Genetic engineering of the forage legume lotus corniculatus using Agrobacterium: mediated transformation systems

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GENETIC ENGINEERING OF THE FORAGE LEGUME *LOTUS CORNICULATUS* USING *AGROBACTERIUM* - MEDIATED TRANSFORMATION SYSTEMS

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DECLARATION

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

To my family and in memory of my Grandfather.

ABSTRACT

Gene transfer vectors based on the *Agrobacterium tumefaciens* Ti plasmid were used to develop a successful disarmed *Agrobacterium tumefaciens*-mediated transformation method for *Lotus corniculatus*.

A binary vector construct, pJIT73, was used during the development of the *Agrobacterium tumefaciens* transformation system due to its selectable (*Aph IV, nos-neo*) and scorable markers.

The effects of the antibiotics geneticin (G-418) and hygromycin B were studied. Use of kill curves and selection delay experiments allowed potentially suitable selection pressure parameters to be proposed. Using such selection during transformation experiments led to further optimisation of this stage of transformation. The influence of plant hormones on the regeneration of *Lotus corniculatus* explants was investigated and a modification of an established protocol using leaf explants was introduced as an attempt to reduce the overall time of regeneration. Various explants were used but leaf pieces were chosen as the most suitable explant on which to focus research.

So, through alteration of various stages, including length of cocultivation and subsequent decontamination within the transformation process, a successful method was developed. Experiments indicated the optimum *Agrobacterium tumefaciens* strain to be used with *Lotus corniculatus* was the disarmed Ach5 type, LBA4404(pAL4404). Transgenic *Lotus corniculatus* plants were produced which expressed the scorable marker ß-Glucuronidase gene (GUS) and the selectable marker for hygromycin B resistance, *AphIV*. Gene transfer was confirmed by Southern blotting. The new Agrobacterium tumefaciens-mediated vector system was used to introduce the cowpea trypsin inhibitor gene (CpTi) into *Lotus corniculatus*. However, although there was evidence for transformed callus development, no shoots were induced.

By the use of previously established *Agrobacterium rhizogenes*-mediated system, an attempt was made to introduce the pea lectin gene (*psl*) into *Lotus corniculatus*. Hairy root regenerants were produced but genetic transfer was unconfirmed and attempted investigation of the plant - *Rhizobium* symbiosis involving *Lotus corniculatus* was not fulfilled.

ABBREVIATIONS

AS	-	Acetosyringone
BAP	-	6-benzylaminopurine
bp	-	base pair
BSA	-	Bovine Serum Albumin (Pentax fraction 5)
DNA	-	deoxyribonucleic acid
DTT	-	dithiothrietol
EDTA	-	ethylenediaminotetra-acetic acid, disodium salt
ELISA	-	enzyme-linked immunosorbant assay
EtBr	-	ethidium bromide
g	-	grammes
G-418	-	Genticin
GUS	-	ß-glucuronidase
hr	-	hour
hygromycin	-	Hygromycin B
kb	-	kilobases
min	-	minute
MU	-	4-methyllumbelliferone
MUG	-	4-methyllumbelliferyl glucuronide
NAA	-	1-napthylacetic acid
Nos	-	nopaline synthase
Npt II	-	neomycin phosphotransferase
SDS	-	sodium dodecyl sulphate
S.E.	-	standard error
X-Gluc	-	5-bromo-4-chloro-3-indolyl glucuronide
$\bar{x}$	-	mean
δ_{n-1}	-	standard deviation

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1.1 GENERAL INTRODUCTION

Genetic engineering of plants may be described as the introduction and expression of foreign DNA in plants. A narrower definition suggested by Cocking (1983), described genetic engineering as being " the uptake and expression of foreign DNA by a cell ". To achieve such manipulation an integrated approach involving various fields of biology has been necessary, including tissue culture, plant physiology, cell and molecular biology. One of the cellular phenomena most crucial to genetic engineering is that of totipotency. This was first defined in the nineteenth century and describes how in plant axenic culture, any living cell or tissue possesses the capacity for the regeneration of a whole plant. However, such regenerative potential has not always been fulfilled, and the ability to regenerate whole plants from cell or tissue culture is often a limiting factor in genetic engineering of many plant species. Although genetic manipulation can be used as a tool in the investigation of gene expression and function, it is in crop improvement that genetic manipulation has become of paramount importance (reviewed by Crocomo, 1989). With this application of genetic engineering, potential problems with population dynamics and ecology will also need to be addressed to satisfy public concern about the effects of this technology.

Plant biotechnology could potentially be used to solve a number of problems inherent in conventional plant breeding. Traditional breeding is based upon the introduction of useful agronomic traits by sexual crosses between plants and has been used in crop improvement for many centuries, with many of our present crop varieties derived from ancestral crosses. However sexual incompatibility, at both the inter- and intra- specific levels, is still a major limitation in such traditional plant breeding. Instability of the novel gene combinations through subsequent plant propagation and the often long sexual reproductive cycles of plants are other problems encountered in plant breeding programmes, except for inbreeding or clonal crops. The latter is accentuated when selection of a new plant variety may involve 5 - 15 cycles of sexual reproduction.

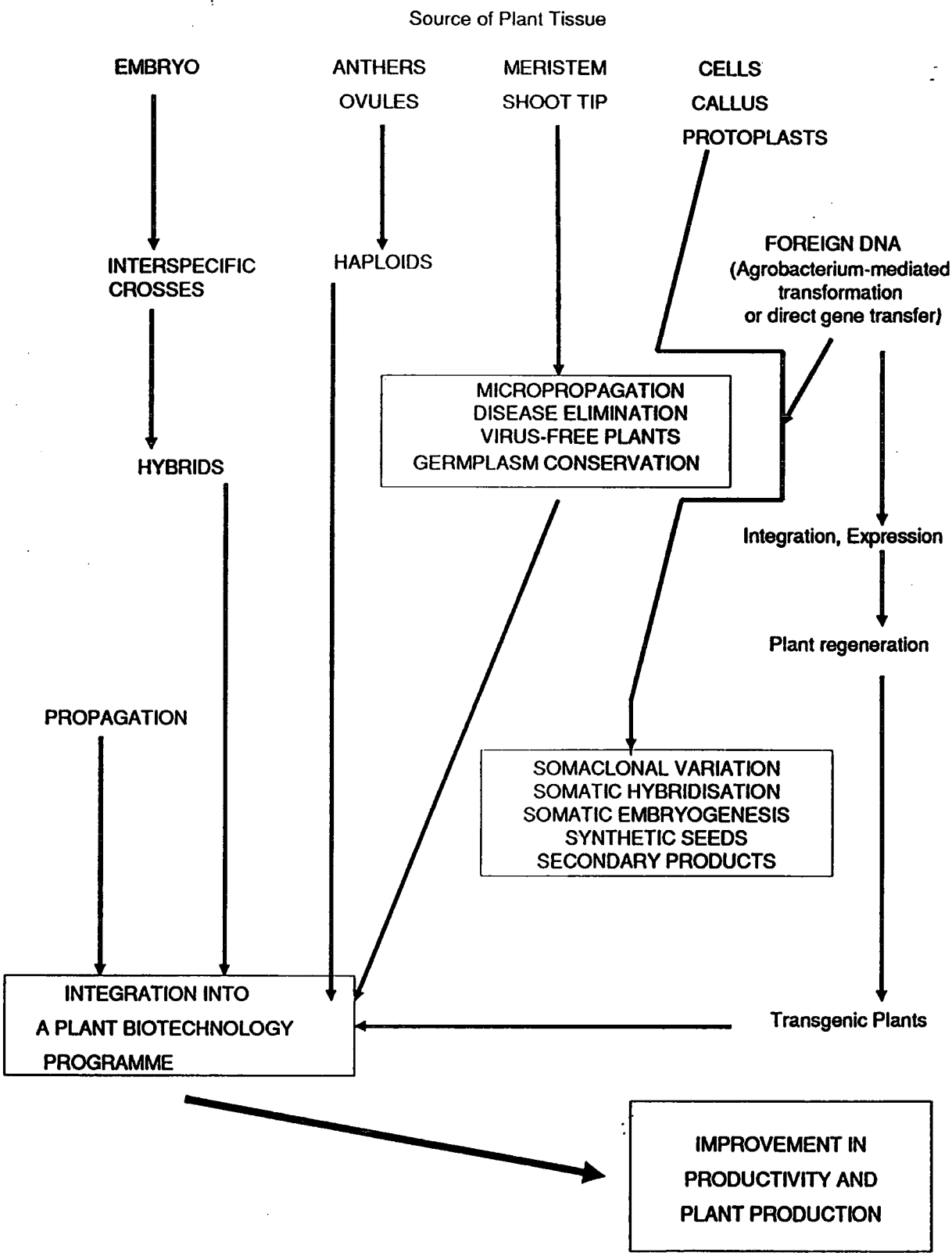
Another constraint on conventional plant breeding is that it is not possible to introduce genes from non plant sources such as microorganisms. Thus development of plant tissue culture and genetic engineering techniques has increased the genetic pool available to scientists, whilst also reducing the space and time requirements needed for the production of new plant varieties. The extension of the useful germplasm pool can be seen to be important when the range and various sources of useful traits is considered. Beneficial characters are present in primitive cultivars, and other wild type cultivars. Such sources of genetic variation may be conserved in germplasm banks and modern techniques used to overcome any sexual incompatibilities. Those traits found in wild varieties include tolerance to various environmental conditions and resistance to pathogens and pests.

1.1.1 Techniques for Gene Transfer

Figure 1.1 outlines techniques which have been developed and used in plant improvement strategies. While the technologies of somatic hybridisation, bacteria-mediated transformation and direct gene transfer allow the incompatibility barriers to be circumvented, hybrid embryo rescue helps overcome post-zygotic incompatibility. Meristem shoot tip culture can be used in the production of disease- and virus- free plants. Production of haploid plant lines through anther and ovule culture has been used in the production of improved crop lines including barley, potato, rapeseed, rice and wheat (Morrison and Evans, 1988). The production of plants from the gametic cells allows the production of novel stable inbred lines in one step, in contrast to the time consuming traditional methods of achieving homozygosity by self-fertilisation or backcrossing. Haploidisation followed by chromosome doubling is an efficient method for achieving homozygous plants and can be used in the eradication of undesirable recessive traits. Such novel technology however does not supercede the traditional methods, but should merely be considered as a tool to be used in conjunction with them.

For many years somaclonal variation has been a source of variation, potentially of use in plant improvement. Especially useful are single gene somaclonal variants

Figure 1.1: Application of Plant Tissue Culture and Genetic Engineering to Plant Biotechnology (modified from Crocomo, 1989).



where the gene involved encodes gross phenotypic effects such as height (Larkin *et al*,1984) or herbicide resistance (Chaleff and Ray, 1984). Such variation between plants derived from a common cell or tissue culture source, will not achieve specific genetic alteration. The mechanisms of genetic change have not been fully elucidated but include changes in ploidy number, chromosome breakage and re-arrangement. The unpredictable nature of somaclonal variation means the more precise methods available in genetic manipulation should yield novel plants more efficiently.

The production of protoplasts from plant tissue by enzyme digestion of the cell wall was pioneered by Cocking in 1960. Following the commercial production of cell wall degrading enzymes, protoplast technology became an important tool for gene manipulation. The formation of the first reported heterokaryons through induced protoplast fusion (Power *et al*, 1970) was followed by the first reported regeneration from mesophyll protoplasts (Takebe *et al*, 1971) and the production of the first somatic hybrid plant, *Nicotiana glauca* x *N.langsdorffi*, (Carlson *et al*, 1972). Totipotency meant that whole "novel" plants could potentially be derived from a single engineered protoplast (Potrykus and Shillito, 1986). Somatic cell hybridisation has also allowed the fusion of two protoplasts from sexually incompatible species (Kumar and Cocking, 1987). Even intergeneric hybridisation is achievable. Protoplast fusion may be achieved by mixing protoplast populations in the presence of polyethylene glycol (PEG) and Ca^{2+} at a high pH or by electrofusion. Electrofusion involves passing brief, high voltage electrical pulses through the protoplasts which become reversibly permeabilised. Protoplasts become aligned in chains and fusion may then take place. Fusion technology also encompasses Asymmetric hybridisation (Dudits and Praznovszky, 1985). Irradiation of one protoplast population results in only a few functional genes being transferred in subsquent fusion with a normal protoplast population. Other alternatives are the fusion of DNA-carrying liposomes or bacterial spheroplasts to protoplasts (Caboche, 1990; Gad *et al*, 1990).

Protoplast technology has been a useful tool for the manipulation of many species including members of the Solanaceae and Cruciferae and also in recent years,

monocotyledons such as wheat, *Triticum monococcum* (Lorz *et al*, 1985) and rice *Oryza* spp. (Uchimiya *et al*, 1986). Somatic hybridisation has allowed manipulation of extrachromosomally inherited traits including male sterility (Izhar and Zelcer, 1986) and herbicide tolerance (Maliga and Menczel, 1986). Development of regeneration culture conditions for protoplasts from each species, is the major difficulty in the universal use of protoplast techniques especially for the cereal crops.

Direct DNA or gene transfer (DGT) into plant cells makes use of the protoplast techniques previously described for the introduction of DNA into plant cells. DGT was developed by Paszkowski *et al*, 1984, as a technique in which protoplasts are incubated with DNA in the presence of PEG. The DNA enters through the plasma membrane and integrates into the host genome (Potrykus *et al*, 1985). DGT has the advantage of no host range barriers, the only difficulty with the system is the need for viable recipient protoplasts. Electroporation has been used successfully on both dicotyledenous and monocotyledonous species (Fromm *et al*, 1985; Hauptmann *et al*, 1987) but it is expensive and the efficiency of transformation is low, due to the high percentage of protoplasts damaged during the procedure. Plants on which DGT has been successful to varying degrees include tobacco (Schöcher *et al*, 1986) where the efficiency was as high as for protoplast-*Agrobacterium* co-cultivation and *Lolium multiflorum* and *Triticum monococcum* (Lorz *et al*, 1985).

Alternative DGT methods are the microinjection techniques discussed by Neuhaus and Spangenberg, (1990); such delivery systems are, typically of DGT, independent from host-range restrictions, but may be technically difficult. A recent advance in DGT techniques has been the use of a particle gun. This involves bombardment of plant tissue with DNA coated microparticles (Tomes *et al*, 1990; Sanford, 1990). This "biolistics" technique appears to be useful for any species or tissue type, though at present efficiency is still relatively low and the requirement of specialised equipment is a further disadvantage.

Other novel techniques under development at present, include direct gene transfer by electric discharge particle acceleration. Christou (1990), demonstrated transformation of soybean meristem tissue via this process. The benefits and problems of this procedure are similar to those of "biolistics".

The use of bacterial plasmid vectors in plant transformation is at present the dominant method in genetic engineering. Most commonly used are vectors based on plasmids found in *Agrobacterium tumefaciens* and *A.rhizogenes;* these bacteria-mediated transformations will be discussed more fully in subsequent sections. These systems are often highly efficient compared to other transformation methods. Agroinfection of plants with viral nucleic acid may prove to be a useful variant of *Agrobacterium*-mediated transformation in the field of plant pathology, in order to elucidate viral life cycles, (Grimsley, 1990).

Other potential vector systems include DNA viruses such as the Cauliflower mosaic (CaMV) (Hohn *et al*, 1982) and geminiviruses as well as RNA viruses such as brome mosaic virus (BMV). These have not fulfilled their expected potential; one important drawback is the high error rate in viral replication which will quickly inactivate any non-selectable foreign gene.

1.1.2 Applications of Genetic Engineering Technology

The production of herbicide-resistant plants was a major target for genetic engineers and was approached in three different ways: overproduction of a target enzyme (Shah *et al*, 1986); production of mutant form of target enyzme (Comai *et al*, 1985) and use of a detoxifying enyzme (De Block *et al*, 1987). Shah *et al*, 1986 introduced the isolated 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase gene in association with 35S promoter and *nptII*, into *Petunia* via *A.tumefaciens* binary vectors. The high expression level of EPSP synthase conferred glyphosate tolerance on the regenerated transgenic plants. Comai *et al*, 1985, introduced a mutant bacterial allele of *aroA* into transgenic tobacco. This mutant *aroA* encodes for a form of EPSP synthase (the cellular target of glyphosate), less sensitive to glyphosate than the wild

type plant enzyme. Thus expression of the mutant *aroA* enhanced glyphosate tolerance in transgenic plants, the tolerance levels correlated with levels of expression of the mutant *aroA*. De Block *et al*, 1987 produced transgenic tobacco, tomato and potato plants completely resistant to the non-selective herbicide phosphinathrin (PPT). The chimeric 35S promoter - PAT gene, encoding phosphinothricin acetyltransferase, was transferred to the transgenic plants.

The development of virus-resistant plants (Bevan *et al*, 1985; Hemenway *et al*, 1988) and insect resistant plants (Fischhoff *et al*, 1987; Hilder *et al*, 1987) are also important goals in agriculture and horticulture. Bevan *et al*, 1985 produced transgenic tobacco callus expressing chimeric 35S cDNA TMV coat protein gene. Attaining such expression of a TMV-RNA encoded protein from cDNA extends DNA analysis techniques to studies of plant RNA viruses. Hemenway *et al*, 1988 studied the role of coat proteins in cross protection. Transgenic tobacco expressing the potato virus X (PVX) coat protein or the corresponding antisense construct were found to be protected from subsequent PVX infection. Fischhoff *et al*, 1987 produced transgenic tomatoes expressing the chimeric insect endotoxin gene from *Bacillus thuringiensis* . Tolerance to lepidopteran larvae was conferred on both F_0 and F_1 plants. A different approach was used by Hilder *et al*, 1987 when tobacco was transformed with the broad range insect resistance gene, CpTi, effective against *Callosobruchus maculatus* and *Heliothis, Spodotera, Diabrotica* and *Tribolium* species.

The genetic engineering of biochemical pathways is a further area of research and has included the transformation of a mutant petunia with a maize gene encoding for an intermediate in pelargonidin biosynthesis, yielding a novel pelargonidin-type red flowered *Petunia* (Meyer *et al*, 1987).

As well as tools for crop improvement, the techniques now available in molecular biology permit studies of gene expression and regulation. For example, the

genes encoding storage proteins expressed during legume seed development (Boulter *et al*, 1987; Shirsat *et al*, 1990) have been investigated via studies involving expression in transgenic plants as have ribulose-1,5-carboxylase small subunit genes (Herrera-Estrella *et al*, 1984; Morelli *et al*, 1985) and many others.

Initial studies of plant gene expression regulation using *Agrobacterium*-mediated transformations include those of Broglie *et al*, 1984 and Herrera-Estrella *et al*, 1984. Broglie *et al*, 1984 transformed *Petunia* using mutation contructs in putative regulatory regions to investigate effect on gene expression. Herrera-Estrella *et al*, 1984 transferred the *Pisum ss* - CAT chimeric gene to *N.tabacum*. Expression of this gene was light-inducible in chloroplast - containing tissue.

DNA sequences required for promoter activity have been defined for the light induced ribulose-1,5-bisphosphate carboxylase small subunit gene *rbcs*-E9 (Morelli *et al*, 1985). Through deletion mutation experiments a conserved 33bp region close to TATA box was found to be sufficient to confer light inducibility of the small subunit gene. The pea chlorophyll a/b binding protein gene light-inducible and tissue-specific expression was investigated by Simpson *et al*, 1985. A 0.4kb sequence was found to confer such expression regulation in transgenic tobacco. Kaulen *et al*, 1986 studied expression of chimeric chalcone synthase (*chs*) - *nptII* gene in transgenic tobacco cells and found that induction of this gene was mainly dependent on UV - B photoreceptor activity and optimum expression occurred using 1.2bp of the 3.9bp *Antirrhinum majus chs* promoter.

Finally, another area of exploitation within plant biotechnology is tissue culture for the production of secondary metabolites. The role such technology has in the pharmaceutical industry as well as in dye and flavouring production is reviewed by Curtin, 1983 and Rhodes *et al*, 1987. Secondary metabolites which have been produced via plant tissue culture include Shikonin, a dye, antibacterial and antiflammatory drug produced in Japan. Compounds such as digoxin from *Digitalis* and the cancer drugs vinblastine and vincristine could be usefully produced by plant tissue culture.

1.2 LOTUS CORNICULATUS

Lotus corniculatus L., commonly called Bird's Foot Trefoil, is a temperate forage legume of the Loteae tribe and a member of the Leguminosae family. The geographical origin of *L.corniculatus* is the Mediterranean basin, but it is now naturalised in other parts of the world where it has been introduced, including N. America, New Zealand, and Australia.

Agriculturally *Lotus* is of more importance in North America than in Britain, with 8000 tonnes of seed sown in North America and 66 tonnes imported and sown in Britain, (MAFF statistics, 1983). Its main use is as a bloat-safe forage crop. Other features of the plant which contribute to its agricultural importance include its ability to give good drought resistant ground cover, even on poor quality pastureland, it is also winter-hardy, long-lived and deeply rooted. *L.corniculatus* has a higher tolerance to poor soil than other leading forage legumes such as *Trifolium repens* (White Clover) and *L.pedunculatus* (Big Trefoil), and is drought resistant (Charlton *et al*, 1978) although it also grows in wet places (Zanstra and Grant, 1968). *L.corniculatus* can be used for pasture soil improvement and erosion control. *L.corniculatus* possesses two defence mechanisms which allows it to withstand herbivore grazing; the presence of cyanide (Jones, 1962) and the presence of tannin. There is a negative correlation between cyanide production and tannin content. One drawback to this crop is that seed production is poor. Two major factors were shown to conribute to low seed production: low proportion of assimilates directed to seed production and indeterminate flowering habit in association with dehiscent pods (McGraw and Beuselick, 1983). In an attempt to reduce the high frequency of pod dehiscence, which may cause 50% loss of seed yield in *L.corniculatus*, embryo rescue techniques were used to produce two new interspecific *Lotus* hybrids: *L.alpinus x L.conimbricensis; L.burtii x L.ornithopodivides* (ODonoughue and Grant, 1988). The reduced shattering trait in the hybrids could be introduced via amphidiploidy into *L.corniculatus*. Phillips and Keim, (1968) hybridised *L.corniculatus x L.conimbricensis* in an attempt to transfer the indehiscent seed pod character of *L.conimbricensis* to *L.corniculatus*. However hybrid plants exhibited low

pollen fertility. Wright *et al*, 1987 achieved the first somatic hybridisation of *L.corniculatus x L.conimbricensis* but hybrids were sterile.

In comparison to other forage legumes such as White Clover (*T.repens*) and Alfalfa (*M.sativa*), *L.corniculatus* also has a good nutritional composition with respect to its possible contribution to the food chain. Forage legumes in general are an important source of phosphorous, iron, and some water-soluble vitamins. However, their protein is deficient in sulphur-containing amino-acids and is low in digestibility. Nevertheless, within animal husbandry, the forage legumes concentrate foliar protein and so there is a higher protein intake than would occur on a grass only diet. The effect of the nitrogen-fixing ability of *L.corniculatus* is also important, its use thus helping to alleviate a requirement for addition of nitrogenous chemicals to the soil.

The most recent model for the ancestry of *Lotus corniculatus* (Ross & Jones, 1985) suggests that it is a hybrid between *L.alpinus* or *L.tenuis* (probably as female parent) and *L.uliginosus* (probably as male parent); chromosome doubling then followed. This hypothesis is based on various characteristics studied, including tannin and phenolic content, morphology, cytogenetics and *Rhizobium* specificity. Raelson and Grant, (1988) made a study of the various proposed hypotheses of the origin of *L.corniculatus* using isoenzymes to establish the relationship between the diploid species *L.alpinus*, *L.japonicus*, *L.tenuis*, *L.uliginosus* and the tetraploid *L.corniculatus*. Previously, all these diploid species were implicated as being an ancestral parent of *L.corniculatus*. They proposed that the combination of *L.japonicus* x *L.alpinus* or *L.tenuis* x *L.alpinus* could be the origin of *L.corniculatus*. Despite the similarities of *L.corniculatus* and *L.uliginosus* with respect to tannin content the latter was discounted as being a parent of *L.corniculatus*.

The modern *Lotus corniculatus* is an out-breeding tetraploid (2n=4x=24) species, pollinated by members of the Hymenoptera. *Lotus corniculatus* provides a well established regeneration system with various explants including leaf, hypocotyl and cotyledon tissue being competent for full regeneration, and so is a suitable forage

legume with which to establish a "model" system for plant biotechnology. Much work has been done on the *in vitro* culture of this species. Anther culture methods were developed by Niizeki and Grant, (1971) with subsequent shoot and root differentiation occuring on a medium developed by Miller, (1963) containing IAA and kinetin or BAP. Hypocotyl, ovary and stem - derived callus was also found to generate shoots, (Tomes, 1976). Following this Tomes, (1979), established a rapid clonal propagation method for *L.corniculatus* in which the presence of the cytokinin, BAP, was indicated as being important. Regeneration from protoplasts derived from several explant types of *L.corniculatus* has been described (Ahuja *et al*, 1983a; Webb, 1987). Investigations have been directed towards levels of variability occurring in *L.corniculatus* callus in culture, with the conclusion that somaclonal variation increases with time in culture, with a concurrent loss of regenerative ability of the tissue (Webb, 1987).

In a comparison of somaclonal variation in regenerated plants derived from leaf explant tissue and leaf or cotyledonary protoplasts, Webb and Watson, (1991), found that similar levels of morphological and cytological changes were found in both protoplast-derived cultures, and that such levels were greater than in the regenerants derived by organogenesis from leaf explants. However, in all cases the majority of regenerants exhibited no major abnormality, more than 90% leaf explant derived plants and more than 80% of protoplast derived plants maintained their chromosome numbers during culture and were morphologically stable.

1.3 GENETIC MANIPULATION OF LEGUMES

1.3.1 Forage Legumes

A major aim of genetic manipulation of forage legumes is to produce bloat-free varieties, since the crops have widespread usage as animal feed both through grazing or as a harvested product. It is hypothesised that condensed tannins present in some forage legumes, including *Lotus corniculatus*, prevent bloat by precipitating soluble leaf proteins and thus are a desirable characteristic.

Leaf tannin production in *Lotus* is inherited by a dominant gene (Ross, 1983). It has been proposed that other useful traits, including resistance to disease, microbial digestion and mechanical damage of seeds, are also transferred when the bloat-safe character is introduced to new hosts. One approach to introducing the bloat-free trait to other legume species has been through interspecific hybridisation, such as that between *T.repens* and *T.arvense* (Bhojwani and White, 1982). Another approach is at the intergeneric level, when *L.corniculatus* and *O.viciifolia* are the donor species (Ahuja *et al*, 1983b; Ghose, 1988). It is interesting to note that even the *Medicago sativa* contains tannins within its seed coat and so may possess some genes necessary for tannin production. Genetic mutation to achieve continued expression of such genes may be an alternative to intergeneric hybridisation to obtain bloat-safe alfalfa (Goplen *et al*, 1980). Other traits which could be usefully transferred within forage legumes are the rhizomatous growth habit found in *Trifolium ambiguum* and *T.medium*, which helps a plant to withstand grazing, and also the disease resistance against *Sclerotinia trifolium* (clover rot) found in *T.alpinum* and *T.suffocatum*. As White clover is of major importance as a forage crop in Europe, it would be beneficial to agriculture if such characteristics could be introduced to this species, though achieving such aims by genetic engineering is limited by the need to isolate the relevant genes.

An initial requirement for genetic manipulation is the development of culture and regeneration procedures for the species concerned. Reviews of legume tissue culture have been published (Mroginzki and Kartha, 1984; Hammatt *et al*, 1986) so this subject will only be briefly addressed here. Within the genus *Trifolium*, whole plant regeneration was obtained via organogenesis of *T.pratense* callus (Phillips and Collins, 1979) and *T.repens* (Gresshoff, 1980). Ahuja *et al*, 1983b established culture conditions for *T.repens* and *Onobrychis viciifolia* protoplasts, however although differentiation did occur from *T.repens*-derived protoplasts, shoots were abnormal. Such inhibition of shoot development had been previously shown by Bhojwani and White, 1982. White, 1984 developed a regeneration system

for *T.repens* cell cultures via meristemoid formation. Under hormone-free solid culture conditions shoot differentiation occurred followed by root production.

Regeneration by embryogenesis is exemplified by *M.sativa* studies. Pezzotti *et al*, 1984, regenerated whole plants from mesophyll, root and cell suspension protoplasts of *M.sativa*. A similar system was established by Atanossov and Brown (1984). In contrast, *M.arborea* was regenerated via meristemmatic organogenesis from mesophyll and rootlet protoplast-derived callus (Mariotti *et al*, 1984a).

Even when successful tissue culture conditions have been determined, a further major limitation to progress in genetic engineering is the development of efficient transformation protocols in many forage legumes. Table 1.1, shows some of the progress achieved in transformation of temperate forage legumes.

The strategy most common in transformation of forage legumes has been to employ *Agrobacterium*-mediated systems. Important factors influencing the success of transformation in legume species include regenerative ability during axenic culture and susceptibility to *Agrobacterium* infection. Initial work used wild-type oncogenic *Agrobacterium* strains, and transformation events were thus easily recognised by the development of galls or hairy roots.

The first successful attempts to genetically transform forage legumes involved *Medicago sativa* (alfalfa), for which tissue culture conditions were established, and used an oncogenic *Agrobacterium tumefaciens* strain. Variation in the phenotypic effects of oncogenic *Agrobacterium* strains on different legume species have been observed. For example, *A.tumefaciens* T37 nopaline strain caused disorganised galls on *M.sativa* whereas on *L.corniculatus* and *Onobrychis viciifolia* disorganised, teratomous (shooty) or rooty tumours were induced by inoculation with various nopaline and octopine *A.tumefaciens* strains (Mariotti *et al*, 1984b; Webb, 1986). The tumours which develop on *Trifolium* species however generally produced root structures. Only shoots from the

Table 1.1: Transformation of Temperate Forage Legumes (modified from Nisbet and Webb, 1990)

Forage Legume	Explant Used	Vector Type	Gene	Result	Refs.
Lotus corniculatus	Plants	*A.tumefaciens* wild-type	—	—	Webb, 1986
	Plants	*A.tumefaciens* & *A.rhizogenes* oncogenic, multitransformation	—	Transgenic plants	Petit *et al*, 1986
	Plants	*A.rhizogenes* wild-type	—	Transgenic plants	Petit *et al*, 1987
	Plants	*A.rhizogenes* oncogenic, cointegrate	Soybean Leghaemoglobin-CAT (Lbc, 5'3' CAT)	Transgenic plants	Stougaard *et al*, 1986
		A.rhizogenes oncogenic, binary	Soybean Leghaemoglobin-CAT (Lbc, 5'3' CAT)	Transgenic plants	Stougaard et al, 1987
	Leaves	*A.tumefaciens* oncotgenic binary	*neo-nos-legA*	Transgenic plants	Nisbet, 1987
	Stem	*A.rhizogenes* oncogenic, binary	*Gln-r*-GUS Gln-ß-GUS	Transgenic plants	Forde *et al*,1989
	Seedlings	*A.rhizogenes* oncogenic	Soybean Leghemaglobin-CAT	Transgenic plants	Jensen *et al*, 1986
	Stem/ hypocotyls	*A.tumefaciens* disarmed, cointegrate	*nos-nptII*	Transgenic plants	Ghose, 1988
		A.tumefaciens carrying pRi	—	Transgenic roots	
		A.rhizogenes oncogenic, binary	*nos-nptII*-GUS	Transgenic plants	Tabaeizadeh, 1989
	Plants	*A.rhizogenes* oncogenic, binary	*Srglb3-GUS*	Transgenic plants	De Bruijn *et al*,1989
Medicago sativa	Plants	*A.tumefaciens* wild-type	—	—	Mariotti *et al*, 1984b
	Stem	*A.tumefaciens* binary	*nos-nptII*	Transgenic plants	Shahin *et al*, 1986a
	Embryo	*A.tumefaciens* binary	lb-cat	Transgenic plants	De Bruijn *et al*, 1989
	Protoplasts	*A.rhizogenes* wild-type	—	Transgenic plants	Spano *et al*, 1987
		A.rhizogenes oncogenic, binary	—	Transgenic plants	Sukhapinda *et al*, 1987
		Microinjection with pTi	—	—	Reich *et al*, 1986
Medicago varia	Stem	*A.tumefaciens* disarmed	*nos-nptII*	Transgenic cells, tissues and plants	Deak *et al*, 1986
	Stem & leaf	*A.tumefaciens disarmed*	*nos-nptII*	Transgenic plants	Chabaud *et al*, 1988
Onobrychis viciifolia	Plants	*A.tumefaciens* wild-type	—	—	Webb, 1986
Trifolium hybridum	Plants	*A.tumefaciens* wild-type	—	—	Webb, 1986
Trifolium pratense	Plants	*A.tumefaciens* wildtype	—	—	Webb, 1986
Trifolium alexandrinum	Stem/ hypocotyls	*A.rhizogenes* oncogenic, binary	*nos-nptII*	Transgenic roots	Ghose, 1988
Trifolium repens	Plants	*A.tumefaciens* wild-type	—	—	Webb, 1986
	Stolon	*A.tumefaciens* disarmed, binary	*nos-nptII*	Transgenic shoots	White & Greenwood, 1987
	Seedlings	*A.rhizogenes* oncogenic, binary	*nptII-psi*	Transgenic roots	Diaz *et al*, 1989
	Stem/ hypocotyls	*A.rhizogenes* oncogenic, binary	*nos-nptII*	Transgenic roots	Ghose, 1988

nopaline teratomous galls of *L.corniculatus* were capable of true development and elongation. The inoculation tests carried out by Mariotti *et al*, (1984b) demonstrated a strong plant variety - bacterial strain specificity of crown gall induction. The *A.tumefaciens* strain Ach5 was the most virulent octopine type *Agrobacterium*, and T37 was the most virulent nopaline type with alfalfa plants. However, investigation also found that after tumorigenesis induced by T37 *A.tumefaciens* strain, transformed cells were unable to regenerate shoots in culture, although such regeneration is exhibited in untransformed tissue. This loss of regenerative ability may stem from the tumorigenicity causing alteration to endogenous hormone levels, so inhibiting totipotency. Host-*Agrobacterium* specificity was further exemplified in *L.corniculatus* and *O.viciifolia* (Webb, 1986). *O.viciifolia* shoot meristems and petiole explants were most responsive to crown gall induction when exposed to C58 nopaline or B6 octopine *A.tumefaciens* strains. All octopine *A.tumefaciens* strains tested resulted in a lower gall induction frequency than the nopaline *A.tumefaciens* strains.

The possible influence of age and type of plant tissue on transformation was also studied (Armstead and Webb, 1987). Six-seven day old axenic cotyledon tissue was found to be particularly responsive to infection by wild-type *Agrobacterium.* When leaf tissue alone was investigated, it was found that a higher proportion of young leaf material developed tumours than older tissue.

The advent of disarmed *A.tumefaciens* binary vector systems has led to further work with *A.tumefaciens*-mediated systems. Forage legumes which had previously demonstrated infectiveness with wild-type *Agrobacterium* would potentially respond to the new disarmed vectors. This, however, has not been found to be true for all the species, including some known to be susceptible to *A.rhizogenes*-mediated transformation, such as *L.corniculatus* (see table 1.1).

The forage legume genus most conducive to transformation by a variety of techniques has been *Medicago*. Shahin *et al*, 1986a used a stem explant cocultivation method in which *M.sativa* explants were exposed to disarmed *A.tumefaciens* LBA4404

M.sativa protoplasts resulted in the transfer of pTiC58 at reproducible transformation frequencies of 15-26% (Reich *et al*, 1986). Thus more than 70 transformed callus lines were obtained. Meanwhile, Deak *et al*, 1986 generated transgenic plantlets from shoot segments of *M.varia A2*. This genotype was chosen as one with high embryonic capacity. Once again the *nos-nptII* gene construct was transferred as part of pGA71 by disarmed *A.tumefaciens* A281 within a prolonged, 3 day, liquid cocultivation. Deak and coworkers also successfully applied their transformation technique to *M.coerulea* and *M.sativa*. A similar transformation technique for *M.varia* A2 was developed by Chabaud *et al*, 1988 with a transformation frequency of >70%. The 4 day cocultivation of leaf and petiole explants utilised a nurse cell culture and acetosyringone *vir* induction. Thus via embryogenesis transgenic plants were again generated. The use of disarmed *A.tumefaciens* vector systems allowed tissue development free of the phytohormone problems previously shown (Mariotti *et al*, 1984). Recently, De Bruijn *et al*, 1989 used a *A.tumefaciens*-mediated system to study soybean leghemoglobin gene expression as part of a CAT-chimeric construct in alfalfa.

Use of *A.rhizogenes*-mediated transformation was similarly successful in *Medicago*. Spano *et al*, 1987 demonstrated wild type *A.rhizogenes* infection of *M.sativa* whilst Sukhapinda *et al*, 1987 used a *A.rhizogenes* based binary vector system to generate transgenic plants. Those plants produced by Spano and coworkers exhibited short, though more numerous, internodes and the increased number of stems had smaller leaves compared to untransformed plants. These pRi T-DNA generated physical features gave overall greater leafiness to the transgenic plants. With such a trait relating to plant productivity and nutritive value Spano *et al*, proposed that *A.rhizogenes* transformation could be used as a method to yield stable, heritable morphological alterations in *Medicago*. *A.rhizogenes*-mediated transformation producing useful morphological traits in *Medicago* was again demonstated by Sukhapinda *et al*, 1987. The fertile, *nos* expressing transgenic plants possessed shallow but highly branched root systems and this character is

associated with the desirable properties winter hardiness and high yield (McIntosh and Miller, 1980).

Thus, *Medicago* species were confirmed as versatile forage legumes for genetic transfer but it is the possible use of *Agrobacterium tumefaciens*-mediated transformation which makes these *Agrobacterium*-plant systems so significant.

Members of the genus *Trifolium* have proved difficult to use in transformation experiments, with the regeneration of whole transgenic plants being a major barrier to progress. Transgenic shoots were obtained by White and Greenwood, 1987 when *A.tumefaciens* LBA4404 and GV3850 vectors were used to inoculate stolon explants from a "high regenerative" genotype of *T.repens*. Four constructs were transferred in the experiments, each carrying kanamycin resistance as the selection parameter. Ghose, 1988 produced transgenic rootsm *T.repens* and *T.alexandrinum* by seedlimg hypocotyl inoculation and stem segment cocultivation.

Agrobacterium rhizogenes-vector systems have also been used in association with *T.repens*. For example, a pBIN19 binary system carrying *psl* was used to investigate the *Rhizobium* - plant specificity in the nitrogen fixation symbiosis (Diaz *et al*, 1989), see also Introduction 1.1. Use of transgenic roots derived from the *A.rhizogenes*-mediated transformation is particularly important in a species so recalcitrant in culture.

A number of techniques similar to those employed with *Medicago*, have been used to transform *L.corniculatus*. A variety of explants have been used during the development of *A.rhizogenes*-mediated transformation of *L.corniculatus* and the systems utilised in investigations of gene expression. Stougaard *et al*, 1986 developed a cointegrate plasmid with a soybean leghaemoglobin *lbc3*-CAT chimeric gene construct inserted into pRi T_L-DNA. Tissue-specific CAT expression was confined to nodule tissue and in *L.corniculatus* transgenic plant developmental regulation of the *lbc3* promoter was maintained. In association with an

"intermediate integration vector" based on pRi T_L-DNA, Stougaard *et al*, 1987 cotransferred binary vector T-DNA into *L.corniculatus* "hairy roots". Normal constitutive or organ-specific expression of transferred kanamycin resistance and *lbc3* 5'3'CAT chimeric genes was maintained in the transgenic plants. *L.corniculatus* was once again used as the host plant (Forde *et al*, 1989) in studies investigating spatial and developmental expression of *Gln-τ* and *Gln-β* genes encoding two isoenzymes of *Phaseolus vulgaris* glutamine synthetase. The *Gln-β* gene is expressed preferentially in roots and *Gln-τ* gene expression is essentially restricted to developing nodule tissue. An *A.rhizogenes*-mediated binary transformation system was used to transfer *Glnτ-GUS* and *Glnβ-GUS* chimeric genes into *L.corniculatus*. It was found that a 2kb fragment from the 5'-flanking region of either *Gln* genes could confer the predicted expression to the reporter GUS gene. The *Gln τ*-GUS activity was selected at only low levels in roots and shoots whilst at a higher level in the nodules.

Studies of the nitrogen fixation symbiosis have also benefited from the application of genetic transformation techniques based on *A.rhizogenes* to forage legume species (Stougaard *et al*, 1986 and 1990, Petit *et al*, 1987, Jorgensen *et al*, 1988 and De Bruijn *et al*, 1989). These studies have investigated the control of expression of nodule specific genes (leghaemoglobin and genes of unknown function) through the use of promoter-reporter gene constructs in transgenic *L.corniculatus*, *M.sativa* and *T.repens* plants.

Investigations aiming to improve and optimise *L.corniculatus* transformation techniques were carriedout (Webb, 1986; Nisbet, 1987; Ghose, 1988; Tabaeizadeh, 1989). Investigations established some of the optimal parameters concerned with its transformation. Webb, (1986) determined that wild type nopaline-producing *A.tumefaciens* strains showed most potential with respect to shoot differentiation following the tumour induction. Nisbet, (1987) attempted to introduce *neo*, *nos* and *legA* genes to *L.corniculatus* using leaf explant co-cultivation with *A.tumefaciens* LBA4404 carrying a Bin19 derived binary construct. Nopaline positive calli were

produced and gene transfer was revealed by DNA analysis showing the presence of *legA* in 3 transgenic plants. However, later tests indicated that the kanamycin selection system was inefficient since no further transformants were regenerated.

Ghose, 1988 achieved limited success with a C58 derived *A.tumefaciens* cointegrate vector-hypocotyl cocultivation technique, with 4% transformation frequency resulting in the regeneration of 8 plants. Also, using an *A.rhizogenes* and *A.tumefaciens* [pRi] system, whole plants were regenerated from inoculated seedling hypocotyl and stem segment derived hairy roots.

Tabaeizadeh, 1989 developed a hypocotyl and stem explant cocultivation technique and produced *A.rhizogenes*-mediated transformation of *L.corniculatus*. The method was over a short timescale, with only a 2 month period between inoculation and the production of transgenic plants. The cocultivation period on solid medium extended over 2-3 days and following decontamination explants were regenerated on a B_5 (Gamborg, 1968) based medium containing additional BAP. The transformed plants regenerated on kanamycin selection were morphologically unaltered by the gene transfer process.

A potential problem to the use of *A.rhizogenes* based transformation systems is phenotypical abnormalities in the transformaed plant due to the presence of the Ri plasmid T-DNA; clearly the use of a disarmed *A.tumefaciens* system would avoid these difficulties.

Transformation is also a useful "tool" for the investigation of complex intra-plant or plant-bacterial interactions such as gene regulation and the legume-*Rhizobium* symbiosis e.g. De Bruijn *et al*, (1989) and Diaz *et al*, (1989). De Bruijn and co-workers studied expression of soybean leghemoglobin (*lb*), in transgenic *M.sativa*. Also the introduction of *S.rostrataglb3* (*slb3*) into *L.corniculatus* indicated that both CAAT and TATA regions are necessary for nodule-specific expression. It was hoped that these *Agrobacterium rhizogenes*-mediated transformation

experiments would help to elucidate the signal transduction pathway concerned with nodule-specific induction of the leghemoglobin genes. Diaz *et al.* investigated the role of root lectins in *Rhizobium*-legume specificity, by introducing the pea lectin gene (*psl*) into *T.repens* using the *A.rhizogenes* vector system. The transgenic white clover plants were inoculated with *Rhizobium leguminosarum bv. viciae* and nodules did develop though delayed and of abnormal morphology. Nodule development was completely absent on untransformed white clover when inoculated with this *Rhizobium* strain. This experiment was the first report of the use of a genetically engineered host plant being used to overcome the normal plant-bacterium specificity interaction.

1.3.2 Other Legumes

Transformation systems developed for other legumes are summarised in table 1.2. Although some success has been obtained with *Stylosanthes*, *Vigna* and *Glycine* spp., in general the legumes are refractory to transformation. Eapen *et al*, 1987 developed a protoplast regeneration system using mesophyll *Vigna aconitifolia* protoplasts. Cocultivation of protoplasts with *A.tumefaciens* carrying *nos-nptII* chimeric gene resulted in 23% of colonies formed exhibiting cotransfer of *nos* and *nptII* genes. Callus production was followed by whole plant regeneration. Subsequently, Manners (1988), used a disarmed *A.tumefaciens* binary vector system to obtain transgenic *Stylosanthes humilis* which expressed *nos* and *nptII*. Although both chimeric and phenotypically abnormal plants were regenerated, the absence of a relationship between the presence of foreign genes and the abnormal phenotype suggests that with selection, genetic manipulation of *S.humilis* is still a viable aim for future research efforts.

Table 1.2: Transformation of Tropical and Sub-tropical Legumes (modified from Nisbet and Webb, 1990)

Legume	Explant Used	Vector Used	Gene	Result	Reference
Stylosanthes spp.	Leaves	*A.tumefaciens* wild-type	-	-	Manners, 1987
S.humilis	Leaves	*A.tumefaciens* oncogenic, binary	*nptII*	Transgenic shoots	
	Leaves	*A.tumefaciens* disarmed, binary	*nos-nptII*	Transgenic plants	Manners, 1988
Sesbania rostrata	Plants	*A.tumefaciens* wild-type	-	-	Vlachova *et al*, 1987
	Plants	*A.rhizogenes* wild-type	-	-	
Vigna unguiculata	Leaves	*A.tumefaciens* disarmed, binary	CPMV mRNA	Transgenic callus	Garcia *et al*, 1987
Vigna aconitifolia	Protoplasts	*A.tumefaciens*	*nos-nptII*	Transgenic plants	Eapen *et al*, 1987
	Protoplasts	PEG-uptake	*nos-nptII*	-	Kohler *et al*, 1987a
	Protoplasts	PEG-uptake	CaMV-*nptII*	-	Kohler *et al*, 1987b
Glycine spp.	Plants	*A.tumefaciens* wild-type	-	-	Byrne *et al*, 1987
	Plants	*A.tumefaciens* oncogenic	*nptII*	-	
G.canescens	Plants	*A.rhizogenes*	-	Transgenic plants	Rech *et al*, 1988
G.max	Plants	*A.tumefaciens* wild-type	-	-	Pederson *et al*, 1987
	Plants	*A.tumefaciens* oncogenic	Rubisco -*nptII*	Inducible expression	Facciotti *et al*, 1985
	Protoplasts	*A.tumefaciens* oncogenic	*nos-nptII*	Transgenic callus	Baldes *et al*, 1987
	Protoplasts	Electroporation	*nos-nptII*	-	Christou *et al*, 1987
	Cotyledons	*A.tumefaciens* disarmed	*nptII*-GUS *nptII*-EPSP	Transgenic plants	Hinchee *et al*, 1988
	Protoplasts	PEG - electroporation	CaMV-CAT-*nos*	Transient and stable expression	Lin *et al*, 1987
	Embryos	Particle acceleration	*nptII*-GUS	Transgenic plants	Christou *et al*, 1988; Christou, 1990; McCabe, 1988.

1.4 AGROBACTERIUM TUMEFACIENS AND TRANSFORMATION

1.4.1 *Agrobacterium tumefaciens* and the Ti-plasmid

Crown gall is a neoplastic disease affecting 150 plant genera, the majority of which are dicotyledons. In 1907, Smith and Townsend first recognised the gram negative bacterium *Agrobacterium tumefaciens* as being the causative agent. However, it was not until 1948 that the involvement of the bacterium in transformation of plant cells was realised (Braun and Mandle, 1948).

In 1952, Braun first introduced the concept of the "tumour-inducing principle", which was subsequently, demonstrated to be equivalent to plasmid DNA, (Van Larebeke *et al*, 1974). This plasmid, known as the tumour-inducing DNA, or Ti-DNA, is 150-250kb in size in wild-type *Agrobacterium* (Shah *et al*, 1987). The conclusive experiments involved curing *Agrobacterium*, rendering them plasmid-less and conjugating such strains with a virulent strain. It was found that the progeny of such a cross were virulent.

The wild-type Ti-plasmid encodes for a number of functions in addition to crown gall induction, which include host range, opine production and replication/transfer of the plasmid. Ti-plasmids may be classified according to the type of amino-acid derivative biosynthesis and catabolism they induce in transformed plant cells, giving octopine, nopaline, agropine and agrocinopine families. The important zones of homology between the Ti-plasmids of the different families are the Transfer DNA (T-DNA) and the virulence, *vir* region. The first of these directs the transfer of DNA to the plant cell and the second determines the ability to transfer the T-DNA.

1.4.1.1. Transfer of Bacterial T-DNA to the Plant Genome

The 13-25kb of DNA which actually becomes integrated into the genome of transformed plant cells is only a small part of the 150-200kb of the conjugative Ti-plasmid. Work using wild-type Ti-plasmids provided a foundation upon which the various transformation systems available nowadays could be developed.

The transfer mechanism requires three components:

(1) T-DNA

(2) Virulence genes on the *Agrobacterium* plasmid

(3) Virulence genes on *Agrobacterium* chromosomes

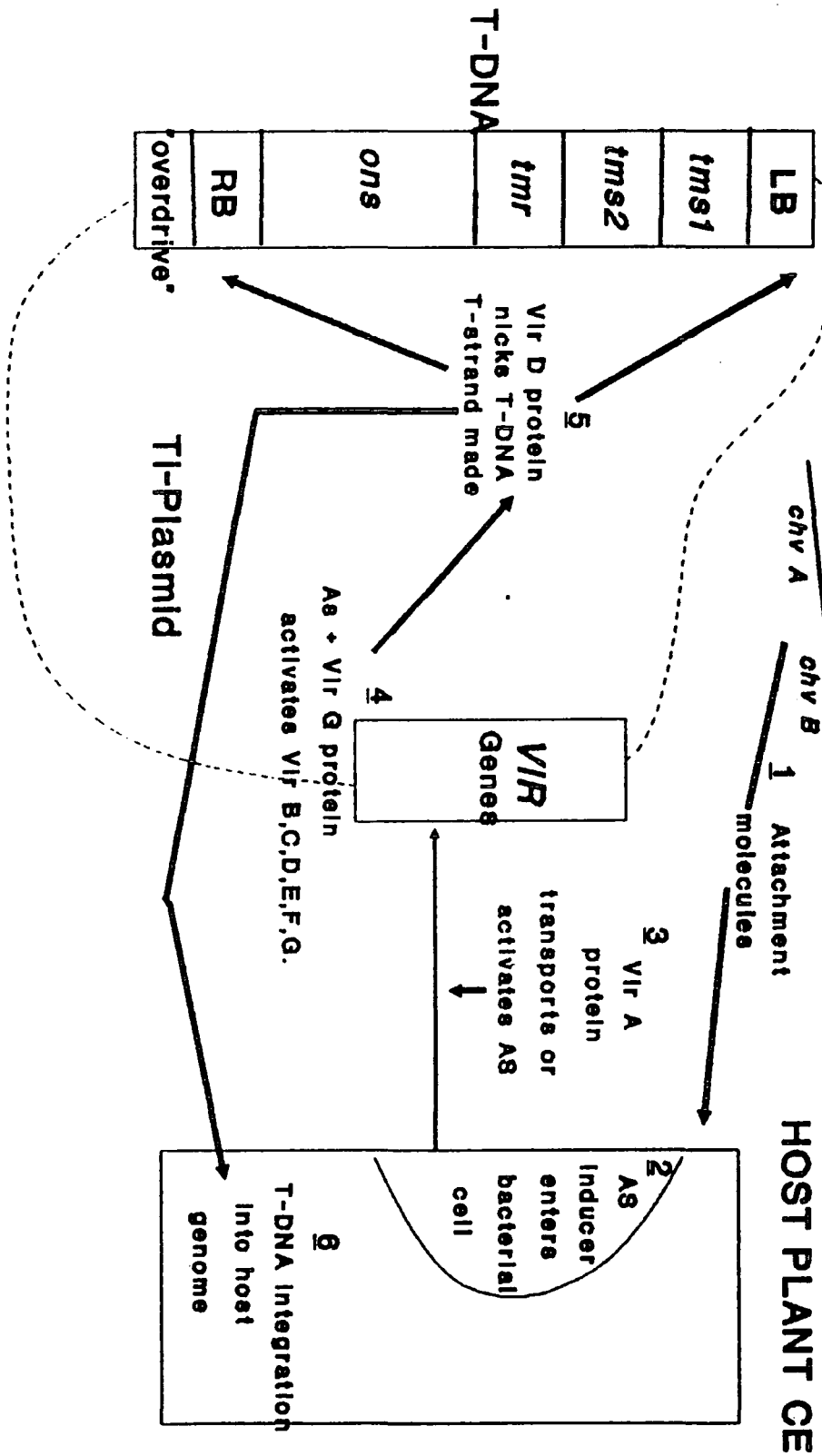
Figure 1.2 illustrates some of the processes involved in the bacterial-plant conjugation (modified from Lichtenstein, 1986).

(1) The T-DNA

The portion of the bacterial plasmid found to be present in the transformed cells is known as transfer DNA or T-DNA. Much of the work done in characterizing this bacterial DNA utilised octopine-type tumours. These are easily produced and the DNA inserted is shorter than that of the nopaline type.

Thomashow *et al* (1980) established that the octopine type T-DNA can be divided into two portions, left (T_L) and right (T_R), the T_L region being 13-14kb and the T_R region 6-8kb in length. At both ends of these segments imperfect repeat border sequences are present (Zambryski *et al*, 1982; Yadav *et al*, 1982; Simpson *et al*,1982). These border sequences are 25bp in length, Machida *et al*, 1986 and are unique *cis* active sites allowing the T-DNA to be inserted into the plant genome. Much research has substantiated the importance of the right hand border in driving T-DNA transfer (Ooms *et al*, 1982; Shaw *et al*, 1984; De Beuckleer *et al*, 1981; Leemans *et al*, 1982). Its activity is polar with T-DNA transfer occurring generally from right to left, hence the dependency upon orientation within the *Agrobacterium* genome, Wang *et al* (1984), so either deletions or a reversal of the right-hand T-DNA border results in failure in T-DNA transfer. Work by Wang *et al*, 1987 indicated that the 25bp direct repeats alone can promote T-DNA transfer but the flanking regions have an important influence on this. With respect to the RH border, the flanking regions improve transfer and enhancement is particularly strong in nopaline derived pTi. At the left T_L borders, these flanking sequences when present, result in attenuated left border activity. Wang *et al*,

Figure 1.2: Generalised Diagram of the Bacterial-Plant Conjugation (modified from Lichtenstein , 1986)



1987 also found an inverse correlation between the degree of nicking events and the relative tumorigenicity of borders.

The difference in relative importance of the left and right hand borders may be influenced not just by positional criteria, but also by the presence of an "enhancer" sequence discovered in recent years. This is a 24 bp sequence, 13 and 14 bp from the right hand border repeats, of the octopine T_L and T_R regions respectively (Peralta *et al*, 1986; Van Haaren *et al*, 1987). Peralta and co-workers designated this region as the "overdrive". This has a 8 bp core which it is proposed enhances interaction between the right-hand border and *vir* proteins. The existence of similar overdrive sequences close to other types of Ti or Ri plasmid is unknown, though Peralta and co-workers, as well as Van Haaren *et al*, (1988), have indicated the presence of sequences homologous to those representing the octopine overdrive sequence in nopaline and agropine Ri plasmid, near to their right hand border sequences.

Although T_R DNA controls T-DNA transfer, this segment is often absent from transformed cells. A highly conserved 9kb region of DNA however is present in both octopine and nopaline types. Transcriptional analysis has shown that 5 or 6 cross-hybridising transcripts of identical size, location and function, exist in both tumour types. Use of site-directed mutagenesis, whereby insertions or deletions are made in the T-DNA, allowed the organisation of the various functions of the DNA to be investigated.

Garfinkel *et al*, (1981) using the octopine plasmid pTi A6NC, demonstrated the presence of 3 loci, based on the phenotypes of tumours containing mutated T-DNA.

(a) *tms* (*aux*) locus (3.1kb)
Transcripts 1 and 2 - designates shoot morphology of the tumour.

(b) *tmr* locus (1.0kb)
Transcript 4 - designates root morphology of the tumour.

(c) *tml* locus (1.25kb)
Transcript 6b - designates size of tumour.

A fourth locus was added by Messens *et al*, (1985).

(d) *ons* locus

Transcript 6a - controls opine secretion.

In 1983, Ream, Gordon and Nester produced evidence that the *tms* and *tmr* loci were concerned with phytohormone production. It has been shown that gene 1 (iaaM) encodes for an enzyme of auxin biosynthesis (Inze *et al*, 1984) and gene 2 (*iaaH*) encodes for indoleacetamide hydrolase (Thomashow *et al*, 1984) on the auxin biosynthetic pathway. Thus the *tms* locus as a whole is responsible for indoleacetic acid production. Barry *et al* (1984) showed that the *tmr* locus (gene 4) encodes isopentenyl transferase, the enzyme involved in cytokinin biosynthesis. Other genes assigned specific functions include gene 6a which involves octopine/nopaline secretion from tumours (Messens *et al*, 1985). Gene 6b has been shown to be one conferring oncogenicity on *Agrobacterium* towards the plant host (Hooykaas *et al*, 1988). The T_L region also contains a gene designated as transcript 3, which encodes the appropriate opine synthase. Gene 5 codes for an enzyme synthesizing indole-3-lactic acid, this is an antagonist to auxin activity. It is thought to modulate auxin activity when at toxic levels in culture media and has no effect on endogenous auxin production. The loci of the T_L and T_R regions of an octopine-type Ti plasmid are illustrated in figure 1.3.

(2) Virulence genes on the Agrobacterium Ti plasmid

The 40kb *vir* region is located adjacent to the T-DNA LB repeat, (Garfinkel and Nester,1980). Work by Krens *et al*, (1985), using direct gene transfer techniques, first indicated that the genes within this segment of the pTi plasmid were not concerned with oncogenicity, and the region is not itself integrated into the plant genome but is necessary in the transfer of T-DNA between bacteria and plant cell. The vir region encodes the majority of the *trans* acting products necessary to promote T-DNA transfer (Hoekema *et al*, 1983). Over the past ten years, the *vir* regions of octopine and nopaline Ti-plasmids have been characterised. Seven transcripts have been designated as being part of the virulence region of an octopine Ach5 Ti plasmid and are named Vir

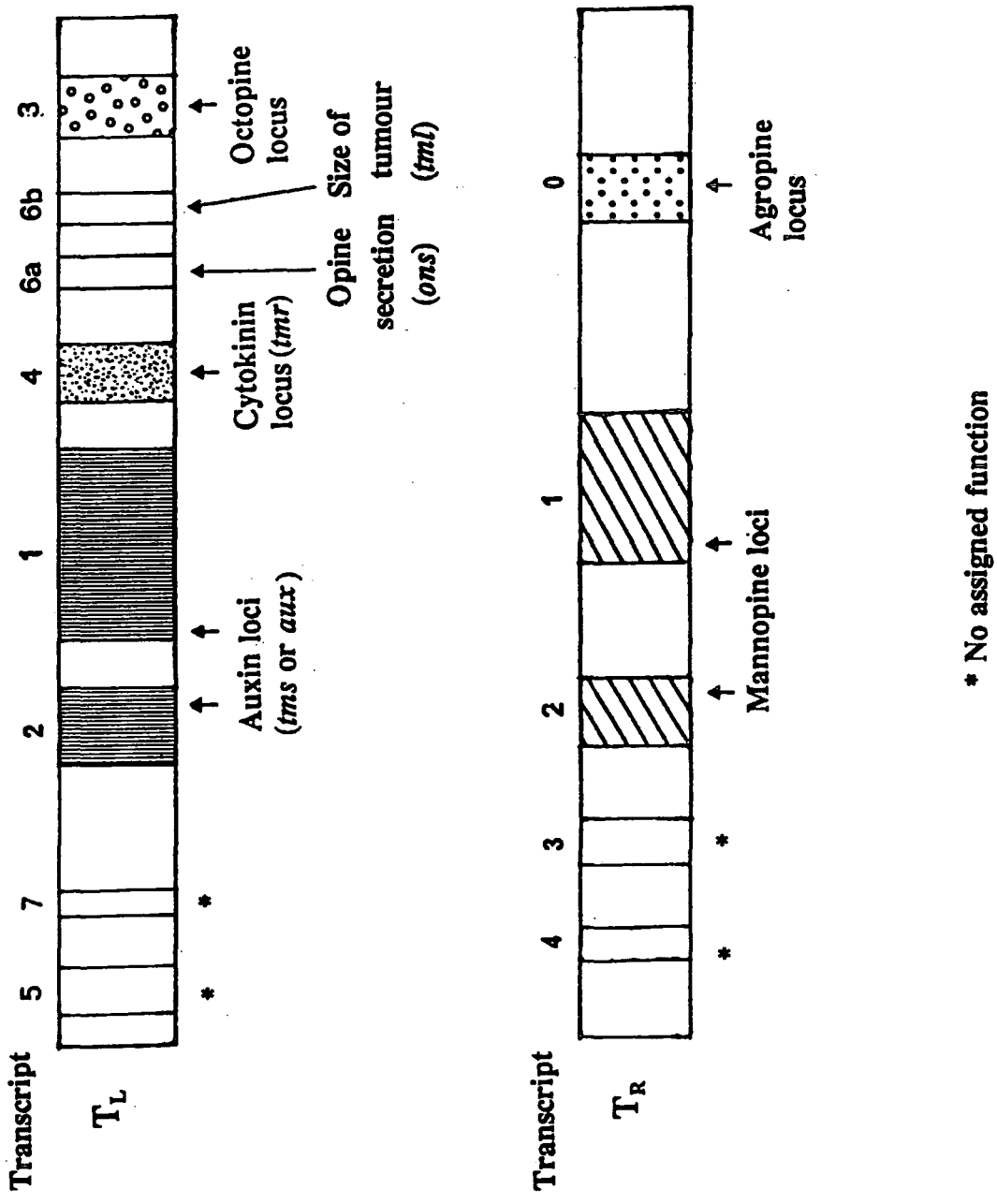
A-G, (Stachel and Nester, 1986). The nopaline C58 strain Ti-plasmid is organised similarly but with only six transcripts, Vir A-E and G, (Rogouski *et al*, 1987).

Mutation analysis showed that the vir loci can be divided into two classes (Melchers and Hooykaas, 1987).

(i) Essential *Vir* loci
Containing genes crucial for tumour induction on all plant species
- *Vir* A, B, D, G.

(ii) Host Range *Vir* loci
Determine host range of the plasmid and enhance the efficiency of transfer in the plant transformation process
- *Vir* C, E, F.

Figure 1.3: Diagram showing genes present on the T_L and T_R Regions of an Octopine Ti-plasmid.



More specifically it has been shown that *vir* E encodes a single-stranded DNA binding protein, which is thought to form a complex with a single-stranded T-DNA during the transfer process. A possible function of *vir* B is the direction of T-DNA transit through the bacterial membrane and *vir* C may enhance *vir* D enzyme production. *Vir D* encodes a site-specific endonuclease cleaving at a unique site within the 24 bp direct repeats at the ends of the T-DNA (Yanofsky *et al*, 1986). Stachel *et al*, (1986) proposed that *Agrobacterium* - *vir* induction is a specific event mediated by plant cell metabolites, released by wounded plant cells. The compounds, such as acetosyringone (AS) and α-hydroxyacetosyringone are specific inducers of these *vir* genes (Stachel *et al*, 1985). A sensor/regulator system has been indicated as existing in *Agrobacterium* with *vir* A encoding the sensor and *vir* G the regulatory component (Stachel and Zambryski, 1986). Stachel and Zambryski (1986) proposed that *Vir A* encodes a transport protein for a plant signal molecule and that *Vir G* encodes a positive regulator protein which acts together with the plant metabolite to activate *vir* expression. Melchers *et al*, (1989), investigated *vir* induction and demonstrated maximum *vir* stimulation after 5-6 hours and proposed that one or both of the regulatory proteins (*vir A* and G) affects the thermo-sensitive step in tumorigenesis.

(3) Virulence genes on Agrobacterium chromosomes

So far, four regions on chromosomes and *Agrobacterium* have been shown to have a direct role in the attachment of the bacteria to plant cells, all encoding for cell wall structural components or surface proteins*: chv* A/*chv* B; *psc*A(*exo*C); *att*. These chromosomal *vir* regions are constitutively expressed.

The precise T-DNA transfer mechanisms are still undefined but the first stages can be seen in part to be analogous to bacterial conjugation (Stachel *et al*, 1986; Stachel and Zambryski, 1986) and the later stages are comparable to virus-cell interactions (Citovsky *et al*,1988). It is thought that the first T-DNA associated molecular reaction in the transfer process is the specific nicking of the border repeats by the enzyme products of *vir* D, and that these nicks are the starting points for the production of

ssT-DNA (Albright *et al*, 1987). Two mechanisms for the production of such a single strand of DNA have been hypothesised:

(i) T-DNA replication on one template strand from RB nick towards LB cleavage; a new complement strand of T-DNA is thus produced, (Stachel *et al*, 1986). 5'- 3' helicase activity (*Vir D* encoded) unwinds the T-DNA from RB nick towards the LB cleavage freeing the new T-strand, which is then transferred to plant cells by a mechanism similar to bacterial conjugation, (Howard and Citovsky, 1990). Potential may exist to improve the efficiency of plant transformation by use of multiple copies of *vir* D genes, causing a reciprocal increase in *vir* induction initiation (Wang *et al*, 1990).

(ii) The involvement of double stranded circular T-DNA. Koukolikova-Nicola *et al*,(1985); Machida *et al*, (1986) suggested *vir D* activity resulted in the production of circular T-DNA. The subsequent transfer of this T-strand then resembles viral infection. Howard and Citovsky suggest the vir E product acts as a coat protein and *vir* D2 has an involvement in transfer as a "pilot protein". So, it is hypothesised that there is recognition between the *vir* D2 and E2 complex and the structural proteins in the bacterial cell wall and membrane. *Vir* B proteins may also have a role in this final process.

1.4.1.2. Integration of T-DNA into the Plant Genome

This process occurs by a random insertion event. Although many transformed plant cells will contain the full complement of T-DNA, deletions do occur as well as multiple copies. It is likely that these T-DNA rearrangements could occur at a number of stages in the transformation, such as in bacterial processing of T-DNA, during T-DNA transfer/integration, or at a post-integration stage via somaclonal variation.

1.5 AGROBACTERIUM RHIZOGENES AND THE Ri-PLASMID

Agrobacterium rhizogenes is the causative agent of hairy root disease in plants. The characteristic response of plants inoculated with *A.rhizogenes* is the proliferation of adventitious "hairy" roots from the infected cells (Hildebrand, 1934).

A large plasmid known as the root inducing, Ri-plasmid carries the virulence functions of the bacterium (White and Nester, 1980) and the genes conferring the hairy root phenotype to the host plant (Moore *et al*, 1979; Cardarelli *et al*, 1987). A range of opine compounds have been found in hairy root tumours, these varying according to the type of Ri-plasmid involved.

1.5.1. The T-DNA of the Ri-Plasmid

Over the last decade there has been much investigation of the T-DNA of the Ri-plasmid. Both agropine and mannopine type *Agrobacterium rhizogenes* strains contain two 15kb sections of T-DNA, separated by 16kb of non-integrating DNA (White *et al*, 1985).

The T_R T-DNA of these strains has been found to be comparable to Ti T-DNA, containing two loci (*aux*) corresponding to the Ti *tms* (White *et al*, 1985). Deletion of these *aux* loci results in *A.rhizogenes* strains becoming avirulent, so illustrating the critical role these genes have in the "hairy root" tumorigenesis. Work by De Paolis *et al* (1985), indicated that the opine genes are orientated in opposite directions in Ti and Ri plasmids.

In contrast to the *A.tumefaciens* system where tumour tissue requires functional auxin genes carried by the T-DNA to result in total hormone autotrophy, the hairy roots produced via the *A.rhizogenes* system only need auxin during the induction of hairy root proliferation, not for their continued growth, (Cardarelli *et al*, 1985).

The root loci present on the pRi T_L-DNA are designated as the *Rol A, B, C* and *D* genes. Work by Spena *et al*, (1987) and Schmulling *et al*, (1988) demonstrated the various phenotypes occurring in the plant regenerants containing different *rol* genes.

Rol A - formation of wrinkled leaves
larger flower size

Rol B - stimulates adventitious root production and tissue becomes more auxin sensitive.

Rol C - stunting/ increased no. of side shoots/ reduction in flower size and pollen production

The effect of *rol* D is less clear.

Rol A, B or *C* can individually induce hairy root formation and there is probably a synergistic action of these three genes with respect to root induction. When *rol B* is present together with *rol A* or *rol C* this root induction is increased according to the expression levels of the genes. It was suggested that the *rol* gene products affect auxin sensitivity of transformed cells. Physiological studies by Shen *et al*, (1988) demonstrated that "hairy roots" are approximately a hundred times more sensitive to exogenous auxin than untransformed tissue.

1.5.2. The "hairy root" Phenotype

As well as T-DNA integration into a plant genome, a transformation using an *A.rhizogenes* vector will also result in co-inheritance of an altered phenotype. So plants regenerated from "hairy roots" exhibit abnormalities involving basic plant morphological characters. Common manifestations of this phenotypic plasticity is wrinkled, chlorotic leaves and stunted or bushy growth (Tepfer, 1984; Levesque et al, 1988).

1.6 DEVELOPMENT OF VECTORS FOR TRANSFORMATION

The feature of *Agrobacterium* species which permits their use as genetic vectors is the form of parasitism to which they subject host plant cells, "genetic colonization" (Schell *et al*, 1979). These bacteria manipulate the genome of the host cell and create a favourable niche for themselves.

The effect of the T-DNA genes on plant development has been an important criterion influencing vector construction. The pioneering work, which resulted in intensive research in the field of plant transformation, was carried out by Hernalsteens *et al*, (1980). The *Agrobacterium* Ti plasmid has been utilised both as a gene vector and as a source of sequences acting as constitutive promoters in plant cells.

There are a number of essential requirements needed in a successful Ti or Ri based transformation system:

- virulent *Agrobacterium* strain, type will vary according to the plant species involved, containing a Ti(Ri) plasmid which has
- functional *vir* region
- T-DNA border regions
- space to insert gene(s) of interest inserted between T-DNA border regions
- a suitable system for the selection or screening of transformed material from a background of untransformed material.

Agrobacterium vector systems have evolved significantly since their conception a decade ago. The use of markers illustrates this rapid progress.

Initially phytohormone production was used as a selectable marker for transformants, the genes encoding their synthesis being present on Ti and Ri plasmids. Transformed plants were able to grow on phytohormone-free media, in contrast to non-transformed tissue. The early vector systems which employed Ti plasmids were termed "oncogenic" because the phytohormone production, encoded by the Ti plasmid T-DNA, alters normal plant differentiation processes and only undifferentiated callus tissue can be produced, or shoots which fail to root. In contrast, regenerants from wild-type *A.rhizogenes* hairy root systems can develop into complete plants. The shoots which differentiate from hairy roots, once excised, can develop their own root system, thus fulfilling a requirement for a viable transformation system - the production of whole transgenic plants. However these F_0 generation transformants still possess the hairy root phenotype and morphologically often exhibit features detrimental to crop improvement, so the progeny needs to be crossed to segregate out the hairy root genotype. However, in early vectors, the genes were placed on T_L DNA so could not be crossed out. More recent progress in co-transformation using binary vector systems now allows segregation of the useful foreign gene and the detrimental oncogenes, after meiosis, (Shahin *et al*, 1986b).

Thus the use of disarmed *A.tumefaciens* vectors lacking the oncogenes responsible for crown gall has been an approach which holds the greatest potential for genetic manipulation of plants.

Chimaeric gene constructs are now of paramount importance because the presence of the selectable markers they carry dispels the need for developmentally detrimental phytohormone biosynthesis as a marker.

1.6.1 Use of Marker Genes

The original "selectable" marker utilized by researchers was the basic phytohormone independence trait possessed by plant cells transformed by wild-type "oncogenic" *Agrobacteria*. Moves were made to introduce suitable bacterial genes into the T-DNA which would be expressed by the transgenic plant host and cells lacking these genes would be a developmental disadvantage. The original attempts to use bacterial and animal eukaryotic genes such as Tn5 neomycin phosphotransferase II gene, conferring kanamycin resistance all failed. So, it appeared necessary to utilize genes known to function in plant cells.

Among the handful of plant genes which had been characterized was the nopaline synthase (*nos*) gene. This gene was well equipped for the role of marker, being actively transcribed in tumour tissue (Willmitzer *et al*, 1983), and constitutively expressed in transformed callus tissue and differentiated shoots lacking in phytohormone synthesis genes. Octopine synthase (*ocs)* and chloramphenicol acetyl transferase (CAT) genes were also used as markers (Herrera-Estrella *et al*, 1983a), with the *nos* promoter being attached, this being known to result in constitutive expression in plant cells. However, this gene is only a scorable marker and putative transgenic tissue must be assayed to detect *nos* activity. So, it was desirable to develop a selectable marker.

The first successful work in the development of selectable chimaeric gene constructs involved the bacterial neomycin phosphotransferase II (*npt II*) gene attached to the *nos* promoter and polyadenylation signals. This gene confers resistance to aminoglycoside antibiotics such as kanamycin, neomycin and G-418. The results of several groups (Bevan *et al*, 1983; Fraley *et al*, 1983; Herrera-Estrella *et al*, 1983b; Horsch *et al*, 1984) all demonstrated the great potential the *nptII* gene has as a dominant, selectable marker for plant transformation systems.

Other selectable markers have more recently been developed. The *dhfr* gene encoding methotrexate-insensitive dihydrofolate reductase within a chimaeric construct

with *nos* promoter can confer resistance to the herbicide, methotrexate, (Herrera-Estrella *et al*, (1983b). The *aphIV* gene encodes for hygromycin phosphotransferase and in tandem with either CaMV 35S, a *nos* or *ocs* promoters and *nos* terminator can confer resistance to the aminoglycoside antibiotic, hygromycin B, (Waldron *et al*, 1985; Van den Elzen *et al*, 1985a).

As previously stated, some genes can be utilized as scorable rather than selectable markers, their expression having to be detected by assays. As well as opine synthesis and CAT genes mentioned above, genes encoding for ß-galactosidase, *lacZ*, (Helmer *et al*, 1984), luciferase (Gould and Subramani, 1988; Scheider *et al*, 1990) and ß-glucuronidase, GUS, (Jefferson *et al*, 1987; Jefferson, 1989) have been used. The *nptII* selectable marker gene can also be classed as scorable, with an assay available to detect its activity. Only the marker or reporter gene encoding for ß-glucuronidase will be discussed in further detail here.

1.6.2.The GUS Reporter Gene

The ß-glucuronidase (GUS) gene has potential as a reporter gene due to the absence of any endogenous activity in a number of species, including tobacco, tomato and wheat.

The enzyme ß-glucuronidase is encoded by the *E.coli nid*A locus (Novel and Novel, 1973) and is especially useful due to the variety of ß-glucuronide substrates with which it can react. It is a hydrolase causing cleavage of substrates yielding ß-glucuronides. Measurement of the chimaeric gene activity is limited only by the properties of the reporter enzyme and the quality of available assays. Work on the use of GUS has been led by Jefferson and his co-workers. Three forms of assay are available for locating the presence of the GUS gene in putative transformants, these are the spectrophotometric, fluorometric and histochemical assays. Of these, the fluorometric assay is the most sensitive but the histochemical method is a useful

qualitative procedure allowing localisation of the GUS activity (Jefferson *et al*, 1987, Jefferson, 1987, 1988, 1989).

The advantage of using the GUS reporter system over the others, such as chloramphenicol acetyltransferase (CAT) (Gorman *et al*, 1982), or neomycin phosphotransferase II (*nptII*) (Reiss *et al*, 1984), includes the relative cost, speed and ease of the assays. Also, Töpfer et al, (1988), compared these 3 reporter gene systems and established that the sensitivity of detection was GUS > *nptII* > CAT, when associated with the CaMV 35S promoter. ß-Glucuronidase then possesses qualities essential to the "ideal" gene fusion system - it yields highly sensitive results both through a quantitative fluorometric assay and a histochemical localisation assay.

1.6.3 Types of Agrobacterium Vector

An important step in the development of plasmid vectors was the deletion of large sections of the wild type T-DNA, containing the genes coding for oncogenesis, phytohormone and opine biosynthesis (Zambryski *et al*, 1983). The border regions were retained due to their function in transfer and subsequent integration into the plant genome.

Two main types of system have been developed; binary and monomeric gene vector systems.

1.6.3.1. The "Binary" Gene Vector System

Observations that led to the development of "binary" or "trans" vectors include the basic fact that foreign DNA inserted in T-DNA becomes incorporated into the host plant genome. Also, it was shown that the T-DNA does not require physical linkage with the virulence region; it simply needs to be maintained within the same cell for transfer to be achieved (Hoekema *et al*, 1983; De Frammond *et al*, 1983) i.e. the regions work in "trans". Thus, binary vector systems where the *Agrobacterium* Ti plasmid had the entire T-DNA deleted and the T-DNA carried on a separate plasmid

were developed. The T-DNA containing plasmids such as Bin 19 (Bevan, 1984); pAGS (Van den Elzen *et al*, 1985b); pGA471 (An *et al*, 1985); pARC (Simpson *et al*, 1986); pEND (Klee *et al*, 1985), are based on a broad-range replication origin. This allows them to be grown and maintained in *E.coli* and transferred to *Agrobacterium* by bacterial conjugations. Suitable antibiotic resistance genes are necessary to allow selection of the desired conjugants. The region of the vector bounded by T-DNA border sequences also contains dominant selectable markers which are constitutively expressed within chimaeric gene constructs and are essential for the selection of transformed plant cells, (see section 1.4.1), allowing these to develop and differentiate at the expense of the untransformed tissue (Herrera-Estrella *et al*, 1983a). Those systems which have proved most useful in establishing transformation systems, are those also containing a non-selectable marker or reporter gene. When placed in specific parts of T-DNA with respect to the selectable marker and the gene of interest, these allow a quick way of ensuring the latter has been transferred, i.e. selectable marker - gene of interest - reporter gene. The independence of binary vectors from specific Ti plasmids means the vector can be introduced to almost any *Agrobacterium* host containing Ti/Ri plasmids, when the vir genes are also present. This is of great importance as *Agrobacterium* strains vary in their ability to transform different plant species.

An interesting aspect of binary vectors is their instability within *Agrobacterium*. A selection pressure must therefore be maintained to ensure retention and function of engineered plasmids and their genes. This then represents biological containment of genetically engineered genetic material, an important concept, in the consideration of the argument of safety of plant genetic engineering.

1.6.3.2. "Monomeric" or "Cis" Vector System

Replacement "*cis*" or Split End Vectors (SEV), have been established as viable systems (Zambryski *et al*, 1983, 1984). These involve the replacement of T-DNA genes through recombination events between plasmids, resulting in the DNA of interest being integrated into a single *Agrobacterium* plasmid which possesses active *vir* functions.

1.7 TYPES OF PLANT TRANSFORMATION TECHNIQUES USING AGROBACTERIUM VECTORS

The design of a successful transformation protocol is dependent on many cellular events in living plant and bacterial cells, as well as the correct design of the transfer vector at the molecular level. For example, the attachment process between the vector *Agrobacterium* and host plant requires the wounding of the plant, but there appears only to be a relatively short period of susceptibility after injury, perhaps only 2 hours, although other data suggests up to 36 hours. Transformation techniques "at the bench" thus involve many apparently arbitrary steps and constraints.

The emphasis on the type of systems used tends to differ according to whether *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* is being used as the vector. Three main classes of approaches will be considered here:-

(a) Whole Plant Infection

(b) Explant Infection

(c) Protoplast co-cultivation

(a) Whole Plant Infection

The use of whole plants as a starting point for *Agrobacterium* infection is commonly used with Ri-transformations. This technique involves wounding of the plant epidermis and inoculation of the site with *Agrobacterium.* If *A.tumefaciens* is to be used, the strains must be oncogenic so that gall tumorigenesis occurs, giving recognition that a transformation event has taken place. Model plants transformed in this way include tobacco (Klee *et al*, 1982) and sunflower (Murai *et al*, 1984).

(b) Explant Infection

Commonly, explants of several origins have been used, such as leaf discs, hypocotyls, cotyledons and stem pieces, in pTi-transformation.

The explants are immersed in a suspension of *Agrobacterium* and after a suitable length of time washed with antibiotics to eliminate the bacteria and placed onto selection plates as appropriate to the chimaeric construct within the T-DNA.

A transformation protocol for leaf disc transformation for tobacco tissue was originated by Horsch *et al,* (1985) and this has been the basis of many successful transformations. Use of leaf material where possible is preferable to other denser tissues such as stem sections, because the arrangement of cells in leaves allows selection antibiotics to penetrate more easily and thus making the selection process more efficient.

(c) Protoplast co-cultivation

Newly dividing protoplasts can be incubated with *A.tumefaciens* for 36-48hours. *Agrobacterium* cells can bind to the protoplasts and T-DNA passes into the protoplast. As with explant culture, antibiotics are used to decontaminate the protoplast suspension from bacteria and the protoplasts are grown on selective medium. This system is useful for biochemical and molecular studies of the transformation process in general, but can only be used to generate transgenic plants with species that have a reliable protoplast regeneration culture system already established.

1.8 AIMS OF THIS PROJECT

The objective of this project has been to establish a new *Agrobacterium tumefaciens* disarmed gene transfer vector system for use with the forage legume *Lotus corniculatus*. The full regenerative capacity exhibited by *L.corniculatus* would make the development of such a system a useful model for forage legumes in general. In order to achieve this objective the aim was to investigate a number of steps in tissue culture and transformation processes, and to optimise the tissue culture regime for *Lotus corniculatus*. In terms of a transformation technique, establishment of an efficient selection system was one of the initial aims, together with the choice of explant used. Ideally, such a system would be able to produce transgenic *Lotus* plants at a high frequency, and the resulting plants should be phenotypically normal and fully fertile.

Once a successful disarmed *Agrobacterium tumefaciens*-mediated system was developed, a further aim of the project was to introduce agriculturally useful genes into regenerants of *L.corniculatus*.

II. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Glassware and Plasticware

All glassware used in nucleic acid manipulations were siliconised and autoclaved. Plasticware and pipetteman tips were autoclaved. All containers were autoclaved for use in tissue culture work. Pipetteman tips were individually wrapped in foil and autoclaved.

2.1.2 Chemicals and Biological Reagents

Reagents, unless listed separately below, were obtained from BDH Chemicals Ltd., Poole, Dorest, U.K. and were of analytical grade or the best available.

Acetosyringone, BSA, DTT, EtBr, RNase A, Pronase P, Proteinase K, BAP, NAA, lysozyme, ampicillin, chloramphenicol, carbenicillin, gentamycin, kanamycin, rifampicin and mannitol were all obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Augmentin was supplied from Beechams Pharmaceuticals, Brentford, Middlesex, U.K., Cefotaxime from Roussel Laboratories Ltd., Uxbridge, U.K.

3MM paper and filter paper were from Whatman Ltd., Maidstone, Kent, U.K.

Nitrocellulose filters were from Anderman and Company Ltd., Kingston-Upon-Thames, U.K..

MS medium and Gambourg's Basal salts without sucrose, kinetin or 2,4-D were

obtained from Flow Laboratories, Rickmansworth, Herts., U.K.. Agar was obtained from Difco, Detroit, Michigan, U.S.A.. Agarose was from Gibco BRL Ltd., Paisley, U.K.. Tissue culture Petri dishes (9cm), 50ml pots and Chloros were supplied by A & J Beveridge Ltd., Newcastle, U.K.. Sterile Acrodisc filter units (0.2μm) were from Gelman Sciences, Ann Arbor MI48106, USA.

Sephadex G-50 and Ficoll-400 were from Pharmacia Fine Chemicals, Uppsala, Sweden. NaCl was from May and Baker Ltd., Dagenham, U.K.. Yeast extract was obtained from Biolife S.r.l., Milan, Italy. Bacto-typtone and beef extract were from Oxoid Ltd., Basingstoke, Hants., U.K., and Trypticase-peptone was from Becton Dickinson Microbiology Systems, USA.

Radiolabelled dCTP and the nick translation kit were from Amersham International Plc., Amersham, Oxon., U.K.. Restriction endonucleases, buffer solutions and DNA modifying enyzmes were from Boehringer Mannheim GmbH., Mannheim, W.Germany, and Northumberland Biologicals Ltd., Cramlington, U.K..

Products for the ß-Glucuronidase assay system (purified bacterial ß-glucuronidase enzyme, 4-methylumbelliferone, substrates 4-Methyllumbelliferyl-ß-D-glucuronide and 5-bromo-4-chloro-3-indoyl-ß-D-glucuronide (X-Gluc)) were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

2.1.3 Plant Material

Wild-type *Lotus corniculatus* cv. Leo seeds were obtained from Dr. K.J. Webb (W.P.B.S., Aberystwyth, U.K.).

2.1.4 Bacterial Strains and Plasmids

Tables 2.1.1 and 2.1.2, outline the bacterial strains and plasmids used.

Table 2.1.1: Characteristics of Bacteria Used

Bacterial Strains	Genotypes/Characteristics	Source or Ref.
E.coli : DH5α	F⁻, ϕ80d, *lac*Z M15, *end*A1, *rec*A1, *hsd* R17 (r^- ,m_k^+), supE44, *thi*-1, d^-, *gyr*A96, (*lac*ZYA-*arg*F),U169	
GJ23	JC2926[R64*drd*11. pGJ28]	Van Haute *et al*, 1978
A.tumefaciens : LBA4404	Avirulent, disarmed ACH5[pAL4404] Rif^R $Strep^R$	Ooms *et al*, 1982
GV3101[pGV3850]	$C58C^1$ Rif^R $Carb^R$, Ti-cured	Edwards, 1988
A.rhizogenes : LBA9402	ACH5[p1855]	Phil Mullineaux
LBA1334 [pRi1855::pBin19Psl]	$C58C^9$ derivative Rif^R, Kan^R	Diaz *et al*, 1989
Rhizobium spp. : *Rh. leguminosarum* 1001		W.P.B.S. *Rhizobium* Collection
Rh. var. Loti 3011		W.P.B.S. *Rhizobium* Collection

Key:
pro. - promoter
ter. - terminator
Rif^R - Rifampicin resistance
Kan^R - Kanamycin resistance
Gm^R - Gentamycin resistance
$Carb^R$ - Carbenicillin resistance
$Strep^R$ - Streptamycin resistance
$Spec^R$ - Spectinomycin resistance

Table 2.1.2: Characteristics of Plasmids Used

Plasmid	Genotype/characteristics	Source or Ref.
pDB007	pBR322 containing nos-neo pro. and CaMV pro. and nos ter. cloned into a multiple cloning site of lac Z coding region. Carries Gm^R and Kan^R.	D.Bown
pEJC1	pUC18Xho containing CaMV 35S promoter, CaMV terminator and GUS coding region. Amp^R	E.Croy
pAL4404	Disarmed Ach5 type. T-DNA SmaI deletion replaced by Tn904. *Vir* region intact.	Ooms *et al*, 1982
pGV3850	pGV3100, pBR322 replacing T-DNA between Hind III fragments 23-10. Rif^R $Carb^R$	Zambryski *et al*, 1983
pRi1334	Agropine type plasmid. $Spec^R$ Rif^R	Diaz *et al*, 1989
pBin19Psl'	pBIN19 containing Hind III-EcoRI fragment consisting of lecA gene. Rif^R	Diaz *et al*, 1989
pJIT73	pBIN19 derivative containing *Aph IV*, *Nos-neo* and GUS marker genes.	Phil Mullineaux
pROK/CpTi+5	pBIN19 containing CaMV 35S pro., nos ter. and CpTi encoding region	Hilder *et al*, 1987 (donated by A.G.C.)

Key: pro. - promoter
ter. - terminator
$Strep^R$ - Streptamycin resistance
Rif^R - Rifampicin resistance
Gem^R - Gentamycin resistance
$Carb^R$ - Carbenicillin resistance
$Spec^R$ - Spectinomycin resistance
Kan^R - Kanamycin resistance

2.2 METHODS

2.2.1 BACTERIAL MANIPULATIONS

2.2.1.1 Storage of Bacteria

Short term storage of bacteria at 4°C on inverted agar plates is possible for up to six weeks, if sealed with Nescofilm.

For long term storage of bacteria, a lawn was grown up on selection agar plates from a single colony and transferred to sterile tubes containing 1ml YEB or L-broth. After thorough mixing by vortexing, 1ml sterile 80% glycerol was added and the contents were again mixed and immediately transferred to storage at -80°C.

2.2.1.2 Bacterial Culture Medium

E. coli cultures were grown in the following media;
YT medium: 8g Bacto-tryptone, 5g yeast extract, 5g NaCl - L^{-1}
LB medium: 10g Bacto-tryptone, 5g yeast extract, 5g NaCl, 1g glucose - L^{-1}

Agrobacterium spp. were cultured on YEB medium:
1g yeast extract, 5g beef extract, 5g peptone typticase, 5g sucrose,
493mg $MgSO_4.7H_20$ - L^{-1}.

Rhizobium spp. were cultured on YMB medium: 10g mannitol, 0.4g yeast extract, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl - L^{-1}, 0.25% (w/v) Red Congo, pH 6.8-7.0.

All media were corrected to pH 7-7.2. When cultured on solid medium, plates contained 1.5% agar. Sterilisation was by autoclaving at 121°C, 15p.s.i.for 20 min. Levels of antibiotics used in maintenance of cultures are shown in table 2.2.1 Any medium was cooled to < 60°C prior to addition of antibiotics.

Other strains produced by triparental matings are shown in table 2.2.2.

Table 2.2.1: Antibiotic Levels for Bacterial Cultures (μg ml^{-1})

Antibiotic → Bacteria ↓	Kanamycin	Rifampicin	Carbenicillin	Gentamycin
E.coli [pJIT73]	50	-	-	-
E.coli [pROK5.CpTi]	50	-	-	-
Agrobacterium tumefaciens LBA4404[pAL4404]	-	100	-	-
A. tumefaciens GV3101[pGV3850]	-	100	100	-
A.rhizogenes LBA[pBin19Psl']	50	100	-	-
A.rhizogenes LBA9402[p1855]	-	100	-	-
A.tumefaciens GV3101[pGV3850.G]	-	100	100	10

2.2.1.3Plasmid DNA Preparation

A. E.coli Plasmid Method

The method used was based on the alkaline lysis method, (Birnboim and Doly, 1979), with modifications as shown below.

E.coli cultures grown at 37°C in 10ml L-broth containing the necessary antibiotics (see Table 2.2.1), were grown to stationary phase. The cells were pelleted by centrifugation at 900g for 5 minutes in a bench top centrifuge in original overnight culture. Most of the supernatant was removed and the cells resuspended in the remaining 0.5-1ml medium. This suspension was transferred to a 1.5ml eppendorf tube and the cells were pelleted in a microfuge, and the last of the supernatant removed. To the cells, 200μl of freshly prepared 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH8.0, 2mg ml^{-1} lysozyme [solution I] was added. After mixing by inversion until the pellet of cells dissolved, the mixture was left on ice for 30 min. 400μl of freshly prepared 0.2M NaOH, 1% SDS [solution II] was added and gently mixed by inversion. A short period of 5 min on ice was followed by the addition of 600μl of 3M sodium acetate pH4.8 and mixing by inversion. The DNA clot could be seen to form at this stage and the tube was left for a further 30 minutes prior to centrifuging for 10 minutes at 900g or until a clear supernatant was produced. This supernatant was transferred to a fresh eppendorf and phenol-extracted twice, followed by a phenol:chloroform (1:1) extraction and finally twice extracted with chloroform. After ethanol precipitation, the DNA was resuspended in 20-50μl sterile double distilled water.

B. *E.coli* high quality "maxi"-preparation.

This method was based on the alkaline lysis method as above but with further modifications of the volumes of solutions used.

After resuspension in 200μl solution I (minus lysozyme) the suspensions were split into two 1.5ml eppendorfs and to each, 100μl of freshly prepared 8 mg ml^{-1} lysozyme added. After mixing, the tubes were left at room temperature for 5

min with the lids open. 600μl of solution II was added, mixed, left on ice for 5 min, followed by the addition of 450μl 3M sodium acetate pH4,8, mixing and a final 5 min on ice. The suspension was again divided between two eppendorfs and the phenol-chloroform extractions carried out. 400μl phenol was mixed by inversion (x50) and 400μl chloroform was mixed in by inversion (x50). This was then spun in a microfuge, 12000g, for 3 min. The DNA was then ethanol precipitated (with no sodium acetate added) and resuspended after drying in 20-50μl double distilled water.

C. Total *Agrobacterium* DNA Preparation

This method was based on that of Dhase *et al*, (1979). The *Agrobacterium* were grown to stationary phase, 24-48 hours, at 27°C and 1.5ml of this culture was transferred to an eppendorf tube and centrifuged for 30 seconds at 12000g. The cells were resuspended by vortexing in 380μl pronase buffer, 50mM Tris-Cl pH8.0, 20mM EDTA, 0.8% Na-lauroyl sarcosinate, then 20μl of a 20mg ml^{-1} stock solution of pronase P, previously self-digested by incubation at 42°C for 2 hours, was added to the cells. These were mixed thoroughly and incubated at 37°C for 1 hour or until colourless. The resulting lysate was sheared by carefully passing it through a syringe needle of 1.1mm bore several times, followed by 0.6mm bore needle, until the viscous suspension becomes more fluid. The DNA was then deproteinized by two phenol extractions; 500μl equilibrated phenol and 400μl sterile distilled water were added to the 400μl of suspension, mixed, spun, and the aqueous layer re-extracted. Four chloroform extractions followed. 3M Sodium acetate was then added in order to give a final salt concentration of 0.3M together with 1000μl 100% ethanol (-20°C). The DNA was immediately pelleted and resuspended in TE and the ethanol precipitation repeated. Finally the pellet was washed twice in 80% ethanol (-20°C), and dried with N_2(g) and dissolved in an appropriate volume of sterile double distilled water.

2.2.1.4Production of Competent Cells

E.coli DH5α were made competent by the following method. A 10 ml YT broth was inoculated with DH5α from a glycerol and grown up overnight at 35°C on a shaker. 2ml of this culture was used as inoculant in 200ml pre-warmed YT in a 1L baffled flask (ratio of cells:YT = 1:100) and placed on a shaker. The absorbance at 550nm was monitored as a measure of cell growth until the cells reached an O.D. of 0.3-0.35and were thus in log phase. At this stage the culture was chilled on ice for 5 minutes before being centrifuged at 2000g for 10 minutes at low temperature. The cells were resuspended in 40% original volume of TfbI buffer; 30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, manganese chloride (pH 5.8-filter sterilized) and 15% glycerol (v/v).

The culture was left on ice for 5 minutes before a further 10 minute spin and resuspended in 4% original volume of TfbII; 10mM MOPS, 75mM calcium chloride, 10mM rubidium chloride (pH 6.5,filter sterilized) and 15% glycerol (v/v). After a further 15 minutes on ice the now competent cells were carefully aliquoted into sterile eppendorfs and immediately frozen in liquid nitrogen before storing at -80°C.

2.2.1.5 Triparental Conjugation

Such a mating involved three bacterial strains, and directed the transfer of a bacterial plasmid from *E.coli* into an *Agrobacterium* strain. Two *E.coli* strains, designated as the donor and mobilizing strains and a single *Agrobacterium* strain designated the recipient were involved in the mating. Table 2.2.3 shows the bacteria involved in these matings.

Antibiotic resistances of all the strains involved were determined and confirmed. *E.coli* strains were grown up on L-agar plates, containing the antibiotic(s) to which they were resistant or the antibiotic(s) to which the *Agrobacterium* was resistant. Similarly,

Table 2.2.2: Triparental Matings Producing Binary Vector Systems

STRAIN → MATING ↓	DONOR	MOBILISING	RECIPIENT	PRODUCT OF MATING
A.	*E.coli* [pJIT73]	*E.coli* [pRK2013]	*A.tumefaciens* LBA4404 [pAL4404]	*A.tumefaciens* LBA4404 [pAL4404::pJIT73]
Resistance Genotype	Kan^R	Kan^R	Rif^R StrepR	Rif^R Kan^R $Strep^R$
Levels used	50μg ml^{-1} kanamycin	50μg ml^{-1} kanamycin	100μg ml^{-1} rifampicin	100μg ml^{-1} rifampicin 50μg ml^{-1} kanamycin
B.	*E.coli* [pJIT73]	*E.coli* [pRK2013]	*A.tumefaciens* GV3101 [pGV3850]	*A.tumefaciens* GV3101 [pGV3850::pJIT73]
Resistance Genotype	Kan^R	Kan^R	Rif^R $Strep^R$	Rif^R $Strep^R$
Levels used	50μg ml^{-1} kanamycin	50μg ml^{-1} kanamycin	100μg ml^{-1} rifampicin 100μg ml^{-1} carbenicillin	100μg ml^{-1} rifampicin 100μg ml^{-1} carbenicillin 50μg ml^{-1} kanamycin
C.	*E.coli* [pJIT73]	*E.coli* [pRK2013]	*A.rhizogenes* LBA9402 [p1855]	*A.rhizogenes* LBA9402 [p1855::pJIT73]
Resistance Genotype	Kan^R	Kan^R	Rif^R	Rif^R Kan^R
Levels used	50μg ml^{-1} kanamycin	50μg ml^{-1} kanamycin	100μg ml^{-1} rifampicin	100μg ml^{-1} rifampicin 50μg ml^{-1} kanamycin
D.	*E.coli* [p208.96.8]	*E.coli* [pRK2013]	*A.tumefaciens* LBA4404 [pAL4404]	*A.tumefaciens* LBA4404 [pAL4404::p208.96.8]
Resistance Genotype	Kan^R	Kan^R	Rif^R	Rif^R Kan^R
Levels used	50μg ml^{-1} kanamycin	50μg ml^{-1} kanamycin	100μg ml^{-1} rifampicin	100μg ml^{-1} rifampicin 50μg ml^{-1} kanamycin

the *Agrobacterium* sensitivity was checked on YEB-agar plates. All plates were sealed, inverted and the *E.coli* grown at 37°C overnight and *Agrobacterium* at 27°C for 24-48 hours.

For the mating procedure itself, overnight cultures of all 3 strains were set up in liquid media under selection. Once grown to stationary phase the bacterial cells were harvested by centrifugation for 10min at 900g and washed by resuspension in 5ml 10mM $MgSO_4$ and then repelleted. A 1ml aliquot of a 1:1:1 mix of the donor:mobilizing:recipient strains was plated onto non-selection L-agar plates. An even spread of the layer was achieved by rocking the plate. This was then incubated for 24-48 hours, non-inverted at 27°C. 1ml of the three individual strains as well as 1ml 10mM $MgSO_4$ as a sterility control, were also plated out. To the lawn of bacterial growth occurring on the plates, 2ml 10mM $MgSO_4$ was added to dislodge the bacteria. The resulting suspension was pipetted off and a further 1ml 10mM $MgSO_4$ added. This 3ml of 1:1:1 suspension was used to make ten-fold dilutions (10^0-10^{-4}). The three individual strains were prepared in a similar way giving a 10^0-10^{-2} series. 100μl of each dilution was plated out and spread onto YEB agar plates. Present in the plates were the required antibiotics needed to select for *Agrobacterium* conjugants containing the required plasmid DNA (see Table 2.2.2). All plates were sealed and inverted at 27°C for 48 hours.

Colonies were picked off onto a master plate and into overnight mYEB-selection cultures. These were then grown overnight and minipreped to obtain DNA (see 2.2.1.3). This DNA was restricted, transferred from gel to nitrocellulose filter and probed to check for the presence of the plasmid.

2.2.1.6Production of a New Cis Vector System

The aim was to integrate the GUS gene into the pGV3850 of *A.tumefaciens* GV3101[pGV3850]. This involves a number of stages:

(a) Transfer of the GUS gene into E.coli

The *E.coli* pDB007 plasmid was used as the initial recipient of the DNA fragment containing the GUS gene. The latter was isolated from the pEJC1 plasmid derived from pUC18 and constructed by E.J. Croy. pEJC1 was restricted with BamH1 which yielded two fragments, the 2.6kb one consisting of the GUS gene plus the CaMV terminator. This fragment was extracted by electroelution and purified in the normal way (see Methods 2.10) and resuspended in sterile distilled water as necessitated by the subsequent ligation. The pDB007 plasmid was restricted with the BamH1 endonuclease restriction enzyme, so exposing ends cohesive to the similarly cut ends of the GUS insert. The restricted recipient vector plasmid was then phenol-chloroform extracted and ethanol precipitated and resuspended in sterile distilled water.

The ligations were set up (2.2.2.4)and used to transform competent DH5α *E.coli* cells (2.2.2.5). The total 200μl DH5α cells were spread onto YT-plates containing 50mgL^{-1} ampicillin and incubated at 37°C. The pDB007 vector DNA confers ampicillin resistance to the DH5α cells. Colonies grown up after 24 hours were counted, and any from the vector + insert ligation transformation were picked off onto nitrocellulose filters overlaid on YTamp agar plates, in duplicate. Similarly a number of colonies from the control self-ligation transformation plates were picked off. Such control colonies were used to demonstrate background hybridisation. The plates were again incubated inverted overnight at 37°C and one

filter was prepared for probing with ^{32}P-labelled GUS-terminator fragment of the donor pEJC1 plasmid, see Methods 2.2.2.7..

Following overnight hybridisation the filter was washed to 0.1xSSC stringency for 10 minutes, air dried and exposed to excited film for 24 hours. A colony which appeared positive was then grown up from the replicate plate in L-broth with 50mgL^{-1} ampicillin selection. This was minipreped to obtain the plasmid DNA, which was then restricted with BamHI, as were pDB007 and pEJC1 plasmids acting as negative and positive controls, see Results 3.2.3.

(b) Formation of E.coli Intermediary Containing Mobilising Plasmids and Required Fragment

A transformation of competent GJ23 cells with purified minipreped transformed plasmid DNA was carried out.

GJ23 cells were cultured overnight in 5ml L-broth with 10μg ml^{-1} tetracycline and 25μg ml^{-1} neomycin selection. 100μl of this was then added to 50ml L-broth with selection in a 250ml flask and grown for 4 hours. The cells were then spun for 10 minutes at 900g and resuspended in 30ml 0.1M Calcium chloride and left on ice for 1 hour. After another spin and resuspending in 1ml 0.1M Calcium chloride the cells were ready for immediate use. 0.05μg DNA was added to 100μl competent GJ23 cells and the transformation allowed to proceed as previously described. Aliquots were plated onto L-agar plates containing 10ug ml^{-1} tetracycline, 25μg ml^{-1} neomycin and 50μg ml^{-1} ampicillin. 100μl of GJ23 cells were also plated as a negative control and all were incubated overnight at 37°C. Efficiency of the selection meant that a restriction analysis of the DNA of colonies which grew was not required.

(c) Diparental Conjugation Between Intermediary and GV3101-pGV3850

A single colony growing on the tetracycline, neomycin and ampicillin selection plates was grown in L-broth under selection and *A.tumefaciens* GV3101{pGV3850] was grown up in YEB with rifampicin and carbenicillin

selection (see Table 2.2.1). Both donor and recipient bacteria cultures were spun at 900g for 5 minutes and resuspended in 5ml phage buffer; 10mM magnesium sulphate, 10mM TrisCl pH 7.5. This spinning and resuspension was repeated twice more to remove all traces of antibiotics from the cells. 100μl of the *E.coli* donor followed by 100μl of the *A.tumefaciens* were spread onto an L-agar plate. 100μl of phage buffer, as check for sterility, and the two bacteria strains were plated out as controls. After 24 hours, 2ml phage buffer was added to each plate and the culture was dislodged and pipetted off. Serial dilutions of the cultures were made; 10^0-10^{-7} of 1:1 mix, 10^0-10^{-4} donor and recipient only. 100μl of each dilution was spread onto YEB agar plates containing 10 mgml^{-1} gentamycin, 100 mgml^{-1} rifampicin and 100 mgml^{-1} carbenicillin. Due to the leaky gentamycin selection, only the larger colonies which grew up on the final selection plates were picked off onto a master plate and overnight cultures set up for further investigation.

The DNA from these putative positives was miniprepped, BamHI restricted, run on a 0.6% agarose gel, Southern blotted and the nitrocellulose filter probed with the ^{32}P-labelled GUS-terminator fragment. The filter was washed to a stringency of 2xSSC for 30 minutes, see Results 3.2.3.

2.2.1.7 Preparation of *Agrobacterium tumefaciens* and *A.rhizogenes*

The *Agrobacterium* strain was grown on a master plate by inoculating a mYEB agar plate containing the necessary antibiotics (see Table 2.2.1) with 50μl of the bacterial glycerol suspension. The inoculant was spread over the surface of the plate and, after sealing the plate, incubated inverted at 27°C for 48 hours.

A loop full of *A.tumefaciens* or *A.rhizogenes* from such a master plate was then used as inoculant in 5mls mYEB with antibiotics (see Table 2.2.1) which was incubated at 27°C with shaking. After 24-48 hours the culture was added to a flask containing 20ml of mYEB with identical antibiotic concentrations and incubated as before, overnight. The bacteria were ready at this stage to be washed unless acetosyringone was to be used.

If acetosyringone was to be used in order to induce activity in the *vir* region then the bacterial suspension was transferred to a sterile universal bottle and centrifuged for 15 minutes, at 900g at room temperature. Once pelleted the supernatant was removed and the *Agrobacterium* were resuspended in 5ml induction medium (see below). This was then transferred to a further 20ml Induction liquid medium and the appropiate antibiotic selection was added together with 1.4mM acetosyringone (AS). The culture was then returned to the previous culture conditions overnight, prior to the washing stage.

Induction Medium: L^{-1} MS 0.47g

Sucrose 3%

Na_2HPO_4(Anhydrous) 12.5mM - 100ml of 17.4 gl^{-1} stock

pH 5.2

After autoclaving 1.4mM AS was added, 200μl of a 35 mg ml^{-1} stock solution in methanol is used per 25 ml overnight.

At the point at which cultures were to be washed flask contents were transferred to sterile universal bottle and centrifuged at 900g for 15 minutes at room temperature to pellet the *Agrobacterium*. The supernatant was removed and the *Agrobacterium* resuspended in 2mM $MgSO_4$ and repelleted by a further 10 minute spin. This washing procedure was repeated twice more in order to remove the antibiotic from the bacterial suspension. The cells were resuspended finally in liquid medium corresponding to the culture medium to be used with the co-cultivated explants, (see Table 2.2.3). In the final transformation procedure(s) developed, liquid B_5O was used.

A.rhizogenes was used directly from an agar selection culture in the seedling inoculation procedure.

2.2.2 DNA MANIPULATIONS

2.2.2.1 Phenol-Choroform Extraction

An equal volume of phenol was added to the volume of resuspended DNA in an eppendorf, vortexed briefly, and spun for 5 minutes (900g) in a microcentrifuge. The resulting upper aqueous layer was removed to a fresh tube. To ensure maximum DNA recovery the original phenol phase was back extracted by the addition of an equal volume of TE pH7.4, vortexed, spun and the aqueous layer was added to that which had been previously removed. To the aqueous phase an equal volume of phenol-chloroform (1:1) was added (the chloroform contained 4% v/v isoamyl alcohol to aid separation). After a brief vortex, the tube was spun for 2 minutes and the aqueous layer once more transferred to a fresh tube to which an equal volume of chloroform was added. The aqueous layer appearing after a spin was transferred to a fresh tube. Generally DNA was taken through two phenol, one phenol:chloroform and two chloroform extractions.

2.2.2.2 Ethanol Precipitation

Unless otherwise stated, one tenth volume 3M Sodium acetate was added to DNA supernatants, together with 3 volumes of 100% ethanol (-20°C). Thus the overall concentration of salt was 0.3M. The tube was placed at -20°C for at least 1 hour and the DNA which precipitated out was spun down to a pellet for 20 minutes at 4°C, the ethanol removed and the pellet washed once with 80% ethanol (-20°C) or 100% ethanol. The precipitate was repelleted after the wash. After drying with N_2(g) or in a vacuum pump, the DNA was resuspended. When required, this can be carried out overnight at 4°C on a rotating wheel in an appropriate quantity of TE pH7.4 or 7.5 or sterile double distilled water.

2.2.2.3 Extraction of DNA from an Agarose Gel by Electroelution

Dialysis tubing was previously prepared by boiling for 10 minutes in a large volume of 1mM EDTA, and washed with sterile distilled water. This was stored in 70% ethanol and when required cut, washed and boiled in distilled water for 10

minutes. When a specific DNA plasmid fragment was required, the relevant band on an agarose gel was cut out using ethanol sterilised instruments. The fragment was placed in dialysis tubing closed at one end with a clip and 500µl TE pH7.5 was added. The tubing was closed off with a second clip making sure no air was left inside. The tubing was then submerged in TBE minigel buffer perpendicular to the direction of current in a minigel apparatus. A 50V voltage for 20 minutes was generally sufficient to draw the DNA into the TE buffer from the agarose (though a longer time was sometimes required) and was followed by a brief 30 second reversal of current. The buffer within the tubing was transferred to an eppendorf and processed through phenol/chloroform extractions and ethanol precipitation.

2.2.2.4 Enzyme Reactions

Restriction with Endonuceolytic enzymes

Enzymes generally used were Hind III, BamHI, EcoRI and these reactions were set up using buffers from commercial sources.

Enzymes were used at a concentration of 2-5U μg^{-1} DNA, higher levels being used on genomic and agrobacterial DNA samples. Plasmid DNA was incubated for 1½-2½ hours, the reaction mixture also contained 100µg ml^{-1} pancreatic RNase (RNase A), previously boiled for 7 min to inactivate DNase activity.

Genomic DNA was incubated for 4-5 hours with RNase present. Improved restrictability was found to occur if all the reaction components except the enzyme were initially added, mixed and left on ice for 1 hour. This ensured the DNA had completely dissolved and mixed in, prior to addition of the enzyme.

DNA ligation

Double stranded DNA molecules with compatible ends were joined covalently by T4-DNA ligase treatment. The reaction was set up in a low volume (10-20µl) of ligase buffer (50mM Tris-HCl pH7.8, 10mM $MgCl_2$, 1mM DTT, 1mM ATP). For the ligation reaction it was important for there to be an excess of insert over vector DNA

molecules at least 1½ : 1. The number of molecules could be estimated by the use of the following approximate equation: $\frac{\mu g\ DNA}{kb\ DNA}$ = no. of DNA molecules.

2.2.2.5 Bacterial Transformation

In order that a maximum of 0.01µg DNA was introduced to 100µl of competent cells, the ligation reaction mixtures were diluted as necessary with L-broth. The competent cells were left on ice to thaw slowly and the maximum of 0.01µg DNA in a small volume <10µl, was added. After a gentle mixing the cells were left on ice for 30 min. After this period the cells were incubated at 37°C for 30 sec followed by a further 2 min on ice.

The cells were then grown up by adding YT to a total volume of 200µl and placed on a rotary shaker at 37°C for 50-60min. Finally the total 200µl aliquot was plated and spread on YT-plates containing 50µg ml^{-1} ampillicin.

2.2.2.6 Agarose Gel Electrophoresis of DNA

The basic techniques for preparation of gels and electrophoresis were as described by Maniatis *et al*, (1982). Generally, throughout this project a 0.7% (w/v) agarose gel was used. The agarose was boiled in Alex's gel buffer (40mM Tris-acetate pH7.7, 2mM EDTA) until the agarose dissolved. 200ml buffer was used if a 190x150x6mm gel was required. Once cooled to 50-60°C, EtBr was added to a final concentration of 0.5µg ml^{-1}. The gel was then poured into a mould previously adhered to an ethanol washed glass plate using silicone grease. The mould also supported a well comb. The mould was removed and the gel on the plate was placed into an electrophoresis tank containing Alex's gel buffer containing 0.5 µg ml^{-1} EtBr, so that the buffer covered the gel by 2-5mm.

DNA was prepared for electrophoresis by adding one tenth volume of loading buffer 1% (w/v) Bromophenol blue, 30% (v/v) glycerol, 10mM Tris-Cl pH8.0, 10mM

EDTA pH8.0) prior to loading into wells. Electrophoresis was generally at 25V, for 14-16 hours overnight. However a gel could be run at a higher current (<120mA) for a reduced period of time. A minigel apparatus was used for small amounts of DNA and to check amount and restrictability of DNA samples. The mould size was 100x80x50mm and Tris-borate buffer (0.089M Tris-borate, 0.089M boric acid, 2mM EDTA) used. The gel was run at 50mA (15V) for 1-3 hours.

DNA on gels was visualised on a 300nm U.V. light box and photographed with a Polaroid MP-4 Land camera through a Kodak 23A Wrattan filter, using a Polaroid type 667 film with exposure of 12s at f16.

2.2.2.7 DNA Hybridisation

(a) Southern Transfer of DNA from Agarose gel to Nitrocellulose filter

The method used was based on that of Southern, (1975). The gel was first treated with 0.25M HCl for 30min. with agitation to depurinate the DNA. It was then transferred to denaturation solution (1.5M NaCl, 0.5M NaOH) and agitated for 1-1½ hours, with changes of solution every 30 min. 1-1½ hours in neutralisation solution (3.0M NaCl, 0.5M Tris-HCl pH7.0, 1mM EDTA) then followed similarly with agitation and changes of solution.

The gel was transferred to a capillary blotting apparatus, consisting of a Whatmann 3MM paper wick system taking the 20xSSC buffer up through the gel, this having been surrounded by cling film to ensure that the buffer could only move upwards through the gel. The nitrocellulose filter was wetted with distilled water and then soaked in 20x SSC prior to being overlayed on the agarose gel. Air bubbles were rolled out from the layers in the apparatus at each stage. Three layers of Whatmann 3MM paper were placed on top, followed by 3 layers of disposable nappies and the whole apparatus weighted down by 0.5-1kg. Transfer was allowed to take place for 16 hours at room temperature.

When disassembled, the gel well positions and gel outline were marked on the

filter. The filter was then baked in a vacuum oven at 80°C for 1½-2 hours, to bind the DNA to the filter and allow storage and further hybridisation processing.

(b) Preparation of Colony Screen Filters

The filter was laid on blotting paper dampened with a series of solutions and dried between each:

10% SDS	3 minutes
Denaturing Solution	5 minutes
Neutralizing Solution	5 minutes
2xSSC	5 minutes

After air drying the filter for 30 minutes, it was baked in the oven at 80°C for 1 hour.

(c) Prehybridisation and Hybridisation

Filters were prehybridised and hybridised according to Maniatis *et al*, (1982), with the exclusion of EDTA from the hybridisation solution. Plasmid DNA filters were prehybridized in a 100ml volume and hybridized in 50ml. Genomic DNA filters could also be processed in 50 and 20ml volumes.

(d) Preparation of Radioactive Probes

^{32}P labelled probes were produced using nick translation Rigby *et al*, 1977, reagents being supplied in a kit (Amersham Co.). The reaction mixture was run down a G-50 Sephadex column which separated unincorporated radioactive label from that incorporated in the DNA. Fractions were collected and analysed on a scintillation counter, so that the specific activity of the fractions could be calculated. Probes were also produced by using the random priming technique (Feinberg and Voelstein, 1984), the reaction mixture was then processed as described for Nick Translation.

(e) Post-Hybridisation Washing

Solutions were based on those of Maniatis *et al*, (1982). The filters were washed in a series of decreasing SSC concentrations; x2, x1, x0.1. The stringency of washing varied, (see results for individual filters).

2.2.2.8 Genomic DNA Extraction from *Lotus corniculatus*

This method was modified from Murray & Thompson, (1980). Lyophilised leaf material was ground in a pestle and mortar with liquid nitrogen. The use of petiole material was avoided. It was necessary to obtain a fine powder, to which approximately 10ml isolation buffer was added per gramme of dry material.

The buffer contained 0.05M Tris, 0.01M EDTA, 10mM sodium bisulphite, 10mM o-phenathroline, 20 $\mu g\ ml^{-1}$ proteinase K, 0.7M sodium chloride, 1% w/v cetyl trimethyl ammonium bromide (CTAB) and was made up freshly just prior to use. After an incubation period of 30 minutes at 60°C, with occasional gentle mixing an equal volume of chloroform:octanol 24:1 was added and mixed. The tube was sealed with both foil and nescofilm prior to inversions and then centrifuged at 15,000g for 10 minutes at room temperature. The aqueous layer was then transferred to a sterile, siliconised beaker and one tenth volume of 5M Ammonium acetate added and mixed gently. Finally, 1-2x volume of isopropanol (-20°C) was added and the resulting DNA, which precipitated out of the solution was spooled out with a sterile, siliconised glass hook and was resuspended in 1ml of sterile double distilled water or TE pH8.0. This large volume was used to ensure the DNA solubilised prior to a phenol/chloroform extraction and ethanol precipitation stages, after which the DNA was resuspended in sterile double distilled water.

2.2.2.9 Quantitative DNA Assay (Thomas & Farquhar, 1978)

The DNA to be assayed, together with duplicate standards of calf thymus DNA; (0, 0.1, 0.5,1.0, 5.0, 10.0 μg), were purified by precipitation. To the samples of DNA, 10μl of a 10 $mgml^{-1}$ solution of BSA was added followed by 250μl of 50mM sodium acetate in 95% ethanol at -20°C. After vortexing, the eppendorfs were left to stand for 15 minutes on ice. The tubes were centrifuged at 4°C for 10 minutes at 12500g and the supernatant removed. The pellets were then washed twice with 75% ethanol and dried at 80°C for 15 minutes in a vacuum oven.

The assay reagent diaminobenzoic acid (DABA) was prepared just prior to use

in distilled water at 400 $mgml^{-1}$. 20µl of this DABA solution was added to each pellet and two blank tubes. The tubes were vigorously vortexed and incubated at 60°C for 30 minutes. After cooling, 1ml 1N HCl was added and vortexed. The solutions were transferred to fluorimeter cuvettes, and the eppendorfs were washed with a further 1ml 1N HCl, which was then added to the cuvettes and the contents mixed prior to reading.

The fluorimeter was set to an emission wavelength of 505nm and excitation wavelength of 400nm and blanked with 2ml 1N HCl. The standard curve was constructed and the quantity of the sample DNA calculated.

2.2.3 PLANT MANIPULATIONS

2.2.3.1 Basic Sterile Techniques for Tissue Culture

A laminar flow hood is the essential tool for working under the stringent sterile conditions required for tissue culture. This was sprayed and wiped thoroughly with 70% ethanol and gloved hands were similarly sprayed regularly during the work. Instruments to be used in manipulation of material were placed in 70% ethanol, flamed and allowed to cool prior to use. All manipulations were carried out with care and speed to reduce the possibility of contamination. After work was completed, the laminar flow hood was once again wiped with 70% ethanol.

2.2.3.2 Preparation of *L.corniculatus* Plant Material

Table 2.2.3 shows the media used during tissue culture manipulations. All medium was autoclaved for 20 min. at 15 p.s.i. (121°C) and cooled to 60°C prior to addition of antibiotics. All media was solidified as necessary with 0.8% agar.

(i) Preparation of seeds.

L.corniculatus cv.Leo seeds were scarified by rubbing between two pieces of paper prior to surface sterilization. This was achieved by immersion in 50% sodium hypochlorite (Chloros) and 0.01%(v/v) polyoxyehylene sorbitan monooleate (Tween 20) for 30 minutes (Dr K.J. Webb, personal communication). After sterilization, the seeds

were washed six times in sterile distilled water and then left over night in fresh sterile water, in order to initiate the period of imbibition. A further wash was then carried out to remove any tannins leached out.

(ii) Germination

Seeds were either germinated in axenic culture on media plates, or in 250ml Beatson jars containing Murashige and Skoog medium, 3% sucrose and 0.8% Bacto-agar (MSO medium), or grown in compost. Those grown in soil in plant pots were sprinkled on top of the soil. The compost was previously autoclaved for 2 hours, and the plant pots and watering trays were autoclaved for 20 min, before introduction to the plant growth room. Plants grown in pots were bottom watered every two days. In both soil and axenic culture an ambient temperature of 25°C and high light intensities (26 lux), were required.

(iii) Preparation of Non-Axenic Explants for Tissue Culture Experiments

Initial surface sterilization was carried out by submersion of stem and leaves in 20% sodium hypochlorite (Chloros) and 0.1%(v/v) Tween 20 for 30 minutes. To reduce damage caused to the plant material by the detergent, the exposed cut end of the stem was sealed with molten dental wax prior to the sterilizing treatment. Following sterilization, the plant material was washed six times with sterile distilled water prior to further manipulations. Leaves were then cut either with a 5mm diameter leaf borer or with a scalpel blade. A petri dish was used as a work surface.

(iv) Preparation of Axenic Explants for Tissue Culture.

A petri dish or sterile ceramic tile was used as a work surface and the required explant cut away. When the hypocotyl was required, it was important to ensure the radicle and plumule were both cleanly removed. When cotyledons were to be used for transformation experiments, they were scored to produce a wounded surface,exposing cells for entry of *Agrobacteria.*

Table 2.2.3: Plant Tissue Culture Medium

All media autoclaved for 20 min. at 15 p.s.i. (121°C) and cooled to < 60°C prior to addition of antibiotics. All media were solidified as necessary with 0.8% agar.

* Constituents of B_5 and MS shown in Table 2.2.4.

MEDIUM	CONTENT	pH	USE
$B_{5.5.5}$	Gamborg salts (B_5)* (Gamborg *et al*, 1968) 3% sucrose 0.5mgl^{-1} NAA 0.5mgl^{-1} BAP	5.6	Shooting medium for *L.corniculatus* leaf discs
$B_5$0	B_5 salts 3% sucrose	5.6	Cocultivation & decontamination washes. Shoot elongation for *L.corniculatus*
½$B_5$1S	½B_5 salts 1% sucrose	5.6	Rooting medium for *L.corniculatus*
B_5H	B_5 salts 2% sucrose 0.05mgl^{-1} BAP	5.6	Shooting medium for *L.corniculatus* hypocotyls and cotyledons
MSO	Murashige & Skoog salts (MS) (Murashige & Skoog, 1962) 3% sucrose	5.8	Seed germination of *L.corniculatus* and *N.tabacum* SR1 and *L.corniculatus* rooted shootlets
Fahreus'	0.7mM $CaCl.2H_2O$ 0.5mM $MgSO_4.7H_2O$ 0.7mM KH_2PO_4 0.4mM $Na_2HPO_4.12H_2O$ 20nM Ferric citrate	-	Culture of *L.corniculatus* plants on nitrogen-free medium for *Rhizobium* inoculation

Table 2.2.4: Constituents of Plant Tissue Culture B_5 and MS Media

Basal salts excluding kinetin, 2,4-D, sucrose.

Consituents:	B_5 - basal salts excluding kinetin & 2,4-D. (Gamborg *et al*, 1968) mgl^{-1}	MS - (Murashige & Skoog, 1962) mgl^{-1}
KNO_3	2500	1900
$CaCl_2.2H_2O$	150	440
$MgSO_4.7H_2O$	250	370
$(NH_4)_2SO_4$	134	-
$NaH_2PO_4.H_2O$	150	-
KI	0.75	0.83
H_3BO_3	3.0	62
$MnSO_4$	10.0	-
$ZnSO_4.7H_2O$	2.0	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_26H_2O$	0.025	0.025
Na_2EDTA	37.3*1	27.85*2
$FeSO_4.7H_2O$	27.8*1	37.25*2
Inositol	100	100
Pyridoxine HCl	1.0	0.5
Thiamine HCl	10.0	0.1
Nicotinic acid	1.0	0.5
Glycine	-	2.0
NH_4NO_3	-	1650
KH_2PO_4	-	170
$MnSO_4.4H_2O$	-	22.3

*1 - Or, in place of both components, FeNaEDTA 40.0 mgl^{-1}

*2 - Or, in place of both components, FeNaEDTA 0.1M

2.2.3.3 The Scoring System for Tissue Response in Culture

In order to assess and analyse variation of parameters in tissue culture and the subsequent transformation process, a subjective scoring system was devised, as outlined below.

Classification	Description	Expressed as Percentage of Leaf Area
H	Healthy-green tissue	-
N	Necrosis	-
B	Bleaching	-
Further divisions used;		
Nl	Limited necrosis	<10% necrotic
Nm	Medium necrosis	10-79% necrosis
Nt	Total necrosis	80-100% necrosis
Bl	Limited bleaching	<10% bleaching
Bm	Medium bleaching	10-79% bleaching
Bt	Total bleaching	80-100% bleaching

Data were represented as percentage of explants exhibiting each classification, individual explants being scored only once. Therefore, Σ(Explant classifications) = 100%. Experiments investigating the effect of phytohormones and decontaminating antibiotics on *L.corniculatus* explants or the subsequent transformation experiments, used the basic classification of healthy (H), necrosis (N) or bleaching (B) tissue. Necrosis was a browning response whilst bleaching involved loss of any coloration in the tissue. However for the study of the effect of selection antibiotics on *L.corniculatus* explants, a more detailed system was used, where the degree of necrosis or bleaching was recorded. In addition, the total explants were classified to indicate the percentage exhibiting callus production and shoot or root production;

Cal	Callus Production
S	Shoot initiation & production
R	Root initiation & production

All subsequent analysis of explant response in culture uses the appropriate scoring

system. When data is displayed in tabulated form, the total number of explants, n, described by this system is shown where possible as (n). Where fungal contamination occurred explants were discarded during the experimental period, the total number of explants (n) decreased. Similarly if explants fragmented during an experimental period the total number of explants (n) increased.

2.2.3.4 Statistical Analysis

Contingency Test

The test of association or independence (G-test) was applied to data collected by use of the classification system outlined in 2.2.3.3.

The null hypothesis states that there is no relationship between treatments and tissue responses.

In algebraic terms:

	Treatment 1	Treatment 2	Σ
Category A	a	b	a + b
Category B	c	d	c + d
Totals: Σ	a + c	b + d	a+b+c+d (n)

Yates' correction must be applied when n = ≤ 200 but only with a 2 x 2 contigency table. When ad - bc is positive then subtract ½ from a and d and add ½ to b and c. When ad - bc is negative add ½ to a and d and subtract ½ from b and c.

Then the following quantities are calculated:

Q1 = Σflnf for cell frequencies (a, b, c and d)

Q2 = Σflnf for row and column totals [(a+b) + (c+d) + (a+c) + (b+d)]

Q3 = nlnn

So, $G_{adj} = 2[Q1 - Q2 + Q3]$

G_{adj} is compared to the critical value of X^2 for appropriate degrees of freedom [(number of treatments - 1) - (number of categories - 1)] If $G_{adj} < X^2$, then null hypothesis is accepted, if $G_{adj} > X^2$, then null hypothesis is rejected.

2.2.3.5 *L.corniculatus* Transformation Protocols

pTi Transformations

(a) Leaf Discs

The general protocol used for transformation of *L.corniculatus* leaf discs is outlined below but conditions used in specific transformations varied as detailed in Results, chapter 3.

Leaf explants were cut with a sterile cork borer, 5mm in diameter or scalpel blade, from sterilised material. Co-cultivation of the explants with *Agrobacterium* consisted of two stages:

(1) Initially explants were placed in a liquid suspension of the *Agrobacterium* and left for a period of time on a slow shaker, 60rpm.

(2) Explants were transferred to media plates, overlaid with filter papers and were left in the dark, sealed, at 25°C for 48hours.

The subsequent decontamination was similarly a two phase event with a series of at least 6 washes in sterile distilled water, followed by an antibiotic wash in liquid plant media on a shaker. The leaf explants were then dried on sterile filter paper and plated on decontamination medium (200mgl^{-1} augmentin) for 48 hours in the light, prior to transfer onto selection media which contained both the decontamination antibiotic and a selection antibiotic.

(b) *In vitro* Hypocotyls (Ghose, 1988)

Hypocotyls were excised from 10 day old seedlings and immersed in the *Agrobacterium* suspension in B_5H liquid. This was left in the dark on a 60rpm slow shaker for 36-48 hours. A thorough wash with sterile distilled water precedes the transfer of the explants to fresh B_5H liquid containing 250 mg l^{-1} cefotaxime in a Beatson jar. This was left for 2 days in the light, shaking at 60rpm. Then the explants were dried on filter paper and placed on selection plates containing 250 mg l^{-1} cefotaxime and 50 mgl^{-1} kanamycin.

Ri-plasmid Transformation

(1) Inoculation of Seedlings

Axenic seedlings were inoculated at day 7-10 with fresh *A.rhizogenes*. The seedlings were inoculated with the fresh *Agrobacterium* and were then wounded on the hypocotyl region, below the cotelydons, with a sterile hypodermic needle of 1.1mm bore.

The plates were resealed with nescofilm and the initial transformation attempts were placed in the dark at 25°C at an angle. Later the method was modified and the method finally adopted was to leave the petri-dishes horizontal.

(2) Stem Co-cultivation (K.J. Webb, pers.comm.)

Lotus stems were sterilised as for leaf pieces and the internodes or nodes were cut (5-10mm long). These were co-cultivated in previously prepared *A.rhizogenes* suspension for various time intervals, (see individual experiments). The pieces were placed onto B_5H media and left in light at 25°C for 3 days. The stem pieces were then placed on the above media containing 300 mgl^{-1} cefotaxime and left for 4 days in the light before being transferred to the media containing 250mgl^{-1} cefotaxime and 30mgl^{-1} kanamycin. This method is similar to that of Tabaeizadeh, (1989).

"Hairy roots" were cultured in liquid or on $\frac{1}{2}B_50$ solid medium.

2.2.3.6 ß-Glucuronidase Assays

(a) Fluorometric Assay (Modified from Jefferson *et al*, (1987))

When plant samples were harvested, non-axenic material was washed with 70% ethanol followed by a rinse with distilled water. The tissue was then ground in a sterile 1.5ml eppendorf with a ground glass rod in 100µl extraction (MUG) buffer (50mM NaH_2PO_4, 10mMEDTA pH6.8/7.0, 0.01% v/v Triton-X100, 10mM mercaptoethanol, 1mM DTT) with several glass beads added for tissue disruption. The contents of the eppendorfs were centrifuged for 10 min, 12500g, 4°C and the supernatant transferred to a fresh tube. The centrifugation step was repeated with the supernatant and 40µl of the resultant supernatant was used in the subsequent assay.

The other components of the final reaction were added to this supernatant; 360µl MUG buffer, 47µl 4-Methylumbelliferyl glucuronide (MUG) substrate (100mM stock made up in buffer). Following vortexing, the reaction was incubated at 37°C.

100µl samples were removed at 15 minute intervals and added to 1900µl 0.2M Sodium carbonate in clear sided cuvette tubes, the reaction was thus halted at this stage. Fluorescence was then assayed on a Fluoripoint (FP100) spectrofluorimeter. The conditions for reading the Methylumbelliferone fluorescence were; excitation 365nm, emission 455nm with slit width 10nm. The fluorimeter was calibrated by assaying the product of the reaction, Methylumbelliferone (MU), at different concentrations, 10µM-0.01nM. It was found that two readings at 1µM and 100nM MU was sufficient. The MU was dissolved in 0.2M Sodium carbonate and diluted as necessary to read a 2ml volume.

The crude units of fluorescence data was then standardised using the product calibration data. Thus the results could be presented in terms of nM MU produced and finally as nM MU produced μg^{-1} protein min^{-1}.

(b) Histochemical Technique

The substrate used in this technique was 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The final product of ß-glucuronidase activity on X-Gluc is a blue precipitate, clearly visible by eye or under a microscope.

The X-Gluc substrate was prepared as a 2mM solution in 0.1M sodium phosphate buffer pH7 (30.5 units 0.2M Na_2HPO_4 + 19.5 units 0.2M NaH_2PO_4). This was then aliquoted into 500µl fractions and stored at -20°C.

The histochemical qualitative assay was carried out in both leaf and root material. The material was sliced up using a scapel and placed in a large eppendorf containing 10µl phosphate buffer and 10µl X-Gluc solution. This was gently mixed, ensuring all the plant samples were covered. The reaction solution was then left at 37°C

overnight. With leaf material, it was necessary to dehydrate the material through a series of ethanol dilutions; 12.5%, 25%, 50%, 75%, 100% - 10 min in each. Alternatively material could be placed in 100% EtOH immediately. This removes the chlorophyll from the material.

The plant tissue was viewed under a binocular microscope as necessary to scan for the presence of any blue precipitate.

2.2.3.7. Determination of Protein Content of Plant Extracts

Material was lyophilised for 18 hours and ground in 1.5ml eppendorf. Tissue was resuspended in extraction buffer (50mM Tris-HCl pH .5) and shaken at 4°C. The homogenate was centrifuged at 900g for 5 min, supernatant removed to a fresh tube and then recentrifuged at 900g for 5 min. The protein content of this supernatant was then determined by a modification of the dye-binding assay of Bradford, (1976).

The protein assay reagent consisted of 20mg Coomassie brilliant blue G-250 dissolved in 10ml 95% (v/v) ethanol. To this solution 20ml 85% (w/v) phosphoric acid was added and the solution diluted with distilled water to a final volume of 200ml.

A calibration curve of 0-500 $\mu g\ ml^{-1}$ BSA standards was set up, together with the test samples, 20µl of each solution was added to 200µl of assay reagent in wells of a microtitre plate. The blank reading wells contained 200µl assay reagent and 200µl 50mM Tris-HCl pH 9.5. Absorbance was read at 595 nm. A standard curve was constructed using the BSA standards, from which the protein content of the samples was extrapolated.

III. RESULTS

III. RESULTS

CHAPTER 1: Development of the tissue culture regime for Lotus corniculatus

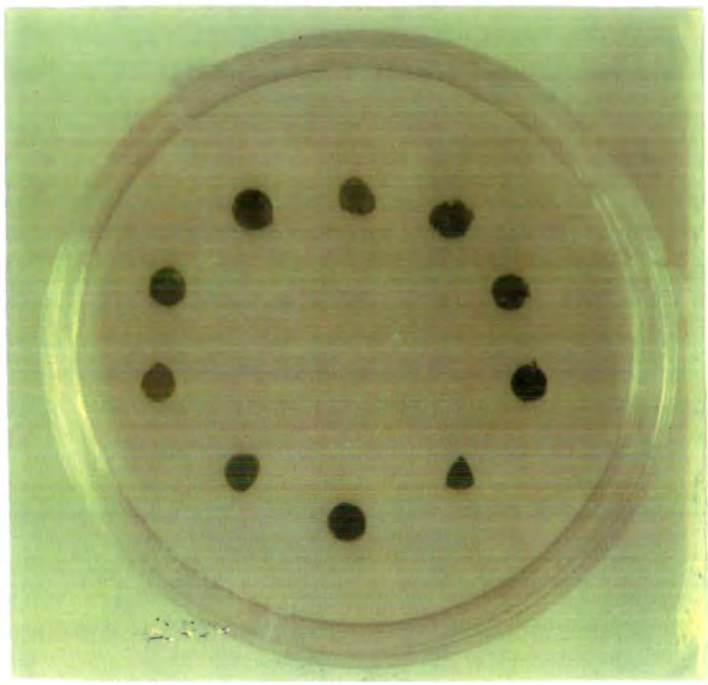
3.1.1 Introduction

Work on the culture conditions for axenic leaf explants of *L.corniculatus* has been based on the technique previously established by Webb, (1986). The initial culture agar medium, $B_{5.5.5}$, (table 2.2.3). $B_{5.5.5}$ medium induces tissue to proliferate as callus, which tends generally to be friable in nature. The differentiation process over time of *L. corniculatus* leaf explants in culture is shown in figure 3.1.1, and can be divided into 4 tissue culture stages. During the first 14 day period leaf tissue adjusts to tissue culture conditions and de-differentiation occurs. The de-differentiated cells begin cell division and this cell proliferation results in the appearance of callus tissue (figure 3.1.1b). During the following 14 days, further production of callus occurs, and finally, after approximately 4 weeks in culture, organ initiation mainly in the form of shoot primordia (figure 3.1.1c), becomes apparent. To a lesser extent, some root organogenesis is also present. Shoots generally take two weeks to grow to more than 5mm in size, which then can develop into viable plantlets on rooting medium.

Rooting was induced on 25ml agar slopes based on Gamborg's B_5 salts, containing no plant hormones, and with a reduced sugar content ($\frac{1}{2}B_5$1S; see table 2.2.3). Roots emerged after two weeks, and after a total of about 4 weeks on the rooting medium, the plants were ready to be transferred to 75ml horizontal agar beds in 250ml Beatson jars. The plants could be maintained in such cultures for many weeks, although once the root system had become well established and the shoot(s) extended, they were usually potted into soil and hardened off, (section 2.2.3). During the early stages, while explants were kept under a low intensity light regime (9 lux) to stimulate shoot production. Individual shoots were excised and placed at a higher light intensity (26 lux), a level which was maintained throughout the rest of the culture process and for soil-grown plants.

Figure 3.1.1: Tissue Culture Stages of *Lotus corniculatus* Leaf Explants
(Number of days corresponds to time in culture)

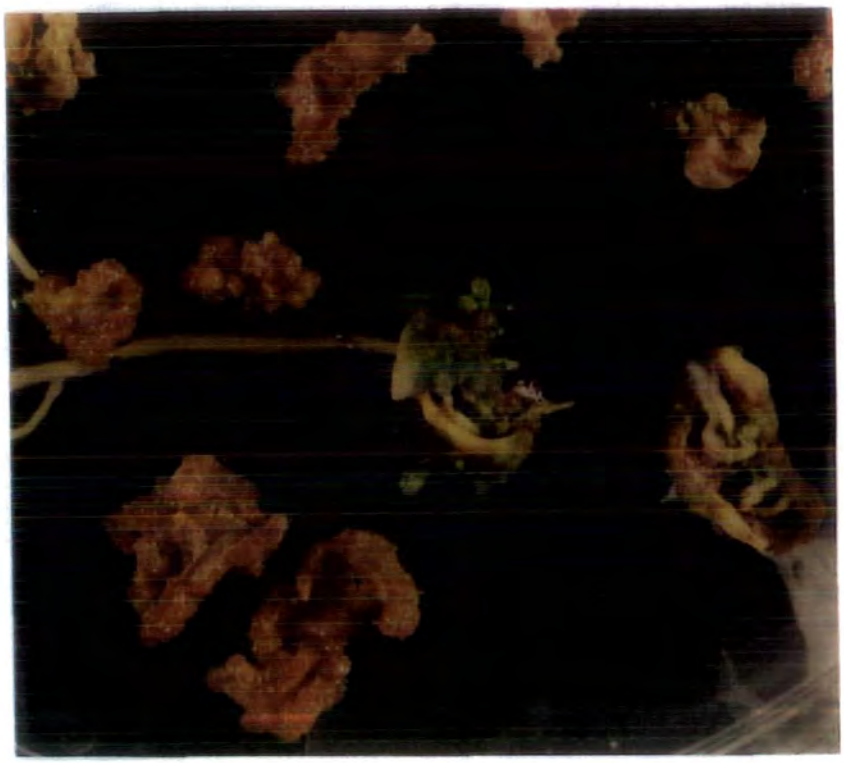
(a) Leaf Explants freshly excised - day 0.



(b) Callus production - from day 14.
Some explants show root differentiation during this early cell proliferation stage.

Figure 3.1.1 continued:

(c) Shoot initiation from callus cells - from day 21.



(d) Individual shoots are excised and transferred to rooting slopes.
Root differentiation takes 2-3 weeks.

Figure 3.1.1 continued:

(e) Plantlets transferred to a larger volume of medium to extend both root and aerial growth.

Figure 3.1.1 continued:

(f) Plants finally potted into compost
Plants exhibit features typical of *L.corniculatus*; Decumbent and erect growth. Trifoliate leaves can be easily seen here.

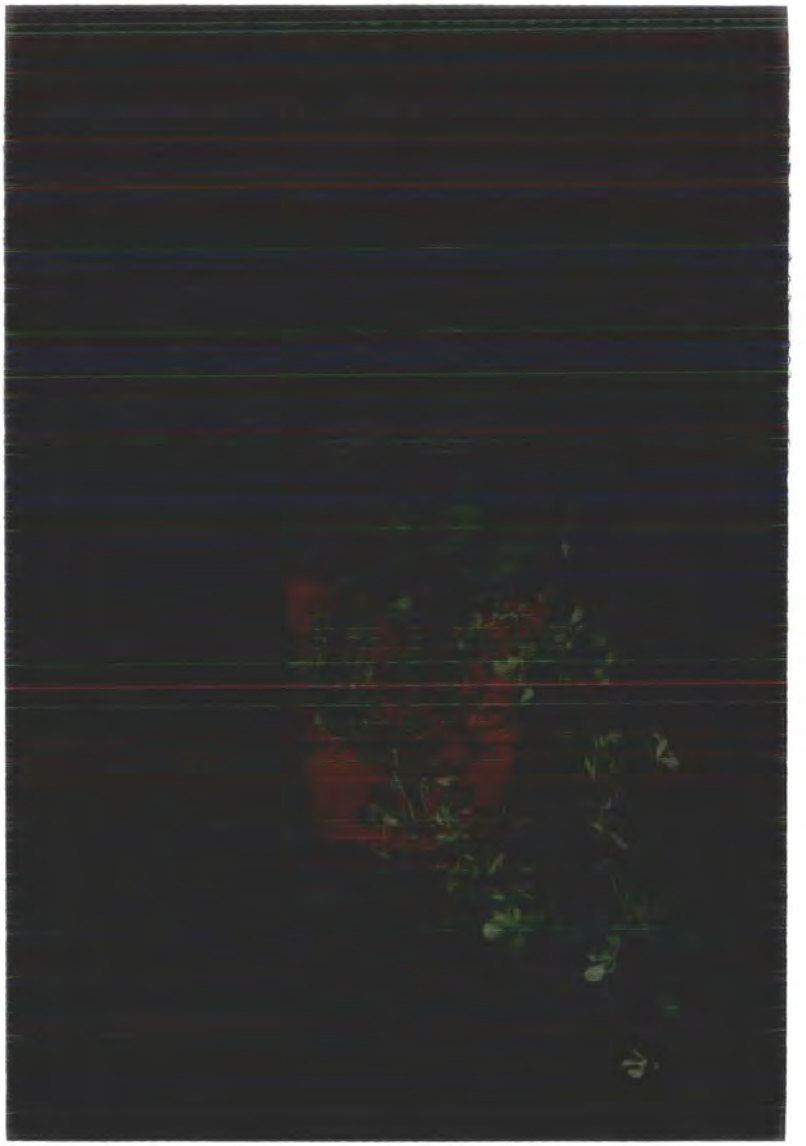


Figure 3.1.2, shows explants exhibiting the different levels of necrosis or bleaching. Differentiating between the classifications was sometimes difficult, with for example, both necrosis and bleaching tissue sometimes present on a single explant. Despite such problems, this system did allow comparisons of plant material and the initial assessment of the success of a transformation experiment. The response of control *L.corniculatus* leaf explants grown on control medium under axenic tissue culture conditions is presented in terms of this classification system in table 3.1.1.

3.1.2 Effect of Hormones on *Lotus corniculatus* in Culture

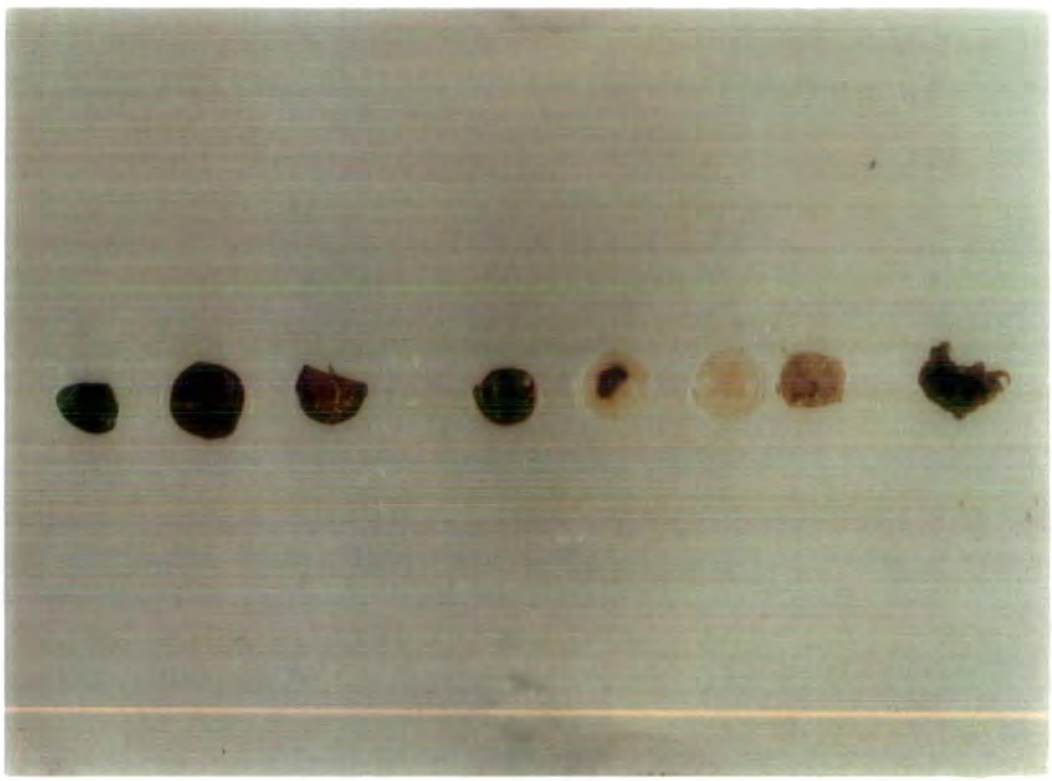
Experiments were carried out in which the hormonal constitution of the culture medium was perturbed in an attempt to reduce the overall timescale of regeneration, from explant to plant. Many exogenous factors affect explant culture, such as the constituents of the basal medium, pH, sugar content and phytohormones. Variation of all or some of these parameters can alter the end product/sequence of regeneration of a plant in culture.

Comparisons of growth of the *L.corniculatus* leaf explants on B_5 media containing a variety of hormones were carried out.

3.1.2.1 Effect of Cytokinins on Shoot Regeneration

In the first experiment in this series, a comparison was made of different cytokinins (BAP and kinetin) on shoot differentiation at 0.5 mgl^{-1}. Data collected over a six week period are summarised in table 3.1.2. This experiment showed that cytokinin in culture medium was essential for shoot regeneration of *L.corniculatus* tissue. Even after only 7 days on hormone-free (B_50) medium, more explants were showing a bleached reaction than those on the normal $B_{5.5.5}$ medium. This deterioration of tissue on B_50 continued with increasing intensity over the 28 day experimental period. Cell proliferation was absent in this material.

Figure 3.1.2: Classification of Explants in Culture



From left to right:

H - Healthy

Nl - Limited necrosis (<10% explant area)

Necrosis Nm - Medium necrosis (10 - 79% explant area) - This example is at the margin between Nm and Nt classifications.

Nt - Total necrosis (80 - 100% explant area)

Bl - Limited bleaching (<10% explant area)

Bleaching Bm - Medium bleaching (10 - 79% explant area)

Bt - Total bleaching (80 - 100% explant area)

Bt - " " (" " ") - This example is difficult to classify as necrotic or bleached, but the lighter tone of the explant suggests a type of bleached response.

Cal - Callus production

Table 3.1.1: Growth of leaf discs of *Lotus corniculatus* cv.Leo on $B_{5.5.5}$ agar with no added antibiotics (Control)

Expressed as a percentage of each classification

DAY	% of total explants exhibiting each classification
7	H 76.7 Nl 23.3 (50)
14	H 38.3 Nl 29.8 Nm 29.8 Bm 2.1 *Cal 85.1* (47)
21	H 35.0 Nl 5.0 Nm 17.5 Nt 42.5 *Cal 100.0* (40)
28	Nm 13.3 Nt 86.7 *Cal 100.0* *S 30.0* (30)

Key
H - Healthy tissue
Nl - Limited (<10% explant area) necrosis
Nm - Medium (10-79% area) necrosis
Nt - Total (80-100% area) necrosis
Bl - Limited (<10% area) bleaching
Bm - Medium (10-79% area) bleaching
Bt - Total (80-100% area) bleaching

Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over period of experiment loses are due to explant contamination.

Similarly, explants cultured on B_5 containing 0.5mgl^{-1} kinetin exhibited no regenerative ability beyond the production of de-differentiated callus. General bleaching and necrosis accompanied the process. Shoot development was apparent on B_5 containing 0.5mgl^{-1} BAP and so this initial experiment confirmed the choice of BAP as the cytokinin for optimal *L.corniculatus* regeneration.

Tissue on the control $B_{5.5.5}$ medium responded as before (table 3.1.1) with callus production by day 14, followed by shoot iniation by day 21. A similar response was seen with explants on B_5 medium containing 0.5mgl^{-1} BAP only. However, callus production was not initiated as fast on all the explants, with only 77.4% of explants on B_5+0.5mgl^{-1} BAP producing callus as compared to 100% of explants on $B_{5.5.5}$ at day 28. So, the presence of 0.5mgl^{-1} NAA significantly enhances callus production on *L.corniculatus* leaf explants (G_{adj}=10.216, $X^2_{.001[1]}$).

When the second important measure of regenerative potential, the frequency of shoot development, was compared there is shown to be no significant variation in shoot proliferation between explants cultured on $B_{5.5.5}$ and B_5+0.5mgl^{-1} BAP media (G_{adj}=1.298; $X^2_{.05[1]}$=3.84).

Although the process of de-differentiation followed by differentiation of shoots were exhibited by explants on B_5 containing 0.5mgl^{-1} BAP only, these explants bleached more than those on $B_{5.5.5}$ which tended to remain healthy or show slight necrosis. So, the presence of both BAP and NAA was necessary to reduce detrimental tissue responses which would cause additional pressure on explants involved in subsequent transformation experiments.

Table 3.1.2: Effect of Cytokinins on L.corniculatus in axenic culture

Expressed as a percentage of each classification

DAY	$B_5 0$	B_5+0.5mgl^{-1} BAP	B_5+0.5mgl^{-1} Kinetin	$B_{5.5.5}$
7	H 65.0 B 35.5 (45)	H 90.0 B 10.0 (50)	H 73.3 B 26.7 (30)	H 78.6 Nl 19.0 Bl 2.4 (84)
14	H 26.1 N 39.1 B 34.8 (46)	H 53.1 N 31.1 B 15.6 *Cal 50.0* (32)	H 30.0 N 40.0 B 30.0 *Cal 30.0* (30)	H 36.8 N 37.7 B 25.5 *Cal 98.9* (65)
21	B 100.0 (31)	-------	H 8.7 N 43.5 B 47.8 *Cal 34.8* (23)	H 52.9 N 47.1 *Cal 98.9* (51)
28	N 18.2 B 81.8 (22)	N 25.8 B 74.2 *Cal 77.4 (24)* *S 45.2 (14)* (31)	N 65.0 B 35.0 *Cal 10.0* (20)	H 26.0 N 74.0 *Cal 100.0 (50)* *S 30.0 (15)* (50)

Key

H - healthy explants; N - necrotic explants; B - bleached explants;

Cal - explants exhibiting callus production; *S* - explants exhibiting shoot development

(n) - Total number of explants, where n decreases over period of experiment loses are due to contamination.

Bold figures in parentheses represent actual number of explants for statistical analysis.

3.1.2.2. Influence of Cytokinin : Auxin Hormone Ratio

The effect of altering the proportions of auxin:cytokinin in the media on the regenerative response of *L.corniculatus* was investigated, since small changes can channel differentiation in the direction of either shoot or root production. When auxin levels are greater than cytokinin levels the equilibrium is pushed towards the initiation of root primordia. When cytokinin levels are greater than the auxin concentration, the equilibrium is pushed towards the production of shoot primordia. So, it would be expected that increasing BAP in relation to NAA would induce greater shoot production.

An initial experiment investigated the effects of (a) decreasing NAA levels but keeping the BAP level constant at $0.5mgl^{-1}$ and conversely (b) increasing levels of BAP with NAA remaining constant at $0.5mgl^{-1}$. The explants were maintained in culture for 14 days and over this period variation in the response of the tissues became apparent. Medium B_5+BAP only showed high levels of bleaching but and there was significant bleaching on all media with NAA content of $<0.3mgl^{-1}$ in comparison to $B_{5.5.5}$ (figure 3.1.3). The increased bleaching response is shown in figure 3.1.3a. The production of callus by explants on reduced NAA content media was variable but no medium results in a significantly higher frequency of callus production than $B_{5.5.5}$ while 40% explants showed callus formation.

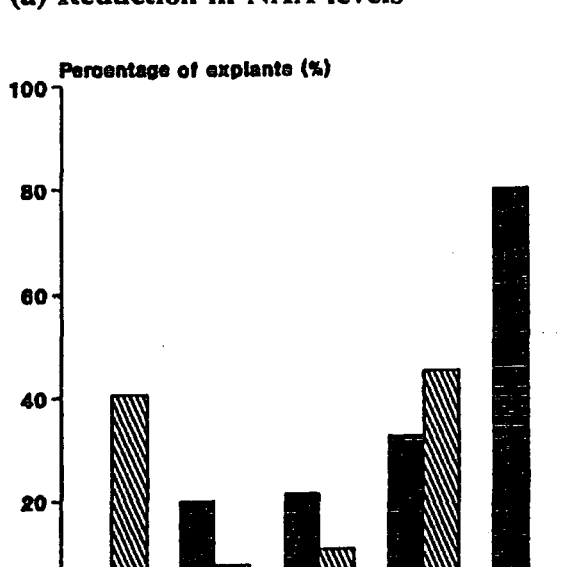
Increased BAP levels with $0.5mgl^{-1}$ NAA gave less consistent results, (figure 3.1.3b). However, every medium except B_5 with $0.9mgl^{-1}$ BAP, resulted in significantly greater levels of bleaching than occurred on the standard $B_{5.5.5}$ medium.

A second experiment was carried out over a longer period to obtain more information on the optimum hormone balance for shoot proliferation. Material was maintained in culture for 4 weeks, with transfer to fresh medium every two weeks.

Figure 3.1.3: Percentage of leaf explants of *Lotus corniculatus* which are Bleached when Cultured on Different Levels of NAA and BAP.

At day 14

(a) Reduction in NAA levels



Statistical analysis of percentage of bleached leaves cultured on different levels of NAA compared to $B_{5.5.5}$.

0.4 mgl^{-1} G_{adj}= 2.97 (not significant)

0.3 mgl^{-1} G_{adj}=3.128 (not significant)

0.2 mgl^{-1} G_{adj}=8.824*

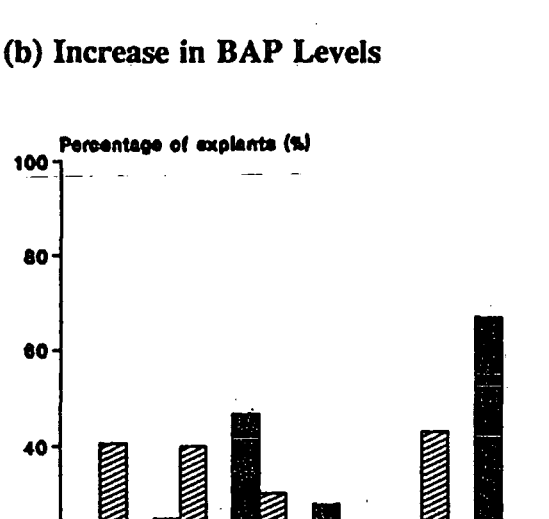
0.0 mgl^{-1} G_{adj}=42.3**

* When $X^2_{.05[1]}$=3.84

**When $X^2_{.001[1]}$=10.83

So, when NAA is present at <0.3mgl^{-1}, bleaching is significantly greater than that present on $B_{5.5.5}$ medium.

(b) Increase in BAP Levels



Statistical analysis of percentage of bleached leaves cultured on different levels of BAP, compared to $B_{5.5.5}$.

0.6 mgl^{-1} G_{adj}=78.148***

0.7 mgl^{-1} G_{adj}=22.544***

0.8 mgl^{-1} G_{adj}=987.002***

0.9 mgl^{-1} G_{adj}=0.182 (not significant)

1.0 mgl^{-1} G_{adj}=29.598***

*****When $X^2_{.001[1]}$=10.83**

So, significantly higher levels of bleaching occurs on the majority of media containing levels of BAP >0.5mgl^{-1}, except 0.9mgl^{-1} BAP.

Figure 3.1.4 shows the frequency of bleaching reaction and shoot production on the *L.corniculatus* explants placed on media with various hormone combinations. The general trend of increasing bleached reaction with reductions in NAA levels as well as an accompanying loss of shooting ability can be seen. The low shooting frequency on the media with raised BAP levels is also apparent.

From these experiments it was concluded that the optimum culture medium for use with *L.corniculatus* leaf explants was $B_{5.5.5}$ containing 0.5mgl^{-1} BAP and NAA as recommended by Webb, (1986).

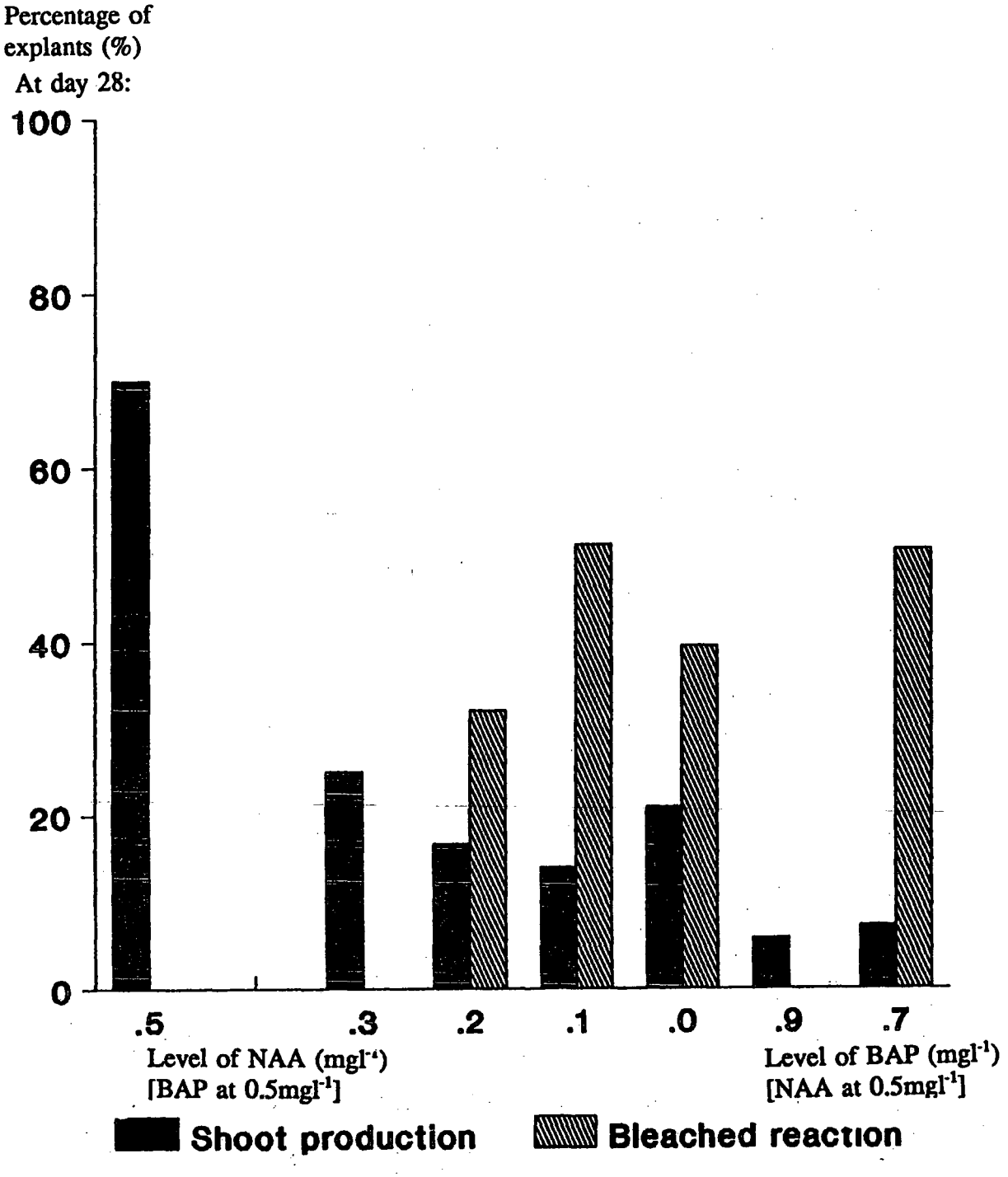
Further observations (data not shown) indicated that shoot initiation required the presence of NAA and BAP but good internodal elongation occurred on hormone-free medium, B_50, when in conditions of high light intensity (26lux). This additional culture stage was adopted to stimulate shoot elongation during transformation experiments after shoot primordia induction.

3.1.3. Effect of Decontamination Antibiotics on Plant Differentiation

The recommended procedure for removing *Agrobacterium* contamination from co-cultivated explants involved the use of two antibiotics, cefotaxime and carbenicillin (Nisbet, 1987). Explants were first washed in liquid culture medium containing 800mgl^{-1} carbenicillin for up to 1 hour. After blotting to remove excess liquid the explants were transferred to solid medium containing either 400mgl^{-1} carbenicillin or 150mgl^{-1} cefotaxime and incubated in the dark for up to 5 days prior to transfer to medium with the selectable marker being used.

However, financial constraints as well as basic microbiological common sense meant that it would be beneficial to utilize only one antibiotic. The expense of using high concentrations of carbenicillin meant cefotaxime, at the increased level of 250mgl^{-1} was used on its own. However, experience showed that some agrobacterial overgrowth did sometimes occur during the subsequent culture process. Also previous work using *N.tabacum* SR1 (Edwards, pers.comm.) indicated the antibiotic augmentin was a

Figure 3.1.4: Frequency of Bleached Reaction and Shoot Production on *Lotus corniculatus* Leaf Explants as Hormone Content is Varied.



possible alternative, as augmentin did not inhibit rooting of excised shoots which cefotaxime had been shown to cause (Edwards, pers. comm.). Experiments were therefore set up in order to establish whether there was any detrimental effect of augmentin and cefotaxime on the ability to regenerate shoots in cultured *L.corniculatus* leaf explants and the subsequent establishment of root systems on the shoots.

3.1.3.1 Effect of Decontamination Antibiotics Upon Shooting Ability

Leaf explants were plated out onto three media: $B_{5.5.5}$ only, $B_{5.5.5}$ + 200mgl^{-1} augmentin and $B_{5.5.5}$ + 250mgl^{-1} cefotaxime, and cultured for 7 weeks. Explants were transferred to fresh medium every 2-3 weeks.

The resulting growth was compared and the data is shown in figure 3.1.5. The explants on the control $B_{5.5.5}$ medium followed the previously described tissue culture cycle, though by day 35, contamination was severe. Cell proliferation, as measured by the number of explants showing callus formation, was not reduced by the presence of the antibiotics. Use of the G-test to show independence of data collected as number of explants producing shoots, showed there was no statistical difference in shooting ability of explants on media containing decontamination antibiotics. When compared to frequency of shoot differentiation on the standard antibiotic-free $B_{5.5.5}$.
$B_{5.5.5}$ media containing either 200mgl^{-1} augmentin (G_{adj}+0.008, where $X^2_{.05[1]}$=3.84) or 250mgl^{-1} cefotaxime (G_{adj}=0.178, where $X^2_{.05[1]}$=3.84) showed minimal variation in shoot production.

3.1.3.2 Effect of Decontamination Antibiotics Upon the Rooting Ability of Shoots

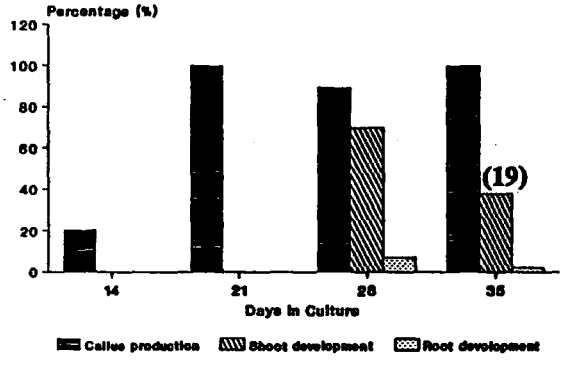
Shoots excised from explants grown on $B_{5.5.5}$ medium were transferred to three types of rooting slopes: ½$B_5$1S, ½$B_5$1S+250mgl^{-1} cefotaxime and ½$B_5$1S+200mgl^{-1} augmentin. The percentages of shoots which produced roots over the 4 week period are shown in graphical form in figure 3.1.6.

Shoots placed in ½$B_5$1S+250mgl^{-1} cefotaxime were significantly less able to establish roots. Shoots on this medium were only half as likely to root than those on

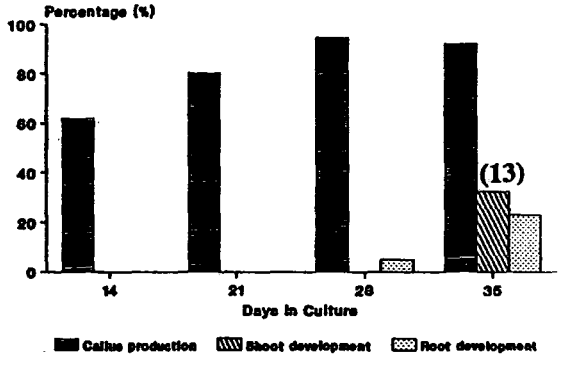
Figure 3.1.5: Effect of Antibiotics on Ability of Explants to Produce Shoots

(n) = Total number of shoots

(a) Differentiation on $B_{5.5.5}$ + 250 mgl^{-1} Cefotaxime



(b) Differentiation on $B_{5.5.5}$ + 200 mgl^{-1} Augmentin



(c) Differentiation on $B_{5.5.5}$ only (Control)

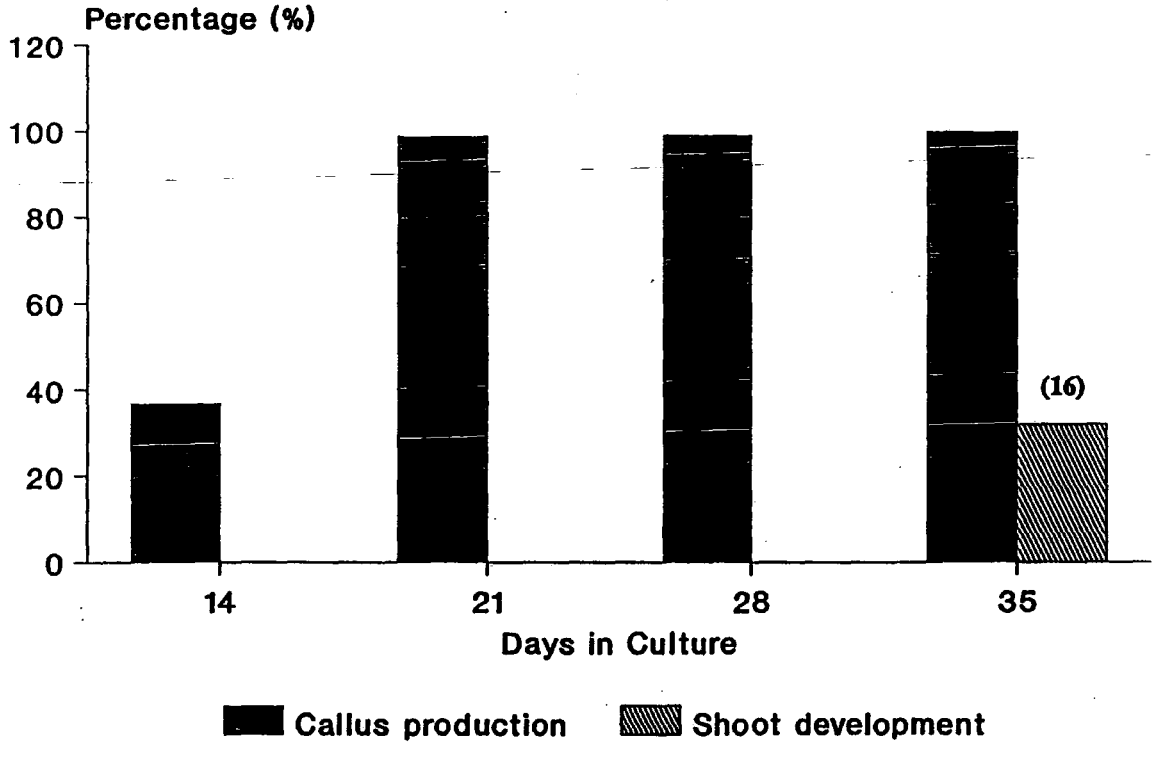


Figure 3.1.6: Effect of Antibiotics on Rooting Ability of Shoots

Key

CON = Shoots on ½$B_5$1S only

CEF = Shoots on ½$B_5$1S + 250mgl^{-1} cefotaxime

AUG = Shoots on ½$B_5$1S + 200mgl^{-1} augmentin

(n) = Number of shoots which rooted, (figure used in statistical analysis).

the control or ½$B_5$1S+200mgl^{-1} augmentin, by day 28. Statistical analysis using the G-test shows significant differences in the rooting potential on the two antibiotic containing media. When analysed, control medium versus augmentin medium, G_{adj} = 0.112, as this is $\leq X^2_{.05[1]}$, 3.84 the null hypothesis is accepted. In contrast, when control medium versus cefotaxime medium results were analysed, G_{adj} = 17.8. Thus the null hypothesis must be rejected as G_{adj} was greater than the critical $X^2_{.001[1]}$. Thus these results demonstrate the severe reduction in the ability of *L.corniculatus* shoots to produce roots when 250mgl^{-1} cefotaxime is present in the rooting medium.

Augmentin was therefore adopted as the decontaminating antibiotic against *Agrobacterium* in later transformation experiments. For the initial liquid wash a concentration of 400mgl^{-1} was used for 2 hours,and then 200mgl^{-1} augmentin in the semi-solid agar plates. This antibiotic was required at least until shoots were excised, and generally until plants were transferred to 250ml Beatson jars.

3.1.4 Establishing a Selection System for *Lotus corniculatus* Explants

The selection of transformed cells in explants co-cultivated with *A.tumefaciens* is an important part of a transformation system: the choice of antibiotic and its concentration are critical for success. Incorporated within the pJIT73 construct are two genes coding resistance to antibiotics neomycin phosphotransferase (*nptII*) and hygromycin phosphotransferase (*aphIV*). *NptII* encodes resistance to the aminoglucosides, kanamycin or geneticin (G-418). *AphIV* encodes resistance to hygromycin B, an aminocyclitol antibiotic.

In order to distinguish which antibiotic would be the most effective to use as a selectable marker for transformation, antibiotic kill curves were set up. Leaf explants were placed onto a series of media containing various antibiotic concentrations. Over the duration of these experiments, it became evident that uniformity was difficult to maintain when describing the leaf disc morphology, according to the classification system previously defined. Nevertheless, callus and shoot production were easy to define and useful results were obtained.

3.1.4.1 Kanamycin Selection

Previous work attempting *A.tumefaciens*-mediated transformation with *L.corniculatus* by Nisbet, (1987) had utilized kanamycin (100 mgl^{-1}) to confer selection pressure on co-cultivated explants. However, the putative transformants, when analyzed at the DNA level, did not contain the *nos-neo* gene and T-DNA incorporation into the host genome had not taken place. Conditions for kanamycin selection were therefore re-investigated.

Data collected over a 28 day period, (table 3.1.3) shows that levels as low as 25mgl^{-1} kanamycin reduced or prevented cell proliferation, with no callus being formed. The main response was necrosis but a degree of bleaching was also observed. The classifications at day 14 were compared at 0mgl^{-1} and 25mgl^{-1} kanamycin, when critical $X^2_{.001[4]}=18.47$; the calculated $G_{adj}=74.192$. Thus 25mgl^{-1} kanamycin to significantly alters the tissue response by reducing the amount of healthy tissue present and causing an increased frequency of bleached reaction.

Similarly, the difference in tissue reaction at increased concentrations of kanamycin can be analysed. Data for 25, 50, 75 and 100mgl^{-1} kanamycin were compared, when critical $X^2_{.05[9]}=16.92$ the calculated $G_{adj}=6.25$. Thus it may be concluded that the level of kanamycin does not affect tissue response of *L.corniculatus* leaf explants. Although these experiments indicated that kanamycin inhibits differentiation of *L.corniculatus* leaf explant tissue, a previous study by Nisbet, 1987 had demonstrated that kanamycin gave "leaky" selection pressure. In the *A.tumefaciens-L.corniculatus* transformation system only untransformed shoots differentiated.

3.1.4.2 G-418 Selection

This second aminoglycoside compound was found to have a much more severe effect on *L.corniculatus* leaf explants than kanamycin. Table 3.1.4,shows the data collected over a 21 day period with a low concentration series, 5-20 mgl^{-1}. Even after 7 days, all leaves were bleached to varying degrees. The 3.3% explants still classed as healthy on 15mgl^{-1} G-418 were larger and thicker tissue sample(s), perhaps accidently

Table 3.1.3: Effect of Different Levels of Kanamycin on growth of *Lotus corniculatus* leaf discs
Expressed as a percentage of each classification

Mgl^{-1}	0	25	50	75	100
DAY					
7	H 100.0 (25)	H 16.7 Nl 30.0 Bl 50.0 Bm 3.3 (30)	H 13.3 Nl 30.0 Bl 53.3 Bm 3.3 (30)	H 23.3 Nl 20.0 Bl 46.7 Bm 10.0 (30)	H 35.0 Nl 15.0 Bl 45.0 Bm 5.0 (20)
14	H 62.5 (15) Nm 37.5 (9) *Cal* 83.3 (20) (24)	Nl 60.0 (18) Bl 36.7 (11) Bm 3.3 (1) (30)	Nl 55.2 (16) Bl 41.4 (12) Bm 3.4 (1) (29)	H 6.7 (2) Nl 56.7 (17) Bl 30.0 (9) Bm 6.7 (2) (30)	Nl 60.0 (12) Bl 35.0 (7) Bm 5.0 (1) (20)
21	H 60.0 Nm 40.0 *Cal 100.0* (10)	Nl 76.7 Nm 10.0 Bl 10.0 Bm 3.3 (30)	Nl 60.0 Bl 36.7 Bm 3.3 (30)	Nl 56.7 Bl 33.3 Bm 10.0 (30)	Nl 70.0 Bl 25.0 Bm 5.0 (20)
28	-----	Nl 60.0 Bl 33.3 Bm 6.7 (30)	Nl 56.7 Bl 40.0 Bm 3.3 (29)	Nl 43.3 Bl 50.0 Bm 6.7 (30)	Nl 75.0 Bl 20.0 Bm 5.0 (20)

Key
H - Healthy tissue
Nl - Limited (<10% explant area) necrosis
Nm - Medium (10-79% area) necrosis
Bl - Limited (<10% explant area) bleaching
Bm - Medium (10-79% area) bleaching
Bt - Total (80-100% area) bleaching

Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over the period of the experiment loses are due to contamination.

Bold figures in parentheses represent actual number of explants for statistical analysis

Table 3.1.4: Effect of Different Levels of G-418 on Growth of Leaf Discs of *Lotus corniculatus*

Expressed as a percentage of each classification

Mgl^{-1}	0	5	10	15	20
DAY					
7	H 100.0 (25)	Bl 85.0 Bm 15.0 (20)	Bl 70.0 Bm 30.0 (30)	H 3.3 Bl 43.3 Bm 50.0 Bt 3.3 (30)	Bl 23.3 Bm 53.3 Bt 23.3 (30)
14	H 62.5 (15) Nm 37.5 (9) *Cal* 83.3 (20) (24)	Nl 10.0 (2) Bl 50.0 (10) Bm 35.0 (7) Bt 5.0 (1) (20)	Bl 13.3 (4) Bm 56.7 (17) Bt 30.0 (9) (30)	Bl 13.3 (4) Bm 50.0 (15) Bt 36.7 (11) (30)	Bm 20.0 (6) Bt 80.0 (24) (30)
21	H 60.0 Nm 40.0 *Cal* *100.0* (10)	Bl 40.0 Bm 35.0 Bt 25.0 (20)	Bm 53.8 Bt 46.2 (26)	Bl 5.0 Bm 50.0 Bt 45.0 (20)	Bm 10.0 Bt 90.0 (20)
	G_{adj}=60.632 $X^2_{.001[5]}$=20.52	G_{adj}=15.072 $X^2_{.01[3]}$=11.35	G_{adj}=0.324 $X^2_{.05[2]}$=5.99	G_{adj}=14.478 X^2.001[2]=13.82	
Null hypothesis states that there is no difference treatments	Rejected	Rejected	Accepted	Rejected	

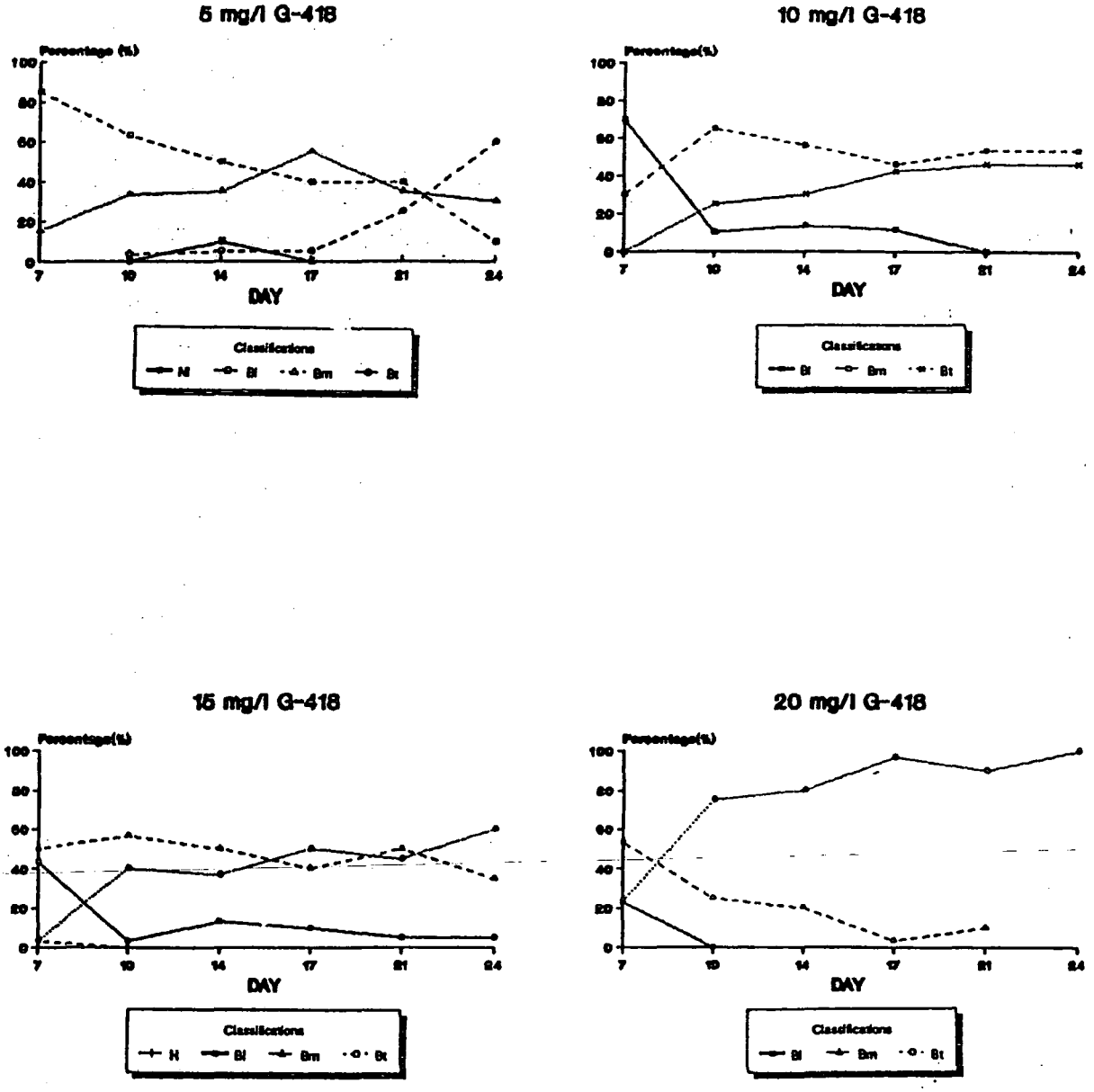
Key
H - Healthy tissue
Nl - Limited (<10% explant area) necrosis
Bl - Limited (<10% explant area) bleaching
Bm - Medium (10-79% area) bleaching
Bt - Total (80-100% area) bleaching
Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period loses are due to contamination
Bold figures in parentheses represent actual number of explants for statistical analysis

layered on transfer. Unlike the effect of kanamycin, there was an observed increase in severity of response at higher G-418 concentrations. After 7 days, some explants on 15 and 20 mgl^{-1} were totally bleached and a higher percentage (≥50%) in category Bm than on 5-10 mgl^{-1} concentrations (≤30%). After 21 days, at the end of the experiment, some explants on 5 mgl^{-1} were still only showing mild bleaching effects (Bl) but on 20 mgl^{-1} all leaf explants were totally bleached (Bt). These results are illustrated in figure 3.1.7. The significance of variation between tissue responses on different concentrations of G-418 can be statistically analysed. A comparison between media with low concentrations of G-418 ($5mgl^{-1}$) and $0mgl^{-1}$ G-418 showed that at day 14 the observed shift from the healthy or necrotic tissue on $0mgl^{-1}$ G-418 to high frequencies of bleaching in those explants on $5mgl^{-1}$ G-418 is significant at $p.001$ level, (G_{adj}=60.632; $X^2_{.001[5]}$=20.52).

At day 14, a significant variation in bleaching response is shown when data from 5, 10, 15 and $20mgl^{-1}$ G-418 are compared, calculated G_{adj}, $52.204 \geq X^2_{.001[9]}$=27.88. Overall the concentration of G-418 also influences the tissue response seen on the different media but the increase in severity of response is not uniform. The significance of the differences between the various levels of G-418 are indicated on table 3.1.4.

10 mgl^{-1} G-418 was chosen for selection of transformed tissue. This concentration should avoid a possible "lethal reaction" between non-transformed, dead cells and adjacent transformed cells. This was exemplified when the result of two transformation experiments were compared: one using $20mgl^{-1}$ (II) and one using $10mgl^{-1}$ G-418 (IV). The second yielded many shoots, whereas the first gave no positive growth (see Results, chapter 3). Subsequent transformation experiments demonstrated the "leakiness" of this antibiotic selection (see chapter 3).

Figure 3.1.7: Effect of G-418 on *L.corniculatus* Leaf Discs



3.1.4.3 Hygromycin Selection

The effect of hygromycin on leaf explants was studied over a range of 10-100 mgl^{-1} for 14 days (table 3.1.5). It appears that this antibiotic is less potent than G-418 causing no total bleaching on 20 mgl^{-1} hygromycin by day 14, in comparison to 80% Bt on 20 mgl^{-1} G-418. Nevertheless no callus was present even on the leaf discs grown on 10mgl^{-1} hygromycin. The significances of differences between treatments are indicated on table 3.1.5. Hygromycin was used to select co-cultivated explants for some transformation experiments, see chapter 3. Levels $\geq$17.5mgl^{-1} hygromycin used in the kill curve resulted in greater bleaching but it was proposed that the fatal Bl and Bm type responses at $\leq$15mgl^{-1} would be sufficient pressure against non-transformed cells, see figure 3.1.8.

The use of 15mgl^{-1} hygromycin led to the establishment of a successful transformation protocol and later results (chapter 3), demonstrate the effectiveness of this selection pressure.

3.1.4.4 Effect of Delaying Selection

Due to the apparent "leakiness" of the G-418 selection, an experiment was devised in order to investigate whether the delay between explants being cut and placed on solid medium and subsequent transfer onto the selection plates causes a reduction in the efficiency of the selection system. Such a delay is an integral part of the transformation procedure, with the co-cultivation stage alone extending over 48 hours.

Twenty explants were placed onto the following media: 12.5 or 15 mgl^{-1} G-418 and 15 or 20 mgl^{-1} hygromycin either immediately or after a 1,2,3 or 4 day delay. The explants were maintained on these plates for 3 weeks. Results collected are shown in table 3.1.6 a/b. The data indicates that there is no significant reduction in efficiency when the explants were subjected to a temporal delay before transferring onto G-418 selective media. The "leakiness" of G-418 selection is not caused by delay in culture, prior to tranfer to selection medium.

Table 3.1.5: Effect of Different Levels of Hygromycin on *L.corniculatus* leaf discs

Expressed as a percentage of each classification

DAY	0 (Mgl^{-1})	10	12.5	15	17.5	20	25	50	100
7	H 100.0 (25)	Bl 12.5 Bm 87.5 (24)	Bl 51.8 Bm 48.2 (27)	Bl 75.0 Bm 25.0 (24)	Bl 33.3 Bm 66.7 (30)	Bl 23.3 Bm 76.7 (30)	H 20.0 Bl 50.0 Bm 30.0 (20)	H 23.3 Bl 36.7 Bm 36.7 Bt 3.3 (30)	Bl 60.0 Bm 40.0 (30)
14	H 62.5 (15) Nm 37.5 (9) *Cal 83.3* (20) (24)	Bm 93.3 (14) Bt 6.7 (1) (15)	Bl 5.3 (1) Bm 84.2 (16) Bt 10.5 (2) (19)	Bl 78.6 (11) Bm 21.4 (3) (14)	Bl 17.2 (5) Bm 79.3 (23) Bt 3.4 (1) (29)	Bl 6.7 (2) Bm 93.3 (28) (30)	Bl 21.1 (4) Bt 78.9 (15) (19)	Bm 80.0 (24) Bt 20.0 (6) (30)	Bm 53.3 (16) Bt 46.7 (14) (30)

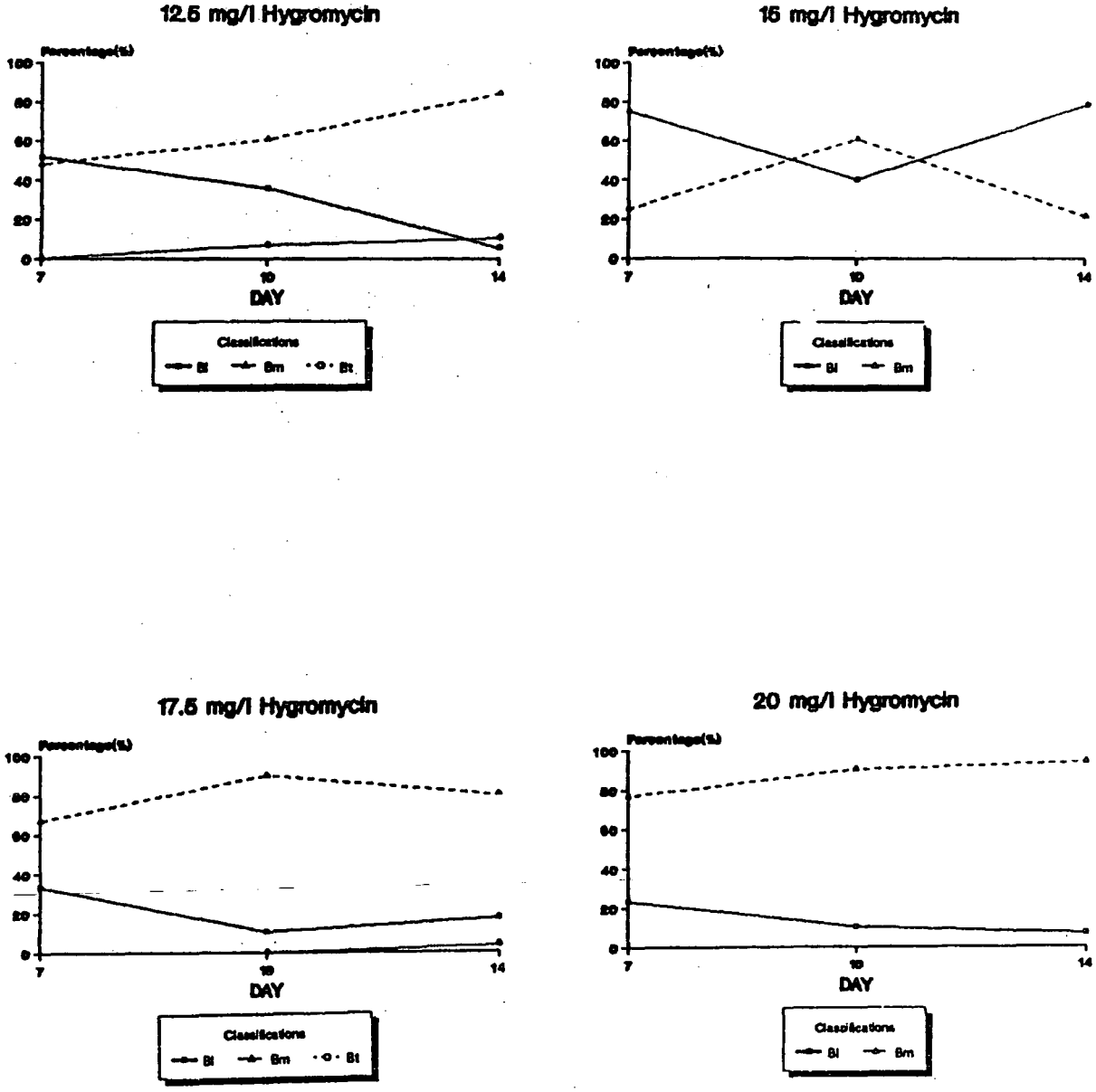
At day 14:

G_{adj}=51.97	G_{adj}1.386	G_{adj}=21.53	G_{adj}=15.794	G_{adj}=3.188	G_{adj}=57.798	G_{adj}=40.312	G_{adj}=3.734
$X^2_{.001[3]}$=16.27	$X^2_{.05[2]}$=5.99	$X^2_{.001[2]}$=13.82	$X^2_{.001[2]}$=13.82	$X^2_{.05[2]}$=5.99	$X^2_{.001[2]}$=13.82	$X^2_{.001[2]}$=13.82	$X^2_{.001[1]}$=10.83
(Rejected)	(Accepted)	(Rejected)	(Rejected)	(Accepted)	(Rejected)	(Rejected)	(Accepted)

The null hypothesis states that tissue reaction is independent of antibiotic treatment.

Key
H - Healthy tissue
Nm - Medium (10-79% explant area)
Bl - Limited (<10% area) bleaching
Bm - Medium (10-79% area) bleaching
Bt - Total (80-100% area) bleaching
Cal - Explants exhibiting callus production; *S* - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period loses are due to contamination
Bold figures in parentheses represent actual number of explants for statistical analysis

Figure 3.1.8: Effect of Different Levels of Hygromycin on *Lotus corniculatus* Leaf Discs



Percentage of each classification. Nl - Limited (<10% explant area) necrotic; Bl - Limited (<10% explant area) bleached; Bm - Medium (10-79% area) bleached; Bt - Total (80-100% area) bleached; (n) - Total number of explants, where n decreases over experimental period loses are due to contamination.

Null hypothesis for statistical analysis states that response is independent of treatment.

(a) G-418

	12.5 mgl^{-1}					15 mgl^{-1}				
Delay → / DAY ↓	0	1	2	3	4	0	1	2	3	4
7	Bt 65.0 Bm 35.0 (20)	Bt 85.0 Bm 15.0 (20)	Bt 21.1 Bm 78.9 (19)	Bt 35.3 Bm 47.1 Bl 17.6 (17)	Bt 10.0 Bm 90.0 (20)	Bt 90.0 Bm 10.0 (20)	Bt 95.0 Bm 5.0 (20)	Bt 90.0 Bm 10.0 (20)	Bt 50.0 Bm 50.0 (20)	Bt 75.0 Bm 25.0 (20)
14	Bt100.0 (20)	Bt100.0 (20)	Bt100.0 (14)	Bt100.0 (15)	Bt100.0 (20)	Bt100.0 (20) (20)	Bt100.0 (20)	Bt100.0 (20)	Bt100.0 (10)	Bt 95.0 (19) Bm 5.0 (1) (20)
21	Bt 95.0 Bm 5.0 (20)	Bt100.0 (12)	Bt100.0 (14)	Bt100.0 (14)	Bt100.0 (-)	Bt100.0 (20)	Bt100.0 (25)	Bt100.0 (20)	Bt100.0 (10)	Bt100.0 (20)

Significance of a 4-day delay at day 14 in culture:
On 12.5 mgl^{-1} G-418, the tissue response was identical after a 4-day delay as when there was no delay.
On 15mgl^{-1} G-418, the difference in response was not significant (Critical $X^2_{.05[1]}$=3.84, G_{adj}=3.504..

(b) HYGROMYCIN B:

	15 mgl^{-1}					20 mgl^{-1}				
Delay → / DAY ↓	0	1	2	3	4	0	1	2	3	4
7	Bt 5.0 Bm 35.0 Bl 60.0 (20)	Bt 20.0 Bm 25.0 Bl 45.0 Nl 10.0 (20)	Bt 25.0 Bm 5.0 Bl 70.0 (20)	Bt 10.0 Bm 10.0 Nl 80.0 (20)	Bt 5.0 Bm 5.0 Bl 55.0 Nl 35.0 (20)	Bt 55.0 Bm 45.0 (20)	Bt 50.0 Bm 25.0 Bl 25.0 (20)	Bt 5.0 Bl 85.0 Nl 10.0 (20)	Bm 25.0 Bl 25.0 Nl 50.0 (20)	Bm 15.0 Bl 45.0 Nl 40.0 (20)
14	Bm 80.0 (8) Bt 20.0 (2) (10)	Bt 50.0 Bm 30.0 Bl 20.0 (10)	Bt 30.0 Bm 40.0 Bl 30.0 (20)	Bl 30.0 Nl 70.0 (10)	Bt 15.0 (3) Bm 55.0 (11) Bl 30.0 (6) (20)	Bt 20.0 (4) Bm 80.0 (16) (20)	Bt 40.0 Bm 25.0 Bl 35.0 (20)	Bt 5.2 Bm 52.6 Bl 42.1 (19)	——	Bm 60.0 (6) Bl 40.0 (4) (10)
21	——	Bt 35.0 Bm 50.0 Bl 15.0 Cal10.0 (20)	Bt 30.0 Bm 10.0 Bl 60.0 (20)	——	——	Bt 55.0 Bm 45.0 (20)	Bt 76.9 Bm 23.1 (13)	Bt 5.0 Bm 66.0 Bl 35.0 (19)	——	

Significance of a 4-day delay at day 14: On 15 mgl^{-1} hygromycin - When critical $X^2_{.05[2]}$= 5.99, G_{adj}=5.598
On 20 mgl^{-1} hygromycin - When critical $X^2_{.001[2]}$=13.82, G_{adj}=12.408
So, there are no significant differences in tissue response after a 4-day delay prior to placing on hygromycin

Table 3.1.6: Effect of Delaying Selection on Leaf Explants

Over the three week culture period of this experiment, the greater the delay in tissue being placed on either of the two hygromycin levels, the less severe the bleaching response that occurred, table 3.1.6b. The 4-day delay between explant cocultivation and hygromycin selection pressure resulted in only a limited reduction in bleaching severity. There was no significant difference in tissue response on $15mgl^{-1}$ but the 4-day delay prior to tissue transfer onto $20mgl^{-1}$ hygromycin caused a significant reduction in bleaching severity. No callus production was apparent and all the transformation experiments, using hygromycin as the basis of selection, demonstrated the efficiency of this antibiotic as a selectable marker, despite the delay inherent in the protocol.

3.1.4.5 Kill Curves for Axenically Grown Explants

Work by Ghose, 1988 using hypocotyls and cotyledons as alternative explants sources used kanamycin ($50\ mgl^{-1}$) as a selection pressure. Table 3.1.7, shows the data collected over a 28 day period, comparing differentiation of both hypocotyls and cotyledons on selection and non-selection (B_5H) medium. Under control conditions, these explants have a more rapid regeneration cycle than leaf tissue, with negligible callus production.

Although there was no significant detrimental effect on hypocotyl or cotyledon tissue regeneration by the presence of $50mgl^{-1}$ kanamycin (table 3.1.7) initially, shoot elongation was suppressed.

These tissues showed a bleached reaction rather than the necrosis evident on kanamycin selection. Shoot differentiation is inhibited even at the lowest hygromycin concentration ($10\ mgl^{-1}$) but the severity of bleaching response caused by the antibiotic on tissue morphology increases as the concentration rises as shown in figure 3.1.9.

This data suggests that hygromycin at a level of $10\ mgl^{-1}$ would be suitable for the selection of transformed cells in hypocotyls.

Table 3.1.7: Growth of Hypocotyls and Cotyledons of *L.corniculatus* on non-selection and selection (50 mgl^{-1} kanamycin) Medium

Expressed as a percentage of each classification

	HYPOCOTYLS		COTYLEDONS	
DAY	**No Selection %**	**Selection %**	**No Selection %**	**Selection %**
4	H 100.0 (8)	H 100.0 (8)	H 83.3 Nl 16.7 (20)	H 100.0 (15)
10	H 75.0 Nl 25.0 *S 50.0* (8)	Nl 90.0 Nm 10.0 *S 10.0* (15)	Nl 88.9 Nm 11.1 *Cal 16.7* (18)	Nl 100.0 (15)
14	Nl 62.5 Nm 37.5 *S 100.0* *R 28.6* (8)	Nl 63.6 Nm 36.4 *S 100.0* *R 27.3* (11)	H 13.3 Nl 73.3 Nt 13.3 *S 13.3* (15)	H 26.7 Nl 73.3 (15)
21	H 100.0 *S 100.0* *R 25.0* (8)	Nl 85.7 Nt 14.3 *S 14.3* (10)	H 18.2 Nl 45.4 Nt 27.3 Bm 9.1 *S 45.5* (11)	Nl 100.0 *S 13.3* (15)
28	H 100.0 (8) *S 100.0 (8)* *R 25.0* (8)	Nt 14.3 (1) Bt 85.7 (6) *S 14.3* (1) (7)	H 10.0 (1) Nl 40.0 (4) Nt 50.0 (5) *S 50.0* (5) (10)	Nl 100.0 (15) *S 13.3* (2) (15)
	G_{adj}=9.174 $X^2_{.01[1]}$=6.64		G_{adj}=2.368 $X^2_{.05[1]}$=3.84	

In the comparison of shooting ability at day 28, the null hypothesis states that tissue regeneration is independent of level of antibiotic treatment. So, the null hypothesis was accepted for the effect of 50mgl^{-1} kanamycin on both hypocotyls and cotyledons.

Key
H - Healthy tissue
Nl - Limited (<10% explant area) necrosis
Nm - Medium (10-79% area) necrosis
Nt - Total (80-100% area) necrosis
Bm - Medium (10-79% area) bleaching
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period loses are due to contamination
Bold figures in parentheses represent actual numbers of explants for statistical analysis

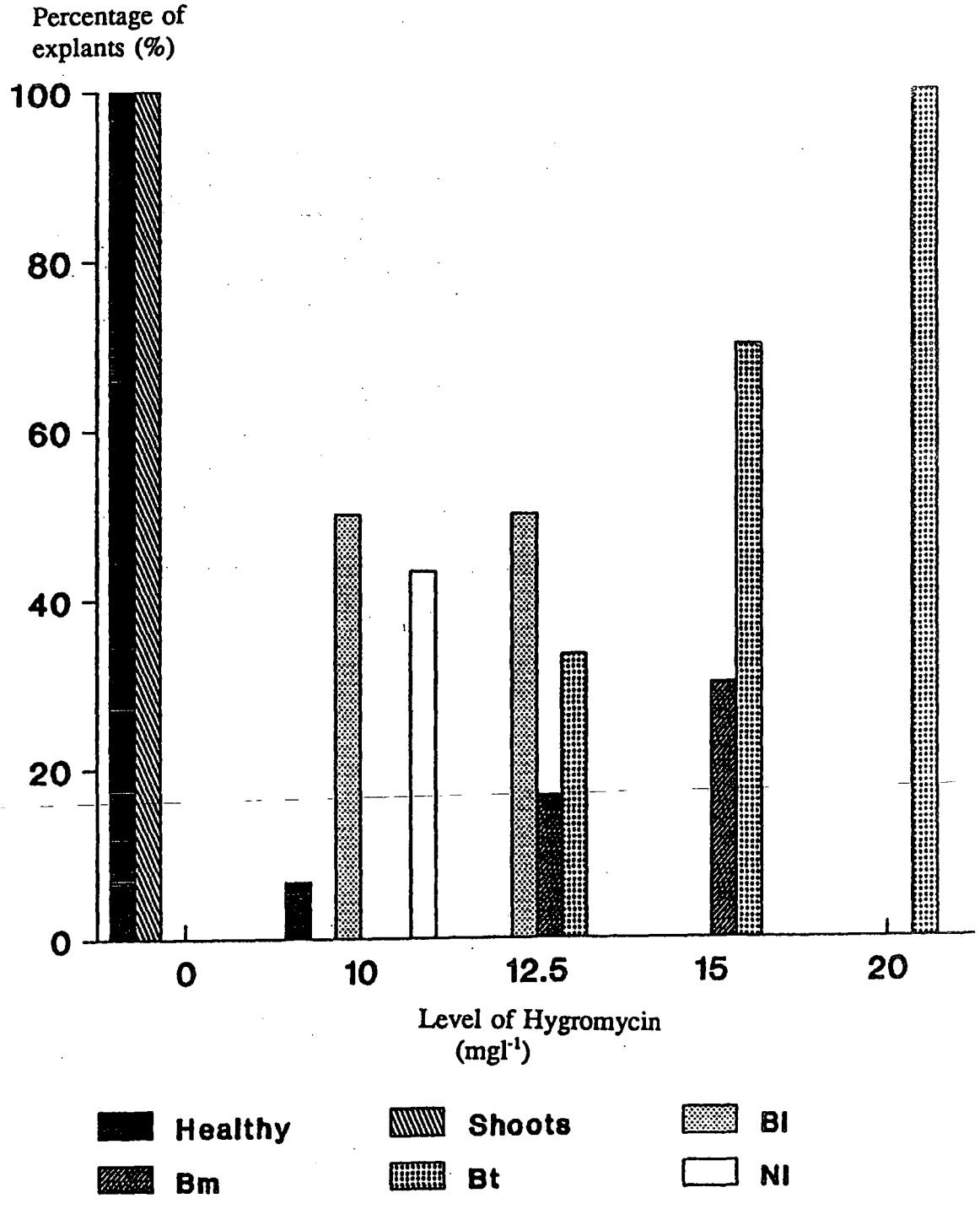
Table 3.1.8: Effect of Hygromycin on hypocotyls of *L.corniculatus*

Expressed as a percentage of each classification

DAY	Hygromycin (mgl^{-1})				
	0	10	12.5	15	20
7	H 100.0 *S 3.3* (30)	H 96.7 Nm 3.3 (30)	H 86.7 Bl 13.3 (30)	H 56.7 Bl 33.3 Bm 6.7 Bt 3.3 (30)	H 3.3 Bl 40.0 Bm 50.0 Bt 6.7 (30)
14	H 100.0 *S 70.0* (30)	H 56.7 Bl 43.3 (30)	H 20.0 Bl 63.3 Bt 16.7 (30)	Bl 26.7 Bm 23.3 Bt 50.0 (30)	Bt 100.0 (30)
21	H 100.0 *S 100.0* (30)	H 6.7 Nl 43.3 Bl 50.0 (30)	Bl 50.0 Bm 16.7 Bt 33.3 (24)	Bm 30.0 Bt 70.0 (30)	Bt 100.0 (30)

Key
H - Healthy tissue
Nl - Limited (<10% explant area) necrosis
Nm - Medium (10-79% area) necrosis
Bl - Limited (<10% explant area) bleaching
Bm - Medium (10-79% area) bleaching
Bt - Total (80-100% area) bleaching
Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period loses are due to contamination

Figure 3.1.9: Hypocotyl Response to Hygromycin (0 - 20 mgl^{-1}) after 21 days on selection



3.2.1 The pJIT73 Plasmid

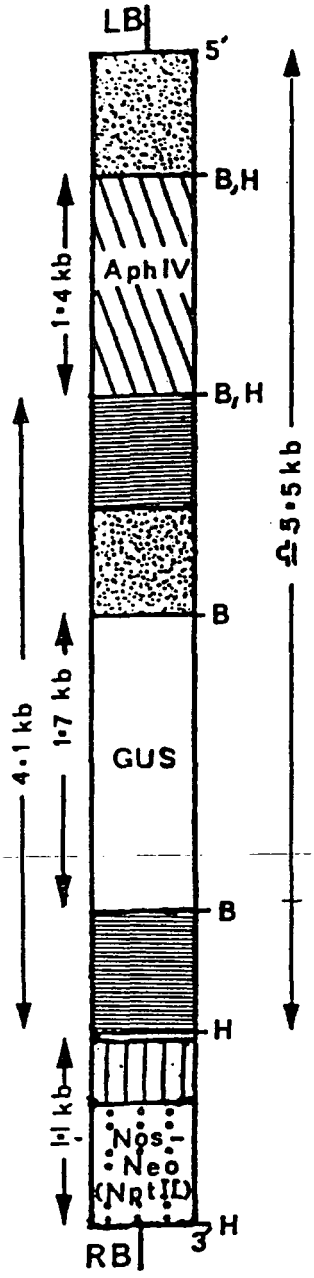
This 17.5kb chimaeric construct, developed by P.Mullineaux, is a pBIN19 based plasmid containing a broad host range replication origin enabling it to replicate in both *E.coli* and *Agrobacterium*. Antibiotic resistance markers and a region of DNA bounded by *Agrobacterium* T_L and T_R T-DNA border repeats. Figure 3.2.1 shows the essential features within the T-DNA. The 5.5kb region shown contains the two genes, hygromycin phosphotransferase (*AphIV*) and GUS, used to demonstrate gene transfer in the successful transformations with *N.tabacum* and *L.corniculatus*. Initially the neomycin phosphotransferase (*npt II*) gene was also utilized.

Another feature of the pJIT73 plasmid, which promoted its use in the development of the *Agrobacterium tumefaciens*-mediated transformation system for *L.corniculatus*, is the attachment of the CaMV 35S promoter sequence to the selectable and scorable markers. Sanders *et al* (1987) have investigated the relative strength of this promoter compared to the commonly used nopaline synthase (*nos*) promoter. The gene studied in their experiment was the *nptII* gene. It was found that expression from the 35S CaMV promoter was more than 30 times higher than from the *nos* promoter sequence.

Like *nos*, the 35S CaMV promoter is not under developmental or strong tissue specific regulation. The functioning of this promoter in transgenic plants has previously been demonstrated, though the level of constitutive expression of the chimaeric genes involved did vary between transformants (Sanders *et al*, 1987). This variation is probably due to positional effects derived from T-DNA integration sites and not due directly to the CaMV promoter. Thus the reliable 35S CaMV promoter is ideal for use in an otherwise unknown system.

Similarly, the choice of the scorable reporter gene was important and here pJIT73 contains the GUS gene encoding ß-Glucuronidase activity. Previous work had shown no endogenous GUS activity in other plants (Jefferson *et al*, 1987), indicating its potential usefulness and subsequent study of control *L.corniculatus* tissue confirmed

Figure 3.2.1: Diagrammatic Representation of the T-DNA from pJIT73



KEY

CaMV 35S promoter

CaMV terminator

poly A

LB - Left-hand border
RB - Right-hand border

Restriction sites used; B - BamHI
H - HindIII

the lack of endogenous activity in this plant species.

It was therefore concluded that the pJIT73 plasmid possessed a useful combination of genes with which the success and efficiency of the novel transformation system being developed could be assessed. Prior to use in experiments with *L.corniculatus* transformation the *in vivo* expression of the T-DNA genes of pJIT73 was tested by transfer of this construct to *N.tabacum* SR1 via *A.tumefaciens* LBA4404[pAL4404::pJIT73] (data not shown). Expression of hygromycin resistance and ß-glucuronidase genes was detected in subsequent transgenic regenerants. Studies of *L.corniculatus* transformation were then initiated.

3.2.2 Production of *Agrobacterium* Binary Vector Systems

(a) *Agrobacterium tumefaciens* LBA4404[pAL4404::pJIT73]
(b) *Agrobacterium tumefaciens* GV3101[pGV3850::pJIT73]
(c) *Agrobacterium rhizogenes* LBA9402[p1855::pJIT73]

The procedure for introducing a broad host range plasmid to *Agrobacterium* by a triparental mating is described in Methods 2.2.1.5. In all cases, the antibiotic resistance phenotypes of *E.coli* strains used, pRK2013 and pJIT73, was confirmed. Both strains grew on 50 μgml^{-1} kanamycin and failed to grow on either 100 μgml^{-1} rifampicin, or 100 μgml^{-1} carbenicillin, these being levels of resistance possessed by the recipient *Agrobacterium* in each case, see table 2.2.1. The reverse situation occurred when the recipient *Agrobacterium* strain was tested.

(a) *Agrobacterium tumefaciens* LBA4404[pAL4404::pJIT73]

The recipient *Agrobacterium* in this triparental mating was a Ach5 octopine derived-type *A.tumefaciens* LBA4404[pAL4404]. This is a non-oncogenic, *A.tumefaciens* strain containing an intact *vir* region but with the T-DNA deleted (Hoekema *et al*, 1983).

After the triparental mating 9 single colonies were picked off the antibiotic

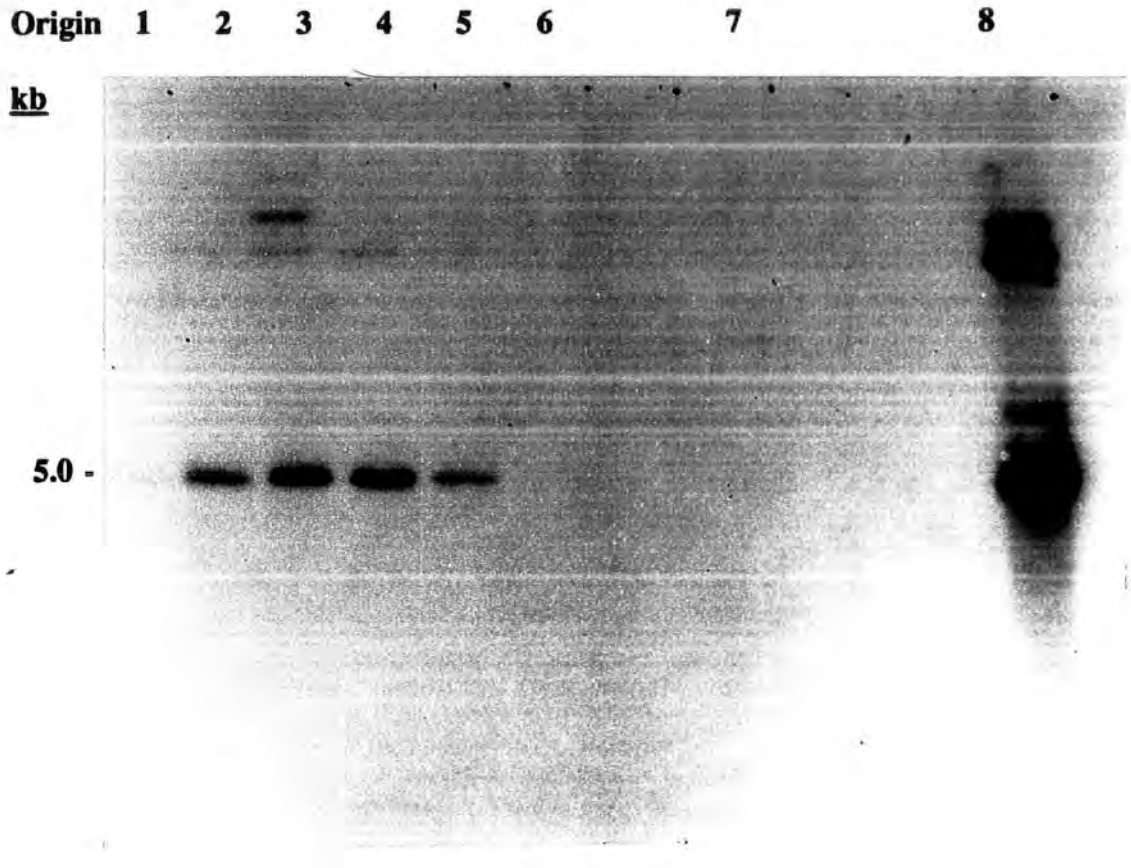
selection plates and grown up over night for the production of minipreps. DNA was extracted from all 9 colonies and was restricted with Hind III, run on 0.6% agarose gel and transferred to a nitrocellulose filter through Southern blotting. The recipient *Agrobacterium* was similarly processed as a negative "background" control and the pJIT73 DNA to act as a positive control in the subsequent probing. The 5kb Hind III probe fragment of pJIT73, containing the GUS gene and *AphIV* terminator was labelled by nick translation to a specific activity of $3x10^6$ count min^{-1} μg^{-1}. The filters were washed to a stringency of 0.1X SSC for 10 min at 65°C. Of the 9 *Agrobacterium* conjugant colonies screened, 4 exhibited hybridisation with the radioactive probe, as did the positive control pJIT73 DNA. No background hybridisation to the recipient *Agrobacterium* DNA was evident after 24 hours. These results are shown in figure 3.2.2. The conjugant *A.tumefaciens* shown in lane 4 was chosen for use in subsequent plant transformation experiments.

(b) *Agrobacterium tumefaciens* GV3101 [pGV3850::pJIT73]

The recipient *A.tumefaciens* strain used was a C58 nopaline type GV3101 (Holsters *et al*, 1980) containing the pGV3850 plasmid (Zambryski *et al*, 1983). pGV3850 is a fully disarmed Ti plasmid with full length pBR322 inserted between the left and right border T-DNA sequences.

After the appropriate triparental mating, 5 single colonies were picked off the conjugation plates and DNA was prepared and restricted with Hind III. The DNA was resolved by electrophoresis and Southern blotting. A probe was produced by nick translation of the Hind III 5kb fragment from pJIT73, containing the GUS gene, and had a specific activity of $4.1x10^7$ cpm μg^{-1}. Washes to a stringency of 0.1X SSC for 15min at 65°C, followed. Of the 5 colonies, 4 showed hybridisation to the probe. The control *A.tumefaciens* recipient GV3101[pGV3850] DNA showed no background and the pJIT73 DNA hybridised to the radioactive probe DNA as shown in figure 3.2.3.

Figure 3.2.2: Autoradiograph of Triparental Conjugants from the Production of the Binary Vector *A.tumefaciens* LBA4404[pAL4404::pJIT73]

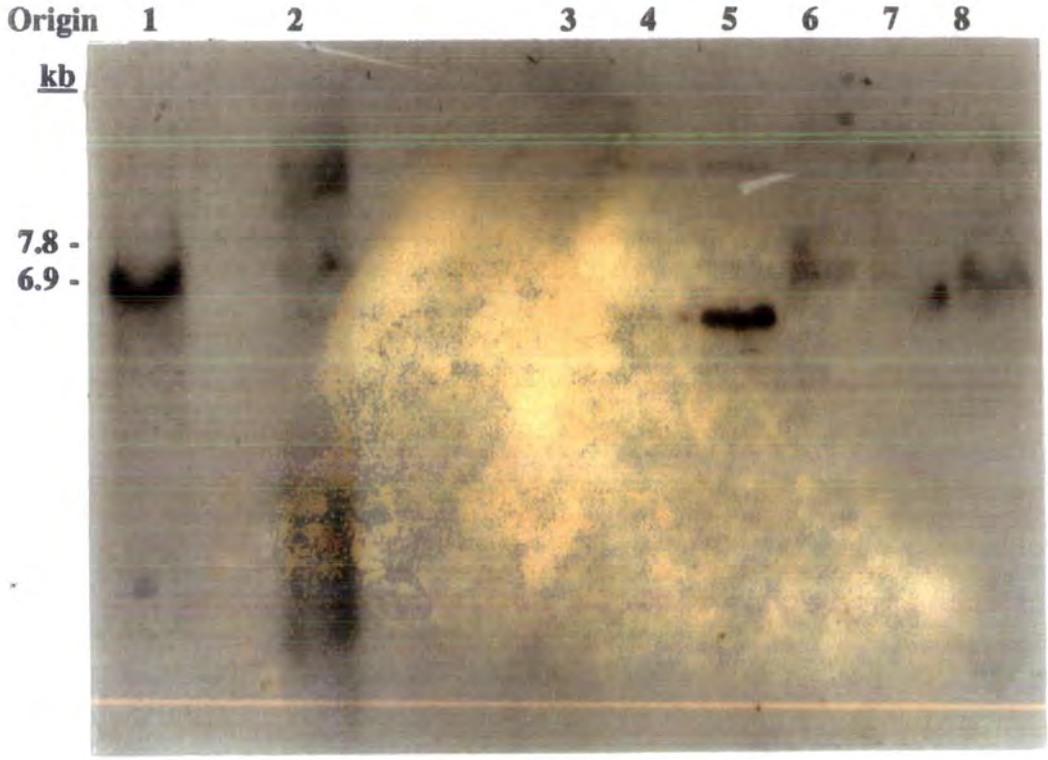


Lane

1-5 - DNA from putative conjugant clones (Clone from lane 4 used susequently)
6 - DNA from recipient *A.tumefaciens* LBA4404 [pAL4404]
7 - Eco RI/Hind III Lambda DNA
8 - pJIT73 DNA

Agrobacterium and plasmid pJIT73 DNA was restricted with Hind III. Probe consisted of ^{32}P-labelled Hind III GUS ter., 5kb fragment of pJIT73. Specific activity = $3x10^6$ counts min^{-1} μg^{-1} DNA. Wash stringency - 0.1xSSC. Exposure - 24 hours.

Figure 3.2.3: Autoradiograph of Triparental Conjugants from the Production of *Agrobacterium tumefaciens* GV3101[pGV3850::pJIT73]



Lane

1 - pJIT73 DNA
2 - pJIT73 DNA
3 - DNA from recipient *Agrobacterium* GV3101 [pGV3850]
4-8 - DNA from putative conjugant clones (clone from lane 5used subsequently)

Agrobacterium and pJIT73 DNA restricted with Hind III. Probe consisted of the ^{32}P-labelled 5kb Hind III fragment of pJIT73. Specific activity = 3.3×10^7 cpm μg^{-1} DNA. Wash stringency - 2xSSC + 0.1% SDS. Exposure - 48 hours.

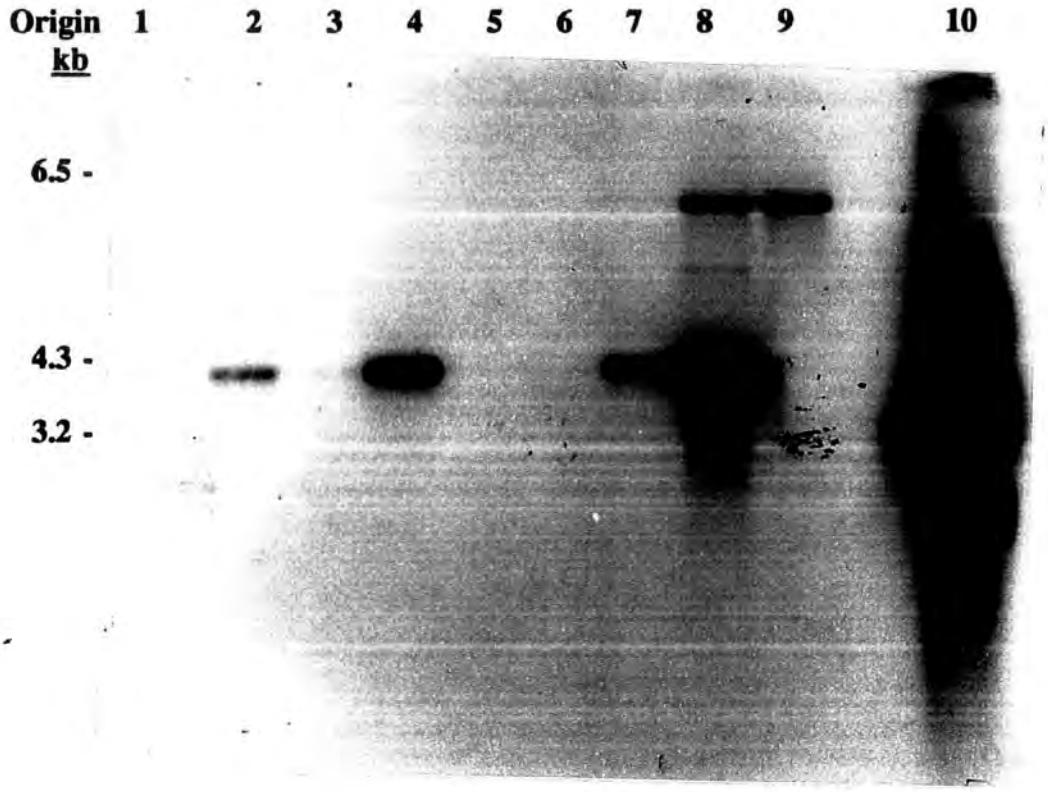
In 3 DNA samples, a 7.8kb band hybridised to the probe (lanes 6, 7, 8) as opposed to the 6.9kb band positive control and DNA from one conjugant (lane 5). Thus, the conjugant from which the DNA shown in lane 5 is derived, was used in subsequent plant transformation experiments. A potential drawback of this strain is

caused by the homology between pJIT73 and the pBR322 region of pGV3850, which means that co-integration may occur, causing the pJIT73 T-DNA to be inserted into the *vir* carrying pGV3850. The original aim, which was to produce a C58 binary vector system, has not necessarily been achieved since recombination could occur at any time, although the resulting recombinant would still constitute a functional transfer system for the pJIT73 T-DNA.

(c) ***Agrobacterium rhizogenes*** **LBA9402[p1855::pJIT73]**

After the triparental mating eight single colonies were picked off the triparental co-cultivation plates and their DNA was prepared and restricted with Hind III. Following gel electrophoresis and blotting, DNA was probed using a nick translated radiolabelled BamHI fragment containing the GUS gene. The specific activity of the probe was 6.8×10^7 cpm μg^{-1}. Following washing to a stringency of 0.1X SSC for 10 min. at 65°C and a 24 hour exposure, 4 clones exhibited hybrisation. Hybridisation was also present in the positive control and absent in the recipient *A.rhizogenes* DNA, as shown in figure 3.2.4. The colony from which DNA in lane 4 was derived was used in subsequent plant genetic manipulations. The recipient *A.rhizogenes* strain LBA9402 is a nopaline Ach5 wild-type strain.

Figure 3.2.4: Autoradiograph of Triparental Conjugants from the Production of *Agrobacterium rhizogenes* LBA9402[p1855::pJIT73]



Lane

1 - DNA from recpient *A.rhizogenes* LBA9402[p1855]
2-9 - DNA from triparental conjugants (Conjugant 4 used subsequently)
10 - pEJC1 DNA (containing GUS)

All *Agrobacterium* and plasmid DNA restricted with Bam HI. Probe consists of ^{32}P-radiolabelled BaM HI GUS-ter. fragment. Specific activity = 6.8×10^7 cpm μg^{-1}. Wash stringency - 0.1xSSC. Exposure - 24 hours.

3.2.3 Production of the Cis Vector System *Agrobacterium tumefaciens* GV3101[GV3850.G]

The aim of producing this vector was to insert the GUS reporter gene between the left and right T-DNA borders of pGV3850, via an intermediate vector (pDB007) which is transferable to *Agrobacterium*, but cannot be maintained.

(a) Transfer of the GUS gene into E.coli

pDB007 (donated by D.Bown) was restricted with BamHI and ligated overnight at 15°C to a 2.6kb BamHI fragment isolated from pEJC1 (donated by E.Croy), which contained the CaMV-GUS chimaeric gene and a terminator derived from the *nos* gene. Ligated DNA (0.005µg) was incubated with 100µl of competent DH5α during the transformation procedure. Similarly, DH5α was transformed with 0.003µg of self-ligated pDB007. From the cells plated out, transformation frequencies of $1.8x10^4$ transformation events μg^{-1} of self ligated DNA, and $2.3\text{-}2.55x10^4$ transformation events μg^{-1} ligated DNA were estimated. Replicate colony screen filters were set up, with 7 colonies of the selfed vector DNA transformation as background controls, and 14 colonies from the experimental line of transformed DH5α cells. Filters were probed with a GUS sequence, radiolabelled by nick translation to a specific activity of $6.1x10^6$ cpm μg^{-1}. Following a 0.1X SSC wash of 10 min at 65°C and a 24 hour film exposure, 2 colonies demonstrated hybridisation to a significantly greater extent than the relatively high levels seen in the control colonies, as shown in figure 3.2.5.

Upon restriction analysis of DNA from controls and both colonies, (figure 3.2.6), only colony I contained the required GUS insert. This was used in subsequent manipulations.

(b) Formation of E.coli Intermediary Containing Mobilising Plasmids and GUS Fragment

Minipreped DH5α-GUS DNA was introduced into the competent intermediary cells GJ23 by transformation. Following transformation, no growth occurred where GJ23 cells only were plated on the selection plates but hundreds of colonies from the DNA transformation GJ23 grew, giving a frequency of $5x10^5$ transformation events μg^{-1}.

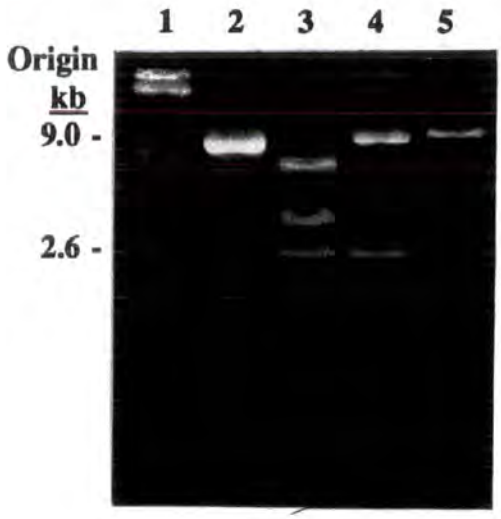
Figure 3.2.5: Colony Screen of Putative Transformants

Control DH5α transformed with self-ligation reaction, showing background hybridisation

Putative transformants

Probe - ^{32}P radiolabelled BamHI restricted GUS-ter. Fragment of pEJC1. Specific activity = $6.1x10^6$ cpm μg^{-1} DNA. Wash stringency = 0.1xSSC. Exposure = 24 hours.

Figure 3.2.6: Restriction Analysis of Positive Colonies



All plasmid DNA, except size markers, restricted with BamHI.

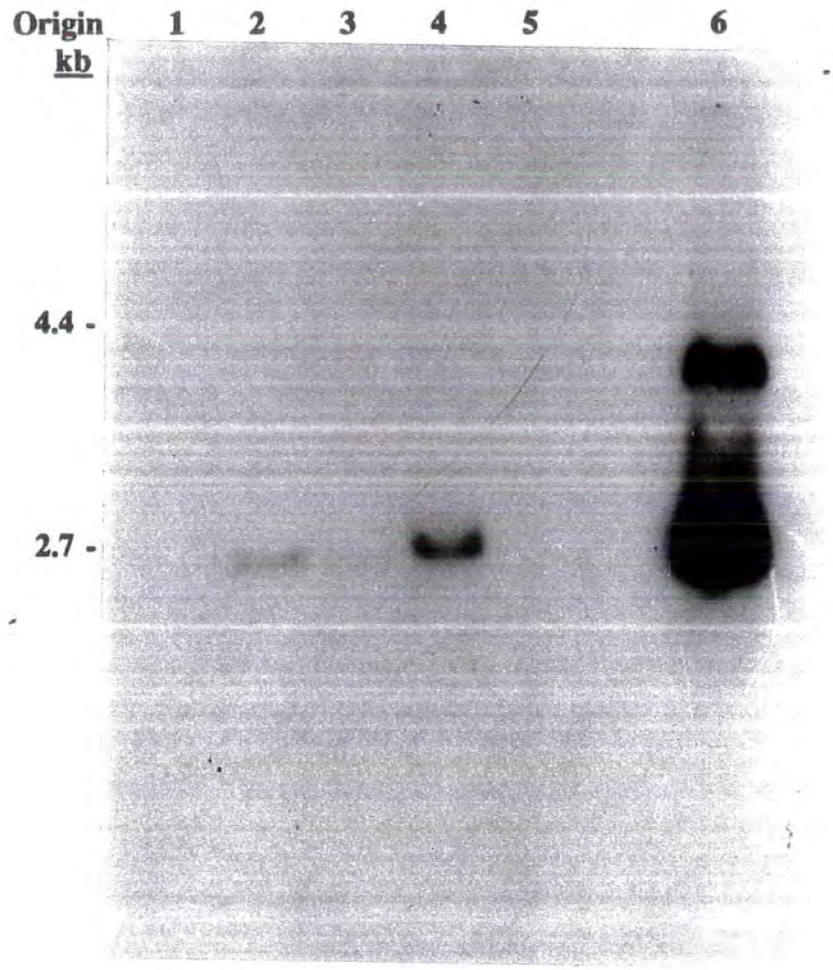
Lane

1 - EcoRI/Hind III restricted Lambda DNA
2 - pDB007 (Vector only)
3 - pEJC1 (Donor plasmid only), note only partial restriction of donor pEJC1 occurred.
4 - Putative transformed clone I (used in subsequent conjugation)
5 - Putative transformed clone II

(c) Biparental Conjugation Between *E.coli* Intermediary and *A.tumefaciens* GV3101[pGV3850.G]

The check on the antibiotic sensitivity of the two components of the mating, indicated the leaky nature of the gentamycin selection on the *Agrobacterium*. Concentration was increased from 5 to 10 $\mu g ml^{-1}$ gentamycin in the selection plates, which also contained 100 $\mu g ml^{-1}$ of both rifampicin and carbenicillin. During the conjugation, growth occurred on the mixture serial dilution plates 10^{0}-10^{-3}. Ten colonies were picked off and processed. The DNA was restricted with BamHI separated by electrophoresis on agarose gel and Southern blotted. The filter was probed with the GUS-terminator fragment labelled by nick translation to a specific activity of $6.4x10^{3}$ cpm μg^{-1}. Washes at 65°C were to a stringency of 0.1X SSC for 10 min. After 24 hours, there was evidence for a positive clone having been produced in lane 4, and after 70 hour exposure, this was confirmed. A second positive in lane 2 also became apparent, as shown in figure 3.2.7. Thus, the "cis" vector system of *Agrobacterium tumefaciens* GV3101 [pGV3850.G] was produced.

Figure 3.2.7: Autoradiograph of Final Biparental Conjugation in the Production of *A.tumefaciens* GV3101[pGV3850.G]



Lanes

1-4 - Putative conjugant clones (clone from lane 4 subsequently used)
5 - Recipient *Agrobacterium tumefaciens* GV3101 [pGV3850]
6 - pEJC1 DNA (Donor plasmid)

All *Agrobacterium* and plasmid DNA restricted with Bam HI. Probe consisted of ^{32}P-labelled BamHI GUS-ter, fragment from pEJC1. Specific activity = 6.4×10^3 cpm μg^{-1} DNA. Wash stringency - 0.1xSSC. Exposure - 70 hours.

CHAPTER 3: Preliminary *Lotus corniculatus* Transformation Experiments

3.3.1 *L.corniculatus* Transformation Systems

Several *L.corniculatus* explants including leaves, stem segments, hypocotyls and cotyledons as well as protoplasts exhibit full regeneration competence and so are potential tissue sources for transformation experiments. Previously,attempts were made using leaf discs (Nisbet, 1987) and hypocotyls (Ghose, 1988). Another variable parameter within transformation protocols is the type of *Agrobacterium* used as the gene transfer vector. Both *A.tumefaciens* (oncogenic or disarmed) and *A.rhizogenes* (oncogenic) may be utilized.

3.3.1.1 *A.rhizogenes* - mediated transformation

Transformations were set up using a binary *A.rhizogenes* vector so that a comparison of the established *A.rhizogenes* system could be made to any new disarmed *A.tumefaciens*-mediated system developed.

Both seedling inoculation and hypocotyl and stem cocultivation techniques (see Methods) were used with *A.rhizogenes* LBA9402[p1855::pJIT73]. The seedling inoculation method was found to be the most effective method of hairy root induction. Direct wounding of the seedling hypocotyl reduced the manipulations required in culture, so the possibility of contamination is lowered. From 19 sites of inoculation, 12 groups of hairy roots were excised, these were then cultured on $\frac{1}{2}B_5 0$ and 3 lines of shoots differentiated. Figure 3.3.1 shows "hairy root" organogenesis. Shoot primordia develop directly from the "hairy root" and the shoots then elongate. These shoots were excised and developed roots. The three plants derived from *A.rhizogenes*-mediated transformation were then tested for GUS activity. Initially, the X-Gluc test showed GUS activity in all three plants but after several weeks, only one plant maintained detectable activity.

8 - 10 day old *L.corniculatus* seedlings were used as the source of hypocotyl explants for cocultivation experiments. The first experiment of this series (i) involved a short 20 min. liquid cocultivation followed by an extended cocultivation period of 3

Figure 3.3.1: Shoot development initiated in a "hairy root" culture

days on plates. Prior to culture on 300 mgl^{-1} cefotaxime for 4 days, explants were washed in sterile distilled water. The final selection consisted of 30 mgl^{-1} kanamycin. After two weeks in culture, "hairy" roots emerged. Roots of two morphological types were identified, those which were "non-hairy" single roots and those which possessed abundant hairs, the "hairy" roots. Both types were negatively geotrophic. However in subsequent culture, only "hairy" roots responded and produced shoots. Fungal contamination prevented further development of those transformants. Repeated experiments where cocultivation in liquid was increased to 1 hour failed to yield any hairy root formation (data not presented).

In transformation experiments ii and iii, both nodal and internodal stem pieces (approx. 15mm long) were cocultivated for 20 min, transferred onto B_5+0.5 mgl^{-1} BAP agar plates, left for 3 days before washing with sterile distilled water and then plated onto media containing 300 mgl^{-1} cefotaxime for 4 days. Final selection was on media containing 250 mgl^{-1} cefotaxime and 30 mgl^{-1} kanamycin.

Transformation experiments iv and v were based on the method of Ghose, 1988. 10 day old seedlings were the source of hypocotyls. No tissue proliferation or shoot initiation occurred at all in either transformation attempts using this method. Tissue response was similar to those shown in table 3.3.1, with the presence of antibiotic preventing initial swelling of the explants and thus inhibiting differentiation.

Table 3.3.1: Axenic Transformations iv and v
Expressed as a percentage of each classification

(a) Hypocotyl Explants:

DAY	Control - No selection	Control - 50 mgl^{-1} kanamycin	Cocultivated experiment (vi) on selection	Cocultivated experiment (vii) on selection
14	N 100.0 *S 100.0* *R 25.0* (8)	N 100.0 *S 100.0* *R 25.0* (8)	N 100.0 (19)	H 2.0 N 98.0 *S 2.0* (101)
21	H 100.0 *S 100.0 (8)* *R 25.0* (8)	N 100.0 *S 12.5 (1)* (8)	N 100.0 *Swollen* 100.0 (10)	-------

(b) Cotyledon Explants

DAY	Control - No selection	Control - 50 mgl^{-1} kanamycin	Cocultivated experiment (vi) on selection	Cocultivated experiments (vii) on selection
14	H 13.3 N 86.7 *S 13.3* (15)	H 26.7 N 73.3 (15)	N 100.0 (24)	N 99.3 B 0.7 (140)
21	H 18.2 N 54.6 B 27.3 *S 18.2* (2) (11)	N 100.0 *S 13.3* (2) (15)	---------	---------

Key
H - Healthy swollen tissue
N - Necrotic tissue
B - Bleaching tissue
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period losses are due to contamination. Bold figures in parentheses represent the actual number of explants used in statistical analysis.

When statistically analysed using the G-test a comparison between shoot development from hypocotyl explants in the presence or absence of 50mgl^{-1} kanamycin (at day 21) yielded $G_{adj} = 10.466$. This value is larger than the critical $X^2_{.01[1]} = 6.64$. So, the null hypothesis that the presence of kanamycin does not alter hypocotyl response in culture can be rejected.

In contrast, when data for regeneration from cotyledons are analysed, the null seedlings and the selection pressure used was 10mgl^{-1} hygromycin. Experiment viii hypothesis that the selection has no significant effect on the subsequent shooting ability of the cotyledons at day 21, must be accepted. The critical $X^{2.05[1]} = 3.84$ and the calculated $G_{adj} = 0.046$.

3.3.1.2*A.tumefaciens* - mediated transformation

Four experiments (vi - ix) based on Ghose, 1988 (see methods) using hypocotyl and cotyledon explants derived from axenic seedlings in cocultivation with A.tumefaciens [pAL4404::pJIT73], were set up (see methods).

In the first experiment (vi) explants were excised from 7-8 day old seedlings. No shoot differentiation was evident on cocultivated explants even after 28 days (hypocotyls) and 14 days (cotyledons) on 50 mgl^{-1} kanamycin selection. Experiment vii repeated the procedure and explants exhibited limited shoot formation by day 14. Experiments viii and ix were carried out using hypocotyls excised from 10 day old seedlings and the selection pressure used was 10 mgl^{-1} hygromycin. Experiment viii involved a 2 hour cocultivation and experiment ix an overnight cocultivation. No transgenic shoot production occurred. Despite the appearance of shoot primordia on hypocotyl tissue, (see table 3.3.2), shoots failed to develop to an excisable size. Repeated attempts using this protocol failed to yield any transgenic plants (data not presented).

Table 3.3.2: Axenic Transformations viii and ix

Expressed as a percentage of each classification

		Selection - 10 mgl^{-1} Hygromycin		
DAY	**Control**	**Control**	**(viii) 2 hour cocultivation**	**(ix) O/N cocultivation**
7	H 100.0 S 5.6 (18)	N 100.0 S 5.3 (19)	N 50.0 B 50.0 (14)	N 100.0 (34)
14	H 100.0 S 5.6 (18)	H 31.6 N 68.4 (19)	N 87.5 B 12.5 (16)	N 100.0 S 20.6 (34)
21	H 72.2 N 27.8 S 27.8 (18)	H 26.3 N 73.7 S 31.6 (19)	N 100.0 S 31.3 (16)	N 100.0 S 50.0 (20)
28	H 44.4 (8) N 44.4 B 11.2 S 61.1 (11) (18)	N 100.0 S 47.1 (8) (17)	N 75.0 B 25.0 S 50.0 (16)	N 100.0 S 47.1 (34)

Key
H - Healthy swollen tissue
N - Necrotic tissue
B - Bleached tissue
S - explants exhibiting shoot development
(n) - Total number of explants
Bold figures in parentheses represent actual number of explants used in statistical analysis

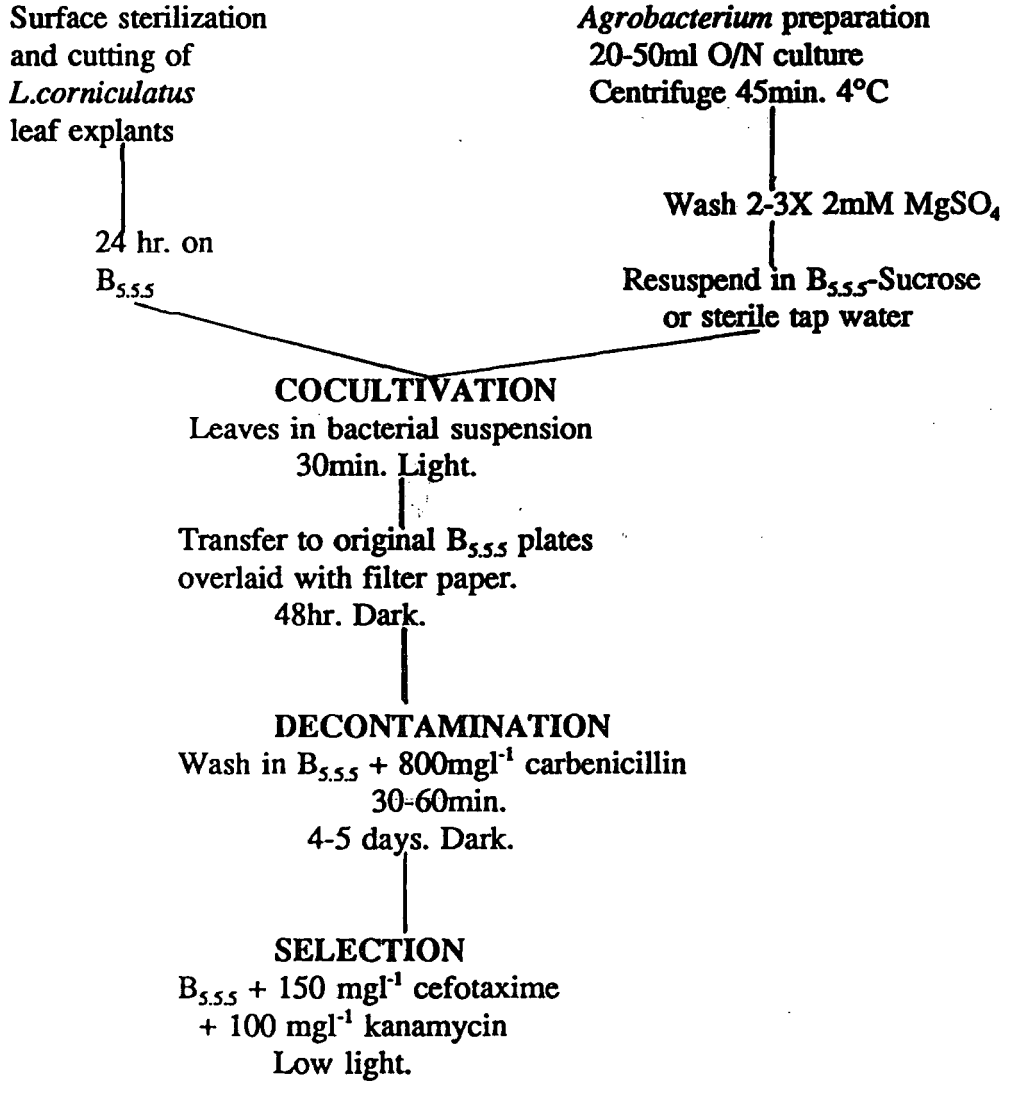
When the G-test is applied to above data regarding shoot development at day 28, a null hypothesis that treatment with hygromcin does not alter differentiation freqency can be accepted. $G_{adj} = 0.246$ which is $< X^2_{.05[1]} = 3.84$.

However there is a significant reduction in the swelling reaction of hypocotyls in axenic culture when under selection pressure. The null hypothesis states that the swelling process of hypocotyls in culture is independent of the presence of antibiotics. With critical $X^2_{.05[1]} = 3.84$, and $G_{adj} = 8.66$ the null hypothesis is rejected. Thus the presence of $10mgl^{-1}$ hygromycin results in a significant reduction in the fequency of swollen hypocotyl tissue after day 28 in culture.

CHAPTER 4: Development of the Disarmed *Agrobacterium tumefaciens* -mediated Transformation System for *Lotus corniculatus* leaf explants.

A protocol first used by Nisbet (1987) was the basis upon which the following experiments were conducted. This original protocol is best described in a flow chart, see figure 3.4.1;

Figure 3.4.1: Flow Chart of Original Transformation Protocol



Modifications to this basic protocol will be discussed in the context of the transformation experiments attempted. The complexity of parameters contributing to a successful plant transformation necessitated multiple modification. The 25 experiments carried out in the development of the modified transformation system can be divided into 4 groups.

3.4.1 GROUP 1: Experiments I-II

These experiments were based directly on the original protocol, using the *A.tumefaciens* LBA4404[pAL4404::pJIT73] binary vector as transforming agent. The only parameters to be varied were the type and level of the decontamination antibiotic used and the selection pressure applied. These experiments were a preliminary test of suitability of the selection pressure applied. In these group 1 experiments, carbenicillin was used during the washing stage at 800 mgl^{-1}. During culture on agar plates, decontamination was at a reduced level of 400 mgl^{-1} carbenicillin and 150 mgl^{-1} cefotaxime. Selection pressures used were: 20 mgl^{-1} G-418 in experiment I, 12.5 mgl^{-1} hygromycin in experiment II.

Experiment I:

The selection used was 20 mgl^{-1} G-418 but by day 14 all the explant replicates were totally bleached. This selection pressure was too severe with no further growth of the limited callus initiated early in the culture period.

Experiment II:

A much reduced level of hygromycin, 12.5 mgl^{-1} was used. This had been shown to be a potentially suitable concentration by the kill curve data (see chapter 1. 3.1.4.3). By day 14, callus had developed on an appreciable proportion of the inoculated explants (table 3.4.1) but shoots initiated on these, had not elongated or grown even by day 42. Nevertheless, this indicated that some genetic transfer may have occurred.

Table 3.4.1: Experiment II
Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]
Selection - 12.5 mgl^{-1} Hygromycin

Expressed as a percentage of each classification

	CONTROL (%)	% EXPLANTS
7	H 63.1 N 26.1 B 10.8 *Cal 2.6* (30)	H 22.5 N 37.5 B 40.0 *Cal 13.3* (120)
14	H 20.0 N 66.7 B 13.3 *Cal 46.7* (-)	H 4.3 N 56.3 B 39.4 *Cal 45.1* (107)
28	----------	H 4.3 N 56.1 B 39.4 *Cal 28.1* (107)
35	----------	H 4.3 N 56.5 B 39.2 *Cal 69.6* (-)
42	----------	N 81.2 B 18.8 *Cal 70.8* *S 14.6* (-)

Key

H - Healthy tissue
N - Necrotic tissue
B - Bleached tissue
Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases loses are due to contamination
(-) - Total number of explants not recorded

The results indicate a greater degree of tissue damage was caused by 20mgl^{-1} G418 (experiment I), compared to 12.5 mgl^{-1} hygromycin (experiment II), with no evidence of regeneration occurring on the former.

These first two experiments had included a 24 hour delay between cutting the explants and their immersion in the *Agrobacterium* suspension. During the first 24 hours *vir* inducer compounds are produced by the wounded plants. However, this added 24 hours to the period of time in culture before selection was applied so to ensure the optimium selection pressure in subsequent protocols, leaf pieces were immersed in the *Agrobacterium* suspension immediately or with only a 30 min delay.

The preparation of sterile explants from soil grown *L.corniculatus* plants was also slightly altered in all subsequent protocols in order to reduce the damage sustained by tissue during sterilization. Whole stem sections were removed, generally those containing 2-4 week old tissue, in preference to the previous technique of using individual trifoliate leaves. The cut end of the stem was sealed with molten wax to prevent the sterilization solution entering internal plant tissue. When any stem accidently had not been closed, a bleaching reaction could be seen in the lower 10mm of the stem. This emphasizes the need to protect the tissue whereas previously no such attention had been given to the individual leaves. The length of delay between cutting and inoculating the leaf explants with *Agrobacterium* was up to 30 min as all excised explants were immersed simultaneously. The cocultivation liquid used was $B_{5.5.5.}$

3.4.2 GROUP 2: Experiment III-VI

This group of experiments used a protocol integrating the above modifications into the original protocol. Decontamination and selection antibiotics used were then altered. All 4 experiments involved *A.tumefaciens* [pAL4404::pJIT73] and cocultivation was still carried out in the light. During the decontamination step, cefotaxime was the only antibiotic being used. The type and concentration of antibiotic used in selection was altered within this group of experiments: III - 10mgl^{-1} G-418; IV - 12.5mgl^{-1} G-418; V - 10mgl^{-1} hygromycin; VI - 15mgl^{-1} hygromycin.

Experiment III

The level of cefotaxime used during decontamination washes was 300mgl^{-1} but this was reduced to 150mgl^{-1} cefotaxime in the agar medium plates. Control non-cocultivated leaf tissue placed on 10mgl^{-1} G-418 medium became severely bleached and no cell proliferation was visible. Those leaf discs which had been cocultivated began to show signs of callus production after 7 days on 10mgl^{-1} G-418 selection. This dedifferentiation phase in regeneration continued so that by day 21, 58.4% of explants were producing callus. Results are shown in figure 3.4.2.

Differentiation was visible by day 14, with 2.4% of explants having shoot primordia. Over the following 3 weeks more than 10% of the explants produced at least one shoot. At day 35, 28 shoots were excised and placed on antibiotic-free rooting slopes, in an attempt to increase the rate of growth, in order to be able to assay for ß-glucuronidase activity as soon as possible. Although, clearly discrete shoots were present on the leaf explants after even 73 days in culture, only a few shoots of these were sufficiently elongated to allow transfer to rooting medium. In order to encourage further shoot expansion leaf explants were placed on $B_5 0$. Finally after day 85 the majority of shoots were transferred to selective rooting medium. Culture on the selection medium would confirm antibiotic resistance of the putative transformants. Shoots from control explants (non-selected) were also transferred and these were found to bleach gradually, eventually dying completely after 10 days on selection. Fifty two shoots were cultured from experiment III, of these, 16 died under selection immediately and 28 were lost through contamination. The remaining 8 shoots were senescent with either bleached or necrotic lower stem regions, and failed to produce roots. This exemplifies the "leakiness" of the G-418 selection system. Photographs of explants derived from experiment III and the effect the antibiotic has on tissue and shoot development are shown in figure 3.4.3.

Vector used: *A.tumefaciens* [pAL4404::pJIT73]

Selection used: 10mgl^{-1} G-418

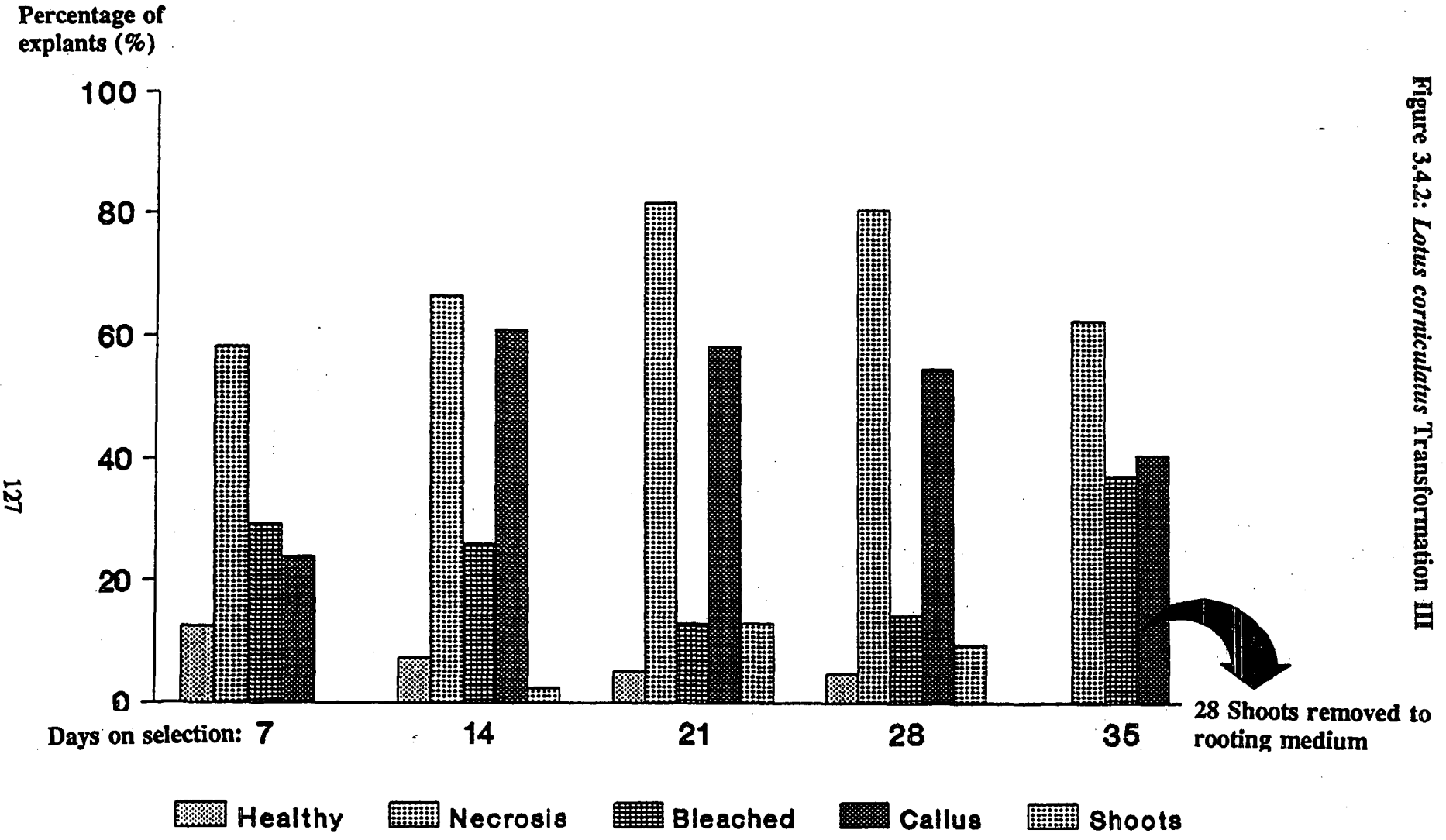


Figure 3.4.2: *Lotus corniculatus* Transformation III

Figure 3.4.3: Abnormal Shoot Differentiation from *Lotus corniculatus* Leaf Explants on Selection Medium

3.4.2.1 Analysis of ß-Glucuronidase Activity in Experiment III Putative Transformants

The original group of 28 shoots removed at day 35, established root systems on the non-selective slopes and fluorimetric analysis of GUS activity was carried out. Of the putative transformed *L.corniculatus* shoots assayed, none exhibited GUS activity very much elevated above background and where possible activity had been detected this proved non-reproducible. Use of the histochemical assay failed to confirm GUS expression in these plants.

3.4.2.2 Testing the Antibiotic Resistance of the Putative Transgenic Plants

Leaf samples from 5 plants which had been previously rooted on non-selective medium were placed on both 15 mgl^{-1} hygromycin and 12.5 mgl^{-1} G-418.

After 2 weeks, bleaching was total in all samples on G-418 and those on hygromycin exhibited a degree of bleaching and all further differentiation was prevented.

If DNA transfer had taken place the antibiotic resistance genes, *aph IV*+*nptII*, failed to be incorporated into a functional position within the host plant genome.

3.4.2.3 Genomic DNA Analysis

DNA was extracted from 12 of the plants derived from experiment III. The samples were restricted with BamHI for 4½ hours at 37°C and resolved on a 0.7% agarose gel overnight. A typical gel of restricted *L. corniculatus* genomic DNA is shown in figure 3.4.4. After DNA transfer by Southern blotting, the resultant filters were probed with ^{32}P radiolabelled pJIT73 (BamHI restricted) DNA. The probe had a specific activity of $7.2x10^8$ cpm μg^{-1}. The autoradiograph obtained is shown in figure 3.4.5. Background homology to the pBIN19 derived regions of the pJIT73 meant further probing utilized the individually prepared GUS and *Aph IV* genes, as this homology may obliterate the specific hybridisation of the T-DNA carried genes. However figure 3.4.6 shows a typical result probing with GUS and *Aph IV* probes only,

no hybridisation was detected. All other hybridisation attempts were similarly negative.

From the total absence of any of the three possible bands (12.5kb *nptII*, 2.55kb *AphIV*, 2.95kb GUS) and the failure to confirm GUS expression in the plants, it was concluded that DNA transfer and incorporation into the genome of these plants had not occurred.

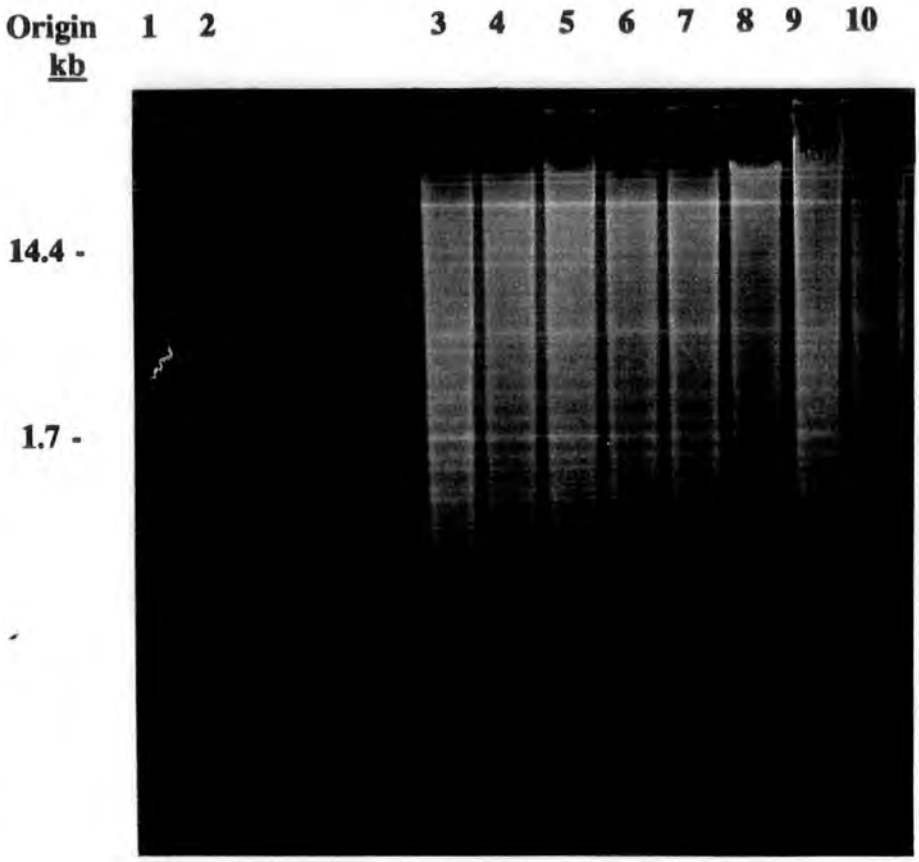
Experiment IV:

The transformation procedure used in experiment III was followed but with an increased level of G-418 selection (12.5 mgl^{-1}) and 250 mgl^{-1} cefotaxime was used in both the decontamination washing and solid culture stages. Forty-one shoots were excised from leaf explants so treated and placed on non-selective rooting medium, others were subjected to 12.5mgl^{-1} G-418 selective rooting medium. Subsequently 34 plants were analysed at the genomic level using radioactive probes. Both GUS activity and hygromycin resistance were studied in 25 plants derived from experiment IV.

3.4.2.4 Analysis of ß-Glucuronidase Activity Experiment IV Putative Transformants

Once again the GUS assay proved inconclusive in determining the GUS activity of the putative transformants, although GUS levels were marginally above background levels in some plants GUS activity could not be confirmed by the histochemical assay.

Figure 3.4.4: Typical *Lotus corniculatus* Genomic Restriction



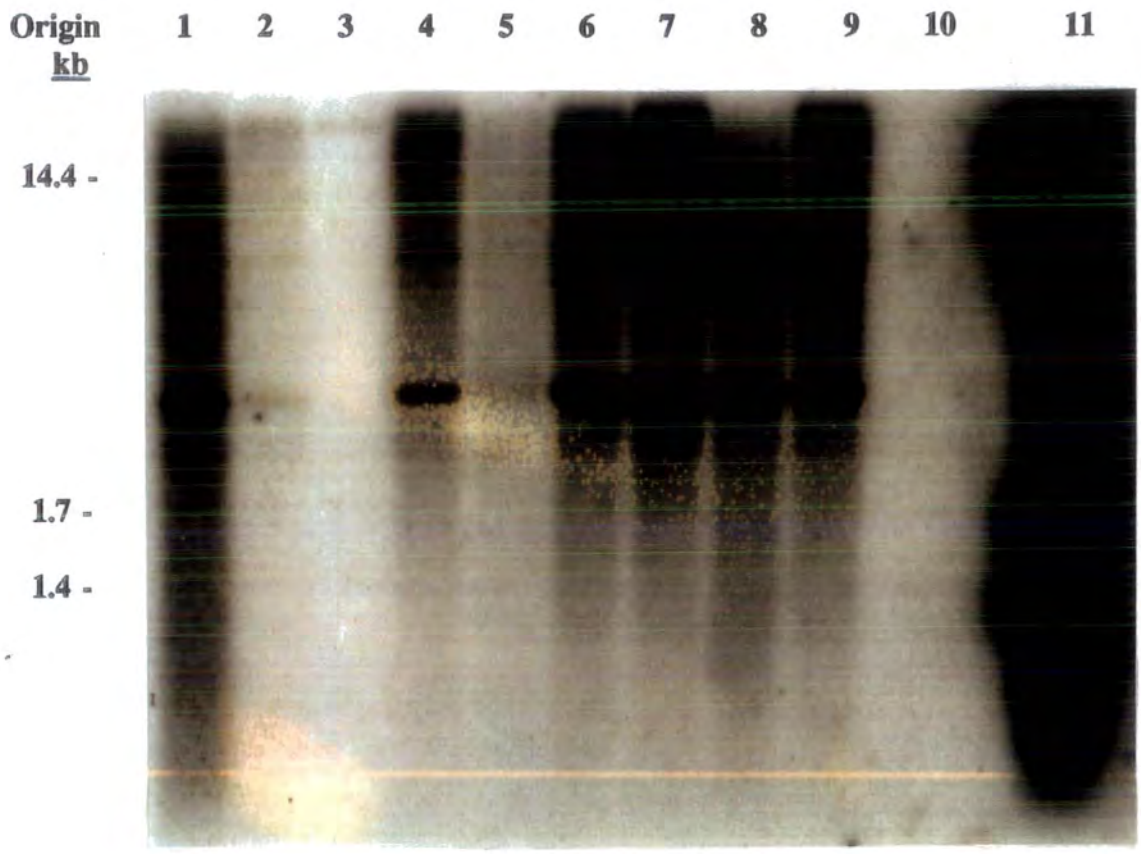
Lane

1 - pJIT73 DNA HindIII restricted

2 - EcoRI/HindIII restricted Lambda DNA

3-9 - *L.corniculatus* Genomic DNA

Figure 3.4.5: Autoradiograph of Putative Transgenic *Lotus corniculatus* Genomic DNA



Lane

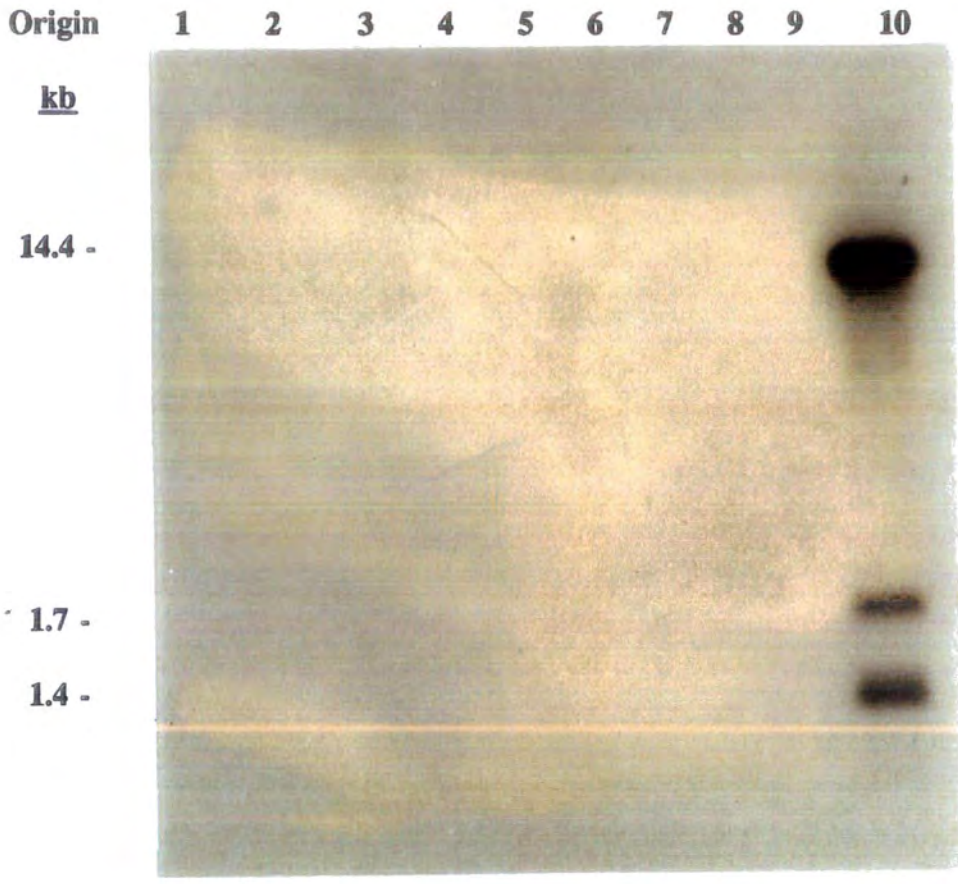
1-9 - Bam HI restricted Genomic DNA of Putative transgenic *L.corniculatus* plant from Experiment IV

10 - Bam HI restricted control *L.corniculatus* DNA

11 - pJIT 73 DNA

Probe - ^{32}P-radiolabelled Bam HI restricted pJIT73 DNA.
Specific activity = 7.2×10^8 cpm μg^{-1}. Wash stringency = 1xSSC. Exposure = 2 weeks.

Figure 3.4.6: Autoradiograph of Putative Transgenic *L.corniculatus* Plants Restricted DNA - Probe ^{32}P-GUS/*AphIV*



Lane

1 - Control *Lotus corniculatus* Genomic DNA.
2-9 - DNA from Putative Transgenic *Lotus corniculatus*
10 - pJIT73 DNA

DNA restricted with BamHI. Probe - GUS fragment radiolabelled to a specific activity of $3x10^8$ cpm μg^{-1}. Wash stringecy = 2xSSC. Exposure = 2 weeks.

3.4.2.5 Testing the Antibiotic Resistance of the Experiment IV Putative Transgenic Plants

The survival of the shoots on rooting medium containing 12.5 mgl^{-1} G-418 was a false indication of the presence of the *nptII* gene in the plants, as subsequent plating of leaf samples onto this level of G-418 resulted in universal bleaching. This further accentuates the "leakiness" of the selection pressure conferred by G-418 on leaf tissue of *L.corniculatus*.

3.4.2.6 Genomic Analysis

DNA from plants derived from experiment IV was analysed in conjunction with samples from experiment III, but no hybridisation indicating transfer of the foreign genes to experiment IV plants was obtained.

Experiment V:

In experiment V, the whole transformation procedure was repeated as for experiment IV, but the final selection pressure applied was 10 mgl^{-1} hygromycin. The response of the cocultivated explants on this selection is shown in table 3.4.2. Despite callus production, only a low percentage of explants showed evidence of shoot primordia development and these then failed to grow. Thus shoot production is severely inhibited and no transgenic shoots were obtained from this transformation experiment.

Experiment VI:

This experiment repeated V but with a higher level of hygromycin (15 mgl^{-1}). As shown in table 3.4.3, a similar tissue response occurred, with no shoot becoming large enough or individually excisable.

The responses of cocultivated leaf tissue were compared between experiment V and VI when two different concentrations of hygromycin were used, significant results regarding the effect of hygromycin can be obtained. Use of 10mgl^{-1} hygromycin resulted in a higher frequency of callus production compared to levels arising from

Table 3.4.2: Experiment V

Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection - 10 mgl^{-1} Hygromycin

Expressed as a percentage of each classification

DAY	Control material on selection (%)	Co-cultivated Explants (%)
7	B 100.0 (30)	H 43.3 N 49.5 B 7.4 *Cal 64.2* (95)
14	 --- 	H 35.8 N 63.1 B 1.1 *Cal 97.9* (95)
21	 --- 	H 23.7 N 75.3 B 1.0 *Cal 100.0 (93)* *S 1.1* (1) (93)
28	 --- 	H 9.4 N 90.6 *Cal 94.1* *S 4.7* (85)
35	 --- 	H 7.2 N 89.8 B 2.9 *Cal 79.7* *S 1.4* (79)

Key

H - Healthy tissue

N - Necrotic tissue

B - Bleached tissue

Cal - Explants exhibiting callus production

S - Explants exhibiting shoot development

(n) - Total number of explants, where n decreases over experimental period losses are due to contamination.

Bold figures in parentheses represent actual number of explants used in statistical analysis.

Table 3.4.3: Experiment VI

Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection - 15 mgl^{-1} Hygromycin

Expressed as a percentage of each classification

DAY	Control (%)	Co-cultivated Explants (%)
14	H 13.0 N 78.0 B 9.0 *Cal 48.0* (100)	H 40.0 N 56.0 B 4.0 *Cal 65.0* (100)
21	N 100.0 *Cal 70.0* **(63)** (90)	H 1.7 N 95.8 B 2.5 *Cal 91.3 (70)* *S 1.3* **(1)** (80)
48	N 95.8 B 4.2 *Cal 64.0* *S 16.7* **(12)** (72)	H 2.9 N 92.8 B 4.3 *Cal 100.0* *S 48.6 (34)* (70)

Key
H - Healthy tissue
N - Necrotic tissue
B - Bleached tissue
Cal - Explants exhibiting callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period losses are due to decontamination
Bold figures in parentheses represent actual number of explants used in statistical analysis

cocultivated explants on 15mgl^{-1} hygromycin at day 21 (G_{adj}=11.89, critical $X^2_{.001[1]}$=10.83). But shoot production was not significantly enhanced on either concentration of hygromycin (G_{adj}=0.388).

The tissue derived from experiment VI also indicated that cocultivated explants showed significantly greater callus proliferation than control explants at day 21 on selection (G_{adj}=6.834, $X^2_{.01[1]}$=6.64). The data is illustrated graphically in figure 3.4.7. This regenerative advantage continues at day 48, where callus production is seen on 100% of cocultivated explants compared to 64% control tissue. Shoot development can be seen to be initiated earlier in culture and there is a greater frequency of shoot development at day 48, on cocultivated material (G_{adj}=15.49, $X^2_{.001[1]}$=10.83).

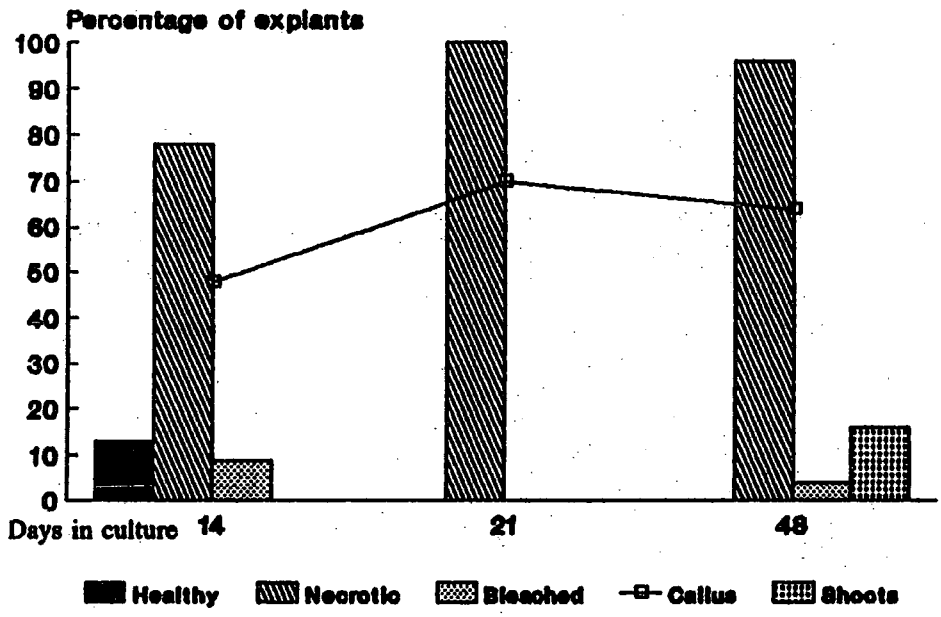
GROUP 3: Experiment VII-XI

The procedures followed in this group were similar to those developed within group 2 but several *Agrobacterium* vectors were used. The early work in the establishment of a leaf explant transformation system for *L.corniculatus* had utilised the octopine type *A.tumefaciens* LBA4404[pAL4404::pJIT73] as the vector.

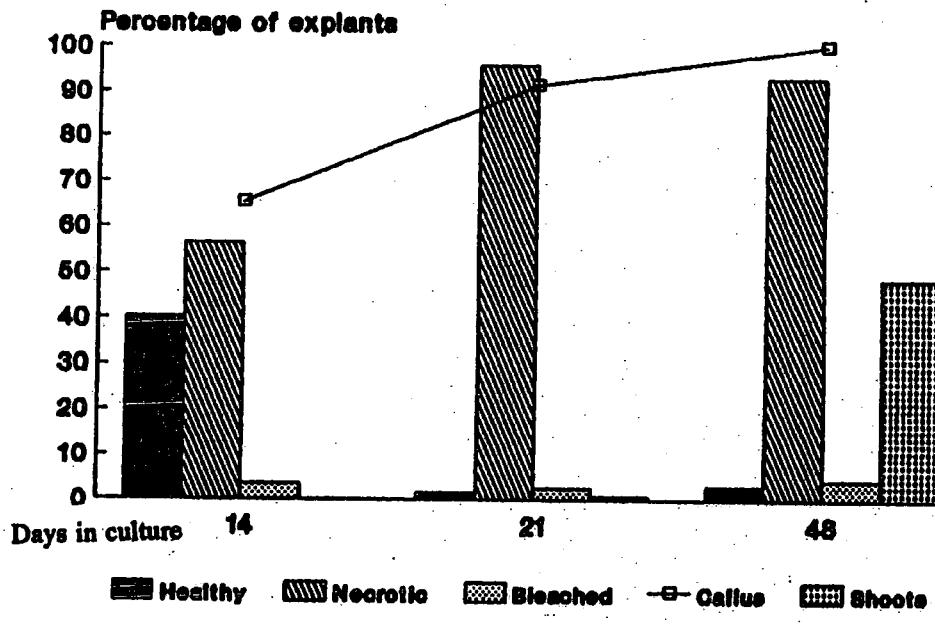
However, the pJIT73 plasmid had also been transferred to the nopaline type *A.tumefaciens* strain GV3101[pGV3850::pJIT73]. Previous work by Webb and co-workers (Webb, 1986) indicated that wild-type nopaline strains had greatest potential to confer differentiation ability to the oncogenic tumour tissue and thus this strain might have a greater efficiency of gene transfer. In these experiments, augmentin was introduced as the sole decontamination antibiotic, as the experiments showing the deleterious effect of cefotaxime on tissue differentiation had been carried out. The antibiotic was present at 400 mgl^{-1} during the liquid wash and 200 mgl^{-1} in the agar-medium, and this was adopted as a standard decontamination procedure throughout future transformation experiments. The first two experiments using this C58 based vector failed to yield any significant results, VII (selection on 15 mgl^{-1} hygromycin) and VIII (selection on 10 mgl^{-1} G-418), with no excisable shoots developing on the cocultivated explants under selection pressure. The *A.tumefaciens* GV3101[pGV3850]

Figure 3.4.7: Tissue response in experiment VI

(a) Control explants



(b) Cocultivated explants



strain was also used as a basis of the "monomeric" vector containing only *npt II* and GUS genes as markers.

Experiment IX and X:

Two experiments (IX and X) using the monomeric system *A.tumefaciens* GV3101[pGV3850.G] with selection on 12.5 mgl^{-1} G-418 were set up. Only experiment X yielded any possible transformed tissue, but relatively few shoots were removed and when these were analysed at the genomic level no evidence was found to show genetic incorporation of T-DNA into the host plant. There is an apparent increase in effectiveness of the G-418 selection in these experiments in comparison to those where *A.tumefaciens* LBA4404 [pAL4404::pJIT73] had been used as the vector.

3.3.4 Group 4 - Experiments A - D

A number of parameters were altered for the subsequent transformations: (i) time in liquid cocultivation was extended to 2 hours and the explants were agitated whilst in the *Agrobacterium* suspension. Cocultivation was also carried out under dark conditions. (ii) the decontamination period on agar plates was reduced to 2 days; (iii) the co-cultivation and decontamination liquid stages used B_50 medium rather than medium containing growth regulators; (iv) following selection on $B_{5.5.5}$ medium, explants were transferred to B_50 agar selection plates after initial shoot induction.

This group of experiments can themselves be further divided and are classified as $A_{(1-4)}$, $B_{(1-4)}$ etc. Each subgroup (1-4) was set up from the same batch of leaf material. Table 3.4.4, shows the conditions used in this set of work.

Two antibiotics, hygromycin and G-418, were used as selection agents in this fourth group of experiments. The hygromycin selection was still considered potentially the most efficient, but G-418 was included again both to confirm its "leakiness" and as a comparison with hygromycin selection. Each experiment used the same sterile leaf source and so four combinations of parameters were set up, as shown in table 3.4.4.

Table 3.4.4: Group 4 Experiments - Parameters Used

				Co-cultivation		Decontamination		
Experiment	Explant	*A.tumefaciens* Srain	Cutting Details	In liquid (hr)	On plates (hr)	Washes	Plates	Selection
$A_{1,3}$ $B_{1,3}$	Leaf strips	LBA4404 [pAL4404::pJIT73]	Cut under suspension	2 hours B_5O Shaker Dark	48 hours Dark	H_2O + 400 mgl^{-1} Augmentin	200mgl^{-1} Augmentin 2 days	$A_1 + B_1$ 15 mgl^{-1} Hygromycin $A_2 + B_2$ 12.5 mgl^{-1} G-418
$A_{2,4}$ $B_{2,4}$	Leaf strips	GV3101 [pGV3850::pJIT73]	Cut under suspension	2 hours B_5O Shaker Dark	48 hours Dark	H_2O + 400 mgl^{-1} Augmentin	200mgl^{-1} Augmentin 2 days	$A_3 + B_3$ 15 mgl^{-1} Hygromycin $A_4 + B_4$ 12.5 mgl^{-1} G-418
$C_{1,3}$	Leaf strips	LBA4404 [pAL4404::pJIT73] with AS *vir* Induction	Immediately introduced to bacteria after cutting	2 hours B_5O Shaker Dark	48 hours Dark	H_2O + 400 mgl^{-1} Augmentin	200mgl^{-1} Augmentin 2 days	C_1 15 mgl^{-1} Hygromycin C_2 12.5 mgl^{-1} G-418
$C_{2,4}$	Leaf strips	GV3101 [pGV3850::pJIT73] with AS *vir* Induction	Immediately introduced to bacteria after cutting	2 hours B_5O Shaker Dark	48 hours Dark	H_2O + 400 mgl^{-1} Augmentin	200mgl^{-1} Augmentin 2 days	C_3 15 mgl^{-1} Hygromycin C_4 12.5 mgl^{-1} G-418
$D_{1,3}$	Leaf strips	GV3101 [pGV3850::pJIT73]	Immediately introduced to bacteria after cutting	2 hours B_5O Shaker Dark	48 hours Dark	H_2O + 400 mgl^{-1} Augmentin	200 mgl^{-1} Augmentin 2 days	D_1 15 mgl^{-1} Hygromycin D_2 17.5 mgl^{-1} Hygromycin

3.4.4.1 Experiments A_{1+2}

A_1 and A_2 were identical except for the final selection step. Both involved LBA4404 [pAL4404::pJIT73] as the vector, but used hygromycin and G-418 respectively as selection (table 3.4.5). The control, non-inoculated *L.corniculatus* material demonstrates the relative effectiveness of the two selection antibiotics. Explants on 15 mgl^{-1} hygromycin developed necrotic callus tissue and only severely stunted and abnormal shoots, similar to those illustrated in figure 3.4.3., emerged. However, although the mass of callus on 12.5 mgl^{-1} G-418 was also necrotic, product of shoots on the replicate explants was extensive and many of these grew to an excisable size.

The most obvious difference between inoculated and non-inoculated leaf tissue on either of the antibiotics was the presence of friable healthy callus (*gr.cal.*) as well as the necrotic form seen previously. Such healthy cell proliferation is only present on the cocultivated explants. When the proportion of healthy, green or necrotic callus present at day 35 in experiments A_1 and A_2 was analysed statistically, $G_{adj}=16.81$, $X^2_{.001[1]}=10.83$. This confirmed that green, healthy callus occurred at a higher frequency on the explants subjected to 15 mgl^{-1} hygromycin (experiment A_1). On plates containing 15 mgl^{-1} hygromycin (A_1), despite this relatively healthy cell proliferation, shoot production remained very low and only two shoots could be excised. These subsequently died on selective medium.

Under 12.5 mgl^{-1} G-418 selection (A_2), a lower proportion of explants contained healthy callus tissue and again shoot elongation was inhibited so that only 6 shoots could be removed. All these A_2 shoots died on further selection after failing to establish a root system and subsequently exhibiting bleaching.

3.4.4.2 Experiment A_{3+4}

These experiments again employed the two selection antibiotics but the C58 derived vector *A.tumefaciens* GV3101[pGV3850::pJIT73] was employed. The response of control material on selection was as shown in table 3.4.6. Once again, healthier

Table 3.4.5: Experiment A_{1+2}

Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection - A_1 15 mgl^{-1} Hygromycin

- A_2 12.5 mgl^{-1} G-418

Expressed as a percentage of each classification

DAY	Control: 15 mgl^{-1} Hygromycin	Experiment A_1 15 mgl^{-1} Hygromycin	Control: 12.5 mgl^{-1} G-418	Experiment A_2 12.5 mgl^{-1} G-418
11	N 100.0 *Cal 45.7* (35)	N 77.1 B 22.9 *Cal 44.8* (105)	N 50.0 B 50.0 (26)	H 0.7 N 69.7 B 29.6 *Cal 32.9* (149)
28	B 100.0 *Cal 62.9* *S 11.4* (35)	B 100.0 *Cal 54.8* *S 1.4* (73)	B 100.0 *Cal 55.3* *S 21.1* (38)	B 100.0 *Cal 92.1* *Green cal. 30.7* (101)
35	B 52.6 N 47.4 *Cal 36.8* (38)	N 56.6 B 43.4 *Cal 88.9* **(88)** *Green cal. 18.3* **(18)** (99)	N 100.0 *Cal 100.0* *S 21.1* (40)	N 92.5 B 7.5 *Cal 92.5* **(124)** *S 14.2* *Green cal. 3.0* **(4)** (134)
42	B 100.0 *Cal 27.8* *S 13.9* (-)	B 100.0 *Cal 69.5* *Green cal. 30.5* (105)	B 100.0 *Cal 100.0* *S 50.0* (40)	N 45.6 B 54.4 *Cal 100.0* *S 14.2* *Green cal. 4.0* (150)
80	N 100.0 *Cal 73.5* *S 59.8* (35)	N 100.0 *Cal 73.5* *S 59.8* (102)	N 100.0 *Cal 100.0* *S 100.0* (20)	N 100.0 *Cal 95.0* *S 78.2* (-)

Key

H - Healthy tissue

N - Necrotic tissue

B - Bleached tissue

Cal - Explants exhibiting callus production (healthy or necrotic)

Green cal. - Explants exhibiting healthy, green callus production

(n) - Total number of explants, where n decreases over experimental period losses are due to contamination. Where n increases during experimental period explants fragmented

(-) - Total number of explants unrecorded

Bold figures in parentheses represent actual number of explants used in statistical analysis.

Table 3.4.6: Experiment A_{3+4}

Vector - *Agrobacterium tumefaciens* GV3101 [pGV3850::pJIT73]

Selection - A_3 15 mgl^{-1} Hygromycin

- A_4 12.5 mgl^{-1} G-418

Expressed as a percentage of each classification

DAY	Control: 15 mgl^{-1} Hygromycin	Experiment A_3 15 mgl^{-1} Hygromycin	Control: 12.5 mgl^{-1} G-418	Experiment A_4 12.5 mgl^{-1} G-418
11	N 100.0 *Cal 45.7* (35)	N 90.9 B 9.1 *Cal 57.6* (66)	N 50.0 B 50.0 (26)	N 87.5 B 12.5 *Cal 27.1* (40)
28	B 100.0 *Cal 62.9* *S 11.9* (35)	B 100.0 *Cal 90.7* *Green cal. 52.0* (75)	B 100.0 *Cal 55.3* *S 21.1* (38)	B 100.0 *Cal 91.2* *Green cal. 10.3* (68)
35	N 47.4 B 52.6 *Cal 36.8* (38)	N 100.0 *Cal 92.1 (70)* *Green cal. 51.3* (39) (76)	N 100.0 *Cal 100.0* *S 52.5* (40)	N 95.0 B 5.0 *Cal 95.0 (95)* *S 12.0* *Green cal. 7.0* (7) (100)
42	B 100.0 *Cal 27.8* *S 13.9* (36)	N 69.2 B 30.8 *Cal 93.8* *Green cal. 52.3* (65)	B 100.0 *Cal 100.0* *S 50.0* (40)	N 45.6 B 54.4 *Cal 100.0* *S 25.6* *Green cal. 25.3* (125)
80	N 100.0 (35)	N 100.0 *Cal 47.9* *S 4.1* (-)	N 100.0 *Cal 100.0* *S 100.0* (20)	N 100.0 *Cal 99.1* (111)

Key

H - Healthy tissue

N - Necrotic tissue

B - Bleached tissue

Cal - Explants exhibiting callus (necrotic and healthy) production

Gr. cal - Explants exhibiting healthy, green callus production

S - Explants exhibiting shoot development

(n) - Total number of explants

(-) - Total number of explants unrecorded

callus tissue was seen on cocultivated explants on both treatments but the relative differences in abundance of shooting were marked.

Tissue grown on 15 mgl-1 hygromycin (experiment A_3) exhibited more healthy callus than that on 12.5 mgl-1 G-418 (experiment A_4) at day 35. If the frequency of green, healthy and necrotic callus present on the leaf explants were compared in experiments A_3 and A_4, significantly more green callus occurred on $15mgl^{-1}$ than on $12.5mgl^{-1}$ G-418 ($G_{adj}=46.478$, $X^2_{.001[1]}=10.83$). Shoot primordia however were not visible until day 80, far later than found in normal control cultures. No shoots were excised from these explants. Those shoots which developed were probably only buffered from the harmful hygromycin selection by the abundance of semi-resistant/hardy callus tissue.

However, as shown in table 3.4.6,25.6% of explants on 12.5 mgl-1 G-418 were producing shoots by day 42 despite the lower proportion of green callus. Forty-six shoots were excised and transferred onto 12.5 mgl-1 G-418 rooting medium; of these 63% died almost immediately and those remaining showed some bleaching. However they also failed to produce roots and were classified as being "moribund", and as failing to survive selection.

This experiment again demonstrates the stronger influence of hygromycin on *L.corniculatus* leaf tissue than G-418, at the concentrations used.

3.4.4.3Experiments B_{1+2}

Experiments B_{1-4} were identical to those of A. However, the response of control material on 15 mgl^{-1} hygromycin was slightly improved compared to previous control samples. Some "healthy" callus occurred between day 28-42 but subsequently total necrosis and bleaching encompassed this material. Shoot initiation was again negligible and shoot extension non-existent. No time course data were obtained for control material on 12.5 mgl^{-1} G-418.

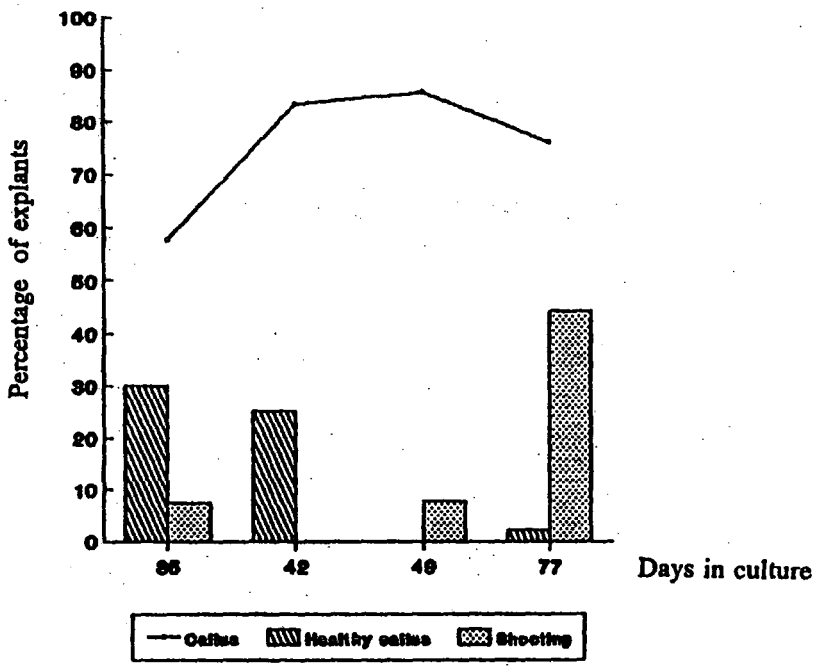
Table 3.4.7: Experiment B_{1+2}
Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]
Selection - B_1 15 mgl^{-1} Hygromycin; B_2 12.5 mgl^{-1} G-418
Expressed as percentage of each classification

DAY	Control- 15 mgl^{-1} Hygromycin (%)	15 mgl^{-1} Hygromycin (%) (Expt. B_1)	12.5 mgl^{-1} G-418 (%) (Expt. B_2)
28	B 100.0 *Cal 48.8* *Gr.cal 4.7* (43)	N 4.2 B 95.8 (120)	H 1.0 B 99.0 *Cal 57.0* *S 21.0* (100)
35	B 100.0 *Cal 53.1* *Gr.cal 10.9* (64)	B 100.0 *Cal 57.5 (23)* *Gr.cal 30.0 (12)* *S 7.5* (40)	B 100.0 *Cal 90.9 (130)* *Gr.cal 33.6 (48)* *S 15.4* (143)
42	B 100.0 *Cal 95.2* *Gr.cal 26.2* (42)	N 100.0 *Cal 83.3 (20)* *Gr.cal 25.0 (6)* (24)	H 2.4 B 97.6 *Cal 73.2 (123)* *Gr.cal 50.6 (85)* *S 11.3* (168)
49	B 100.0 *Cal 89.1* *S 2.2* (92)	N 100.0 *Cal 85.7* *S 7.9* (63)	N 7.0 B 93.0 *Cal 71.5* *S 21.5* (186)
56	B 100.0 *Cal 75.0* (88)	N 100.0 *Cal 80.4* *Gr.cal 45.7* *S 8.7* (46)	N 10.5 B 89.5 *Cal 77.0* *S 27.2* (191)
77	B 100.0 *Cal 100.0* (35)	N 100.0 *Cal 76.1* *Gr.cal 2.3* *S 44.3* (88)	N 100.0 *Cal 96.5* *S 45.6* (114)
91	B 100.0 *Cal 100.0* (14)	N 100.0 *Cal 97.1* *S 52.9* (70) Many S abnormal	N 100.0 *Cal 100.0* *S 93.6* (110)

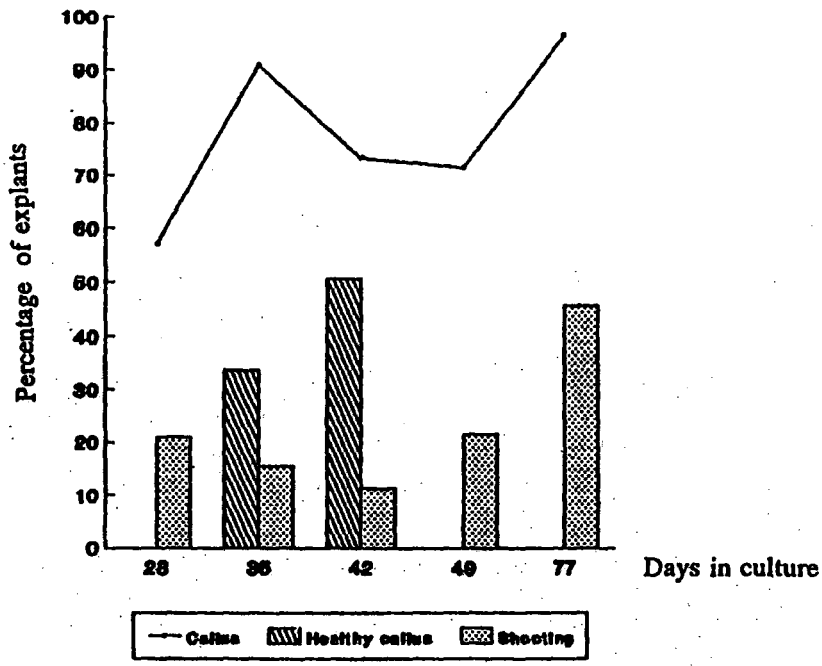
Key
H - Healthy tissue; N - Necrotic tissue; B - Bleached tissue; *Cal* - Explants exhibiting callus (necrotic or healthy) production; *Gr.cal.* - Explants exhibiting healthy, green callus production; *S* - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over the experimental period losses are due to contamination. Where n increases over time explants fragmented. Bold figures in parentheses represent actual numbers of explants used in statistical analysis.

Figure 3.4.8: Callus Production and Shoot Differentiation on leaf explants in Experiments B_1 and B_2

Experiment B_1 Vector: *A.tumefaciens* LBA4404 [pAL4404::pJIT73]
Selection: 15 mgl^{-1} Hygromycin



Experiment B_2 Vector: *A.tumefaciens* LBA4404 [pAL4404::pJIT73]
Selection: 12.5 mgl^{-1} G-418



Transformation was attempted using the *A.tumefaciens* LBA4404 [pAL4404::pJIT73] vector, and explants were placed on either 15 mgl^{-1} hygromycin (B_1) or 12.5 mgl^{-1} (B_2). Table 3.4.7 and figure 3.4.8 shows the data collected for these two experiments.

On 15 mgl^{-1} hygromycin some healthy callus was generated on explants and shoot differentiation occurred but in contrast to previous experiments there was more healthy, green callus on leaf explants subjected to 12.5mgl^{-1} G-418 (experiment B_2) compared to those on 15mgl^{-1} hygromycin (experiment B_1), however this only became significant at day 42, (G_{adj}=9.358, $X^2_{.01[1]}$=6.64). Prolific shoot production was delayed and many shoots emerged during these later weeks which were morphologically abnormal, as found in previous transformatiom attempts. Only shoots with a normal appearance were excised and recorded as having passed through the initial selection regime. In all, 70 shoots were transferred to ½$B_5$1S rooting medium containing 15 mgl^{-1} hygromycin, and by 6 months in culture after the co-cultivation step, 33% of these had survived. Further analysis of these is described in chapter 5. The overall shoot proliferation was greater for explants plated on 12.5 mgl^{-1} G-418 but of 112 excised shoots, none survived subsequent selection. Resistance to hygromycin B is a strong, efficient selectable marker.

3.4.4.4 Experiments B_{3+4}

A.tumefaciens GV3101[pGV3850::pJIT73] was used as the vector but otherwise these experiments followed the transformation procedure as for B_{1+2} and shared the same control plates. Only limited shoot initiation was induced on 15 mgl^{-1} hygromycin selected explants, and there were associated high levels of tissue damage either causing necrosis or bleaching. Results are shown in table 3.4.8 and figure 3.4.9. As in experiments B_{1+2}, greater healthy callus developed on 12.5mgl^{-1} (experiment B_4) compared to 15mgl^{-1} hygromycin (experiment B_3) at day 35 on selection (G_{adj}=19.89, $X^2_{.001[1]}$=10.83).

Predictably, greater shooting ability was evident on 12.5 mgl^{-1} G-418 selected

Table 3.4.8: Experiment B_{3+4}
Vector - *Agrobacterium tumefaciens* GV3101 [pGV3850::pJIT73]
Selection - B3 15 mgl^{-1} Hygromycin; B_4 12.5 mgl^{-1} G-418

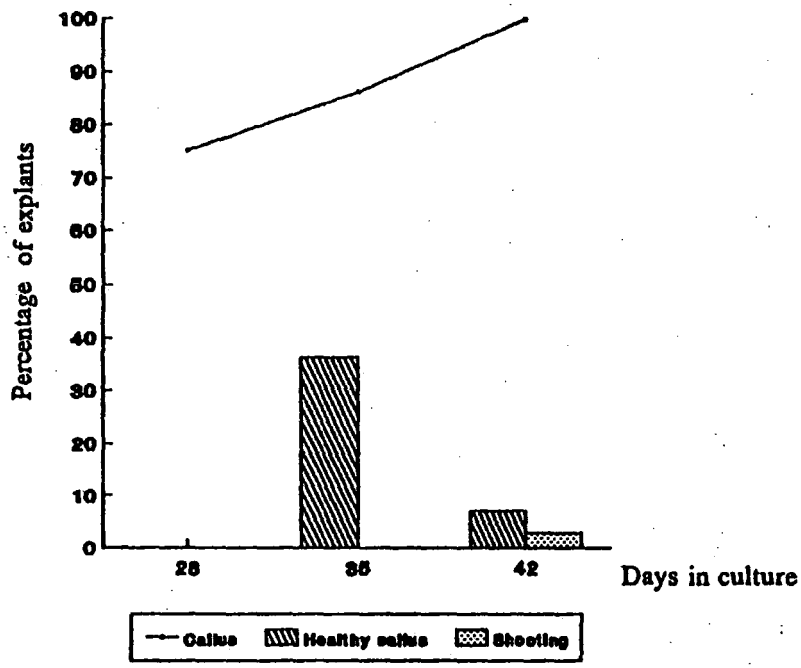
Expressed as a percentage of each classification

DAY	Experiment B_3 15 mgl^{-1} Hygromycin (%)	Experiment B_4 12.5 mgl^{-1} G-418 (%)
28	N 41.7 B 58.3 *Cal 75.0* (36)	B 100.0 *Cal 45.8* *S 4.2* (24)
35	N 36.3 B 63.7 *Cal 86.2 (50)* *Green cal. 36.2 (21)* (-)	B 100.0 *Cal 64.3 (18)* *(Green)* *S 28.6* *R 3.6* (28)
42	H 4.2 B 95.8 *Cal 100.0* *S 2.8* *Green cal. 7.0* (71)	N 100.0 *Cal 76.3* *S 34.2* *Green cal. 50.0* (38)
49	N 73.4 B 26.6 *Cal 86.7* *S 0.7* *Green cal. 6.3* (143)	B 100.0 *Cal 84.1* *S 15.9* *Green cal. 45.5* (44)
56	Bt 100.0 *Cal 84.4* *S 1.1* *Green cal. 4.4* (90)	N 100.0 Cal 100.0 S 100.0 (12)
77	N 96.4 B 3.6 *Cal 87.4* *S 4.5* *Green cal. 0.9* (111)	----------

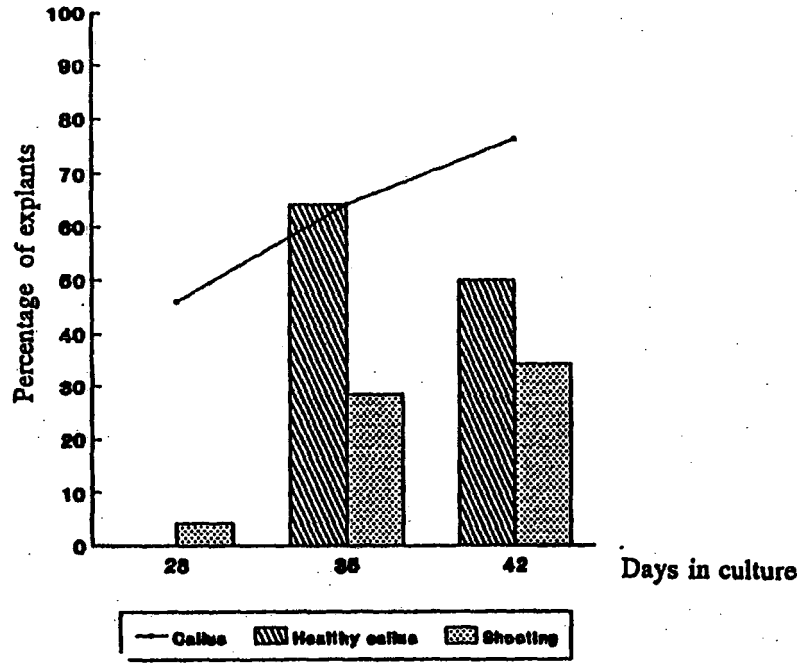
Key
H - Healthy tissue
N - Necrotic tissue
B - Bleached tissue
Cal - Explants exhibiting callus (necrotic and healthy) production
Gr.cal. - Explants exhibiting healthy, green callus production
S - Explants exhibiting shoot development
(n) - Total number of explants, where n decreases over experimental period losses are due to contamination. Increases in n were due to explant fragmenting.
Bold figures in parentheses represent actual numbers of explants used in statistical analysis

Figure 3.4.9: Callus Production and Shoot Differentiation on leaf explants in Experiment B_3 and B_4

Experiment B_3 Vector: *A.tumefaciens* GV3101 [pGV3850::pJIT73]
Selection: 15 mgl^{-1} Hygromycin



Experiment B_4 Vector: *A.tumefaciens* GV3101 [pGV3850::pJIT73]
Selection: 12.5 mgl^{-1} G-418



tissue. The ineffectiveness of this antibiotic to impose a selection pressure on *L.corniculatus* leaf explants, was shown by the death of the 8 shoots cut off and placed on selective rooting medium.

A feasible transformation system had been developed, which could be improved. The failure however of the A_1 experimental explants to respond, compared to B_1 explants may relate to the virulence of the *A.tumefaciens* LBA4404[pAL4404::pJIT73] culture being enhanced in the second attempt.

Experiment C:

Experiment C closely followed the previous methods, with the modification that the pieces of leaf were not cut whilst submerged, but after cutting were immediately suspended in the *Agrobacterium* culture shown in experiment III. Also *vir* induction using acetosyringone (AS) during the *Agrobacterium* culture process was introduced. A noticeable difference in growth pattern of the *Agrobacterium* when grown in the presence of acetosyringone could be seen, cultures then contained many aggregated clumps of *Agrobacterium*. Following the usual two overnight cultures, the *Agrobacterium* was spun down and resuspended in induction medium. 1.4mM AS was then added and the bacteria incubated overnight at 27°C. Growth does not occur at this stage as the culture will already have reached stationary phase. This extra culture step is solely concerned with induction of the *vir* region.

There were early indications that this transformation attempt had been successful. Those explants incubated with the *A.tumefaciens* LBA4404 [pAL4404::pJIT73] strain and then selected on 15 mgl^{-1} hygromycin (C_1), table 3.4.9 and figure 3.4.10, showed shoot initiation more quickly than in previous attempts and a higher percentage of the callus was relatively healthy with areas of green tissue present. Those explants subjected to 15mgl^{-1} hygromycin (experiment C_1) exhibited significantly greater healthy callus than explants on 12.5mgl^{-1} (experiment C_2), G_{adj}=6.448 where $X^2_{.05[1]}$=3.84. If the differentiation process in experiment C_1 is compared to that of control, non-selected explants as in table 3.1.1, it can be seen that

Table 3.4.9: Experiment C_{1+2}

Vector - *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection - C_1 15 mgl^{-1} Hygromycin; C_2 12.5 mgl^{-1} G-418

Expressed as a percentage of each classification

DAY	15 mgl^{-1} Hygromycin (%) (Expt. C_1)	12.5 mgl^{-1} G-418 (Expt. C_2) (%)
14	N 100.0 *Cal* *8.8* (80)	H 45.6 N 36.7 B 17.7 *Cal* *75.9* (79)
21	H 6.1 N 10.2 B 83.8 *Cal* *62.9* *Gr.cal* *31.0* *S* *20.3* (197)	N 100.0 *Cal* *95.9* *Gr.cal* *39.2* (74)
28	N 46.1 B 53.9 *Cal* *100.0* *Gr.cal* *41.7* *S* *13.9* (180)	N 66.7 B 33.3 *Cal* *96.5* *Gr.cal* *36.8* *S* *12.6* (87)
35	N 18.0 B 82.0 Cal 80.5 (103) Gr.cal 43.8 (56) S 13.3 (128)	N 14.6 B 85.4 *Cal* *84.4 (81)* *Gr.cal* *29.2 (28)* *S* *6.3* (96)
63	H 3.7 N 96.3 *Cal* *81.5* *S* *40.7* (135)	N 100.0 *Cal* *100.0* *S* *36.0* (75)

Key

H - Healthy tissue

N - Necrotic tissue

B - Bleached tissue

Cal - Explants exhibiting callus (necrotic and healthy) production

Gr.cal - Explants exhibiting healthy, green callus production

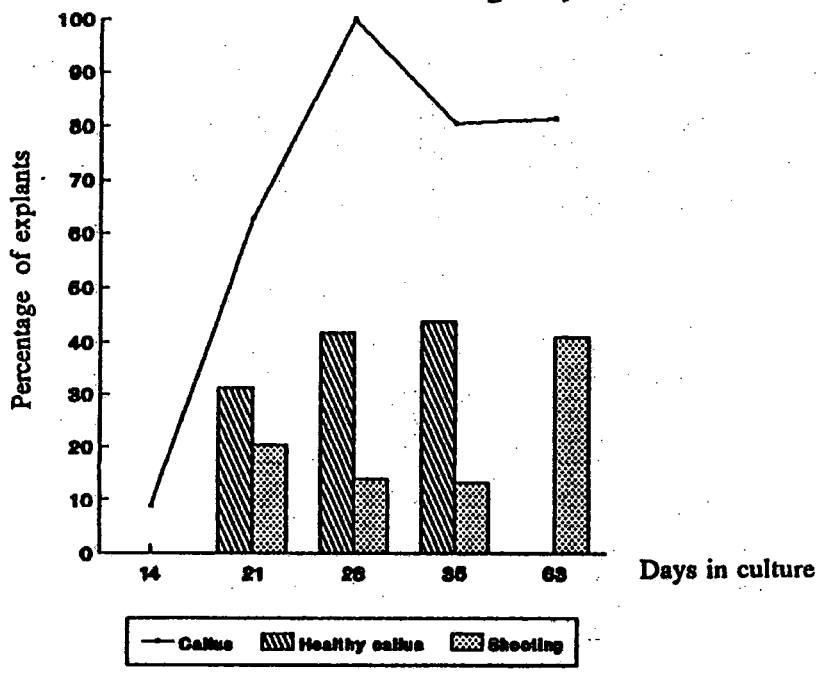
(n) - Total number of explants, where n decreases over experimental period losses are due to contamination. Increases in n are due to explant fragmenting.

Bold figures in parentheses represent actual number of explants used in statistical analysis

Figure 3.4.10: Callus Production and Shoot Differentiation on Leaf Explants in Experiments C_1 and C_2

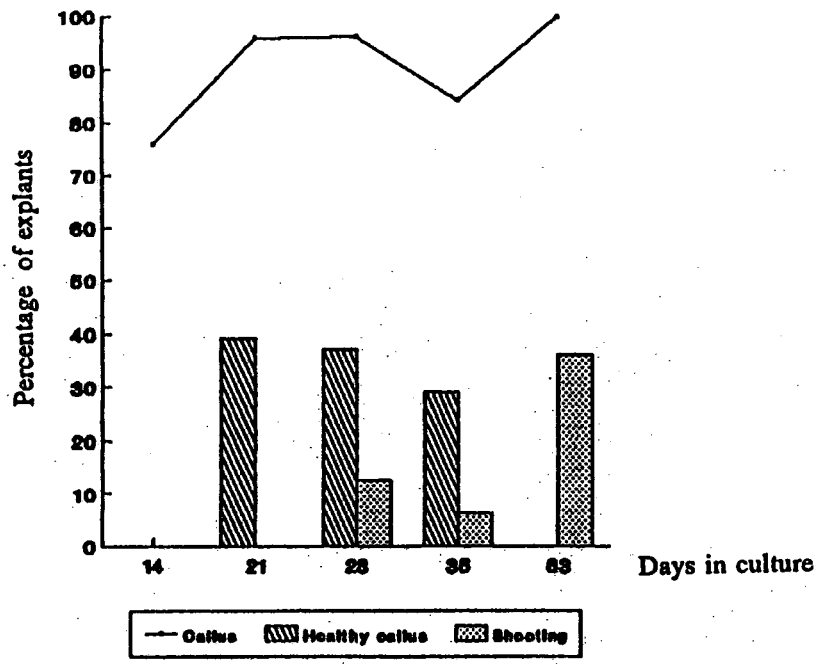
Experiment C_1 Vector: *A.tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection: 15 mgl^{-1} Hygromycin



Experiment C_2 Vector: *A.tumefaciens* LBA4404 [pAL4404::pJIT73]

Selection: 12.5 mgl^{-1} G-418



these co-cultivated explants exhibit the "model" development pattern over time, when the 4 extra days in culture prior to selection are considered. The presence of untransformed tissue either as callus or as the stunted and abnormal shoots which were present had a detrimental effect on the development of the transgenic shoots. Neverthless shoots were excised from these explants. A total of 40 shoots were removed by 5 months in culture, though only 7 of these had been cut by 4 months. The shoots were transferred onto selective-slopes to assay expression of the *Aph IV* gene.

By month 4: Total excised - 7; Survived - 6; 85.7% hygromycin-resistant.
By month 5: Total excised - 40; Survived - 29; 72.3% hygromycin-resistant.

Fourteen control shoots all died when exposed to hygromycin in rooting medium, the bleaching being a relatively rapid event. For those from the experimental explants, events leading to death were sometimes more involved with shoot tissue becoming moribund and failing to root. Leaf absciscion occurred over a period of a month and the shoot eventually degenerated.

Those explants subjected to the *A.tumefaciens* LBA4404[pAL4404::pJIT73] strain and selected on 12.5 mgl^{-1} G-418 (experiment C_2), showed similar tissue response to the C_1 type (table 3.4.9 and figure 3.4.10). However, the 64 shoots excised over the same period as those on 15 mgl^{-1} hygromycin all died on the subsequent rooting selection.

Neither of the groups of explants incubated with *A.tumefaciens* strain GV3101[pGV3850::pJIT73] (experiments C_3 and C_4) yielded antibiotic-resistant plants (table 3.4.10). Those selected on 12.5 mgl^{-1} G-418 became contaminated with a fungal infection, although 6 shoots were excised. All these were sensitive to G-418 and died on selection rooting medium. Despite a proportion of explants exhibiting callus production (40%), no shoot differentiation was visible on those plated on 15 mgl^{-1} hygromycin. These also became infected with fungus.

Table 3.4.10: Experiment C_{3+4}

Vector - *Agrobacterium tumefaciens* GV3101 [pGV3850::pJIT73]

Selection - C_3 15 mgl^{-1} Hygromycin; C_4 12.5 mgl^{-1} G-418

Expressed as a percentage of each classification

DAY	Experiment C_3 15 mgl^{-1} Hygromycin (%)	Experiment C_4 12.5 mgl^{-1} G-418 (%)
7	N 100.0 (55)	N 100.0 (20)
14	H 51.9 N 40.4 B 7.7 *Cal 96.2* (52)	H 20.0 N 66.7 B 13.3 (15)
21	N 50.0 B 50.0 *Cal 70.0* *Green cal. 40.0* (40)	------

Key

H - Healthy tissue

N - Necrotic tissue

B - Bleached tissue

Cal - Explants exhibiting callus (necrotic and healthy) production

Gr.cal. - Explants exhibiting healthy, green callus production

(n) - Total number of explants, where n decreases over experimental period losses are due to contamination

Experiment D

In this experiment the method of experiment B was repeated, with *A.tumefaciens* GV3101[pGV3850::pJIT73] as the transfer system used in a final attempt to obtain genetic transfer using this vector. Only hygromycin was used as the selection either at a concentration of 15 mgl^{-1} (D_1) or 17.5 mgl^{-1} (D_2). Despite the absence of shoot development on the explants selected on 15 mgl^{-1} hygromycin, the callus did appear more healthy than the necrotic control tissue. Of the shoots visible on the explants selected on 17.5 mgl^{-1} hygromycin, only 1 shoot was transferred and survived on the rooting medium. This plant was then subsequently assayed as for those of experiments B_1 and C_1 (see Results, chapter 5). Further work using *vir* induced C58 type *Agrobacterium* may prove useful in the future.

A feature of *Lotus corniculatus* tissue regeneration unique to experiments within group 4, was the proliferation of healthy, green callus in contrast to the previously recorded necrotic tissue. When the degree of transgenic shoot development is considered across all the transformation attempts true transgenic plants were only initiated from healthy, green callus. So the frequency of explants with healthy callus, were compared between the experiments using the two strains of *A.tumefaciens* in order to establish whether the type of vector used influenced the subsequent development of such callus on explants. The results at day 35 indicated that there was a significant increase in green callus proliferation in those experiments (A_{3+4}, B_{3+4}) using *A.tumefaciens* GV3101 [pGV3850::pJIT73] to experiments A_{1+2}, B_{1+2}, using *A.tumefaciens* LBA4404 [pAL4404::pJIT73], [G_{adj}=13.676, $X^2_{.001[1]}$=10.83].
However the production of a higher proportion of green callus by explants exposed to *A.tumefaciens* GV3101 [pGV3850::pJIT73] did not extend to successful transgenic shoot proliferation.

In a similar way, the effect of acetosyringone *vir*-induction on the *Agrobacterium tumefaciens* LBA4404[pAL4404::pJIT73] can be seen from the data collected from experiments A_{1+2}, B_{1+2}, C_{1+2}. Here the higher level of healthy callused tissue at day 35 found on explants cocultivated with AS-pretreated *A.tumefaciens*

(experiments C_{1+2}) was significant, (G_{adj}=30.34, $X^2_{.001[1]}$=10.83). The production of callus in general was not influenced by the treatment of *Agrobacterium*, therefore proliferation of necrotic callus doesnot represent evidence of genetic transfer having taken place.

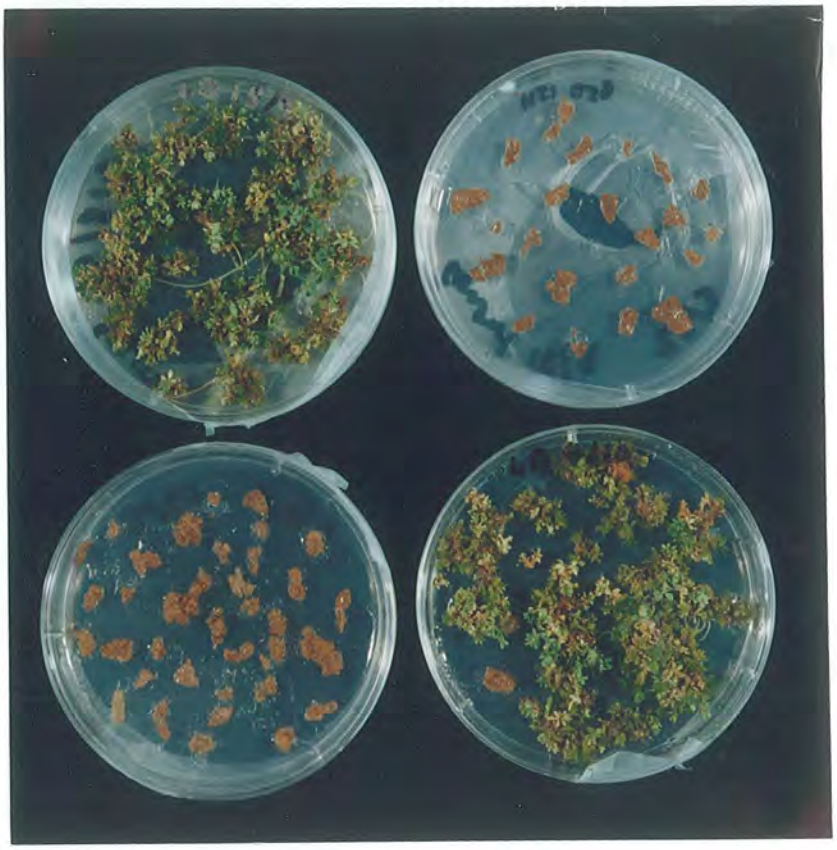
The group 4 series of experiments also helped exemplify the efficiency of hygromycin B as selection agent with *L.corniculatus* leaf explants. Figure 3.4.11 illustrates the response of both non-cocultivated explants and *A.tumefaciens* LBA4404[pAL4404::pJIT73] cocultivated explants on 15mgl^{-1} hygromycin.

Meanwhile however, the emergence of shoots from cocultivated leaf explants subjected to selection pressure is also not a definitive indication of transformation events, only the survival of excised shoots under further selection is a measure of genetic transfer. The proportion of shoot survival on rooting selective medium of excised shoots derived from the Group 4 experiments may be compared. Results shown in figure 3.4.12, indicate the significantly greater shoot survival on 15mgl^{-1} hygromycin after use of *vir*-induced *Agrobacterium* in experiment C_1 compared to non-induced *Agrobacterium* in experiment B_1, (G_{adj}=18.2;$X^2_{.001[1]}$=10.83). Use of AS saw no improvement in shoot survival when G-418 was the selection antibiotic. The detrimental affect of the selection antibiotics used in experiments B_2, B_4 and C_2 was absolute. Thus the inefficiency of G-418 as the selection antibiotic has been clearly demonstrated.

Figure 3.4.13 shows the final protocol established for the transformation of *Lotus corniculatus* with disarmed *A.tumefaciens*. This method and its development will be further discussed in section IV.

Figure 3.4.11: Explants from Group 4, experiments B_1 and C_1 on Selection.
All explants are plated on B_50 + 200 mgl^{-1} Augmentin + 15 mgl^{-1} Hygromycin B.

Top left - B_1 Explants **Top right - Control Explants**
Bottom left - Control Explants **Bottom right - C_1 Explants**



Clear morphological effects of hygromycin B at 15mgl^{-1} on control leaf explants can be seen. Although control tissue may show some callus production, total necrosis aborts further differentiation. This is in contrast to the apparent uninhibited regenerative ability of cells within the cocultivated explants. Tissue from which the transgenic shoots still develop in general, becomes necrotic but transformed cells appear able to continue differentiation against this moribund background. Only shoots with normal phenotypic appearance were excised and transferred onto the rooting stage. Abnormal

Figure 3.4.12 illustrates a similarly clear difference between responses in culture of *L.cornicultus* control shoots on rooting slopes containing hygromycin and those with no additions. Under normal culture conditions *L.corniculatus* shoots initiate root development rapidly and after approximately 2 weeks a single root is visible. Secondary branching over the subsequent 2 weeks establishes an extensive, penetrating system.

In contrast, in the the presence of 15 mgl^{-1} hygromycin, control tissue becomes completely bleached and fails to initiate root development. The bleaching reaction usually occurs over a 10 day period.

Forty-one shoots from B_1 and C_1 experiments, surviving the selection during rooting were subsequently analysed for GUS activity and 5 from experiment C_1 at the genomic DNA level.

Figure 3.4.12: Development of Excised Shoots (Control and Transgenic) on 15 mgl^{-1} Hygromycin

Excised shoots were all placed on rooting slopes containing 200 mgl^{-1} Augmentin and 15 mgl^{-1} Hygromycin

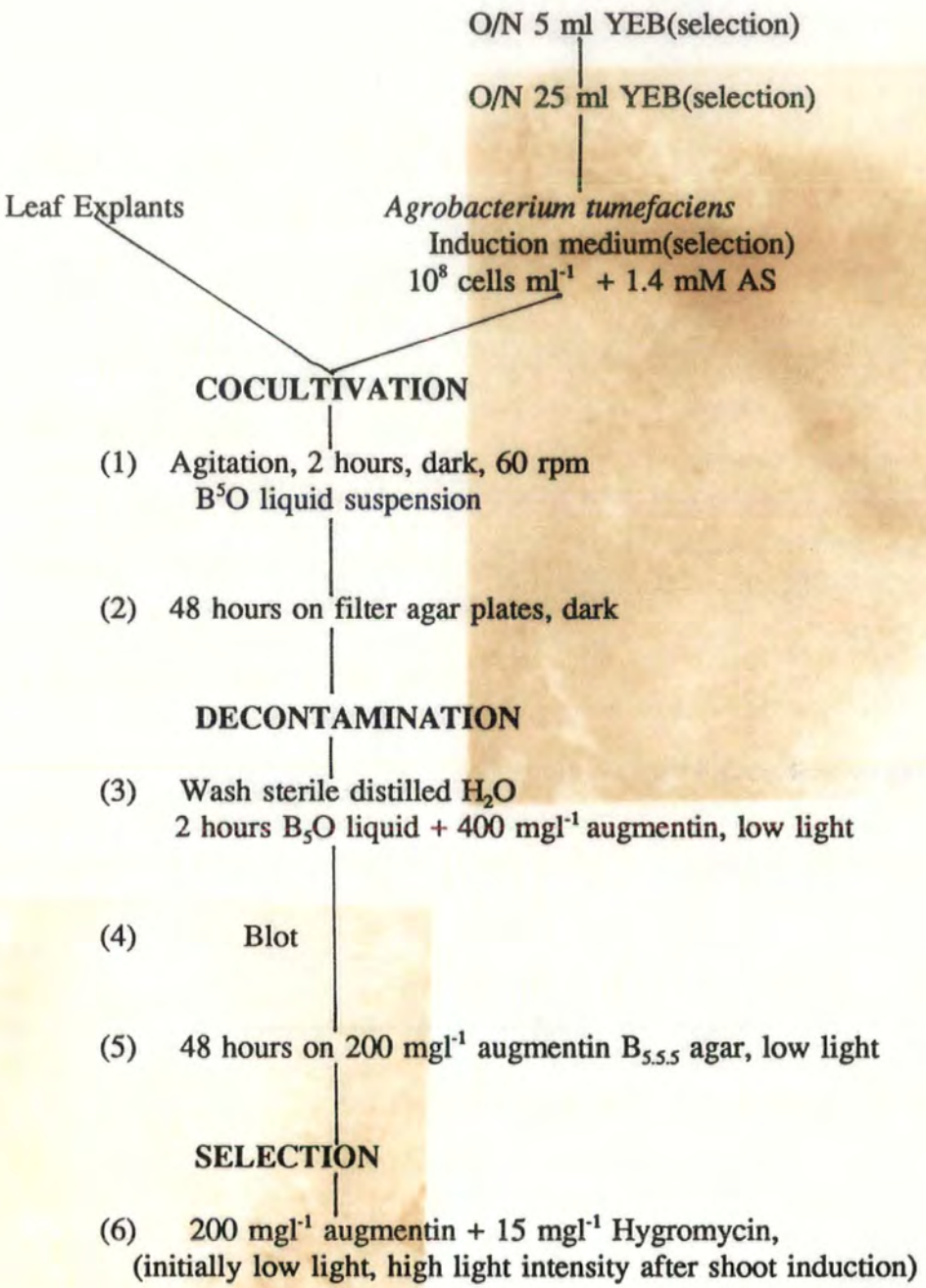
(a) Reaction of Control Shoots

Bleaching begins immediately with total bleaching of tissue after 10 days on selection.

(b) Reaction of Transgenic Shoots

Normal development of a root system occurs over 2-3 weeks. Aerial parts of plant also remain healthy.

Figure 3.4.13: Final *Agrobacterium tumefaciens*-mediated Transformation Protocol Developed for *Lotus corniculatus* Leaf Explants



CHAPTER 5: Analysis of Putative Transformed Lotus corniculatus Shoots

3.5.1 Introduction

Table 3.5.1 summarises the data collected during the study of the transgenic material produced. Both antibiotic resistance and ß-glucuronidase activity are utilised as indicators of gene transfer. Initially, the selection pressure imposed, see chapter 4, constitutes the most important aspect of assessing the presence or absence of gene transfer. Confirmation of GUS activity can follow together with the DNA analysis and a secondary investigation of antibiotic resistance.

3.5.2 ß-Glucuronidase Assay

A total of 42 hygromycin-resistant plants were assayed for GUS activity; 20 from experiment B_1, 21 from experiment C_1 and one derived from experiment D_2. At least three leaf piece replicates of each plant were assayed. With the GUS gene under the control of the CaMV 35S constitutive promoter, GUS would be expected to be produced in all tissue, thus, due to ease of harvesting leaf tissue was used in this gene activity assay. The assay procedure followed that outlined in Methods 2.2.3.4,and readings were taken at 15, 30 and 45 min after the start of the assay. Consistently low values were measured at time zero (figure 3.5.1). The relative levels of expression over time is comparatively stable with the kinetics of enzyme reaction being close to linear over the 15-45 min. period. Thus the comparison of GUS activity recorded over this time period for the plants tested is suitable. Taking this stability into account and the additional problem of starting the assay and taking large numbers of samples at time 0, it was decided always to start readings after 15 min incubation.

Results are presented in terms of the production of 4-Methylumbelliferone (MU) occurring during ß-glucuronidase reaction, nM MU produced μg^{-1} protein min^{-1} (Jefferson *et al*, 1987). Table 3.5.2 shows the levels of "background" GUS activity found in control *L.corniculatus* leaf tissue samples.

Table 3.5.1: Confirmation of Transformation

n = number of plants excised or plants tested.

ASSESSMENT:	Experiment B_1	Experiment C_1	Experiment D_2
Resistance to 15 mgl^{-1} hygromycin (*Aph IV*) of excised shoots	33% *Aph* ^{R} (70)	72.3% *Aph* ^{R} (40)	100% *Aph* ^{R} (1)
GUS Assays of *Aph* ^{R} plants			
(a) Fluorometric assay	60% GUS$^+$ (20)	81.0% GUS$^+$ (21)	100% GUS$^+$ (1)
(b) Histochemical assay	45% GUS$^+$ (20)	81.0% GUS$^+$ (21)	100% GUS$^+$ (1)
Percentage of original shoots excised showing both *Aph* ^{R}, GUS$^+$	19.8% (20)	58.6% (21)	100% (1)
Antibiotic resistance of regenerated plants			
Aph IV	Resistance to at least 100 mgl^{-1} hygromycin (2)	Resistance to at least 100 mgl^{-1} hygromycin (2)	-------
Npt II	No resistance shown to even 12.5 mgl^{-1} G-418 (2)	No resistance shown to even 12.5 mgl^{-1} G-418 (2)	No resistance shown to even 12.5 mgl^{-1} G-418 (2)
Genomic DNA Analysis	-------	DNA from all 4 plants tested showed hybridisation to GUS and *Aph IV*. 3 plants = GUS$^+$ *Aph*^{R} 1 plant = GUS$^-$ *Aph*^{R} No hybridisation to a non T-DNA sequence of pBIN19.	GUS$^+$ *Aph* ^{R} plant confirmed to contain both genes

Figure 3.5.1: Graph showing the Fluorescence over Time Derived from Transgenic *L.corniculatus* tissue

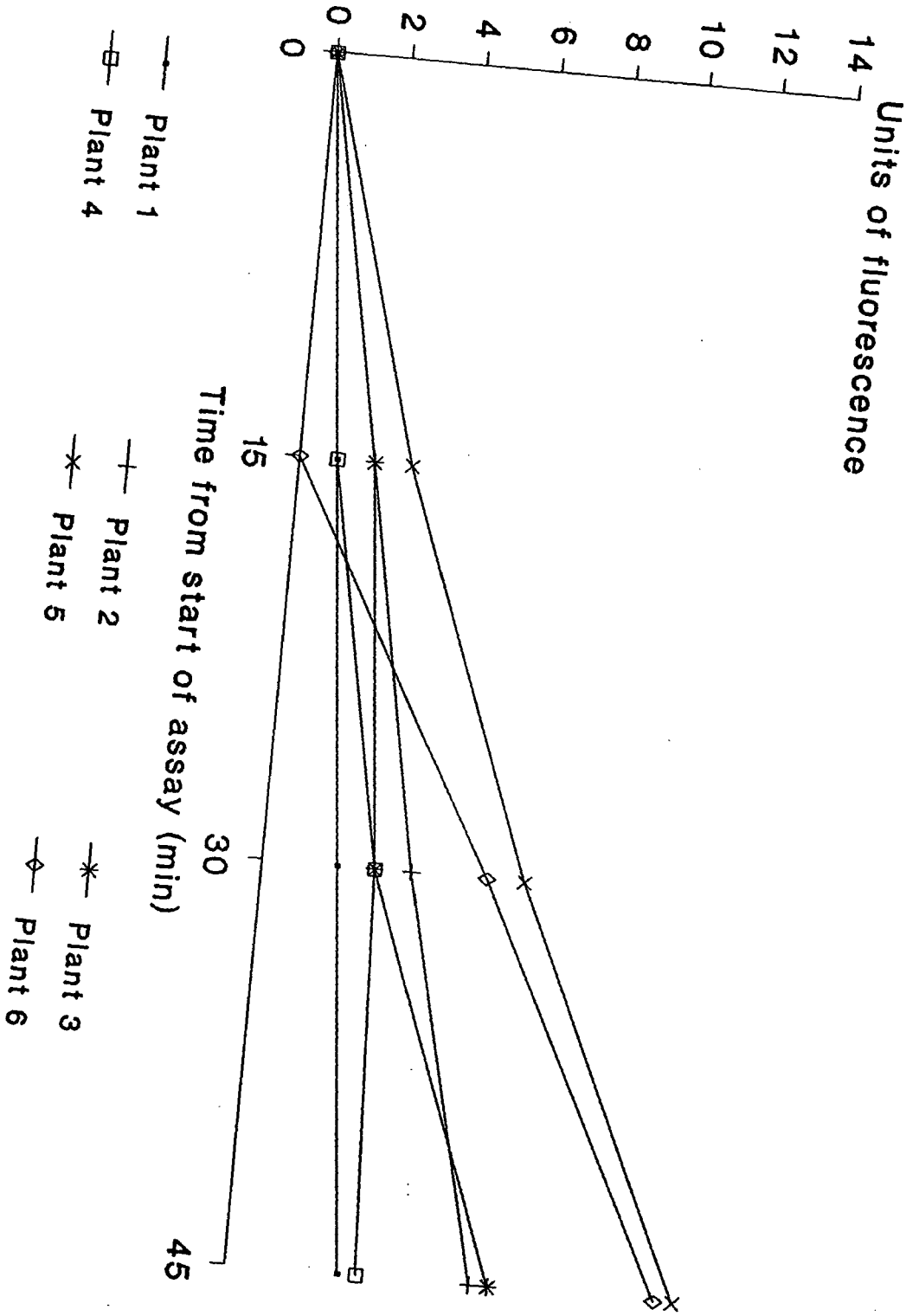


Table 3.5.2: Endogenous GUS Activity in Control *Lotus corniculatus* Tissue

Plant	Calculated nM MU Produced min^{-1} μg^{-1} Protein	Plant	Calculated nM MU Produced min^{-1} μg^{-1} Protein	Plant	Calculated nM MU Produced min^{-1} μg^{-1} Protein
1	0	16	$1.1x10^{-4}$	31	0
2	$2.64x10^{-4}$	17	$1.1x10^{-4}$	32	0
3	$2.64x10^{-4}$	18	$1.1x10^{-4}$	33	0
4	0	19	$1.1x10^{-4}$	34	$8.5x10^{-5}$
5	0	20	0	35	$8.5x10^{-5}$
6	0	21	$1.1x10^{-4}$	36	$1.44x10^{-4}$
7	0	22	$7.2x10^{-5}$	37	0
8	0	23	0	38	$8.5x10^{-5}$
9	$3.84x10^{-5}$	24	0	39	$1.15x10^{-4}$
10	$1.1x10^{-4}$	25	$1.1x10^{-4}$	40	0
11	$1.1x10^{-4}$	26	0	41	0
12	0	27	0	42	0
13	$1.1x10^{-4}$	28	0	43	0
14	0	29	0	44	0
15	$1.1x10^{-4}$	30	$6.1x10^{-5}$		

nM MU produced μg^{-1} protein min-1; $\bar{x} = 5.25x10^{-5}$; $\delta_n = 6.82x10^{-5}$; S.E. = $1.03x10^{-5}$

Figure 3.5.2: Micrographs of Tissue Incubated with X-Gluc

All tissue was dehydrated after exposure to the substrate

(a) Control Tissue (x460) - total colour loss after dehydration

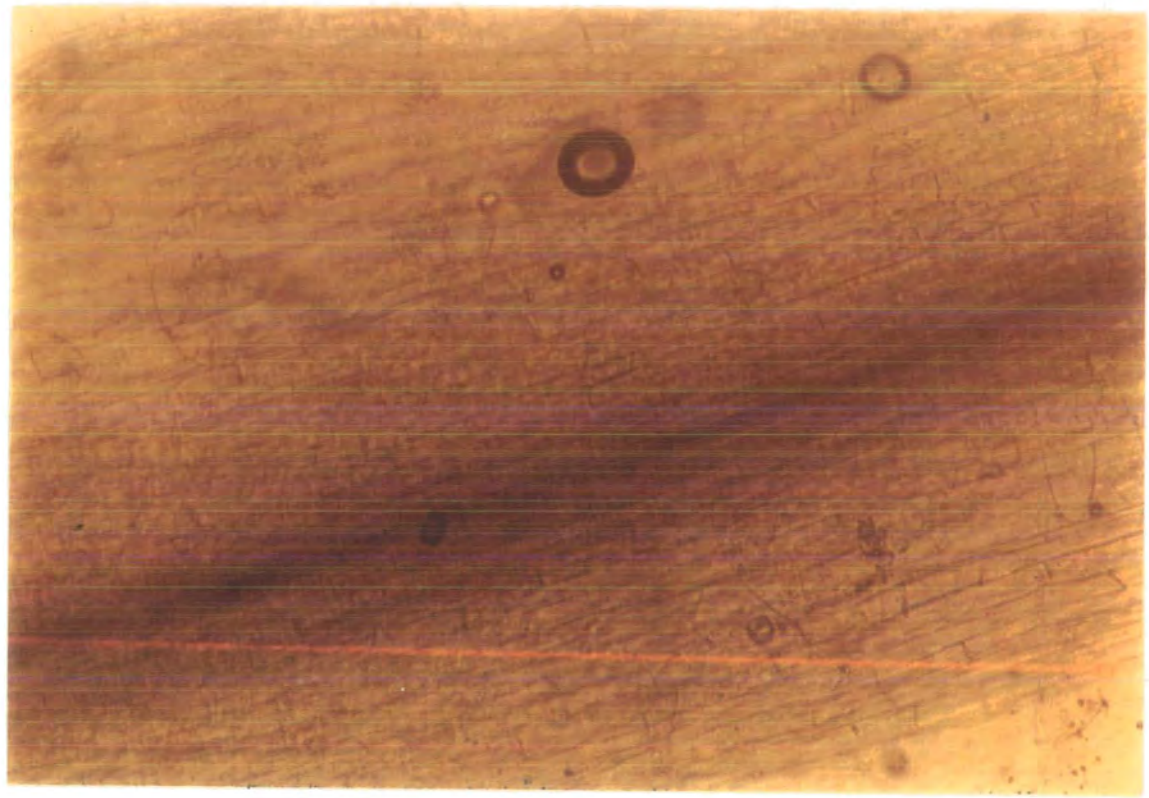
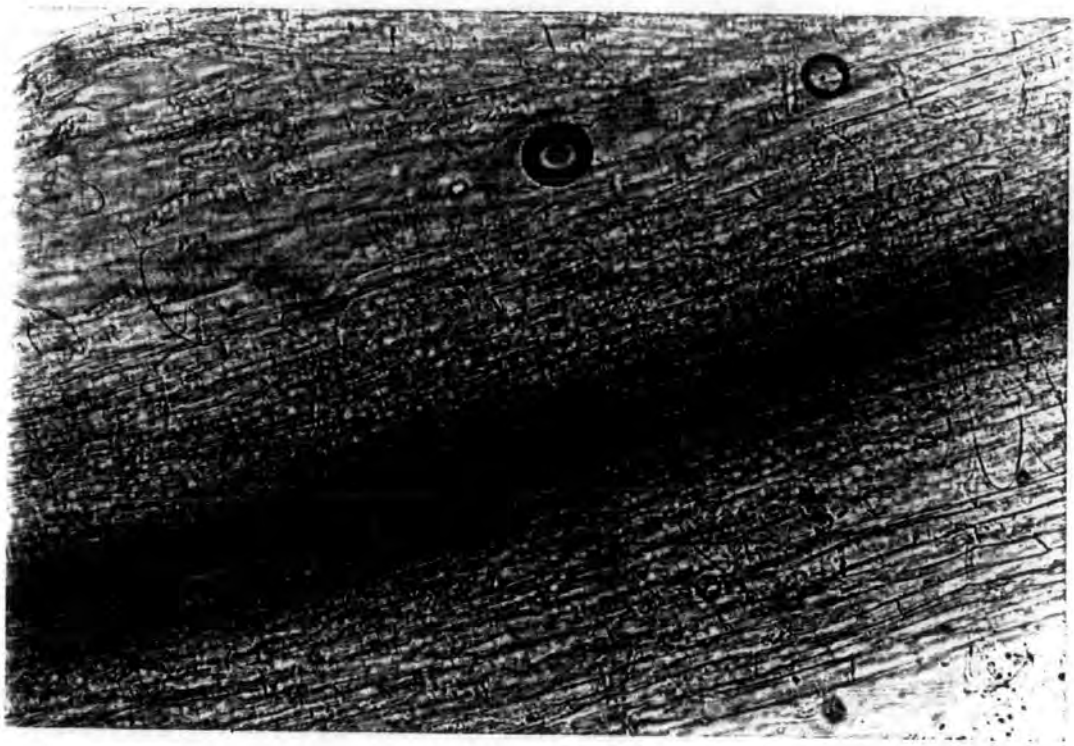


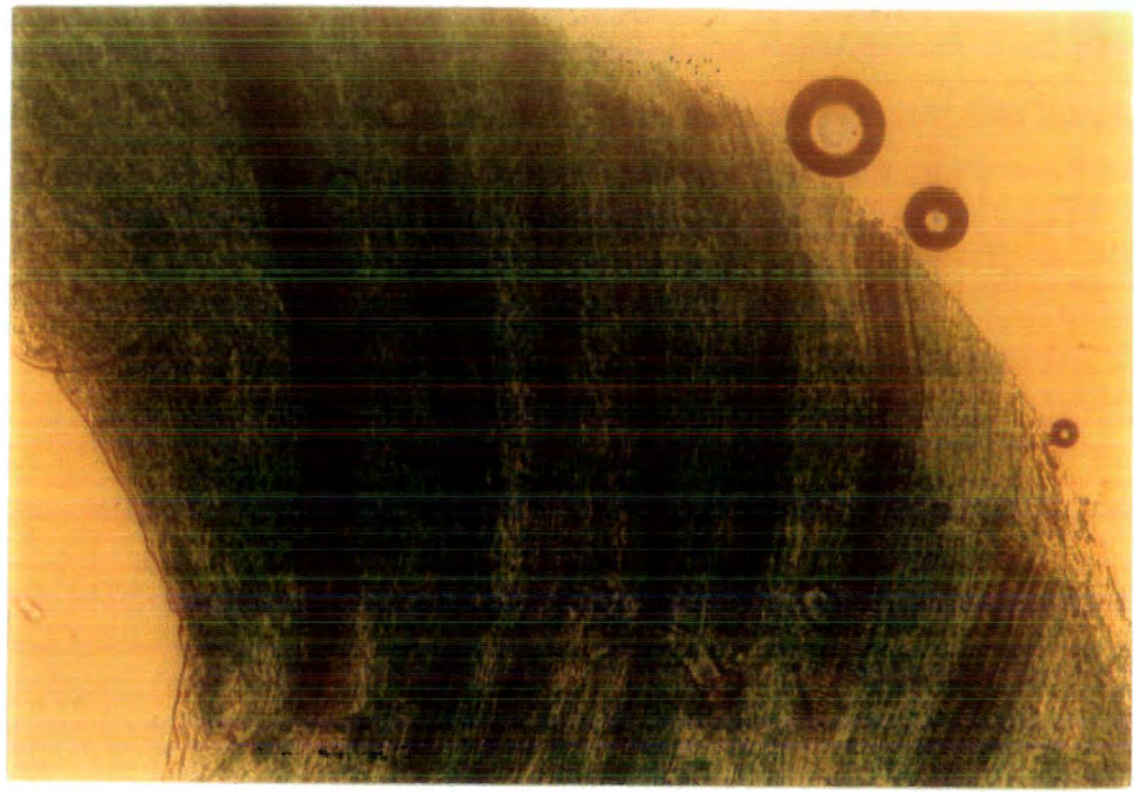
Figure 3.5.2: Micrographs of Tissue Incubated with X-Gluc

All tissue was dehydrated after exposure to the substrate

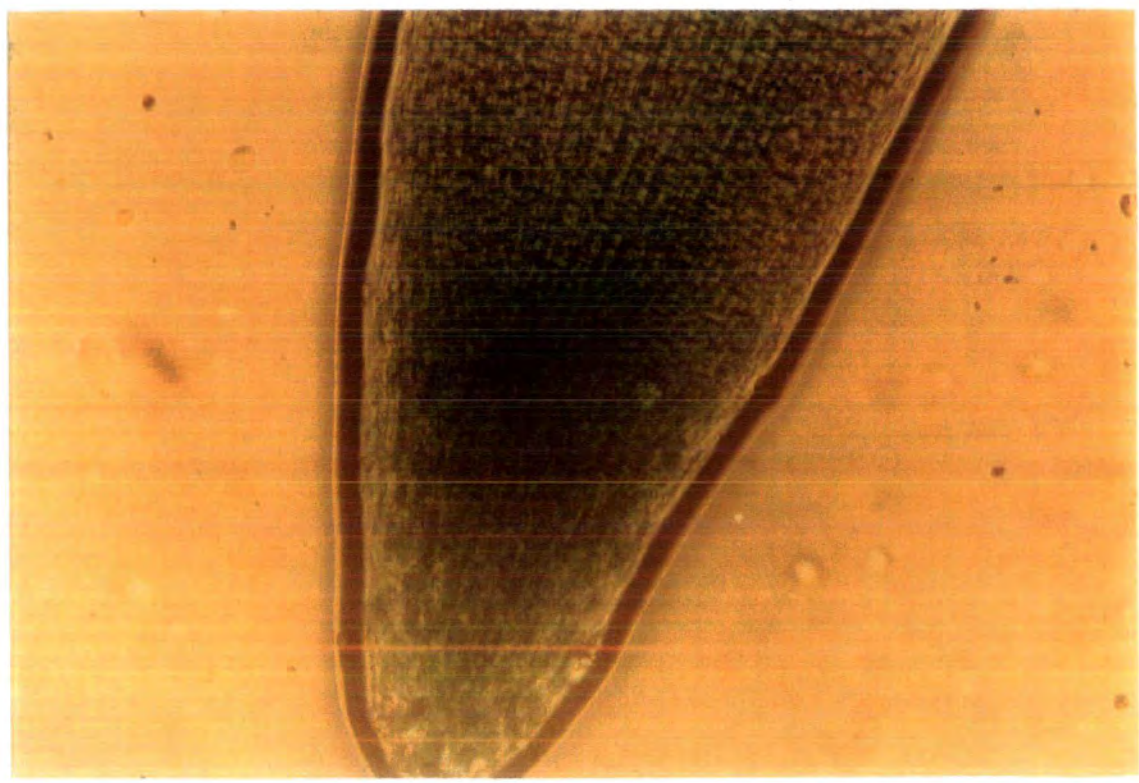
(a) Control Tissue (x460) - total colour loss after dehydration



(b) Squashed transgenic stem material (x460) - Blue precipitate left in GUS expressing cells. General, non-specific expression in tissue.



(c) Squashed transgenic root (x460) - Evidence of GUS expression. But expression is strongest at the apical meristem. Expression much reduced at root tip and older tissue.



These data allow a lower limit for measured GUS expression in putative transgenic plants to be set as $5.25x10^{-5}$ +/- $1.0x10^{-5}$ nM MU produced μg^{-1} protein min^{-1}.

The histochemical assay was a useful aid to confirming GUS activity. Figure 3.5.2 shows control transgenic material after incubation with X-Gluc substrate. Blue precipitate, the oxidised product of the ß-glucuronidase reaction with X-Gluc substrate, is visible only in GUS expressing transgenic tissue.

Putative Transgenic Plants from Experiment B_1

The calculated units of GUS activity as nM MU μg^{-1} protein min^{-1} in putative transformants from experiment B1 are shown in table 3.5.3. Within replicates of a single plant, variation may indicate chimaeric tissue or assay artefacts. These plants are indicated by "c" in the table. In all cases, this assessment is made by comparing the mean, $\bar{x}$, of replicates corrected for S.E., to control data. Figure 3.5.3illustrates this productivity data graphically. This assay has identified 7 plants (2, 3, 9, 10, 11, 12, 13) exhibiting GUS expression at all times of sampling. Nine plants (4, 7, 8, 14, 15, 17, 18, 19) failed to show any significant GUS activity above that of background. Four plants (1, 5, 16 and 20) were shown to possess GUS activity in some assays, but not in others.

The GUS positive plants can be divided into groups with respect to their MU productivity. One group, plants (1, 2, 5, 11, 13, 20) produced low but significant levels of MU and the second group, plants (3, 9, 10, 12) produced high levels of MU. Although plant 16 exhibits GUS activity, the mean level of expression was not significantly above that of the control level due to the non-universal GUS expression. However the positive reading obtained was confirmed as true GUS activity rather than an experimental artefact by use of the histochemical assay. So, 60% of the regenerated hygromycin-resistant plants tested also exhibited GUS activity.

Table 3.5.3: GUS Activity in Putative Transgenic Plants Regenerated from Experiment B_1

PLANT	GUS Activity (nM MU μg^{-1} Protein min^{-1})	$\bar{x}$ nM MU μg^{-1} Protein min^{-1}	S.E.	Possible Tissue Makeup
1	$2.2x10^{-3}$/0/ $1.2x10^{-3}$	$1.1x10^{-3}$	$6.5x10^{-4}$	c (+)
2	$5.5x10^{-4}$/$4.3x10^{-4}$/ $2.0x10^{-3}$	$9.9x10^{-4}$	$5.1x10^{-4}$	+
3	$6.6x10^{-3}$/$2.5x10^{-3}$/ $8.8x10^{-3}$	$6.0x10^{-3}$	$1.9x10^{-3}$	+
4	0/$1.9x10^{-5}$/ $2.5x10^{-5}$	$1.5x10^{-5}$	$7.7x10^{-6}$	c (-)
5	0/0/$4.7x10^{-4}$/ $6.2x10^{-4}$/$8.5x10^{-6}$/ $1.9x10^{-5}$	$1.9x10^{-4}$	$1.2x10^{-4}$	c (+)
6	0/$1.8x10^{-5}$/ $2.5x10^{-5}$	$1.4x10^{-5}$	$7.6x10^{-4}$	-
7	$2.5x10^{-6}$/$4.2x10^{-4}$/ $3.0x10^{-5}$	$1.5x10^{-4}$	$1.4x10^{-4}$	c (-)
8	$6.0x10^{-6}$/ $4.3x10^{-5}$/ $2.6x10^{-5}$	$2.5x10^{-5}$	$1.1x10^{-5}$	-
9	$4.6x10^{-3}$/$2.0x10^{-3}$/ $7.6x10^{-3}$	$4.7x10^{-3}$	$1.6x10^{-3}$	+
10	$7.7x10^{-3}$/$2.6x10^{-3}$/ $7.8x10^{-3}$	$6.0x10^{-3}$	$1.7x10^{-3}$	+

Key

+ = Constitutive GUS expression
- = No detectable GUS expression
c = Non-continuous GUS activity

Table 3.5.3 continued:

PLANT	GUS Activity (nM MU μg^{-1} protein min^{-1})	$\bar{x}$ nM MU μg^{-1} protein min^{-1}	S.E.	Possible tissue makeup
11	$6.0x10^{-4}$/ $8.0x10^{-4}$/ $7.9x10^{-5}$	$4.9x10^{-4}$	$2.2x10^{-4}$	+
12	$1.4x10^{-3}$/ $7.1x10^{-3}$/ 0.01	$6.2x10^{-3}$	$2.6x10^{-3}$	+
13	$3.2x10^{-3}$/ $1.1x10^{-3}$/ $3.6x10^{-4}$	$1.6x10^{-3}$	$8.7x10^{-4}$	+
14	$1.1x10^{-5}$/ $1.9x10^{-5}$/0	$1.0x10^{-5}$	$5.6x10^{-6}$	-
15	$1.1x10^{-5}$/0 $4.3x10^{-5}$	$1.8x10^{-5}$	$1.3x10^{-5}$	-
16	$1.2x10^{-3}$/ $1.0x10^{-5}$/ $1.9x10^{-5}$	$4.1x10^{-4}$	$4.0x10^{-4}$	+
17	$6.0x10^{-6}$/ $6.0x10^{-6}$/ $3.7x10^{-5}$	$1.6x10^{-5}$	$1.0x10^{-5}$	-
18	$1.9x10^{-5}$/ $1.9x10^{-5}$/0	$1.3x10^{-5}$	$6.5x10^{-6}$	-
19	0/0/0	0	0	-
20	$6.0x10^{-6}$/ $1.0x10^{-4}$/ $4.8x10^{-4}$/ $9.6x10^{-4}$	$3.9x10^{-4}$	$2.2x10^{-4}$	+

Key

+ = Constitutive GUS expression
- = No detectable GUS expression
c = Non-continuous GUS activity

Putative Transgenic Plants from Experiment C_1

Table 3.5.4 shows the calculated results from C_1 putative transformants. GUS activity is shown in figure 3.5.3. Of the 21 plants assayed only 3 plants, 36, 39 and 41, did not exhibit GUS activity. Seventeen plants were classed as transformants expressing GUS and these again exhibit a range of expression levels, but in general show higher activity than transgenic plants from experiment B_1. Three plants showed noncontinuous activity (28, 34, 40). These transgenic C_1 plants can be grouped in relation to their GUS activity less easily than those derived from experiment B_1. Highest activity is seen in plants 25 and 37 with a further seven plants (21, 24, 27, 30, 33, 38, 40) exhibiting moderate GUS activity. Eight plants showed low but significant levels of GUS activity (plants 22, 23, 26, 29, 31, 32, 35). Although the mean activity of plant 34 was not significantly above control levels due to non universal activity at the time of the assays, when GUS expression occurred it was significantly above background activity. So, 81.0% of regenerated hygromycin-resistant plants tested also exhibited GUS activity.

Putative Transgenic Plant from Experiment D_2

The plant derived from experiment D_2 was assayed for GUS expression and the calculated data from 3 replicate assays gave this plant, designated 42, a mean GUS activity of 2.04×10^{-3} nM MU produced μg^{-1} protein min^{-1} (S.E. = 6.95x10-4).

Analysis of Variability of GUS Activity in Different Leaves of some Plants

In view of the possibility of generation of some chimaeric plants in these transformation events, an experiment was set up to investigate GUS expression distribution in different plant organs to attempt to determine whether this indicated that the plants were chimaeric. Three B_1 and 7 C_1 GUS positive plants were chosen for this more detailed analysis. Tissue samples were assayed from the first and third open leaves, lowest leaf, stem (between first and third nodes) and roots of each plant (data not presented). Replicate samples were not assayed in this experiment and no statistical analysis of the organ specificity of GUS can be made.

Table 3.5.4: GUS Activity in Experiment C_1 Putative Transgenic Plants

PLANT	GUS Activity (nM MU μg^{-1} protein min^{-1})	$\bar{x}$ nM MU μg^{-1} protein min^{-1}	S.E.	Possible Tissue Makeup
21	$3.0x10^{-3}$/ $5.5x10^{-3}$/ $6.0x10^{-4}$	$3.0x10^{-3}$	$1.4x10^{-3}$	+
22	$1.9x10^{-4}$/ $5.5x10^{-3}$/ $1.2x10^{-3}$	$2.3x10^{-3}$	$1.7x10^{-3}$	+
23	$3.4x10^{-3}$/ $2.0x10^{-3}$/ $1.2x10^{-4}$	$1.8x10^{-3}$	$9.7x10^{-4}$	+
24	$2.8x10^{-3}$/ $5.5x10^{-3}$/ $8.8x10^{-5}$	$2.8x10^{-3}$	$1.6x10^{-3}$	+
25	0.012/ 0.023/ $2.5x10^{-3}$	0.013	$6.0x10^{-3}$	+
26	$2.6x10^{-3}$/ $1.3x10^{-3}$	$2.0x10^{-3}$	$6.6x10^{-4}$	+
27	$1.8x10^{-3}$/ $2.0x10^{-3}$/ $7.4x10^{-3}$	$3.7x10^{-3}$	$1.9x10^{-3}$	+
28	$1.44x10^{-3}$/ $2.5x10^{-3}$/ 0	$1.3x10^{-3}$	$7.4x10^{-4}$	c (+)
29	$9.6x10^{-4}$/ $1.2x10^{-4}$/ $8.4x10^{-4}$	$6.4x10^{-4}$	$2.7x10^{-4}$	+
30	$6.6x10^{-3}$/ $2.4x10^{-4}$/ $8.4x10^{-4}$	$2.6x10^{-3}$	$2.1x10^{-3}$	+

PLANT	GUS Activity (nM MU μg^{-1} protein min^{-1})	$\bar{x}$ nM MU μg^{-1} protein min^{-1}	S.E.	Possible Tissue Makeup
31	$1.9x10^{-3}$/ $1.3x10^{-3}$/ $6.0x10^{-4}$	$1.3x10^{-3}$	$3.8x10^{-4}$	+
32	$2.3x10^{-3}$/ $7.2x10^{-3}$/ $8.4x10^{-4}$	$3.4x10^{-3}$	$2.0x10^{-3}$	+
33	$1.6x10^{-3}$/ $2.6x10^{-3}$/ $1.2x10^{-3}$	$1.8x10^{-3}$	$4.2x10^{-4}$	+
34	$4.8x10^{-4}$/ 0/ $4.3x10^{-3}$	$1.6x10^{-3}$	$1.4x10^{-3}$	c (+)
35	$1.6x10^{-3}$/ $1.4x10^{-3}$/ $1.6x10^{-3}$	$1.5x10^{-3}$	$6.8x10^{-5}$	+
36	$4.7x10^{-6}$/ $4.7x10^{-6}$/0	$3.1x10^{-6}$	$1.6x10^{-6}$	-
37	$7.7x10^{-3}$/ $3.7x10^{-3}$/ $8.2x10^{-3}$	$6.5x10^{-3}$	$1.45x10^{-3}$	+
38	$2.3x10^{-3}$/ $1.2x10^{-3}$/ $4.7x10^{-3}$	$2.7x10^{-3}$	$1.0x10^{-3}$	+
39	$5.2x10^{-7}$/ $1.1x10^{-5}$/ $4.3x10^{-6}$	$5.3x10^{-6}$	$3.1x10^{-6}$	-
40	$4.0x10^{-3}$/ $1.9x10^{-3}$/ $2.0x10^{-5}$	$2.0x10^{-3}$	$1.2x10^{-3}$	c (+)
41	$1.4x10^{-5}$/ $1.1x10^{-5}$/ $4.7x10^{-6}$/0	$7.4x10^{-6}$	$3.1x10^{-6}$	-

Key: +, Constitutive GUS expression; -, No detectable GUS activity, c, Non-continuous GUS activity

Figure 3.5.3: GUS Activity Detected in Putative Transgenic *Lotus corniculatus*

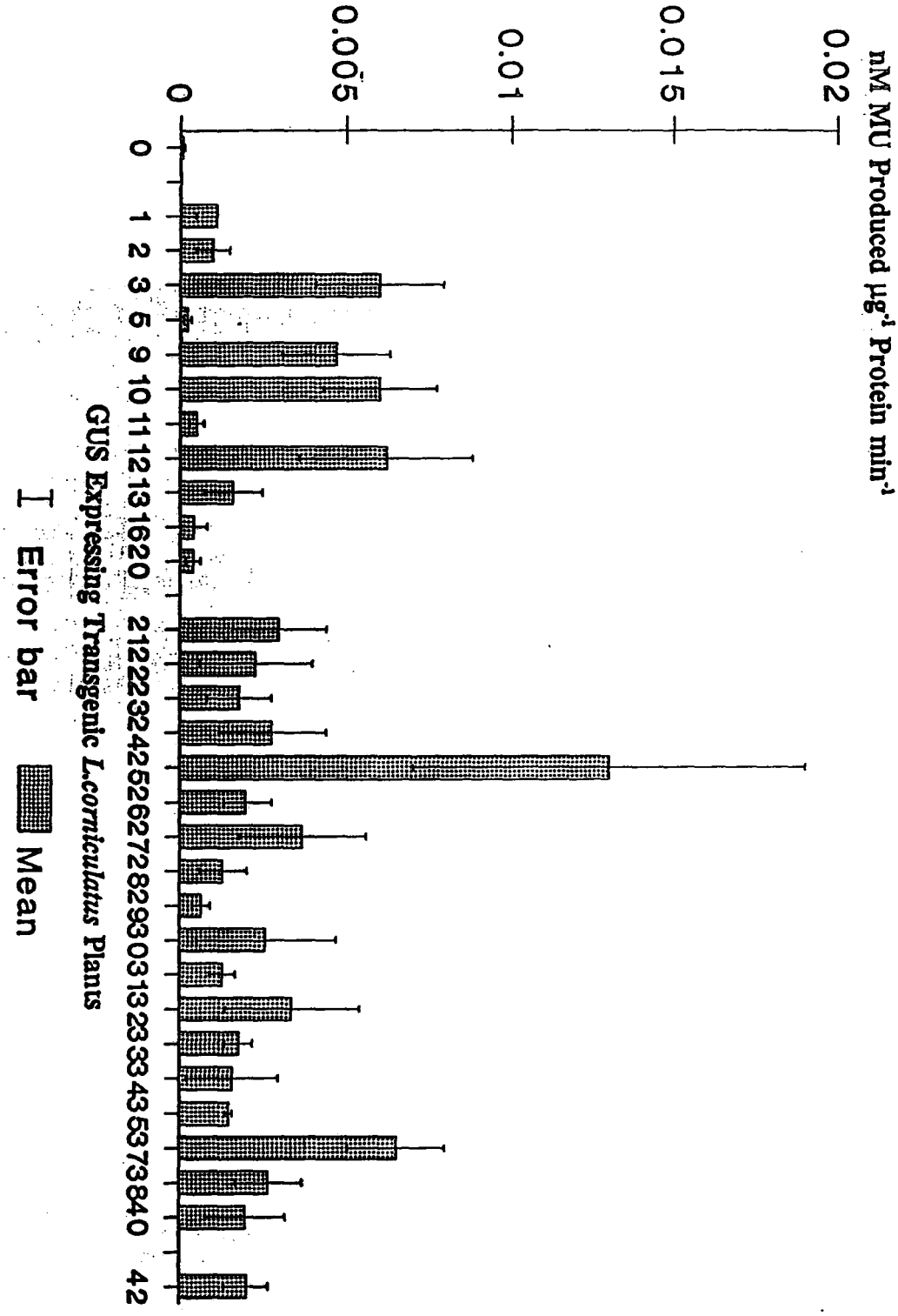
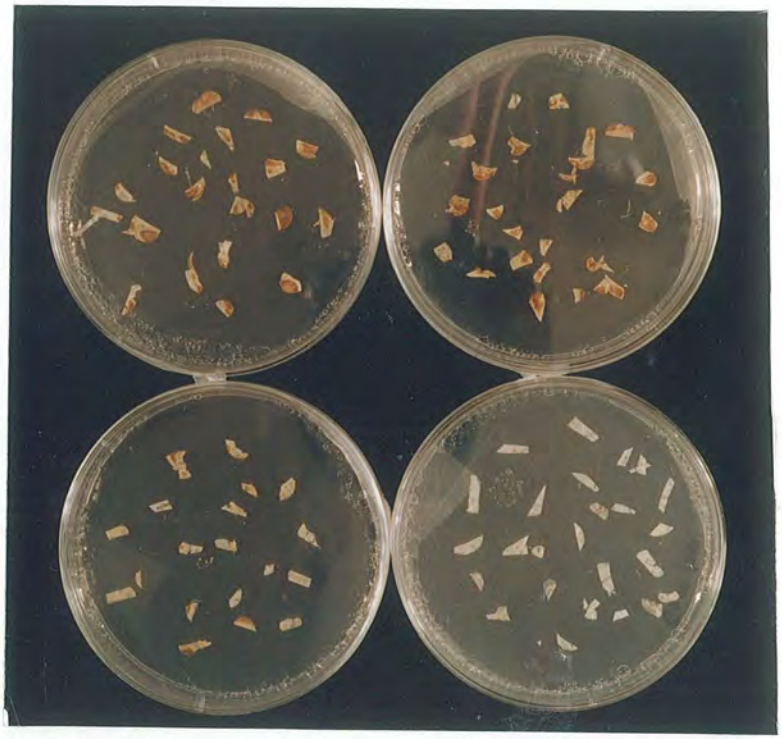


Figure 3.5.4: Response of Transgenic Tissue on 100 mgl^{-1} Hygromycin

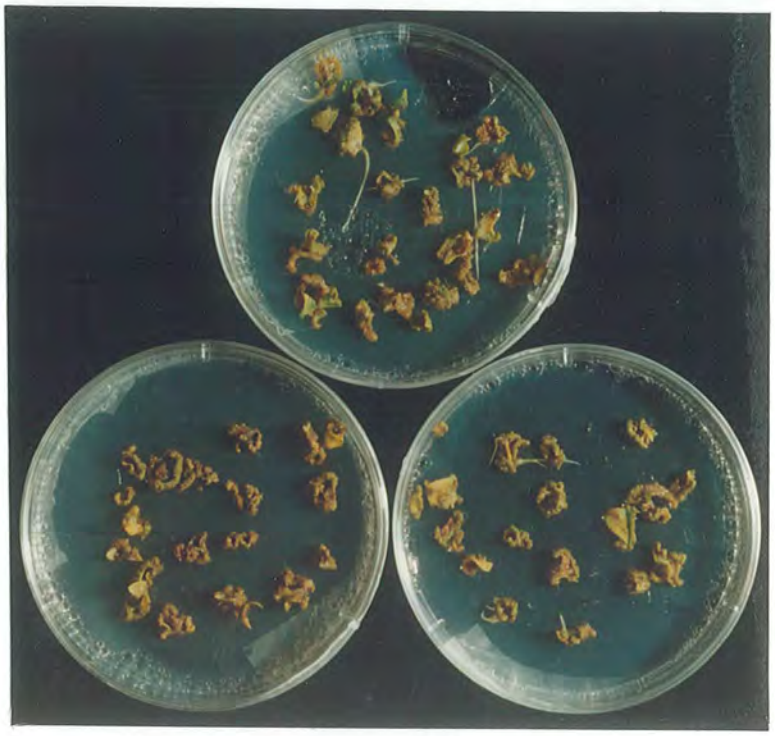
(a) Control explants on Hygromycin Selection (Day 28)

Top Left - 15 mgl-1 **Top right - 25 mgl^{-1}**
Bottom left - 50 mgl^{-1} **Bottom right - 100 mgl^{-1}**



(b) Explants From Transgenic Plants on 100 mgl^{-1} Hygromycin Selection (Day 28)

Top - Plant 4; Bottom Left - plant 28; Bottom Right - Plant 32
Material generally necrotic but later results showed shoot development.



3.5.3 Antibiotic Resistance

The percentage of excised shoots exhibiting hygromycin resistance were detailed in Results, chapter 4. To take this further, leaf tissues from selected transgenic plants were plated onto a series of hygromycin concentrations, (0, 15, 25, 50, 100 mgl^{-1}) over a 28 day period, to assess the effectiveness of the hygromycin resistance conferred by the *Aph IV* gene transferred from pJIT73. Tissues were also plated on a series of G-418 concentrations to investigate the presence/expression of *nptII* gene.

Over the experimental period, all non-transgenic leaf explants became totally bleached and no callus production occurred. However, explants from the four transgenic plants, 3, 4, (experiment B1) and 28, 32 (experiment C1), all survived and exhibited callus proliferation even on $100mgl^{-1}$ hygromycin. Tissue response on $100mgl^{-1}$ hygromycin is shown in figure 3.5.4. Of the 4 plants tested here, one plant (4) was GUS negative and three GUS positive (+), so confirming that in some cases only partial T-DNA transfer had occurred (see "Genomic DNA analysis" and discussion).

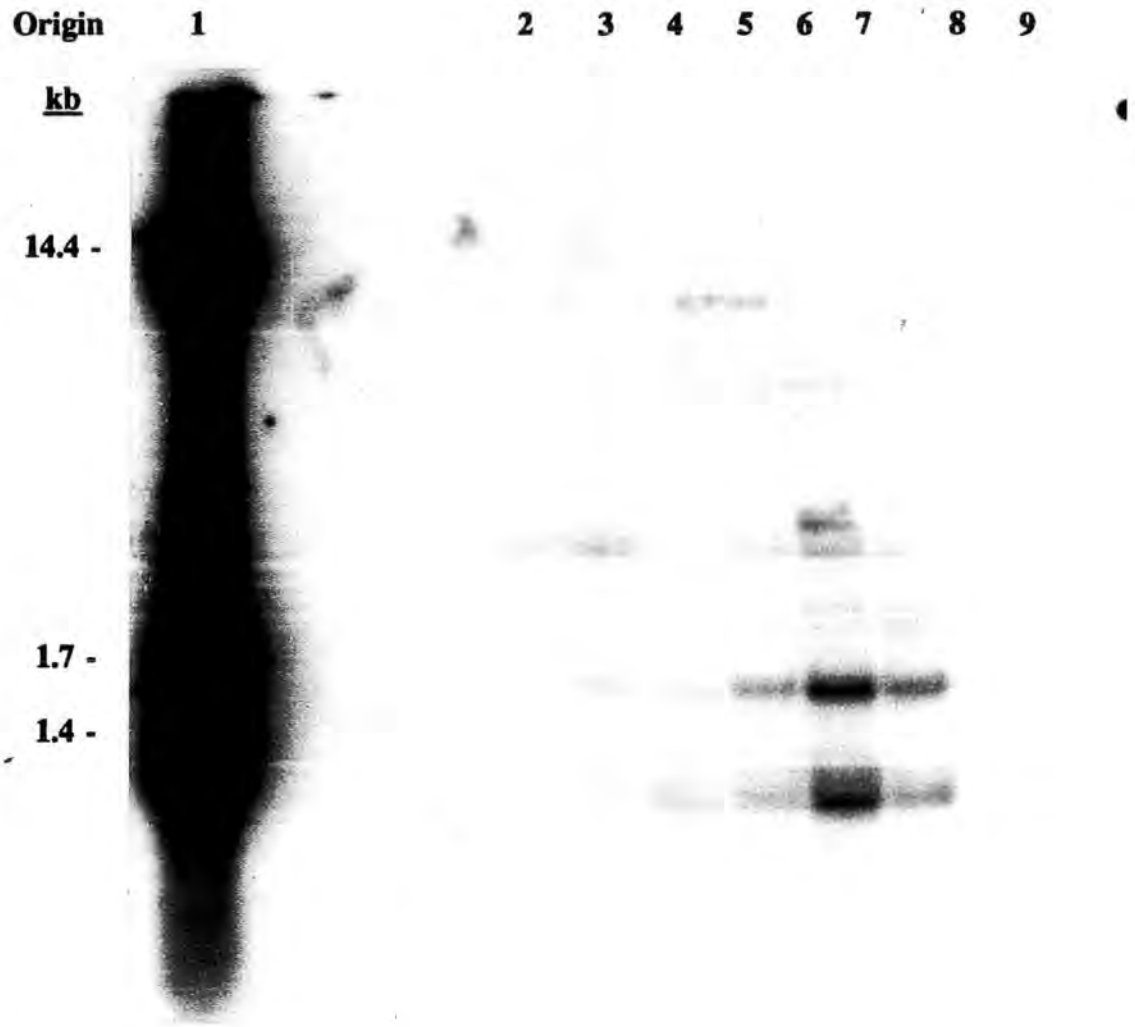
In contrast, samples from the four transgenic plants resulted in the same bleached reaction as control tissue on all concentrations of G-418. These plants were not expressing the *nptII* gene even if it was present in the host genome.

3.5.4 Genomic DNA Analysis

Plants 21, 22, 29 and 36 derived from experiment C_1 and plant 42 from experiment D_2, were analysed by Southern blotting. DNA was extracted from these plants, restricted with BamHI and resolved by electrophoresis a 0.7% agarose gel.

The probe used consisted of a Hind III fragments containing GUS and *aphIV* genes of pJIT73, ^{32}P-radiolabelled by a random priming giving a specific activity of $1.8x10^8$ cpm μg^{-1}. Exposure of 14 days showed specific hybridisation to fragments in the genomic DNA of all transgenic plants except plant 36. The hybridisation corresponded to the 1.7 and 1.4 kb restricted fragments of pJIT73,

Figure 3.5.5: Autoradiograph of Transformed *Lotus corniculatus* Genomic DNA Probed with Radiolabelled GUS and *Aph IV* genes

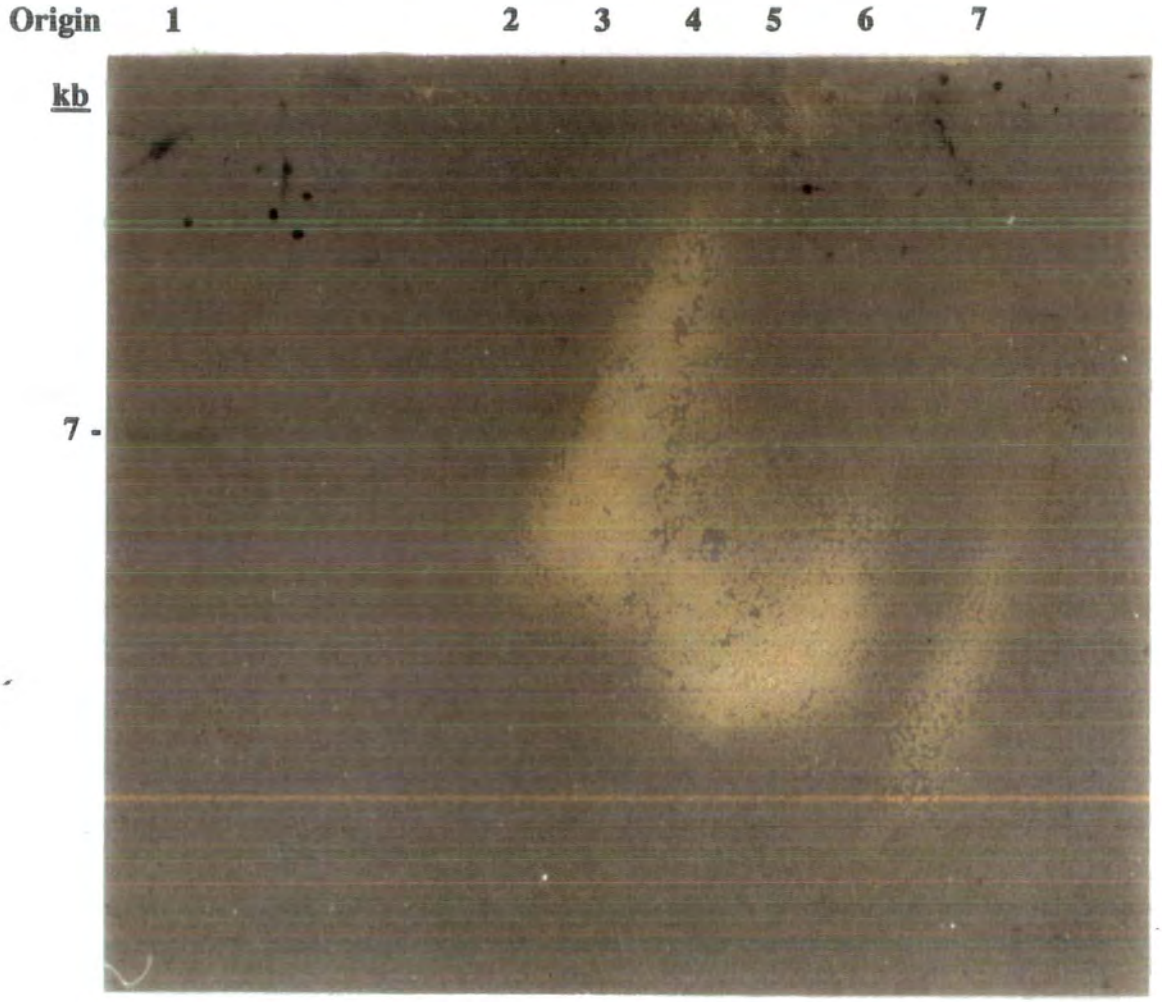


Lane

1 - pJIT73 restricted with BamHI
2 - Control *L.corniculatus* DNA
3-9 - Putative Transgenic DNA;
3 - Plant unknown
4 - Plant 44
5 - Plant 22
6 - Plant 29
7 - Plant 21
8 - Plant 36
9 - Control *L.corniculatus* DNA

Genomic DNA restricted with BamHI.
Probe - ^{32}P-radiolabelled GUS and *Aph IV* HindIII fragments of pJIT73.
Specific activity = 1.6×10^8 cpm μg^{-1} DNA. Wash stringency = 0.1xSSC.

Figure 3.5.6: Autoradiograph of Transformed *Lotus corniculatus* Genomic DNA Probed with pBIN19 DNA with T-DNA deleted



Lane

1 - 5pg of pJIT73 DNA
2 - Control *L.corniculatus* Genomic DNA
3-7- Transgenic Genomic DNA from Experiment C_1 Plants
3 - Plant 36
4 - Plant 28
5 - Plant 32
6 - Plant 33
7 - Plant 27

Plasmid and genomic DNA restricted with BamHI.
Probe - ^{32}P-radiolabelled 7kb BglI fragment of pBIN19 (T-DNA deleted).
Specific activity = 1.4×10^7 cpm μg^{-1} DNA. Wash stringency = 0.1xSSC.
Exposure = 2 weeks.

(see figure 3.5.5). Some evidence of re-arrangement was observed in plant 29, shown in lane **6**. This plant possessed relatively low levels of GUS expression perhaps due to such rearrangement. Plant 36 showed hybridisation to the 1.4kb fragment only, demonstrating that the lack of ß-glucuronidase activity in this plant was due to the absence of the GUS in the plant genome.

To demonstrate that the GUS activity exhibited by the majority of the transgenic plants produced was due to gene expression in the plant genome rather than external *Agrobacterium* contamination, a further hybridisation experiment was performed. Figure 3.5.6shows the result of probing transgenic *L.corniculatus* DNA with the *Agrobacterium* DNA excluding the T-DNA. No hybridisation was detected with this probe even in GUS expressing plants in lanes 4 - 7, showing the absence of contaminating bacteria.

3.5.5 Seed Production In Transgenic Plants

Prior to analysing F_1 seed for gene segregation, 10 reciprocal crosses were made to normal, control plants (K.Webb, pers.comm.). Gross morphological abnormalities in the flowers of the majority of the plants tested (K.Webb pers. comm.). The following plants flowered: 14, 16 (experiment B_1); 23, 24, 26, 37, 38, 39, 40 (experiment C_1). However of these only plant 38 exhibited flowers with a normal morphology (see figure 3.5.7)in which seed set occurred whether plant 38 was the male or female parent. The abnormalities of flower development in the other 9 plants is also shown in figure 3.5.7. Abnormalities include exposed styles and stigmas caused by telescoped and reduced petals which in combination results in desiccation of style and pollen. However, the pollen showed viability (FDA test used). Seed was only produced in crosses with plant 38 and there seeds will be tested for hygromycin and G-418 resistance and GUS activity.

Figure 3.5.7: Flower Morphology and Seed Production in Transgenic *Lotus corniculatus* Plants

(a) Comparison of *Lotus corniculatus* Flower Morphology

Flower Typical of the Abnormal Flowers on other Transgenic Plants Tested

Transgenic Plant 38

Control Flower

(b) Transgenic Plant 38, Exhibiting Seed Pod Development

(c) Further Comparison of *Lotus corniculatus* Flower Morphology

Control Flowers

Abnormal Transgenic Flowers - Features include reduced petal development, extended style and under developed stigma.

CHAPTER 6: The Use of *Agrobacterium*-mediated transformation systems to introduce agriculturally important genes to *L.corniculatus*

An important aspect of the development of a successful transformation system is its subsequent use in the transfer of genes of agricultural importance into that plant. Both the previously established *A.rhizogenes*-mediated transformation system and the new *A.tumefaciens*-mediated system described in chapter 4, were thus used in order to attempt to introduce (a) the insect resistance gene, CpTi and (b) the pea lectin gene *psl* into *L.corniculatus*.

3.6.1 Introduction of insect resistance to plants

At present plant breeding and biological control are used to reduce the high proportion, 13% of total crop production lost annually by insect damage. However, genetic engineering could allow resistance genes from unrelated donor plants to be incorporated into crops requiring protection. Such a transfer is unattainable using conventional present breeding technology. A limitation to the use of genetic engineering is the necessity to develop regeneration and transformation systems for each plant species. Those species which have been successfully transformed include maize, rice and 14 other food crops (Gasser and Fraley, 1989). The success of forage legume transformation was previously outlined in the introduction.

There are at present, two new approaches to genetically engineered crop protection against insect predators. The first approach is the use of genes such as the cowpea trypsin inhibitor (CpTi) which possess activity against a broad spectrum of insects (Hilder *et al*, 1987). The second utilizes more specific resistance genes, such as *Bacillus thuringiensis* endotoxin genes (Fischhoff *et al*, 1987).

When considering the use of engineered biological control in crop protection some important advantages exist for this novel technology over traditional methods of breeding and biological control. Protection is not dependent on seasons or weather; is conferred to all plant tissue; the protection can be targeted against specific insects and the active factor is biodegradable thus there is no residual environmental contamination.

The CpTi gene is part of the pest defence mechanism of the cowpea, *Vigna ungiculata* L.(Walp). Work by Gatehouse and collaborators implicated such inhibitors in field resistance to *Callosobruchus maculatus*, the major storage pest (Gatehouse and Hilder, 1988). The protein gene product is toxic to developing larvae. Its activity is targeted at the catalytic site of an enzyme, thus minimizing the probability of insects being able to evolve a complementary resistance mechanism by mutation.

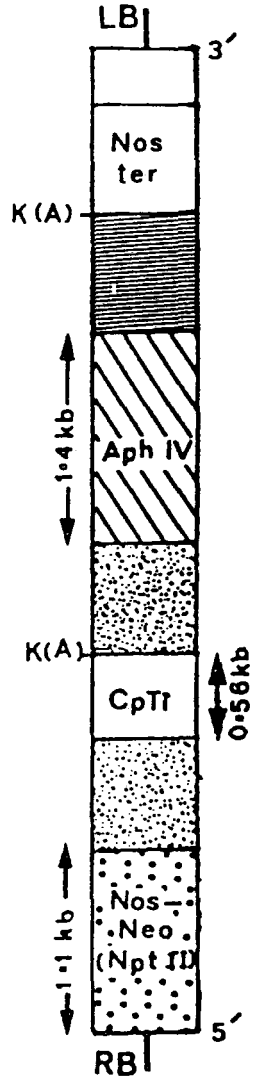
A suggested problem with genetic engineering has been the effect on plant yield of new genes. The introduction of CpTi into tobacco gave high gene expression levels and no loss in yield was observed (Hilder, *et al* 1990). The novel resistance is effective in the field as well as in a controlled laboratory environment. It was decided that the insect resistance gene, CpTi, was to be introduced into *L.corniculatus* using the *Agrobacterium*-mediated system.

3.6.2 Results

3.6.2.1 Production of *Agrobacterium tumefaciens* LBA4404[pAL4404::p208.96]

The chimaeric construct pRok.5+CpTi containing *aph IV* was designated as p208.96 and was 17.5kb in size. The *aphIV*, hygromycin resistance gene was introduced into the pROK.5+CpTi plasmid (donated by A.G.C.). Figure 3.6.1 illustrates the T-DNA of this construct and shows the relative positioning of the antibiotic resistance gene to the CpTi insect resistance gene. Use of Asp718I endonuclease allowed the unique 2.4kb fragment containing *aphIV* with promoter and terminater, to be cut from pJIT73 plasmid. This was inserted into the Asp 718 site in pROK/CpTi+5 next to the LH border of the T-DNA, by shotgun cloning. As was shown by expression studies in the transgenic *L.corniculatus* containing the pJIT73 plasmid, (chapter 4), transgenic plants showing hygromycin resistance should also then contain the insect resistance gene. After the shotgun cloning step the conjugants were selected on LA-kanamycin ($50mgl^{-1}$) agar plates. 169 colonies were streaked and screened using a probe based on *aphIV*, of $6.8x10^6$ cpm μg^{-1} specific activity. DNA was prepared from 10 positive colonies, by the "maxi" *E.coli* method, see2.2.1.3. The DNA was restricted with BamHI and Asp718 in order to check that the aph IV gene and its promoter and

Figure 3.6.1: Diagrammatic representation of the T-DNA of p208.96



KEY

CaMV 35S promoter

CaMV terminator

LB - Left-hand border
RB - Right-hand border

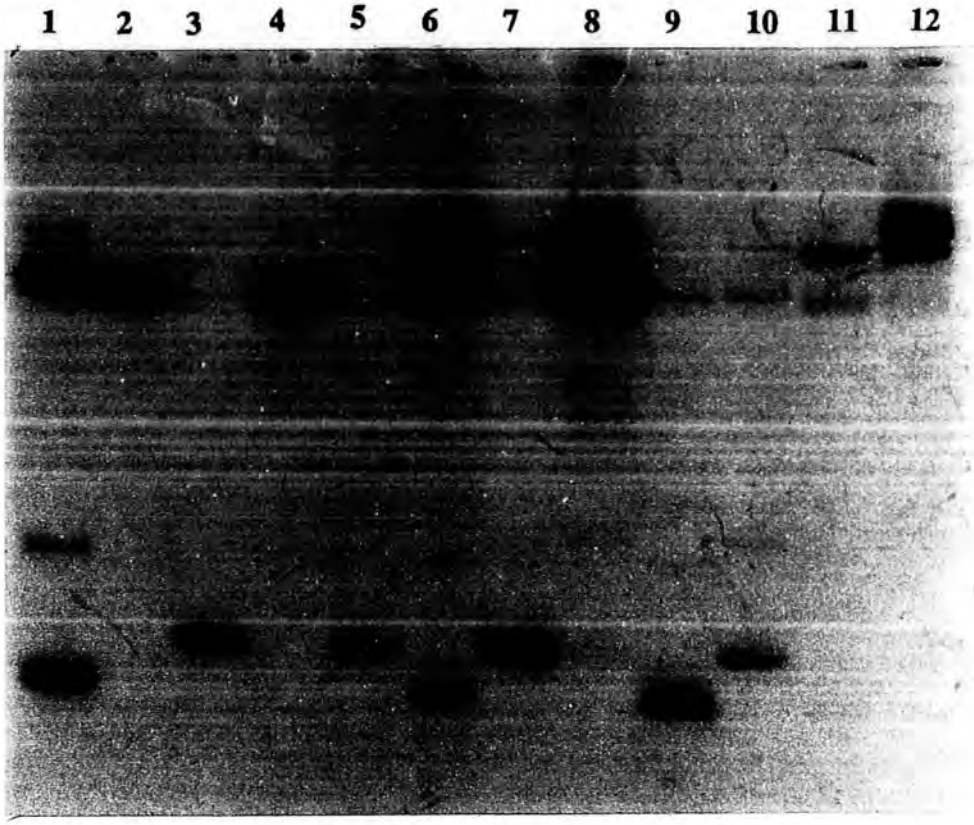
Restriction sites used; K - KpnI
(A) - Asp718I

terminator had been inserted in the correct functional orientation. The DNA samples, after being run on agarose gel and blotted, were probed with a ^{32}P radiolabelled PstI fragment of pUSSRC312, this contained the CpTi gene. This was produced by an overnight random priming reaction and had a specific activity of $1.1x10^7$ cpm μg^{-1}.

Figure 3.6.2 shows the different hybridisation response of DNA containing the insert in the possible orientations. When restricted with BamHI, the correct orientation of the insert will generate fragments of 1.3 kb and 0.9 kb; the incorrect (reverse) orientation generates two 1.3 kb fragments. The presence of hybridisation to a 0.9kb band, determined that three conjugant colonies were suitable for subsquent transfer into *A.tumefaciens* Ti-plasmid. The DNA samples were also restricted with Asp718, to check that the original Asp718 pJIT73 fragment containing the *AphIV* segment had been regenerated. This fragment was confirmed on gel as being 2.6kb. This new construct was designated as p208.96, conjugant 96 shown in lane 10 was subsequently used in the introduction of this construct to *Agrobacterium.*

A triparental mating was carried out to introduce this new construct into *A.tumefaciens* LBA4404. Antibiotic resistances of the three strains were confirmed (table 2.2.1) and at the final plating of serial dilutions, 140 colonies appeared after 48 hours at 10^{-3} and 35 at 10^{-4}. Some smaller colonies (39 and 16) were observed from the undiluted 10^0 and 10^{-1} dilution of the donor *E.coli* p*208.96* and 2 on the undiluted 100 plating of the mobilizing *E.coli* pRK2013, but these were assumed to be mutations. Ten large *Agrobacterium* colonies from the 1:1:1 10^{-4} dilution plate were minipreped and 5 samples restricted with BamHI, together with control *A.tumefaciens* LBA4404 DNA and DNA from donor *E.coli* strain.

Figure 3.6.2: Autoradiograph Showing Confirmation of *Aph IV* Orientation Within pROK.5 + CpTi

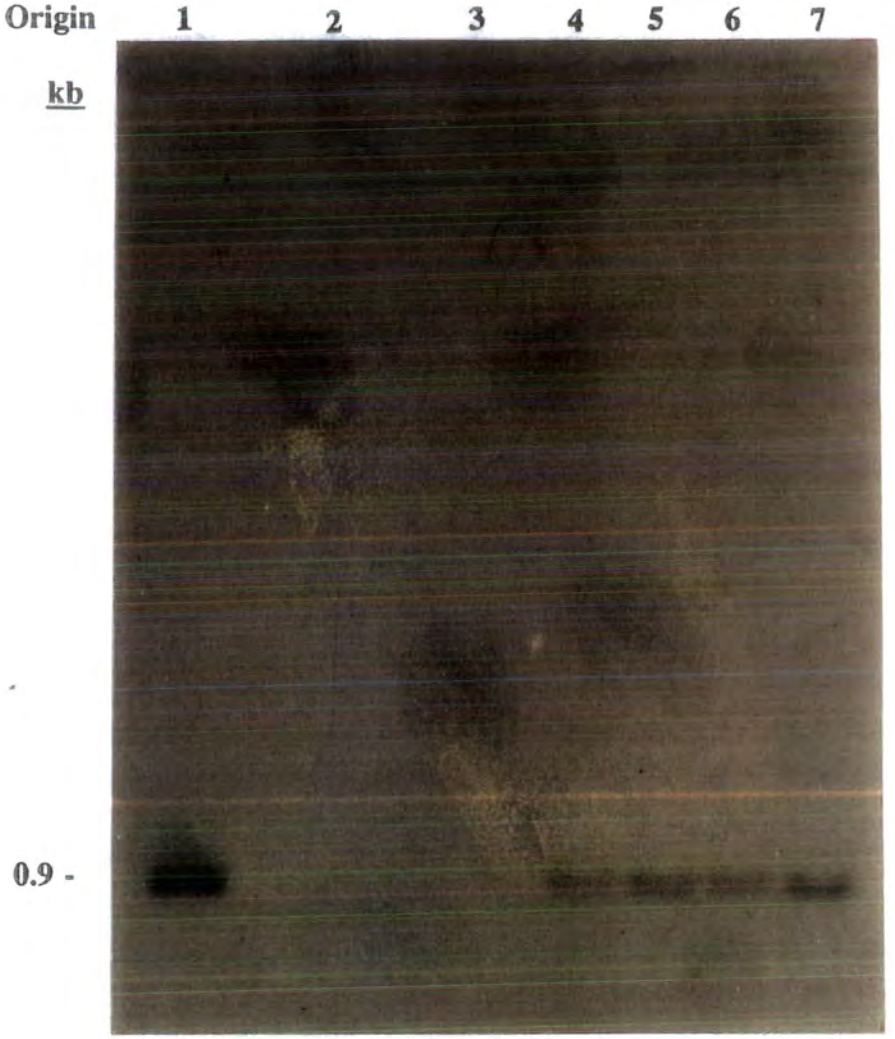


Lane

1 - Conjugant 98 - Bam HI restricted
2 - Conjugant 98 - Asp 718 restricted
3 - Conjugant 117 - Bam HI restricted
4 - Conjugant 117 - Asp 718 restricted
5-12 - Conjugants all restricted with Bam HI

Probe consisted of ^{32}-radiolabelled Pst I fragment containing CpTi gene. Specific activity = 1.1×10^7 cpm μg^{-1}. Wash stringency - 2xSSC. Exposure - 4 hours.

Figure 3.6.3: Autoradiograph of Triparental Producing *Agrobacterium tumefaciens* LBA4404 [pAL4404::p208.96]



Lane

1- p208.96 DNA

2 - DNA from *A.tumefaciens* LBA4404 [pAL4404] - Recipient

3 - 7 - DNA from putative conjugants (Conjugant from lane 8 used subsequently)

All DNA restricted with Bam HI. Probe consisted of ^{32}P-radiolabelled Pst I fragment containing CpTi gene. Specific activity = 1.1×10^7 cpm μg^{-1}. Wash stringency - 1xSSC. Exposure - 48 hours.

After blotting, the DNA was probed with the same ^{32}P radiolabelled fragment of pROK as before. The filter was washed to 1xSSC for 30 min prior to a 24 hour exposure to film. Figure 3.6.3 shows the resulting hybridisation with four of the five putative conjugants containing the p*208.96* construct. Colony 8 was chosen for use in subsquent plant transformation experiments.

3.6.2.2 Introduction of the CpTi Gene into *Lotus corniculatus*

The critical gene contained on the new p208.96 T-DNA was the *AphIV* gene conferring resistance to hygrogromycin B. This gene was previously shown to be an efficient selectable marker by the series of experiments described in chapters 3-5. The reporter gene was shown to be redundant if a gene of interest was placed between the right hand border and the *AphIV* gene, which is positioned adjacent to the left hand border. Thus the new construct p208.96 in the *Agrobacterium tumefaciens* LBA4404 strain, should possess all the features necessary for a successful transformation of *L.corniculatus* leaf explants.

All attempts at introducing the insect resistance gene, CpTi into *L.corniculatus* were unsuccessful. The protocols used were those developed in Group 4 experiments of chapter 4. The first experiment used non-induced *Agrobacterium* in the cocultivation suspension. Results are shown in table 3.6.1a. In total 126 leaf explants were cut and co-cultivated with *A.tumefaciens* LBA4404 [pAL4404::p208.96]. Other experiments were aborted due to fungal contamination. However the response of these explants when placed on selection medium of 15 mgl^{-1} hygromycin, was similar to that of control tissue. Though a degree of callusing did develop on co-cultivated explants, this apparent resistance soon disappeared as the material succumbed to total necrosis. Shoot primordia were seen to develop on a few leaf pieces but only after 8 weeks and these failed to develop beyond a very small abnormal looking growth. Such development then became bleached.

In the second experiment, *Agrobacterium* pre-conditioned with AS was used, as in the previous Group 4-C experiments. Due to fungal contamination 22 explants were maintained on selection. Concurrently a transformation was set up using the

Table 3.6.1: Response of Explants on 15 mgl^{-1} Hygromycin

Expressed as a percentage of each classification

(a) No *Vir* Induction - Day 30

Control - non cocultivated	Cocultivated with *A.tumefaciens* LBA4404 [pAL4404::p208.96]
N 84.3 B 15.7 *Cal 63.1* (76)	H 16.7 N 40.6 B 42.7 *Cal 35.4* (96)

(b) *Vir* Induction with Acetosyringone

DAY	Control (No selection)	Cocultivated with *A.tumefaciens* LBA4404 [pAL4404::p208.96]	Cocultivated with *A.tumefaciens* LBA4404 [pAL4404::pJIT73]
14	H 53.8 N 46.2 *Cal 100.0* (13)	H 100.0 *Cal 95.5* (22)	H 100.0 *Cal 96.4* (28)
21	H 100.0 *Cal 100.0* (8)	H 100.0 *Cal 100.0* (7)	H 100.0 *Cal 46.4* (28)
35	H 100.0 *Cal 100.0* (7)	N 100.0 *Cal 100.0* (6)	N 100.0 *Cal 100.0* (13)

Key

H - Healthy, swollen tissue

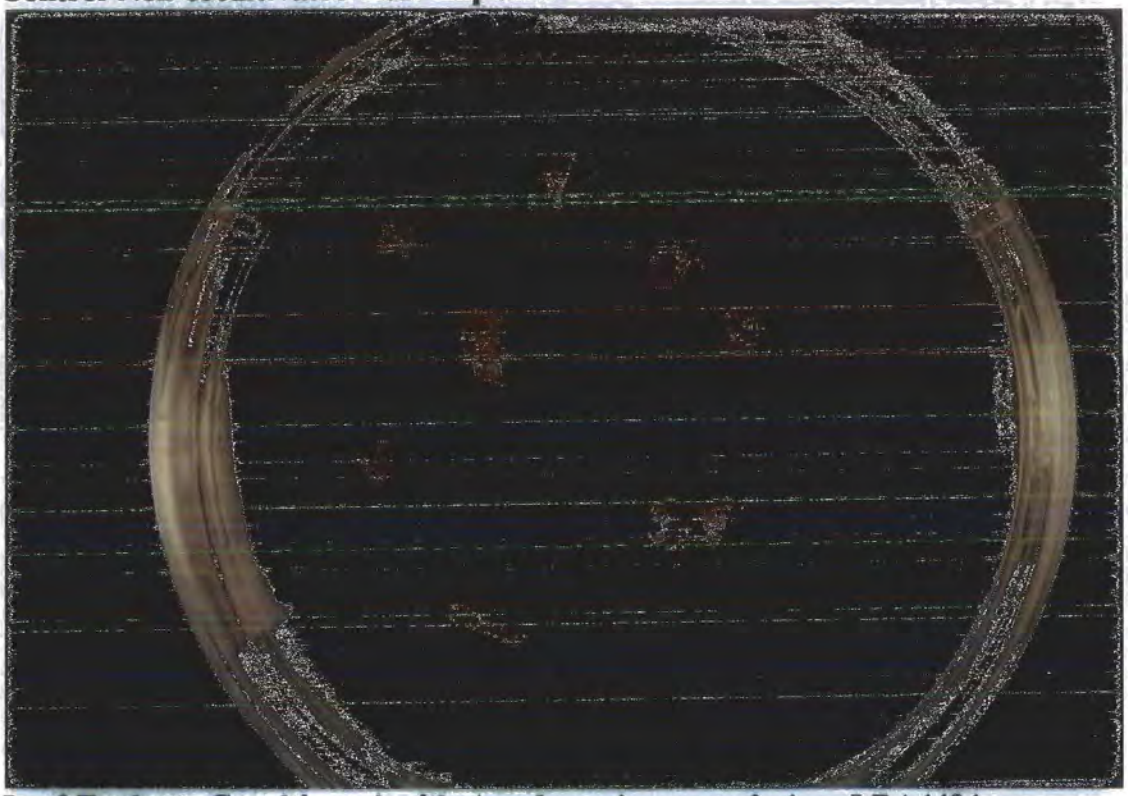
N - Necrotic tissue

Cal - Explants exhibiting shoot development

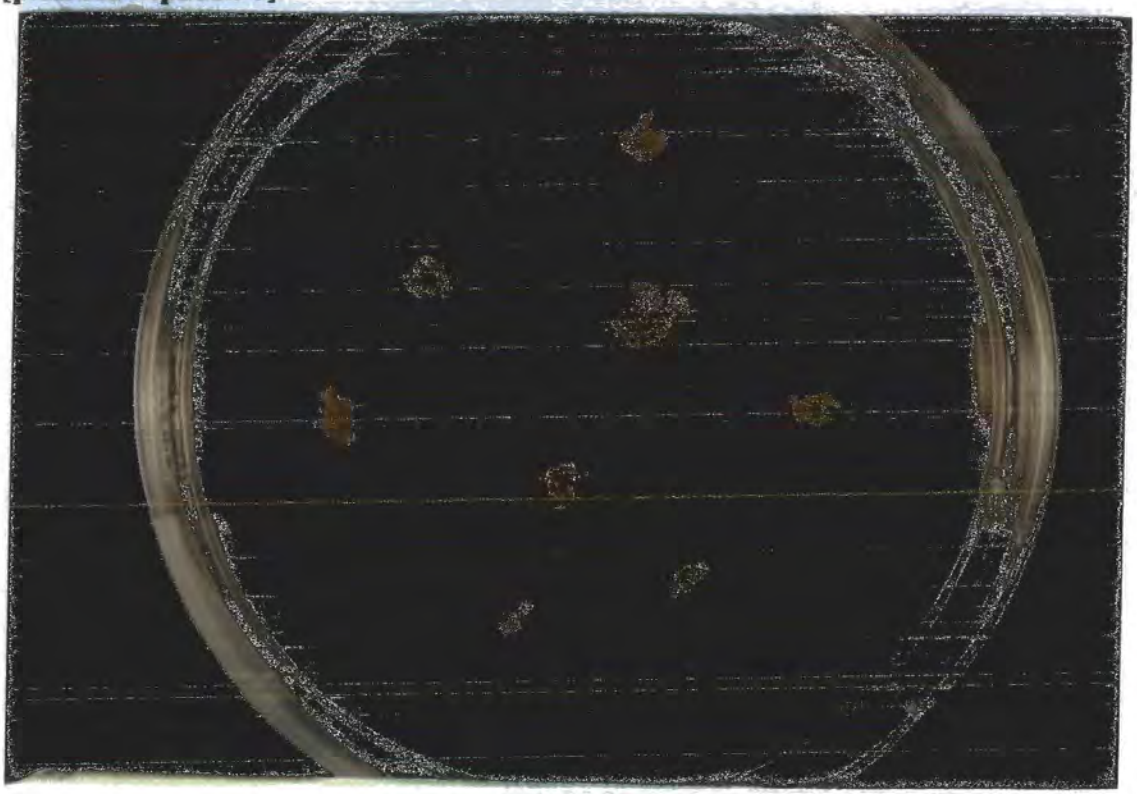
(n) - Total number of explants, where n decreases over experimental period losses are due to contamination

Figure 3.6.4: Reaction of Cocultivated *Lotus corniculatus* Explants on 15 mgl^{-1} Hygromycin Selection

(a) Control Non-cocultivated Leaf Explants



(b) Leaf Explants Cocultivated with *Agrobacterium tumefaciens* LBA4404 [pAL4404::p208.96]



Agrobacterium tumefaciens LBA4404[pAL4404::pJIT73]. This was used as a positive comparison having previously shown to result in transgenic plants.

Table 3.6.1b shows the response of the leaf explants on selection after this experiment. This reaction on selection is also illustrated in figure 3.6.4. Neither transformation gave potentially transformed shoots.

So, despite promising yields of transgenic plants previously resulting from the same protocol and vector type, the attempts to introduce the CpTi gene into *L.corniculatus* failed.

3.6.3 Introduction of the pea lectin gene *psl* to *Lotus corniculatus*

Lectins are carbohydrate-binding proteins which have been implicated in various plant-microorganism interactions. The possible role lectins may have in conferring nitrogen-fixation ability to legume, will be discussed here.

The Lectin Recognition Hypothesis was proposed by Albersheim and Anderson-Prouty in 1975. This hypothesis suggests that plant lectins are involved at the molecular level of infection specificity. Verification of this hypothesis experimentally, has however, proved difficult. Pioneering work giving credence to the hypothesis was carried out by Bohlool and Schmidt, (1974) using soybean. Some puzzling results arose from their study. For example, some *Rhizobium* strains capable of soybean nodulation did not bind to the purified soybean lectin. Also, no positive evidence for the presence of a lectin on soybean root hairs was found.

Important information was derived by studies of the *Trifolium repens-Rh.trifolii* symbiosis. Dazzo *et al*, 1978 identified a protein, "trifoliin". This molecule possessed lectin-like properties and was present on the surface of the roots and in exudate. A question to be cleared up concerned the level of homology between root and seed lectins. Antigenic homology between soybean root and seed lectins was shown by Pueppke *et al*, 1978. Localization studies showed that these molecules are restricted to epidermal cells. A strong positive correlation between the presence of lectin and

susceptibility to infection by *Rh.leguminosarum* was also apparent.

However, conflicting data has also been collected illustrating how the assessment of the role of root surface lectins in rhizobia-legume symbiosis has been limited by problems obtaining quantitative data. The introduction of ELISA "enzyme-linked immunoassays" has meant this difficulty has now been overcome. Inconsistency between purity levels in lectin preparations is another problem. Further complexity occurs through variation in *Rhizobium* culture conditions thus making comparison of studies non-viable, such conditions having been identified as being a critical influence upon lectin binding properties of *Rhizobium*.

More recent work has helped elucidate the question of the root/seed lectin relationship. Kaminski *et al*, 1987 outlined the information available about the "genetics" of the pea lectin. This is encoded by a multigene family of four genes. Of these only one, *psl* results in the production of a functional pea lectin. Kijne *et al*, 1980 indicated structural and immunological similarities existed between the seed isolectin 2 and the root lectin in pea.

Temporal expression studies of the root lectin in relation to rhizobia infection period in germinating seeds were carried out (Buffard *et al*, 1988). There was increasing expression of the root lectin over the 4-10 days after sowing, and a peak occurred on the tenth day just prior to rhizobia infection. This provided further circumstantial evidence for lectins having a role in the initial infection steps between bacteria and host.

Work by Diaz *et al*, 1989 produced strong evidence that the host-range of the bacterial-legume symbiosis is at least in part determined by the symbiont-root lectin interactions. The pea lectin (*Psl*) gene was introduced into white clover via *A.rhizogenes*-mediated binary transformation system. Using transgenic white clover roots, Diaz found that *Rh.leguminosarum bv.* infection resulted not only in root-hair curling but also in infection thread induction. These experiments were the first example of genetic engineering of a host plant causing altered host-bacterial specificity. The novel lectin was found on the root hairs but despite initial infection processes taking

place, nodulation, if it occurred at all was delayed. The majority of nodules produced were abnormal. This exemplifies the complexity of the rhizobia-legume symbiosis.

Transformation of a legume with a gene for a lectin from another member of the Leguminoseae family and experiments on transgenic plants produced, could potentially yield important information about the *Rhizobium*:legume symbiosis.

3.6.4 Results

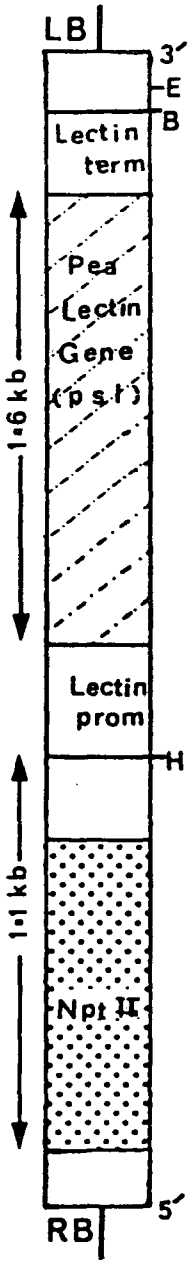
The *psl* gene was introduced, under the control of its own promoter, within a construct carried in the *A.rhizogenes* LBA9402 [p1855]. This was donated by C.Diaz. Figure 3.6.5 shows the T-DNA incorporated in this binary vector.

Since the gene is under the control of its own promoter, it will be relatively weakly expressed in a cell/plant even if incorporation of the gene into the plant genome had occurred. This has therefore led to problems of detection, the ELISA test not being sensitive enough and a genomic Southern blot was not possible because of the small quantities of plant tissue available to sample.

The *A.rhizogenes* vector containing the p*lecA* construct, was used to inoculate 10 day old axenic seedlings by the method described in Methods II. Once again "hairy roots" were induced at points of inoculation and epidermal damage as for the *A.rhizogenes* LBA4404 [pAL4404::pJIT73] experiment. But in contrast no antibiotic selection was used during subculture of these hairy roots. The relative positions of the *nptII* and *psl* genes did not permit the assumption that antibiotic resistance indicates the presence of *psl* also. Several different culture lines were established and shoots were initiated from these. Once such shoots had rooted on normal 171 ½$B_5$1S rooting medium they were used in the subsequent nodulation experiments.

170 seedlings were hypocotyl inoculated and from these 13 "hairy root" lines were generated over 19 days. Culture on hormone-free, B_5O resulted in extensive hairy root proliferation and then shoot development occurred on 10 of these lines after a further 3 weeks.

Figure 3.6.5: Diagrammatic Representation of T-DNA of pBin19*psl*



KEY
term - terminator
prom - promoter
LB - Left-hand border
RB - Right-hand border

Restriction sites: E - EcoRI
B - BamHI
H - HindIII

Shoots were continued to be excised from these subcultured roots and a series of shoots were derived for each hairy root line. These were then rooted on ½$B_5$1S slopes. Plantlets at this stage were then transferred to nitrogen-free Fahreus' agar plates. These plants then were immediately inoculated with a suspension of *Rhizobium* culture. Drops of suspension were placed on the plant roots, either *Rhizobium* var. *loti* strain 3011 or *Rh. leguminosarum* strain 1001 were used. Plates were then incubated upright with the root regions covered with black plastic.

Control *Lotus corniculatus* seedlings were similarly processed, so that confirmation of the natural *L.corniculatus-Rhizobium* specificity could be obtained. It was expected that nodulation would only occur when *Rhizobium var. loti* was used to inoculate control tissue. In contrast it was hoped that transgenic *L.corniculatus* plants carrying the p*lecA* gene would respond to *Rh. leguminosarum.* The effect of the new pea lectin gene on the normal *L.corniculatus-Rh. var. loti* symbiosis would perhaps become evident upon inoculation of the transgenic plants with *Rhizobium var. loti.*

However, inoculation of more than 20 control seedlings with the *Rhizobium var. loti*, failed to result in nodule development. The only response to be seen was the proliferation of many short, adventitious hairy roots. So, new *Rhizobium* cultures were used to inoculate 25 fresh seedlings but still nodulation failed to occur.

Similarly, transgenic plants regenerated from "hairy" roots failed to nodulate with either *Rhizobium* strain. In total 9 different lines of regenerated shoots were inoculated as shown below. Numbers shown in brackets (), represent the total number of plantlets of this line inoculated in this way.

Inoculated with *Rhizobium loti* 3011: Transformed line a(1), c(9), d(5), e(4), g(1), h(3), i(4), k(3), l(3).

Inoculated with *Rhizobium leguminosarum* 1001: Transformed line a(1), c(4), d(7), e(14), g(1), h(2), i(3), k(2), l(2).

In an attempt to investigate the reason for the failure of nodule formation, *Rhizobium* were restreaked onto YMB-agar plates from the roots of inoculated plants. *Rhizobium* grew from this inoculum, and thus it can be concluded that the *Rhizobium* cultures had not died. Nodulation of soil potted pea plants was also attempted with the culture of *Rh.leguminosarum* and still no nodulation occurred. Further reasons for this failure to nodulate will be discussed in the Discussion, section IV.

IV. DISCUSSION

IV. DISCUSSION

4.1 Introduction

The objective of this research was the development of a new gene transfer system for the transformation of *Lotus corniculatus*, using disarmed *Agrobacterium tumefaciens* vectors. The production of transgenic plants without any phenotypic abnormalities associated with the use of *A.rhizogenes* could then be achieved.

Previously hypocotyl, stem segments, protoplasts and leaf tissue have all been used in pRi or pTi transformation experiments (see Introduction 1.3). My research concentrated on the use of leaf tissue from pot-grown *L.corniculatus* as the target of transformation experiments, this source of material was abundant and easy to handle. Leaves from axenically grown plants were smaller and although not requiring surface sterilisation prior to use, were both more difficult to manipulate and yielded fewer leaf pieces than leaves derived from pot-grown plants. Previously only limited success was achieved with disarmed vector transformation of leaf material (Nisbet, 1987) so establishment of such a system would complement the hypocotyl and protoplast methods developed by Ghose, 1988. With this in view, the extended tissue culture regime of leaf explant regeneration to whole potted plants (20 - 24 weeks) compared to hypocotyl and internode regeneration (6 - 8 weeks) could be beneficially reduced. With the incorporation of an overall shortened regeneration period, any new leaf explant transformation system, which was of reasonable efficiency, would be competitive in comparison with the previously developed systems.

4.2 Tissue Culture Conditions

Although ultimately transformation success is measured in terms of the number of transgenic plants produced, an alteration in tissue response under culture may indicate decreased susceptibility to antibiotics as a result of gene transfer having taken place. Thus the classification system was used as an aid to observation of the tissue reactions in experiments and to compare the effect of different hormones, antibiotics or transformation protocols upon the leaf explants.

The system gave a general description of explant reactions in culture and an indication of both de-differentiation (callus production) and of shoot and root differentiation.

Slight modifications were made to the tissue culture technique used for *L.corniculatus* leaf explants developed by Nisbet, (1987). It was found that the main period of delay which occurred during regeneration from cocultivated explants was between the point of shoot initiation and shoot elongation on the callus tissue. Such a delay could ultimately reduce the feasibility of using any transformation method developed. However, care had to be taken to avoid increasing overall rapidity of regeneration at the expense of the selection. If a "tight" selection pressure was not maintained then the production of false positive plants is a further waste of resources both of time and space. Because of this, work concentrated on reducing the time between the initiation of shoot primordia and the development of these shoots to an excisable size. The fulfillment of totipotency is greatly influenced by plant hormones and so the experiments shown in 3.1.2 were envisaged to optimize the shoot differentiation from *L.corniculatus* leaf explants.

Investigation of hormonal constituents of the culture medium confirms the use of $B_{5.5.5}$ medium(Webb, 1986) for *L.corniculatus* leaf explant regeneration. Although an increased proportion of cytokinin to auxin levels would be expected to enhance shoot development, shoot production was lowered compared to $B_{5.5.5}$ when either BAP levels were raised or NAA levels were reduced. Endogeneous phytohormone production with the *L.corniculatus* explants may then be altering the expected hormone balance. The B_5H medium used in *L.corniculatus* hypocotyl culture (Ghose, 1988) was an auxin-free medium containing only $0.05mgl^{-1}$ BAP. Further hormonal experiments, using such low concentrations of phytohormones, may have yielded another plant medium conducive to prolific and rapid shoot development on leaf explants, especially as shoot primordia initiated on $B_{5.5.5}$, were found to elongate more rapidly on hormone-free medium, B_50 under high light intensities. Thus experiments held under different light intensities may also have resulted in more positive enhancement of shoot regeneration.

During the post-cocultivation culture period the presence of the decontamination antibiotics are an important influence on organogenesis. The effect of the decontamination antibiotics, cefotaxime and augmentin, upon shoot or root differentiation in *L.corniculatus* leaf explants was similar to the response found in tobacco tissue by Edwards, (1988). Shoot development was not shown to be detrimentally affected by either antibiotic. However a severe effect on rooting ability of excised shoots, caused by cefotaxime was apparent, there being a 50% reduction in numbers of rooting shoots on this antibiotic in comparison to antibiotic-free medium. Augmentin, whilst still efficient at decontaminating the culture, caused no adverse effects on root development. Therefore augmentin was used in preference.

Within transformation procedures one of the most critical steps is that of selection. Of the hundreds of cells present in an explant, only a fraction at the cut surfaces will be susceptible to foreign DNA uptake, allowing their transformation to take place. So the majority of cells would be sensitive to subsequent antibiotic selection and would react accordingly by undergoing necrosis or bleaching. Transformed cells are thus subjected to a doubly stressful environment with the presence of the antibiotic as well as the moribund cellular background. Careful choice of both tissue culture conditions and antibiotic concentration can maximise the production of transformed shoots while minimising the number of "escapes" where untransformed shoots evade the antibiotic selection. To this end, use of the scoring system as a method of assessing explants, allowed comparison between experiments of both general tissue response and time to differentiation.

Kill curve experiments are a useful method of establishing correct conditions for viable antibiotic selection pressure. In the present work, they were used to indicate whether selection based on the transfer of bacterial *nptII* (neomycin phosphotransferase; resistance to kanamycin and G-418) or *aphIV* (hygromycin phosphotransferase; resistance to hygromycin) would be a viable selectable marker for transgenic *Lotus*. As indicated by Nisbet, (1987) the level of kanamycin expected to be a suitable selection for *L.corniculatus*, 100 mgl^{-1}, is lethal to

untransformed *Lotus*. Indeed, the kill curve set up during this present project to re-investigate kanamycin selection indicated that no cell proliferation or differentiation occurred even at 25 mgl^{-1} kanamycin. However, the results of Nisbet (1987) showed that kanamycin was not an efficient selectable marker. The present results gave no indication why the use of kanamycin previously failed to fulfill its potential as selection pressure.

G-418, the second antibiotic to which resistance is encoded by *npt* II, causes tissues to become severely bleached. Low concentrations of this antibiotic were therefore used in the kill curve and both 10 and 20 mgl^{-1} G-418 were later used in transformations. However, G-418 also failed to maintain a tight selection pressure on leaf explants, but on excision and transfer to rooting agar slopes ($\frac{1}{2}B_5 1S$) containing G-418 untransformed shoots are killed. An explanation for the emergence of untransformed shoots from explants on the original selection medium, could be the buffering of a few cells within the explant by other cells, so reducing the effect of G-418 selection. Some cells are sheltered from the detrimental affect of G-418. This is similar to the plant defence hypersensitivity reaction, where cells near the point of infection die and protect other tissue from the disease.

The delay between the excision of explants and their being subjected to a selection pressure was considered a reason why regenerative ability was still exhibited by untransformed cells on selection, after the cocultivation and decontamination phases of a transformation. However on investigation, even a delay of 4 days did not give explants immunity to the subsequent addition of antibiotic.

The severity of hygromycin selection on *L.corniculatus* leaf discs was unknown, thus the large range of concentrations, 0-100 mgl^{-1} was necessary for its kill curve. This antibiotic resulted in a bleached rather than the browning necrotic reaction in the tissue. No callus production was evident at levels ≤ 15 mgl^{-1} and so 15 mgl^{-1} hygromycin was chosen as a basis of *L.corniculatus* leaf explant

selection.

Only limited work was carried out using other explant types. The leaf explant was favoured, due to the ease of harvesting and abundance of leaf tissue from a pot grown plant. Use of axenically derived explants introduced further culture space and time requirements, so growth of non-sterile material was more attractive. Ten mgl^{-1} hygromycin was suggested by the kill curves as a suitable selection for hypocotyl and cotyledon explant selection, in addition to the previously efficient selection system established by Ghose, (1988), of 50 mgl^{-1} kanamycin.

4.3 Transformation of *L.corniculatus*

4.3.1 Introduction

The construct selected for use in the gene transfer experiments with *L.corniculatus* was pJIT73. The T-DNA in pJIT73 containing several marker genes could potentially be utilised during development of a new protocol. During the experiments which followed, the *nptII* and *aphIV* genes demonstrated their relative suitab ilities as the selectable marker. The reporter gene, GUS aided in obtaining cotransfer details important in future useful foreign DNA insertions.

In pJIT73 *aph IV* is strategically better positioned adjacent to the left-hand border, as T-DNA transfer occurs from the right to left borders. Therefore, if the gene located towards the left border was found to have been integrated into the plant genome then it might be assumed that the gene found nearer to the right border has a high probability of being transferred. The analysis of the transgenic *Lotus corniculatus* derived from the *Agrobacterium tumefaciens*-mediated transformation system, supports this hypothesis although the cotransformation frequencies obtained were varied. 60% of hygromycin resistant plants of experiment B_1 also exhibited GUS activity and 81% of hygromycin-resistant plants derived from experiment C_1 also possessed GUS activity. However the absence of ß-glucuronidase activity is not necessarily due to absence of the GUS gene in the plant host genome but due to rearrangment or positional effects. Nevertheless, as the ultimate aim is to achieve foreign gene activity in transgenic *L.corniculatus*

plants the expression of both the selectable marker and reporter genes is a viable representation of transformation efficiency.

Since the decision to use the pJIT73 construct, research has shown the presence of a point mutation in *npt II, (Anon.)*. This would cause a reduction in expression of this antibiotic resistance gene in a plant genome, thus fewer transgenic shoots survive selection. So, this mutation in *npt II* cannot account for the "leakiness" of the kanamycin or G-418 selection when used against *L.corniculatus* tissue.

Other genes both in the Ti plasmid and on the chromosome of *A.tumefaciens* may be crucial to the success of transformation experiments. Although preliminary work using wildtype *A.tumefaciens* strains demonstrated *L.corniculatus* to be most susceptible to nopaline C58 strains (Webb, 1986), vectors based on both disarmed *A.tumefaciens* LBA4404[pAL4404] and GV3101[pGV3850] were produced. Indeed, Ghose, 1988 obtained transgenic *L.corniculatus* via a C58 derived gene vector system and Nisbet, 1987 utilised *A.tumefaciens* LBA4404 based system.

In constructing the C58 nopaline-derived vector as a comparison to the Ach5 octopine-type, the pBR322 region within the left and right borders of the pGV3850 could allow recombination events with pJIT73. Thus, when studying the relative virulence of C58 (nopaline) and Ach5 (octopine) vectors with *L.corniculatus* it must be acknowledged that this may not be a comparison of two binary vectors. However, it was shown by the production of the single *L.corniculatus* transgenic plant from experiment D_2, that the two important genes, *aph IV* and GUS were still functional in *A.tumefaciens* GV3101 [pGV3850::pJIT73]. Further evidence for this was shown when this vector was used to transform *Arabidopsis thaliana*. Efficiency and frequency of transformation were then found to be high (K. Evans, pers. comm.). So, it can be proposed that the relatively unsuccessful use of *A.tumefaciens* GV3101 [pGV3850::pJIT73] with *L.corniculatus* leaf explants, is an example of the typical specificity of *Agrobacterium* strain - plant species found

during transformation. Further confirmation of this could be obtained if the true binary vector *A.tumefaciens* C58 C1 was to be used.

As well as the comparison between the "binary" vectors described above, a *cis* vector incorporating only the GUS reporter gene was produced. But since the requirement for hygromycin selection became evident during the early stages of this project, work concentrated on the "binary" vectors and only limited experimental resources were given to the use of the *cis* vector, *A.tumefaciens* GV3101 [pGV3850.G]. The use of hygromycin B as selection on *L.corniculatus* leaf tissue was critical to the development of the efficient transformation protocol finally achieved. This illustrates that a *cis* vector lacking a useful selectable marker would not be conducive to the production of a new transformation system. Use of a *cis* vector containing *aph IV* may yet be beneficial to *L.corniculatus* transformation.

4.3.2 Transformation Experiments

The use of the previously established naturally oncogenic *Agrobacterium rhizogenes* vector system to introduce the pJIT73 T-DNA into *Lotus corniculatus* was a useful comparison to the new disarmed *Agrobacterium tumefaciens* system. However this comparison could only be a general one as full investigation of the hairy root transgenic plants was not carried out. A higher frequency of transgenic plants were regenerated from disarmed *A.tumefaciens* cocultivated leaf explants, than from *A.rhizogenes* inoculated seedlings. The low frequency of "hairy root" transformation could be in part due to the viability of the *A.rhizogenes* culture rather than due to an innate inefficiency of the inoculation. Although time of regeneration of a plant from the transgenic hairy root is much reduced compared to full differentiation from *A.tumefaciens* cocultivated leaf explants, the presence of hairy root phenotype characters in the former necessitates a genetic backcross to segregate out such detrimental characteristics. Such a process extends the overall period between transformation and regeneration of a morphological normal plant. For example, upon flower fertilisation, seed production takes 6 weeks to obtain the F_1 generation. These plants would then need to be outcrossed and gene

segregation in the F_2 seed analysed.

However, Webb *et al*, 1990 carried out a more extensive investigation of the use of *A.rhizogenes* vectors with *L.corniculatus* as well as *Trifolium* species. This study confirmed *A.rhizogenes*-mediated transformation as a viable method for genetic transfer in *L.corniculatus* with only limited changes occurring to morphology, cytology and physiology of plants after transformation.

Similarly, attempts to transform *L.corniculatus* hypocotyl tisue through the technique developed by Ghose, 1988 was necessary in an attempt to assess the relative merits of any new pTi vector system developed. However, this method proved non-reproducible in my hands.

The previously used transformation method for *Lotus corniculatus* (Nisbet, 1987) was the basis for the development of a more efficient protocol. In view of the fact that kanamycin and G-418 were the selection antibiotics used previously, work initially concentrated on improving the efficiency of selection for transformed cells in leaf explants. The first indications that hygromycin was an efficient antibiotic became evident during the group I experiments. Kill curve data had shown that low levels of hygromycin significantly restricted the differentiation process in control material. So, the fact that shoots were initiated on cocultivated explants in experiment II suggested that transformation had occurred. Continued shoot development was subsequently inhibited on that medium. However, the relatively low proportion of leaf pieces exhibiting shoot primordia, $<15\%$, suggested that if transformation had occurred, it had only done so at a low frequency, thus transformed cells would exist in a highly necrotic background, which could then affect the growth of transformed tissue. The length of explant decontamination was 4 days and so early cellular tissue culture processes were well established before selection pressure was applied. More extensive cell proliferation was permitted within the explants after transfer to selection medium, than would have been expected if selection had been applied earlier. This appears to contradict the previously discussed experiment where selection was delayed.

However, in the latter experiment only non-transformed tissue was present so no assessment could be made with regard to any inhibitory effect of untransformed callus on transformed cells, the delay experiment having been set up to investigate the "leakiness" of selection rather than retardation of shoot development.

An important modification made to the transformation system at this point was the cocultivation of explants immediately after excision without the 24 hour delay used for the first 3 experiments. This delay had been devised to allow the wound-induced *Agrobacterium vir* induction compounds to be produced and so enhance *Agrobacterium* - plant attraction and thus increase the opportunity for gene transfer. However use of AS could achieve such *vir* induction in the *Agrobacterium* cultures used and reductions in selection pressure caused by an additional delay would be overcome. Use of AS was later introduced during group 4 experiments.

The effects of changing decontamination parameters were tested in the experioments designated group 2. The only antibiotic used here to kill *Agrobacterium* was cefotaxime. Of the 4 experiments (IV - VI), only two produced excisable shoots (experiments III and IV), which all emerged from explants subjected to G-418 as selection for transformation. Further analysis of these putative transgenic shoots failed to yield conclusive evidence of genetic transfer. Those experiments utilising hygromycin as selection for transformation exhibited preliminary evidence of transformation with the initiation of shoot primordia, but these shoots failed to continue development. Two reasons for this could be proposed: (i) that even these relatively low levels of hygromycin were too severe a selection pressure on transformed cells; (ii) that transformation had not occurred and so this selection method was not strong enough to inhibit shoot initiation completely, though it does prevent continued extension of shoot primordia from untransformed tissue.

Group 3 experiments differed from the previous work with respect to the *Agrobacterium tumefaciens* strain acting as the gene transfer vector. The study showing the influence of decontamination antibiotics on root development of

shoots, meant that these experiments incorporated the use of the new decontamination procedures, augmentin at 400 mgl^{-1} for a 2 hour liquid wash and 200 mgl^{-1} in subsequent agar culture. The first two attempts at transformation, using *Agrobacterium tumefaciens* GV3101 [pGV3850::pJIT73] were unsuccessful and no confirmation of genetic transfer in shoots regenerated from explants exposed to the *cis* vector, *Agrobacterium tumefaciens* [pGV3850.G] was obtained. No further work using this *cis* vector was carried out because of the failure to generate transgenic plants. Nevertheless, hygromycin still demonstrated the greatest potential for selection efficiency with G-418 showing apparent leakiness.

The final successful protocols emerged during the group 4 series of experiments. The length of time that leaf explants were on decontamination plates in the absence of selection had been reduced, and cocultivation in liquid culture occurred in the absence of plant hormones. Both of these factors could have been critical to the final success achieved. Explants being subjected to selection earlier may have given transformed cells a better competitive advantage over the nontransformed tissue, with the reduced time without selection ensuring that untransformed tissue would not develop a buffering capacity to the selection antibiotic.

The improvement of transformation frequency when the liquid culture medium used during the first 2 hours of cocultivation, contained only Gamborg salts and sucrose (B_5O) is interesting. The plant hormones, BAP and NAA, present in $B_{5.5.5}$, may have had a detrimental effect on the *Agrobacterium* when this medium was used during the liquid cocultivation phase. The plant hormones may have acted as antagonists to either the induction of the *vir* region or the chemotatic response of *Agrobacterium* to the wound induced compounds produced by the excised explants (Alt-Moerbe *et al*, 1988; Ashby, 1988). Aggregation of the *Agrobacterium* to each other was more marked in $B_{5.5.5}$ than in $B_5 0$ liquid cultures but as such aggregation was also in evidence after AS had been used in some of the group 4 experiments, so a link between aggregation and failure of transformation cannot be established. Work by Alt-Moerbe and coworkers (1988)

found that secretion of iP-type cytokinins by *Agrobacterium* correlated with *vir*-region induction. Either the additional presence of both exogenous sources of auxin and cytokinin interferes with the normal induction mechanism or simply the auxin source was detrimental.

These experiments finally confirmed hygromycin B to be the optimum antibiotic for the selection of transformed *L.corniculatus* in preference to G-418. Once again the severity of the selection was evident, as even when shoot primordia developed, if untransformed, only abnormal differentiation would occur. Although use of AS was not essential to the achievement of transformation, a marked improvement in frequency and efficiency were to be seen with the use of the *vir* inducer compound.

Work from this group 4 series demonstrated that the optimum *Agrobacterium tumefaciens* strain for use in *Lotus corniculatus* leaf disc transformation was an Ach5 type *Agrobacterium tumefaciens* (LBA4404) in comparison to the C58 derived strain. The results suggest that future transformation of *Lotus corniculatus* leaf pieces should utilise *A.tumefaciens* LBA4404 as the disarmed gene transfer vector. However further studies may indicate other *A.tumefaciens* strains to which *L.corniculatus* leaf explants have even greater susceptibility.

4.3.3 Confirmation of Transformation

With the regeneration of transgenic plants from B_1, C_1 and D_2 experiments in group 4, a detailed investigation of transformation efficiency and characteristics exhibited by the transgenic plants was carried out. Several different methods were used to study the transgenic plants, using both the scorable and selectable marker genes present on the T-DNA of the bacterial plasmid.

Testing for resistance to hygromycin was the first method of checking that excised shoots were transformed. Tissue from some fully regenerated plants was cultured on hygromycin giving further evidence of the selection efficiency.

Additional work testing more plants on 100 mgl^{-1} hygromycin and also at higher concentrations of the antibiotic would establish whether all of the hygromycin resistant plants possessed a uniform level of resistance or whether variation in expression exists. Such variation may become evident through differences in differentiation occuring on the explants.

The absence of G-418 resistance in the transgenic plants again demonstrates the importance of *aph IV* as the basis of a selection system in *Lotus corniculatus*. Further analysis of transgenic plant genomic DNA would, however, be necessary to determine whether *npt II* had been transferred into the host plant and was nonfunctioning, or whether only partial pJIT73 T-DNA transfer had occurred and *npt II* located adjacent to the right-hand border had failed to become incorporated.

The GUS assay was a critical tool in the assessment of transformation. The pJIT73 construct was a model system and future work would insert a gene of interest into a similar position in T-DNA as the GUS reporter gene. Thus it was useful to study the efficiency of its transfer and expression. Both the fluorometric and histochemical assays were utilised. For more detailed expression information the fluorometric assay was used, but further work using the histochemical test could show localisation of expression that may exist in the transformed plants, either in chimaeras or in tissue specific expression, despite the use of the CaMV 35S promoter. Benfey *et al*, (1990) demonstrated that this gene consists of several domains which can potentially encode tissue-specific or developmentally regulated expression of a gene. When deletions are made from the CaMV 35S promoter then tissue-specific expression can occur, whereas the complete 35S promoter remains constitutive. Further genomic DNA analysis would be interesting, in order, for example, to assess whether some of the transformed plants which did not express GUS contained GUS genes which were not functioning, or had been rearranged or partially deleted. This could affect the efficiency of the system as the GUS reporter gene here represents a possible future gene of interest. This more extensive genomic DNA analysis was not carried out, due to problems in achieving hybridisation even in plants known to possess novel phenotypic features.

Although the genomic DNA extraction method produced DNA that was consistently restrictable, yield remained poor. Total DNA extracted from 2g fresh weight *L.corniculatus* stem/leaf material was required for subsequent restriction. Thus approximately 10 μg DNA was obtained from a harvested pot grown plant. It was then necessary to wait several weeks before harvesting again from a single plant, and such problems limited the DNA analysis currently achieved. Use of polymerase chain reaction (PCR) could potentially allow genomic analysis to be carried out even when only low yields of genomic DNA could be obtained. However, other problems become accentuated by this method. For example, when briefly attempted during this project, contamination of control samples with GUS sequences proved difficult to eradicate. Also, as for traditional radioactive hybridisation experiments, homology of plant genomic DNA to the primer sequences used, can complicate interpretation of the resulting DNA agarose gel produced.

The ultimate confirmation of transformation would be a study of segregation of GUS and *aph IV* genes in F_1 seed, derived from outcrossing original transgenic plants to control plants. Only 1 of the 10 plants from experiments B_1 and C_1 which were induced to flower, developed morphologically normal and fertile flowers. Seed production could be attained with this plant acting as the female parent in a reciprocal cross. This may illustrate molecular disruption of normal flower development in the transgenic *L.corniculatus*, though viable pollen was collected from the other 9 plants. Whether this is due to the events of genetic transformation or through somaclonal variation has yet to be determined. Shoots were excised from explants which had been in culture for several months, so the probability of somaclonal variation would be increased over time in culture. However, the high frequency of similar abnormalities would indicate that another event accounts for the changes in morphology. An extensive investigation of tissue culture regeneration of *L.corniculatus* explants was carried out by Webb and Watson (1991) and this found that >90% of leaf explant regenerants on $B_{5.5.5}$ medium were morphologically normal.

The presence of incompletely deleted oncogenic phytohormone genes on the

T-DNA and thus in the recipient plant genome could be another reason for the resulting abnormalities. To test the hypothesis, further hybridisation experiments using phytohormone genes as probes are necessary.

4.4 Introducing Genes of Interest into *Lotus corniculatus*

Genetic transfer had been achieved in 3 different experiments using the new disarmed *Agrobacterium tumefaciens*-mediated transformation system. Despite this reproducibility, transfer of the CpTi gene into *L.corniculatus* was unsuccessful using this new method. The new construct p208.96 is then implicated as being the reason for this failure, the functioning of the *Aph IV* gene inserted into pROK.5+CpTi had not been tested prior to its "transfer" into *L.corniculatus*. However, in parallel with the second attempt to introduce the T-DNA of p208.96 into *L.corniculatus*, some explants were simultaneously placed in an *Agrobacterium tumefaciens* LBA4404 [pAL4404::pJIT73] suspension. It was hoped this experiment would confirm the viability of the protocol used, but no transformation was found to occur. Thus the failure to transfer CpTi to *L.corniculatus* may not be due to faults in the construct. Further attempts to achieve incorporation of CpTi into the *Lotus corniculatus* genome together with a model species such as tobacco, would perhaps clarify the reasons for these initial failures. As both experiments with *Agrobacterium tumefaciens* LBA4404 [pAL4404::p208.96] and [pAL4404::pJIT73] were unsuccessful, a possible factor influencing them may have been age of leaf material used.

An *Agrobacterium rhizogenes*-mediated transformation system was used for the work involving the introduction of the pea lectin gene, *psl*, into this legume. The aim of these experiments was to investigate the *L.corniculatus* - *Rhizobium* symbiosis. Unfortutnately although *psl* was introduced to *Lotus corniculatus* with the "hairy root" phenotype acting as the marker for transformation, no confirmation could be obtained showing incorporation of the T-DNA into regenerated transgenic plants. Because the *psl* gene was under the control of its own lectin promoter the expression levels are extremely low and thus could not be detected by an ELISA assay. Detection of the genes by genomic hybridisation was not possible with the

transgenic plants still relatively small in axenic culture. An alternative procedure would be the use of PCR in order to amplify the limited DNA resources. Nevertheless, the problem of achieving nodulation of the transgenic plants either with the compatible *Lotus Rhizobium loti* or pea *Rhizobium leguminosarum* could not have been solely due to false positives having been generated by the initial transformation step.

Even the normal *L.corniculatus* - *Rhizobium* symbiosis failed to cause nodule development, so inhibition of the process through the presence of nitrogen in the culture medium was a possibility. Previously though, *L.corniculatus* had been induced to nodulate on the same medium without difficulty (R.Teverson, Pers. comm.). The failure of soil grown pea plants to nodulate with *Rhizobium leguminosarum* culture, then implicated the *Rhizobium* cultures in the nodulation difficulties. Despite relatively rapid growth on the specified medium the *Rhizobium* cultures apparently had lost the *nod* and/or *nif* gene functions. This then was the conclusion drawn from these experiments. Recent research indicated that both nodulation and nitrogen fixation genes were linked on the same plasmid, (Hombrecher *et al*, 1981). So, the evidence that *nif* genes were absent on the *Rhizobium loti* strain 3011 (K.Webb, Pers.comm.), was probably indicative of a similar lack of *nod* genes, thus no nodulation would occur.

4.5 Conclusions

Despite the difficulties encountered during the development of the new *A.tumefaciens* disarmed vector system for *L.corniculatus*, the protocol is potentially important. As shown in table 1.1, the majority of successful *L.corniculatus* transformations were initiated by *A.rhizogenes*-mediated gene transfer systems although crown gall was induced by wild type *A.tumefaciens* (Webb, 1986). In comparison to the protocol utilising axenic explants (Ghose, 1988), the transformation frequencies obtained by the use of leaf pieces and *A.tumefaciens* LBA4404[pAL4404::pJIT73] showed greater efficiency. Adaptation of this system to mature axenic tissue would be a possibility, though additional pressure would then be placed upon the culture resources available.

Immediate extension of the previously described work relates to more detailed morphological and cytological investigations of the transgenic *L.corniculatus*, together with further genomic hybridisation experiments. For example, details of the copy number of inserted genes would be of interest. Investigation of the gene segregation in seed derived from the transgenic plants, where possible, is also an essential experiment for future studies. Such information would help in a comparison of the *Agrobacterium tumefaciens* and *A.rhizogenes*-mediated gene transfer systems. Also, the overall strategy of the *Lotus corniculatus* disarmed *A.tumefaciens*-mediated transformation system developed could be compared to that previously developed for *Medicago sativa* (Shahin *et al*, 1986b). Experiments utilising the different successful techniques on alternative host forage legume species and genotypes may result in further advances in genetic engineering of forage legume species.

A major requirement for the continuation of the research presented would be further demonstration of genetic transfer into *Lotus corniculatus* using the newly developed method, by for example, the introduction of the insect resistance gene, CpTi, into this legume.

Meanwhile, further work on the tissue culture procedures followed could be beneficial. Shoot extension was still slow, overall improvement of the transformation procedure would be achieved if time from explant to novel flowering plant could be reduced. With this in mind a fuller comparison between the previously proposed hypocotyl transformation technique (Ghose, 1988) and the new leaf explant technique may be useful. The former has a more rapid regeneration time course.

Much work of importance is carried out through *A.rhizogenes*-mediated transformation regarding the nitrogen fixation symbiosis. Transgenic "hairy roots" are particularly useful for such studies, as shoot regeneration from "hairy root" is rapid and the roots contain the genetically engineered root and nodule specific genes.

Overall the temperate forage legumes are relatively recalcitrant to transformation via disarmed *A.tumefaciens* but continued research in this field could yielded further transformation protocols. The economic importance of legumes means that improvement in yield, pest resistance and the bloat-free character could all profitably be introduced into these plants, thus a new *A.tumefaciens*-mediated transformation protocol is particularly useful. Any future extension of the work in this project could be potentially successful in contributing to the use of transformation technology in forage legumes.

V. REFERENCES

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