable element *Ac*, and was successfully used to monitor its movement in transgenic tobacco cells (Baker *et al.*, 1987).

5.1.5 Regulation of *Mu1* transposition in transgenic plants

Although the molecular basis of the regulation of transposition is not fully understood yet, there are data that suggest the role of certain conditions in influencing these events. Environmental background, stresses (environmental and genetic), DNA damage and repair mechanisms, have been correlated with increased mutability in plants, and have been hypothesised to be responsible for activating transposable elements (McClintock, 1984). These inducing conditions are often inter-related thus, proving which one causes the inactivation or activation of the element directly is very difficult. A strong correlation has, in particular, been demonstrated between DNA methylation and loss of transposition ability of *Ac* and *Mu1* (Chomet *et al.*, 1987; Chandler and Walbot, 1986). This is the only recent observation which gives some indications of the molecular regulation of transposition. DNA methylation has also been shown to occur in prokaryotes (reviews by Marinus, 1987). Two types of methylations, 6-

methyladenine and 5-methylcytosine, are produced by post replicative modification of residues in specific DNA sequences by the action of three methylases: the *hsd* (host-specificity), *dam* (DNA adenine methylation), and *dcm* (DNA cytosine methylation) gene products. Methylation of eukaryotic or prokaryotic DNA sequences can affect gene expression. DNA methylation also influences transposition of Tn5 and Tn903 both of which have *dam* sites in transposase-promoter regions. Methylation of promoter regions decreases initiation of transcription.

For the purpose of these studies, these conditions might be manipulated to regulate the activity of transposable element. Molecular studies have strongly indicated that inactivated elements are often methylated (Chomet *et al.*, 1987; Chandler and Walbot, 1986). Accordingly, an hypothesis can be made that any treatment which can cause demethylation of the element may reactivate the element. Studies in animal cells have shown that UV irradiation can cause complete demethylation of modified DNA during subsequent DNA replication or repair (Lieberman *et al.*, 1983). It is thought that such treatments which have deleterious effects on DNA will evoke DNA repair pathways and subsequent demethylation and activation of transposable elements. Demethylation of a modified inactive element into an active one can also be achieved by treatment with azacytosine. 5-Azacytosine (5-azaC) is a potent inhibitor of DNA cytosine methylases. Its inhibition is due to the

formation of complexes in which the enzyme is tightly bound to the 6-position of the 5-azaC residues in DNA (Santi *et al.*, 1983; Friedman, 1986). Thus, some kind of regulation can be devised, by methylation and demethylation of DNA.

The environmental conditions under which plants are grown often introduce environmental or genetic stresses upon the plants. Since plants are not mobile, they have to develop a means for adaptation to these conditions. One way is through rapid genomic changes. These genomic changes can be stimulated through mechanisms such as gene amplification and depletion as a result of unequal exchange, internal gene rearrangements, altered methylation patterns, gene conversion and DNA transposition (Scowcroft *et al.*, 1983; Walbot and Cullis, 1985; Brettel *et al.*, 1986). These mechanisms which often operate when cells are grown under stressful conditions, such as in culture, can produce heritable variations among some species, flax being one example. The underlying mechanism of these variations, termed somaclonal variation, has been linked to DNA transposition induced by de-differentiation of cultured cells. Peschke *et al.*, 1986 recovered an active *Ac* element in maize plants regenerated from tissue culture of explants which originally did not have *Ac* activity. Hence, it is possible that the same mechanism which causes these phenomena will also activate a transposable element which is stably integrated in a transgenic plant. In addition, studies on the *Ac* element, carried out concurrently with this project, have shown that the

element transposes soon after transformation in transgenic tobacco and *Arabidopsis* cells which have undergone dedifferentiation (Baker *et al.*, 1986, 1987; Van Sluys *et al.*, 1987).

5.1.6 Stability of *Mu1* in transgenic plants

To explore the possibility of using the transposable element *Mu1* as a mutagen and gene tag in plants other than maize, first the element has to be stably integrated in the plant genome. There are numerous wide host range, non-oncogenic, Ti plasmid based vectors with multicloning sites and dominant selectable marker genes, that can be used for this purpose (Draper *et al.*, 1988). Inserting *Mu1* sequences into the cloning sites will enable the element to be co-transferred with the marker gene. The selectable marker gene will allow independent verification of the transformed status of tissues growing on selection medium, and give an indication of which cells have recieved the transposable element.

Since the start of this project there have been several reports suggesting that *Mu1* can be successfully inserted into the nuclear genome of tobacco, tomato and *Arabidopsis* (Lillis *et al.*, 1985; Zhang and Somerville, 1987). In all cases, Ti plasmid-mediated transformation procedures were used to achieve transfer of the element into the transgenic plant genomes. *Mu1* sequences were found at significantly higher levels than the accompanying T-DNA

sequences in the transformed tobacco tumour cells. A different pattern of restriction fragments which hybridised to *Mu1*, from that expected of the transforming vector was observed. However, it was not that expected for simple transposition of *Mu1*. The results suggest that rearrangements or modifications had taken place in some copies of the *Mu1* element (Lillis *et al.*, 1985). Analysis of transformed, *Mu1*-containing tomato roots showed that the element has been transferred intact into the tomato DNA, without rearrangement (Lillis *et al.*, 1985). Similar results were obtained when *Mu1* was transferred into *Arabidopsis thaliana* (Zhang and Somerville, 1987). *Mu1* was stably integrated and transmitted intact through several generations in the *Arabidopsis* genome. The *Mu1*-containing line was reported to carry approximately 2-5 copies of *Mu1*.

5.1.6.1 Transfer of *Mu1* to transgenic flax plants

The transformation systems established in Chapter 4 provide means of stably inserting *Mu1* into the flax genome. An independent and random transfer of *Mu1* can be achieved at a reasonably high frequency without affecting the totipotency of the competent flax cells. If transposition of *Mu1* occurs in flax in the same way as the element moves in maize, it may be possible to isolate genes from flax by procedures described in Chapter 1, and demonstrated in maize (O'Reilly *et al.*, 1985). Alternatively, transposition might be induced by one of the several schemes described in section 5.1.5.

The binary vector pBin19 described in Chapter 4,

carries a polylinker with an array of restriction enzyme sites suitable for cloning (Bevan, 1984). An ~2.9 kb *Bam* HI/*Hind* III restriction fragment containing the whole of *Mu1* was isolated from plasmid pMJ9 (a gift from Dr. Mike Freeling) and is inserted into pBin19 cut with *Bam* HI and *Hind* III (Fig. 5.2). After conjugation with the *Agrobacterium tumefaciens* non-oncogenic virulence helper strain LBA4404, the *Mu1*-carrying plasmid was then ready for transformation experiments. *Mu1* transposition in this case could only be monitored by Southern blot analysis.

5.1.6.2 Examination of *Mu1* behaviour in transgenic flax

The strategy described in section 5.1.4 and more recently by Baker *et al.*, (1987), provide the phenotypic assay to aid detection of *Mu1* movement in transgenic flax easily. A vector has been constructed *in vitro*, in which *Mu1* is inserted into the untranslated leader sequence of a chimaeric *nos-npt-II* gene, in a Ti plasmid based vector to form pMuNK14 (a gift from R.B Simpson and S.T.W Bright). Fig. 5.3 shows the structure of the DNA between the T-DNA borders in pMuNK14. Excision of the transposable element *Mu1* would restore the function of the chimaeric gene and cells exhibiting *Mu1* transposition would be kanamycin resistant. A restriction fragment internal to the T-DNA borders will hybridise with probes homologous to the *npt-II* gene and *Mu1* sequences. Thus, any separation of *Mu1* and *npt-II* sequence on Southern analysis may confirm that a transposition

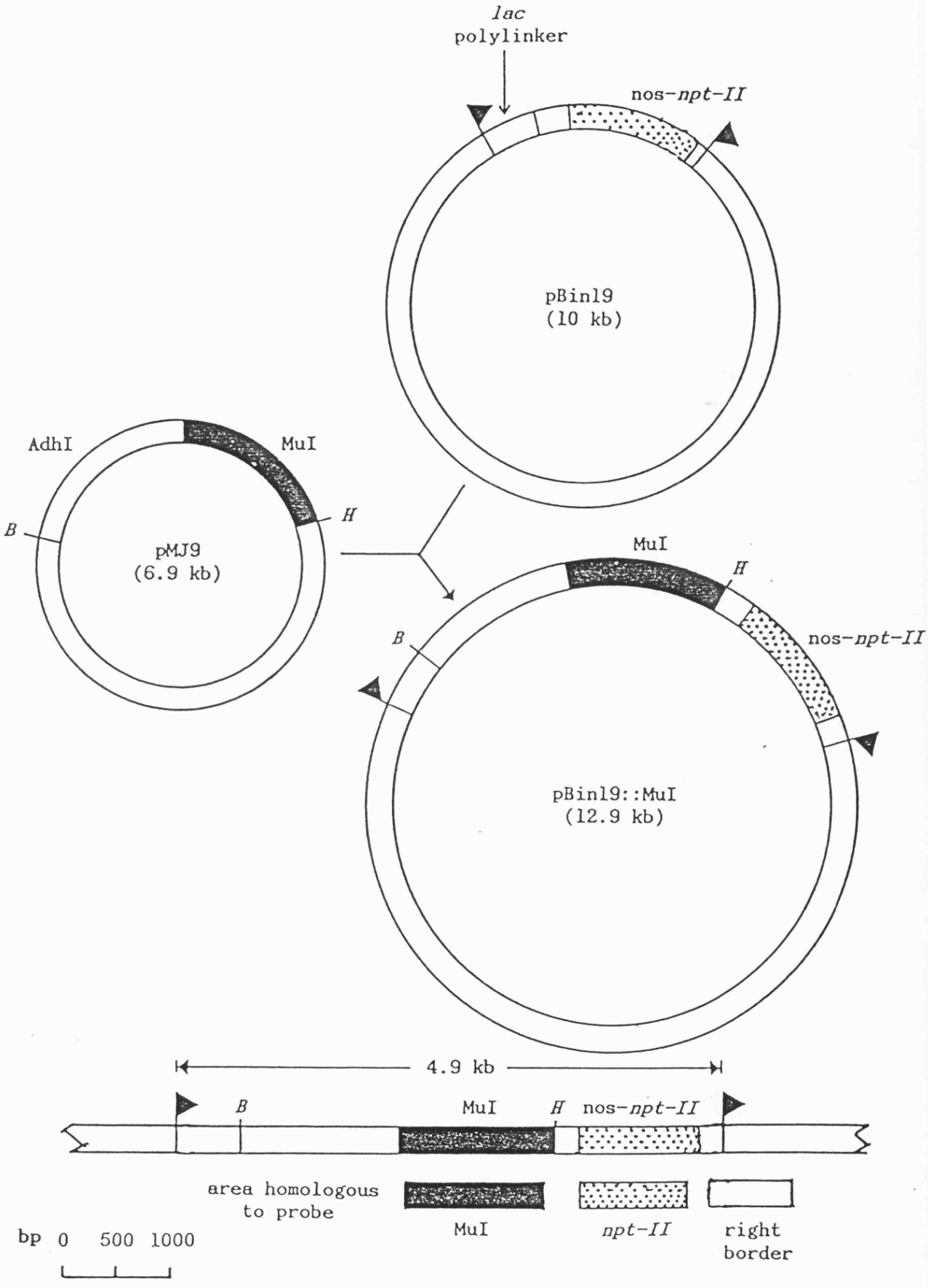
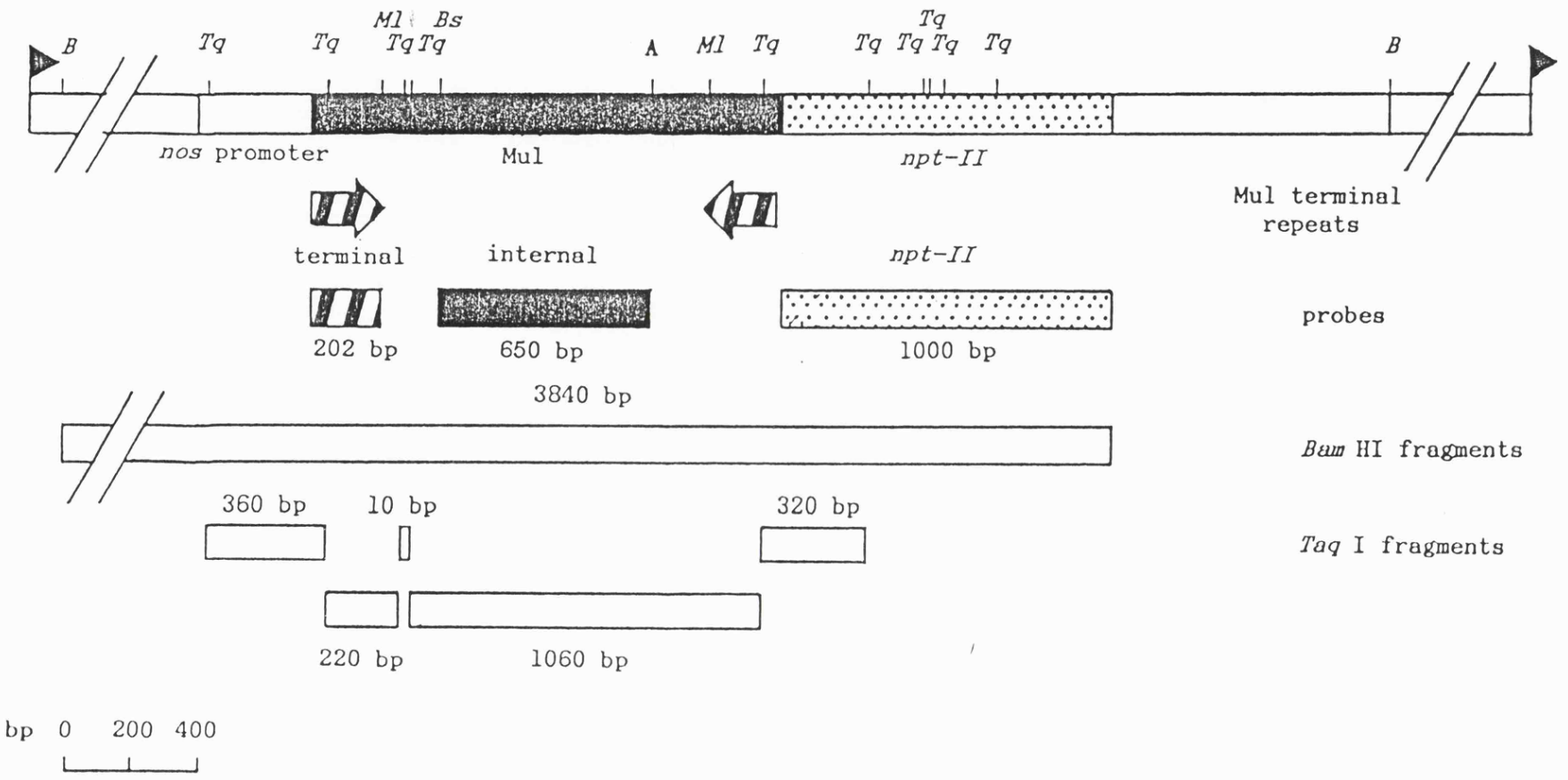


Fig 5.2 Construction of pBinl9::MuI and detail of T-DNA structure

Fig 5.3 Structure and restriction map of pMUNK14



event has occurred (see Fig. 5.4).

The pMuNK14 plasmid is based on the *Agrobacterium tumefaciens* binary vector pARC8 (Simpson *et al.*, 1986). However, it has been transferred into an *Agrobacterium rhizogenes* vir helper strain A4 which acts *in trans* to transfer the T-DNA region of pMuNK14. The similarity in function of the *vir* genes in *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Huffman et al., 1984) allows T-DNA transfer from non-oncogenic binary vectors derived from *Agrobacterium tumefaciens* into *Agrobacterium rhizogenes* strains (Simpson et al., 1986; Trulson *et al.*, 1986; Sukhapinda *et al.*, 1987; Hamill *et al.*, 1987). During transformation, T-DNA from the binary vector is co-transferred with the T-DNA from the *Agrobacterium rhizogenes* wild type Ri plasmid, into the plant genome (Simpson *et al.*, 1986; Shahin *et al.*, 1986; Hamill *et al.*, 1987). Thus, in this case putitive *Mu1* transformation events can be detected theoretically by selection of individual transformed roots (which are clonal in origin) on medium containing kanamycin.

In the present chapter, the stability of *Mu1* in transgenic flax cells is examined by two methods. Firstly by looking at stability of *Mu1* sequence by Southern blotting. Secondly by using pMuNK14 to utilise the restoration of the Km^r phenotype as an indication that excision has occurred under conditions that might induce transposition.

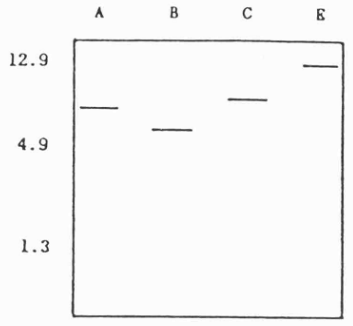
Fig. 5.4 Southern blot patterns of *Mu1*-containing tissues which would result from the three most likely 'events'

I) *Hind* III digest of pBin19::*Mu1* transformants produce unpredictable size fragments. The figure shows possible fragments that can be detected by the three probes. Changes in the restriction fragment patterns as a result of the three 'events' could be detected if the same lines are analysed at different periods of development.

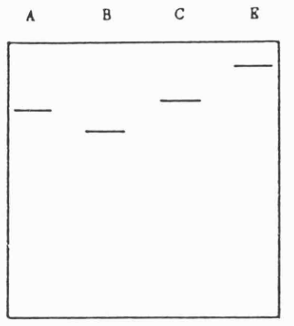
II) *Bam* HI and *Taq* I digest of pMuNK14 transformants, hybridised with the *npt-II* and *Mu1* probes. The figure shows the different characteristics of fragments produced as a result of the three 'events'.

pBin19::Mul

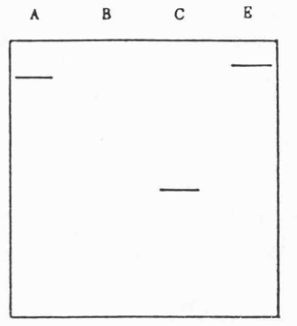
Hind III/*npt*-II



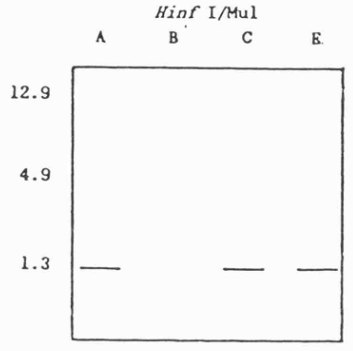
Hind III/right border



Hind III/MuI



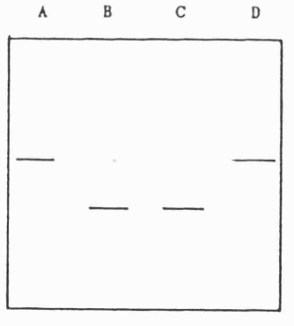
Hinf I/Mul



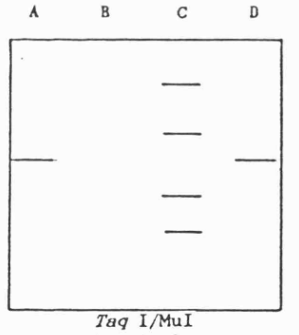
A No change
B Mu loss
C Mu transposition
D pMUNK14 (control)
E pBin19::Mul (control)

pMUNK14

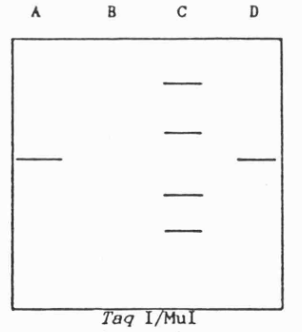
Bam HI/*npt-II*



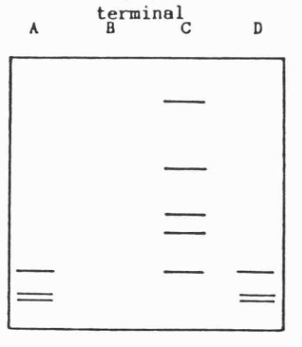
Bam HI/Mul terminal



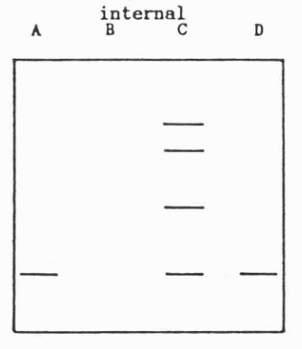
Bam HI/Mul internal



Taq I/Mul terminal



Taq I/Mul internal



5.2 RESULTS AND DISCUSSION

5.2.1 Integration of *Mu1* in transgenic flax cells by *Agrobacterium*-mediated transformation.

5.2.1.1 Transformation of flax

Previous studies have shown that flax could be transformed with oncogenic strains of *A. tumefaciens* (Hepburn *et al.*, 1983) and with 3850::1103, a *cis*- type non-oncogenic disarmed Ti plasmid (Basiran *et al.*, 1987; Chapter 4). When the transformation system was changed to use the general purpose, wide host range binary vector pBin19, a similar response was observed, but with a much higher transformation frequency. The reason for the higher transformation frequency was not determined. It may possibly be due to the different *vir* helper plasmid or other slight differences in culture conditions which made the cells more competent. The vector (pBin::*Mu1*) used for all transformation procedures in this experiment carried the *Mu1* element inserted in the multicloning site of pBin19 (Fig. 5.2, section 5.1.6.1). The induction of kanamycin resistant callus and shoots was found to be dependent not only on the initial medium in which the explants were incubated, but also on the accessibility of the transforming vector to the more totipotent cells. These results are summarised in Table 5.1 and 5.2. In every case, several independently transformed tissues from both cut ends were obtained from each hypocotyl segment.

Irrespective of the treatments given, the explants

TABLE 5.1

RESPONSE OF HYPOCOTYL SEGMENTS ON REGENERATION MEDIUM WITH, AND WITHOUT KANAMYCIN

Treatment	MSD4x2 + Cx	MSD4x2 + Cx + Km
LBA4404	All explants formed callus and regenerated shoots	All explants bleached
LBA4404:pBin19:Mu1	All explants formed callus and regenerated shoots	All explants formed callus but only 46.4% have shoots
Uninoculated	All explants formed callus and regenerated shoots	All explants bleached

TABLE 5.2

RESPONSE OF INDUCED CALLUS AND REGENERATED SHOOTS ON SELECTION

Treatment	Initial medium	Growth on selection medium B	
		Callus	Shoots
LBA4404	Medium A	100.0% bleached	100.0% bleached
	Medium B	none	none
LBA4404:pBin19:Mu1	Medium A	100.0% bleached	100.0% bleached
	Medium B	100.0% growth	73.1% growth
Uninoculated	Medium A	100.0% bleached	100.0% bleached
	Medium B	none	none

showed a similar response on medium A (MSD4x2 + cefotaxime) in each case (Table 5.1). These observations have been described in detail in Chapter 4. Upon selection on medium B (MSD4x2 + cefotaxime + kanamycin), all the induced calli and regenerated shoots from explants initially incubated on medium A, bleached after more than 2 weeks.

However, if the explants were incubated on medium B (MSD4x2 medium containing cefotaxime and kanamycin), the response was observed to be very dependent on the treatment given (Table 5.1). Total growth inhibition was observed in all of the controls (LBA4404-inoculated and non-inoculated). Although initial cell expansion was observed, the explants subsequently bleached after about two weeks. Meanwhile, out of 56 explants inoculated with the vector carrying the kanamycin resistant marker gene and *Mu1* element (pBin19::*Mu1*) which were incubated on medium B, 109 green Km^r calli were induced (~97% frequency) at one or both ends of the explants. Unless it was very clear that the calli originated at different sites of the explant, callus emerging from one cut end was counted as a single transformant. It was not possible to determine if the transformed callus originated from a single transformed cell or otherwise. Out of these, only 19 of the callusing explants regenerated shoots (Table 5.1). These shoots were observed to arise either directly from the middle of expanding explants or from regions at the end of the explant in very close proximity to the the induced callus. All of the

induced Km^r calli remained green and proliferated into a mass of friable callus upon further selection on the same medium. More than 25% of the regenerated shoots were found to be kanamycin sensitive and bleached after further selection (Table 5.2). Only those arising from areas in the vicinity of the site of origin of the Km^r callus proved resistant to kanamycin.

The Km^r regenerated shoots were separated from the callus, propagated and rooted as described in Chapter 3. Shoots regenerated from callus were soft, vitrified, and had tiny leaves. Inducing them to form roots was very difficult. Only 6 Km^r shoots were successfully rooted in 1/2 B5 medium without any hormones. The regenerated shoots gradually lost their vitrified nature and the stem became more sturdy after rooting. Once an extensive root system had developed and was able to support further growth, the plantlets were transferred to soil. During the first week after transfer from culture into soil (vermiculite:compost mixture), the young plants were covered in a perforated plastic bag to prevent desiccation while the plants were adapting to photoautotrophic growth in the Saxil cabinet. Regular weekly watering with Hoagland's solution (Appendix 2) accelerated growth and made the plants sturdy. When the plants had adapted to the conditions and were strong enough to grow photoautrophically, the plastic bag was removed. All of the plants appeared to grow as normal and looked like wild-type flax plants with no visible morphological differences. They started flowering after 8 weeks following

transfer into soil. In some cases, the plants formed one or two fertile flowers prematurely (after about 1 week) but later stopped and the plants grew as normal and flowered again after around 8 weeks. This phenomenon was mainly observed on those plantlets which were induced from callus that had undergone subculture for a few months. Only four plantlets were grown to maturity in the soil. One of these was found to be sterile. The other three grew vigorously into mature, normal-looking, fertile plants. They were propagated and grown to maturity till they set seed and were designated lines $9AR_0$, $10AR_0$ and $23BR_0$.

5.2.1.2 Molecular analysis of transformants

Transformed, kanamycin resistant and control non-transformed tissues were screened by hybridisation to *Mu1* sequences isolated as a *Hinf* I fragment from the plasmid pMJ9 (a gift from Dr. Mike Freeling). The presence of *Mu1*-homologous sequences in the plant genome in relation to the accompanying *npt-II* gene and right hand border sequences was determined by hybridisation with the respective probes (Fig. 5.2). If a normal, single insertion of T-DNA took place during transformation, a *Hind* III digestion of DNA extracted from transformed tissues should produce two fragments. Each fragment consists of part of the T-DNA and some plant DNA (Fig. 5.5). One fragment will hybridise with the *Mu1* probe, and the other with the *npt-II* and right hand border probes. A *Bam* HI digestion on the other hand, will produce a single fragment

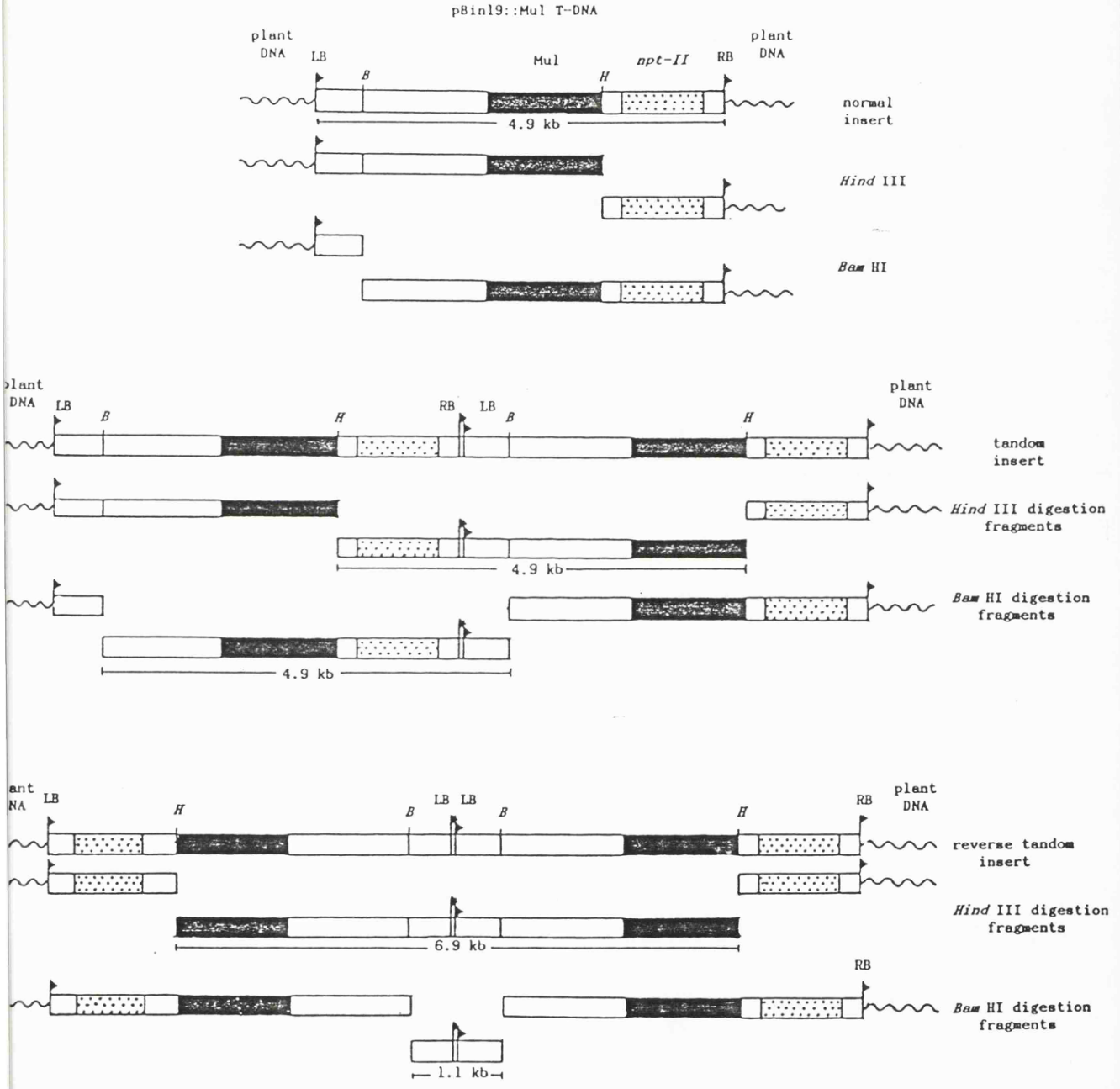


Fig 5.5 Expected fragments from *Hind* III and *Bam* HI digestion of pBinl9::Mul transformed tissues

consisting of part of the T-DNA and some plant DNA, which hybridises to all three probes (Fig. 5.5).

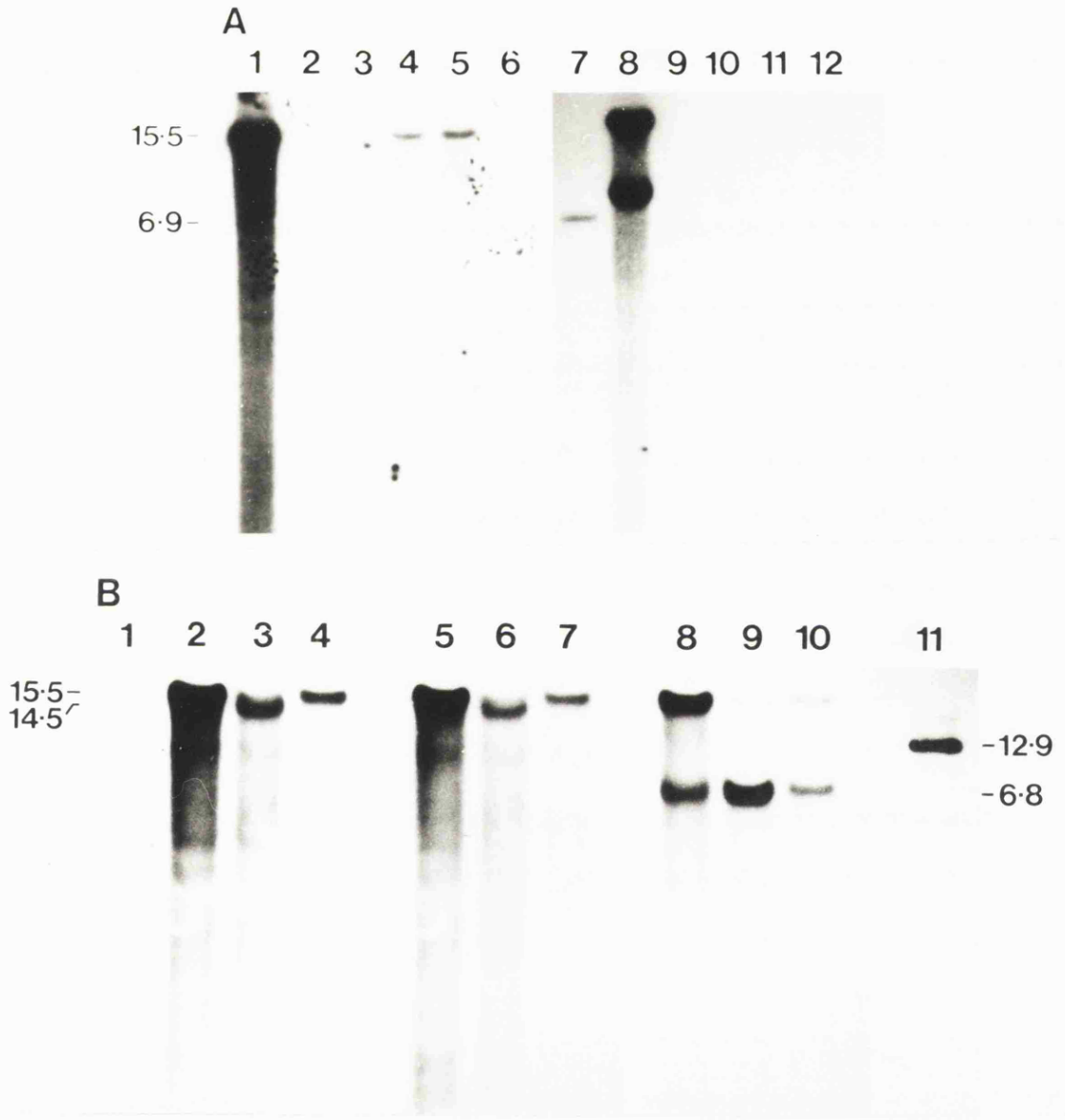
DNA was extracted from several independently-transformed kanamycin resistant callus lines (9A, 10A, 23A, 24A and 53A) after the second subculture following excision from the original explant and subjected to Southern blot analysis (Fig. 5.6A). A *Hind* III band of 15.5 kb hybridising with both *Mu1* and *npt-II* probes was common to lines 53A, 9A and 10A (Fig. 5.6A; lanes 1, 4 and 5). This band was found at a copy number of approximately 1-2 per genome in 9A and 10A. Other faint bands hybridising to the probe were found in lines 23A and 24A (Fig. 5.6A; lanes 2 and 3). The 15.5 kb *Hind* III fragment gave a much stronger signal in line 53A, corresponding to approximately 10 copies per genome (Fig. 5.6A; lanes 1 and 8). This band was accompanied by a lower molecular weight *Hind* III fragment of 8.1 kb that hybridised with an equal intensity to only the *Mu1* probe (Fig. 5.6A; lane 8). DNA from non-transformed plants lacked homology to any of the probes (Fig. 5.6; lane 6). These results were difficult to interpret as none of the *Hind* III restriction fragments had the expected size that would result from tandem, or reverse tandem inserts (see Fig. 5.5). The callus was thus subcultured further to provide sufficient material for a larger DNA extraction and more detailed Southern analysis.

DNA was extracted a second time from three surviving callus lines (9A, 10A and 23B) after four months of subculture.

Fig. 5.6 Analysis of T-DNA in callus transformed by LBA4404 (pBin19::*Mul*)

A) DNA extracted after the second subculture on selection medium. DNA restricted with *Hind* III and 5 μg used for each lane. Lanes 1-6, *npt-II* probe; lane 7-12, probed with *Mul* probe. Lanes 1 and 8, line 53A; lanes 2 and 9, line 23A; lanes 3 and 10, line 24A; lanes 4 and 11, line 9A; lanes 5 and 11, line 10A; lane 6, control, non-transformed flax callus; lane 7, *Hind* III digest of pMJ9 containing the whole of *Mul*.

B) DNA extracted after four months of subculture on selection medium. DNA restricted with *Hind* III and 5 μg used for each lane. Lanes 1-4, *npt-II* probe; lanes 5-7, right hand border probe; lanes 8-11, *Mul* probe. Lane 1, DNA from non-transformed callus; lanes 2, 5 and 8, line 9A; lanes 3, 6 and 9, line 10A; lanes 4, 7 and 10, line 23B; lane 11, *Hind* III-digested pBin19::*Mul*.


A
1 2 3 4 5 6 7 8 9 10 11 12
15·5
6·9
B
1 2 3 4 5 6 7 8 9 10 11
15·5
14·5
12·9
6·8

It is evident from Fig. 5.6B that sequences homologous to *Mu1* and *npt-II* were still present in all lines. However, the restriction fragment patterns were not as expected. Two *Hind* III fragments of 15.5 and 6.8 kb were observed in all three lines (Fig. 5.6B; lanes 8-10) that hybridised strongly to the Mu1 probe. The 15.5 kb band was probably that found in the same callus lines soon after selection. However, the lower band of 6.8 kb had not been detected previously in lines 9A and 10A. The lower band in these lines was present at a copy number from 5-10 per genome. The 15.5 kb band was present at the original level (1-2 copies per genome) in line 10A, whilst the intensity of this band had increased to a copy number of around 10 in line 9A. The T-DNA pattern in 23B (which had previously not been analysed) was similar to 9A and 10A.

The increased intensity of the 15.5 kb band in line 9A and appearance of a new 6.8 kb band in both 9A and 10A may be the result of recombination and amplification events occurring during subculture. Alternatively, this result could reflect the selection of a subpopulation of transformed cells containing *Mu1* sequences at a new site. Such cells may have a selective advantage on kanamycin-containing medium if the *Mu1* amplification events also generated further copies of the *npt-II* gene, as it has been shown previously (Chapter 4) that there may be a relationship between copy number and NPT-II levels in flax transformants (Basiran *et al.*, 1987). In order to test this idea, the hybridisation filter was washed to strip the *Mu1*

probe and then reprobed separately with the *npt-II* and right-hand border probe (Fig. 5.2). In all lines the 15.5 kb restriction fragment hybridised to both *npt-II* and right hand border probes (Fig. 5.6B; lanes 2-7). In line 10A the two probes hybridised strongly to a new band of around 14.5 kb that had no homology to *Mu1* sequences (Fig. 5.6B; lanes 3 and 6). Other, smaller molecular weight fragments hybridised weakly to the *npt-II* and border probes in all three lines. These results are consistent with the fact that amplification of sequences with homology to the *npt-II* had also occurred, together with recombination events which separated *Mu1* from border and *npt-II* sequences.

As in the original study (Fig. 5.6A), these hybridisation patterns were not easy to interpret and so further analysis was performed using DNA digested with *Bam* HI (which only cuts once within the T-DNA and should not separate *Mu1*, *npt-II* or right hand border sequences), and *Hinf* I which should release a 1.3 kb internal fragment of *Mu1* if the transposable element is still intact. When *Bam* HI was used to cleave DNA extracted from lines 9A, 10A and 23B only one fragment of 15.1 kb was found at a copy number of 5-10 per genome (Fig. 5.7A; lanes 3-5) after hybridisation with the *Mu1* probe. The fact that large numbers of border fragments were not found in these transformed tissues (Fig. 5.7A; lanes 3-5), coupled with the observation that the 15.5 kb *Hind* III fragment found in all lines also had homology to *Mu1* sequences suggests

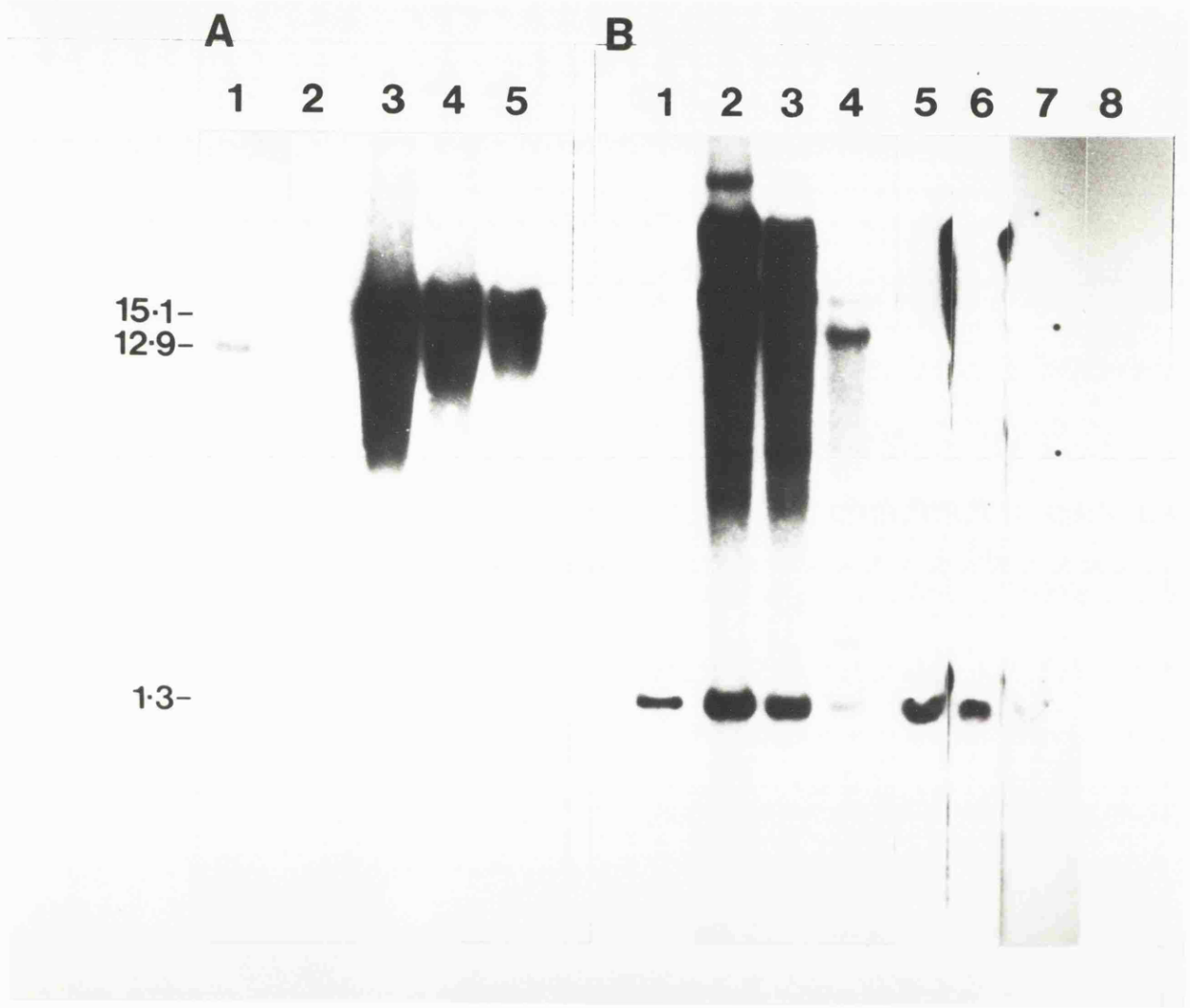
Fig. 5.7 Analysis of T-DNA and *Mul* internal fragments in callus and regenerated plants derived from tissues transformed by LBA4404 (pBin19::*Mul*)

Both blots (A and B) hybridised against the *Mul* probe.

A) DNA extracted after 4 months of subculture on selection medium and digested with *Bam* HI. Lane 1, *Bam* HI digest of pBin19::*Mul*, xl copy reconstruction of *Mul* sequences; lane 2, control DNA from non-transformed plant tissue; lanes 3-5, lines 9A, 10A and 23B respectively.

B) Lanes 1-4, DNA extracted from callus after 4 months of subculture on selection medium and digested with *Hinf* I. Lane 1, *Hinf* I disgestion of pBin19::*Mul*, x5 copy reconstruction for internal *Mul* sequences. Lanes 2, 3 and 4, lines 9A, 10A and 23B respectively.

Lanes 5-7, DNA extracted from plants regenerated from pBin19::*Mul*-transformed callus, digested with *Hinf* I. Lanes 5, 6 and 7, plants 9ARO, 10ARO and 23BRO respectively. Lane 8, control transformed plant lacking *Mul* sequences.


A
1 2 3 4 5
15·1–
12·9–
1·3–
B
1 2 3 4 5 6 7 8

that at least some of the T-DNA copies may have originally been present as tandem inserts. In lines 9A and 23B the T-DNA inserts may have been integrated as direct tandem repeats, whilst in line 10A the data is consistent with a reverse tandem organisation. However, the molecular weights of the fragments which hybridised with the probes, and those predicted from the proposed transformation events (Fig. 5.5) are not consistent. The lengths of the expected composite fragments are in most cases too long and thus it is possible that the original T-DNA inserts were separated by a small amount of plant DNA, as has been found previously in other studies (Holsters *et al.*, 1983).

There are no convenient restriction sites within pBin19::*Mu1* which can be used to further analyse the gross structure of the T-DNA in these lines. However, without knowing for certain the T-DNA structure it was interesting to note that after repeated subculture the stoichiometry of *Mu1* to *npt-II* signal was altered in 9A and 10A. This suggested that *Mu1* sequences might have amplified in the flax genome, possibly by replicative transposition events. However, a *Hind* III digest of the flax DNA would have been expected to produce random fragments with *Mu1* homology if transposition and reinsertion had occurred. This was not the case and so the origin of the 6.8 kb band with homology only to *Mu1* sequences (Fig. 5.6B; lanes 8-10) remains a mystery. It was hoped that digestion of DNA from the transformed flax lines with *Hinf* I should release a single internal fragment of 1.3 kb (Fig. 5.7B;

lane 1) with homology only to *Mu1* sequences, and thus enable an accurate estimation of the number of *Mu1* copies present in each line. As can be seen in Fig. 5.7B (lanes 2-4) several bands greater than 1.3 kb were also found in the callus DNA. It is known that *Hinf* I is sensitive to methylation but it is not certain in this instance whether the DNA was simply underdigested. DNA was also extracted from plants regenerated from these callus lines (R_0), and in all cases a 1.3 kb *Hinf* I fragment was observed, indicating the presence of intact *Mu1* sequences in these tissues (Fig. 5.7B; lanes 5-7). Unfortunately this DNA was isolated only in a small amount from flowering plants and was very degraded, making pointless any further analysis examining expected restriction fragments of much higher molecular weight.

Plants regenerated from the above callus lines ($9AR_0$, $10AR_0$ and $23BR_0$) were grown to maturity and selfed; all further work was carried out with R_1 seedlings. The seeds were germinated in the presence of 350 µg/ml of kanamycin (see Table 5-3) and from each line ($9AR_0$, $10AR_0$ and $23BR_0$) three kanamycin-resistant seedlings (R_1) were grown in soil. These progeny were designated ($9AR_1$-1, $9AR_1$-4, $9AR_1$-6; $10AR_1$-1, $10AR_1$-8, $10AR_1$-9; $23BR_1$-1, $23BR_1$-3, $23BR_1$-10). Callus was induced on leaf sections from each of these lines on MSD4x2 and subcultured once.

A Southern blot analysis was performed to examine the organisation of T-DNA and *Mu1* sequences in leaves and leaf-

TABLE 5.3

INHERITANCE OF KANAMYCIN-RESISTANCE PHENOTYPE IN R_1 SEEDLINGS

Km^r R_1 lines	R_o seeds germinated	R_1 seedlings	
		Km^r	Km^s
9A	14	4	10
10A	15	4	11
23B	15	6	9

Km^r, kanamycin resistant; Km^s, kanamycin sensitive.

derived callus from R seedlings. A *Bam* HI digestion was performed on DNA extracted from leaves of each of the nine progeny. After Southern blotting, the hybridisation filter was probed with a 650 bp *Mlu* I-*Bst* NI fragment of internal *Mu1* sequences (Fig. 5.3). It can be seen in Fig. 5.8A that all the three R_1 seedlings from each separate R_0 plant contained a similar fragment pattern. An identical pattern was also found in the leaf-derived callus tissues (Fig. 5.8B). Four fragments of 22, 18.5 kb, 15.1 kb and 12.9 kb had homology to the *Mu1* probe. The 18.5 kb restriction fragment is common to all progeny of $9AR_0$ and $10AR_0$. The 15.1 kb fragment is common to the progeny of $9AR_0$ and $23BR_0$. The R_1 seedlings from $23BR_0$ have a fragment of 12.9 kb which almost co-migrates with *Bam* HI-linearised vector DNA (Fig. 5.8A and B; lanes 9-12).

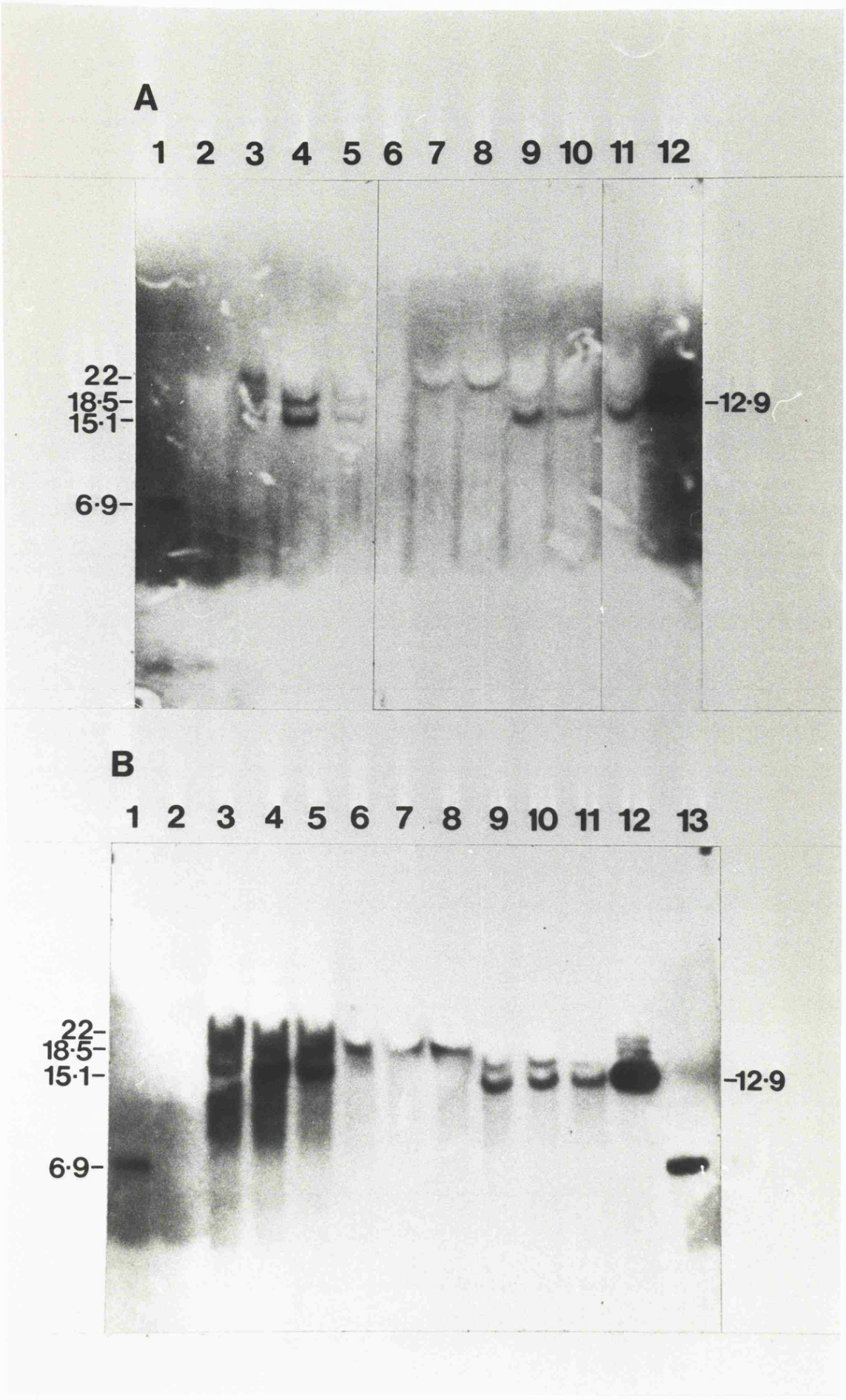
The data in Fig. 5.8 indicates two important points. Firstly, R_1 seedlings derived from the same R_0 plant each contain an identical T-DNA pattern with regard to *Mu1* sequences. It is therefore very likely that the R_0 plants had the same T-DNA organisation and that this was stably inherited by the R_1 population. However, it is immediately obvious that the hybridisation patterns were different from those found in the callus line from which the R_0 plants were derived. The 15.1 kb fragment was still visible, but at a reduced intensity, in two of the three types of R_1 seedlings ($23BR_1$ and $9AR_1$), whilst in $10AR_1$ seedlings this band had disappeared (Fig. 5.8). It is possible that the new bands of 12.9 and 18.5 kb with *Mu1*

Fig. 5.8 T-DNA structure in R_1 seedlings and seedling-derived callus from plants transformed with pBin19::*Mul*

Both blots (A and B) were hybridised against the *Mul* probe.

A) DNA extracted from R_1 seedlings and restricted with *Bam* HI. Lane 1, plasmid pMJ9 cut with *Hind* III, x 5 copy number reconstruction for *Mul* sequences. Lane 2, control, non-transformed flax seedling DNA. lanes 3-11, DNA from R_1 seedlings $9AR_1$-1, $9AR_1$-4, $9AR_1$-6, $10AR_1$-1, $10AR_1$-8, $10AR_1$-9, $23BR_1$-1, $23BR_1$-3 and $23BR_1$-10 respectively. Lane 12, *Bam* HI digest of pBin19::*Mul*, T-DNA reconstruction.

B) DNA of callus derived from R_1 seedlings restricted with *Bam* HI. Lane 1 and 13, plasmid pMJ9 cut with *Hind* III, x 5 and x10 copy number reconstruction for *Mul* sequences. Lane 2, control, non-transformed flax seedling DNA. lanes 3-11, DNA from callus derived from R_1 seedlings $9AR_1$-1, $9AR_1$-4, $9AR_1$-6, $10AR_1$-1, $10AR_1$-8, $10AR_1$-9, $23BR_1$-1, $23BR_1$-3 and $23BR_1$-10 respectively. Lane 12, *Bam* HI digest of pBin::*Mul* (T-DNA reconstruction).


A
1 2 3 4 5 6 7 8 9 10 11 12
22-
18·5-
15·1-
6·9-
-12·9
B
1 2 3 4 5 6 7 8 9 10 11 12 13
22-
18·5-
15·1-
6·9-
-12·9

homology may be derived from new transposition events in cells giving rise to R_0 shoots. In this respect, it is important to note that in the R_0 plants the 1.3 kb internal *Hinf* I fragment of *Mu1* was still present (Fig. 5.7B; lanes 5-7). This suggested that intact *Mu1* elements were still present in the flax genome, but whether all the signal is derived from unaltered T-DNA inserts or transposed *Mu1* elements cannot be known for certain. One of the most important observations is the fact that the pattern of bands with *Mu1* homology did not alter in callus derived from R_1 plants (compare Fig. 5.8B with 5.8A). This may point towards the fact that reversion to a differentiated state (shoot organogenesis) may stabilise *Mu1* inserts, possibly by methylation. However, much more analysis would be required before this question can be addressed properly.

5.2.1.3 Inheritance of introduced DNA

On the basis of the intensity of the hybridisation signal obtained with *Mu1* DNA as the probe, the *Mu1*-containing lines, 9A, 10A and 23B carried from 2-10 copies of *Mu1* (Figs. 5.6-5.8). The inheritance of the introduced DNA in all three lines was followed for one generation by scoring for antibiotic resistance in germinated seedlings. The concentration level of kanamycin needed to kill wild type flax seedlings was determined by growing pre-germinated flax seeds in vermiculite wetted with Hoagland's solution containing kanamycin with concentration levels ranging from 0-500 µg/ml. As the

constructed kanamycin sensitivity curve in Fig 5.9 shows, the 'killing' level is 350 µg/ml. Fifteen randomly chosen R_2 progeny (seedlings germinated from seeds of selfed R_1 plants of each line) were scored for kanamycin resistance. The seeds were initially germinated for two days on wet filter paper, to ensure that spurious problems with seed viability did not bias the results, and then transferred to pots containing vermiculite soaked with Hoagland's solution (Appendix 2) plus 350 µg/ml kanamycin.

The number of surviving plants were scored after more than two weeks and the results are summarised in Table 5.3. Because the population screened is small, it is not statistically possible to conclude if the segregation ratios fit any acceptable (resistant/susceptible) pattern of inheritance. The results may reflect the complexity of the transformation events that had taken place. It suggests in some cases, that plants produce more gametes lacking a functional Km^r gene that would be expected. A single insertion of T-DNA which would be expected to behave as a single dominant Mendelian locus and give a 3:1 segregation ratio was not observed with any population.

5.2.1.4 Transposition of integrated *Mu1* element in transgenic flax plants

Although the maize transposable element *Mu1*, has previously been transferred into tobacco tumour cells, tomato

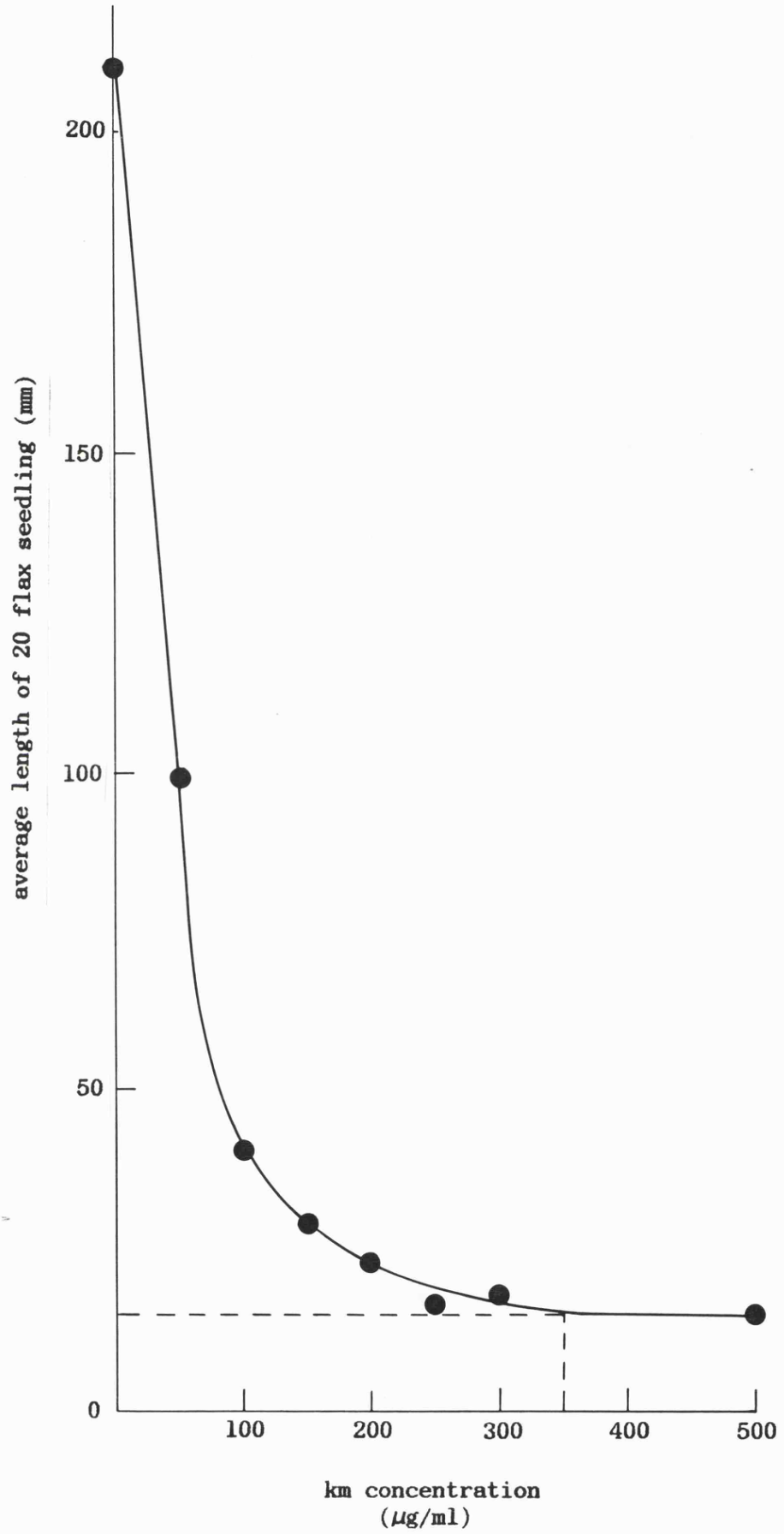


Fig 5.9 Kanamycin sensitivity curve

root cells and *Arabidopsis thaliana*, its transposition could not be unambiguously proved (Lillis *et al.*, 1985; Zhang and Somerville, 1987). A similar experiment performed here has shown that *Mu1* can be integrated into the flax genome by Ti plasmid-mediated transformation procedures. Fertile flax plants carrying intact copies of the element have been successfully regenerated from transformed *Mu1* containing callus lines. There are definite changes in the T-DNA organisation between original transformed tissue, subcultured transformed callus and regenerated shoots. Much of this data suggested that recombination events resulting sometimes in amplification of the T-DNA were occurring. The *Mu1* element had also been shown to undergo rearrangements in transformed tobacco tumour lines (Lillis *et al.*, 1985). The *Mu1* hybridisation pattern of the tobacco tumour lines were different and at a higher copy number than the T-DNA of the transforming vector. In flax however, once intact plants had been generated, the hybridisation pattern of restriction fragments with homology to *Mu1* sequence remained stable. Similar observations were made in *Arabidopsis thaliana*, where *Mu1* was stable for at least four generations (Zhang and Somerville, 1987).

The results of the molecular analyses do not give any real evidence of *Mu1* transposition in the transformed flax cells. An attempt to induce transposition by inducing callus from *Mu1*-containing R_1 seedlings was unsuccessful. Results of *Bam* HI digestion on DNA from leaf-derived callus showed a

similar hybridisation pattern to the leaf DNA (Fig. 5.8). It may be that *Mu1* is methylated, and a longer period of dedifferentiation is needed to activate the element. The callus lines were subcultured only once and thus not all explant cells would have undergone many divisions. This was evident from the appearance of the callus, in which more than 50% still consisted of expanded explants instead of friable callus.

The *Mu1* element in maize has been shown to transpose late during development, possibly during meiosis. However, no such event was detected in all 9 lines of transgenic flax R1 seedlings which contain the element. As the frequency of transposition is correlated with copy number in maize (Barker, 1984; Chandler *et al.*, 1986), it may be that the copy number of *Mu1* in the transformants obtained is too low. Alternatively, if the transposition events do occur at a very low frequency, then a higher number of transformed seedlings may have to be screened in order to detect such an event.

5.2.2 Examination of *Mu1* mobility in transgenic flax

The experiments examining the stability of *Mu1* in flax described in section 5.2.1 suggested that, in general, the transposable element did not excise by a normal transposition event after transformation. As outlined in the introduction to this section (5.1.4), it is possible to use a 'reporter' system to both detect low frequency transposition events and also to select cell lines in which transposition has occurred (Baker *et al.*, 1987). In the following experiments the vector (pMuNK14) was designed such that excision of *Mu1* would result in the induction of kanamycin resistance in the cells concerned. In pMuNK14 (Fig. 5.3) a *Mu1* element has been engineered into the 5' untranslated region of a *nos-npt-II* gene.

The experiments described below aimed firstly to see if kanamycin resistance could be induced in tissues transformed with A4 (pMuNK14). Factors affecting the frequency of transposition were investigated. A study was also made to compare molecular aspects of *Mu1* excision and reintegration in transgenic flax tissue with the transposition of *Mu1* in its normal host, maize.

5.2.2.1 Induction of kanamycin resistant callus on hypocotyl sections by transformation with A4 (pMuNK14)

Hypocotyl sections of flax seedlings are not only very responsive to tissue culture manipulations, but also competent for transformation by *Agrobacterium tumefaciens*. It was thought that if hypocotyls were equally responsive to

Agrobacterium rhizogenes, then *Mu1* mobility could be studied soon after transformation in clonal 'hairy root' tissues. This should aid the selection of rare kanamycin resistant events and give useful frequency data. Flax seedlings were grown aseptically for two days, 1.0-2.0 mm hypocotyl sections were excised and infected with the wild-type *Agrobacterium rhizogenes* strain A4, or with A4 carrying the binary vector pMuNK14, whose structure is shown in Fig. 5.3. Following inoculation, the hypocotyl sections were incubated in different MS-based media (see Appendix 1), and selection on kanamycin applied immediately after co-incubation period, or after a further 20 days growth.

Table 5.4 shows that the responses observed were dependent on the presence of hormones in the medium. Infection with *Agrobacterium rhizogenes* results in the production of auxins by the transformed tissues which normally stimulates 'hairy root' tumour induction at wound sites. This indeed proved to be the case when hypocotyl sections were inoculated with A4 on media lacking phytohormones (MSO). After three weeks on MSO some tumorous rooty callus also developed at the cut ends of the hypocotyl explants (Fig. 5.10A). However, on MSD4x2 medium, which contains high levels of cytokinins and low levels of auxin, most hypocotyl explants inoculated with A4 produced callus with occasional shoot primordia that did not develop further to give normal adventitious shoots (Fig. 5.10B). After more than 3 weeks of culture, a few roots (less

TABLE 5.4

RECOVERY OF KANAMYCIN RESISTANT CALLUS

FROM HYPOCOTYL SECTIONS

Medium	Vector	
	A4	A4(pMuNK14)
MSO	Roots + rooty callus	Roots + rooty callus
+Km (0 day)	All bleached (roots)	All bleached (roots)
+Km (20 day)	All bleached (roots)	All bleached (roots)
MSD4x2	Predominantly callus	Predominantly callus
+Km (0 day)	All bleached (callus)	11.8% resistant callus
+Km (20 day)	All bleached (callus)	9.0% resistant callus

Fig. 5.10 Transformation of flax hypocotyl explants by A4 and A4 (pMuNK14)

A) and (B); flax hypocotyl segments 3 weeks after inoculation with *A. rhizogenese* A4, on MSO and MSD4x2 medium respectively.

C) Km^r callus growing out of flax hypocotyl explant on MSD4x2 + Cx + Km medium, 6 weeks after inoculation with A4 (pMuNK14).

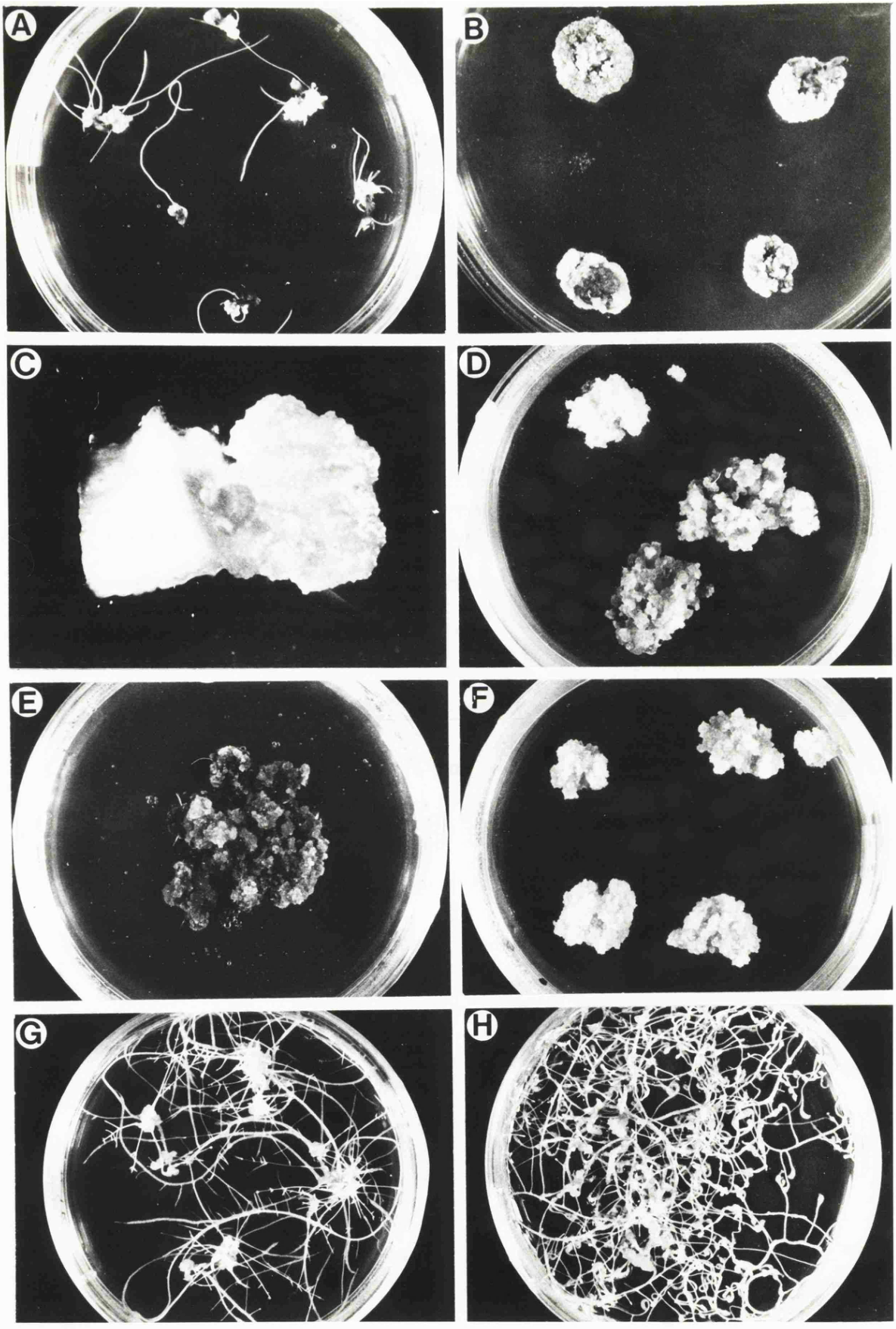
D) Callus from (C) continues to grow when subcultured on selection medium.

E) Ist category of Km^r tissue derived from flax hypocotyls inoculated with A4 (pMuNK14); green, fast-growing callus.

F) Second category of Km^r tissue derived from flax hypocotyls inoculated with A4 (pMuNK14); friable, yellowish callus with green sectors.

G) Hairy root regeneration from Km^r callus lines grown on MSO plus 1.0 mg/l 2,4-D and 100 μg/ml Km.

H) Roots regenerated from callus in (G) continue to grow on MSO plus 100 μg/ml Km.


A
B
C
D
E
F
G
H

than 5) may be induced from the predominant proliferating callus tissue.

Both callus and roots induced on MSD4x2 and MSO were subcultured onto the same medium containing kanamycin. Most were bleached and stopped growing (Table 5.4). However, kanamycin resistant callus was successfully recovered at a low frequency from hypocotyl sections inoculated with A4 (pMuNK14) and incubated on MSD4x2 medium. Kanamycin resistant calli were recovered from such experiments at a frequency of around 10% when selection was applied either immediately, or 20 days post-inoculation. Thus, the induction of kanamycin resistant events on MSD4x2 medium does not depend on the timing of selection pressure.

Kanamycin resistant callus first appeared on hypocotyl sections inoculated with A4 (pMuNK14) after approximately six weeks culture on MSD4x2. A total of 13 antibiotic resistant callus lines on independent explants were recovered from 110 inoculated explants stressed on kanamycin. This response is much slower, and at a much lower frequency than that observed for the selection of kanamycin resistant tissues resulting from the inoculation of flax hypocotyls with vectors containing a constitutively expressed *nos-npt-II* gene (see Chapter 4 and section 5.2.1.1). After about 8-9 weeks when the calli were >5.0 mm in diameter, they were excised from the bleached explants and subcultured onto fresh selection medium. These kanamycin resistant callus lines could be

grouped according to their appearance and growth behaviour in the presence of kanamycin.

In one group, the callus first appeared at one end of the bleached explant as a tiny 'green island' of tissue, which slowly grew out of the explant, eventually producing a large lump of green, kanamycin resistant callus (Fig. 5.10C). On subculture, these tissues grew vigorously as predominantly green, friable callus, with occasional yellow/green patches (Fig. 5.10D). The yellowish section of the callus often bleached or turned brown after a couple of weeks. During subculture, these areas were discarded, allowing the greener, more healthy-looking callus to grow vigorously on the selection medium (Fig. 5.10E). On a few occasions, one or two roots developed from these callus lines, but they were not propagated. Such roots formed a green callus whenever they came into contact with the medium.

In a second category of transformants, yellowish callus grew out of one end of the bleached hypocotyl sections. This type of callus was friable, yellowish and probably consisted a mixture of kanamycin sensitive and resistant cells (Fig. 5.10F). Very often, after two subcultures, some sections of these yellowish callus lines differentiated roots. However, this capacity to form organised root tissues was generally lost after several more subcultures and a friable green callus gradually began to dominate the tissue. These friable green regions were subcultured and were found to be more resistant to

kanamycin and grew more vigorously than the yellowish tissues from which they were derived.

In certain experiments, callus tissues with a few regenerating roots were isolated from the yellowish callus lines and subcultured onto MSO medium supplemented with 1.0 mg/ml 2,4-D and containing 100 µg/ml kanamycin. Kanamycin resistant roots that continued to grow (Fig. 5.10G) were then excised from such calli and grown as root cultures on MSO containing kanamycin. Callus was often induced on the original root explant as it elongated and developed a network of secondary roots (Fig. 5.10H). The ability of the roots to grow on kanamycin indicates that they may have been derived from a single kanamycin resistant cell.

It is very clear from this data that the recovery of kanamycin resistant tissues is associated with the formation of callus. Roots induced directly on inoculated hypocotyl sections on MSO medium were all found to be sensitive to kanamycin (Table 5.4). Kanamycin resistant tissues were recovered mainly as callus; kanamycin resistant roots only being formed on callus after a number of subcultures. Continuous selection on medium containing antibiotics seemed to enrich for kanamycin resistant cells. This may be due to enrichment for a subpopulation of more kanamycin resistant cells which outgrew less resistant, or sensitive cells in the original callus.

5.2.2.2 Induction of 'hairy roots' on explants from mature plants

It should be possible to generate clonally-derived transformed tissues by inducing 'hairy roots' on explants inoculated with *Agrobacterium rhizogenes* (Hamill *et al.*, 1987; Simpson *et al.*, 1986). Independently transformed roots can then be selected on kanamycin to screen for *Mu1* transposition events from pMuNK14 T-DNA. In this way it was hoped that a relatively accurate value for excision frequency could be obtained.

Different explants, taken from 1-2 month old flax plants grown in a 'Saxil' cabinet, were inoculated with A4 (pMuNK14) on different media to determine their capacity to initiate root formation. Table 5.5 shows that roots are most readily obtained on leaf explants incubated on MSD4x2 and MSO medium. Root formation on MSD4x2 was always preceded by cell expansion and division at the wound site (Fig. 5.11A). On MSO medium, roots developed directly at the wounded sites on explants without an intervening callus phase (Fig. 5.11B).

5.2.2.3 Monitoring *Mu1* activity by induction of kanamycin resistant roots/callus on flax leaf sections

Before hairy root tissues could be used to monitor the excision of *Mu1* from the pMuNK14 T-DNA it was important to check the co-transfer frequency of oncogenic Ri T-DNA and binary vector T-DNA in transformed tissues. Roots were therefore induced on leaf explants by *Agrobacterium rhizogenes*

TABLE 5.5

RESPONSE OF MATURE FLAX EXPLANTS INOCULATED WITH pMuNK14 ON DIFFERENT MEDIA

Explants	Media		
	MSP1	MSD4x2	MSO
Leaf	semi compact callus at cut edges	callus at cut edges, followed by root formation	Roots induced directly from explant without callus formation.
Stems	semi compact callus, followed by induction of a few roots on callus	callus at cut edges and few roots	none

Fig. 5.11 Transformation of flax leaf explants by A4 and A4 (pMuNK14)

A) Development of hairy roots after 2 weeks on MSD4x2 plus Cx, following incubation of leaf explants with A4 (pMuNK14). Note the swelling of the explant and a limited amount of callus formation at the cut edges.

B) Development of hairy roots after 2 weeks on MSO plus Cx, following incubation of leaf explants with A4 (pMuNK14). Note the absence of callus formation at the cut edges.

C) Kanamycin resistant callus developing in sectors on roots derived from leaf explants inoculated with A4 (pMuNK14) and incubated on MSD4x2 + Cx + Km.

D) Continued growth of root-derived callus from (C) on MSD4x2 + Cx + Km.

A
B
C
D

strain 9402 (pDLT) which contained a wild type Ri plasmid (p9402) and the binary vector pDLT which carried a constitutively expressed *nos-npt-II* gene (Dr. C. Lichtenstein; personal communication). As a further control, inoculations were also performed with a wild type *Agrobacterium rhizogenes* strain A4, in order to check that no spontaneous kanamycin resistant tissues are generated under the experimental conditions used.

In the preliminary experiments it was found that control, uninoculated leaf explants did not produce any roots on either MSO or MSD4x2. On MSD4x2, uninoculated leaf pieces formed prolific callus growth at wound sites, whereas no growth was seen at all on MSO. Leaf explants inoculated with A4 developed roots directly from wound sites on MSO and produced roots via a callus phase on MSD4x2. As expected, none of the transformed tissue resulting from inoculations with A4 proved to be kanamycin resistant.

All of the roots induced by 9402 (pDLT) on leaf explants were able to continue growth on MSD4x2 medium when excised from the original explant (Table 5.6). Culture of similar roots on MSO was less successful with only 45-66% of the roots able to survive. Hairy roots derived from flax tissues grew as organised root cultures on MSO and as a friable callus on MSD4x2. If kanamycin selection is applied after root formation, the co-transfer of Ri and binary vector DNA appears to be around 90%. However, even if kanamycin selection is

TABLE 5.6

MONITORING *MU1* ACTIVITY BY INDUCTION OF KANAMYCIN RESISTANT ROOTS/CALLUS ON FLAX LEAF SECTIONS

9402:pDLT inoculations [constitutively expressed nos/npt-II gene]

Medium	Root/callus induction (% of explants with roots/callus)	Growth of subcultured roots (% of root tissues continuing growth)	
		+Km	-Km
MSO	67.0% (roots)	65.0%	45.0%
MSO + Km	40.0% (roots)	56.0%	66.7%
MSD4x2	63.0% (rooty callus)	89.9%+	100.0%+
MSD4x2 + Km	12.0% (rooty callus)	100.0%+	100.0%+

TABLE 5.6 Contd.

A4 (pMuNK14) inoculations [nos/npt-II with Mu1 inserted in leader sequence]

Medium	Root/callus induction (% of explants with roots /callus)	Growth of subcultured roots/callus (% of tissues continuing growth)	
		+Km	-Km
MSO	65.0% (roots)	0.0%	91.0%
MSO + Km	0.0%	0.0%	0.0%
MSD4x2	95.0% (rooty callus)	MSO 0.0% MSD4x2 24.0%*	MSO 48.0% MSD4x2 100.0%+
MSD4x2 + Km	2.5% (callus only)	100.0% (callus)	none

+Roots callus on MSD4x2 and continue growth mainly as undifferentiated cells

*Km^r callus growing out of hairy roots

imposed immediately after inoculation then the majority of hairy roots continue growth on MSD4x2 in the presence of kanamycin after excision from the original explant. This data indicated that the kanamycin resistant phenotype could be used as a stringent selection marker for clonal root lines expressing a *nos-npt-II* gene.

Transformation of leaf pieces by A4 (pMuNK14) on MSO medium (which does not support the growth of callus in flax explants) gave rise to hairy roots only in the absence of kanamycin (Table 5.6). Roots developing on explants cultured on MSO were unable to grow when subcultured to MSO containing kanamycin. This observation is consistent with results obtained from inoculated hypocotyl sections on MSO medium (Table 5.4).

Leaf pieces inoculated with A4 (pMuNK 14) gave rise to hairy roots via a callus phase on MSD4x2, only if selection was imposed 2-3 weeks after transformation. 24% of these roots produced callus which was resistant to kanamycin (Table 5.6). Thus, the roots developed only from explants which had undergone expansion and callus formation before transfer to medium containing kanamycin.

The kanamycin resistant calli usually developed from swellings deep within the root explants, either in the root tip region, or randomly along the roots cultured on MSD4x2 + Km (Fig. 5.11C). These swellings initially appeared globular, then burst out through the organised root tissues to produce a

yellowish callus. Some regions of the dividing calli were found to be kanamycin sensitive. Occasionally, the calli regenerated a few roots which usually bleached before subculture to fresh selection medium (Fig. 5.11D). The presence of kanamycin sensitive cells affected the growth rate of the more resistant cells and so only the more kanamycin resistant regions of the calli were propagated. Eventually, after several subcultures, larger regions of green, kanamycin resistant callus became more prominent and most of the yellowish tissue disappeared. Kanamycin resistant callus could be induced directly on inoculated leaf explants cultured on MSD4x2 + Km at a frequency of 2.5% of that obtained using a vector with a constitutively expressed *nos-npt-II* gene. The callus developed only at the wounded area of the explant, was often not friable and appeared vividly green.

The fact that kanamycin resistant callus can be recovered from these experiments suggests that *Mu1* sequences have been excised from the leader sequences of the *nos-npt-II* gene in the pMuNK14 T-DNA in transformed tissues. The frequency at which kanamycin resistant tissues are obtained is increased by an order of magnitude if selection is not applied immediately after transformation. This data suggests that the majority of *Mu1* excision events do not occur during the transformation process. There is a strong correlation between callus formation on the explant and the frequency of recovery of kanamycin resistant events. The data suggest that *Mu1*

excision (possibly by a precise transposition) may occur at a reasonably high frequency only in cells that have dedifferentiated. This could be consistent with the fact that these cells are rapidly synthesising DNA which is unlikely to be methylated; it has been shown that methylation is primarily responsible for the inactivation of transposable elements (including *Mu1*) in maize (Chandler and Walbot, 1986; Schwartz and Dennis, 1986; Chomet et al., 1987).

5.2.2.4 Analysis of NPT-II activity in kanamycin resistant callus derived from inoculation of flax explants with A4 (pMuNK14)

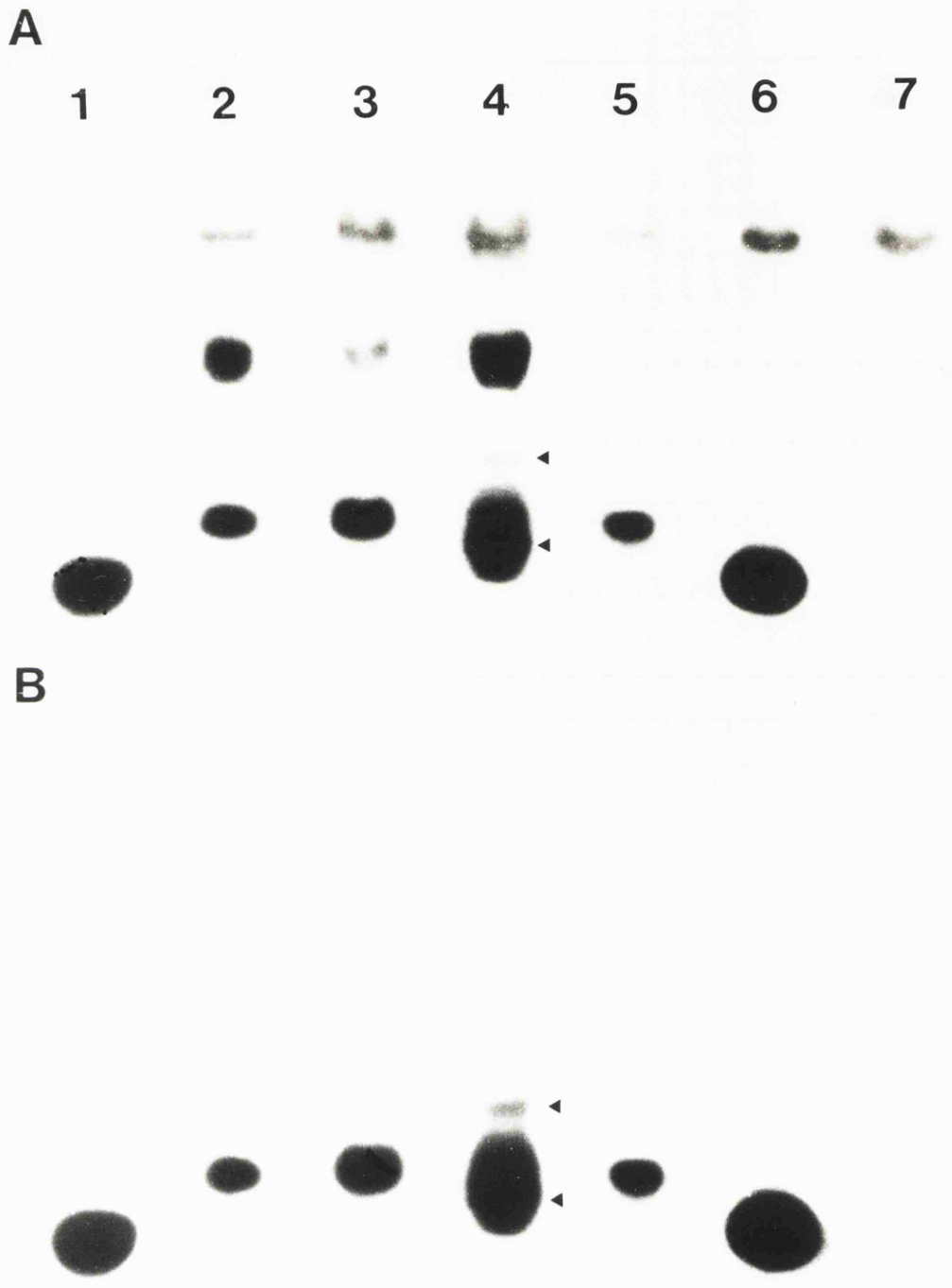
The NPT-II activity in a selection of kanamycin resistant lines was analysed using a non-denaturing polyacrylamide gel *in situ* activity assay. Although Km^r tissues have never been found to arise spontaneously from cultured flax explants it was important to be certain that the expression of the Km^r phenotype was due to NPT-II activity. The results were very consistent in that all of the Km^r calli resulting from inoculation of explants with A4 (pMuNK14) contained reasonable levels of NPT-II activity (Fig. 5.12). The amount of activity, although not quantified accurately, was noticably less (only 15-20%) than that found in Km^r tissues generated by transformation of flax explants with a constitutively expressed *nos-npt-II* gene (section 4.2.4).

In all NPT-II assays the enzyme consistently migrated more slowly than bacterial NPT-II (Fig. 5.12, lane 1), or NPT-II extracted from flax tissues transformed with a constitutive *nos-npt-II* construct (Fig. 5.12, lane 6). Each A4 (pMuNK14) transformant contained an NPT-II band of a similar molecular weight. One line contained, in addition, two other bands of greater size than native NPT-II (Fig. 5.12, lane 4; arrowed). This data suggests that alternative AUG's are being utilised, upstream of the normal translation initiation codon, thus giving rise to a larger protein. The fact that an enzyme of a

Fig. 5.12 NPT-II expression in Km^r resistant colonies derived from transformation of flax hypocotyls with *A. rhizogenes* strain A4 (pMuNK14)

A) P81 paper not treated with protease. B) P81 paper treated with trypsin prior to washing.

Lane 1, bacterial NPT-II extract (1/10 dilution of normal control sample). Lanes 2-5, pMuNK14 transformants; note the two extra bands of NPT-II activity of abberant mobility (arrowed) in lane 4. Lane 6, NPT-II activity in a callus tissue transformed by vector C58 (pGV3850::1103) which has a constitutively-expressed *nos-npt-II* gene (note the relative increase in NPT-II activity). Lane 7, protein extract from non-transformed flax callus.


A
1
2
3
4
5
6
7
B

consistent molecular weight is found in all transformants may indicate that the same AUG is being utilised in each case and that the recombination events reconstituting the *nos-npt-II* gene are also very similar.

It is interesting to note that several ATG codons are present at the extremities of the 200 bp inverted repeat sequences at the ends of *Mu1* (Fig. 5.1), at least one of which is 'in-frame' with the npt-II coding sequence. If an aberrant *Mu1* excision event was to leave behind the extreme ends of the terminal repeat sequence, this latter ATG codon could be used for translation initiation and would result in an NPT-II protein with an extra 14 amino acid residues at the N-terminal; an increase in size possibly consistent with the aberrant mobility of NPT-II in these tissues.

5.2.2.5 Rationale for the characterisation of T-DNA in tissues transformed with A4 (pMuNK14)

Southern analysis was performed on DNA extracted from kanamycin resistant calli derived from inoculated hypocotyl sections ('hypocotyl-derived callus'), root cultures derived from 'hypocotyl-derived callus' and kanamycin resistant callus derived from kanamycin sensitive roots ('root-derived callus'). All tissues were continuously subcultured in the presence of 100 µg/ml of kanamycin. DNA extracted from from these tissues was analysed by Southern blotting in order to answer the following questions:

(A) Is the Km^r phenotype the result of *Mu1* excision from

the untranslated leader sequence of the *nos-npt-II* gene?

(B) What is the origin of the NPT-II with a higher molecular weight in Km^r tissues?

(C) If *Mu1* elements have excised, have they inserted elsewhere in the transformed flax cell genome?

Fig. 5.3 illustrates the probes used in the Southern blot study and Fig. 5.4 outlines the bands expected to hybridise with each probe if the T-DNA is unaltered, if *Mu1* excises and if *Mu1* inserts at a new site in transformed cells.

Four probes were used in this study. The majority of *Mu1* was isolated as a *Hinf* I fragment from plasmid pMJ9 (Bennetzen *et al.*, 1984). Internal *Mu1* sequences were isolated on a 650 bp *Ava* I-*Bst* NI fragment of *Mu1* that was subsequently blunt ended and cloned into pUC8 as an *Sma* I fragment to give pA/B5 (Chandler *et al.*, 1986). The internal *Mu1* probe was released from this plasmid as a 650 bp fragment by digestion with *Eco* RI and *Bam* HI. The *Mu1* terminal repeat probe consisted of a 202 bp *Hind* III/*Mlu* I fragment subcloned into pUC8 to give pDTE1 (Chandler *et al.*, 1986). This probe contained most of one terminal repeat and 15 bp of *Adh1* sequence and can be released from the vector by digestion with *Hind* III and *Eco* RI. The *npt-II* probe was as described in Chapter 4.

As the structure of pMuNK14 and *Mu1* are known in detail, then specific restriction fragments can be predicted if *Mu1* excises precisely, and if it inserts elsewhere in the flax

genome (Fig. 5.4). A *Bam* HI digest of pMuNK14 releases a 3.8 kb restriction fragment which will hybridise with an equal intensity to all four probes. A 2.5 kb *Bam* HI fragment will be generated in the case of a precise excision of *Mu1* from the pMuNK14 T-DNA in transformed tissues. This band contains the reconstituted *nos-npt-II* gene and should hybridise only to the *npt-II* probe. Any *Bam* HI restriction fragments greater than 1.3 kb that only hybridise to *Mu1*-specific probes could result from new insertions of *Mu1* in the flax genome, as the *Mu1* element is around 1.3 kb and does not contain a *Bam* HI site. That transposition has occurred may be confirmed by analysing *Taq* I restriction fragments in the transformant DNA. *Taq* I digestion of pMuNK14 produces a 1.06 kb band that hybridises only to the internal *Mu1* probe, and fragments of 360, 320, 220 bp hybridising only to the terminal *Mu1* probe. If *Mu1* moves to a new position in the flax genome, the 1.06 kb and 220 bp internal fragments should still be present and will light up with the internal and terminal *Mu1* probes respectively. However, the 360 and 320 bp bands will be lost and replaced with bands of unpredictable size that hybridise only to the terminal *Mu1* probe.

5.2.2.6 Presence of unaltered T-DNA internal fragments in kanamycin resistant transformed callus

The results of hybridisation with all four probes described in 5.2.2.5 showed, somewhat unexpectedly, that around

60% of the Km^r cell lines contained internal fragments typical of an unaltered pMuNK14 T-DNA. For example, callus lines 1 and 146 (Fig. 5.13, lanes 2 and 7), root cultures derived from these calli (1/D, 1/G and 146/A; Fig. 5.14, lanes 3, 4 and 6 respectively) and almost all lines of the root-derived Km^r calli (Fig. 5.15 A-C, lanes 1-4 and 6; D, lanes 3-6 and 8) contained a *Bam* HI fragment of 3.8 kb that co-migrates exactly with the pMuNK14 reconstruction band and hybridises, as expected, with all four probes.

There appears to be a correlation between the presence of these unaltered T-DNA fragments and the nature of the kanamycin resistant calli from which the DNA was extracted. These lines are all in the second category of Km^r calli described in 5.2.2.1, which consisted initially of mainly yellowish, kanamycin resistant callus that often regenerated roots. Some of these lines, especially all of the root-derived callus, need regular subculture for continued growth in the presence of kanamycin. This is probably due to the fact that a large proportion of the callus still appears to be sensitive to kanamycin, and its presence affects the growth rate of the resistant cells on selection plates.

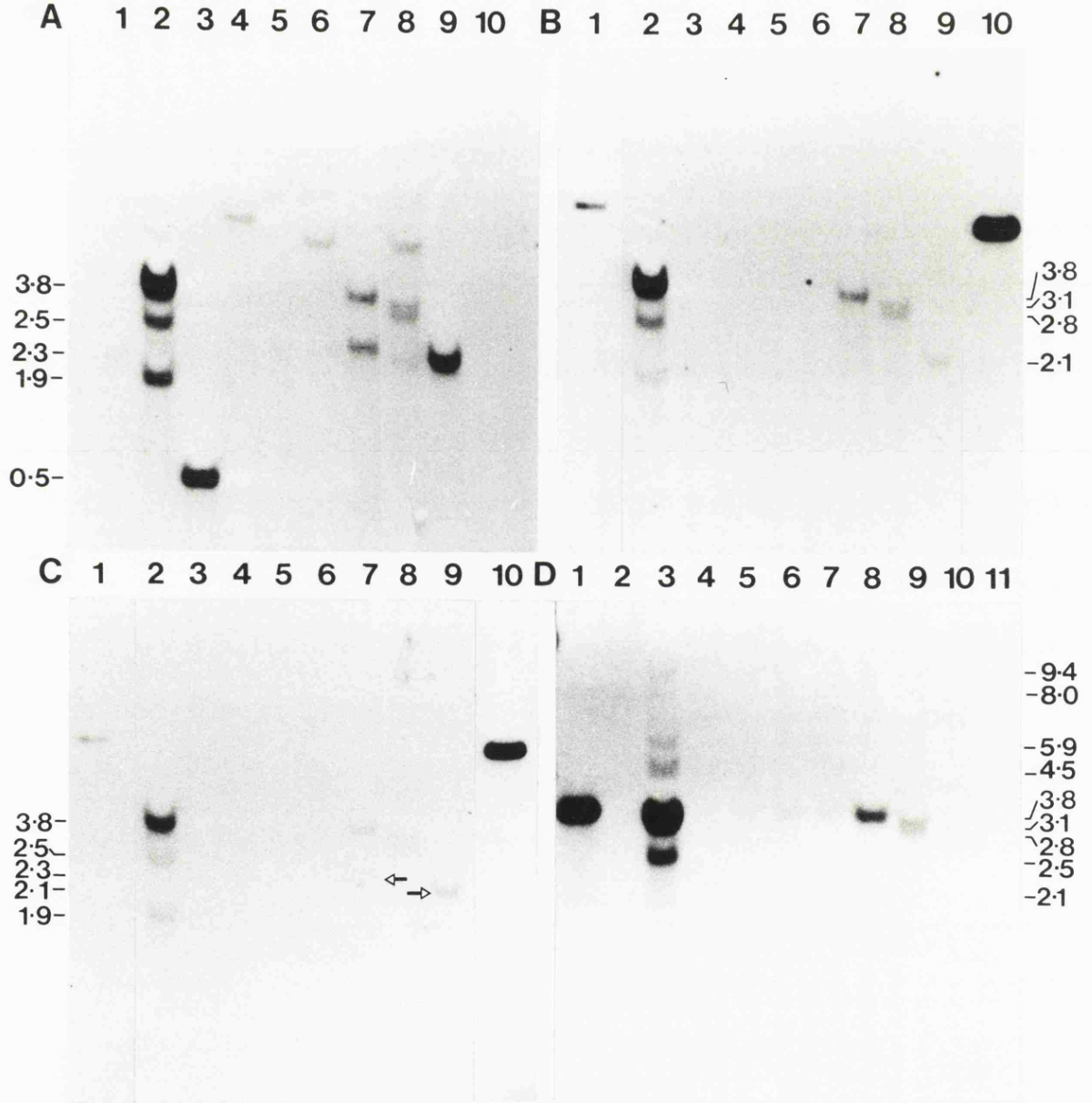
These observations might suggest that the callus lines are not clonal and that the unaltered T-DNA in these tissues results from the presence of sub-population of transformed, but kanamycin sensitive cells. If this theory was correct it would be expected that roots clonally-derived from

Fig. 5.13 T-DNA structure in hypocotyl-derived callus derived from transformation by A4 (pMuNK14)

DNA in all lanes, unless otherwise stated was digested with *Bam* HI. Blots probed with (A), *npt-II*; (B), whole *Mu1*; (C), terminal *Mu1* and (D), internal *Mu1* sequences respectively.

A)-(C) are re-probes of the same Southern. Lanes 1 and 10 are pMJ9 cut with *Hind* III, x1 and x10 copy number reconstructions of *Mu1* sequences respectively. Lanes 2-9 contain DNA from independent hypocotyl-derived callus transformed by pMuNK14, lines 1, 2, 6, 8, 143, 146, 148 and 149 respectively.

D) Lane 1, *Bam* HI digestion of pMuNK14, x10 copy number reconstruction. Lane 2, nontransformed flax callus; Lanes 3-10 contain DNA from independent hypocotyl-derived callus transformed by pMuNK14, lines 1, 2, 6, 8, 143, 146, 148 and 149 respectively.


A
1 2 3 4 5 6 7 8 9 10
3·8
2·5
2·3
1·9
0·5
B
1 2 3 4 5 6 7 8 9 10
3·8
3·1
2·8
2·1
C
1 2 3 4 5 6 7 8 9 10
3·8
2·5
2·3
2·1
1·9
D
1 2 3 4 5 6 7 8 9 10 11
9·4
8·0
5·9
4·5
3·8
3·1
2·8
2·5
2·1

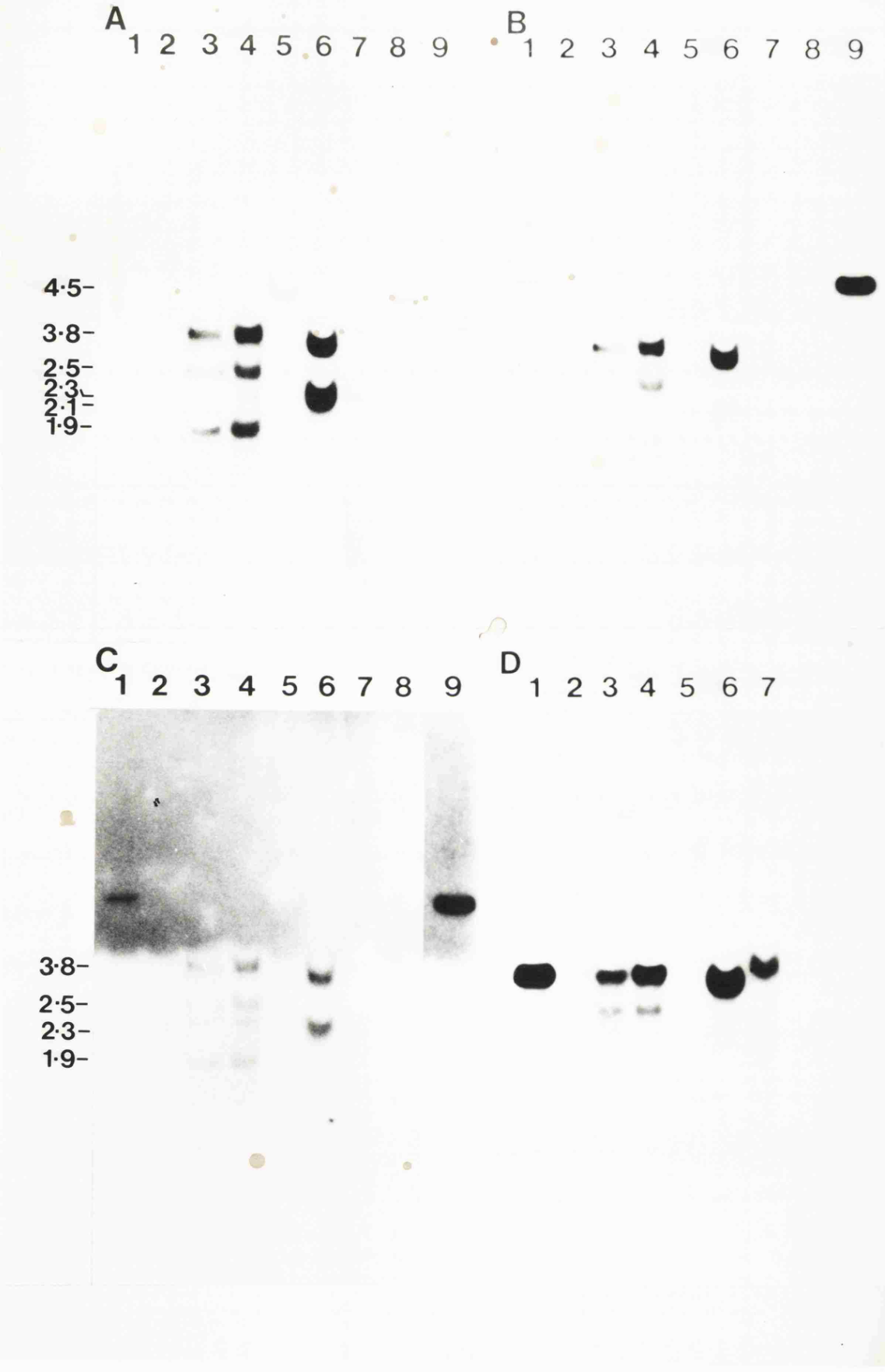
such tissues should contain sub-populations of the total T-DNA restriction pattern. In fact, this may not be the case, as in each root clone examined a qualitatively similar hybridisation pattern to the parent callus lines was observed (e.g lines 1/D, 1/G and 146/A; Fig. 5.14, lanes 3, 4, and 6 respectively). However, there are differences in the copy number of individual bands in the clonal root tissues when compared to each other and to the Km^r calli from which they were derived. For instance, the intensity of the 3.8 kb *Bam* HI T-DNA internal fragment in the clonal root line 146/A (Fig. 5.14; lane 6) is approximately four times greater than the signal detected in the original callus (5.13A-C;lane 7), or in a second root clone 146/B (Fig. 5.14, lane 7) derived from the same callus. The copy number difference could have resulted from an amplification event, or could be due to the presence of more than one population of cells in the Km^r callus. Whichever of these theories proves to be correct, the presence of the intact 3.8 kb *Bam* HI internal fragment, together with the other bands hybridising to the *npt-II* probe suggests that *Mu1* has excised from only one or two sites in the multiple T-DNA copies found in these tissues, or that other recombination events have occurred that reconstituted the *nos-npt-II* gene and the Km^r phenotype in these cells. Thus, the kanamycin resistance phenotype is restored as long as *Mu1* sequences are removed from the untranslated leader sequence of the *nos-npt-II* gene in at least one of the T-DNA copies.

Fig. 5.14 T-DNA structure in root cultures from hypocotyl-derived callus

DNA in all lanes was digested with *Bam* HI unless stated otherwise. Blots A-D were hybridised with *npt-II* (A), whole *Mul* (B), terminal *Mul* (C) and internal *Mul* probes respectively.

A)-(C) are re-probes of the same Southern. Lanes 1 and 10 are pMJ9 cut with *Hind* II, x1 and x10 copy number reconstructions of *Mul* sequences respectively. Lane 2, empty. Lanes 3-8 contain DNA of independent root lines from hypocotyl-derived callus transformed by pMuNK14, lines 1/10, 1/G, 143/B, 146/A, 146/B, 148/B respectively. Lane 9, non-transformed root tissue.

D) Lane 1, *Bam* HI digestion of pMuNK14, x10 copy number reconstruction of T-DNA fragments. Lane 2, non-transformed flax callus; Lanes 3-7 contain DNA of independent root lines from hypocotyl-derived callus transformed by pMuNK14, lines 1/10, 1/G, 143/B, 146/A, 146/B, 148/B respectively.


A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
4·5–
3·8–
2·5–
2·3–
2·1–
1·9–
C
1 2 3 4 5 6 7 8 9
D
1 2 3 4 5 6 7
3·8–
2·5–
2·3–
1·9–

Fragments representative of unaltered T-DNA were absent in approximately 40% of the Km^r tissues examined. These tissues were generally green, reasonably friable calli and much more healthy-looking in the presence of kanamycin. These lines may represent clonally-derived tissues in which *Mu1* has excised from the T-DNA, or the T-DNA may have been recombined before, during, or soon after transfer. This latter explanation may account for several restriction fragments which hybridised to one, or most, of the probes, but do not have the characteristics of fragments expected if *Mu1* excises precisely.

5.2.2.7 *Mu1* excision and the reconstitution of the *nos-npt-II* gene

If *Mu1* excises from the pMuNK14 T-DNA a characteristic *Bam* HI fragment of 2.5 kb is expected which will hybridise only with the *npt-II* probe. A hybridising band with these characteristics has not been found in any of the tissues so far examined. However, in all Km^r tissues there were *Bam* HI fragments which had homology to the *npt-II* gene but none to *Mu1* sequences, thus indicating the separation of these sequences in the kanamycin resistant lines. For example, *Bam* HI digestion of DNA extracted from hypocotyl-derived kanamycin resistant callus transformed with A4 (pMuNK14), yielded fragments of 0.5 and 2.3 kb (Fig. 5.13A; lines 1, 2, 6, 8, 143 and 148) which had homology only to the *npt-II* probe. This conforms to an expected situation if *Mu1* excised in an aberrant fashion from the T-DNA. Other larger fragments with homology to *npt-II* are

seen in lines 6, 8, 143, 148 (Fig. 5.13A-C; lanes 4, 5, 6 and 8). Four hypocotyl-derived callus lines (1, 148, 146, and 149) contain restriction fragments which have homology to both *npt-II* and *Mu1* probes. In line 1, two fragments had this characteristic, but only one co-migrated with the internal T-DNA fragment of 3.8 kb in the pMuNK14 reconstruction lane. The other fragment is smaller in size, only about 2.5 kb. Similar fragments were found in lines 146 and 148 with a size of 3.8, 3.1 and 2.8 kb respectively (Fig. 5.13B; lane 7 and 8). In line 149, a band of around 2.1 kb has homology to *npt-II* and only the terminal repeat sequences of *Mu1* (Fig. 5.13A and C; lane 9).

A *Bam* HI digestion of DNA extracted from Km^r root cultures regenerated from Km^r hypocotyl-derived callus yielded broadly similar restriction fragment patterns to those found in the callus of origin (Fig. 5.14). However, as outlined above (5.2.2.6), in some root clones there were distinct differences in restriction pattern and T-DNA copy number, suggesting that the Km^r callus was not of clonal origin. It can also be seen that root clone 148/B (Fig. 5.14; lane 7) did not have the 3.1 kb and 2.8 kb fragments which were present in the original hypocotyl callus line 148 (Fig. 5.13A and B; lane 8). This suggests again that there are at least two types of transformed cells in this callus line which have different restriction fragments with homology to *npt-II*. The 2.3 kb fragment in lines 1/D and 1/G, as well as the 2.1 kb fragment in lines

143/B and 148/B, hybridised only to the *npt-II* probe, producing a low intensity signal. The 3.8 kb *Bam* HI band in lines 1/D, 1/G, and 146/A hybridised to all probes at an approximately equal intensity (Fig. 5.14A, B and D; lanes 3, 4 and 6), suggesting that in these lines some *Mu1* sequences were still associated with *npt-II* sequences exactly as in the pMuNK14 T-DNA.

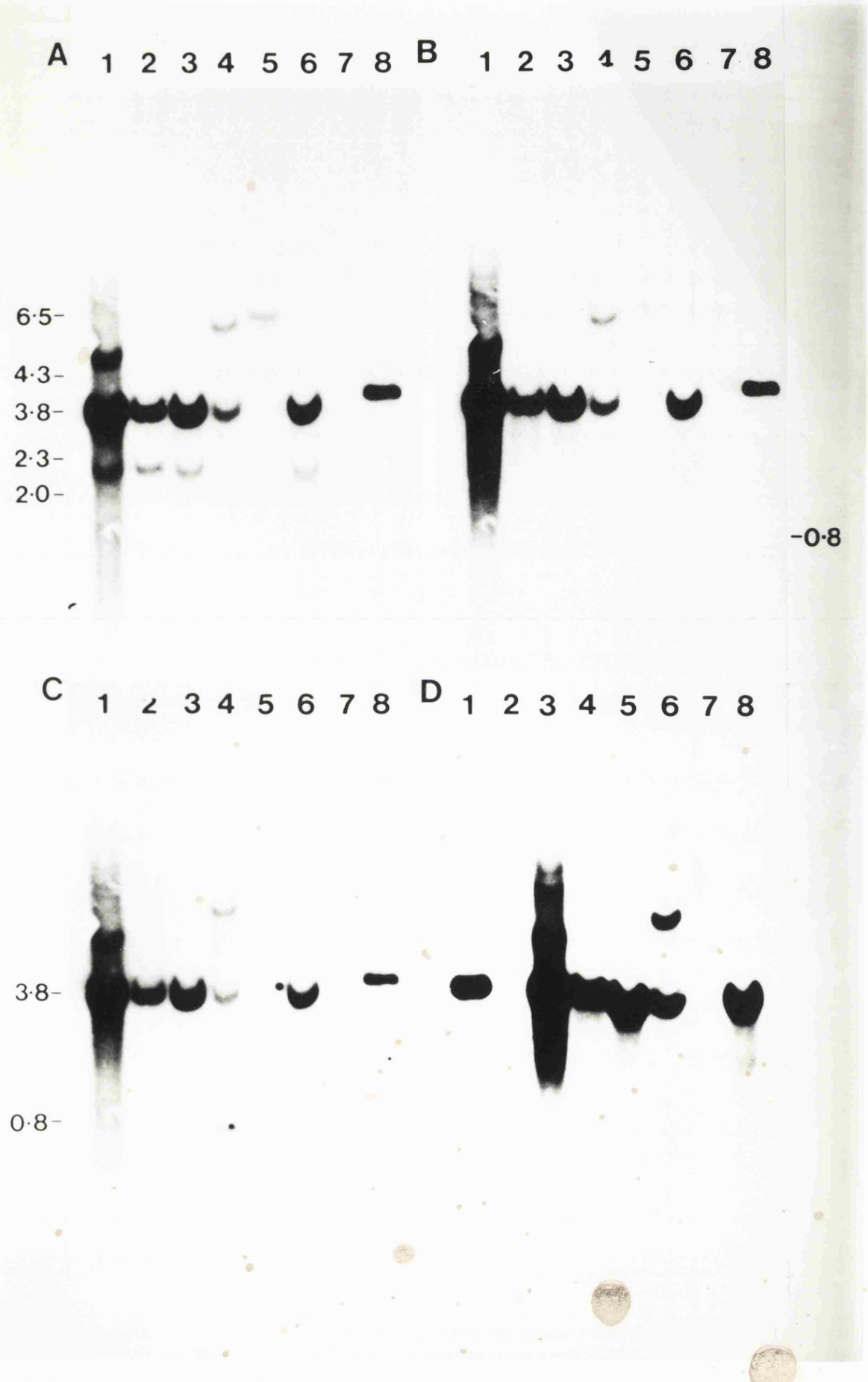
The same analysis was performed using DNA isolated from Km^r root-derived callus. The results were similar to those described above; hybridisation with the *npt-II* probe revealing the expected 3.8 kb T-DNA internal fragment, together with a second, lower intensity fragment, which in each line was of an approximately equal size (2.1-2.2 kb; Fig. 5.15A). With the exception of line 1/30 (Fig. 5.15; lane 6) these fragments only showed homology to the *npt-II* probe. In Km^r lines derived from hypocotyl callus which lacked the 3.8 kb *Bam* HI fragment, it was generally observed that hybridising bands, with homology to both *npt-II* and the three *Mu1* probes, had much more homology to *npt-II* sequences. For example, a fragment of 1.9 kb in line 1 (Fig. 5.13 A-C; lane 2) and its corresponding regenerated root lines 1/D and 1/G (Fig. 5.14 A-C; lanes 3 and 4), a fragment of 2.1 kb in line 149 (Fig. 5.13A-C; lane 9) and a fragment of 2.3 kb in line 146 (Fig. 5.13A; lane 7) and a corresponding regenerated root line 146/A (Fig. 5.14A-C, lane 6), all hybridised more strongly to *npt-II* than the two *Mu1* probes. It was interesting to note that the 2.3 kb band in 146

Fig. 5.15 T-DNA structure in root-derived callus from A4 (pMuNK14)-transformed tissues

DNA in all lanes was digested with *Bam* HI unless stated otherwise. Blots A-D were hybridised with *npt-II* (A), whole *Mul* (B), terminal *Mul* (C) and internal *Mul* probes respectively.

A)-(C) are re-probes of the same Southern. Lanes 1-6 contain DNA from root-derived callus, lines 1/1, 1/13, 1/14, 1/20, 1/24, and 1/30 respectively. Lane 7, control non-transformed flax callus. Lane 8, *Bam* HI digestion of pMuNK14 (x10 copy number reconstruction of T-DNA fragments).

D) Lane 1, *Bam* HI digestion of pMuNK14 (x10 copy number reconstruction of T-DNA fragments); lane 2, control, non-transformed flax callus; lanes 3-8, lines 1/1, 1/13, 1/14, 1/20, 1/24, and 1/30 respectively.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
6·5
4·3
3·8
2·3
2·0
0·8
C
1 2 3 4 5 6 7 8
D
1 2 3 4 5 6 7 8
3·8
0·8

and 1.9 kb band in line 1 had no homology to the internal sequence of *Mu1* (Fig. 5.13D; lane 8 and 3) but hybridised to a reasonable extent with terminal *Mu1* sequences (Fig. 5.13C; lanes 7 and 2 [arrowed]). These results may indicate that an internal portion of *Mu1* has been excised, leaving behind a small part of the terminal repeat sequences. These type of bands are mostly detected in the hypocotyl-derived callus lines and in corresponding regenerated root clones. The majority of bands had very little homology to internal *Mu1* sequences in all of the lines which lacked the internal 3.8 kb *Bam* HI T-DNA fragment.

These results suggest that in most of the kanamycin resistant lines analysed, the majority of the *Mu1* sequence had been excised from the untranslated leader region of the *nos-npt-II* gene in the T-DNA. At least two types of events were observed to be occurring; loss of the whole *Mu1* element (ie lack of any *Mu1* homology in the DNA) or loss of the internal portion of *Mu1* element. Most of the lines examined contained a *Bam* HI DNA fragment that had homology only to the *npt-II* probe, or to both *npt-II* and terminal *Mu1* sequences; a characteristic expected of a band if most of *Mu1* has excised. These fragments, which range from 2.1 to 2.3 kb, are likely to be the result of an aberrant *Mu1* excision as the expected size of such bands after a precise excision is 2.5 kb. The fact that such bands all fall within this narrow size range in each tissue so far examined, coupled with the consistent size of the higher

molecular weight NPT-II protein in these cells may be consistent with a common mechanism for the loss of *Mu1* sequences.

5.2.2.8 T-DNA rearrangements in kanamycin resistant callus lines

Some of the Km^r lines induced by the transformation of flax explants by A4 (pMuNK14) contain *Bam* HI fragments which have homology with *npt-II*, but are either very much smaller, or larger, than the expected 2.5 kb fragment containing the reconstituted *nos-npt-II* gene after *Mu1* excision, or the unaltered 3.8 kb internal T-DNA fragment. A 0.5 kb fragment in line 2 and several other fragments larger than 3.8 kb with homology to *npt-II* (Fig. 5.13A) could be the result of recombination events that are possibly not linked to *Mu1* excision. These fragments are also present in the corresponding regenerated root tissue from each callus line (e.g. a fragment of 4.5 kb in lines 143/B, 148/B; Fig 5.14; lanes 5 and 8). It is not possible to determine, from these results, if recombination occurred during an aberrant excision of *Mu1*, or during transformation. Certain fragments have homology only to *npt-II*, which suggests that recombination accompanied an aberrant excision event. However, there are other fragments which have homology to all probes, which could indicate a different mechanism of recombination resulting in the scrambling of T-DNA (e.g. fragments of 3.1 and 2.8 kb in

line 148; Fig 5.13A; lane 8).The majority of lines having such fragments also have a *Bam* HI fragment of 2.1 - 2.3 kb that contains only *npt-II* sequences and DNA homologous to *Mu1* terminal repeat regions. It is therefore likely that the former, heterogeneous group of bands contain a functional *nos-npt-II* gene, only if the latter type of band is absent.

5.2.2.9 Is *Mu1* excision accompanied by integration of the transposable element elsewhere in the flax genome?

In the majority of lines, the Southern blot analysis showed the presence of *Mu1* homologous sequences outside of its original position in the *Bam* HI 3.8 kb internal T-DNA fragment. Some of these hybridising bands lack any homology to *npt-II* and any such restriction fragment greater than approximately 1.5 kb could result from *Mu1* excision and reintegration. To examine in more detail, the organisation of *Mu1* homologous sequences in these transformed cell lines, the DNA was digested with *Taq* I and probed with both internal and terminal *Mu1* probes in order to locate border fragments generated when *Mu1* inserts at a new site in genomic DNA (see Fig. 5.3 for *Taq* I restriction map). A *Taq* I digestion performed on DNA extracted from Km^r lines yielded numerous restriction fragments with homology to *Mu1* sequences (Fig. 5.16 and 5.17). Line 146/A (Fig. 5.16A, lane 1) had 5 additional bands with homology to internal *Mu1* sequences that have a higher molecular weight than the *Taq* I restriction fragments found in the pMuNK14 T-DNA reconstruction lane (Fig. 5.16A; lane 4). The same bands also light up with

Fig. 5.16 Analysis of *Mu1* terminal sequences in root cultures from hypocotyl-derived callus transformed by A4 (pMuNK14)

DNA in all lanes was digested with *Taq* I unless otherwise indicated. Blots (A) and (B) were probed with the internal and terminal *Mu1* probes respectively.

A) Lanes 1-3, DNA from callus-derived Km^r root cultures; lines 146/A, 146/B and 148/B respectively. Lane 4, pMuNK14 T-DNA reconstruction. Lane 5, *Hind* III digest of pMJ9 (*Mu1* control DNA).

B) Lanes 1-3, DNA from callus-derived Km^r root cultures, lines 146/A, 146/B and 148/B respectively. Lane 4, non-transformed flax roots. Lane 5, pMuNK14 T-DNA reconstruction.

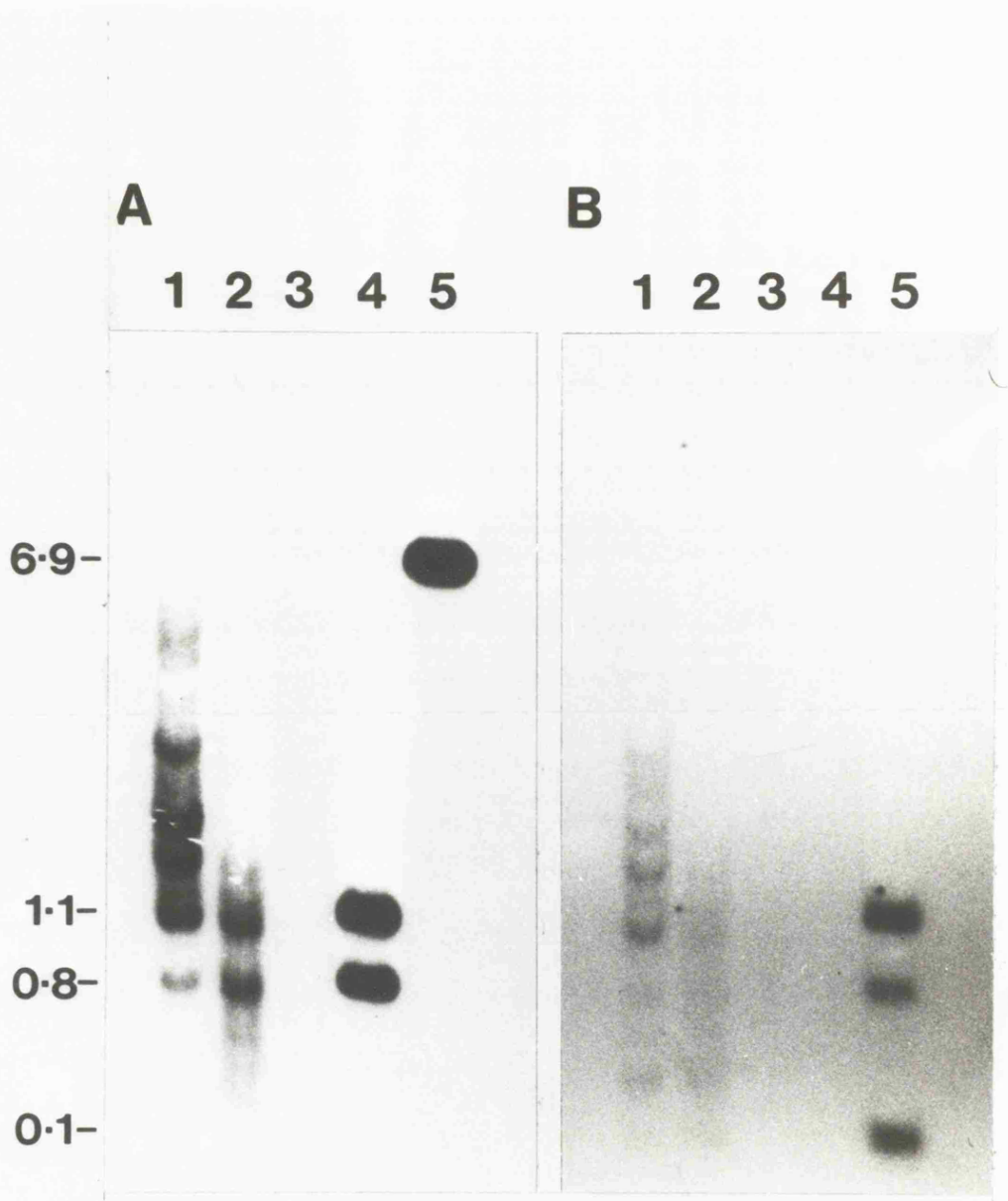
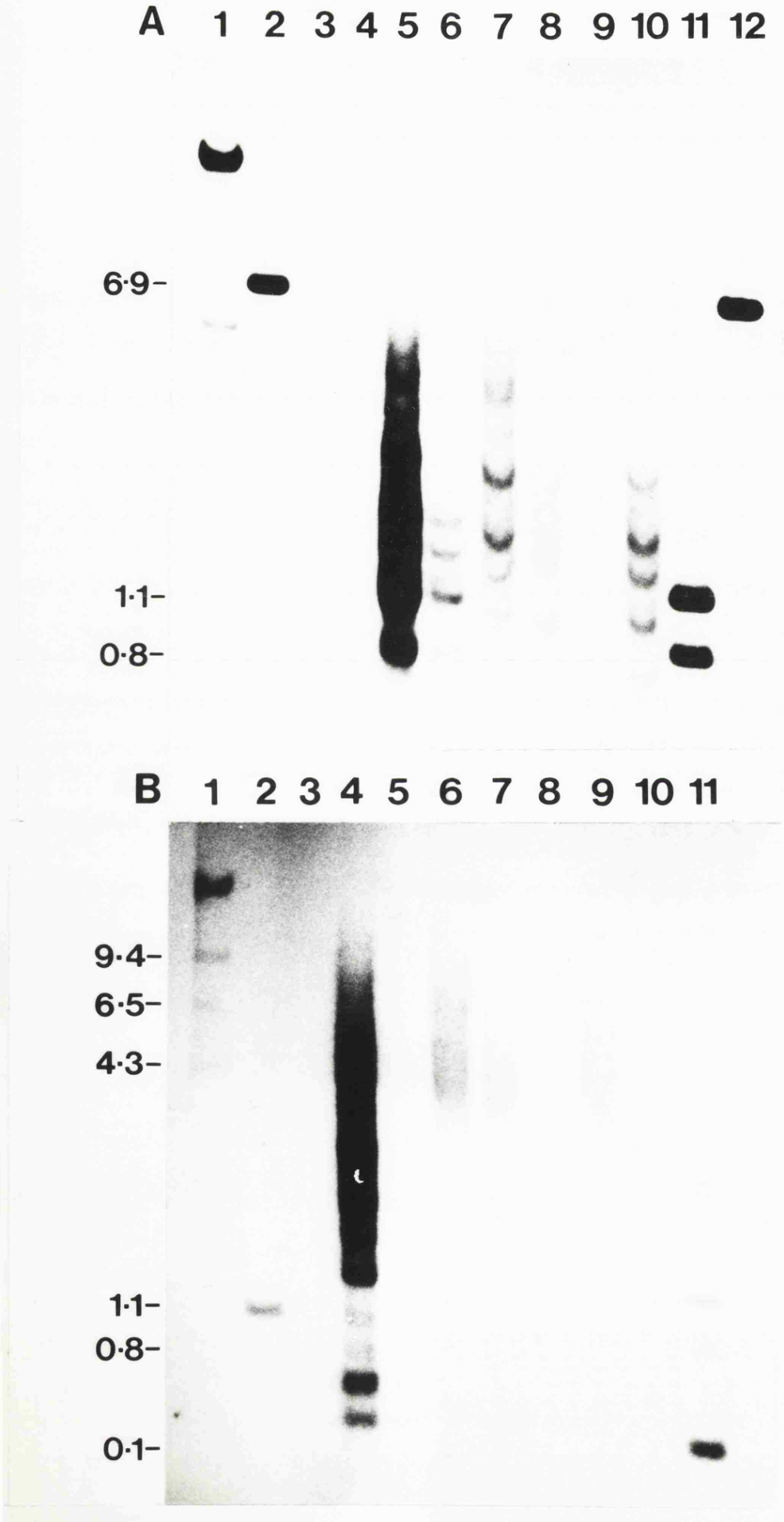

A
B
1 2 3 4 5
1 2 3 4 5
6·9–
1·1–
0·8–
0·1–

Fig. 5.17 Analysis of *Mul* terminal sequences in root-derived callus cultures transformed by A4 (pMuNK14)

DNA in all lanes was digested with *Taq* I unless otherwise indicated. Blots (A) and (B) were probed with the internal and terminal *Mul* probes respectively.

A) Lane 1, radioactive λ-marker DNA cut with *Hind* III and *Eco* RI. Lanes 2 and 12, *Hind* III digest of pMJ9 (x5 and x10 *Mul* copy number reconstructions respectively). Lanes 3 and 4 are control, non-transformed flax root and callus DNA respectively. Lanes 5-10 contain DNA from root-derived callus, lines 1/1, 1/13, 1/14, 1/20, 1/24, and 1/30 respectively. Lane 8, *Bam* HI digestion of pMuNK14, x10 copy number reconstruction of T-DNA fragments. Lane 11, pMuNK14 T-DNA reconstruction.

B) Lane 1, radioactive λ-marker DNA cut with *Hind* III and *Eco* RI. Lane 2, *Hind* III digest of pMJ9 (*Mul* x5 copy number reconstruction). Lane 3, control, non-transformed flax callus DNA. Lanes 4-9 contain DNA from root-derived callus, lines 1/1, 1/13, 1/14, 1/20, 1/24, and 1/30 respectively. Lane 10, control, non-transformed flax root tissue. Lane 11, pMuNK14 T-DNA reconstruction.


A
1 2 3 4 5 6 7 8 9 10 11 12
6·9–
1·1–
0·8–
B
1 2 3 4 5 6 7 8 9 10 11
9·4–
6·5–
4·3–
1·1–
0·8–
0·1–

the terminal *Mu1* probe. A similar situation is found in Km^r calli derived from roots transformed by A4 (pMuNK14). In the majority of these lines there were 5-7 bands with homology to *Mu1* that did not co-migrate with internal *Taq* I restriction fragments of pMuNK14, but which had homology to both internal and terminal *Mu1* sequences (Fig. 5.17A and B). As *Taq* I is insensitive to DNA methylation, this data suggests that these bands are not the result of *Mu1* insertion at a different site, but are more likely to be due to abberant excision and recombination events. In a few lines, 1/1 and 1/13 (Fig. 5.17B; lanes 4 and 5), 146/A and 146/B (Fig. 5.16B, lanes 1 and 2) there are low molecular weight bands which only hybridise to *Mu1* terminal sequences. However, in the absence of the 2.5 kb *Bam* HI fragment expected after a precise *Mu1* excision, it is very unlikely that these fragments result from insertion of *Mu1* at new sites within the flax genome.

5.2.3 Summary

The Southern blot analyses in transformed Km^r lines derived from A4 (pMuNK14) inoculations were complex and generally showed little evidence that *Mu1* had excised precisely to reconstitute the Km^r phenotype. With the exception of the 3.8 kb *Bam* HI internal T-DNA fragment, the origin of any hybridising bands still remains largely a subject of conjecture. Genomic cloning and sequencing is required before the structure and origin of each unpredicted band can be

evaluated. However, it is reasonable to suggest that, in each of the selected Km^r tissues, there is sufficient evidence to indicate that *Mu1* and *npt-II* sequences have been separated in a manner that reconstitutes the expression of the *nos-npt-II* gene. The fact that similar-sized hybridising bands with strong homology to *npt-II* and *Mu1* terminal repeat sequences are found in all lines makes it very likely that the experimental conditions have selected Km^r lines generated by a common recombination event, possibly involving an aberrant transposition event. It is also feasible that many other types of recombination events also occur, but obviously the experimental system used will only select those events which reconstitute the *nos-npt-II* gene and allow expression of Km^r phenotype.

Possible recombination and modification associated with *Mu1* has also been reported in *Agrobacterium tumefaciens* transformed tobacco tumours (Lillis *et al.*, 1985). In other transgenic plants, such as *Arabidopsis thaliana* and tomato however, *Mu1* integration was observed to be stable (Zhang and Somerville, 1987; Lillis *et al.*, 1985), but in these studies only organised tissues were examined. Imprecise excision is also a common phenomena in other plant transposable elements, especially the *Tam* systems of *Antirrhinum majus*, since the target duplication is normally retained in a modified form (Bonas *et al.*, 1984; Sommer *et al.*, 1985; Coen *et al.*, 1986; Carpenter *et al.*, 1987). Modifications often include deletions

and/or inverted duplication, possibly due to action of exonucleases and repair enzymes (Saedler and Nevers, 1985).

In one case, deletion occurred in the adjacent sequences at the site of integration of *Mu1*, but the element itself was retained (Taylor and Walbot, 1985). Deletion of the coding sequence plus the intervening sequence and the target duplication caused by insertion of *Mu1* resulted in consequent loss of alcohol dehydrogenase activity in maize (Taylor and Walbot, 1985).

In *Tam* systems, particularly *Tam3*, often the element was removed together with adjacent sequences (Schwarz-Sommer *et al.*, 1985; Coen *et al.*, 1986; Carpenter *et al.*, 1987). One bi-directional deletion has also been described in the *Tam2* system in *Antirrhinum majus* (Krebbers *et al.*, 1987). Depending on the extent of the deletions and rearrangements associated with the excision events, both quantitative and spatial variation in gene expression can be generated. In this experiment however, only events that lead to kanamycin resistance at the level of selection can be recovered.

Activation of other transposable elements has also been associated with dedifferentiated cultured cells. For example, a higher degree of reversion of mutable allele of alfalfa was observed in tissue culture than *in planta* (Bingham *et al.*, 1987). Movement of the *Ac* element in transgenic tobacco was detected also in dedifferentiated protoplast-derived callus (Baker *et al.*, 1986, 1987). The *Ac* element, unlike *Mu1* in

these experiments however, excised in a similar manner to maize and was shown to re-insert into a new chromosomal location (Baker *et al.*, 1986). Activation of silent elements in tissue culture has also been correlated with a high frequency of chromosomal structural alterations seen in regenerated maize plants (Pescke *et al.*, 1986).

The activation of transposable elements during dedifferentiation may be through rapid DNA synthesis, repair mechanisms or chromosomal breakages which cause demethylation. Transposable elements, including *Mu1* and *Ac*, preferentially insert into hypomethylated DNA which also tends to be single copy DNA (Bennetzen *et al.*, 1987b). DNA modification by methylation of the element leads to the loss of transposable element activity (Chandler and Walbot, 1986; Bennetzen, 1987; Chomet *et al.*, 1987). Methylation may also account for the *Mu1* activity observed in flax. As mentioned previously in *Arabidopsis thaliana* and tomato 'hairy roots', the *Mu1* element was stable in differentiated tissue which may have their DNA highly methylated (Zhang and Somerville, 1987; Lillis *et al.*, 1985). The same mechanisms may be responsible for the imprecise excison of *Mu1* in flax as in the *Tam* systems. Further work is needed to verify this possibility and also to check the degree of methylation in the *Mu1* containing kanamycin resistant tissues.

CHAPTER 6

'HAIRY ROOT' INDUCTION AS A TOOL IN INSERTION MUTAGENESIS

6.1 INTRODUCTION

6.1.1 Insertion mutagenesis by T-DNA

The previous chapters have described the requirements and problems involved in insertion mutagenesis and the potential systems developed for such a purpose using flax cells. Briefly summarised, an ideal system needs: 1) target cells - large populations of clonal cells, preferably haploid, which can be easily manipulated in culture; 2) mutagen treatment - an exogenous DNA delivery system which gives high frequency, random and independent single insertions, plus specific and effective screening and selection procedures for transformants; 3) target gene - easily selectable phenotype giving recessive or dominant mutants, preferably at single loci; and finally 4) selection system - a non-leaky, simple, effective and efficient selection system for the desired mutant phenotype.

6.1.2 Induction of clonal transformants

As described in earlier chapters, large numbers of haploid target cells for mass transformation have to be generated through the use of cell suspensions, isolated cells or protoplasts. In flax there is always the difficulty of maintaining the haploid status of suspension cultured cells which may prove to be a problem. An alternative route to this objective, is to use 'hairy roots' induced by *Agrobacterium rhizogenes* on explants taken from haploid flax plants. The

mode of A. *rhizogenes* infection is similar to A. *tumefaciens* (Hoekema *et al.*, 1984; Huffman *et al.*, 1984; Jouanin, 1984). Hairy root induction normally occurs at wound sites. Wounding provides access for the bacterial infection, induces divisions which make plant cells competent for transformation and stimulates the production of wound-associated compounds such as acetosyringone which attract *Agrobacterium* and induce the *vir* genes required for T-DNA transfer (Klee *et al.*, 1983; Lundquist *et al.*, 1984; Stachel *et al.*, 1986; Stachel and Zambryski, 1986). Adventitious 'hairy roots' produced at the site of inoculation contain and express the Ri (root-inducing) T-DNA, and can be grown in axenic culture. The excision of transformed tissue from 'hairy root' tumours is relatively simple, as the neoplastic cells grow out of the explant. Individual roots from hairy root tumours are known to be of clonal origin (David *et al.*, 1984; Petit *et al.*, 1986). The transfer of Ri T-DNA is also known to be random. Often the roots are capable of regenerating into whole plants, which transmit the Ri T-DNA to their progeny in a Mendelian fashion (Jensen *et al.*, 1986; Trulson *et al.*, 1986; Guerche *et al.*, 1987). A suitable *Agrobacterium rhizogenes* strain containing a wildtype Ri plasmid can supply *vir* functions *in trans* to act on non-oncogenic binary vectors carrying the DNA sequence to be delivered into the plant cells (Simpson *et al.*, 1986; Hamill *et al.*, 1987). A number of useful vectors have been designed that contain selectable marker genes for selection of transformants

and can be used to deliver any desired gene into plant cells (Bevan, 1984; An *et al.*, 1985; Van den Elzen *et al.*, 1985; Simpson *et al.*, 1986).

A mutagenesis protocol using large explants of haploid flax tissue can be envisaged by exploiting the following properties of the 'hairy root' transformation system: i) the roots are of clonal origin and so any mutant root will not be chimaeric and will grow as a distinct transformed tissue clone; ii) they are neoplastic and thus media can be used that gives little callus formation, so that the transformed tissues are not masked by undifferentiated cells. This means that specialised media are not required; iii) they are very easy to recognise and grow out of the explant into the medium, thus no selection is required; iv) 'hairy roots' are very sensitive to antibiotics and other selection agents and should provide an ideal type of tissue for mutant selection. Bearing in mind these properties, mutagenesis could be achieved, by either insertion of the T-DNA of the Ri (root-inducing) plasmid, or exogenous DNA sequences in binary vector T-DNAs co-transferred by *Agrobacterium rhizogenes*.

Several factors have to be investigated to evaluate the feasibility of this approach to insertion mutagenesis. However, if successful, the technique can be used with species such as flax, with poorly developed single cell/protoplast systems. It is also important to note that explants from propagated haploid plants will contain mainly haploid cells

unlike dedifferentiated, fast dividing, haploid cell cultures which are the only other suitable alternative. Optimum conditions have to be discovered that allow large numbers of transformed roots to be produced from inoculated explants. This will increase the efficiency of the system in generating insertion mutations. Several parameters may influence the root induction; the competence of target cells, virulence of the *Agrobacterium* strain, presence of phytohormones in the medium, and time of inoculation. Once optimum conditions are established, the most competent type of explants can be inoculated and incubated until roots develop. After inoculation the agrobacteria are killed by using suitable antibiotics.

When a lot of independently transformed roots have developed at the wound site of the explant, they can then be excised and grown in an appropriate medium for selection of any desired mutant phenotype. Alternatively, whole explants may be moved onto selection medium. By using explants taken from haploid plants clonal haploid roots can be induced. This will simplify selection of recessive mutants. In addition, as mentioned earlier, explant tissue culture is relatively simple compared to the more tedious and sometimes difficult protoplast isolation or generation of cell suspension cultures. However, this system could prove very laborious when large numbers of explants are involved.

6.1.3 Target gene

In view of the random nature of transformation events

(Horsch *et al.*, 1985; De Frammond *et al.*, 1986; McCormick *et al.*, 1986) and a potential low frequency of mutation induction, success in the recovery of desirable mutations largely depends on the application of efficient selection techniques to large numbers of independently transformed cells. To avoid screening of a large number of transformed cells, a selectable phenotype is needed. If a recessive mutant is to be detected, it is best if the target gene is present at a single copy per haploid nucleus. Several mutant traits which might be used for selection have been previously well studied. They include nitrate reductase-deficient (NR^-), alcohol dehydrogenase (Adh) and amino-acid analog resistant lines. Of these traits the induction of chlorate resistant NR^- mutants possibly represents the most efficient, non-leaky selection system and is easy to perform.

6.1.4 Nitrate reductase deficient mutants

Besides being the first auxotrophic mutant to make a major contribution to plant somatic cell genetics, nitrate reductase-deficient (NR^-) mutants are also one of the most frequent traits among nutritional mutants in higher plants. The first mutant cell lines totally deficient in NR activity were isolated from amphihaploid tobacco cell cultures (Müller and Grafe, 1978). By now, NR^- mutants have been isolated in cell cultures of several species (review, Maliga, 1984), mostly in *Nicotiana plumbaginifolia* (de Vries *et al.*, 1986; Mendel *et*

al., 1986), and *Nicotiana tabacum* (Mendel *et al.*, 1984). Similar mutants have also been isolated and characterised in *Hyoscyamus muticus* (Strauss *et al.*, 1981; Gebhart *et al.*, 1981, 1983), *Daucus innoxia* (King and Khanna, 1980), *Petunia* (Steffen and Schieder, 1984), and *Rosa damascena* (Murphy and Imbrie, 1981). Using intact plants NR^- mutants have been isolated in seedlings of *Arabidopsis thaliana* (Oostindiër-Braaksma and Feenstra, 1973), barley (Kleinhofs *et al.*, 1980) and pea (Feenstra and Jacobsen, 1980). The variant lines can be selected from untreated cells, although mutagen treatment can increase the variation frequency in haploid material by 25 fold in some cases (Márton *et al.*, 1982). An induced mutation frequency of $\sim 3 \times 10^{-5}$ was achieved by UV or gamma irradiation of haploid *Nicotiana plumbaginifolia* protoplasts (Gabard *et al.*, 1987).

6.1.4.1 Selection of nitrate reductase deficient mutants

The availability of diverse selection systems using chlorate resistance, nitrate non-utilisation and requirement for reduced nitrogen sources, makes NR^- mutants useful subjects for studies, not only of nitrate metabolism but also genetic engineering in plant cells. Nitrate reductase (NR) performs a critical step in the pathways of nitrate assimilation and is highly regulated. Nitrate reductase-deficient (NR^-) cells are unable to utilise nitrate, and growth is dependent on the availability of amino-acids or urea as a nitrogen source. NR^- lines are commonly selected by their resistance to chlorate, an

analog of nitrate (Müller and Grafe, 1978). The absence of NR, by diminishing reduction of chlorate to toxic chlorite, confers chlorate resistance. Chlorate resistance may be due to lower uptake of chlorate or lower reduction rate of chlorate because of low levels of nitrate reductase. NR^- lines have been characterised biochemically and genetically (de Vries *et al.*, 1986; Mendel *et al.*, 1986; Gabard *et al.*, 1987) and have been isolated in several cell culture systems by selection for chlorate resistance (Grafe and Müller, 1983; Steffen and Schieder, 1984; Biasini and Màrton, 1985; Gabard *et al.*, 1987), or by total isolation (Strauss *et al.*, 1981; Frankhauser *et al.*, 1983).

6.1.4.2 Characterisation of nitrate reductase deficient mutants

Mutants leading to the loss of NR activity are genetically and biochemically well characterised in the fungus *Aspergillus nidulans* (Cove, 1979). NR is considered to consist of two identical 4S haemoflavoprotein subunits, exhibiting cytochrome-c-reductase activity, kept together by a Mo-bearing co-factor and so forming the functional 8S holoenzyme. In fungi as well as in plants, the Mo-factor is common to another enzyme, xanthine dehydrogenase (XDH). Figure 6.1 shows the genetic basis of nitrate reductase activity.

NR^- mutants can be classified in two distinct phenotypic categories. One mutant type (*nia*) is defective in

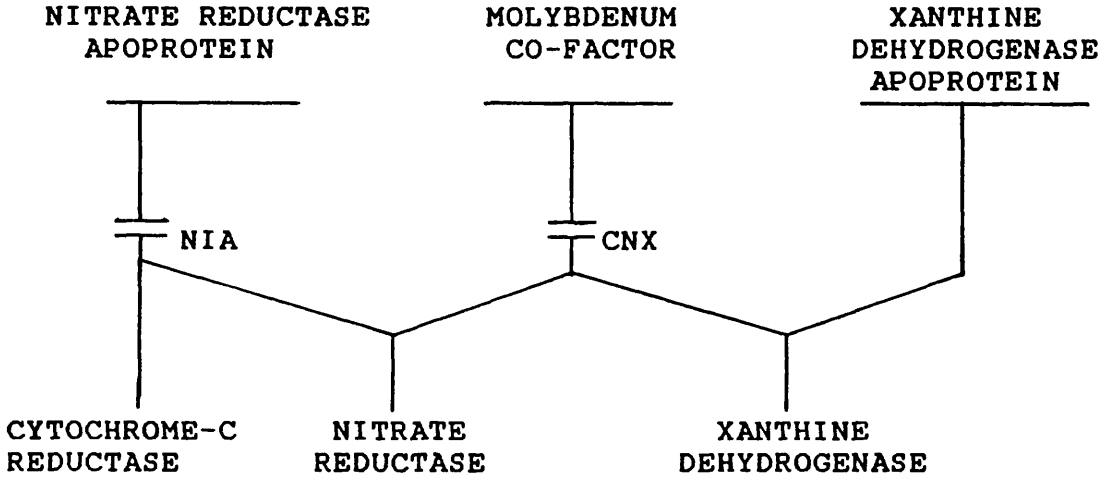


Fig. 6.1 Genetic basis of nitrate reductase activity: (1) mutations in the *nia* allele inactivate the nitrate reductase apoprotein, (2) mutations in the *cnx* allele inactivate the molybdenum cofactor necessary for both nitrate reductase and xanthine dehydrogenase activities.

the NR apoprotein, and the other (*cnx*) is characterised by an impaired molybdenum cofactor (Mo-co) and therefore simultaneously lacking XDH activity. In the *nia* group, it is thought that only one structural gene is involved in the synthesis of the apoenzyme (de Vries *et al.*, 1986). The *nia* mutants are all allelic. The amphihaploid *N. tabacum* carries duplicate loci (*nia1*, *nia2*) coding for the apoprotein of NADH-NR; therefore the appearance of NR^- cells was the result of two independent mutations. Gabard *et al.* (1987) found that *nia* mutants of *Nicotiana plumbaginifolia* were monogenic recessives. It was also shown that the *nia* complementation group corresponds to the structural gene of nitrate reductase and some of the mutations affecting the gene result in the overproduction of inactive nitrate reductase. The results suggest a feedback regulation of the level of the apoenzyme in the wild type. Immunological techniques were used to confirm that *nia* mutants are indeed altered in the structural gene of the nitrate reductase apoenzyme.

The cofactor deficient mutants (*cnx*), could be divided into two groups according to restoration, or lack of restoration of NR activity when physiologically high molybdate concentrations supplemented the culture medium. By intra- and interspecific somatic hybridisation, three complementation groups of the *cnx* mutants could be distinguished; the *cnxA* (Mo-restorable), *cnxB* and *cnxC* (Mo-non-restorable) and *cnxD* (Mo-non-restorable) (Mendel *et al.*,

1986; de Vries *et al.*, 1986; Dirks *et al.*, 1985). Nevertheless, Mendel *et al.* (1986) found three common properties in at least the *cnxA*, *cnxB*, and *cnxC* mutants:

1) simultaneous loss of of NADH-NR and xanthine dehydrogenase activity due to the defect of Mo-co which is common to both of these molybdoenzymes (this is the definition of a *cnx* mutant).
2) the functioning of the mutant NR apoprotein as acceptor for Mo-co of homologous origin (*nia* mutants) or heterologous origin (animal xanthine oxidase), thereby restoring NADH-NR activity *in vitro*.
3) cellular molybdenum concentrations similar to those found in wild type or *nia* cells which makes it unlikely that *cnxA*, *cnxB*, and *cnxC* are mutations of the molybdenum uptake mechanism.

By complementation analysis and genetic crosses both *nia* and *cnx* mutants were shown to be non-allelic, double recessive mutants (Glimelius *et al.*, 1978; Müller 1983; Grafe and Müller, 1983). Negrutiu *et al.* (1983) isolated three types of NR^- cell lines in *Nicotiana plumbaginifolia* and demonstrated by genetic crossing that NR^- deficiency was inherited as a single, recessive nuclear gene. The mutants isolated from *Arabidopsis thaliana* were also found to be monogenic and recessive, the F_2 from crosses between the wild type and the mutants yielded a 3:1 ratio for chlorate sensitivity and chlorate resistance (Oostindiër-Braaksma and Feenstra, 1973).

In summary NR^- mutants may posess all these characteristics: chlorate resistant, normal nitrate uptake, lack of nitrate-reductase and xanthine-dehydrogenase or cytochrome-c-reductase activities, strict dependence on a reduced nitrogen source such as a mixture of amino acids, and *in vitro* nitrate reductase complementation with a molybdenum cofactor source.

6.1.5 Cloning of a nitrate reductase mutant gene by gene tagging

The characteristics of nitrate reductase deficient mutants as described above, make it an attractive choice as a marker for gene transfer experiments in plant tissue culture. Cloning of the gene(s) coding for nitrate reductase will broaden its application as a marker in species other than those from which it has so far been isolated. For this purpose, the spontaneous frequency of reversion has to be sufficiently low. The reversion frequency for *nia* and *cnx* mutants in *Nicotiana plumbaginifolia* varies from 10^{-6}-10^{-7} (Dirks *et al.*, 1986).

Commére *et al.* (1986), reported *in vitro* translation of nitrate reductase messenger RNA from maize and tobacco. Poly(A)$^+$ RNA was isolated from maize leaves, translated and immunoselected by antibody to nitrate reductase. NR mRNA was found to represent 10^{-4}-10^{-5} of maize mRNA. Calza *et al.* (1987) has also successfully cloned DNA fragments complementary to tobacco nitrate reductase mRNA.

Cloning of the nitrate reductase gene by gene tagging may be possible by the scheme described in Chapter 1. When the induced mutants have been selected and characterised, its linkage to the inserted exogenous DNA may be proven. In this chapter the NR- phenotype is used as an example target gene(s) system in the evaluation of the *Agrobacterium rhizogenes* 'hairy root' system as a technique for insertion mutagenesis.

6.2 RESULTS AND DISCUSSION

6.2.1 Determination of optimum conditions for hairy root induction in flax

The parameters chosen to determine the optimum condition for hairy root induction include, the type of explant, *Agrobacterium* strain, time of inoculation and presence of phytohormones in the medium. Mature haploid plants were used as a source of explants as each plant can provide much identical material. Moreover, the haploid status can be determined by root-tip squashes as outlined in Chapter 3. Explants were inoculated after different periods of culture to allow cells to expand and dedifferentiate and become more responsive to transformation.

Leaf and stem explants taken from vigorously growing, pre-flowering haploid flax plants, were surface sterilised and cut into 1.0-2.0 mm sections. These sections were incubated on medium without hormones (MSO) and medium containing cytokinin and auxin (MSD4x2), at 27°C with a 16 h photoperiod. Inoculation with a 1/10 dilution of overnight *Agrobacterium* cultures was performed after 0, 4, and 8 days of culture. The strains used were A4, 9402 (pDLT), and A4 (pMuNK14). Leaf and stem sections expanded on MSD4x2 medium after 2 days of culture and had formed some callus along the cut edges of the sections after 8 days. Non-inoculated explants did not form any roots on either MSO or MSD4x2. After 4 weeks on MSD4x2 the explants had expanded and formed lots of callus. The hormones in MSD4x2

medium induced some shoot regeneration from the axillary buds pesent on the stem sections. There was not much growth observed on MSO medium. Leaf sections were not able to sustain growth without phytohormones after 4 days of culture. Some of the stem sections expanded and formed a little callus after 8 days on MSO medium.

Independent of the time at which *Agrobacterium* was applied, roots started to regenerate about 1 week after inoculation. Tables 6.1 and 6.2 show that the average number of roots induced per explant is dependent on the *Agrobacterium* strain, presence of hormones in the medium and the time when inoculation was performed. Generally, roots were easily induced on leaf pieces cultured on the hormone-containing medium, MSD4x2. More roots were regenerated if inoculation was performed several days after explanting. From the data, it was decided that leaf pieces inoculated with A4 (pMuNK14) 4 days after culture will give large numbers of independently-transformed roots. These conditions were later used in all experiments to generate transformed roots for mutagenesis studies.

6.2.2 Induction of nitrate reductase deficient mutants by T-DNA insertion

In this preliminary study of T-DNA insertion mutagenesis, 'hairy roots' were induced under optimum conditions as determined above. Leaf pieces taken from about a

TABLE 6.1

AVERAGE NUMBER OF INDEPENDENTLY TRANSFORMED ROOTS INDUCED PER EXPLANT ON HORMONE-CONTAINING MEDIUM (MSD4X2)

Agrobacterium strains	Time of inoculation					
	Day 0		Day 4		Day 8	
	stem	leaf	stem	leaf	stem	leaf
A4 (pMuNK14)	3.7	3.4	15.1	22.8	4.2	18
A4	0.67	7.0	4.7	6.4	2.4	2.7
9402 (pDLT)	3.8	5.8	18.9	17.5	1.2	11.5

TABLE 6.2

AVERAGE NUMBER OF INDEPENDENTLY TRANSFORMED ROOTS INDUCED PER EXPLANT ON MEDIUM LACKING HORMONES (MSO)

Agrobacterium strains	Time of inoculation					
	Day 0		Day 4		Day 8	
	stem	leaf	stem	leaf	stem	leaf
A4 (pMuNK14)	22.2	0.4	Most explants were not			
A4	1.4	none	able to grow further			
9402 (pDLT)	2.5	none	without any hormones			

month old haploid flax plant (*Linum usitatissimum* cv. RA91), were cultured on MSD4x2 medium (Fig. 6.2A). A total of 5 plates were incubated, each containing an average of 80 explants. After 4 days of culture, the leaf pieces were inoculated with A4 (pMuNK14). After about 2-3 weeks, when the majority of roots regenerated on inoculated explants were about 2 to 5 mm long (Fig. 6.2C), they were excised and transferred onto AA-chlorate selection medium (Appendix 4) containing 20 mM chlorate. Within one week, most of the regenerated roots were bleached (Fig. 6.2D). Some showed initial callus growth which later bleached too. Out of 1240 roots selected, 4 expanded and formed small yellowish calli (Fig. 6.2D). These were divided into two halves and subcultured onto fresh AA medium and AA-chlorate medium. These presumptive chlorate-resistant calli bleached within a week of culture on the AA-chlorate medium. Out of the other halves of the callus lines, only one line continued to grow on the non-selective AA medium. The callus appeared yellowish initially but some regions of green callus appeared gradually. Due to time constraints no further analysis was carried out on this callus. It would be interesting to find out whether this callus line showed any resistance to different levels of chlorate in the future.

Several different control experiments were performed to make sure that flax cells do not show any chlorate resistance at the level of selection (ie 20 mM of chlorate). In one experiment about 2000 uninoculated leaf pieces were

Fig. 6.2 Hairy root induction on flax leaf explants and root development in the presence of chlorate

A) and B) Leaf pieces from a 1 month old haploid flax plant incubated on MSD4x2 and AA medium plus chlorate respectively.

C) Leaf pieces from a 1 month old haploid flax plant inoculated with A4 (pMuNK14) after 2 weeks on MSD4x2 + Cx.

D) Roots from leaf pieces shown in (C) after one week on AA medium plus chlorate.


A
B
C
D

cultured on the AA-chlorate selection medium. These uninoculated leaf sections bleached after two days on the AA-chlorate medium (Fig. 6.2B). In the other experiment performed, leaf explants were cultured on the non-selective AA-medium for about two weeks. Growth of the explants on this medium was very slow. After more than 6 weeks, most of the explants expanded and then turned brown. Less than 3% were able to form green callus at different sites on the edges of the explant. When these developing calli were subjected to selection on the AA-chlorate medium, all were bleached within a week.

6.3 Summary

From these data it may be concluded that flax cells so far examined showed no background level of nitrate reductase deficiency. Thus, 20 mM chlorate may be used as a non-leaky selection level to recover induced nitrate reductase mutants from flax cell mutagenesis. However, some further work is still required to get the correct conditions allowing normal root/callus growth on a medium to replace non-selective AA medium.

CHAPTER 7

GENERAL CONCLUSION

7.0 GENERAL CONCLUSION

At the time of finishing this thesis, insertion mutagenesis in higher plants was still a wide open field and largely unexplored. The selection of calli, shoots or seedlings derived from cells mutated by DNA insertion in transgenic transposable elements in species such as *Arabidopsis* (which has a rapid life cycle and produces lots of seed), and the isolation of mutants induced by transposition, by screening large M_2 seed populations, has still not been reported and, in the long run, may not prove to be the most productive way forward for insertion mutagenesis studies. One major problem with tranposable elements is that transposition cannot readily be controlled in a transgenic host. Although some progress has been made in the present study towards developing insertion mutagenesis systems for higher plants, the future of such techniques still depends critically on the generation of efficient methods for controlling transposition and the selection of specific mutant traits.

7.1 Target plant system

This study has shown that flax has some attributes that make it a useful target system for gene isolation by insertion mutagenesis. In particular it has a small genome, it performs well in tissue culture and is relatively easy to transform.

The experiments with *Mu1* indicated that T-DNA inserts

containing intact elements were certainly not stable. However, taking note of previous work on the flax genome, it could be argued that the apparent recombination and amplification events involving *Mu1* sequences may be a feature peculiar only to flax genomic DNA, and may not be related to the presence of a transgenic transposable element in such tissues. It is therefore important that similar experiments are carried out in other species. If flax does prove to be unusual in this respect then it may ultimately not be the system of choice for future insertion mutagenesis studies.

Perhaps one of the greatest attributes of the flax system is the facile method for obtaining a regular supply of haploid seedlings. This tissue is extremely valuable for any kind of mutagenesis work. In future it may be productive to spend some time re-investigating the possibility of developing a reliable protoplast system from these seedlings.

7.2 Insertion mutagenesis systems using T-DNA

If haploid protoplasts of flax can be obtained in the future in large numbers, then the use of T-DNA as a random mutagen is extremely attractive. The present system, using quasi-haploid cell suspensions, is worth pursuing, but will involve a lot of work to continuously produce fresh suspension cultures, and will also need large amounts of culture dishes and manpower to cope with the enormous numbers of plating units required for mutagenesis. In fact, one of the most attractive systems for immediate exploitation is the use of *A. rhizogenes*

to induce 'hairy roots' on haploid leaf explants. Again, there is a requirement for a large number of repetitive manipulations to produce, transform and stress the explants. However, roots grow out of the explant on media that do not allow much callus formation and are very sensitive to many stressful agents. Thus, if the roots are indeed mainly clonally-derived, each containing random Ri and also binary vector T-DNA insertions, then the effort may be worthwhile. In this case there will not be a need to continuously produce dedifferentiated haploid cells and hope that the ploidy level does not change before transformation.

7.3 Transposable element-mediated gene mutagenesis

It should be realised that the range of mutants induced by transposition in cultured tissues are probably quite different to those produced by random transposition in intact plants. Many types of biochemical mutants will be much easier to select *in vitro* on appropriate media. On the other hand, many important developmental and physiological mutant phenotypes can only be screened/selected for,using populations of intact seedlings or mature plants. In this latter situation, the scope of the types of mutants possible can be equated to the kind of mutants previously generated by somaclonal variation (Larkin and Scowcroft, 1981; Scowcroft *et al.*, 1983). It is important to stress that the two different approaches (selection *in vitro* using dedifferentiated tissues,

roots or shoots; compared to selection imposed on segregating seedling populations) are not entirely independent in their requirements and so any information gained using one system may be useful if applied to the other. Thus, in conclusion, both of these approaches which use transgenic plants and transposable elements are probably worth pursuing in the future.

Whichever, system is employed, it is necessary that the genetics and biochemistry of the target phenotype is known in as much detail as possible in order that sensible insertion mutagenesis and selection strategies may be designed. In this respect, transgenic transposable elements may be useful in the creation of genetic maps of plant species using restriction fragment length polymorphism (RFLP) techniques. There is a lot of evidence that many transposable elements transpose most frequently to positions in the same chromosome, and even the same chromosome arm, and only for short, defined distances (Dellaporta *et al.*, 1985). Thus, it could be imagined that inserts into a particular genome might first be mapped to get at least one transposable element in each linkage group. Then, if transposition occurs as in maize, the transposable elements might be induced to hop successively up or down the chromosome, and DNA sequences flanking the elements cloned after each round of transposition for use as RFLP markers.

The experiments outlined in this thesis provided little evidence that the element *Mu1* can transpose normally in

transgenic flax plants. However, two points are worth noting. Firstly, T-DNA inserts lacking *Mu1* sequences (eg. 3850::1103; Chapter 4) were all co-linear with the T-DNA in the *Agrobacterium* strain and were stable in transformed callus over many months of subculture. In this case, perhaps only a subset of transformants with normal T-DNA inserts that possessed a functional *nos-npt-II* might have been selected. However, this is very unlikely, as most of the transformants contained several T-DNA copies; so there was plenty of scope for T-DNA rearrangements to have occurred that left only a proportion of the *nos-npt-II* genes intact. In experiments with the pBin19::*Mu1* vector, again Km^r events were selected; in this case very few of the transformed tissues, selected at random, appeared to have a T-DNA structure that was both similar to that found in the original *Agrobacterium* strain and that was also stable when the transformed tissue was growing as dedifferentiated callus. These observations do suggest that the presence of *Mu1* affects the stability of the T-DNA. Similar results were observed in tobacco callus (Lillis *et al.*, 1985).

In experiments with pMuNK14, it might be argued that only very rare recombination events, having very little to do with transposition, resulted in the restoration of the Km^r phenotype. There was no real evidence again to suggest that this was indeed not the case. However, circumstantial evidence, in that the NPT-II activity in each independently-

derived line resulted from the expression of an identical aberrant form of this enzyme, suggests that the recombination events are specific. This suggestion is reinforced by the fact that many independent Km^r lines have similar T-DNA patterns. The proposed mechanism alluded to in Chapter 5 suggested that most of the internal portion of *Mu1* may be excised, leaving behind only small fragments of the terminal repeat sequences to separate the *nos* promoter from the *npt-II* gene. In maize terminal sequences of *Mu1*, and other elements, are found more frequently in the genome than internal sequences (Chandler *et al.*, 1986) and it is possible that our observations show similar phenomena to be occurring in flax. These observations may suggest that *Mu1* is actually a 'slave' (non-autonomous) element and it is possible that it may have two methods of excision. Firstly, a normal, non-replicative transposition, involving precise excision and reinsertion, that depends on *trans*-acting factors found in *Mutator* lines of maize. Secondly, an independent, autonomous, but aberrant mechanism of excision may operate that involves loss of most of the internal sequences of the element to leave behind 'relic' fragments of the terminal repeats. This second proposed mechanism may possibly be able to function in any genetic background. Bearing in mind the proposed behaviour of *Mu1*, it is very unlikely that it can ever be used as a mutagenic agent in plants other than *Mutator* lines of maize. For future work, autonomous elements, such as *Ac*, which are now known to excise

cleanly and reintegrate normally, may be much more suitable.

Whichever element is actually chosen, the problem still remains of how to control its activity in transgenic plants. To be useful in insertion mutagenesis studies, it has to be possible to induce transposition, and then to stabilise the insert, so that a specific cellular genetic clone can be grown to provide enough biomass for DNA isolation and the production of a genomic library. The observation that *Mu1* activity can be controlled by the state of cell differentiation in flax tissues could prove to be very useful, as it represents an easy way of controlling transposition. Thus, an important experiment will be to see if similar methods can be used to control the transposition of other elements, such as Ac, in transgenic plants. Indeed, Brettel *et al.* (1986) isolated a number of *Adh* mutants induced by *Ac* movement in somaclonal variation experiments. Thus, the link between transposition and some classes of somaclonal mutations generated in dedifferentiated cells have been proven conclusively.

There are a range of alternative methods for controlling transposition, by engineering transposable elements, which have been used with some success in other eukaryotes. For example, mutated P-elements in *Drosophila*, have been engineered that lack a transposase gene and can only transpose if transposase functions are supplied *in trans* (Karess, 1985). This element can then be included on a vector which has a suitable transposase coding sequence driven by an

inducible promoter, such as that from a heat shock gene. Thus, transposition is dependent upon the activity of the 'helper' transposase which is inducible and therefore can be specifically controlled (personal communication, Dr. Steve Schofield, PBI).

In order to study the induction of transposition, it may be better in future to use 'reporter' systems that do not rely on the selection of antibiotic resistant calli, as these only provide information on transposition many cell generations after the event. Thus, making constructs which have transposable elements splitting a screenable marker gene from its promoter may be productive. In particular, the *gus* gene (ß-glucuronidase) may be useful in this respect, as single cells or small groups of cells, expressing GUS activity can be visualised using histochemical staining techniques on tissue sections (Jefferson *et al.*, 1987).

In the final analysis, several potential systems for insertion mutagenesis have been developed for flax. If other transposable elements, such as *Ac*, can be induced to transpose normally and subsequently be stabilised, then insertion mutagenesis methods for gene cloning may well be worth attempting. Alternatively, the road seems clear for the mutagenesis of haploid leaf sections by *Agrobacterium*-based transformation systems.

The best model system still probably involves the generation of nitrate reductase null mutations, as the

selection systems are so powerful. In future a productive line may be to induce useful mutations by traditional methods (eg. herbicide resistance) and then attempt to knock out this new function with a transgenic transposable element or T-DNA insertion, making it possible to clone the mutant gene sequences flanking the insert. In the same way, any other pre-existing useful traits could be mutagenised in order to produce probes to eventually clone the wild-type gene. Of course, these techniques are not limited to the analysis and cloning of known genes in any way; the beauty of an insertion mutagenesis system is that previous knowledge of the genes functioning to give rise to a complex phenotype are not required. The genes in some way involved with any new phenotypes that are generated will always be flanking the inserted DNA sequences and can be cloned by standard recombinant DNA procedures.

It should be added in conclusion that the backbone of all the experiments aimed at cloning flax genes by insertion mutagenesis is the ability to reproducibly transform several types of flax explants, and produce normal-looking, fertile, transgenic plants. Flax is an important crop plant and in future the techniques developed in this thesis may be used for the genetic engineering of flax varieties using recombinant DNA procedures.

APPENDICES AND REFERENCES

APPENDIX 1. Media used in the experiments.

Ingredients (mg/l)	MS	B5
$(NH_4)2SO_4$		134
$(NH_4)NO_3$	1650	
KNO_3	1900	2500
$CaCl_2.2H_2O$	440	150
$MgSO_4.7H_2O$	370	250
KH_2PO_4	170	
$NaH_2PO_4.H_2O$		150
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
$MnSO_4.4H_2O$	22.3	
$MnSO_4.H_2O$		10
$ZnSO_4.7H_2O$	8.6	2
H_3BO_3	6.2	3
KI	0.83	0.75
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Myo-inositol	100	100
Nicotinic acid	0.5	1.0
Pyridoxine.HCl	0.5	1.0
Thiamine.HCl	0.1	10.0
Glycine	2.0	
Sucrose	30,000	20,000
pH	5.7-5.8	5.5

APPENDIX 2.

Hoagland's 2nd. solution modified by Johnson *et al.*, 1957.

Compounds	Concentration of stock (M)	Concentration of stock (g/l)	Volume of stock solution/l of final solution (ml)
Macronutrients			
KNO_3	1.00	101.10	6.0
$Ca(NO_3)_2.4H_2O$	1.00	236.16	4.0
$NH_4H_2PO_4$	1.00	115.08	2.0
$MgSO_4.7H_2O$	1.00	246.49	1.0

Compounds	Concentration of stock (mM)	Concentration of stock (g/l)	Volume of stock solution/l of (ml)
Micronutrients			
KCl	50.0	3.728	
H_3BO_3	25.0	1.546	
$MnSO_4.H_2O$	2.0	0.338	
$ZnSO_4.7H_2O$	2.0	0.575	1.0
$CuSO_4.5H_2O$	0.5	0.125	
H_2MoO_4 (85% MoO)	0.5	0.081	
FeEDTA	20.0	6.922	1.0

pH 5.0-7.0

Appendix 3

Oligo-labelling Buffer (OLB) is made by combining 3 separate solutions:

Solution A:

Component	µl
2 M Tris-HCl	625
5 M $MgCl_2$	25
dH_2O	350
2-mercaptoethanol	18
dATP	5
dTTP	5
dGTP	5

(Dissolve each nucleotide separately in 3 mM Tris-HCl pH 7.0, 0.2 mM EDTA, at a concentration of 0.1 M. This solution should be stored at -20°C

Solution B: (2 M HEPES pH 6.6). For 100 ml: Dissolve 47.66 g of solid in 75 ml of dH O, titrate to pH 6.6 with NaOH and make to volume. Store at 4°C.

Solution C:

Hexadeoxyribonucleotides (Pharmacia cat. no. 27-2166-01).

These should be evenly suspended (they do not completely dissolve) in 3 mM Tris-HCl, 0.2 mM EDTA pH 7.0 at 90 OD_{260} units/ml and stored at -20°C.

To make OLB mix A, B and C in the ratio 2:5:3. OLB stored at -20°C remains stable for at least 3 months with repeated freeze/thawing.

Appendix 4

Amino Acid medium (AA-medium) for nitrate reductase mutants callus growth

Compounds	(mg/l)
$CaCl_2.2H_2O$	440
KCl	2940
KI	0.83
$CoCl_2.6H\ O$	0.025
KH_2PO_4	170
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$MgSO_4.7H_2O$	370
$MnSO_4.4H_2O$	22.3
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.6
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Sucrose	20,000
<u>Vitamins</u>	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.1
Thiamine HCl	0.5

Hormones

2,4-D	1
Kinetin	0.2
GA	0.1
Agar	8 g

pH 5.8
Volume made up to 90% and autoclaved for 20 min at 121°C. Amino acid solution is added after autoclaving to make up for the final volume.

Amino acid solutions

	mg/l
L-glutamine	877
L-arginine	228
L-glycine	75
L-aspartic acid	266

pH 5.8

Volume made up to 10% and filter sterilised.

For AA-chlorate medium, KCl is replaced with 3.66 g/l of $KClO_3$ to give 20mM chlorate.

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Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase

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ABSTRACT

Genetic transformation of flax (*Linum usitatissimum*) has been achieved using an *A. tumefaciens* strain carrying a non-oncogenic Ti plasmid-derived vector containing a chimaeric *npt-II* gene and a wild type nopaline synthase gene. Fertile, transformed shoots were most easily obtained from Km^r callus developing on hypocotyl sections. The totipotency of the Km^r callus was dependent upon its origin. T-DNA was visualised by Southern blotting in all Km^r tissues. Efficient expression of nopaline synthase and the chimaeric *npt-II* gene was found in transformed Km^r callus and regenerated shoots.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is a dicotyledonous plant of the family Linaceae. Besides being an oil seed crop in countries with temperate climates, flax is an important source of natural fibres and its seeds contain significant amounts of protein. Tissue culture techniques have been developed in flax mainly with the aims of obtaining new variants, through somatic hybridisation and somaclonal variation, as well as for propagation (Gamborg and Shyluk, 1976; Barakat and Cocking, 1983; McHughen and Swartz, 1984). Plantlet regeneration has been obtained from *in vitro* cultured hypocotyls (Gamborg and Shyluk, 1976), mature stem explants (Murray *et al.*, 1977), cotyledons (Rybczynski, 1975), and cotyledon protoplasts (Barakat and Cocking, 1983). Crown gall induction by wild type Ti plasmids of *Agrobacterium tumefaciens* has been shown to be possible by Hepburn *et al.*, (1983).

The nuclear genome of flax is small compared with many other plants (1.4 pg/2C nucleus) with a chromosome number of 2n=30 (Bennet and Smith, 1976). In addition, haploid plants can be generated readily from some flax varieties. The properties of totipotency, small genome, availability of haploids and susceptibility to transformation by *Agrobacterium* would recommend flax as a favourable system for mutagenesis studies by random insertion of T-DNA or transgenic transposable elements.

In this paper, we describe the transfer of a chimaeric kanamycin resistance gene into flax cells and the regeneration of fertile transformed plants. Some general properties of the transformation system are discussed.

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MATERIALS and METHODS

Plant material

Commercial flax seeds (*Linum usitatissimum* L. cv. Antares; Sharpes Seeds) were surface sterilized by immersion for 20 min in 10% commercial bleach followed by rigorous washing with sterile distilled water. Sterile seeds were then placed in a Kilner jar, on sterile vermiculite wetted with a modified Hoagland's solution (Johnson *et al.*, 1957) and germinated at 27°C with a 16 h photoperiod. After two days, 1.0–2.0 mm sections of hypocotyls were excised and used in the transformation experiments. Callus formation and shoot regeneration were induced on agar-solidified (0.8%) MSD4x2 medium comprising of MS basic salts and vitamins (Flow-labs) supplemented with 1.0 mg/l BAP, 0.1 mg/l NAA and 3% sucrose.

Bacterial strains

The *Agrobacterium* strain used in all experiments was C58C1 (pGV3850:1103) (Hain *et al.*, 1985). This strain harbours a modified version of the disarmed Ti-plasmid vector pGV3850 (Zambryski *et al.*, 1983) containing a nos-*npt-II* chimaeric gene to allow selection of kanamycin resistant transformed plant cells and a functional nopaline synthase gene. Agrobacteria for transformation were prepared by growing a single colony, picked from a selection plate, overnight at 27°C in 5.0 ml of nutrient broth containing 50 µg/ml carbenicillin on a rotary shaker.

Transformation

Hypocotyl sections from flax seedlings were soaked in 1:50 dilution of the overnight *A. tumefaciens* culture in liquid MSD4x2 medium for 1–2 h at room temperature. The sections were then removed to agar-solidified MSD4x2 medium and placed at 27°C in the dark overnight. The explants were then transferred onto 5 ml of either medium A (MSD4x2 + 200 µg/ml cefotaxime), or medium B (MSD4x2 + 200 µg/ml cefotaxime + 100 µg/ml kanamycin), both solidified with 0.8% agar, and contained in 50 mm Petri dishes. Four explants were placed in each dish and incubated at 27°C with a 16 h photoperiod. Control (uninoculated) explants were treated in the same way.

After 20–30 days, regenerated shoots of about 1.0–2.0 cm in height were separated from the hypocotyl explants and proliferating callus and

subcultured onto propagation + selection medium comprising of MS salts plus 0.05 mg/l kinetin, 200 µg/ml cefotaxime and 100 µg/ml kanamycin. The remaining hypocotyl explants and induced kanamycin resistant callus, were transferred onto fresh medium B for further growth and selection of transformed tissue. Subsequent subculture of kanamycin resistant callus lines was performed using medium B without cefotaxime.

When 4.0-5.0 cm, putatively transformed shoots were transferred to root induction medium consisting of half-strength B_5 medium (Gamborg *et al.*, 1968) without phytohormones containing kanamycin (100 µg/ml).

Molecular analyses on Km^r tissues

Nopaline synthase, NPT-II and Southern blot analysis was carried out as described by Scott and Draper (1987). *Eco* RI-digested DNA was probed for the kanamycin resistance gene using a 1.45 kb *Hind* III/*Sal* I fragment of the *npt-II* gene.

RESULTS

The different responses shown by the hypocotyl explants under different culture and inoculation regimes are summarised in Table 1.

On MSD4x2 and medium A, hypocotyl sections expanded considerably during the first two weeks of culture followed by appearance of swellings on the surface near to one end and compact callus at both cut ends (Fig 1a). These swellings developed into leaf primordia and subsequently produced buds and shoots, during which phase the callus growth at both ends was reduced. After 3-4 weeks of culture the hypocotyl sections were full of leaf primordia with several dominant shoots growing out (Fig 1b). The presence of the antibiotic cefotaxime in the medium, or inoculation of the explants with *Agrobacterium*, did not affect the shoot regeneration capacity of the hypocotyl sections (Table 1). However, shoot regeneration from control (uninoculated) hypocotyl sections is completely inhibited in the presence of kanamycin.

TABLE 1

Response of *Agrobacterium*-inoculated and non-inoculated hypocotyl explants to different media

pGV3850 ::1103	Medium	No. of explants	No. of explants with shoots
-	MSD4x2	20	20
-	A	30	30
-	B	40	0
+	A	70	70
+	B	60	5

Initial experiments concentrated on producing shoots from inoculated explants without selection pressure. Once these shoots were large enough to handle easily, they were cut at the base and transferred into propagation and selection medium. After about 4 weeks in this medium, these shoots elongated and began to bleach at the basal end and none were found to be kanamycin resistant.

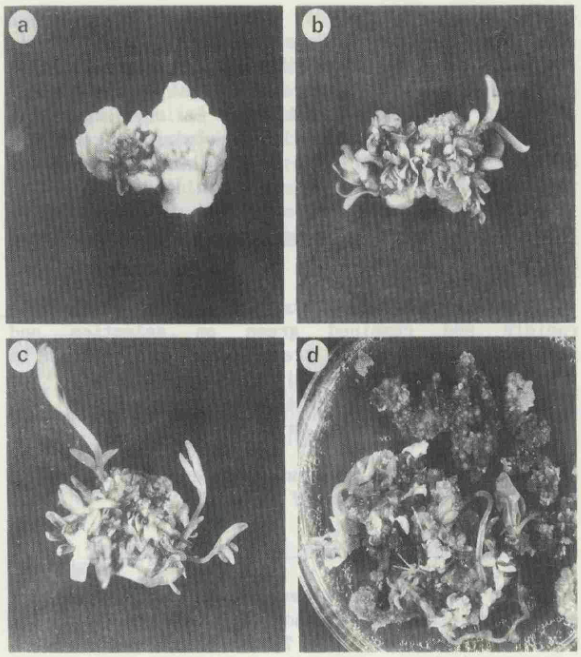

Figure 1. *Agrobacterium*-mediated transformation of flax hypocotyl sections. a) Initiation of callus and shoot primordia on hypocotyl sections after 1-2 weeks culture on MSD4x2; b) appearence of similar explant after 3-4 weeks on MSD4x2; c) production of green callus and shoot primordia from bleached 1° callus following subculture on medium B; d) regeneration of shoots from Km^r green callus on medium B.

Two different approaches were then used in an attempt to produce transformed shoots, and these differed in the timing of application of selection pressure following inoculation of the explants with agrobacteria.

Method 1: Inoculated explants plated originally on regeneration medium lacking kanamycin (medium A)

Following the removal of well developed shoots, hypocotyl sections which still contained leaf primordia and some callus were cut into small pieces and placed in medium B for the selection of transformed cells and regeneration of further shoots. Callus formation resumed and some green 2° primordia developed in the vicinity of the bleached 1° primordia after 3-4 weeks on selection medium. Once subcultured into fresh medium B, green Km^R callus proliferated from the region of these green 2° leaf primordia and shoots were subsequently regenerated from the callus (Fig 1c). In one experiment eighteen independent lines of green, kanamycin resistant callus were isolated and three of them regenerated shoots. 2° primordia never arose from parts of the original explant which failed to produce 1° primordia during the initial phase of culture on medium A.

Method 2: Inoculated explants cultured directly on regeneration medium containing kanamycin (medium B)

All the hypocotyl sections, including those undergoing primordia formation, enlarged and then bleached on medium B. However, after 3-4 weeks green callus developed at the cut ends of some of the bleached hypocotyl sections and green 2° shoot primordia developed. Some of these calli later bleached while others remained green and

continued to grow rapidly upon subculture to fresh selection medium. Regeneration of Km^r shoots (Fig 1d) proved to be possible only from green callus produced on explants which had initially showed signs of shoot primordia formation. In one experiment twenty independent callus lines growing on kanamycin were isolated and two of them subsequently regenerated Km^r shoots.

Selection, propagation and rooting of regenerated shoots

Shoots regenerated from Km^r callus which grew rapidly and remained green on selection and propagation medium were transferred into $^1/_2$ B_5 + Km for root induction and further selection of transformants. Cefotaxime was omitted since this tended to slow down root development. All Km^r shoots formed roots, whilst all shoots derived from the control hypocotyl sections failed to root and bleached in the presence of kanamycin.

Nopaline synthase activity in Km^r flax tissues

Seven independent lines of kanamycin resistant callus obtained by Method 2, were assayed for nopaline synthase. Three different shoots regenerated from two of the callus lines were also screened for nopaline synthase activity.

Nopaline was detected in all of the kanamycin resistant callus lines (Fig 2, lanes 3-9) and regenerated shoots (Fig 2, lanes 10-12). It is interesting to note that the amount of nopaline in these tissues was elevated as compared to transformed tobacco tissue produced using the same vector (unpublished observations).

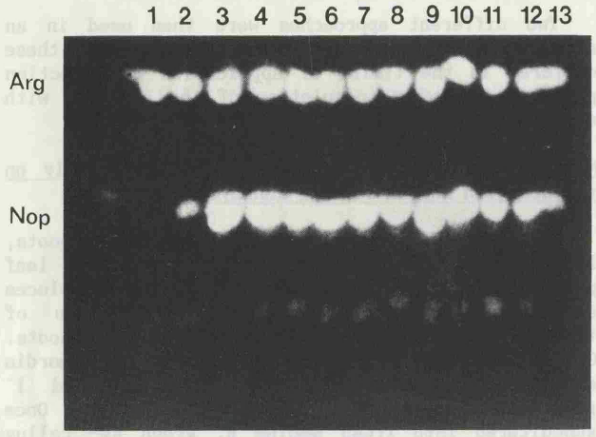

Figure 2. Detection of nopaline in kanamycin resistant calli and shoots. Lane 1, control callus; lane 2, control callus plus standard nopaline; lanes 3-9, kanamycin resistant calli; lanes 10-12, regenerated kanamycin resistant shoots; lane 13, standard nopaline and arginine.

NPT-II activity in transformed flax tissues

All of the Km^r callus lines contained NPT-II activity (Fig 3, lanes 1-7) which comigrated with authentic *E. coli* NPT-II (lane 9). This activity was not present in control callus protein extract (Fig 3, lane 8). It is interesting to note that the amount of NPT-II activity varied between the different callus lines.

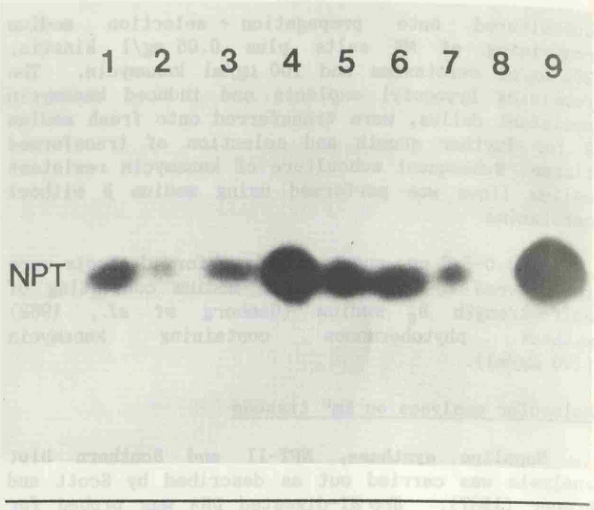

Figure 3. Detection of NPT-II activity in kanamycin resistant calli. Lanes 1-7, independent kanamycin resistant calli; lane 8, control, kanamycin sensitive callus; lane 9, bacterial NPT-II.

T-DNA analysis of transformed flax tissues

Eco RI restricted DNA of all of the transformed callus lines contained fragments which hybridized to the neomycin phosphotransferase gene probe (Fig 4, lanes 2-8). Two hybridising fragments of 9.4 and 7.2 kb are common to all the lines and to the vector DNA within *Agrobacterium* (Fig 4, lane 10). Lines 4 and 5 and lines 6 and 7 contain an extra restriction fragment with homology to *npt-II*.

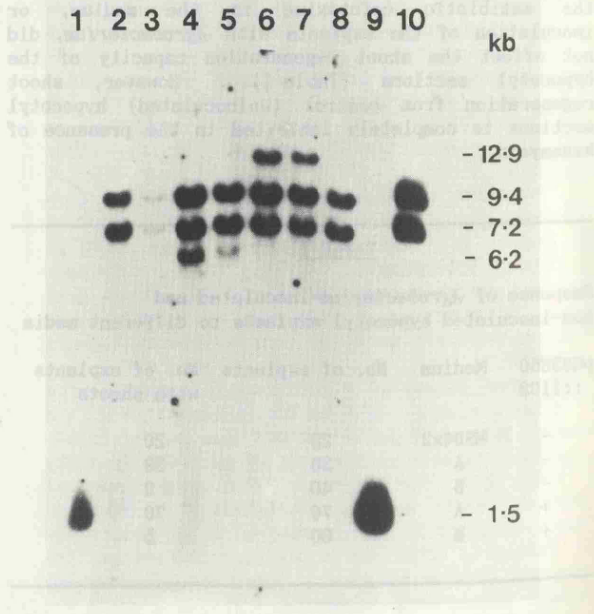

Figure 4. Southern blot analysis of transformed callus. Lanes 1 and 9, 1x and 5x copy number reconstructions respectively of the *npt-II* gene; lanes 2-8, *Eco* RI restricted DNA from independent kanamycin resistant calli (lines 1-7 respectively); lane 10, *Eco* RI-digested DNA from *Agrobacterium* strain pGV3850::1103.

DISCUSSION

In this paper we demonstrate the transformation of flax hypocotyl sections by *Agrobacterium* using a disarmed Ti-plasmid and the subsequent regeneration of Km^r plantlets from the transformed callus lines. After 3-4 weeks primordia had formed on the original explant tissues, but not from the callus induced at the cut ends of the hypocotyl sections. The response of explants was markedly polar, so that primordia were formed only at the cotyledon end of the initial explant. Leaf primordia, subsequently formed buds and shoots are thought to be wholly derived directly from totipotent epidermal cells (Link and Eggers, 1946) without dedifferentiation. However, it is very likely that cell dedifferentiation is required for transformation and this may explain why none of the shoots regenerating directly from inoculated explants were found to be transformed. Transformed shoots were recovered only from green callus proliferated from explants which had produced 1' primordia. Thus, a likely sequence of events leading to the production of transformed shoots would appear to be that cells at the cut end of the explant in the region of developing shoot primordia pass through a period of dedifferentiation during which time they are permissive to transformation by *Agrobacterium*. Subsequently these transformed cells establish Km^r callus which is competent for shoot regeneration. It may be significant in this respect that cefotaxime had to be maintained in the medium for several subcultures before all agrobacteria were finally eliminated and it may be these persistant bacteria which actually accomplish transformation of the newly permissive cells.

The results of the T-DNA analysis were complicated by the fact that rearrangements of the T-DNA region appeared to have occurred in the *Agrobacterium* strain, as we would have expected an *Eco* RI digestion of pGV3850::1103 (Hain *et al.*, 1985) to release a single internal fragment of 7 kb containing the *npt-II* gene. The cointegration of plasmids into pGV3850 results in the duplication of pBR322 sequences and it is likely that recombination between these homologous regions was responsible for the rearrangement of vector sequences. Further analysis is required to determine accurately the structure of the T-DNA in these transformed flax lines. From the bacterial reconstruction, it is estimated that there are from <1 to 3 copies of the *npt-II* gene integrated in the flax genome.

The presence of nopaline and expression of the *npt-II* gene in the transformed callus lines further proves the integration of the T-DNA in the flax genome. Experiments are in progress to determine the stability and inheritance of the transferred DNA in the transformed shoots.

Thus, in summary, under suitable conditions a *cis*-acting, disarmed Ti-plasmid vector is capable of transforming flax cells without affecting their morphogenic potential. The transformation system for flax described here is simple, reliable and can produce large numbers of transformed shoots. Experiments are currently underway to utilise this system for the mutagenisation of the flax genome by the transposable elements Mul and Ac.

ABBREVIATIONS

npt-II,	neomycin phosphotransferase II gene;
NPT-II,	neomycin phosphotransferase II;
nos,	nopaline synthase gene promoter;
Km^r,	kanamycin resistant;
BAP,	6-benzylaminopurine;
NAA,	α-naphthaleneacetic acid;
MSD4x2,	medium D4x2 based on Murashige & Skoog medium (see Scott & Draper, 1987).

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